Cathepsin D is involved in the regulation of transglutaminase 1 and epidermal differentiation

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Summary

We previously demonstrated that the aspartate protease cathepsin D is activated by ceramide derived from acid sphingomyelinase. Increased expression of cathepsin D in the skin has been reported in wound healing, psoriasis and skin tumors. We explored specific functions of cathepsin D during epidermal differentiation. Protein expression and enzymatic activity of cathepsin D increased in differentiated keratinocytes in both stratified organotypic cultures and in mouse skin during epidermal barrier repair. Treatment of cultured keratinocytes with exogenous cathepsin D increased the activity of transglutaminase 1, known to cross-link the cornified envelope proteins involucrin and loricrin during epidermal differentiation. Inhibition of cathepsin D by pepstatin A suppressed the activity of transglutaminase 1. Cathepsin D-deficient mice revealed reduced transglutaminase 1 activity and reduced protein levels of the cornified envelope proteins involucrin and loricrin. Also, amount and distribution of cornified envelope proteins involucrin, loricrin, filaggrin, and of the keratins K1 and K5 were significantly altered in cathepsin D-deficient mice. Stratum corneum morphology in cathepsin D-deficient mice was impaired, with increased numbers of corneocyte layers and faint staining of the cornified envelope only, which is similar to the human skin disease lamellar ichthyosis. Our findings suggest a functional link between cathepsin D activation, transglutaminase 1 activity and protein expression of cornified envelope proteins during epidermal differentiation.

Key words: Barrier function, Cathepsin D, Cornified envelope, Epidermal differentiation, Involucrin, Loricrin, Filaggrin, Keratin, Sphingomyelinase, Transglutaminase

Introduction

The goal of epidermal differentiation is to form the epidermal permeability barrier, which prevents excessive water loss and entry of harmful substances into the body. The barrier is localized in the stratum corneum, a two-compartment system of protein-enriched terminally differentiated keratinocytes (corneocytes) and lipid-enriched intercellular bilayers (Elias and Friend, 1975; Downing, 1992).

The insoluble protein envelope located beneath the plasma membrane of the keratinocytes is cross-linked by cellular transglutaminase 1 (TG1) during terminal differentiation of the epidermis. Involucrin, an early marker of terminal differentiation, is a soluble protein precursor of the cross-linked cornified envelope (CE) and is synthesized in the keratinocytes of the upper spinous layers (Watt, 1983; Eckert et al., 1993; Kanitakis et al., 1987; Negi et al., 1981; Rice and Green, 1979; Steinert and Marekov, 1997). In hyperproliferative diseases like psoriasis, premature expression of involucrin is found in the lower spinous layers (Thewes et al., 1991). Another main component of the CE is loricrin. It is expressed in a later stage of differentiation (Watt, 1983) and is cross-linked to other epidermal proteins such as cystatin, elafin or filaggrin.

The extracellular lipids of the barrier, predominantly ceramides, are synthesized in the keratinocytes, stored in the epidermal lamellar bodies and secreted into the intercellular space of the stratum corneum (Elias, 1983). Beside its structural role, ceramide is known as an important intracellular signal mediator for various cytokines, in particular tumor necrosis factor (TNF) (Liu et al., 1997; Perry and Hannun, 1998). TNF binding to the p55 TNF receptor (TNF-R55) results in activation of an endolysosomal acid sphingomyelinase (Wiegmann et al., 1994; Krönke et al., 1996). Increase in intracellular ceramide levels is followed by different cellular responses depending on cell type and degree of activation such as differentiation, proliferation and programmed cell death (apoptosis) (Krönke et al., 1996; Jarvis et al., 1994; Kolesnick and Golde, 1994; Geilen et al., 1997; Wakita et al., 1994). We suggested a functional role of TNF and ceramide derived from acid sphingomyelinase in cutaneous permeability barrier repair after experimentally induced injury of the skin. We detected high levels of sphingomyelinase and ceramides after barrier disruption as well as a significant delay in barrier repair in TNF-R55-deficient mice (Jensen et al., 1999). In understanding the various biological effects of ceramides, it is important to know their direct intracellular targets.

Recently, we found that the aspartatic protease cathepsin D

(CTSD) is a novel specific intracellular binding protein for ceramide, derived from acid sphingomyelinase (Heinrich et al., 1999). Ceramide enhances the proteolytic activity of CTSD. CTSD is the main aspartatic protease of endolysosomes. It is synthesized and translocated into the endoplasmic reticulum as an inactive pre-proenzyme (52 kDa), processed into an enzymatically active, intermediate proenzyme (48 kDa) and finally converted into the mature form of 32 kDa in the lysosomes (Fujita et al., 1991). CTSD is involved in the proteolytic activation as well as proteolytic degradation of intracellular proteins (Diment et al., 1988; Lazarus et al., 1974; Sato et al., 1997). Increased levels of CTSD are correlated with tumor cell invasion and metastasis in malignant melanoma, squamous cell carcinoma and human breast cancer (Kageshita et al., 1995; Kawada, 1997). Reports suggest that CTSD may play a role in the metastasizing process of malignant cells because of their destructive effects on the extracellular matrix. Therefore, CTSD activity was used to predict recurrence in breast cancer (Tandon et al., 1990). In HeLa cell cultures CTSD is a mediator of programmed cell death induced by various cytokines. Overexpression of CTSD induced cell death without any external stimuli and the CTSD inhibitor pepstatin A suppressed cell death in this system (Deiss et al., 1996). Some of these findings were supported by in vivo studies in CTSD-deficient mice, generated by gene-targeting. These mice developed normally during the first 2 weeks. Afterwards they exhibited progressive atrophy of the intestinal mucosa, followed by massive intestinal necrosis and profound destruction of lymphoid cells. The mice died in a state of anorexia at the age of 4 weeks, though lysosomal bulk proteolysis was maintained, possibly due to compensatory activation of related lysosomal proteases (Saftig et al., 1995).

In the skin CTSD plays a role in both extracellular and intracellular catabolism. In hyperproliferative skin disorders such as psoriasis, increased expression of the mature form of CTSD has been reported, returning to normal after resolution of the psoriasis by psoralen and long-wave ultraviolet radiation (PUVA) light treatment (Kawada et al., 1997; Chen et al., 2000). It was suggested that CTSD may be involved in the control of cell differentiation during normal development. Horikoshi et al. found increased expression and increased activity of CTSD isoforms in the skin depending on the stage of epidermal differentiation (Horikoshi et al., 1998). CTSD enzyme is active at an acid pH (maximum at pH 3) and may work at the transition of the stratum granulosum to stratum corneum, because the stratum corneum produces an acid environment (pH 5.5) (Öhmann and Valquist, 1994). Despite these results, function and cellular substrates of CTSD in epidermal differentiation are still unknown.

In the present study we explored the functional role of CTSD in the skin during permeability barrier repair and epidermal differentiation using keratinocyte cell culture, wild-type and CTSD-deficient mice. In particular, we examined the functional role of CTSD in the activation of keratinocyte TG1 as a key enzyme for the processing of CE proteins such as involucrin, loricrin and filaggrin during epidermal differentiation.

Materials and Methods

Mice and cells

Male hairless mice (Crl: (hr/hr)BR) 4-12 weeks of age were supplied by Charles River, Sulzfeld, Germany. CTSD-deficient mice (C57BL/6) were obtained by gene targeting as described before (Saftig et al., 1995). Progeny of heterozygous CTSD-deficient mice were genotyped by PCR of genomic DNA from tail biopsies. The animals were maintained conventionally under standardized conditions. The study protocols were approved by the University of Kiel, Committee of Animal Care. Normal human keratinocytes and stratified keratinocytes (organotypic, raft cultures) were obtained from human foreskins. Primary keratinocytes were maintained in serum-free keratinocyte growth medium (KGM, Clonetics, San Diego, USA), supplemented with 0.07 mM calcium, and grown to 60-80% confluency. Also, immortalized HaCaT keratinocytes were used (Boukamp et al., 1988). Human raft cultured epidermis was prepared as described previously (Steude et al., 2002). Briefly, primary dermal fibroblasts and keratinocytes were prepared from foreskin and grown as described previously (Mielke et al., 1990). Third passage fibroblasts ($5\text{-}7\times10^5$) were resuspended in ice cold collagen solution containing 5.3 mg collagen type I, 1× Dulbecco minimal Eagle's medium (DMEM; Gibco-BRL), 2 mM L-glutamine (Gibco-BRL), 0.5% $NaHCO_3$, 66.7 mM Hepes and 0.03 M NaOH, and submerge cultured in DMEM + 10% fetal bovine serum for 5 days. Third passage keratinocytes (5×10^5) were seeded onto these collagen lattices and submerge cultured for 4 days in keratinocyte growth medium (KGM Bullet kit; BioWhittaker Europe, Belgium) + 5% fetal bovine serum. Raft cultures were lifted to the air-medium interphase and incubated in keratinocyte growth medium without bovine pituitary extract and EGF, but with additional 5% fetal bovine serum and 1.25 mM $CaCl_2$. Growth factors were added into the medium. The medium including growth factors was renewed every 2-3 days. Raft cultures were harvested and frozen at –70°C.

Permeability barrier disruption

Disruption of the permeability barrier was induced in hairless mice by tape stripping (Tesafilm®, Beiersdorf, Hamburg, Germany) to remove cells from the stratum corneum, resulting in a superficial wound, until a 20- to 30-fold increase in transepidermal water loss (TEWL), as a marker of barrier disruption, was achieved (Meeco® electronic water analyzer, Meeco® Inc., Warrington PA) (Spruit and Malten, 1966; Grubauer et al., 1989). Barrier recovery was calculated as follows: Barrier recovery (%)=100 – ($_x$h – baseline)100/($_0$h – baseline). ($_x$h=TEWL × hours after barrier disruption; $_o$h=TEWL directly after barrier disruption; baseline=normal TEWL of the skin without barrier disruption). In the hairy CTSD-deficient mice the fur was carefully removed by shaving prior to barrier disruption. The shaving did not result in irritation or barrier disruption. For each experiment, at least three animals were treated and at least three animals served as control. At different times after barrier disruption (0-24 hours), TEWL, as a marker of barrier repair, was measured and skin samples of about 4 cm^2 were obtained.

Measurements of the basal TEWL

Measurements of the basal TEWL were performed using the Tewameter® (Courage and Khazaka, Germany) as described previously (Jensen et al., 2000). (The sensitivity of the Tewameter® for the small changes in TEWL as seen under basal conditions is higher than that of the Meeco® water analyzer.)

Topical application of pepstatin A to normal mouse skin

Immediately after barrier disruption, 100 μl of pepstatin A (0.1%) in propylene glycol/isopropanol 7:3 (v/v) was applied topically. Vehicle application served as control. TEWL was determined at different time points after barrier disruption (0-24 hours) and skin samples (about 4 cm^2) were taken.

Isolation of epidermal samples after acute barrier disruption

Flank skin of the treated or untreated sites were excised and immediately placed epidermal-side down onto a covered Petri dish containing crushed ice. The skin pieces were scraped with a scalpel blade to remove subcutaneous fat and immersed at 37°C for 40 minutes in 10 mM EDTA in calcium- and magnesium-free phosphate-buffered saline (PBS). Thereafter, the epidermis was peeled off the dermis by gentle scraping with a scalpel blade. ~15-20 mg epidermis were disrupted in 350 ml buffer H [150 mM KCl, 5 mM NaF, 1 mM phenylmethylsulfonylfluoride, 20 μM pepstatin, 20 μM leupeptin, 20 μM antipain (Boehringer Complete, 1:100) in Hepes pH 7.4] with an electric glass homogenizer (Potter S®, Braun, Melsungen, Germany) at 600 rpm for 4 minutes. Cells were homogenized by passing through a 28 G needle followed by sonication three times for 10 seconds. To analyze the involucrin and loricrin expression the entire skin sample was excised, the subcutaneous fat removed and the skin disrupted with the Ultrathurrax® (IKA Labortechnik, Staufen, Germany) in Tris-buffer (85 mM NaCl, 50 mM Tris, pH 7.4) and homogenized with the Potter S® as described. Subsequently the probes were boiled for 10 minutes and lysates were cleared by centrifugation for 5 minutes (20,000 ***g***).

Isolation of culture cells

The keratinocyte culture cells were harvested by incubation with trypsin-EDTA, scraped into PBS, pelleted by centrifugation (5 minutes, 800 ***g***), homogenized in buffer H and sonicated. After centrifugation all supernatants were stored at –80°C.

Determination of CTSD, TG1, involucrin and loricrin

Protein concentration was measured by the bicinchoninic acid protein assay (Pierce, Rockford, USA). Equal protein samples were electrophoresed on 7.5% or 12.5% polyacrylamide gels and transferred onto nitrocellulose filters at 100 V for 45 minutes (Mini-Transblot Biorad, Munich, Germany). CTSD in cell cultures was detected by incubation for 1 hour at room temperature with a polyclonal rabbit anti-human CTSD antibody at a dilution of 1:1000 in TBST (Calbiochem, Oncogene Science, USA), CTSD in mice was detected with a rabbit anti-mouse CTSD antibody (kindly provided by R. Pohlmann, University Münster, Germany), TG1 by a goat anti-human polyclonal antibody [kindly provided by S. Y. Kim and P. Steinert, NIH, Bethesda MD (see Kim et al., 1995)] and involucrin and loricrin were detected by rabbit anti-mouse antibodies (PRB 142C and PRB 145P, respectively, Covance Inc., CA, USA distributed by Hiss Diagnostics, Freiburg, Germany). Secondary antibody complexes were visualized using a chemiluminescent detection system (ECL; Amersham, Braunschweig, Germany), and quantified by densitometry (PC-BAS TINA software).

CTSD assay

To estimate the activity of cellular CTSD an assay was performed using parathyroid hormone (PTH) as a specific substrate as previously described (Heinrich et al., 1999). Digestion of PTH results in cleavage of the hormone between Phe34 and Val35 yielding PTH (1-34) and PTH (35-84) fragments. PTH proteolysis was detected by immunoblotting using a monoclonal antibody and could be blocked by the aspartate-protease inhibitor pepstatin A, a selective inhibitor of CTSD. 2 μg lysate-protein were incubated for the indicated times with 50 ng PTH at 37°C in a volume of 20 μl acidic buffer (100 mM sodium acetate, 100 mM KCl, pH 4.2). To demonstrate CTSD specificity of the reactions, 0.5 μM pepstatin A was added to the assay as indicated. One sample containing PTH in acidic buffer but without lysate served as control. Reactions were stopped by boiling the samples for 3 minutes with Tris-tricine-SDS sample buffer (2% β-mercaptoethanol, 12% glycerol, 50 mM Tris pH 6.8, 4% sodium dodecyl sulfate, 0.01% Coomassie G). Proteins were separated on 15% SDS-PAGE and transferred onto nitrocellulose filters. Immunoblotting was performed using anti-PTH mouse antibody specific for fragment 1-34 (Biogenesis) and anti-mouse secondary horseradish-peroxidase conjugate. Blots were developed using the ECL detection reagent (Amersham).

TG1 assay

Mouse epidermis was disrupted with the Ultrathurrax® (IKA Labortechnik, Staufen, Germany) and homogenized with the Potter S®, as described, in a buffer containing 20 mM sodium phosphate, pH 7.2, 0.5 mM EDTA, 10 mM dithiothreitol, 50 μg/ml phenylmethylsulfonylfluoride. Epidermal cells were lysed in the same buffer by sonication. Epidermal TG1 activity was measured as described previously (Hohl et al., 1998). Briefly, 5 μg of tissue extract was added to 95 μl of a solution containing 0.5 M sodium borate pH 9, 5 μl 10 mM EDTA pH 8, 5 μl of 100 mM $CaCl_2$, 20 μl of dimethylcasein (10 mg/ml), 2.5 μl of 10% Triton X-100, 0.5 μl of 1 M dithiotreitol, 2.8 μl of 100 μM putrescine, 1 μl of [1,4 (n)-^{3}H] putrescine dihydrochloride (1 mCi/ml, 10-30 Ci/mmol; NEN) and 48.2 μl H_2O. After incubation at 28°C for 30 minutes, 80 μl were applied to cellulose filter papers (Whatman) and washed sequentially in 10% TCA and 0.1% putrescine, 5% TCA and 0.05% putrescine and 95% ethanol. Radioactivity was determined by liquid scintillation counting. TG1 activity is expressed as pmol [^{3}H]putrescine incorporated into dimethylcaseine per hour and mg protein. For evaluation of the effects of CTSD on TG1 activity, membrane fractions were prepared from the lysates: homogenates were centrifuged at 25,000 ***g*** at 4°C for 30 minutes. The pellet was re-extracted by sonication with the same buffer supplemented with 1% Triton X-100. After 10 minutes incubation at 37°C, the lysate was centrifuged as above and the supernatant (membrane fraction) collected. TG1 activity in the membrane fraction was measured as described above for whole tissue extracts.

Skin histology

Chemical fixation and embedding for light and electron microscopy were as follows. Skin samples were prefixed overnight in modified Karnovsky's medium (Elias and Friend, 1975) at 4°C, washed twice with 0.2 M sodium cacodylate buffer for 10 minutes each, and postfixed with 1% (w/v) OsO_4 in 0.133 M sodium cacodylate buffer containing 0.5% (w/v) $K_4Fe(CN)_6$ at 4°C for 45 minutes. Subsequently, specimens were dehydrated in an ethanol series and embedded in Epon 812 (Luft, 1961). Polymerization was carried out overnight at 60°C. Semi-thin sections were cut on an ultra-microtome (Leica UCT, Leica Bensheim, Germany) and after staining investigated in a Zeiss Axioskop 40 (Zeiss, Göttingen, Germany) with transmission mode. For electron microscopy, ultra thin sections were cut and post-stained according to the method of Reynolds (Reynolds, 1963) and subsequently investigated in a Philips CM 10 electron microscope.

Immunohistochemistry

Skin samples were fixed in formaldehyde and embedded in paraffin. 5 μm sections were incubated with 3% H_2O_2 for 5 minutes to block endogenous peroxidase activity, rinsed, and microwave irradiated at 650 W for antigen detection according to the method of Hazelbag et al. (Hazelbag et al., 1995). After blocking unspecific antibody binding by incubation with 20% pig serum (DAKO, Germany), the primary antibodies were applied for 30 minutes at room temperature. The primary antibodies: anti-keratin K1 (1:500), anti-keratin K5 (1:1000), anti-keratin K6 (1:500), anti-involucrin (1:1000), anti-loricrin (1:500) and anti-filaggrin (1:1000) (Hohl, 1993; Rosenthal et al., 1992). All primary antibodies were purchased from Hiss Diagnostics, Germany.

A strep AB complex/HRP was used as third antibody, followed by incubation with diaminobenzidine as substrate for the peroxidase.

Results

Increased protein levels and activity of CTSD in differentiated keratinocyte cultures

To investigate the functional role of CTSD during epidermal differentiation, we first investigated the amount and enzyme activity of CTSD in primary undifferentiated keratinocytes and in differentiated, stratified keratinocyte cell cultures (Steude et al., 2002; Asselineau et al., 1986) by western blotting and a specific bioassay. In primary keratinocytes the three known isoforms of CTSD (52 kDa, 48 kDa and 32 kDa) were detected with the 48 kDa form being the main product. In 20-day-old stratified cultures, expressing various signature proteins of keratinocyte, cornification and differentiation (Steude et al., 2002), we found a significant increase of the 52 kDa and the 48 kDa CTSD forms (Fig. 1A). The 52 kDa protein represents the enzymatically inactive pre-pro CTSD form, while the 48 kDa protein is the active membrane-bound enzyme (Fujita et al., 1991).

The enzymatic activity of CTSD in primary and differentiated keratinocytes correlated with the amount of CTSD protein and was significantly enhanced in the 20-day stratified cultures as estimated by cleavage of the CTSD-specific substrate PTH 84 amino acid polypeptide (Heinrich et al., 1999) resulting in generation of the 34 amino acid fragment The amount of PTH decreased (upper part of Fig. 1B), while CTSD activity, calculated as the amount of PTH cleaved/hour, was increased in differentiated keratinocytes (Fig. 1B). These results indicate that both CTSD protein and enzymatic activity correlate with the stage of keratinocyte differentiation in vitro.

Increased epidermal expression and increased activity of CTSD after experimental injury to the skin in hairless mice

To evaluate the possible role of CTSD in the skin during epidermal differentiation in vivo, we determined the protein levels and activity of CTSD during epidermal barrier repair following experimental barrier disruption by tape-stripping. Following experimental skin injury the expression of the active, intermediate 48 kDa proenzyme (Fig. 2A, black bars) and the mature 32 kDa form (Fig. 2B, black bars) was significantly increased at 3 hours and 5 hours (+185% and +215%, $P<0.05$, $n=4$ for 48 kDa form and +204% and +260%, $P<0.05$, ($n=4$) for the 32 kDa form, respectively) as estimated by western blotting

Inhibition of CTSD by topical application of the CTSD inhibitor pepstatin A suppressed an increase in the amount of both the intermediate and the mature enzyme after barrier disruption (Fig. 2A,B, grey bars).

The increase of CTSD protein was paralleled by enhanced CTSD enzyme activity at 3 hours and 5 hours after skin injury as determined by PTH cleavage assays (Fig. 3). Topical application of pepstatin A resulted in a decrease in enzymatic activity at 6 hours after treatment (data not shown). Together, these data demonstrate increased protein expression and increased enzyme activity of CTSD during skin repair and epidermal differentiation.

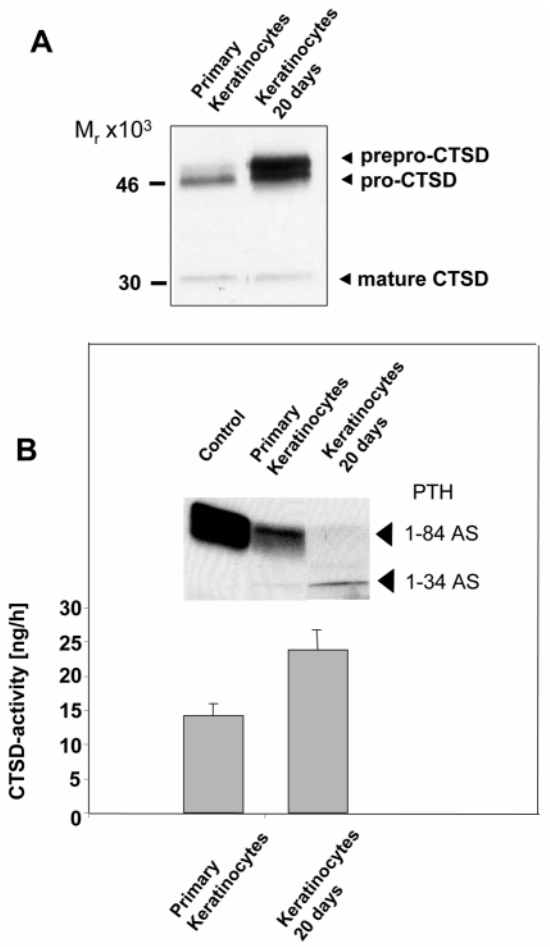

Fig. 1. Increase in protein expression and activity of CTSD in differentiated keratinocyte cultures. (A) Protein expression of CTSD isoforms in the primary and differentiated keratinocytes was determined in cell lysates by western blotting using anti-CTSD antibodies. There was an increase in the prepro and enzymatically active pro forms in differentiated keratinocytes. (B) CTSD activity was measured by an in vitro enzyme assay of keratinocyte lysates using parathyroid hormone (PTH) as a CTSD-specific substrate. The amount of PTH in the absence of sample protein was used as a control. The level of PTH protein was determined by western blotting using anti-PTH mAb (peptide 1-34) and quantified by two-dimensional laser scanning densitometry (Molecular Dynamics Personal Densitometer). CTSD activity, calculated as the amount of PTH cleaved/hour, was increased in differentiated keratinocytes.

TG1 activity is stimulated by exogenous CTSD in keratinocytes in culture

During epidermal differentiation the CE proteins involucrin, loricrin and filaggrin are cross-linked by the formation of ε-(γ-glutamyl)lysine isodipeptide bonds catalyzed by TG1 (Kim et al., 1995). In addition, it has been described that TG1 catalyzes covalent ester binding of ω-hydroxyceramide to involucrin (Nemes et al., 1999). Thus this enzyme mediates key functions in epidermal differentiation. Since CTSD may be involved in the regulation of the activity of TG1 (Negi et al., 1981; Negi et al., 1990) we investigated a possible direct involvement of CTSD in the proteolytic activation of TG1. Since the high molecular mass TG1 precursor protein is membrane bound

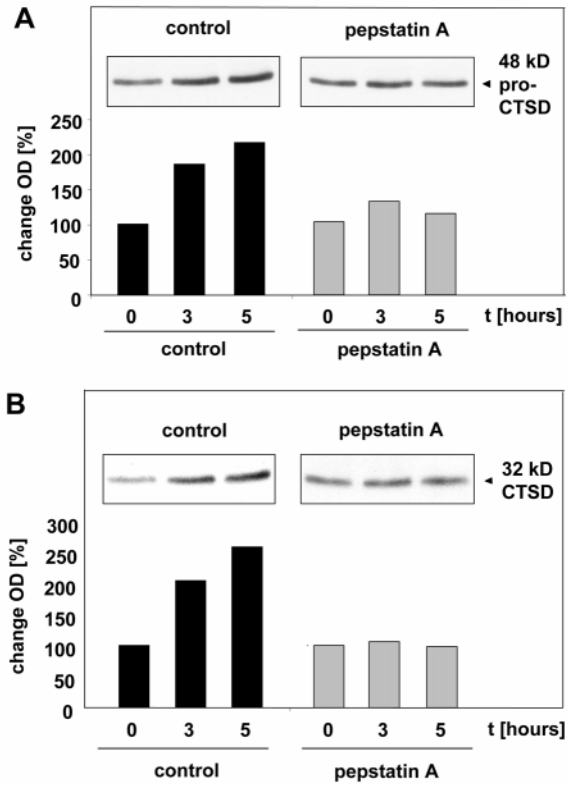

Fig. 2. Increased epidermal expression of CTSD after experimental skin injury. Acute disruption of the permeability barrier was induced by tape-stripping. Immediately, pepstatin A or the carrier solution was applied and skin samples were obtained at different times. The expression of the active, intermediate (A) and the mature form (B) of CTSD were examined by SDS-PAGE and western blotting using polyclonal anti-CTSD antibody and quantified by two-dimensional laser scanning densitometry.

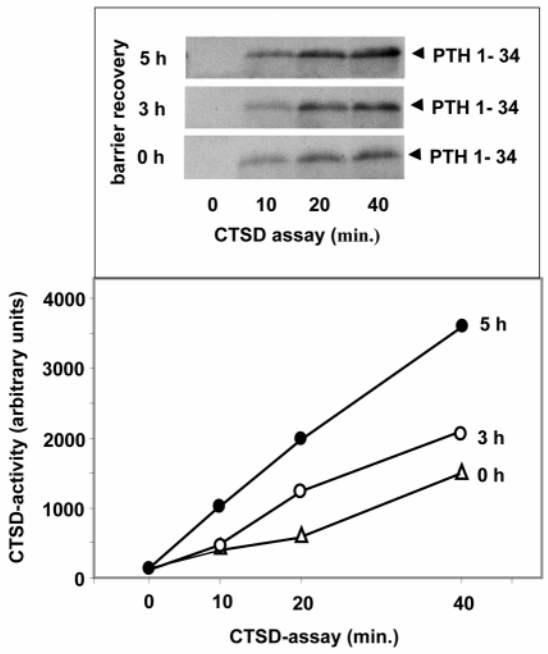

Fig. 3. Increased activity of CTSD after experimental skin injury. Acute disruption of the permeability barrier was induced by tape-stripping. Immediately after barrier disruption, pepstatin A or the carrier solution was applied and skin samples were obtained directly after tape-stripping (0 hours) or after 3 and 5 hours. CTSD activity was measured by specific parathyroid hormone (PTH) enzyme assays. The level of PTH protein was determined by western blotting using anti-PTH mAb (peptide 1-34). The amount of PTH in the assay at the starting point was used as a control and CTSD activity was calculated as the amount of PTH cleaved/hour.

(Chakravarty and Rice, 1989; Steinert et al., 1996b), and upon terminal differentiation of keratinocytes TG1 is cleaved at two sites, leading to a more active form (Rice et al., 1990; Kim et al., 1995; Steinert et al., 1996a; Steinert et al., 1996b), we prepared a membrane fraction from lysates of HaCaT cells as starting material. The enzymatic activity of TG1 was measured in the membrane preparation after addition of exogenous CTSD in the absence and presence of pepstatin A in an in vitro TG1 assay based on the cross-linking of [^{3}H]putrescine to dimethylcasein as substrate (Hohl et al., 1998). As shown in Fig. 4, TG1 activity was increased by exogenous CTSD and the enzymatic activation was blocked by pepstatin A. This result suggests a CTSD-mediated proteolytic activation of a (membrane-bound) TG1 precursor molecule leading to enzymatically active TG1 fragments. This observation supports a functional link between CTSD and TG1 activities.

Topical application of the CTSD inhibitor pepstatin A or of TG inhibitor monodansyl cadaverin significantly delayed permeability barrier repair

To investigate the physiological significance of CTSD in epidermal differentiation, we next examined barrier recovery following experimental skin injury and topical application of the CTSD inhibitor pepstatin A. At different times after barrier disruption by tape-stripping (0-24 hours), TEWL, as a marker of barrier repair, was measured (Grubauer et al., 1989). After experimental barrier disruption endogenous barrier repair commenced. A rapid decrease in TEWL leading to about 60% barrier recovery occurred in hairless mice within 5 hours. This was followed by slower kinetics of barrier recovery within the next 24 hours. Topically application of the CTSD inhibitor pepstatin A immediately after barrier disruption led to a significant delay in barrier repair at 1, 3, 5, 7 and 24 hours after treatment (Fig. 5A).

In addition, the functional role of TG1 in cutaneous differentiation and permeability barrier repair was examined by topical application of the TG1 inhibitor monodansyl cadaverin after experimental skin injury. At different times after barrier disruption by tape-stripping, TEWL was again determined as a marker of barrier repair. After application of monodansyl cadaverin, we found a significant delay in barrier repair at 1, 3, 5, 7 and 24 hours after treatment (Fig. 5B). These results show that inhibition of CTSD or TG1 activity influences epidermal differentiation and delays permeability barrier repair.

Reduced TG1 enzymatic activity and defective TG processing in CTSD deficient mice

Based on our observation that CTSD is able to activate TG1 in

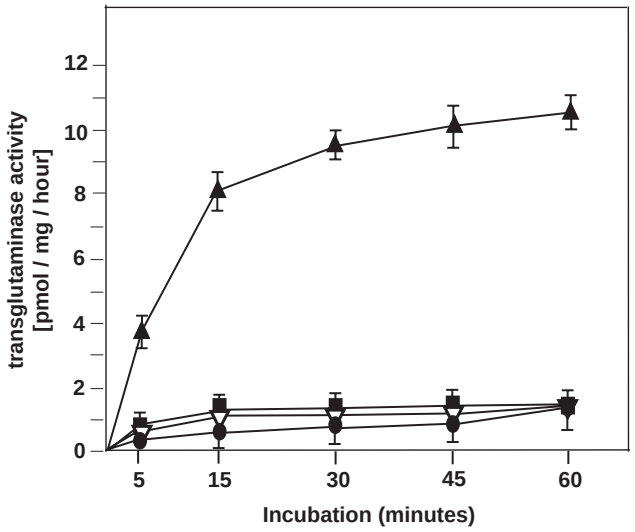

Fig. 4. CTSD stimulates transglutaminase 1 (TG1) activity in primary keratinocytes in vitro. Isolated membranes from HaCat-cells were left untreated (black squares), treated with pepstatin A (black circles), or with purified CTSD in the absence (black triangles) or presence of pepstatin A (white triangles) for 30 minutes and were subsequently measured for TG1 enzymatic activity by an in vitro enzyme assay using dimethylcasein and [1,4(n)-3H] putrescine as substrates. Results of three experiments performed in triplicate are shown (mean±s.e.m.).

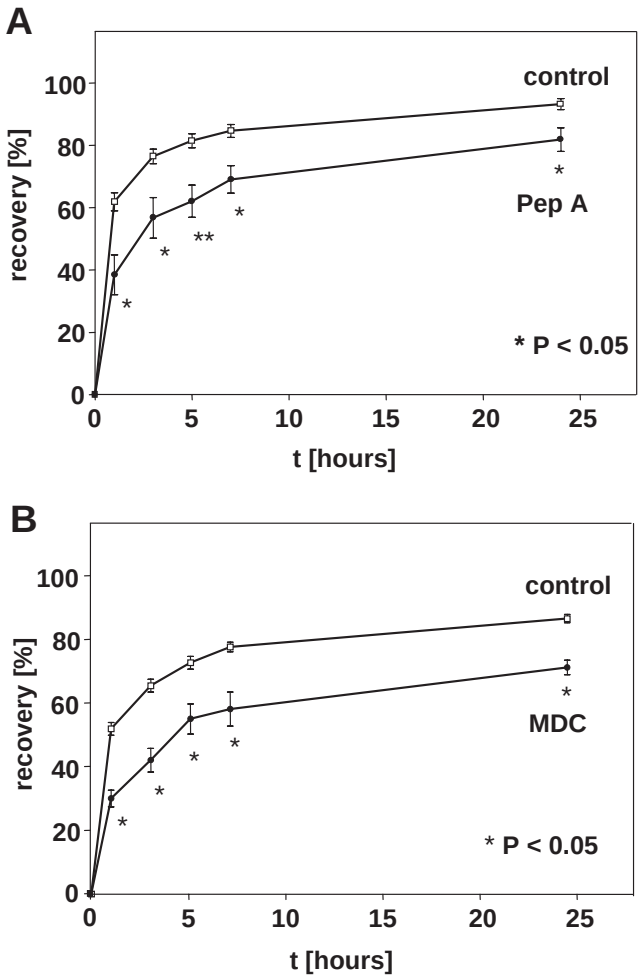

Fig. 5. Topical application of the CTSD inhibitor pepstatin A (A) and of the TG inhibitor monodansyl cadaverin (MDC; B) significantly delays permeability barrier repair. Acute disruption of the permeability barrier was induced by tape-stripping until a 20- to 30-fold increase in TEWL (transepidermal water loss/transcutaneous water loss) occurred. Immediately after barrier disruption, pepstatin A, monodansyl cadaverin or the carrier solution was applied and recovery in TEWL was determined at different times after treatment. Results of three experiments performed in triplicates are shown.

vitro (Fig. 4), we next explored the possible role of CTSD in the regulation of TG1 expression in CTSD-deficient mice in vivo. Using a specific TG1 enzyme assay, we found significantly decreased TG1 activity in the epidermis of heterozygous ($CTSD^{(+/-)}$) mice which was further reduced in homozygous ($CTSD^{(-/-)}$) mice (Fig. 6). In order to investigate whether this decreased TG1 activity in $CTSD^{(-/-)}$ mice is caused by a defective processing of a TG1 precursor molecule, we analyzed the distribution of TG1 protein by western blotting using a specific anti-TG1 antibody. A strongly band of approximately 35 kDa was detected in the epidermis from wild-type mice, but this band was significantly decreased in the skins of CTSD-deficient heterozygous and even more in homozygous mice (Fig. 7). In $CTSD^{(-/-)}$ mice, a 150 kDa protein was strongly expressed instead, which was also seen in $CTSD^{(+/-)}$ mice, but completely absent in wild-type mice. These finding suggest a defective processing of a 150 kDa TG1 precursor protein in the epidermis of CTSD-deficient mice and points to a functional role of CTSD in the maturation of a 150 kDa TG1 precursor to an enzymatic active 35 kDa form in vivo.

Reduced levels of involucrin and loricrin in CTSD deficient mice

We next investigated whether CTSD deficiency also results in changes in the expression of involucrin as an early marker, and loricrin as a late marker, of epidermal differentiation (Watt, 1983; Yoneda et al., 1992; Steinert and Marekov, 1997). In epidermal samples from wild-type mice, involucrin (Fig. 8A) and loricrin (Fig. 8B) were expressed as 65 kDa and 50 kDa proteins, respectively, as estimated by western blotting. In $CTSD^{(+/-)}$ mice, the levels of these proteins were clearly reduced and in $CTSD^{(-/-)}$ mice completely absent, suggesting a crucial function of CTSD for the appearance of 65 kDa involucrin and 50 kDa loricrin in the epidermis.

Changes in the immunohistology of differentiation-related proteins in $CTSD^{(-/-)}$ mice

To examine the protein expression and localization of differentiation-related epidermal proteins, we performed immunohistology using specific antibodies. As shown in Fig. 9 keratin K1 staining in healthy skin is only found in suprabasal layers of the epidermis, whereas keratin K5 is only expressed in epidermal basal cells. In $CTSD^{(-/-)}$ mice there is a focal extension of K1 staining to the basal layer. Staining for K5 is focally extended to the upper epidermal layers in $CTSD^{(+/-)}$ mice and the entire nucleated epidermis is stained in $CTSD^{(-/-)}$ mice. Also, the thickness of the epidermis (stratum

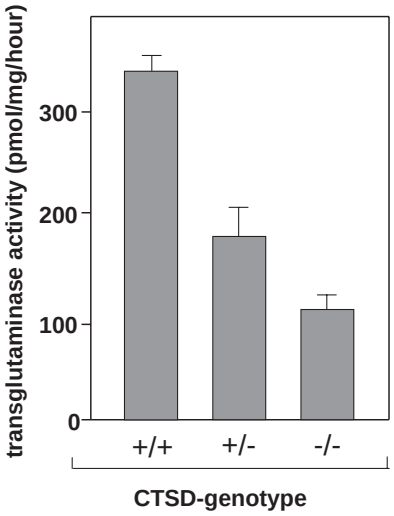

Fig. 6. Reduced transglutaminase 1 (TG1) activity in CTSD-deficient mice. TG1 enzymatic activity was determined in epidermal lysates from wild type, heterozygous and CTSD-deficient mice by an in-vitro assay using dimethylcasein and [1,4(n)-3H] putrescine as substrates. Results of three experiments performed in triplicate are shown (mean±s.e.m.).

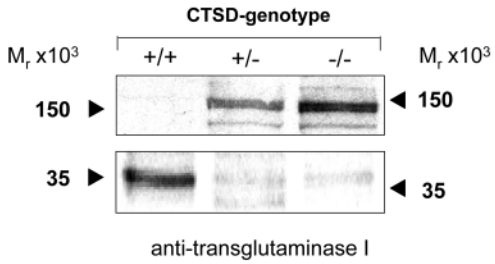

Fig. 7. Absence of 35 kDa TG1 protein in CTSD-deficient mice. TG1 protein levels were determined in epidermal samples from wild-type, heterozygous and homozygous CTSD-deficient mice by western blotting using an anti-TG1 antibody and quantified by densitometry.

granulosum, stratum spinosum and stratum basale) was reduced, whereas, a thickening of the stratum corneum (hyperkeratosis) was evident in CTSD$^{(-/-)}$ mice. These results show changes in the protein expression of basal and differentiation related keratins in CTSD-deficient mice.

Keratin K6 is known to be involved in proliferation and shows faint, probably unspecific, staining in normal mouse skin. No staining was found in heterozygous or homozygous mouse skin. This reveals that the abnormal cornification as seen by light microscopy in CTSD$^{(-/-)}$ mice is not related to hyperproliferation.

The involucrin antibody showed strong continuous staining of the upper stratum spinosum and the stratum granulosum in wild-type mice. In CTSD$^{(+/-)}$ and CTSD$^{(-/-)}$ mice involucrin staining was markedly reduced, the band-like staining was locally interrupted.

The loricrin antibody produced strong staining of the stratum granulosum. In CTSD$^{(+/-)}$ and in CTSD$^{(-/-)}$ mice we found a focally reduced staining.

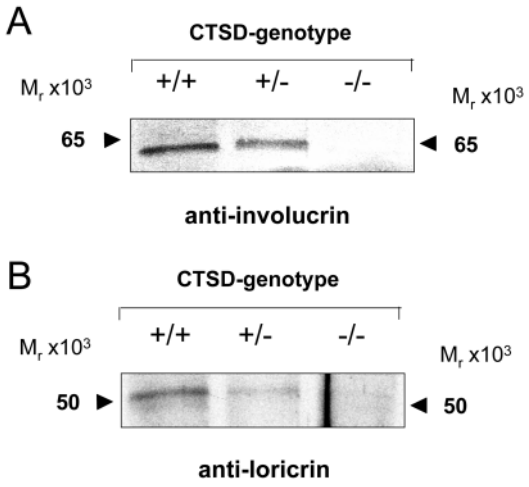

Fig. 8. Reduced CE protein expression in CTSD-deficient mice. Skin sections from wild-type, heterozygous and homozygous CTSD-deficient mice were obtained. Expression of involucrin (A) and loricrin (B) were determined in the epidermal samples by western blotting using anti-involucrin and anti-loricrin antibodies.

Filaggrin, similar to involucrin, stained strongly in the upper stratum spinosum and the stratum granulosum in wild-type mice. A slight reduction in the staining intensity was found in CTSD$^{(+/-)}$ mice. Staining intensity was clearly reduced and staining was focally absent in CTSD$^{(-/-)}$ mice. This reveals reduced expression of CE proteins.

These studies show a functional link between CTSD activity and expression of epidermal differentiation-related proteins.

Structural changes in the stratum corneum and the transition of stratum granulosum to stratum corneum and changes in TEWL in CTSD deficient mice

The biological consequences of CTSD deficiency, reduction of TG1 activity and alterations in CE protein levels were analyzed by histological examination of semi-thin skin sections derived from wild-type and CTSD$^{(-/-)}$ mice. The epidermis of wild-type (and heterozygous mice, data not shown) exhibited the well known regular arrangement of corneocytes in the stratum corneum. In contrast, the stratum corneum of the CTSD$^{(-/-)}$ mice was irregular in structure. The different layers of the stratum corneum were disrupted and the singular corneocytes were undulated. Furthermore, there were more stratum corneum layers (Fig. 10). Part of these changes are also evident in Fig. 9. The ultrastructure of the epidermis was analyzed by electron microscopy, revealing distinct changes in the morphology of the stratum corneum and in the transition of stratum granulosum to stratum corneum in CTSD$^{(-/-)}$ mice. In wild-type mice we found normal distances between the stratum corneum layers and a normal CE as seen by the dark lines around the corneocytes (Fig. 11A, arrow). In CTSD$^{(-/-)}$ mice, the distances between the stratum corneum layers are broader, with only a faint staining of the CE and thickened corneocytes in the axial direction (Fig. 11B, arrow). Measurements of the TEWL under basal conditions in mice at the age of 20 days (the CTSD$^{(-/-)}$ mice have a life expectancy of only 28 days) with the Tewameter® revealed a small increase (not significant)

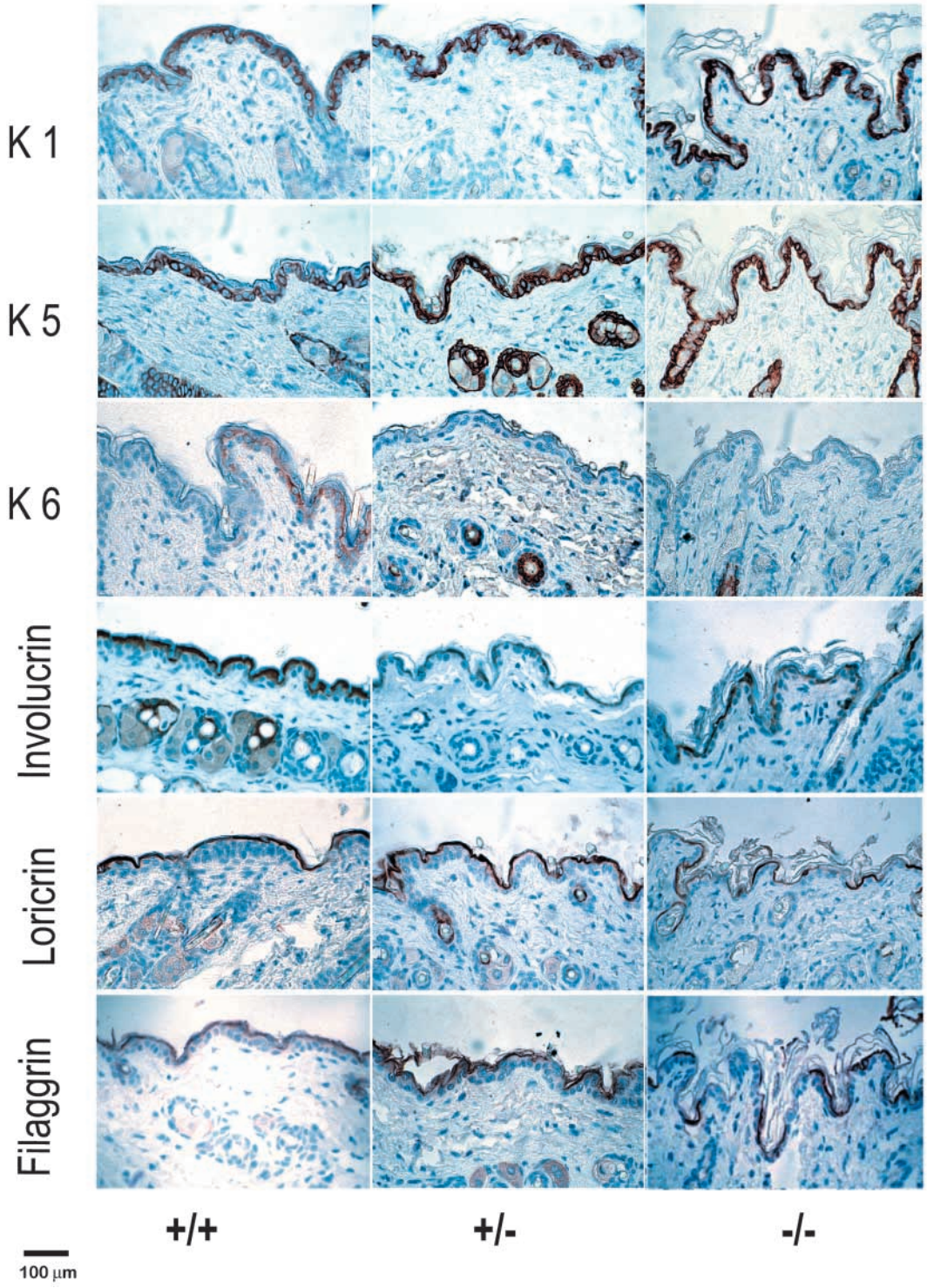

Fig. 9. Immunohistology revealed distinct changes in the expression of keratins and CE proteins in CTSD-deficient mice. Keratin K1 staining in healthy skin is only found in suprabasal layers of the epidermis, whereas keratin K5 is only expressed in epidermal basal cells. In CTSD$^{(-/-)}$ mice there is a focal extension of K1 staining to the basal layer and a focal extension of K5 to the upper epidermal layers. Keratin K6 was faintly stained in normal mouse skin, but not in CTSD$^{(+/-)}$ or CTSD$^{(-/-)}$ mouse skin. Involucrin and filaggrin antibodies showed strong staining of the upper stratum spinosum and the stratum granulosum, whereas the loricrin antibody showed staining of the stratum granulosum solely in wild-type mouse skin. For all three antibodies staining intensity was reduced in CTSD$^{(+/-)}$ mice and even more reduced in CTSD$^{(-/-)}$ mice.

in TEWL in CTSD$^{(+/-)}$ mice (+17%) and CTSD$^{(-/-)}$ mice (+11%): wild-type mice – TEWL 10.9±1.7 g/m^2/h, n=13; CTSD$^{(+/-)}$ – TEWL 12.7±1.2 g/m^2/h, n=10; CTSD$^{(-/-)}$ – TEWL 12.2±1.7 g/m^2/h, n=6. Together, these results show an increase in the thickness and number of stratum corneum layers with ultrastructural changes in CTSD$^{(-/-)}$ mice. The ichthyotic skin phenotype in CTSD$^{(-/-)}$ mice largely compensates for the defect in protein expression, shown by a small increase in basal TEWL, only.

Discussion

Recently, we demonstrated the activation of a TNF signal transduction pathway including TNF-R55, acid sphingomyelinase and the ‘second messenger’ ceramide during skin permeability barrier repair (Jensen et al., 1999). TNF and acid sphingomyelinase are involved in cell signaling for growth, differentiation and apoptosis (Aggarwal and Natarajan, 1996). In vitro, we identified the endolysosomal aspartate protease CTSD as a specific ceramide-binding protein.

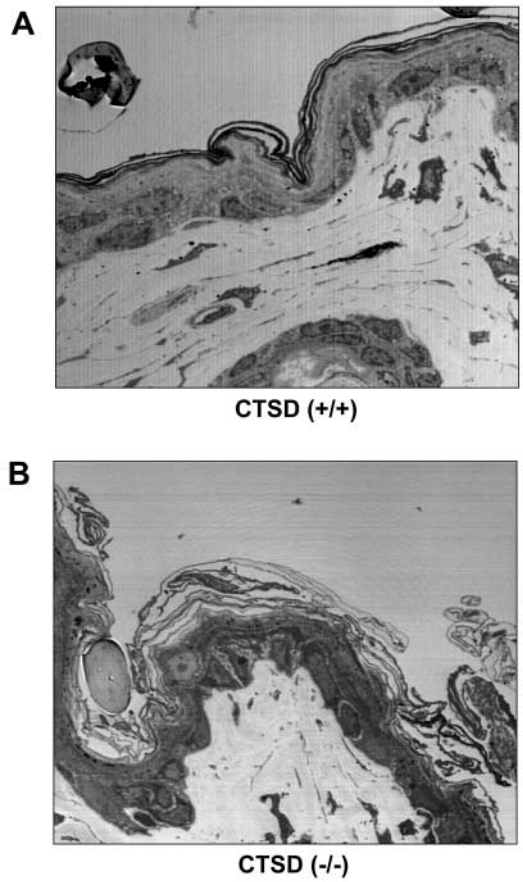

Fig. 10. CTSD-deficient mice exhibited impaired stratum corneum morphology. Microscopic analysis of semi-thin skin sections from CTSD wild-type mice (A) revealed normal stratum corneum morphology. CTSD-deficient mice (B) have a disrupted stratum corneum and an increased number of corneocyte layers.

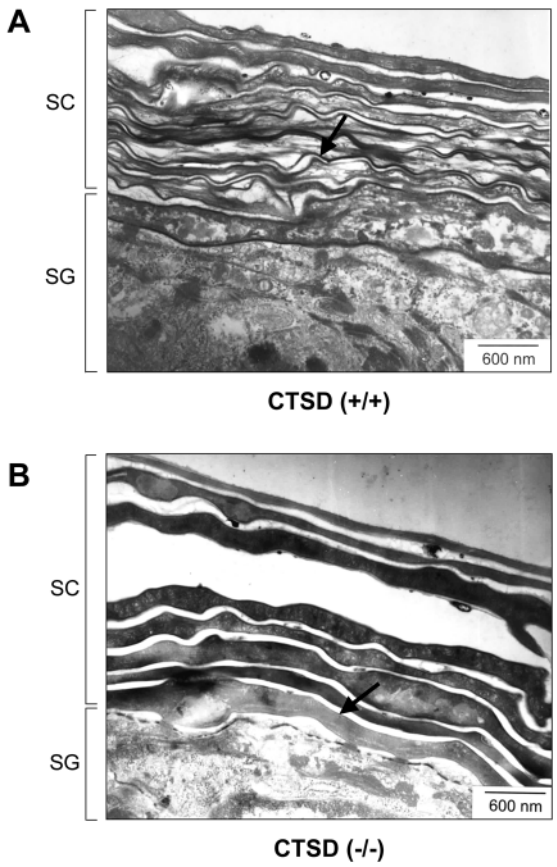

Fig. 11. Ultrastructural changes of the stratum corneum and the transition of stratum granulosum to stratum corneum in CTSD$^{(-/-)}$ mice. Electron microscopy shows that in wild-type mice (A) the cornified envelope (CE) is clearly visible as dark lines around the corneocytes (arrow). In CTSD$^{(-/-)}$ mice (B) there is a broadening of the intercellular spaces in the stratum corneum (SC), only a faint staining of the CE (arrow) and the corneocytes are thickened in the axial direction. SG, stratum granulosum.

Ceramide enhances CTSD proteolytic activity (Heinrich et al., 1999). The existence of CTSD in the skin was shown previously, but the function has not been elucidated. Increased activity of CTSD isoforms depending on the stage of epidermal differentiation has been described (Horikoshi et al., 1999). Also, an increased expression of the mature form of CTSD has been reported (Kawada et al., 1997) in psoriasis, a disease that is characterized, in addition to inflammation, by epidermal hyperproliferation and altered differentiation. Furthermore, in psoriasis premature expression of the CE protein involucrin is known (Thewes et al., 1991).

We examined the role and specific targets of CTSD in epidermal differentiation. First, we performed in vitro studies determining the protein expression and activity of CTSD in primary and in differentiated, stratified (organotypic) cultured keratinocytes. We found a significant increase in CTSD protein levels and an increase in the enzymatic activity of CTSD in differentiated compared to primary keratinocytes, suggesting a function of CTSD during epidermal differentiation in vitro.

To explore a possible link between CTSD and epidermal differentiation in vivo, we investigated the epidermal expression and the enzymatic activity of CTSD after experimental skin injury during permeability barrier repair in wild-type mice. We found significantly increased epidermal expression of the active intermediate as well as the mature form of CTSD 3 hours and 5 hours after permeability barrier disruption, caused by increased processing and increased synthesis of the enzyme. In accordance, we noted a significant increase of epidermal CTSD enzyme activity at different times after skin injury. Topical application of pepstatin A, an inhibitor of CTSD (Heinrich et al., 1999), prevented an increase in the protein expression and in the activity of CTSD and significantly delayed permeability barrier repair after experimental disruption. These results clearly show involvement of CTSD in the epidermal repair process after injury.

The kinetics of CTSD processing and activation, as demonstrated by increased expression at 1-5 hours after treatment, corresponded to the activation of sphingomyelinase and the amount of epidermal ceramides after barrier perturbation (Jensen et al., 1999). In our previous study, a significant increase in acid sphingomyelinase activation and an elevated epidermal ceramide content 1-4 hours after barrier disruption was demonstrated (Jensen et al., 1999), thus acid

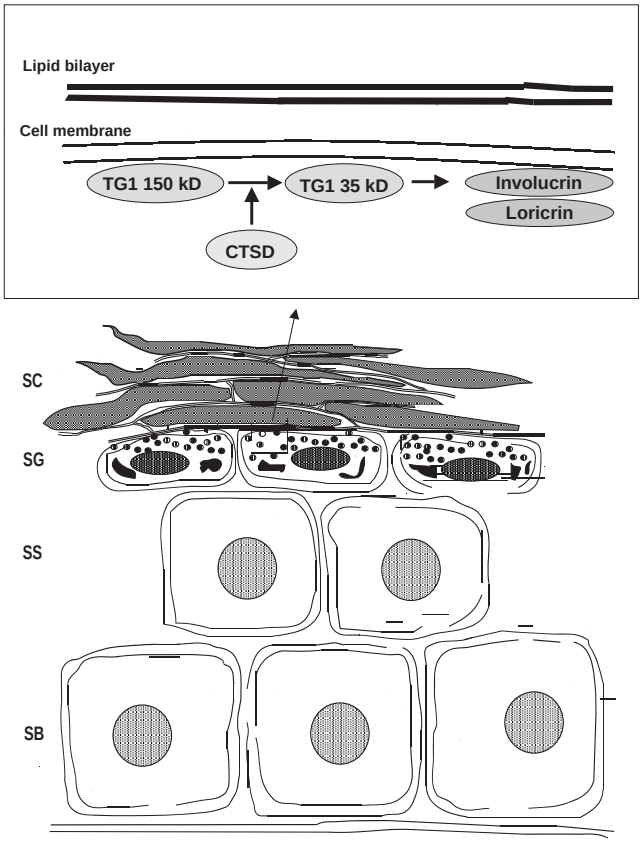

Fig. 12. Model of the role of CTSD in epidermal differentiation. During epidermal differentiation, the aspartate protease CTSD in the stratum granulosum (SG) is activated, cleaves the membrane-bound 150 kDa precursor of transglutaminase 1 (TG1) producing the active 35 kDa form. TG1 in turn mediates cross-linking of the cornified envelope (CE) proteins involucrin and loricrin to the CE. SC, stratum corneum; SS, stratum spinosum; SB, stratum basale.

sphingomyelinase-derived ceramide may be linked to CTSD activity during permeability barrier repair.

The epidermis follows a programmed transformation of keratinocytes from the proliferating basal cells to the spinous, then to the granular and to the horny layers where the permeability barrier resides and finally desquamation occurs. Starting in the granular layer, aspartatic proteinases such as CTSD are activated in the lysosomes where they participate in the massive degradation process accompanying cornification. Recent observations identified CTSD to be located in lamellar bodies within the keratinocytes (Ishida-Yamamoto et al., 2004). Involucrin and loricrin are cross-linked by TG1 to form the insoluble and rigid CE (Steinert and Marekov, 1997). CTSD was reported to activate TG1 and thereby contribute to the enzymatic processes during cornification (Negi et al., 1981; Horikoshi et al., 1999). These findings were supported and extended by our in vitro and in vivo results: we found CTSD-dependent activation of TG1 in a keratinocyte membrane preparation and, furthermore, the activity of TG1 was severely diminished in the epidermis of CTSD$^{(-/-)}$ mice when compared to wild-type mice. The enzymatic activity of TG1 correlated with the expression of a 35 kDa protein in wild-type mice, as detected by immunoblotting. This 35 kDa protein was undetectable in the epidermis of CTSD$^{(-/-)}$ mice, where we observed the expression of a 150 kDa TG1 protein instead. There was less of this protein in CTSD$^{(+/-)}$ mice and none in wild-type control mice. These observations suggest the involvement of CTSD in the processing of a higher molecular weight precursor to generate an enzymatically active 35 kDa TG1 form (see model in Fig. 12). A recent report by Iizuka et al. (Iizuka et al., 2003) studied proteolytically activated TG1 in the epidermis by using cleavage-site-directed antibodies. A 33 kDa fragment was identified by western blotting that was mainly found in the cytosol of keratinocytes, in differentiated cells and in the stratum corneum of the skin (Iizuka et al., 2003). A second fragment resided at the plasma membrane of keratinocytes and in regions of the skin including suprabasal layer, spinous layer and granular layer, but not the stratum corneum. The differentiation-related 33 kDa TG1 fragment of this study could be the same protein as the 35 kDa fragment we detected in CTSD$^{(+/+)}$ mice, but not in CTSD$^{(-/-)}$ mice.

The functional significance of TG1 for epidermal homeostasis was demonstrated by a delay in permeability barrier repair after inhibition of TG1 by monodansyl cadaverin. Similar effects were observed after inhibition of CTSD by pepstatin A or inhibition of acid sphingomyelinase by imipramine (Jensen et al., 1999), suggesting the importance and possible associated function of all three enzymes for barrier formation and differentiation.

Epidermal differentiation including the formation of the CE proteins involucrin and loricrin, are crucially involved in permeability barrier repair (Ekanayake-Mudiyanselage et al., 1998). The soluble CE protein involucrin is expressed in the spinous layer at an early stage in keratinocyte differentiation. Loricrin is an insoluble CE precursor, expressed later in the differentiation process in intracellular granules.

The link between CTSD-dependent activation of keratinocyte TG1 and the expression of CE proteins was confirmed in CTSD$^{(-/-)}$ mice. In these mice, we found significantly reduced involucrin and loricrin protein levels after experimental skin injury in parallel to a reduced activity of TG1. These results are in agreement with the effects observed after application of the CTSD inhibitor pepstatin A (data not shown). Since a high molecular mass TG1 precursor protein was present in CTSD$^{(-/-)}$ but not in wild-type mice, CTSD appears to mediate the proteolysis of the enzymatically inactive TG1 to the active enzyme. Thus, the deficient mice also reveal a functional link between CTSD expression, maturation and activation of TG1, and the appearance of involucrin and loricrin in the epidermis.

Morphologically, the CTSD$^{(-/-)}$ mice exhibited epidermal hyperkeratosis as a sign of disturbed epidermal differentiation. The skin symptoms may be explained by a diminished ability of the corneocytes to bind intercellular lipids, caused by the reduced expression of involucrin and loricrin in the stratum corneum. Previously, it was shown that the CE proteins, in particular involucrin, covalently bind ω-hydroxy ceramides. These ceramides form a scaffold for the attachment of free ceramides, cholesterol and free fatty acids that provides stratum corneum lipid bilayers for the permeability barrier function (Downing, 1992; Steinert and Marekov, 1997). Morphological disruption of the corneocyte layers was found in the CTSD$^{(-/-)}$ mice (Fig. 10B). These disruptions started at the interface of the stratum granulosum/stratum corneum, the

place where normally the lipid bilayers extrude their lipids into the intercellular space. Ultrastructural analysis of the epidermis in CTSD$^{(-/-)}$ mice revealed broadening of the distances between the SC layers, with only a faint staining of the CE and thickened corneocytes in the axial direction. This demonstrates the influence of CTSD on corneocytes and possibly on lipid organization in the stratum corneum. Together, the morphological findings suggest an important role for CTSD in epidermal differentiation.

To evaluate the consequences of CTSD deficiency and reduced TG1 activity in more detail, we analyzed the distribution of various epidermal keratins and CE proteins by immunohistology. An extended expression of basal and differentiation-related keratins K5 and K1 as a sign of a disturbed differentiation was found in CTSD-deficient mice. In previous studies we have found that expression of the differentiation marker K1 is disturbed after acute (tape-stripping) or chronic (metabolic, essential fatty acid deficient diet, EFAD) barrier disruption. However, in these models we also found an increase in the expression of the proliferation-associated keratin K6 (and also K16) (Ekanayake-Mudiyanselage et al., 1998). Expression of K6 (besides changes in basal and differentiation related keratins) is also known in psoriasis (Hagemann and Proksch, 1996). In CTSD$^{(-/-)}$ mice K6 was absent, though a thickening of the horny layer was clearly visible. Therefore, the CTSD$^{(-/-)}$ mice may have similarities to so called 'retention hyperkeratosis', such as ichthyosis, and not to so called 'hyperproliferation-associated keratosis', such as psoriasis. In accordance, mice heterozygous for a truncated keratin K10, as a model for the ichthyosis called epidermolytic hyperkeratosis, which is caused by point mutations in the suprabasal K1 or K10 (Cheng et al., 1992; Chipev et al., 1992; Rothnagel et al., 1992; Yang et al., 1997; Suga et al., 1998; Ishida-Yamamoto et al., 2000), did not show K6/K16 protein expression in flank skin (Porter et al., 1998). Ichthyosis is a group of skin disorders with different causes, showing disturbed epidermal differentiation with variable effects on the permeability barrier (Lavrijsen et al., 1993).

The expression of involucrin and loricrin, as well as filaggrin, were significantly reduced in heterozygote and homozygote epidermis in the CTSD-deficient mice. Filaggrin deficiency is well known in ichthyosis vulgaris (Gunzel et al., 1991). Defective interaction between keratin and filaggrin is also seen in epidermolytic hyperkeratosis. Abnormal distribution of loricrin has been detected in genetic diseases involving loricrin (loricrin keratoderma, Vohwinkel's syndrome) (Maestrini et al., 1996; Suga et al., 2000). Loricrin mutation in Vohwinkel's keratoderma is unique to the variant with ichthyosis (Korge et al., 1997). Defects in cross-linking of loricrin are detected in TG1-deficient mice, the animal model of lamellar ichthyosis (Matsuki et al., 1998).

Evaluation of the barrier function in our CTSD$^{(-/-)}$-mice by measurements of TEWL revealed no constitutive defect. We suggest that development of ichthyosiform skin compensates for defective stratum corneum morphology and deficiency in differentiation-related proteins. Recently, similar results have been reported in mice lacking TG1 (Matsuki et al., 1998). Skin from TG1-deficient mice, which die as neonates, lacks the normal insoluble CE and has impaired barrier function. Neonatal TG1$^{(-/-)}$ mouse skin was taut and erythrodermic, but transplanted TG1$^{(-/-)}$ mouse skin resembled that seen in severe ichthyosis, with epidermal hyperplasia and marked hyperkeratosis. Abnormalities in those barrier structures remained, but TEWL was decreased to control levels in the ichthyosiform skin. The authors suggested that the ichthyosiform skin phenotype in TG1 deficiency develops massive hyperkeratosis as a physical compensation for the defective cutaneous permeability barrier (Kuramoto et al., 2002). A compensatory mechanisms maintaining skin barrier function in the absence of a major CE protein was also described in a loricrin-deficient mouse model (Koch et al., 2000). Also, targeted ablation of the murine involucrin gene did not show defects in barrier function. These mice developed normally, possessed apparently normal epidermis and hair follicles and generated CEs that could not be distinguished from those of wild-type mice (Djian et al., 2000; Jensen et al., 1999b). The complexity and redundancy of epithelial barrier function has been discussed by Steinert (Steinert, 2000). Our CTSD$^{(-/-)}$ mouse model has a broader impact on skin morphology than either involucrin or loricrin deficiency. Notably, there are similarities between the skin of CTSD$^{(-/-)}$ mice, the skin of TG1$^{(-/-)}$ mice and the human skin disease lamellar ichthyosis. In lamellar ichthyosis a mutation in the TG1 gene resulting in a reduction in epidermal involucrin was described (Hohl et al., 1993; Huber et al., 1995). The similarities are explained by the strongly reduced TG1 expression in the skin of CTSD$^{(-/-)}$ mice.

In the initial characterization of the CTSD-deficient mice atopic changes of the ileal mucosa leading to an insufficient mucosal barrier were observed. The limit between epithelium and central connective tissue normally formed by a basement membrane was undetectable in these mice (Saftig et al., 1995). Together, these results prove the importance of CTSD for barrier function and for epithelial differentiation in different organs.

In summary, our in vitro and in vivo results suggest a crucial involvement of the aspartate protease CTSD in the activation of keratinocyte TG1 and in the regulation of the CE protein expression during epidermal differentiation, which is summarized in a model shown in Fig. 12. Our findings may be important for the development of new treatment modalities in skin diseases with an altered epidermal differentiation pattern.

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