Evidence that unrestricted legumain activity is involved in disturbed epidermal cornification in cystatin M/E deficient mice

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Homozygosity for Cst6 null alleles causes the phenotype of the ichq mouse, which is a model for human harlequin ichthyosis (OMIM 242500), a genetically heterogeneous group of keratinization disorders. Here we report evidence for the mechanism by which deficiency of the cysteine protease inhibitor cystatin M/E (the Cst6 gene product) leads to disturbed cornification, impaired barrier function and dehydration. Absence of cystatin M/E causes unrestricted activity of its target protease legumain in hair follicles and epidermis, which is the exact location where cystatin M/E is normally expressed. Analysis of stratum corneum proteins revealed a strong decrease of soluble loricrin monomers in skin extracts of ichq mice, although normal levels of loricrin were present in the stratum granulosum and stratum corneum of ichq mice, as shown by immunohistochemistry. This suggested a premature or enhanced crosslinking of loricrin monomers in ichq mice by transglutaminase 3 (TGase 3). In these mice, we indeed found strongly increased levels of TGase 3 that was processed into its activated 30 and 47 kDa subunits, compared to wild-type mice. This study shows that cystatin M/E and legumain form a functional dyad in epidermis in vivo. Disturbance of this protease–antiprotease balance causes increased enzyme activity of TGase 3 that could explain the observed abnormal cornification.

INTRODUCTION

Over the past decade, significant progress has been made in our understanding of the molecular basis of disorders of epidermal differentiation (1–4). A variety of genes have been identified that underlie inherited forms of ichthyosis, keratoderma and skin fragility. The encoded proteins have diverse cellular functions, ranging from structural properties (loricrin, keratins), cell–cell adhesion (desmoplakin, desmoglein-1, plakophilin, plakoglobin), lipid metabolism and/or trafficking (steroid sulfatase, fatty aldehyde dehydrogenase, lipoxigenases, ABCA12), regulation of post-translational protein processing (transglutaminase 1, cathepsin C, LEKT1), intercellular communication (connexins) and calcium signaling (ATP2C1, ATP2A2). Despite these advances, a number of genodermatoses characterized by disturbed cornification remain unexplained at the molecular level. These include, for example, forms of lamellar ichthyosis that are not caused by known mutations, ichthyosis vulgaris (OMIM 146700) and harlequin ichthyosis (OMIM 242500). Harlequin ichthyosis (HI) is a severe congenital skin disorder usually leading to a stillborn fetus or early neonatal death. Its clinical features at birth include ectropion, eclabium, ear dysmorphology and a thickened fissured epidermis (5). We have recently demonstrated that the phenotype of the ichq mouse, a model for human HI, is caused by a null mutation in the Cst6 gene leading to deficiency for the epidermal cysteine protease inhibitor cystatin M/E (6). The ichq mice phenotype has morphological and

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biochemical similarities to human HI, which includes excessive epidermal and follicular hyperkeratosis, abnormal large mitochondria, and a characteristic keratin expression pattern in the interfollicular epidermis. Although we have so far excluded CST6 mutations in humans as a major cause of harlequin ichthyosis type 2, CST6 and genes that have a role in biological pathways that are controlled by cystatin M/E, should be considered as candidate genes for human disorders of cornification of unknown etiology (7). The function of cystatin M/E in normal epidermal homeostasis is unknown and the mechanism that leads to the observed pathology in ichq mice remains unresolved. Clearly, the identification of its target protease(s) \textit{in vivo} would be an essential step in answering these questions.

A biochemical study on the novel asparaginyl endopeptidase legumain has indicated that cystatin M/E \textit{in vitro} binds to this protease with high affinity and could possibly represent a physiological target enzyme, although no biological evidence has been provided so far (8). Legumain or asparaginyl endopeptidase (AEP, EC 3.4.22.34) belongs to clan CD of the cysteine proteases and has a strict specificity for hydrolysis of asparaginyl bonds. Initially, the enzyme was purified as a cysteine protease responsible for the maturation of seed storage proteins and designated vacuolar processing enzyme (VPE) (9). Subsequently, legumain has been described in mammals (10); it is found at low levels in many tissues, but is particularly abundant in kidney. Very recently, the generation of legumain deficient mice has been reported (11). Limited functional studies have shown disturbed biosynthetic processing of cathepsins and accumulation of macromolecules in the lysosomes in the proximal tubule cells of the kidney.

In the present study, we have investigated the mechanism by which absence of cystatin M/E causes epidermal abnormalities in ichq mice. We report that legumain is a physiologically relevant target protease of cystatin M/E \textit{in vivo}. Cystatin M/E deficiency in ichq mice leads to free cutaneous legumain activity at exactly the location where cystatin M/E is normally present in wild-type mice. These mice show increased transepidermal water loss and neonatal death, probably due to dehydration. We provide evidence that disturbed cornification is caused by abnormalities in loricrin processing, caused by uncontrolled legumain activity.

RESULTS

Skin barrier function is severely disturbed in cystatin M/E deficient mice

Cystatin M/E deficient mice have defects in epidermal cornification and die between 5 and 12 days of age (6,12). Autopsy on neonatal ichq mice strongly suggested that these mice died of dehydration. This observation and the gross abnormalities in the stratum corneum prompted us to measure the rate of transepidermal water loss (TEWL) of ichq mice, compared to phenotypically normal littermates. As shown in Figure 1 (left hand y-axis), we found increased TEWL levels (up to \(\sim 3\)-fold) from day 9 onwards. Figure 1 (right hand y-axis) also shows progressive weight loss in the cystatin M/E deficient mice starting at the time point that transepidermal water loss was apparent. At day 11, body weights were \(\sim 50\%\) compared to normal littermates and most mice died shortly thereafter.

Legumain is expressed in epidermis and hair follicles

The antiprotease activity of cystatin M/E has been investigated previously (8,13–15). Cystatin M/E inhibits the asparaginyl endoprotease legumain with a high affinity (Fig. 2A). In addition to cathepsin B tested by others, we examined the inhibition of cathepsins C, H, L and S. None of these proteases was appreciably inhibited by cystatin M/E (Fig. 2B). These data indicate that legumain is a likely physiological target of cystatin M/E in skin \textit{in vivo}, although nothing is known so far on the presence of legumain in cutaneous tissues. Using anti-legumain antibodies for immunohistochemical analysis of mouse tissues we found legumain expression in the entire epidermis of adult, wild-type mice (Fig. 3A) and in the inner root sheet of the hair follicle (Fig. 3B), most abundantly at the junction of dermis and subcutaneous fat (Fig. 3C). In addition to the known presence in kidney, legumain expression was further detected in the ciliated epithelium of the trachea and in bronchial epithelium (Fig. 3D and E). Immunohistochemical staining of dorsal skin in cystatin M/E deficient ichq mice revealed normal epidermal legumain expression (Fig. 3F). These results demonstrate that legumain is indeed expressed in skin and hair follicles, as well as in other mouse tissues, some of which were previously shown to express cystatin M/E (6). These findings were confirmed at the mRNA level in mouse and human skin by reverse transcriptase PCR analysis, and subsequent sequencing of the PCR product (data not shown).

Free \textit{in situ} legumain activity in skin of cystatin M/E deficient mice but not in wild-type mice

Immunohistochemical localization of legumain cannot be equated with legumain activity because legumain can be present in an inactivezymogen form or complexed with endogenous inhibitors. In order to demonstrate legumain enzymatic activity in tissue sections, we developed a cytochemical assay, based on hydrolysis of a specific fluorescent substrate that could be precipitated by nitrosalicylicdehyde \textit{in situ}. As it was known from literature that kidney is a rich source of free legumain (as measured by biochemical assays) we used this tissue as a positive control. Enzymatic legumain activity is detected \textit{in situ} as a green fluorescent precipitate in the proximal tubulus epithelial cells of pig kidney (Fig. 4A). Specificity of this reaction was confirmed using Cbz-Ala-Ala-Aasn-EPCKOEt, which completely blocks proteolysis of the substrate (Fig. 4B). This synthetic inhibitor is highly specific for legumain (16) and shows similar affinity and specificity as the natural inhibitor cystatin M/E. For comparison, immunohistochemical staining of pig kidney for legumain shows the presence of legumain in epithelial cells of the proximal tubuli, whereas staining of the glomeruli was weak to absent (Fig. 4C); this is in accordance with the immunohistochemical localization of legumain in rat kidney reported by others (17), and it shows colocalization with enzymatically active legumain as demonstrated in Figure 4A. We subsequently used the cytochemical legumain assay to examine free legumain activity in the skin of wild-type and cystatin M/E deficient mice.
Figure 4D visualizes free legumain activity in the stratum granulosum and in the hair follicles at the level of the infundibulum in the cystatin M/E deficient mice. No legumain activity could be demonstrated in the skin of wild type littermates (Fig. 4E), which indicates that the presence of cystatin M/E normally regulates legumain activity in skin in vivo. For comparison, immunohistochemical cystatin M/E staining of dorsal skin is shown in wild-type and cystatin M/E deficient mice. This confirms the absence of cystatin M/E expression at the protein level in the ichq mice (Fig. 4F), whereas the wild-type skin shows cystatin M/E expression in the infundibular epithelium of the hair follicle and the stratum granulosum of the interfollicular epidermis (Fig. 4G). The localization of free legumain activity in the infundibular part of the hair follicle in cystatin M/E deficient mice coincides with the reported localization of tissue pathology in these mice such as excessive cornification (6,12), which leads to plugging of the hair follicle and ichthyosis as shown before.

Absence of loricrin monomers in cystatin M/E deficient mice

Abnormal cornification in ichq mice could be the result of abnormal expression or processing of structural components of the cornified envelope. In previous studies, no abnormalities were found in structural proteins such as cytokeratins 1 and 14, involucrin and filaggrin (18). We now analyzed extracts of ichq and wild-type mice for the presence of loricrin and involucrin, two major components of the stratum corneum. We could confirm the reported normal presence of involucrin in ichq skin (data not shown), but remarkably, loricrin monomers and dimers were nearly absent in cystatin M/E deficient mice as revealed by western blot analysis (Fig. 5B). We took care to prepare the skin extracts in buffer with and without the specific synthetic legumain inhibitor Cbz-Ala-Ala-Aasn-EP-COOEt to preclude the action of excess free legumain in the cystatin M/E deficient skin during extraction. No low molecular weight degradation products of loricrin were detected in lanes 1 and 2 of Figure 5B, nor did we observe high molecular weight loricrin complexes, although these probably cannot be extracted, due to their poor solubility. Immunohistochemical analysis, however, showed that loricrin was abundantly present in the upper layers of the interfollicular epidermis of dorsal skin of cystatin M/E deficient mice (Fig. 5C), arguing against loricrin degradation as a mechanism to explain the findings on western blot. Moreover, in the epidermis of the mutant mice, three of five cell layers were found positive for loricrin expression, whereas in only one of three cell layers, positive staining was detected in the epidermis of wild-type mice (Fig. 5D).

In Figure 5A, a Coomassie blue stained blot is shown to check for equal loading of the samples in Figure 5B. No gross differences were seen in most of the high molecular weight bands, which represent the major soluble structural proteins of mouse skin. Interestingly, two abundant protein bands of ~13 and 15 kDa were observed in the extract of cystatin M/E deficient mice (boxed in Fig. 5A), which were less pronounced in wild-type skin. In order to exclude the possibility that these bands represented accumulated loricrin breakdown products not detected by the anti-mouse loricrin serum (directed against a single epitope), we subjected these bands to in-gel tryptic digestion followed by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry. The lower 13 kDa band was identified as S100 calcium binding protein A9 (or calgranulin B). This is a protein known to be induced in epidermis during inflammation (19), and it can also act as a modulator of intracellular calcium homeostasis during follicular differentiation (20). The upper 15 kDa band was identified as epidermal fatty acid binding protein (E-FABP). Interestingly, E-FABP has been described as an epidermal protein that is strongly upregulated following skin barrier disruption (21), which is in accordance with the observed increase in transepidermal water loss described above. These findings also demonstrate that these low molecular weight bands are not derived from breakdown of loricrin monomers and dimers.

Increased processing of TGase 3 in skin of ichq mice could explain abnormal cornification

The strongly diminished presence of loricrin monomers and dimers in skin extracts of ichq mice, and the observation that
this phenomenon is not due to degradation, led us to consider misregulated crosslinking as an alternative explanation. It is known that loricin and small proline-rich proteins (SPRs) are cross-linked by cytosolic TGase 3 enzyme primarily to form homodimers and heterodimers (22). Proteolytic cleavage of TGase 3 is required to achieve maximal specific activity of the enzyme (23). We now addressed the question of whether aberrant processing of the TGase 3 zymogen in the interfollicular epidermis of cystatin M/E deficient mice could be responsible for the observed defects in epidermal cornification. TGase 3 activation during keratinocyte differentiation involves cleavage of the 77 kDa zymogen by a hitherto unknown protease resulting in the release of 30 and 47 kDa fragments (Fig. 6A), which then associate non-covalently to form the active enzyme (24). To confirm the aberrant processing of the TGase 3 zymogen due to unrestricted legumain activity in the interfollicular epidermis of cystatin M/E deficient mice, we used a cleavage-site-specific antibody that was generated against a synthetic peptide (FGATS) that corresponds to the cleavage site of mouse TGase 3 (Fig. 6A). Western blot analysis revealed the abundant presence of proteolyzed (and hence activated) TGase 3 in skin extracts of cystatin M/E deficient mice at day 6, whereas no appreciable levels of this 30 kDa fragment could be detected in skin extract of wild-type littersmates (Fig. 6B). This indicates that premature, high levels of activated TGase 3 are present at the time when the skin lesions develop.

The next step was to investigate if legumain could process human recombinant TGase 3 in vitro into its active form by proteolysis of the 77 kDa zymogen into the 30 and 47 kDa fragments. As we were unsuccessful in generating active recombinant legumain, and most tissues do not contain

Figure 2. Protease inhibition by recombinant cystatin M/E. (A) Inhibition of free legumain activity in human kidney extract. Nanomolar concentrations of recombinant cystatin M/E and the specific synthetic legumain inhibitor inhibited hydrolysis of the fluorogenic substrate. Representative curves of three experiments are shown. (B) Hydrolysis of the fluorogenic substrate by the plant protease papain was almost completely inhibited by nanomolar concentrations of recombinant cystatin M/E. Inhibition of a panel of lysosomal cathepsins was incomplete, even when 2 μM recombinant cystatin M/E was used.
measurable free legumain activity, we used human kidney extract that is reported by others as a tissue where free legumain is relatively abundant. As shown in Figure 6C (lanes 1 and 4), recombinant human TGase 3 was slowly processed to its active form by human kidney extract as detected by the monoclonal antibodies C2D and C9D that are directed against the human zymogen and the 30 and 47 kDa fragments, respectively. For comparison, we show cleavage of TGase 3 by dispase, a neutral bacterial protease that is commonly used to activate TGase 3 in vitro. Dispase generated a fragment (Fig. 6C, lane 3) with the same electrophoretic properties as those generated using kidney extract (Fig. 6C, lane 4). Proteolytic processing of TGase 3 by kidney extract could be completely inhibited by the specific legumain inhibitor Cbz-Ala-Ala-Aasn-EP-COOEt (Fig. 6C, lanes 2 and 5). As it is known that legumain affects processing of lysosomal cathepsins (11), we considered the possibility that legumain mediates processing of TGase 3 in an indirect manner. Indeed, when we used E-64, a synthetic broad-spectrum inhibitor of cysteine proteases that is not effective against legumain, a complete inhibition of TGase 3 processing was also found (Fig. 6C, lane 6). This finding was addressed in further detail by investigating the in vitro processing of TGase 3 by a panel of purified lysosomal cysteine proteases. Figure 6D shows that cathepsin S completely processed TGase 3 in 30 min (lane 7) similar to the positive control dispase (lane 2). Partial processing of TGase 3 was found using cathepsin B and cathepsin L (Fig. 6D, lanes 3 and 6), whereas cathepsins C and H were not able to do this (Fig. 6D, lanes 4 and 5). The addition of E-64 to the reaction mixtures completely blocked the processing of the TGase 3 zymogen (data not shown).

DISCUSSION

In this report, we provide evidence that the asparaginyl endopeptidase legumain is a physiologically relevant target protease of cystatin M/E in skin. No detectable free legumain activity was found in the skin of wild-type mice whereas in ichq mice, legumain activity was found at exactly the same location where cystatin M/E is normally expressed. Absence of cystatin M/E and appearance of legumain activity colocalize with the observed pathology in ichq mice (i.e. excessive cornification in hair follicles and epidermis).

Cystatin M/E is an unusual cystatin in its biochemical properties, chromosomal localization and restricted tissue distribution. Whereas most cystatins are extracellular inhibitors of cysteine proteases, cystatin M/E appears to function more locally and also intracellularly, as will be discussed below. Little is known on the specific biological functions of cystatin family members. Deficiency for cystatin B in humans causes myoclonic epilepsy (25); deficiency for cystatin C in mice causes no obvious spontaneous phenotype (26). In ichq mice, deficiency for cystatin M/E causes a dramatic, apparently tissue-specific phenotype, with pronounced morphological and ultrastructural abnormalities in epidermis and hair follicles. We considered a number of known lysosomal cysteine proteases as likely candidate target enzymes for cystatin M/E. Only the recently discovered asparaginyl endopeptidase legumain was found to be inhibited at physiologically relevant concentrations. Mammalian legumain is found in late endosomes and is postulated to have a regulatory role in the biosynthesis of lysosomal enzymes, as recently demonstrated using legumain deficient mice (11). The body weights of the legumain deficient mice were significantly decreased, however, they were normally...
born and fertile. Disruption of the legumain gene led to the enlargement of lysosomes in the proximal tubule cells of the kidney, which suggests that materials to be degraded are being accumulated within the lysosomal compartments. It appeared that the processing of the lysosomal proteases, cathepsins B, H and L, from the single-chain forms into the two-chain forms was defective in the kidney cells of these legumain deficient mice. This observation confirms the earlier assumption that legumain could act as a protease responsible for processing of lysosomal cathepsins into their active forms (10). Interestingly, an increase of lysosomal cysteine protease activity has been observed in the terminal differentiation process of keratinocytes (27,28). Recent studies have reported increasing evidence that lysosomal proteases play important roles in physiological processes not restricted to lysosomes only (29). Examples of a disturbed protease–antiprotease balance involving lysosomal cathepsins, leading to abnormal cornification, include Papillon–Lefevre syndrome (cathepsin C deficiency) and the furless mouse (cathepsin L deficiency) (30,31). However, the exact role for these cathepsins in epidermal differentiation and desquamation is unknown. Our findings on the legumain dependent, cathepsin-mediated TGase 3 processing fit very well with the proposed role of legumain in other cell types. Although it has to be verified experimentally, we assume that legumain regulates lysosomal cathepsin processing and TGase 3 processing in normal skin, a process that is apparently under tight control of cystatin M/E. Deficiency of cystatin M/E, as found in ichq mice, unleashes legumain activity thereby causing the observed phenotype.

In epidermis there is a balanced regulation of protease activity that, when disturbed, could lead to faulty cornification processes in the epidermis and upper part of the hair follicle. In epidermis there is a balanced regulation of protease activity that, when disturbed, could lead to faulty cornification processes in the epidermis and upper part of the hair follicle. Examples of a disturbed protease–antiprotease balance involving lysosomal cathepsins, leading to abnormal cornification, include Papillon–Lefevre syndrome (cathepsin C deficiency) and the furless mouse (cathepsin L deficiency) (30,31). However, the exact role for these cathepsins in epidermal differentiation and desquamation is unknown. Our findings on the legumain dependent, cathepsin-mediated TGase 3 processing fit very well with the proposed role of legumain in other cell types. Although it has to be verified experimentally, we assume that legumain regulates lysosomal cathepsin processing and TGase 3 processing in normal skin, a process that is apparently under tight control of cystatin M/E. Deficiency of cystatin M/E, as found in ichq mice, unleashes legumain activity thereby causing the observed phenotype.

Much of the barrier function of human epidermis against the environment is provided by the cornified cell envelope (CE)
which is assembled by TGase mediated cross-linking of several structural proteins during the terminal stages of normal keratinocyte differentiation. Surprisingly, recent studies on mice in which major CE components were knocked out (e.g. loricrin, envoplakin and involucrin), have shown no discernable phenotype (35–37). This suggests that there are compensatory backup systems and additional unidentified components involved that maintain the skin barrier function. However, mutations or absence of desmosomal or cytoskeletal proteins in differentiated keratinocytes often leads to severe pathology and disturbance of barrier function (38–42). Deficiency for regulatory enzymes as TGase1 and steroid sulfatase also leads to disease in humans (43–45). The severe phenotype of cystatin M/E deficient mice provides an example of a disturbed protease–antiprotease balance that causes faulty differentiation processes in the epidermis and hair follicle. Another example is the recent identification of the serine protease inhibitor Kazal-type 5 (SPINK5) as the defective gene in Netherton syndrome (NS, OMIM 256500) (46). It was shown that the proteolytic processing and distribution of the protein product of SPINK5, LEKT1, is disturbed in NS patients (46,47). NS is a congenital ichthyosis associated with erythroderma, a specific hair shaft defect and atopic features. It was hypothesized that defective inhibitory regulation by LEKT1 result in increased protease–antiprotease balance that causes faulty differentiation processes in the epidermis and hair follicle. Another example is the recent identification of the serine protease inhibitor Kazal-type 5 (SPINK5) as the defective gene in Netherton syndrome (NS, OMIM 256500) (46). It was shown that the proteolytic processing and distribution of the protein product of SPINK5, LEKT1, is disturbed in NS patients (46,47). NS is a congenital ichthyosis associated with erythroderma, a specific hair shaft defect and atopic features. It was hypothesized that defective inhibitory regulation by LEKT1 result in increased protease–antiprotease balance that causes faulty differentiation processes in the epidermis and hair follicle. Another example is the recent identification of the serine protease inhibitor Kazal-type 5 (SPINK5) as the defective gene in Netherton syndrome (NS, OMIM 256500) (46). It was shown that the proteolytic processing and distribution of the protein product of SPINK5, LEKT1, is disturbed in NS patients (46,47). NS is a congenital ichthyosis associated with erythroderma, a specific hair shaft defect and atopic features. It was hypothesized that defective inhibitory regulation by LEKT1 result in increased protease–antiprotease balance that causes faulty differentiation processes in the epidermis and hair follicle. Another example is the recent identification of the serine protease inhibitor Kazal-type 5 (SPINK5) as the defective gene in Netherton syndrome (NS, OMIM 256500) (46). It was shown that the proteolytic processing and distribution of the protein product of SPINK5, LEKT1, is disturbed in NS patients (46,47). NS is a congenital ichthyosis associated with erythroderma, a specific hair shaft defect and atopic features. It was hypothesized that defective inhibitory regulation by LEKT1 result in increased protease–antiprotease balance that causes faulty differentiation processes in the epidermis and hair follicle.

Although this might seem paradoxical at first glance, both loss and inappropriate gain of TGase activity could explain ichthyotic changes, although the mechanisms could be different. Excessive TGase 3 activity could lead to retention of scales by hypercross-linked corneocytes, whereas deficiency for TGase 1 could lead to irregular scaling due to lack of attachment of involucrin to the ω-hydroxyceramides (50). There are, however, other forms that are clinically similar to lamellar ichthyosis but are not linked to the locus of the TGase 1 gene. The non-redundancy of TGase 3 is indicated by knockout mice that show an early embryonic-lethal phenotype (51), but no mutations in this gene have been found in humans thus far. New loci for autosomal recessive ichthyosis have recently been identified (reviewed in 51), but they do not link to the gene for TGase 3. This leaves open the possibility that mutations in genes that are involved in the processing of TGases into the active form might be causative for other forms
of ichthyosis. We suggest that cystatin M/E is a candidate gene for heritable human skin disorders that show faulty cornification and desquamation.

In conclusion, the data presented in this study have identified cystatin M/E and legumain as a functional dyad in skin, and we provide evidence that legumain and lysosomal cysteine proteases are involved in the proteolytic activation of TGase 3 during terminal epidermal differentiation.

**MATERIALS AND METHODS**

**Animal studies**

Mice were housed in specific pathogen-free facilities at the Central Animal Laboratory, University of Nijmegen, The Netherlands. Genotyping of the mice was performed as described previously (6). TEWL of cystatin M/E deficient mice (ichq/ichq) and phenotypically normal littermates (+/+) and ichq/+/ was measured using a Tewameter TM 210, in accordance with current guidelines (52).

**Recombinant proteins**

Recombinant human cystatin M/E was produced both in a bacterial expression system as a GST fusion protein and as a fully processed protein in an eukaryotic system using the baculovirus in insect cells as described previously (15). Recombinant human TGase 3 was produced as a full length zymogen in a baculovirus system and as a bacterially expressed His6-tagged fusion protein as previously described in detail (53).

**Antibodies**

Purified GST-cystatin M/E fusion protein was used to immunize a New Zealand White rabbit. Antisera raised against the GST-cystatin M/E fusion protein were purified by affinity chromatography as described previously (15). Anti-mouse legumain antibodies were prepared by immunizing rabbits with a bacterially expressed His6-tagged fusion protein of legumain as previously described (11). Purified human recombinant TGase 3 from Escherichia coli was used to immunize mice for the establishment of monoclonal antibodies (C2D and C9D) as described in detail by Hitomi (53). Affinity purified polyclonal rabbit anti-mouse FGATS antibodies were generated as described previously (54). Affinity purified polyclonal rabbit anti-mouse loricrin antibodies (AF-62) were purchased from BAbCO (BAbCO, Richmond, CA).
**Extraction of proteins and immunoblotting**

Human kidney and mouse skin biopsies were frozen in liquid nitrogen and subsequently ground using a Micro Dismembrator U (B. Braun Biotech International, Melsungen, Germany). Proteins were extracted in buffer containing 50 mM citrate (pH 5.8), 1 mM EDTA, 0.1 M NaCl and 2 mM DTT, followed by mild sonification of the lysate for 1 min at 4°C. Mouse skin proteins were extracted in the absence and presence of Cbz-Ala-Ala-Asn-EP-COOEt. Samples were diluted with SDS sample buffer (containing a reducing agent) and boiled for 3 min. These protein samples were separated by SDS–PAGE on a 12% Bis-Tris-Gel and blotted onto polyvinylidenedifluoride (PVDF) membrane using the NuPAGE system (Invitrogen, Carlsbad, CA), according to the protocol provided by the manufacturer. Membranes were stained with Coomassie blue, or incubated with polyclonal antibodies directed against mouse loricrin (1:5000), mouse anti-FGATS (1:100), and the C2D (1:1000) and C9D (1:4000) monoclonal antibodies against human TGase 3. Proteins were detected with the Phototope-HRP Western Blot Detection Kit (Cell Signaling Technology, Beverly, MA), according to the manufacturer’s protocol.

**MALDI-TOF mass spectrometry analysis**

In-gel digestion coupled with mass spectrometry analysis was used for the identification of proteins extracted from mouse skin. To perform digestions on Coomassie blue stained protein bands that were abundantly found in the skin extract of cystatin M/E deficient mice, the In-Gel Tryptic Digestion Kit (Pierce, Rockford, IL) was used in accordance to the protocol provided by the manufacturer. For MALDI-TOF mass spectrometry, 0.3 μl of the tryptic digest was spotted on the target plate followed by 0.3 μl of matrix solution (a saturated solution of α-cyano-4-hydroxy-cinnamic acid in a 50:50 mixture of acetonitrile and 0.1% TFA, v/v). Positive mass ion spectra were collected in the reflectron mode, with pulsed ion extraction on a Biflex III instrument (Bruker-Franzen, Bremen, FRG). Mass calibration was performed externally using a mixture of peptide standards (Sigma, St Louis, MO). A total of 150 single laser shots were accumulated for each sample spot. For identification of proteins, peptide mass lists were used to search protein databases with the Mascot software system (Matrix Science, London, UK).

**TGase 3 processing**

Baculovirus expressed recombinant TGase 3 zymogen was processed by treatment with dispase, cathepsins and by a protein extract from human kidney. To prepare the proteolyzed form, 0.6 μg zymogen was treated with 5 mM dispase (Roche Diagnostics, Mannheim, Germany) in the presence of 5 mM CaCl2 at 37°C for 30 min. The same reaction conditions were used to test proteolytic activity of human cathepsin B (Sigma), bovine cathepsin C (Sigma), human cathepsin H (Calbiochem, San Diego, CA), human cathepsin L (Sigma) and bovine cathepsin S (Calbiochem). To test whether the TGase 3 zymogen could be processed by free legumain activity, 0.6 μg zymogen was treated with protein extracts from human kidney in the presence of 5 mM CaCl2 at 37°C for 0.5 and 24 h. For controls the same reaction mixtures were prepared but with the addition of Cbz-Ala-Ala-Asn-EP-COOEt, or compound E-64 [trans-epoxysuccinyl-L-leucylamido-(4-guanidino)butane]. The reaction mixtures were subjected to SDS–PAGE and electro-blotted onto PVDF membrane. The membrane was incubated with the monoclonal mouse anti-human TGase 3 antibodies (C2D and C9D) and detection was established as described above.

**In situ assay for legumain activity**

This assay is based on data that have shown that 5-nitrosalicylaldehyde forms an insoluble fluorescent complex with NH2NapOMe, which has been used to visualize cathepsin B in fibroblasts (55). Unfixed cryostat sections (6 μm) of mouse skin and pig kidney were air-dried for 10 min. These tissue sections were subsequently incubated with 100 μl legumain in situ assay buffer under a coverslip. Enzyme activity was measured after an incubation period of 30 min at 37°C with the selective fluorescent Suc-Ala-Ala-Asn-4-methoxy-2-naphthylamide (Suc-Ala-Ala-Asn-NHNapOME) substrate. This substrate was originally synthesized for the measurement of legumain in colorimetric and fluorimetric microplate assays as described previously (56). Legumain in situ assay buffer was prepared by the addition of 5-nitro-salicylaldehyde and Suc-Ala-Ala-Asn-NHNapOME substrate to a final concentration of 1 and 2 mM, respectively, in a buffer that contained 121 mM phosphate (pH 5.8), 39.5 mM citric acid, 1 mM EDTA, 1 mM DTT and 0.01% CHAPS. The specificity of the reaction was verified by incubation in the absence of substrate or in the presence of Cbz-Ala-Ala-Asn-EP-COOEt.

**Fluorimetric enzyme assays**

Protease inhibitory activity of recombinant cystatin M/E against cysteine proteases was determined by measuring the inhibition of papain and lysosomal cathepsins (B, C, H, L and S) using fluorogenic synthetic substrates, essentially described by Abrahamson (53). Protease inhibitory activity of recombinant cystatin M/E and the synthetic aza-peptide epoxide inhibitor against legumain was determined by measuring the inhibition of free legumain activity in human kidney extract. Kidney extract was titrated in the absence and presence of increasing concentrations of recombinant cystatin M/E or the specific legumain inhibitor as a positive control. Preincubation time of kidney extract and inhibitor was 30 min at room temperature. Enzyme activity was measured after an incubation period of 30 min at 37°C with the selective fluorescent Z-Ala-Ala-Asn-MCA substrate (Peptides International, Louisville, KY). The buffer that was used contained 0.1 M phosphate (pH 5.7), 2 mM EDTA, 1 mM DTT and 2.7 mM L-cysteine.

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