Analysis of Proteins with Caseinolytic Activity in a Human Stratum Corneum Extract Revealed a Yet Unidentified Cysteine Thiol Protease and Identified the So-Called “Stratum Corneum Thiol Protease” as Cathepsin L2

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Desquamation is described as a protease-dependent phenomenon where serine proteases with a basic pH optimum play a key role. Recently proteases with an acidic pH optimum were identified in the stratum corneum and associated with desquamation, e.g., cathepsin D and the stratum corneum thiol protease. The purpose of this study was to investigate if human stratum corneum contains proteases different from the above, exhibiting similar properties. After gel filtration, we identified four distinct proteolytic activities in a human stratum corneum extract, a cathepsin-E-like activity (80 kDa), a cathepsin-D activity (40 kDa), a yet unknown cathepsin-L-like form (28 kDa) exhibiting the highest caseinolytic activity, and a chymotrypsin-like protein (24 kDa) containing the acidic activity of the well described stratum corneum chymotryptic enzyme. We named the new 28 kDa protease stratum corneum cathepsin-L-like enzyme. Characterization of stratum corneum cathepsin-L-like enzyme provided clear evidence that this new protease, despite its membership to the cathepsin-L-like family, is distinct from cathepsin L and from the recently described stratum corneum thiol protease. Its ability to hydrolyze corneodesmosin, a marker of corneocyte cohesion, was in favor of a role of stratum corneum cathepsin-L-like enzyme in the desquamation process. A more detailed analysis did not allow us to identify stratum corneum cathepsin-L-like enzyme at the molecular level but revealed that stratum corneum thiol protease is identical with the recently described cathepsin L2 protease. Reverse transcription polymerase chain reaction studies and the use of a specific antibody revealed that, in contrast to earlier reports, expression of stratum corneum thiol protease in human epidermis is not related to keratinocyte differentiation. Our results indicate that the stratum corneum thiol protease is probably expressed as a pro-enzyme in the lower layers of the epidermis and in part activated by a yet unidentified mechanism in the upper layers during keratinocyte differentiation.

Key words: cathepsin D/cathepsin L2/desquamation/proteases/SCCE/SCCL/SCTE/SCTP J Invest Dermatol 120:592–600, 2003

A balance between cell shedding at the skin surface and keratinocyte proliferation in the basal layer of the epidermis is necessary for the maintenance of a functional normal stratum corneum (SC) assuring an efficient physiological barrier for our organism against a hostile environment. The desquamation process is a precisely controlled cascade of events, the molecular mechanisms of which are only known in part and have to be put correctly in place to resolve the puzzle. It is now generally accepted that SC cohesion is essentially mediated through desmosome derived structures, called corneosomes or corneodesmosomes (Serre et al., 1991; Walsh and Chapman, 1991). The entry of corneocytes into the stratum disjunctum is characterized by a dramatic loss of non-peripheral corneodesmosomes, resulting in an intercorneocyte adhesion restricted to the overlapping edges. The selective degradation of the nonperipheral corneodesmosomes and the preservation of the edge structures might indicate that, at this stage, the lateral structures are protected against proteolysis, possibly by distinct carbohydrate entities (Walsh and Chapman, 1991).

The key event that eventually results in desquamation (cell shedding) is the complete proteolysis of all corneodesmosomes. Among the corneodesmosomal proteins, desmoglein I, a member of the cadherin adhesion molecules family, is believed to be a major player, even though its degradation remains difficult to identify (Rawlings et al., 1995). Other corneodesmosomal components like desmocollins (King et al., 1993), desmoplakins (Bernard et al., unpublished results), and corneodesmosin (Serre et al., 1991) are progressively degraded during desquamation (Lundström et al., 1994). Corneodesmosin has recently been demonstrated to be the preferred substrate of two SC serine proteases (Simon et al., 2001).

Walsh and Chapman (1991) described desquamation as a sequential action of glycosidases and proteases on corneodesmosomes. They demonstrated that (i) the addition of exogenous proteases induced no major changes in corneodesmosome structure and composition, (ii) the addition of exogenous glycosidases alone, or added after proteases treatment, caused only a mild
fragmentation of the adhesive structures, and (iii) only glycosidase treatment and the subsequent addition of exogenous proteases caused dramatic degradation of the corneodesmosomal structure. These findings indicate that sugar moieties are present, protecting the corneodesmosomal protein structures from proteolysis. Walsh and Chapman further demonstrated that endoglycosidases were more effective than exoglycosidases. We recently succeeded in finding evidence for the first time of the existence of endoglycosidase activity in the human SC (Bernard et al., 2001).

Based on zymographic analysis, several laboratories have described the presence of proteases in the intercellular regions of the SC: (i) a chymotrypsin-like protease named stratum corneum chymotryptic enzyme (Engelrud and Lundström, 1991); (ii) a trypsin-like protease named stratum corneum trypsin-like enzyme (Engelrud and Lundström, 1990; Suzuki et al., 1994; Ekholl et al., 2000); (iii) a protease of the corneocyte protease family named stratum corneum thiol protease (SCTP) (Watkinson, 1999); and (iv) the aspartic protease cathepsin L (Horikoshi et al., 1998; Böcker et al., 1989). The purpose of this study was to investigate if proteases other than the above are present in human SC and might be a part of the desquamation puzzle.

MATERIALS AND METHODS

Materials Pre-made sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) homogeneous or gradient gels, Rainbow™ molecular weight markers, a silver staining kit, a gel filtration G75 HR3/30 column, a MonoS HR5/5 column, and a chromatography molecular weight standard protein kit were from Amersham Pharmacia Biotech (Uppsala, Sweden). Taq polymerase and secondary antibody coupled to peroxidase were from Sigma (St. Louis, MO). The RNA preparation kit RNasy was obtained from Qiagen (Hilden, Germany). Oligonucleotides were synthesized by Genset (Evry, France). Enzcheck protease assay kit "green fluorescence" and Z-(Phe-Arg) 2R110 (ZPA) were from Calbiochem (La Jolla, CA). Sheep antihuman cathepsin L antibody cat. no. 1911-0507 was from Biogenesis (Poole, U.K.). Human cathepsin L cat. no. H49793 from ICN (Orsay, France), and antirabbit biotinylated secondary antibody to Roche Diagnostics (Meylan, France). VivoTag™ and Sartocon micro™ ultrafiltration filters were from Sartocon (Göttingen, Germany). Gelstar™ was from BMA (Rockland, ME).

Extraction of SC proteases SC proteins were collected by the so-called scraping method. The scraping was carried out on the anterior surface of the reconstructed SC. The area was washed with 200 ml of buﬀer composed of 50 mM sodium phosphate buﬀer, pH 7, 5 mM ethylenediamine tetraacetic acid (EDTA), 150 mM NaCl, and 0.1% Tween 20. Thereafter, the washed area was scraped with the edge of a microscope slide and continuously rinsed with the above buﬀer. The buﬀer containing the corneocytes was collected in a container placed underneath the leg and passed first over a Whatman No. 4 paper, thereafter over a Millipore™ 0.45 μm filter. The resulting SC suspension was clarified by centrifugation (12,000 g, 10 min) and the resulting pellet was extracted with 100 ml of boiling Laemmli buﬀer containing 200 mM DTT. After centrifugation (10,000 g x 10 min) and the resulting pellet was extracted with 100 ml of boiling Laemmli buﬀer containing 200 mM DTT. After centrifugation (10,000 g x 10 min), the supernatant was recovered and assayed for protein content using the BioRad protein assay, using bovine serum albumin as a standard. The protein concentration of the extract was about 0.5 mg protein/ml, a total of 3 mg per person. Each experiment was performed with a pooled extract from at least three volunteers. Informed consent was obtained from all participants and the non-evasive sampling technique didn't require the approval of an ethical committee.

Gel filtration Purification was performed at 4°C on a Biologic™ chromatography station (Bio-Rad, Marnes la Coquette, France). 250 μl of the extract were directly loaded on a G75 HR3/30 column. G75 HR3/30 column was equilibrated with the extraction buﬀer (flow rate of 0.5 ml per min), where Tween 20 was substituted with the low-ultraviolet-absorbing form of Triton X100 (buﬀer GF). Protein elution was monitored by recording the OD280nm and 150 μl fractions were collected, 14 min after injection of the sample, into 96-well plates ready for the analyses of enzymatic activities or electrophoretic analysis. The apparent molecular weights were calculated with the calibration curve obtained after injection of a mixture of standard proteins from the low molecular weight protein kit (Amersham Pharmacia Biotech).

Preparation of extracts for Western blotting Reconstructed human epidermis after 13 d of culture on SC or plantar SC obtained from human volunteers was homogenized in Triton buffered saline (TBS), pH 7.2, containing 0.5% (vol/vol) Triton X100 on an ice bed (50 μl buﬀer per 1 mg tissue weight) with a polytron for 1 min before centrifugation (10,000 g x 10 min). The protein contents of the respective supernatants were determined using the Bio-Rad protein assay. Samples were adjusted to a protein concentration of 0.5 mg per ml before performing the protease assays or Western blot analysis.

Reconstructed human epidermis Reconstructed human epidermis with normal human keratinocytes was prepared as described previously (Tinois et al., 1993). The reconstructed epidermis was examined in a live/dead staining using 37°C (days 1–15).

Western blotting The proteins were submitted to SDS-PAGE on prefabricated gel band PAG film-supported gels (12.5% acrylamide). The molecular weight standard used was a 1 : 1 mix of low and high molecular weight prestained Rainbow™ standards from Amersham Pharmacia Biotech. Proteins were transferred to a PVDF membrane.
Immobilon P, Millipore, Saint Quentin en Yvelines, France) in the presence of 20 mM Tris–HCl pH 8.3, 192 mM glycine, 0.1% (wt/vol) SDS, and 20% (vol/vol) methanol using a trans-blot cell (Bio-Rad) with a constant voltage of 60 V for 4 h. The filters were blocked with TBSTL at room temperature for 1 h. Thereafter, the filters were incubated overnight at 4°C with the primary antibody. The filters were then washed twice for 5 min in TBSTL and incubated at room temperature for 2 h with the second antibody coupled to peroxidase, diluted in TBSTL. The filters were washed twice for 5 min in TBS before the detection procedure. The bands were visualized using the ECL + substrate system (Amersham Pharmacia Biotech) on a FluorSmax™ imager (Bio-Rad). Pre-stained Rainbow™ molecular weight markers served as standards and molecular weights were calculated using Quantity-One™ software (Bio-Rad).

Immunoprecipitation SC protein extract (500 μl) was incubated overnight at 4°C with 10 μl of a sheep antihuman cathepsin L antibody (2.6 mg per ml). The resulting immunocomplex was precipitated using protein G (protein G kit from Roche Molecular). After centrifugation of the protein G agarose beads, 250 μl of the supernatant was applied to the gel filtration column. A control without antibody was run in parallel. The functionality of the immunoprecipitation procedure was checked by incubating 0.25 μg of purified human cathepsin L with 500 μl extraction buffer following the same protocol as for the SC protein extract. Cysteine protease caseinolytic activity was detected as described above.

Purification and identification of the SCTP The purification steps were performed at 4°C on a BiologicTM chromatography station (Bio-Rad). SC soluble proteins from three healthy donors were obtained as described above (9 mg protein in 18 ml buffer). The buffer was exchanged with econo-Pac™ 10DG columns (Bio-Rad) in cation exchange buffer: 50 mM acetate buffer pH 5.0, 350 mM NaCl, 1 mM EDTA, 0.1% reduced Triton X100 (buffer A). The resulting protein extract (9 mg protein in 24 ml buffer) was loaded on a Mono S™ HR5/5 column (Amersham Pharmacia Biotech) equilibrated with the same buffer at a flow rate of 1 ml per min. The column was washed with five column volumes of buffer A. Elution was performed using buffer A containing 550 mM NaCl. Proteins were eluted with a linear NaCl gradient ranging from 0 to 100% in 40 column volumes. The flow rate was 1 ml per min. Fractions (0.42 ml) were collected starting at the beginning of the gradient. Protein elution was monitored by recording the OD280nm.

After gel chromatography, the eluted proteins were submitted to SDS-PAGE on prefabricated gelbond PAG film-supported gels (G8-18) using a 1 : 2 dilution with 2/3 Laemmli sample buffer without DTT and silver stained with the silver nitrate kit (Amersham Pharmacia Biotech). SCTP activity was revealed using the gelatin and the ZPA zymography

Figure 1. Caseinolytic activities of SC proteases separated by gel filtration. The caseinolytic activities were detected with the Enzchek kit (Molecular Probes) used at pH ranging from 4 to 8.25 on fractions after gel filtration of 125 μg protein extracted from human SC. Very low activities were detectable at basic pH (a, arrow 1 on two-dimensional view). The highest activities were detectable at acid pH split into three groups (b): group A with an 80–90 kDa molecular weight and pH optimum at 5.5; group B with a 40 kDa molecular weight and pH optimum between 5.25 and 5.5; group C with a 25 kDa molecular weight and pH optimum between 5.25 and 5.5. After addition of cysteine, a new caseinolytic group D was revealed with a molecular weight of 28 kDa and pH optimum of 5–5.5 (b). Compared to the results without cysteine, group C is masked by this new group. Group A and B proteases are still visible. Data obtained without cysteine (a) were subtracted from the data obtained with cysteine (b). This differential analysis shows the group D activity (c).
methods. SCTP of the most active fractions were pooled, precipitated (10% trichloroacetic acid) and sequenced (Edmann sequencing) as previously described (Bernard et al., 2001).

**Human cathepsin L2 antibodies** The anti-cathepsin L2 polyclonal antibody was produced by CovalAb (Lyon, France) by immunizing rabbits with selected peptides from the cathepsin L2 sequence. One peptide was retained as potentially immunizing: DBcat15CE (CFRNQKFRKGKVFRE amino acid residues 94–108 from Swissprot sequence 060911). The rabbit were immunized with a proprietary protocol (CovalAb) and the resulting antibodies were affinity purified using a peptide immobilized column. Antibody concentration obtained after the immunopurification step was 0.5 mg per ml. For Western blot analysis, the DBcat15CE antibody was diluted 1 : 1000.

**Reverse transcription polymerase chain reaction (RT-PCR) analysis** Total RNA from reconstructed human epidermis was prepared using an RNeasy kit. Briefly, two samples of the reconstructed epidermis (1 cm² each) were homogenized in 300 μl of lysis buffer (included in the kit) and the cell debris was removed using a QIA shredder column. cDNA was produced from purified total RNA using the first-strand cDNA synthesis kit. Expression analysis of cathepsin L2, loricrin, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was performed by RT-PCR using equivalent amounts of total RNA (0.1 μg per assay), taq polymerase, and different primers (for cathepsin L2, 5’-ctcacacattggggtagctggc-3’ and 5’-cttcccaaatctgtggattg-3’; for loricrin, 5’-accacgga-ggcgaaggagt-3’ and 5’-ctgggggtggagtctttggtgtg-3’; for GAPDH, 5’-aatcccatccc-3’ and 5’-gtcatatatggaggtt-3’) under the following conditions: 1 cycle for 2 min at 95°C, 25–30 cycles (94°C for 30 s, 55°C for 30 s, 72°C for 30 s), and 1 cycle at 72°C for 2 min in parallel. The resulting PCR products were visualized by Gelstar™ staining after separation on 2%–3% (wt/vol) agarose gels.

**Histochemistry** Thin cryosections (5 μm) of skin from plastic surgery were obtained with a Microm HMS500M microtome. Sections were fixed in acetone at −20°C for 5 min and air dried. Immunohistochemical detection of cathepsin L2 was performed with streptABComplex/HRP kit from Dako and AEC detection kit from Sigma according to the manufacturer’s instructions except that the incubation steps were extended to 1 h using DB15CE diluted 1 : 2 in phosphate-buffered saline/Tween 20 (0.05% [PBST]) as primary antibody and an antirabbit biotinylated secondary antibody from Roche Molecular diluted 1 : 200 in PBST. Nuclei were counterstained with hematoxylin.

**RESULTS AND DISCUSSION**

**Caseinolytic activity of an SC extract** Gel filtration of the SC protein extract resulted in the separation of two distinct caseinolytic activities, one around pH 8.0 and the large majority at pH 5–5.5 (Fig 1a–c), a pH characteristic for the surface of the
SC (Öhman and Vahlquist, 1994). As proteases involved in the desquamation process are likely to have an acidic pH optimum, we decided to further characterize the acidic activity. Based on the calculated molecular weight, the responsiveness to class specific inhibitors, and the zymograph profile, we distinguished four distinct groups within the acidic active part of the extract.

Group A, with a calculated average molecular weight of 80 kDa (Fig 1a), and completely inhibited by the classic aspartic protease inhibitor pepstatin (Fig 2.3), was considered to comprise part of the cathepsin-E-like family. Even though, to our knowledge, this type of protease activity has not yet been described in the SC, we did not perform any further characterization, as its caseinolytic activity was very low.

Group B, characterized by an average molecular weight of 40 kDa (Fig 1a), a total inhibition by pepstatin (Fig 2.3), and a broad pH optimum, was supposed to represent the cathepsin D activity. The very high caseinolytic activity in our SC extract that was not yet described in the SC, we did not perform any further characterization, as its caseinolytic activity was very low.

Group C, exhibiting a molecular weight of 24 kDa (Fig 1a), with a sensitivity to chymostatin and aprotinin (Figs 2.1, 2.2), comprised the chymotrypsin-like activity. As it perfectly converted (data not shown) the synthetic substrate S2586 (Egelrud, 1993), we assumed that this group represents mainly the acidic activity of the chymotryptic enzyme (SCCE), described by Hansson et al (1994).

Group D attracted our particular interest for various reasons. It represented the main caseinolytic activity in the SC extract studied. It revealed its activity only in the presence of cysteine (Fig 1b, c), required the presence of mild reducing agents, and exhibited a distinct inhibitory response to specific inhibitors. Almost insensitive to pepstatin (1 µM) and aprotinin (6 µM) (Figs 3.5, 3.4), the activity was completely inhibited by E64 (1.4 µM) (Fig 3.1) and not affected by the specific cathepsin B inhibitor CA074 (1 µM) described by Inubushi et al (1994) (Fig 3.6). This particular inhibition profile excluded the presence of cathepsin B, and its sensitivity to leupeptin (1 µM) and chymostatin (2.5 µM) (Figs 3.2, 3.3) ruled out a cathepsin-H-like activity (Schwartz and Baret, 1980). So far, all of the above data suggested the presence of a cathepsin-L-like protease of the papain-type family. The fact that cathepsin-L-specific antibodies failed to immunoprecipitate the proteolytic activity in group D, however, clearly indicated that this activity cannot be attributed to cathepsin L itself but is exerted by another member of this family (data not shown).

Figure 3. SC caseinolytic cysteine-dependent (group D) protease profiles at pH 5.5 and effect of specific inhibitors. The curves presented were obtained by subtracting the activities obtained with and without cysteine. Group D protease activity was tested in the presence of (1) 1.4 µM E64, (2) 1 µM leupeptin, (3) 2.5 µM chymostatin, (4) 6 µM aprotinin, (5) 1 µM pepstatin, (6) 1 µM CA074.
We therefore first checked whether the recently described SCTP (Watkinson, 1999), another member of the cathepsin L family, is involved. Superimposing the caseinolytic and gelatinolytic activity, detected in the fractions of the SC extract, showed that SCTP activity did not match group D activity (Fig 4).

At this stage of investigation, we decided to name the protease present in group D, stratum corneum cathepsin-L-like protease (SCCL). Due to the uniform response to specific inhibitors we were convinced that we were dealing with a single protease. To evaluate whether this new protease is implicated in the degradation of corneodesmosomes, we assayed corneodesmosin degradation by SCCL. Exposing corneocytes to purified SCCL followed by a Western blot immunodetection of the characteristic 33 kDa corneodesmosin fragment (Simon et al., 2001) revealed an efficient degradation of corneodesmosin (Fig 5). As expected from the inhibition profile (see Fig 3), leupeptin reduced, and E64 completely inhibited, SCCL-dependent degradation of corneodesmosin. Interestingly the amount of intact corneodesmosin increased in the presence of E64, indicating that this inhibitor probably blocks other cysteine proteases present in this extract. This observation can be explained by E64 inhibition of endogenous cysteine proteases associated with the corneocytes.

Attempts to purify SCCL protease for further characterization by gel filtration, cation exchange, and hydrophobic interaction chromatography enabled us to dramatically increase its specific activity but did not provide sufficient material to sequence the protein. Efforts are in progress to obtain sufficient material to sequence SCCL, a protease particularly interesting because of its surprising high specific activity.

Figure 4. Comparison of SCTP (gelatinase) and group D cysteine-dependent protease activity after gel filtration chromatography. Human SC extract separated by gel filtration chromatography was analyzed for its cysteine-dependent activity. The activity detected on the gelatin zymogram (SCTP) did not correlate with the caseinolytic dependent activity (group D).

Figure 5. Western blot analysis of corneodesmosins (antibody G36.19) extracted from human SC after exposure to semi-purified group D protease at pH 5 plus 5 mM cysteine. Control, SC incubated without addition of protease; +D, with protease and without inhibitor; +D + L, with protease and with leupeptin 1 μM; +D + E64, with protease and with E64 1.4 μM. The major SC forms of corneodesmosin are also indicated by their molecular weight (kDa).

Figure 6. Purification of SCTP by cation exchange chromatography. 5 mg of protein extracted from human SC were loaded on a cation exchange chromatography column at pH 5. Proteins were eluted with a gradient ranging from 350 to 550 mM NaCl in 40 column volumes. Fractions (0.42 ml) were collected in a 96-well plate. Fractions 7 to 54 were analyzed for SCTP activity by gelatin zymography (GZ) and ZPA zymography (ZPAZ). Proteins of these fractions were separated by SDS-PAGE (Eph) on a G8-18 gradient and silver stained. Arrows indicate the molecular weights calculated for the detected activities and the molecular weight markers for the SDS-PAGE gel.

Figure 7. Coupling zymography and protein staining for the identification of purified SCTP. Fractions 32–37 from the cation exchange step supporting SCTP activity (Fig 6) were pooled and concentrated to 250 μl S, untreated sample; (TCA), trichloroacetic acid precipitated sample. The same gel was used in (1) to detect its cathepsin-L-like activity performing a ZPA zymogram (ZPAZ) and (2) Coomassie blue stained to reveal the band supporting the enzyme activity (indicated by double-headed arrows). Arrows on the right indicate the molecular weights of the protein associated with the activity (35 kDa) and of two other stained proteins. The 35 kDa (SCTP) band was then cut and sequenced.
During our detailed analysis of the various protease activities, we performed experiments to make sure that SCCT activity is distinct from SCTP activity. The gelatin zymograms and cascinolytic profiles always identified SCTP and SCCT activity in distinct but neighboring fractions (data not shown). To provide unequivocal evidence that what we called SCCT is distinct from SCTP, the gelatinolytic activity was purified. We succeeded in a one-step identification by cation exchange chromatography (Fig 6). The most active fractions detected by gelatin zymogram and ZPA zymogram, i.e., fractions 32–37, were pooled and concentrated to perform another ZPA zymogram of the fractions obtained after gel filtration.

Table I. Amino acid sequences obtained by Edmann sequencing of the purified SCTP and sequence chosen for the generation of antibody

<table>
<thead>
<tr>
<th>Amino acid sequence</th>
<th>aa position extracted in SwissProt sequence for hCatL2 (accession 060911)</th>
<th>aa correspondence in alignment with hCatL2 sequence. (SwissProt accession P07711)</th>
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<tbody>
<tr>
<td>Edman sequence n° 1</td>
<td>XYQWK</td>
<td>TKWK</td>
</tr>
<tr>
<td>Edman sequence n° 2</td>
<td>LYGANEEGWR RR</td>
<td>LYGANEEDGW RR</td>
</tr>
<tr>
<td>Edman sequence n° 3</td>
<td>FSATGALEQMF RR</td>
<td>FSATGALEQMF RR</td>
</tr>
<tr>
<td>Edman sequence n° 4</td>
<td>NLDHGVLVGYGFEGANSNNSK</td>
<td>DMDHGVLVGYGFESTEDNNSK</td>
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<tr>
<td>Peptide used for rabbit immunisation</td>
<td>CFRNQKFRKKGKVFRE</td>
<td>GFOKNRKPFRKGKVFQE</td>
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*Sequences obtained by Edmann sequencing of peptides released by trypsinolysis of a 35 kDa band supporting SCTP activity. These sequences have 100% homology with the sequence published for cathepsin L. The sequence used to produce the anti-peptide antibody is given. Highlighting in bold and underlined letters depict the differences between the sequences obtained and the corresponding sequences in human cathepsin L. X is an unidentified residue.

A detailed analysis of the cathepsin L2 sequence together with the results obtained in Western blots using a specific antibody revealed some discrepancy with earlier reports concerning the processing of cathepsin L2 and the putative active form of the enzyme. Adachi et al. (1998) reported that the full-length cDNA encodes a peptide of 334 amino acids with a potential peptide signal (aa 1–17) and a putative activation peptide (aa 18–113) plus two potential N-glycosylation sites. Brome et al. (1999) described a recombinant heterogeneous pro-form of 38 and 45 kDa and an autocatalytic processing, resulting in a 31 and a 35 kDa mature form. Even though the latter result confirms the activation site predicted by Adachi, our results clearly demonstrate a distinct processing in the epidermis: (i) sequence analysis of the purified catalytic active form of cathepsin L2 comprised the aa sequence 30–33 and 39–49 (see Table I) and (ii) an antibody recognizing the cathepsin L2 aa sequence 94–108 decorated both the pro-form and the active form of the enzyme. Furthermore, this polyclonal antibody decorated cathepsin L2 in Western blots and did not cross-react with purified cathepsin L (data not shown). This antibody was used to further characterize the epidermal forms of cathepsin L2.

Western blot analysis  A comparative Western blot analysis of cathepsin L2 expression in SC extracts obtained from plantar SC and nonpalmoplantar SC (Fig 8) confirmed the presence of the 39 kDa pro-form and the mature 34 and 35 kDa forms in nonpalmoplantar SC. In addition, a 33 kDa band, probably a further degradation product, was detected. The plantar SC only contained the 39 kDa inactive pro-form of the enzyme. This is the reason why Watkinson, who analyzed plantar SC only for its catalytic gelatinolytic enzyme activity, could not detect the presence of this enzyme and concluded that it was not expressed in plantar SC.

A more detailed Western blot analysis combined with a gelatin zymogram of the fractions obtained after gel filtration of the SC
Table II. Proteases implicated in the desquamation process

<table>
<thead>
<tr>
<th>Name</th>
<th>PROTEASE CLASS</th>
<th>Precursor MW (kDa)</th>
<th>Proposed activation pathway</th>
<th>MW (kDa) of the active epidermal form(s)</th>
<th>optimum pH</th>
<th>Tissue expression</th>
<th>Differentiation related expression?</th>
<th>Genbank n*</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCCE/KLK7</td>
<td>Serine protease</td>
<td>27</td>
<td>Trypsin-like protease</td>
<td>25</td>
<td>basic</td>
<td>Skin &gt;&gt; Brain, Kidney</td>
<td>yes</td>
<td>AF166330</td>
</tr>
<tr>
<td>SCTE/KLK5</td>
<td>Serine protease</td>
<td>37</td>
<td>Trypsin-like protease</td>
<td>30</td>
<td>basic</td>
<td>Skin Brain, Breast, testis</td>
<td>yes</td>
<td>AF135028</td>
</tr>
<tr>
<td>Cathepsin D</td>
<td>Aspartic protease</td>
<td>52</td>
<td>Cysteine protease</td>
<td>33, 48</td>
<td>acidic</td>
<td>Ubiquitary</td>
<td>yes</td>
<td>M11233</td>
</tr>
<tr>
<td>*Cathepsin L2/SCTP</td>
<td>Cysteine protease</td>
<td>39</td>
<td>Autocatalytic</td>
<td>34, 35</td>
<td>acidic</td>
<td>Thymus, Testis, cornea &gt;&gt; Skin</td>
<td>Not but activation is probably diff. related</td>
<td>AF070448</td>
</tr>
<tr>
<td>*SCCL</td>
<td>Cysteine protease</td>
<td>?</td>
<td>?</td>
<td>28</td>
<td>acidic</td>
<td>Unknown</td>
<td>not determined</td>
<td>not determined</td>
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<tr>
<td>*Cathepsin E-like</td>
<td>Aspartic protease</td>
<td>?</td>
<td>?</td>
<td>80</td>
<td>acidic</td>
<td>Unknown</td>
<td>not determined</td>
<td>not determined</td>
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*The information presented here is obtained essentially from the Swissprot database and from papers discussed in this publication. SC proteases identified and/or characterized for the first time in this publication are marked with an asterisk.

Figure 11. Immunolocalization of cathepsin L2 in human epidermis. The rabbit polyclonal antibody DBcat15CE was used as the primary antibody. Nuclei were counterstained with hematoxylin. E, epidermis; D, dermis. Scale bar: 100 μm.

We thank Dr. Jacques d’Alayer from the Laboratory of Microsequencing of Proteins, Institut Pasteur, Paris, for the protein sequencing. We are also grateful to M. Torrisi, E. Boivin, and Dr. de Lacharrière for providing the human epidermis and Dr. Michel Kernici for helpful discussions.

REFERENCES


epidermis is often hampered by the strong hydrophobic environment.

In conclusion, the analysis of the SC extract from human skin revealed the presence of a new cysteine protease, which we named SCCL, exhibiting a surprisingly high specific activity. Moreover, the purification, sequencing, and detailed analysis at the molecular level of another cysteine protease present in the SC extract demonstrated that what was described earlier to be the “stratum corneum thiol protease” (SCTP) is not a distinct epidermal enzyme but identical with cathepsin L2. A view of these latest results in relation to other existing data concerning proteases involved in the desquamation process is presented in Table II. The fact that SCCL degrades cornodesmosin, a key component of the cornodesmosomes, indicates a possible active function of this enzyme in the desquamation process. Its purification and further characterization are in progress.

Cathepsin L2 expression and epidermal differentiation The reconstructed human epidermis model was used to analyze cathepsin L2 expression during keratinocyte differentiation. RT-PCR analysis was performed at different time intervals during the reconstruction of the epidermis, days 1–13 (Fig 10). The time course clearly evidenced that cathepsin L2 expression is independent of keratinocyte differentiation. Experiments to identify at which level of the stratified epidermis the pro-form of cathepsin L2 becomes activated are in progress. Immunolocalization of cathepsin L2 in normal human skin with the DB15CE antibody confirms the presence of cathepsin L2 throughout the living layers of the epidermis (Fig 11). The reduced staining at the intermediate layers remains to be explained. The negative staining of the SC for the enzyme is less surprising, as it is known that immunostaining in this part of the