Analysis of Proteins with Caseinolytic Activity in a Human Stratum Corneum Extract Revealed a Yet Unidentified Cysteine 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.
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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 physiochemical 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 nonperipheral 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

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Abbreviations: SCCL, stratum corneum cathepsin-L-like enzyme; SCTP, stratum corneum thiol protease; ZPA, Z-$(Phe\text{-}Arg)_2$rhodamine110.

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fragmentation of the adhesive structures, and (iii) only glycosidase treatment and the subsequent addition of exogenous proteases caused dramatic degradation of the corneodesmosomal structures. 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 (Egelrud and Lundström, 1991); (ii) a trypsin-like protease named stratum corneum tryptic enzyme (Egelrud and Lundström, 1990; Suzuki *et al*, 1994; Ekholm *et al*, 2000); (iii) a protease of the cysteine protease family named stratum corneum thiol protease (SCTP) (Watkinson, 1999); and (iv) the aspartic protease cathepsin D (Horikoshi *et al*, 1998; 1999). 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 HR10/30 column, a MonoS HR5/5 column, and a chromatography molecular weight standard protein kit were from Amersham Pharmacia Biotech (Uppsala, Sweden). Taq polymerase and second antibody antirabbit coupled to peroxidase were from Sigma (St. Louis, MO). The RNA preparation kit RNeasy was obtained from Qiagen (Hilden, Germany). Oligonucleotides were synthesized by Genset (Evry, France). Enzchek protease assay kit "green fluorescence" and Z-$(Phe\text{-}Arg)_2$R110 (ZPA) substrate were from Molecular Probes (Eugene, OR). Mouse monoclonal antibody G36.19 against corneodesmosin was a gift from G. Serre (Toulouse, France). Sheep antihuman cathepsin L antibody cat. no. 1911-0507 was from Biogenesis (Poole, U.K.), human cathepsin L cat. no. 194973 from ICN (Orsay, France), and antirabbit biotinylated secondary antibody from Roche Diagnostics (Meylan, France). Vivaspin™ and Sartocon micro™ ultrafiltration filters were from Sartorius (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 leg. Before scraping, the area was washed with 200 ml of a 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, and finally over a Millipore 0.22 μm filter. The resulting SC extract was concentrated to 6 ml by tangential ultrafiltration on a Sartocon micro™ ultra filter with a cut-off of 10 kDa at 4°C, applying a counter pressure of 1 bar and a filtrate exit flow rate of 2 ml per min using the K.BL apparatus (Sartorius). Protein content was evaluated with the Bio-Rad protein assay, using bovine serum albumin as a standard. The average protein concentration of the extract was about 0.5 mg per ml, i.e., a total of 3 mg per volunteer. 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 HR10/30 column, previously 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 OD_{280nm} 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).

Protease assays and zymograms To detect the caseinolytic activities, we used the Enzchek kit from Molecular Probes, applying the procedure proposed by the manufacturer, maintaining a 10 μl/200 μl sample to substrate buffer ratio. Incubations were performed at 37°C for 18 h. pH studies were performed with the following buffers: buffer A (acetate buffer 0.1 M, pH 4.0–5.75) or buffer B (phosphate buffer 0.1 M, pH 5.75–8.25) complemented with 5 mM EDTA and 0.1% Triton X100 each.

Protease activities in the first 48 fractions obtained after gel filtration were measured with buffers A and B at pH intervals of 0.25. In parallel the same experiment was performed using the buffer containing 5 mM cysteine. To exclude a possible buffer effect on the fluorescence detection, a standard curve with the fluorescent reaction product Bodipy-FL (BPFL) was run with each buffer. Activities are expressed in fmoles released BPFL per min per μl sample. Detection of fluorescence was performed on a Biolumin™ fluorescent plate reader from Bio-Rad (exc. 485 nm/em. 535 nm). Cathepsin-L-like protease activities were detected using the substrate ZPA previously described by Assfalg-Machleidt *et al* (1992) at a final substrate concentration of 10 μM after a 3 h incubation time with a 10 μl/200 μl sample to substrate buffer ratio. The inhibition profiles of the caseinolytic activities, detected at pH 5.5, were studied using the following inhibitors: 1.4 μM E64, 1 μM leupeptine, 2.5 μM chymostatine, 1 μM CA074, 1 μM pepstatine, and 6 μM aprotinine.

The gelatin zymography was performed as described by Watkinson (1999), except that the acrylamide concentration used was 12.5% instead of 11%.

Cathepsin-L-like zymography was developed in our laboratory. After horizontal SDS-PAGE separation on prefabricated gelbond PAG film-supported gel (12.5% acrylamide homogeneous gels or gradient G8-18 gels) of the samples diluted 1 : 2 in Laemmli buffer without dithiothreitol (DTT), a re-naturation step for 30 min at room temperature in buffer A containing 2.5% Triton X100 was introduced. Thereafter the gel was soaked for 1 min in buffer A with 5 mM cysteine and 10 μM ZPA. Detection of the fluorescence was performed on a Fluorimager™ laser scanner (Amersham Pharmacia Biotech).

Corneodesmosin degradation Fraction no. 40, obtained after gel filtration chromatography and exhibiting the highest caseinolytic cysteine-dependent activity, was dialyzed against 0.1 M acetate buffer, pH 5. The dialyzed fraction (100 μl), adjusted to 5 mM cysteine, was added to 1 mg dry weight of acetonic SC powder prepared as previously described (Méhul *et al*, 2000). Two more assays were run in parallel, one with 1 μM leupeptine and another with 1.4 μM E64. The control did not contain proteases. After 24 h at 37°C, the acetonic powder was centrifuged at $10{,}000 \times g$ for 10 min and the resulting pellet was extracted with 100 μl of boiling Laemmli buffer containing 200 mM DTT. After centrifugation ($10{,}000 \times g$ for 10 min), the supernatant was recovered and assayed for protein content using the Bio-Rad protein assay. Proteins were adjusted to 1 mg per ml before Western blot analysis. Mouse monoclonal antibody G36.19, directed against corneodesmosin (Serre *et al*, 1991; Simon *et al*, 1997) was used after 1 : 12,500 dilution in 25 mM Tris–HCl, pH 7.2, 150 mM NaCl, 0.05% (vol/vol) Tween 20 containing 0.5% nonfat milk (TBSTL). Corneodesmosin degradation was assessed by quantification of the 43 kDa, 33 kDa, and 19 kDa SC degradation products of the native cytosolic 52 kDa protein.

Preparation of extracts for Western blotting Reconstructed human epidermis after 13 d of culture or SC or plantar SC obtained from human volunteers was homogenized in Tris 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 ($10{,}000 \times g$ for 10 min). The protein contents of the respective supernatants were quantified 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 Reconstruction of human epidermis with normal human keratinocytes was performed as described previously (Tinois *et al*, 1991). The reconstructed epidermis was examined at different time intervals during its reconstruction (days 1–13).

Western blotting The proteins were submitted to SDS-PAGE on prefabricated gelbond 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 Biologic™ 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 OD_{280nm}.

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 × 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

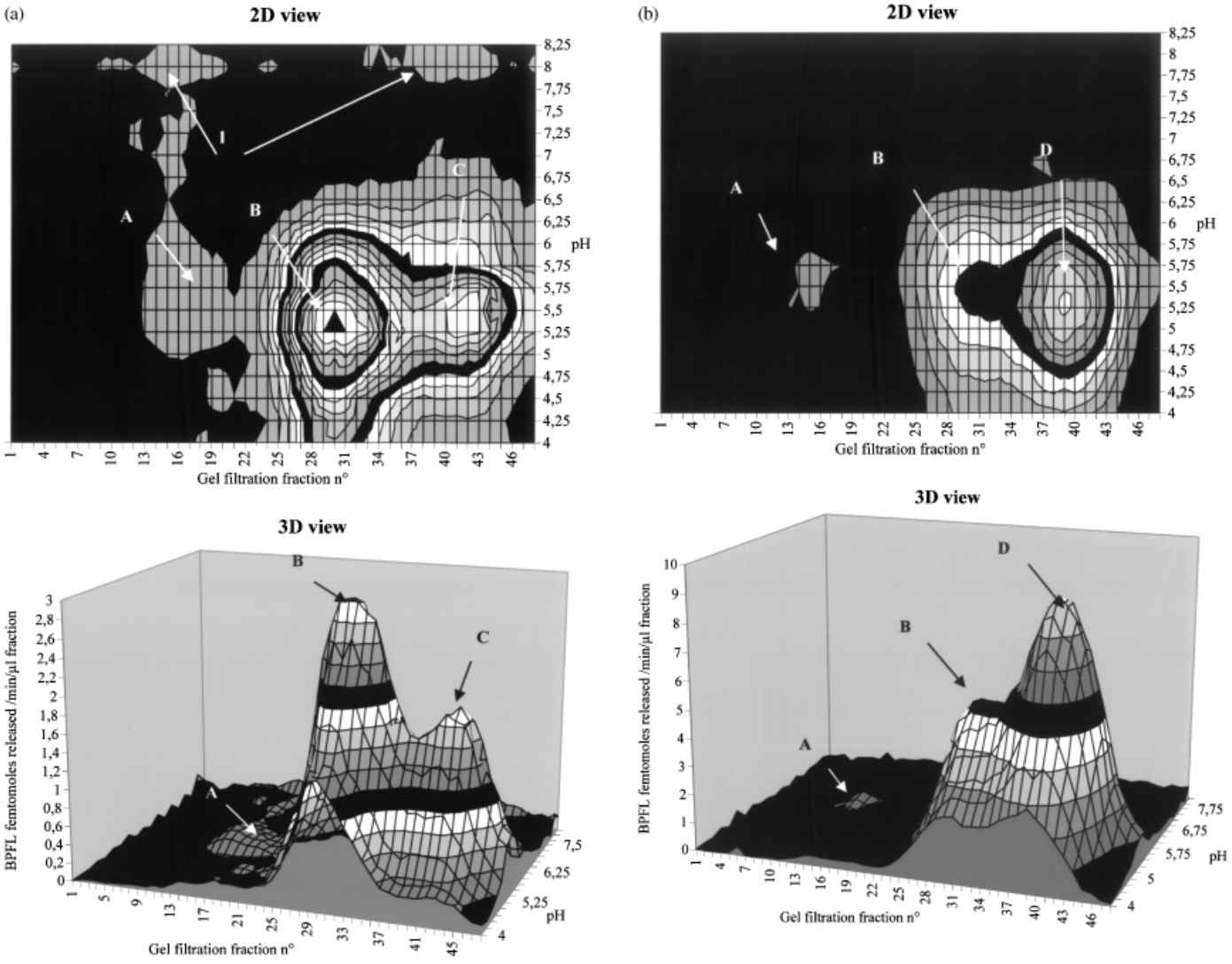

Figure 1. Caseinolytic activities of SC proteases separated by gel filtration. The caseinolytic activities were detected with the Enzchek kit (Molecular Probes) used at pH ranging from 4 to 8.25 on fractions after gel filtration of 125 µg protein extracted from human SC. Very low activities were detectable at basic pH (*a*, *arrow 1* on two-dimensional view). The highest activities were detectable at acid pH split into three groups (*a*): 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 kD 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*).

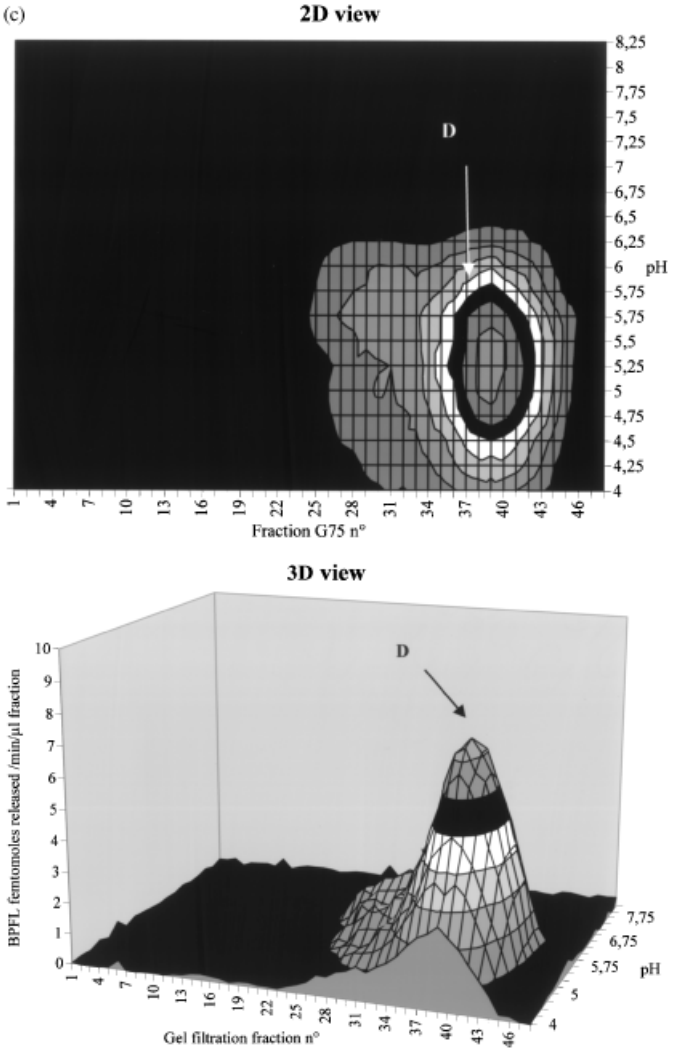

Figure 1. Continued.

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 immuno-purification 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 cm^2 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′-ctcacacatt-ggggtagctggc-3′ and 5′-cttcccaaatctgtggattg-3′; for loricrin, 5′-accacgga-ggcgaaggagtt-3′ and 5′-ctggggttgggaggtagttg-3′; for GAPDH, 5′-aatcc-catcaccatcttcca-3′ and 5′-gtcatcatatttggcaggtt-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 HM500M 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 1*a–c***), a pH characteristic for the surface of the

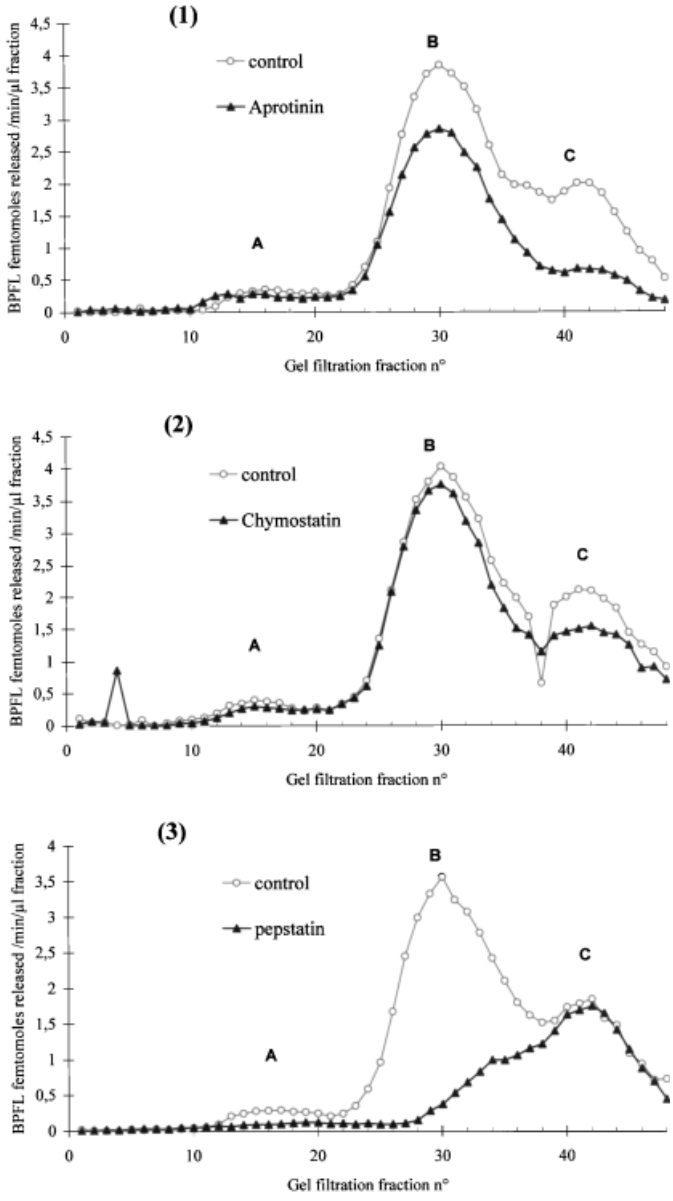

Figure 2. SC caseinolytic protease profiles obtained at pH 5.5 and the effect of specific inhibitors. Group A proteases were essentially affected by 1 µM pepstatin (3). Group B proteases were totally inhibited by 1 µM pepstatin (3) and partially inhibited by 6 µM aprotinin (1). Group C was strongly inactivated by 6 µM aprotinin (1) and to a lesser extent by 2.5 µM chymostatin (2).

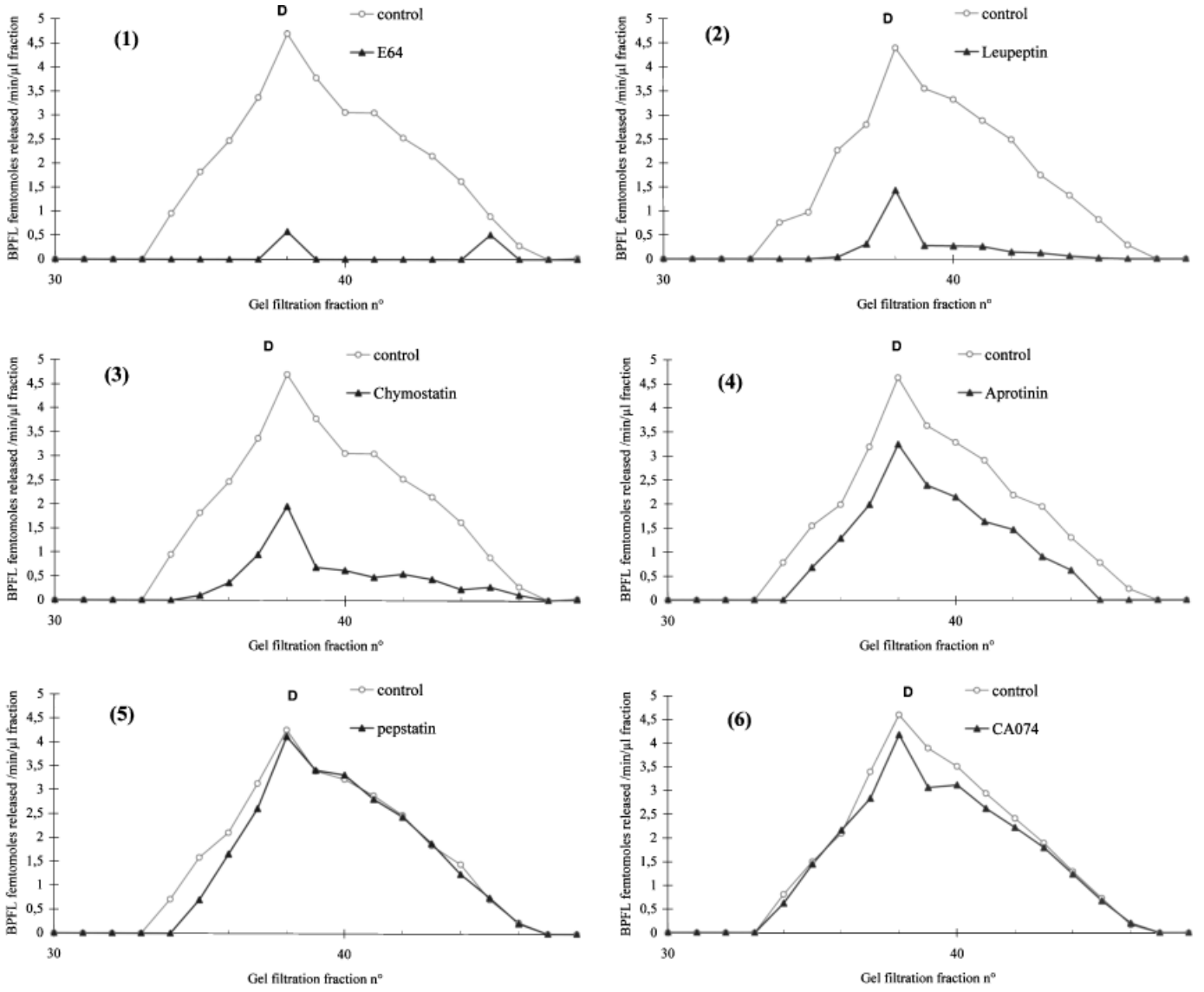

Figure 3. SC caseinolytic cysteine-dependent (group D) protease profiles at pH 5.5 and effect of specific inhibitors. The curves presented were obtained by subtracting the activities obtained with and without cysteine. Group D protease activity was tested in the presence of (1) 1.4 µM E64, (2) 1 µM leupeptin, (3) 2.5 µM chymostatin, (4) 6 µM aprotinin, (5) 1 µM pepstatin, (6) 1 µM CA074.

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 1*a***), 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 1*a***), 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 was not surprising, as Horikoshi *et al* (1998; 1999) described cathepsin D as the major SC proteolytic activity, possibly implicated in desquamation.

Group C, exhibiting a molecular weight of 24 kDa (**Fig 1*a***), 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 1*b*, *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 Barett, 1980). So far, all of the above data suggested the presence of a cathepsin-L-like protease of the papaïn-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).

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.

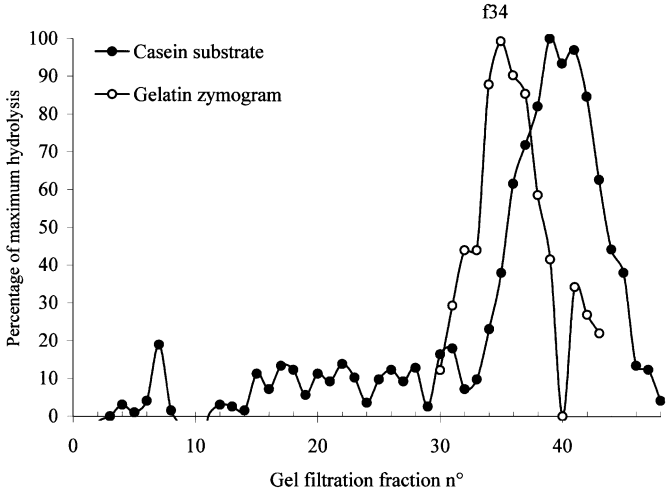

Figure 4. Comparison of SCTP (gelatinase) and group D cysteine-dependent protease activity after gel filtration chromatography. Human SC extract separated by gel filtration chromatography was analyzed for its cysteine-dependent activity. The activity detected on the gelatin zymogram (SCTP) did not correlate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

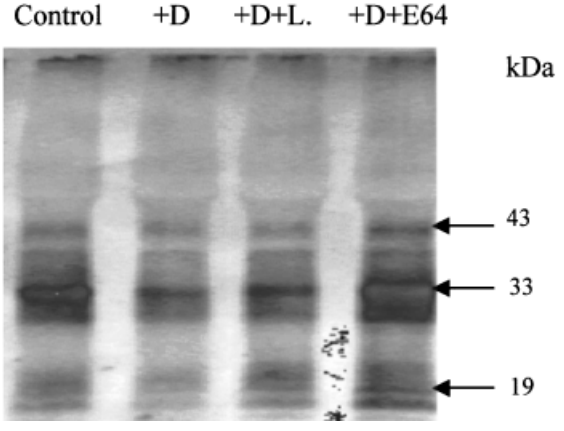

Figure 5. Western blot analysis of corneodesmosins (antibody G36.19) extracted from human SC after exposure to semi-purified group D protease at pH 5 plus 5 mM cysteine. Control, SC incubated without addition of protease; + D, with protease and without inhibitor; + D + L, with protease and with leupeptin 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).

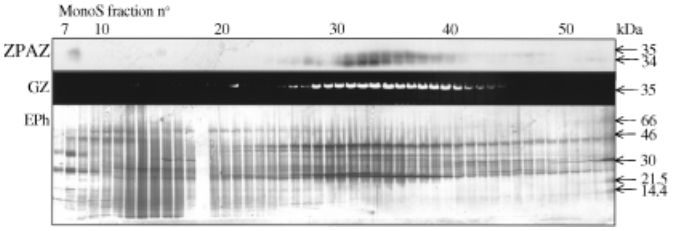

Figure 6. Purification of SCTP by cation exchange chromatography. 5 mg of protein extracted from human SC were loaded on a cation exchange chromatography column at pH 5. Proteins were eluted with a gradient ranging from 350 to 550 mM NaCl in 40 column volumes. Fractions (0.42 ml) were collected in a 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.

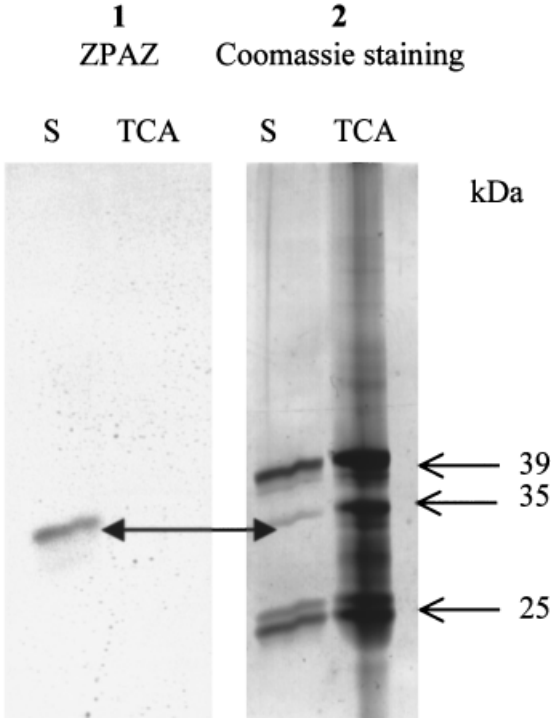

Figure 7. Coupling zymography and protein staining for the identification of purified SCTP. Fractions 32–37 from the cation exchange step supporting SCTP activity (Fig 6) were pooled and concentrated to 250 μl. (S), untreated sample; (TCA), trichloroacetic acid precipitated sample. The same gel was used 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.

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

	Amino acid sequence	aa position extracted in SwissProt sequence for hCatL2 (accession 060911)	aa correspondence in alignement with hcatL sequence. (SwissProt accession P07711)
Edman sequence n*1	XYQWK	30–33	**TK**WK
Edman sequence n*2	LYGANEEGWRR	39–49	LYG**M**NEEGWRR
Edman sequence n*3	FSATGALEGQMFRK	141–154	FSATGALEGQMFRK
Edman sequence n*4	NLDHGVLVVGYGFEGANSNNSK	274–295	**DM**DHGVLVVGYGFE**STE**S**D**N**N**K
Peptide used for rabbit immunisation	CFRNQKFRKGKVFRE	94–108	**G**F**Q**N**R**K**P**RKGKVF**Q**E

*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 L2. 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.

During our detailed analysis of the various protease activities, we performed experiments to make sure that SCCL activity is distinct from SCTP activity. The gelatin zymograms and caseinolytic profiles always identified SCTP and SCCL activity in distinct but neighboring fractions (data not shown). To provide unequivocal evidence that what we called SCCL 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 with the objective of obtaining sufficient material for sequencing. **Figure 7** clearly shows that SCTP-related activity is associated with the 35 kDa protein. The band was cut, trypsin digested, and submitted to Edmann sequencing. To our surprise, the resulting peptide sequence revealed a 100% homology with the recently described cathepsin L2, also named cathepsin U or V protease (Adachi *et al*, 1998; Santamaria *et al*, 1998; Itoh *et al*, 1999; Kinoshita *et al*, 2001). These results indicate that SCTP and cathepsin L2 are two distinct names for the same enzyme. Cathepsin L2 is expressed in colorectal and breast cancer cell lines as well as in tumors of diverse origins, including ovarian and renal carcinomas (Santamaria *et al*, 1998), and in testis, thymus, and cornea (Itoh *et al*, 1999; Kinoshita *et al*, 2001).

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. Brömme *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

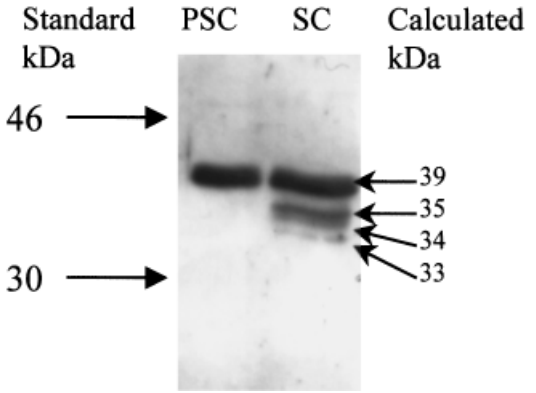

Figure 8. Western blot analysis using the polyclonal anti-cathepsin L2 antibody DBcat15CE. Plantar SC and SC protein extracts (3.7 μg per each lane) were run on a 12.5% acrylamide SDS gel. After transfer to a PVDF membrane, immunoreactive proteins were detected with the ECL method. *Arrows* on the right indicate the calculated molecular weights. *Arrows* on the left indicate the position of two molecular weight markers.

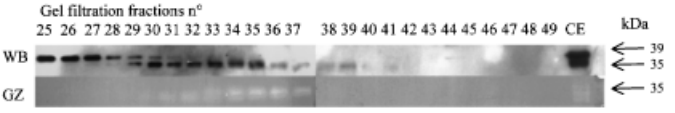

Figure 9. Comparative analysis by Western blot with DBcat15CE antibody and gelatin zymography of SC fractions obtained by gel filtration. (WB), Western blot revealing the 39 kDa inactive form of cathepsin L2 and its 35 kDa active form; (GZ), gelatin zymography revealing the 35 kDa protein supporting cathepsin L2 activity (identical with the 35 kDa in the Western blot). *Arrows* on the right indicate the calculated molecular weights of the detected bands.

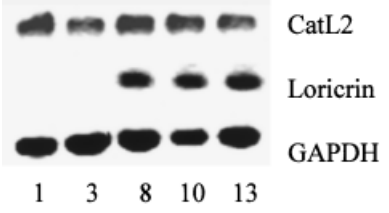

Figure 10. RT-PCR analysis of cathepsin L2 expression during the reconstruction of human epidermis. The figure shows the band intensity of specific amplicons generated by a set of primers corresponding to a differentiation marker (loricrin) and a housekeeping gene (GAPDH) compared with cathepsin L2 (CatL2) (see *Materials and Methods*).

Table II. Proteases implicated in the desquamation process

Name	PROTEASE CLASS	Precursor MW(kDa)	Proposed activation pathway	MW (kDa) of the active epidermal form(s)	optimum pH	Tissue expression	Differentiation related expression?	Genbank n*
SCCE/KLK7	Serine protease	27	Trypsin-like protease	25	basic	Skin ≫ Brain, Kidney	yes	AF166330
SCTE/KLK5	Serine protease	37	Trypsin-like protease	30	basic	Skin Brain, Breast, testis	yes	AF135028
Cathepsin D	Aspartic protease	52	Cysteine protease	33, 48	acidic	Ubiquitary	yes	M11233
*Cathepsin L2/SCTP	Cysteine protease	39	Autocatalytic	34, 35	acidic	Thymus, Testis, cornea > Skin	Not but activation is probably diff. related	AF070448
*SCCL	Cysteine protease	?	?	28	acidic	Unknown	not determined	not determined
*Cathespin E-like	Aspartic protease	?	?	80	acidic	Unknown	not determined	not determined

*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.

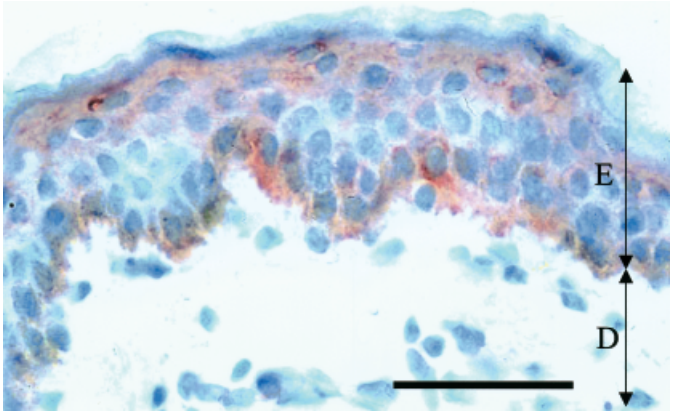

Figure 11. Immunolocalization of cathepsin L2 in human epidermis. The rabbit polyclonal antibody DBcat15CE was used as the primary antibody. Nuclei were counterstained with hematoxylin. E, epidermis; D, dermis. *Scale bar*: 100 μm.

extract revealed the presence of two bands (**Fig 9**), one with a calculated molecular weight of 39 kDa and devoid of catalytic activity in fractions 25–30, and the second with a molecular weight of 35 kDa and catalytic activity evidenced in fractions 29–39. These results clearly show that our antibody recognizes the 39 kDa pro-form as well as the 35 kDa active form of epidermal cathepsin L2. An additional set of experiments was performed to characterize the pattern of cathepsin L2 expression in human epidermis.

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 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 corneodesmosin, a key component of the corneodesmosomes, indicates a possible active function of this enzyme in the desquamation process. Its purification and further characterization are in progress.

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