In the course of a large scale analysis of late-expressed genes in the human epidermis, we identified a new member of the \( \alpha_2 \)-macroglobulin (\( \alpha_2 \)M) protease inhibitor family, \( \alpha 2ML1 \) (for \( \alpha_2 \)-macroglobulin-like 1). Like \( \alpha 2 \)M and PZP, \( \alpha 2ML1 \) is located on chromosome 12p13.31. \( \alpha 2ML1 \) encodes a protein of 1454 amino acids, which fits the characteristics of \( \alpha 2 \)Ms: 1) strong conservation in amino acid sequence including most of cysteine positions with \( \alpha 2 \)M; 2) a putative central bait domain; 3) a typical thiol ester sequence. Northern blot and reverse transcriptase-PCR studies revealed a single 5-kb \( \alpha 2ML1 \) mRNA, mainly in the epidermis granular keratinocytes. \( \alpha 2ML1 \) is also transcribed in placenta, thymus, and testis. By Western blot analysis, \( \alpha 2ML1 \) is detected as a monomeric, \( \sim 180- \)kDa protein in human epidermis. In vitro keratinocyte differentiation is associated with increased expression levels. By immunohistochemistry, \( \alpha 2ML1 \) was detected within keratinosomes in the granular layer of the epidermis, and as a secreted product in the extracellular space between the uppermost granular layer and the cornified layer. Recombinant \( \alpha 2ML1 \) displayed inhibitory activity toward chymotrypsin, papain, thermolysin, subtilisin A, and to a lesser extent, elastase but not trypsin. Incubation with chymotrypsin and the chymotrypsin-like kallikrein 7 protease indicated that \( \alpha 2ML1 \) binds covalently to these proteases, a feature shared with other members of the family. Therefore, \( \alpha 2ML1 \) is the first \( \alpha 2 \)M family member detected in the epidermis. It may play an important role during desquamation by inhibiting extracellular proteases.

Regulation of proteolytic enzyme activity is essential for cell and tissue homeostasis. In epidermis, proteolysis of adhesive structures is a prerequisite for desquamation. Several members of the four protease classes with suggested roles in desquamation have been described in the human epidermis (reviewed in Ref. 1). A wide variety of protease inhibitors is also present in the intercellular spaces of the stratum corneum and participates in the regulation of desquamation-associated proteolysis. Disturbance of the protease-antiprotease balance may have dramatic consequences as demonstrated by the discovery of the serine protease kallikrein 7 (KLK7), also known as stratum corneum chymotryptic enzyme (SCCE), results in a severe phenotype (3). Conversely, a null mutation in the mouse cystatin M/E gene (Cst6), encoding a cysteine protease inhibitor, induces neonatal lethality and abnormalities in cornification and desquamation, highlighting the essential role for protease inhibitors during the final stages of epidermal differentiation (4).

In the course of a large scale search for genes specifically expressed by the last transcriptionally active keratinocytes in the granular layer of the human epidermis, we identified a new member of the \( \alpha \)-macroglobulin (\( \alpha \)M) superfamily, which we named \( \alpha 2ML1 \) (\( \alpha_2 \)-macroglobulin-like 1).

The \( \alpha \)M superfamily comprises both protease inhibitors and components of the complement. The \( \alpha \)M protease inhibitor family, typified by the human tetrameric \( \alpha_2 \)-macroglobulin (\( \alpha 2 \)M), is a class of protease inhibitors with broad specificity (for review, see Refs. 5–7). Because of their abundance in plasma (up to 10% of total serum proteins), they are considered as backup protease inhibitors although their precise function remains incompletely defined.

The ability of \( \alpha \)Ms to inhibit all four protease classes resides in their unique mechanism of inhibition by steric hindrance called the "trap mechanism" (8). The \( \alpha \)M subunits harbor a central bait domain sensitive to proteolytic cleavage (9). Cleavage of the bait region by a protease induces a major conformational change in the \( \alpha \)M and as a consequence, the entrapment of the protease. Thereby, the access of possible substrates to the protease active site is hindered (6, 10). Because of physical constraints, entrapped protease molecules are unable to hydrolyze large substrates but retain almost full activity against small ones (11, 12). Concomitantly to the entrapment, the internal thiol ester bond, distinctive of the \( \alpha \)M, becomes highly reactive and mediates covalent binding with the attacking protease via e-lysyl-g-glutamyl bonds (13, 14). Moreover, the conformational change of the \( \alpha \)M exposes their C-terminal domain, allowing binding to specific receptors such as the \( \alpha 2 \)M receptor/low density lipoprotein receptor-related protein 1 (15) and hence clearance of the \( \alpha \)M-protease complexes. In addition to proteases, \( \alpha 2 \)M binds non-covalently to various distinct proteins, such as inflammatory cytokines, growth factors, \( \beta \)-amyloid peptide, or apolipoprotein E (16–20). The biological importance of \( \alpha 2 \)M in regulating the activity of cytokines and growth factors has been largely documented (16, 21).

Tetrameric, dimeric, and monomeric \( \alpha \)M protease inhibitors have

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been identified in a wide variety of organisms including both invertebrates and vertebrates (22–24). In human, two αM have been described, the tetrmeric α₅-macroglobulin (α2M), and the dimeric pregnancy zone protein (PZP), both being plasma protease inhibitors synthesized predominantly in the liver. Monomeric αM inhibitors have been described in rodents (named muringlobulins) but so far no monomeric form has been described in humans.

**EXPERIMENTAL PROCEDURES**

**Materials**—Trypsin, chymotrypsin, bovine pancreatic elastase, thermolysin, papain, subtilisin A, and human α2M were from Sigma. N-Glycosidase F, O-glycosidase, and neuraminidase were purchased from Roche Applied Science. Anti-V5 monoclonal antibody was from Invitrogen. Human recombinant pro-KLK7 and KLK7 were a generous gift from T. Egelrud (25).

**Biological Materials**—Protein extracts, total RNA, and cDNA mini-libraries were prepared from a plastic surgery specimen of normal abdominal human skin. Paraffin-embedded sections were prepared from breast skin. Primary keratinocytes were from human foreskin. All human specimens were kindly provided by Professor J. P. Chavoin (Chirurgie plastique, Hôpital Rangueil, Toulouse) after informed consent of the patients and in accordance with Helsinki principles.

**Preparation of a Granular Layer Keratinocyte-enriched Cell Population and EST Production**—The procedure to recover total RNA from human epidermis fragments enriched with either basal (sample T1) or granular keratinocytes (sample T4) was described in detail elsewhere (26). Briefly, dermo-epidermal cleavage of the abdominal skin sample was performed after thermolysin incubation. Iterative trypsin incubation and EST Production.

**Analysis of A2ML1 mRNA Expression**—Northern blotting of total RNA extracted from human epidermis after dermo-epidermal cleavage was performed with the digoxigenin technology, according to the manufacturer’s protocol (Roche Applied Science). An A2ML1 probe was produced with the PCR digoxigenin synthesis kit using primers designed from A2ML1 on exons digoxigenin 18 and 19. After hybridization, detection was performed with an anti-digoxigenin alkaline phosphatase-conjugated antibody (digoxigenin Nucleic Acid Detection kit).

For reverse transcriptase-PCR experiments, a primer pair chosen on different exons to avoid amplification of potential contaminating DNA, generated amplicons of 206 nucleotides (exon 29 to 30 of A2ML1). The primer sequences were designed using Primer3 software (28) and Blast analysis (29) for the absence of similarity to any other human sequence. Human Multiple Tissue cDNA (MTC) panels I and II obtained from BD Biosciences were used as templates for PCR analysis. A control reaction with epidermis cDNA was carried out in parallel. The reactions were conducted for 35 cycles in standard conditions. Normalization of the samples was assessed after 22 cycles of amplification using glyceraldehyde-3-phosphate dehydrogenase primers provided in the kit.

For quantitative real-time reverse transcriptase-PCR experiments, reverse transcription was performed by standard procedures, starting from 100 ng of total RNA of T1 and T4 samples and using a mixture of oligo(dT) and random primers. A2ML1 expression was quantified using two pairs of specific primers amplifying exons 29 to 30 and exons 27 to 28. Assays were performed with the ABI prism 7000 Sequence Detection System and analyzed with the corresponding software (Applied Biosystems, Foster City, CA) using the Platinum SYBR Green qPCR SuperMix-UDG kit (Invitrogen). The arbitrary defined number of PCR cycles in which each PCR amplification graph is in the linear range corresponds to the cycle threshold (Ct). The relative amount of A2ML1 to LGALS7 internal control and the -fold induction were calculated by using the equation 2^{ΔΔCt}, where ΔCt = C_{A2ML1} - C_{LGALS7} and ΔΔCt = C_{T4 sample} - C_{T1 sample}. Samples were analyzed in triplicate and differences between the values within triplicates were lower than 0.3 cycles. Amplicons for KRT14, encoding cytokeratin 14 and for KLK7 were analyzed in parallel as additional controls (data not shown).

**Production of Rabbits Antiserum**—A peptide was synthesized according to the predicted amino acid sequence of human α2ML1 in the N-terminal region (547IFYFYLMGKGSVM561). Anti-peptide antibodies were produced by injecting the synthetic peptide conjugated via an added C-terminal cysteine residue to keyhole limpet hemocyanin. Anti-peptide antibodies titer was determined by enzyme-linked immunosorbent assay (Millengen, Toulouse, France). The antiserum was affinity-purified using the peptide coupled to an agarose-activated affinity column (Sulfolink® kit, Pierce).

**Production of Recombinant α2ML1**—The full-length coding sequence of AL832139 (DFKZp686O1010) was cloned into pCEP4 (Invitrogen) in C-terminal fusion with the V5-His epitope and was expressed in stable pools of 293/EBNA cells (Invitrogen). Transfection was performed with JetPEI reagent (QBiogen, Likhir, France). Cells were grown in the presence of 150 μg/ml hygromycin for 2 weeks. After cell washing, serum-free media was conditioned for 48 h, centrifuged at 2000 × g for 5 min, and concentrated on Vivaspin centricrons (Vivascience AG, Hannover, Germany). The α2ML1 protein was then purified by metal chelate affinity chromatography (Ni-NTA Spin Columns, Qiagen). In parallel, control medium, conditioned from mock-transfected cells was submitted to identical purification procedures. The purified protein was monitored after SDS-PAGE by staining with Protogel (BB International, Cardiff, UK).

**Epidermal Extracts, Keratinocyte Extracts, Immunoblotting Experiments, and Deglycosylation**—Epidermal proteins were extracted in TENP-40 lysis buffer (40 mM Tris/HCl, pH 7.5, and 10 mM EDTA containing 0.5% Nonidet P-40 and protease inhibitors). Primary keratinocytes were isolated from human foreskin and grown either in Rheinwald and Green medium (30) or in KGM medium (Promocell). To induce differentiation, sub-confluent cells were either maintained 48 h in the medium of Green (30) or switched into KGM medium supplemented with Ca²⁺ at a final concentration of 1.5 mM. Total proteins were extracted in TENP-40 lysis buffer. For Western blotting, Laemmli buffer with or without β-mercaptoethanol was added to the extracts. The blots were probed with the purified polyclonal antiserum or mouse monoclonal anti-V5 antibody. For deglycosylation experiments, N-glycosidase F, neuraminidase, O-glycosidase, or both neuraminidase and O-glycosidase were added to purified denatured recombinant α2ML1, for 3 h at 37 °C in the conditions recommended by the manufacturer.

**Immunohistochemistry and Immunoelectron Microscopy**—Immunohistochemistry was performed on Bouin-fixed skin samples embedded in paraffin using the peroxidase-labeled streptavidin-biotin amplification method. Immunoelectron microscopy using ultrathin cryosections of normal non-palmoplantar human epidermis were performed as previously described using the purified polyclonal antiserum and gold particle-conjugated anti-rabbit IgG (31). Negative controls consisted in incubations in the presence of secondary antibody alone or with an unrelated primary antibody.
**Characterization of the Human A2ML1 Gene**

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**FIGURE 1. Localization of the A2ML1 gene on human chromosome 12.** The A2ML1 gene is located in a telomeric position relative to A2M and PZP genes on chromosome 12p13.31. Arrows indicate the direction of transcription. Box, genomic alignment of mRNAs from GenBank transcribed from the A2ML1 gene, taken from the UCSC Genome Browser (33).

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**FIGURE 2. Phylogenetic analysis of the a2M superfamily genes.** The accession numbers of the nucleotide sequences and the species are indicated. When already annotated, the gene name is noted on the side. The tree was generated from a multiple alignment of the deduced amino acid sequences using the Multalin algorithm (35). Distances were calculated according to the Dayhoff PAM250 matrix. PAM, percent of accepted point mutation.

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**A2ML1**

**A2M**

**PZP**

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Hide Powder Azure Assay—Human α2M and affinity-purified recombinant α2ML1 were compared for their ability to prevent the various proteases from digesting the "hide powder azure" substrate, essentially as previously described (12). Concentration of α2ML1 was estimated by Protogold staining with respect to various quantities of human α2M (in the nanogram range) loaded on the same gel. α2ML1 molarity was calculated assuming its monomeric status. Purified recombinant α2ML1 protein (0.6–15 pmol) was reacted with 2 pmol each of trypsin, chymotrypsin, elastase, and papain, 1.9 pmol of subtilisin A, and 3 pmol of thermolysin. At least two different concentrations of inhibitor with respect to each protease were tested. Enzyme solutions were freshly prepared and assumed to be 100% active by weight. In parallel, increasing amounts from 0.2 to 2 pmol of human α2M were reacted with each of the enzymes. Papain was activated by adding dithiothreitol (2 mM) and incubating at room temperature for 5 min. A final concentration of dithiothreitol of 0.02 mM was shown not to interfere with α2M inhibition. Reactants were preincubated for 5 min at room temperature in 0.2 ml of 50 mM Tris-HCl, pH 8, 100 mM NaCl, 0.1 mM EDTA (or 5 mM CaCl₂ instead of EDTA when thermolysin was used) before 0.8 ml of the same buffer containing 5.5 mg of hide powder azure substrate was added. Incubation was performed under agitation for 30 min at 37 °C. The reactions were stopped by placing on ice. The high molecular weight substrate was pelleted by centrifugation at 4°C and proteolytic activity was determined by measuring the A₅₉₅ optical density of the supernatants. Each protease was tested with both α2M and α2ML1 in the same experiment. Reactions were performed in duplicates and repeated at least twice. The data were collected from two independent experiments and analyzed by using the StatSoft STATISTICA program, version 6. To compare α2ML1 inhibitory capacities to that of α2M, we determined R₅₀ as the molar ratio of each inhibitor with respect to protease, whereby the proteolytic activity of the protease is decreased to 50%.

**Binding Assays**—For zymography assay, 20 ng of chymotrypsin were incubated with 15 ng of purified α2ML1 or control medium for 10 min at room temperature in phosphate-buffered saline. α2ML1 or control medium were incubated in parallel without addition of chymotrypsin. Non-reducing Laemmli buffer was added to the samples and the reactants were separated by electrophoresis on an SDS-polyacrylamide gel containing 1% casein. The gel was renatured in 0.1 M Tris-HCl, pH 8, 2.5% Triton X-100 for 1 h, then incubated in 0.1 M Tris-HCl, pH 8, 0.2% Triton X-100 overnight at 37 °C, and finally stained with Coomassie Blue (Bio-Safe® Coomassie, Bio-Rad). Casein digestion was evidenced as white, unstained bands. Covalent binding to KLK7 was checked using affinity-purified recombinant α2ML1 (50 ng or 0.3 pmol) incubated with either active KLK7 or the inactive pro-KLK7 (10 ng each or 0.3 pmol) for 10 min at 22 °C or 5 min at 37 °C in 40 mM Tris-HCl, pH 7.4, 150 mM NaCl. The reactions were stopped by adding 0.1 mM phenylmethylsulfonyl fluoride and Laemmli buffer. Reactants incubated on ice were used as controls. Control medium was used as an additional control. The reactants were separated by SDS-PAGE and transferred onto nitrocellulose membrane. The membrane was probed with a polyclonal antibody recognizing both pro-KLK7 and KLK7 (32).
FIGURE 3. cDNA and derived amino acid sequence of α2ML1. The amino acid sequence of α2ML1 is deduced from the sequence of the AL832139 mRNA. The signal sequence is underlined. The bait region is marked by a dashed line. The thiol ester sequence is boxed. Potential N-glycosylation sites are denoted by stars.
Characterization of the Human A2ML1 Gene

RESULTS

Characterization of A2ML1—To identify epidermis late-expressed genes, successive rounds of proteolysis of normal human skin were performed to isolate four batches of cells that roughly represent the successive steps of keratinocyte differentiation. The last batch, mostly containing granular keratinocytes and corneocytes, was used to produce EST mini-libraries by the open reading frame EST method (27). One EST showed complete identity with a full-length cDNA (GenBank accession number AL832139) encoding a hypothetical protein displaying strong conservation with α2M. Thus, in agreement with the HUGO gene nomenclature committee, we named this new gene A2ML1 (for α2M-like 1). The alias name of A2ML1 is CPAMD9 (for C3 and PZP-like α2-macroglobulin containing domain 9). According to the May 2004 human genome assembly (33), the A2ML1 gene is located on chromosome 12p13.31, telomeric to the two other human genes of the αM family, A2M and PZP (Fig. 1). The locus of A2ML1, A2M, and PZP encompasses a 385-kb region. The predicted A2ML1 exon-intron organization (36 exons spanning 54 kb) is similar to that of A2M (36 exons spanning 48 kb) and PZP (36 exons spanning 59.5 kb). A notable difference is the opposite orientation of A2ML1. Twelve mRNA matched the A2ML1 gene (Fig. 1, box), among which AK057908, referred to as the predicted gene FLJ25179, spans only the 3′ part of the A2ML1 transcript. The corresponding hypothetical protein of 158 amino acids (NP653271) was recently classified as a member of the αM family (34). To note, AK122624 mRNA lacks the 3′ part of exon 20, exons 21–26, and the 5′ part of exon 27. The gene has two alternative, non-coding last exons.

Searching for mRNA and EST originating from putative orthologs of A2ML1 resulted in the identification of three mRNA from chimpanzee, dog, and pig that appeared closely related to a2ML1 by using the Multalin algorithm (35) (Fig. 2). No mRNA or EST from rat or mouse was identified as transcribed from a potential A2ML1 ortholog. As illustrated by the phylogenetic tree, even if a2ML1 and the cluster α2M/PZP arose from a common ancestor, they diverged long ago. The cluster of the monomeric murinogulobulins, which evolved after divergence of the rodents and therefore are not present in the human genome (34), were also distinct from a2ML1.

The A2ML1 mRNA encodes a putative protein of 1454 amino acids that displays a similar organization to that of the prototyped α2M (Fig. 3). The α2ML1 protein displays a putative signal peptide of 17 amino acids, a divergent bait domain near the center of the molecule (residues 695–726), and a typical thiol ester sequence (Gly-Cys-Gly-Glu-Gln-Gln) with the putative thiol ester bond formed by Cys278 and Gln973. Of the 10 potential N-glycosylated residues, eight showed striking conservation of position with α2M. The disulfide bridge pattern of α2M as determined by Jensen and Sottrup-Jensen (36) contains 25 cysteinyl residues, 23 of which were found in conserved positions in α2ML1, as illustrated in Fig. 4. However, the two cysteinyl residues involved in the formation of interchain disulfide bridges for α2M were missing in α2ML1. The conservation scores between α2ML1 and the main prototypes of the α2M family assessed by the Clustal W algorithm (37) are presented in Table 1. The best overall amino acid sequence identity was found with human α2M (40% of identity), a score higher than, albeit very similar, to those obtained with human PZP, mouse α2M, or rat α1B. As expected, the identity score was greatly reduced when the bait domains were compared, in agreement with the typical divergence of these regions.

Analysis of the Expression of A2ML1 Transcripts in Epidermis and Other Human Tissues—The expression of A2ML1 was analyzed by Northern blot performed on total RNA extracted from normal human epidermis. A single 5-kb band was detected, consistent with the size expected from the AL832139 cDNA sequence (Fig. 5A). To further investigate the transcription of A2ML1 during epidermal differentiation, we quantitatively analyzed the expression of A2ML1 in epidermal samples representative of the basal (T1) and granular (T4) layers. The cell separation method was validated by a previous work (26) through the analysis of two genes highly specific to the basal and granular layer, KRT14 and KLK7, respectively. Expression levels were calibrated using LGALS7, a gene previously shown by in situ hybridization to be expressed in all epidermal layers (38). A2ML1 displayed a 10-fold T4/T1 expression ratio (Fig. 5B), and is thus clearly an epidermis late-expressed gene.

Human cDNA were used as PCR templates to study the expression of A2ML1 in adult tissues. A representative amplification after 35 cycles is shown in Fig. 5C. A2ML1 was detected in epidermis, placenta, testis, and thymus, but not in epithelia of kidney, lung, small intestine, or colon. The expression of the A2ML1 transcript variant AK122624 was checked on both epidermis and the MTC panels. Using three different specific pairs of primers, we failed to detect any mRNA corresponding to AK122624 (originally cloned from tongue tumor tissue) in any of the tested tissues (data not shown).

α2ML1 Is Secreted as a Monomer and Is Induced in Keratinocytes Differentiated In Vitro—We transfected 293/EBNA cells with a full-length, V5/His-tagged A2ML1 cDNA and analyzed the conditioned medium by Western blot using an anti-V5 monoclonal antibody. A band with 180 kDa apparent molecular mass was detected, most probably corresponding to the full-length protein (Fig. 6A, left panel). This result is consistent with the theoretical molecular mass of 160 kDa tak-
Characterization of the Human A2ML1 Gene

TABLE 1
Pairwise comparison of α2ML1 with α2M, PZP, or α113 proteins using the ClustalW algorithm

|                | Full-length sequence* | Bait domain |
|----------------|------------------------|-------------|
| Human α2M      | I = 40, S = 65         | I = 17, S = 47 |
| Mouse α2M      | I = 39, S = 64         | I = 18, S = 44 |
| Human PZP      | I = 37, S = 63         | I = 9, S = 36 |
| Rat α113       | I = 37, S = 64         | I = 12, S = 23 |

* I, % of identity; S, % of similarity.

FIGURE 5. Detection of A2ML1 transcripts in adult human tissues. A, detection of A2ML1 transcripts in human epidermis by Northern blot. Ten μg of total RNA from normal human epidermis was analyzed using a central A2ML1 probe. A single transcript of 5 kb was detected. B, detection of A2ML1 transcripts by quantitative real-time reverse transcriptase-PCR analysis in epidermal cell samples T1 and T4. Two sets of primers were used to generate amplicons, noted as 1 and 2. The relative amount of A2ML1 to LGALS7 internal control was calculated as ΔCt. The difference of A2ML1 amount between T4 and T1 was calculated as ΔΔCt. The T4/T1-fold induction of A2ML1 was calculated by using the Equation 2. −ΔΔCt, C, detection of A2ML1 transcripts by reverse transcriptase-PCR in human tissues. Epidermal cDNA were produced as described under “Experimental Procedures.” Controls were performed with water and without cDNA. *Other cDNAs were from MTC panels. Besides epidermis, A2ML1 transcripts were detected in testis, placenta, and thymus. **PBL, peripheral blood leukocytes. Bottom panel, glyceraldehyde-3-phosphate dehydrogenase (G3PDH) control amplification leading to a 983-bp fragment.
The treatment increases the mobility of both 180- and 116-kDa (from the backbone)causing scission into two proteolytic fragments of 120 and 60 kDa detected by SDS-PAGE only under reducing conditions (6). Presence of 10 potential N-glycosylated residues, α2ML1 is an N-glycosylated protein. when α2M is heated in the presence of denaturant the internal β-cysteinyl-γ-glutamyl thiol ester bonds react with the polypeptide backbone causing scission into two proteolytic fragments of 120 and 60 kDa detected by SDS-PAGE only under reducing conditions (6). Although this point was not specifically addressed here, the ~55-kDa C-terminal fragment detected by the anti-V5 antibody on reducing SDS-PAGE and the ~116-kDa band detected by the polyclonal antibody (Fig. 6, A and D, arrows) are of the expected size for being the products of the heat-induced hydrolysis of the internal β-cysteinyl-γ-glutamyl thiol ester bond of α2ML1. The ~55-kDa C-terminal fragment observed in reducing conditions was not detected in nonreducing conditions (Fig. 6C, lane 2), suggesting that disulfide bonds connect the two fragments generated upon heating as it is the case for α2M.

Expression Analysis of α2ML1 in Vivo—To localize α2ML1 in the human epidermis, sections of Bouin-fixed skin were analyzed by immunohistochemistry. The labeling signal was predominantly observed within the granular layer at the apical edge of the keratinocytes (Fig. 7A). On parallel control sections incubated in the absence of primary antibody, no significant reactivity was observed (Fig. 7B). The subcellular location of α2ML1 was determined by cryoimmunoelectron microscopy analysis of normal non-palmoplantar human epidermis. α2ML1 localized in small cytoplasmic vesicles containing lamellar structures, corresponding to keratinosomes (Fig. 8, A and B). α2ML1 labeling was also detected in the extracellular space between the uppermost granular...
Characterization of the Human A2ML1 Gene

Protease-inhibiting capacities of α2ML1 and human α2M

Proteases were titrated in a hide powder azure assay with recombinant α2ML1 or human α2M. The R50% values (in bold) were determined as the molar ratio of the inhibitor with respect to protease whereby the proteolytic activity of the protease is decreased to 50%. Data plots were analyzed by using the StatSoft STATISTICA program, version 6. In parentheses are given the 95% confidence interval values.

| Inhibitor      | Thermolysin | Papain | Chymotrypsin | Subtilisin A | Pancreatic elastase | Trypsin |
|----------------|-------------|--------|--------------|--------------|---------------------|---------|
| α2ML1          | 1.24 (1.16–1.32) | 0.08 (0.065–0.1) | 0.77 (0.6–0.94) | 6.5 (5–8.5) | ND*                  | ND*     |
| α2M            | 0.024 (0.023–0.026) | 0.075 (0.071–0.08) | 0.24 (0.16–0.31) | 0.23 (0.19–0.27) | 0.14 (0.14–0.14) | 0.24 (0.19–0.29) |

* ND, not determined. 50% of the proteolytic activity was not reached in the assay. The proteolytic activity was decreased to 70% for a molar ratio of 2.

* No inhibition was observed for a molar ratio of 7.5.

DISCUSSION

Our approach, aiming to characterize the transcriptome of the most differentiated epidermal keratinocytes, led to the description of a novel gene, A2ML1, and its protein expression in the epidermis. Indeed,
Characterization of the Human A2ML1 Gene

A2ML1 encodes a new member of the α2M family mainly differing from A2M by its restricted expression pattern and its monomeric conformation. A2ML1 expression in the epidermis is restricted to the granular layer that corresponds to the last keratinocyte differentiation step still exhibiting transcriptional and translational activity. a2ML1 thus constitutes a new late marker of epidermal differentiation. As expected for epidermis late-expressed genes, A2ML1 expression is up-regulated during the differentiation of normal human keratinocytes induced by Ca²⁺ or by confluence.

We detected A2ML1 transcripts in epidermis, placenta, testis, and thymus. Among the 12 A2ML1 mRNA sequences from GenBank, five arise from tongue tumor, three from cervix carcinomas, two from brain, and one each from normal tongue and testis. Among the 38 EST reported in the UniGene cluster Hs.334306, 17 arise from tissues expressing a squamous epithelia-like differentiation program: nine from tongue, seven from hypopharynx, and one from skin squamous cell carcinoma. Nine additional EST were from colon carcinoma, and the rest were cloned only once or twice from a variety of other tissues. Thus, the origin of mRNA and EST from GenBank confirms the restricted transcription pattern of A2ML1, mainly in normal and tumors stratified epithelia.

The 180-kDa α2M1 protein was detected by Western blot and/or immunohistochemistry in keratinocytes and epidermis. However, it was not detected in commercially available extracts from placenta or testis (data not shown). We assume that in these tissues the α2ML1 protein, if any, is present in scant amounts even though transcripts were evidenced by PCR. Thus, α2ML1 exhibits expression features similar to those of known markers of the terminally differentiating keratinocyte, such as corneodesmosin (45) and SPINK5 (46).

Dimeric and tetrameric αMs are known to be more efficient protease inhibitors than monomeric αMs. Indeed, the inhibitor/trypsin stoichiometry needed for full inhibition is 1:2 for the tetrameric α2M, but as high as 10:1 for the rat monomeric prototype α1I3 (12). For all tested proteases, higher inhibitor/protease molar ratios were necessary to reach 50% inhibition of the proteases using α2ML1 as compared with α2M, although the exact binding ratio of the reactions remains to be quantified. However, our data clearly establish that α2ML1 preferentially inhibits chymotrypsin rather than trypsin, suggesting that chymotrypsin-like proteases may be physiological targets of α2ML1 in vitro. Moreover, α2ML1 also clearly inhibits papain and subtilisin. Subtilisins are chymotrypsin-like serine proteases expressed by various Bacillus species and are known to contribute to host cell invasion. The activity of α2ML1 may be involved in the mechanism of defense against invading pathogens, a role earlier suggested for αMs (10).

Along with the trypsin-like kallikrein 5 (KLK5) also known as stratum corneum trypsin enzyme (47), KLK7 is a pivotal protease synthesized by the terminally differentiating keratinocytes (25). KLK7 is thought to be involved in the proteolysis of the desmosomal proteins corneodesmosin and desmoglein (32). We report that α2ML1 can form specific complexes with KLK7 in vitro, suggesting covalent binding via reaction of the internal thiol ester of α2ML1 with KLK7 lysine residues. This hypothesis is supported by previous reports demonstrating that, unlike tetrameric αMs, monomeric αMs absolutely require covalent binding to target proteases (12). In vivo, both α2ML1 (this study) and KLK7 (44) localize in the keratinocyte secretory vesicles (keratinosomes) before secretion into the extracellular space. Overall, these findings suggest that α2ML1 physically interacts with KLK7. A possible consequence could be the inhibition of proteolysis activity with respect to large substrates because of steric hindrance, whereas accessibility to small substrates would still be effective. Alternatively, binding to α2ML1 could protect KLK7 either from hydrolysis by other proteases or from inhibitors such as the serine protease inhibitor SPINK5, which plays a pivotal role in controlling KLK5- and KLK7-like activities in the upper epidermis (48, 49). Beside KLK7, cathepsin L2, also called stratum corneum thiol protease and cathepsin L-like are papain-like cysteine proteases that are thought to be involved in desquamation (50). The high efficient inhibition of papain by α2ML1 suggests that cathepsin L2 and cathepsin L-like may also be physiological targets of α2ML1 in vivo. Binding to KLK7 and preferential inhibition toward chymotrypsin-like and papain-like proteases support the hypothesis that α2ML1 is a key regulator of desquamation.

Similarly to α2M, α2ML1 is also expected to bind growth factors such as TGF-β1 (19). The growth factor-binding site in α2M for TGF-β1, platelet-derived growth factor-BB, and nerve growth factor-β is located within a short peptide adjacent to the bait domain (51). A peptide, derived from human α2M sequence, has been shown to directly bind to TGF-β1 and block its interaction with TGF-β1 type I and II receptors (52). This peptide sequence (ETWIWLVLV), rich in hydrophobic and acidic residues, is quite conserved in α2ML1 (ETWLWLFLPG). TGF-β1 is synthesized by the upper differentiated layers (53) and is a potent inhibitor of keratinocyte proliferation (54). Therefore, we speculate that α2ML1 binding to such growth factors might play a role in epidermis homeostasis. Moreover, binding of α2M to proteases, growth factors, or cytokines can induce clearance and catabolism of the entire complexes via binding to the low density lipoprotein receptor-related protein 1 receptor (16), although its expression in the epidermis is still controversial (55, 56).

Overall, our findings reveal the presence of a new member of an important class of protease inhibitors at the stratum granulosum-stratum corneum interface. Other members of this class are known to regulate not only protease activities but also the catabolism of proteases, growth factors, and cytokines. α2ML1 might thus play a central and regulatory role not only in the desquamation process but also in the homeostasis of the human epidermis.

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