Cystatin M/E Expression is Restricted to Differentiated Epidermal Keratinocytes and Sweat Glands: a New Skin-Specific Proteinase Inhibitor that is a Target for Cross-Linking by Transglutaminase


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Using serial analysis of gene expression on cultured human keratinocytes we found high expression levels of genes putatively involved in host protection and defense, such as proteinase inhibitors and antimicrobial proteins. One of these expressed genes was the recently discovered cysteine proteinase inhibitor cystatin M/E that has not been characterized so far at the protein level with respect to tissue distribution and additional biologic properties. Here we report that cystatin M/E has a tissue-specific expression pattern in which high expression levels are restricted to the stratum granulosum of normal human skin, the stratum granulosum/spinosum of psoriatic skin, and the secretory coils of eccrine sweat glands. Low expression levels were found in the nasal cavity. The presence of cystatin M/E in skin and the lack of expression in a variety of other tissues was verified both at the protein level by immunohistochemistry or western blotting, and at the mRNA level by reverse transcriptase polymerase chain reaction or northern blotting. Using biotinylated hexapeptide probes we found that cystatin M/E is an efficient substrate for tissue type transglutaminase and for transglutaminases extracted from stratum corneum, and that it acts as an acyl acceptor but not as an acyl donor. Western blot analysis showed that recombinant cystatin M/E could be cross-linked to a variety of proteins extracted from stratum corneum. In vitro, we found that cystatin M/E expression in cultured keratinocytes is upregulated at the mRNA and protein level, upon induction of differentiation. We demonstrate that cystatin M/E, which has a putative signal peptide, is indeed a secreted protein and is found in vitro in culture supernatant and in vivo in human sweat by enzyme-linked immunosorbent assay or western blotting. Cystatin M/E showed moderate inhibition of cathepsin B but was not active against cathepsin C. We speculate that cystatin M/E is unlikely to be a physiologically relevant inhibitor of intracellular lysosomal cysteine proteinases but rather functions as an inhibitor of self and non-self cysteine proteinases that remain to be identified.


Cystatins are natural and specific inhibitors of endogenous mammalian lysosomal cysteine proteinases, such as cathepsins B, L, H, and S (Turk and Bode, 1991; Abrahanson, 1994), and exogenous microbial cysteine proteinases (Bjorck, 1990). Several studies have indicated that cystatins provide important regulatory and protective functions against uncontrolled proteolysis by cysteine proteinases from host, bacterial, and viral origin (Bobek and Levine, 1992). A disturbed balance between proteinases and their inhibitors can lead to irreversible damage as in chronic inflammatory reactions (Henskens et al, 1996) and tumor metastasis (Calkins and Sloane, 1995). In addition to their proteinase inhibitory activity, some of the cystatins (C and S) were shown to have antimicrobial activity against bacteria (Bjorck et al, 1989; Blankenwoorde et al, 1998) and viruses (Korant et al, 1986; Bjorck et al, 1990). Cystatins are members of a superfamily of evolutionarily related proteins and can be divided into three major families (Rawlings and Barrett, 1990): family 1 cystatins and (or steffins A and B), family 2 cystatins (C, D, S, SN, and SA), and the kininogens, which belong to family 3 cystatins. A new member of the human cystatin superfamily, named cystatin M, was recently identified and characterized (Sotiropoulou et al, 1997). The gene was identified by differential display, comparing mRNAs from primary and metastatic breast
tumor cells, and was found to be downregulated in metastatic cells. The same cystatin was independently found to be expressed in activated human keratinocytes, was found at considerable levels in these cells, and was found to be downregulated in metastatic cells. We demonstrate that cystatin M/E is a secreted protein and, in addition to its reported antiproteinase activity, has at least one additional biologic property by acting as an acyl acceptor in Tgase-mediated reactions, thereby becoming a structural part of the CE.

**MATERIALS AND METHODS**

**Keratinocyte culture** Human keratinocytes were obtained from shave biopsies of adult epidermis (lower back) and were primary cultured according to the Rennie-Wall-Green system (Rennie-Wall and Green, 1975). First passage cells were seeded in keratinocyte medium (KGM; BioWhittaker, Walkersville, MD) as described by van Ruisen et al. (1996). Differentiation of keratinocytes was induced by switching confluent keratinocyte cultures for 72 h to KGM supplemented with 5% fetal bovine serum (FBS).

**Cloning of cystatin M/E in baculovirus** Based on the cDNA sequence of cystatin M/E (Sotiropoulou et al, 1997) we designed two oligonucleotide primers that could amplify a partial cDNA, encoding amino acid residue Pro2 to the stop codon at position 150 (cystatin M/E forward primer, 5′-GAGATCTCCACGCGATGCCC-3′; cystatin M/E reverse primer, 5′-CGGATTCTCAGATCATGCACACAG-3′), excluding the amino acid residues corresponding to the hydrophobic signal peptide (Sotiropoulou et al, 1997). Polymerase chain reactions (PCR) were carried out using a DNA thermal cycler (PTC-200, Biozym, Landgraaf, The Netherlands) in 25 µl mixtures. The following buffer conditions were used: 10 mM Tris-HCl, pH 9.0, 1.5 mM magnesium chloride, 50 mM potassium chloride, 0.1% Triton X-100, all four dNTPs (each at 200 µM), 1 unit of Taq DNA Polymerase (Promega, Madison, WI), and 20 pmol of each primer. After an initial incubation of 6 min at 94°C amplification was conducted for one cycle of 1 min at 94°C, 1 min at 47°C, and 2 min at 72°C, followed by 34 cycles with an annealing temperature of 60°C. An additional 10 min at 72°C was added for the last cycle. As template for the PCR we used cystatin M cDNA (Sotiropoulou et al, 1997). The amplified fragment was cloned in frame into the pFastbac-HtB baculovirus expression vector, a component of the Bac-to-Bac Baculovirus Expression System (Life Technologies). After the recombinant pFastbac-HtB donor plasmid has been determined to be correct, the DNA is transformed into competent DH10Bac E.coli cells for transposition into a baculovirus shuttle vector (bacmid), according to the protocol provided by the manufacturer. High molecular weight mini-prep DNA is prepared from selected E.coli clones containing the recombinant bacmid. These clones were screened for the presence of the desired sequences, and then used to transfect insect cells.

**Production of recombinant baculovirus** Recombinant bacmid was transfected into Spodoptera frugiperda (Sf) cells using Cellefectin Reagent (Life Technologies) according to the protocol provided by the manufacturer, followed by infection of insect cells with recombinant baculovirus particles. The infected cells were harvested and lysed, the cell debris was removed by centrifugation, and the supernatant, which contains the recombinant (His6)-tagged cystatin M/E protein, was stored at 4°C.

**Purification of recombinant cystatin M/E** We purified recombinant cystatin M/E with a Tris-based buffer system according to the protocol provided by the manufacturer (Bac-to-Bac Baculovirus Expression System, Life Technologies), with the exception that 2-mercaptoethanol and glycerol were omitted from all buffer solutions. To obtain recombinant cystatin M/E without the polyhistidine tag, the baculovirus expressed protein was digested with tobacco etch virus (TEV) protease according to the protocol provided by the manufacturer (Life Technologies).

**Production and purification of recombinant glutathione S-transferase (GST)–cystatin M/E** The recombinant plasmid pGEX-2T/cystatin M/E was used to produce and purify recombinant GST–cystatin M/E fusion protein as described previously (Sotiropoulou et al, 1997). To obtain recombinant cystatin M/E without the GST tag, the bacterially expressed fusion protein was digested with thrombin according to the protocol provided by the manufacturer (Sigma).

**Polyclonal antibodies** The purified GST–cystatin M/E fusion protein was used to immunize a New Zealand White rabbit and a Duncan Hartley guinea pig, which was carried out at the Central Animal Laboratory, University of Nijmegen, The Netherlands. Antiserum raised against the GST–cystatin M/E fusion protein were purified by affinity chromatography using recombinant fusion protein coupled to CNBr-activated Sepharose 4B (Pharmacia Biotech).

**Autopsic material, skin biopsies, and tape stripping** Autopsic material was obtained from the Department of Pathology, University of Nijmegen, The Netherlands. The following tissues were studied with respect to cystatin M/E expression: skin, tongue, gingiva, palatum,
pharynx, nasal cavity, sole of the foot, esophagus, ileum, colon, stomach, bronchus, lung, trachea, ureter, kidney, bladder, pancreas, liver, heart, spleen, skeleton muscle, lymph node, aorta, cartilage, mammary gland, and uterus. Biopsies of normal skin and from psoriatic lesions were taken under local anesthesia with a keratome as described by Alkemade et al. (1994).

Immunohistochemistry (IHC) Human skin biopsies and autopsy material were processed for IHC as previously described (Latijnhouwers et al., 1996). The sections were subsequently incubated with affinity-purified polyclonal rabbit anticystatin M/E antibodies at a 1:50 dilution followed by IHC staining as described previously (Latijnhouwers et al., 1996).

RINA isolation Total RNA from human cultured keratinocytes and human tissues was isolated with RNA extraction solution (Wigens et al., 1999). mRNA was isolated using the Quickprep Micro mRNA Purification Kit (Pharmacia Biotech), according to the manufacturer’s protocol.

Reverse transcriptase PCR (RT-PCR) amplification of the cystatin M/E transcript First strand cDNA was generated from mRNA as described previously (Zeeuwen et al., 1997). The reverse transcriptase reaction was performed with SuperScript II (Gibco-BRL, Gaithersburg, MD) to obtain cDNA to be used for PCR amplification. The following buffer conditions were used: 10 mM Tris-HCl, pH 9.0, 50 mM potassium chloride, 0.1% Triton X-100, all four dNTPs (each at 200 \( \mu \)M), 1 unit of Taq DNA Polymerase (Promega), and 20 pmol of each primer. After an initial incubation of 6 min at 94°C amplification was conducted for 35 cycles as follows: 1 min at 94°C, 1 min at annealing temperature, and 2 min at 72°C. An additional 10 min at 72°C was added for the last cycle. Annealing temperatures were 58°C when using the cystatin M/E primers and 47°C for the hARP primers. PCR products were analyzed by agarose gel electrophoresis.

Northern blot analysis Northern blot analysis was carried out as described by van Rassen et al. (1996). Cystatin M/E cDNA (Sotropoulou et al., 1997) and hARP cDNA were labeled with \( \alpha ^{32}P \)-dCTP using the Oligolabeling Kit (Pharmacia Biotech) according to the protocol provided by the manufacturer.

Analysis of cystatin M/E inhibitory activity by enzyme assays Papain and cathepsin B assay Protease inhibitory activity of recombinant cystatin M/E was determined by measuring the inhibition of papain (Sigma) and cathepsin B (ICN Pharmaceuticals, Costa Mesa, CA) essentially described by Abrahamson (1994), using the fluorogenic synthetic substrate Z-Phe-Arg-AMC (Sigma). Papain/cathepsin B was titrated in the absence and presence of increasing concentrations of recombinant cystatin M/E.

Cathespin C assay Extracts of normal human skin were tested for cathepsin C activity by measuring the hydrolysis of fluorogenic substrate H-Gly-Gly-Phc-AMC (Bachem, Bubendorf, Switzerland) using a modified protocol (Toomes et al., 1999). The amount of fluorescence signal produced from the substrate by skin extract was measured in sodium phosphate buffer (0.1 M, pH 5.5) containing 100 mM NaCl and 2 mM diethiothreitol. Substrate hydrolysis was measured after an incubation of 30 min at 37°C. Skin extract (containing cathepsin C) was titrated in the absence and presence of increasing concentrations of recombinant cystatin M/E.

Enzyme-linked immunoabsorbent assay (ELISA) for measurement of cystatin M/E levels Cystatin M/E concentrations were measured in supernatant of cultured keratinocytes and human sweat and nasal mucus, using a sandwich-type ELISA. Microtiter plates (96 flat bottom wells) were coated overnight with purified rabbit anticystatin M/E antisera [1:1600 dilution in phosphate-buffered saline (PBS)]. The incubation steps in this protocol are carried out for 45 min at 37°C, and every incubation step in the protocol is followed by a washing step with PBS containing 0.05% Tween-20 (Biorad, Richmond, CA). After washing, the microtiter plates were blocked with bovine serum albumin (BSA, 10 mg per ml in PBS) with 1% normal goat serum and probed with dilutions of test samples and standards (recombinant baculovirus expressed cystatin M/E). As second antibody we used guinea pig anticystatin M/E (1:6000 dilution in PBS, 0.05% Tween-20, 0.1% BSA) with 1% normal rabbit serum. Subsequently, the microtiter plates were incubated with biotinylated goat antimouse pig IgG (Vector Laboratories, Burlingame, CA) with 1% normal rabbit serum, followed by an incubation with the ABC complex. Finally o-phenylenediamine dihydrochloride (Pierce, Rockford, IL) was added as chromogenic substrate for 15 min at room temperature. This enzyme reaction was stopped by the addition of 4 M H2SO4. Data were read with a Biorad ELISA reader at 492 nm and at 655 nm.

Antimicrobial assays Purified recombinant cystatin M/E baculovirus-expressed) without the polyhistidine tag was tested for antimicrobial activity against the bacteria Staphylococcus aureus 42D and the yeast Candida albicans UC 820 in an antimicrobial assay as described previously (Wigens et al., 1998). Recombinant SLPI (a kind gift of Dr. R.C. Thompson, Synergen, Boulder, CO) and cystatin C (a gift from the Department of Oral Biochemistry, VU Amsterdam, The Netherlands) were used in comparison experiments with cystatin M/E.

Preparation of protein extracts Normal skin and epidermal scales from a psoriatic patient were homogenized in buffer containing 50 mM Tris-HCl, pH 7.8, and 100 mM sodium chloride. The protein extracts were centrifuged for 30 min at 25,000 \( \times \) g and the supernatant was subsequently dialyzed against PBS and stored at -20°C until further use.

Cross-linking of cystatin M/E to stratum corneum proteins by endogenous TGase For cross-linking experiments from a psoriatic scale extract were used with 2 \( \mu \)g of baculovirus-expressed purified recombinant cystatin M/E. Buffer and reaction conditions were as previously described (Zeeuwen et al., 1997). Reaction mixtures containing 20 mM ethylenediamine tetraacetic acid (EDTA), pH 7.8, were used as controls. The reaction mixtures were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by electroblotted onto polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA). The membrane was incubated with affinity-purified polyclonal rabbit anticystatin M/E antibodies at a 1:15,000 dilution. This step was followed by incubation with swine antirabbit horseradish peroxidase (SWARPO; Dako, Glostrup, Denmark) at a 1:1000 dilution. Cross-linked cystatin M/E was detected with the Phototope-HRP Western Blot Detection Kit (New England Biolabs, Beverly, MA). This assay uses LumiGLO chemiluminescent reagent and peroxide. The light emitted by destabilized LumiGLO reagent is subsequently captured on X-Omat S1 films (Kodak), and processed using the Imagemaster data image system (Pharmacia).

Cross-linking of biotinylated peptides to cystatin M/E by exogenous TGase Four hexapeptides, based on the TGase substrate motifs of human SKALP/eflin (Zeeuwen et al., 1997), with an N-hy- terminal biotin followed by a C6-spacer were synthesized: GQDPVK, GQDPVR, GNDPVK, and GNDPVR (Eurosequence, Groningen, The Netherlands). For cross-linking experiments 6 \( \mu \)g of baculovirus-expressed purified recombinant cystatin M/E was used with 0.5 \( \mu \)g of biotinylated peptide and 3 \( \mu \)l (0.0313 units per ml) of guinea pig liver TGase (Sigma). The reaction mixtures were blotted directly onto PVDF membranes by a slot blot manifold. The membrane was incubated with an HRP-linked antibiotin antibody, and biotinylated proteins were detected with the Phototope-HRP Western Blot Detection Kit as described above.

Purification of cystatin M/E from human skin Affinity-purified polyclonal rabbit anticystatin M/E antibody was coupled to CNBr-activated Sepharose 4B, and this antibody–agarose conjugate was subsequently used to purify the native cystatin M/E protein from psoriatic scale extracts according to the protocol provided by the manufacturer. The collected proteins were subjected to SDS-PAGE and electroblotted onto PVDF membrane. The membrane was incubated with affinity-purified polyclonal rabbit anticystatin M/E and native cystatin M/E was detected with the Phototope-HRP Western Blot Detection Kit as described above.

SDS-PAGE and western blotting Samples (TGase assay reaction mixtures, purified native protein) were diluted with SDS sample buffer (containing dithiothreitol) and boiled for 2 min. These protein samples were separated by SDS-PAGE on a 15% Tris-glycine Ready gel (Biorad) using Tris-glycine as electrophoresis buffer (Laemmli, 1970).
RESULTS

Cystatin M/E is expressed by epidermal keratinocytes in vitro and in vivo

Keratinocyte gene expression was comprehensively examined by means of SAGE on cultured human keratinocytes. In our SAGE library a total of 25,694 tags were analyzed, corresponding to 10,224 genes, many of which were not known to be expressed in keratinocytes (for details of this analysis see Jansen et al., 2001). A large number of keratinocyte-specific transcripts, coding for proteins involved in epidermal differentiation and skin barrier function, were identified (6% of all transcripts). Surprisingly, a significant part of the identified transcripts accounted for genes involved in host protection such as antimicrobial proteins and proteinase inhibitors (2% of all transcripts). High expression levels were found for members of the cystatin family, SLPI and SKALP/elafin. Cystatin M/E, a recently described family 2 cystatin not previously known to be expressed by adult human keratinocytes, was found at intermediate levels in the SAGE library of cultured keratinocytes (0.04% of all transcripts). On the basis of its expression in our SAGE library we decided to investigate whether cystatin M/E was also expressed in human skin in vivo. Affinity-purified polyclonal antibodies against recombinant cystatin M/E were used for a comprehensive expression study on a large panel of human tissues (see Materials and Methods for a complete list of tissues examined). We found that cystatin M/E showed a tissue-specific expression pattern that was limited to epithelial cells in skin and nasal cavity. Figure 1 shows the immunolocalization of cystatin M/E in these tissues. In normal, nonpalmoplantar skin, cystatin M/E is highly expressed in the stratum granulosum and weakly in the spinous layers (Fig 1a). Positive staining for cystatin M/E was seen in several layers of the suprabasal spinous cells (Fig 1b), whereas the granular cells are not stained as strongly as in normal skin. Differentiated keratinocytes of the infundibular epithelium of the hair follicle were also found to be positive for cystatin M/E (Fig 1c). In plantar skin, positive staining for cystatin M/E was seen in several layers of the suprabasal spinous cells (Fig 1d), which resembles the pattern in psoriatic skin. In the epidermis of the foot sole, a duct of a sweat gland is positively stained for cystatin M/E (Fig 1d, arrowhead). The secretory coils of eccrine sweat glands that are found in the deep dermal part of the foot sole (Fig 1e) are also positively stained for cystatin M/E (arrow). In contrast with the
epidermal part of the sweat gland duct, the dermal part of the duct is almost negative (arrowheads). A slight cystatin M/E staining is found in mucous glands in the nasal cavity (Fig 1f, arrows). No expression of cystatin M/E was seen in any of the other human tissues examined by IHC (data not shown). To examine the expression at the mRNA level we performed RT-PCR analysis on almost the same panel of human tissues. As shown in Fig 2, this analysis confirmed that cystatin M/E gene expression is restricted to human skin, which is demonstrated by the 496 bp PCR product, generated using cystatin M/E specific oligonucleotides. Sequencing of this 496 bp product was performed to verify that it was indeed cystatin M/E. Because the availability of the mRNA of the various tissue samples was limited, we could not perform northern blot analysis on all of them, which would give a more quantitative measure for mRNA levels. Northern blot analysis was performed on skin biopsies (n = 7), foot sole (n = 1), oral epithelia (n = 1), and peripheral blood leukocytes (n = 5), which revealed a strongly hybridizing band of 0.6 kb in the lanes loaded with RNA from skin (Fig 3, lanes 1–7). A weak signal was seen in foot sole but this lane was clearly underloaded compared to normal skin (lane 8), whereas oral epithelia (lanes 9–11) and all blood samples were negative for cystatin M/E (lanes 12–16).

Isolation and characterization of recombinant cystatin M/E To further investigate the functional properties of cystatin M/E we produced recombinant protein, both in a bacterial expression system as a GST fusion protein and as a fully processed protein (lacking the signal peptide) in an eukaryotic system using the baculovirus in insect cells. The purified GST fusion protein was detected on SDS-PAGE as a single ~40 kDa band as previously described (Sotropoulou et al, 1997) (Fig 4, lane 1). Cystatin M/E cleaved from the GST–cystatin M/E fusion protein by thrombin migrates with an apparent size of ~14 kDa (Fig 4, lane 2). The purified baculovirus-expressed protein was found on SDS-PAGE as a doublet, of respectively, ~14 and ~16 kDa, where the upper band was suspected to be a glycosylated species of cystatin M/E (Ni et al, 1997) (Fig 4, lane 3). To test whether both recombinant proteins are functional proteinase inhibitors, cystatin M/E inhibitory activity was measured by titration of papain activity in the absence and presence of increasing concentrations of inhibitor, as described by Sotropoulou et al (1997). It was found that baculovirus expressed (not shown) and bacterially expressed cystatin M/E both displayed inhibitory activity against the cysteine proteinase papain. The inhibitory activity of cystatin M/E cleaved from the GST–cystatin M/E fusion protein is shown in Fig 5. Papain hydrolysis of the fluorogenic substrate Z-Phe-Arg-AMC was almost completely inhibited in the presence of 5 nM cystatin M/E (Fig 5a), whereas recombinant GST did not have any effect on papain activity (not shown). Using the same assay we found a moderate inhibitory activity against cathepsin B as cystatin M/E was used up to micromolar concentrations (Fig 5b). As deficiency for cathepsin C was recently described to be the cause of Papillon–Lefèvre syndrome (Hart et al, 1999), cathepsin C is likely to be an important proteinase for epidermal homeostasis. For this reason we were interested to know if cystatin M/E could inhibit cathepsin C. Based on the pH-dependent activation of cathepsin C by chloride ions (Cigic and Pain, 1999), and using a cathepsin C specific synthetic substrate (H-Gly-Phe-AMC), we could demonstrate the presence of cathepsin C in normal human skin extracts. No inhibition of substrate hydrolysis could be detected, however, in the presence of increasing amounts of recombinant cystatin M/E (Fig 5c). In addition to its proteinase inhibitory activity, we tested cystatin M/E for possible direct antimicrobial activity as was described for other known proteinase inhibitors. Recombinant baculovirus expressed cystatin M/E, which was digested with TEV proteinase to remove the polyhistidine tag, showed no detectable bacterial killing against the tested gram-positive bacterium S. aureus, whereas SLPI, a well-known broad-range antimicrobial protein, effectively inhibits growth of this bacterium (data not shown). Additionally, the growth of C. albicans was completely inhibited by SLPI, whereas cystatin M/E and cystatin C appeared to be ineffective.

Cystatin M/E is a target for cross-linking by TGase The expression pattern of cystatin M/E raised the possibility that this protein could be involved in barrier function in human skin. Furthermore, its presence in terminally differentiated keratinocytes as demonstrated with IHC suggested that it could be a structural part of the stratum corneum, e.g., by TGase-mediated cross-linking. In earlier work we reported that the serine proteinase inhibitor SKALP/elafin possesses TGase substrate motifs that can act as an anchoring sequence to cornified envelope proteins (Zeeuw et al, 1997). To investigate if cystatin M/E is a possible substrate for TGase, we used biotinylated hexapeptide probes based on the TGase substrate motif of human SKALP/elafin in cross-linking reactions that were catalyzed by exogenous tissue type 2 TGase. The TGase cross-linking reaction is based on a Ca2+-dependent exchange of primary amines for ammonia at the γ-carboxamide group of glutamine residues. Peptide-bound lysine residues or polyamines serve as the primary amines to form either ε-[γ-glutamyl]lysine or [γ-glutamyl]polyamine bonds between proteins (Folk and Finlayson, 1977), which are highly resistant to chemical and enzymatic degradation (Folk, 1983). Recombinant cystatin M/E was shown to be an efficient substrate for tissue type 2 TGase (Fig 6a). Cross-linking was demonstrated using the GQDPVK hexapeptide (an acyl donor and acceptor probe) and the GQDPPVR hexapeptide (an acyl donor probe). No cross-linking was observed, however, when the GNPDPPVK hexapeptide (an acyl acceptor probe) or GNPDPPVR hexapeptide (a probe in which both Q and K have been replaced, and which is no longer a TGase substrate) were used, indicating that cystatin M/E acts as an acyl acceptor. Specificity was checked by the addition of excess EDTA, as a control for the Ca2+-dependent reaction (not shown). In addition to cross-linking of cystatin M/E to the biotinylated hexapeptides, we also used an aqueous extract of stratum corneum derived from a psoriatic patient as a source of natural substrate proteins and endogenous TGases (Fig 6b). Analysis by SDS-PAGE showed incorporation of cystatin M/E in proteins predominantly between 30 and 100 kDa (lane 4), whereas the addition of excess EDTA prevents cross-linking of cystatin M/E (lane 5). Lane 3 is a blank of stratum corneum extract without cystatin M/E added. To check self cross-linking, recombinant cystatin M/E was incubated with exogenous TGase type 2

Figure 3. Northern blot analysis of cystatin M/E expression in human tissues. Ten micrograms of total RNA were loaded and probed with 32P-labeled cystatin M/E cDNA. hARP was used for control hybridization to check for equal mRNA loading. Lanes 1, 4, and 7 represent normal skin, and lanes 2, 3, 5, and 6 represent psoriatic skin. Foot sole (lane 8) and oral epithelia (lanes 9–11), which represent, respectively, tongue, gingiva, and pharynx, are negative for cystatin M/E expression. Also, several samples of RNA derived from peripheral blood leukocytes were negative for cystatin M/E (lanes 12–16).
without the scale extract (lane 2), but no additional bands were detected.

**Induction of cystatin M/E in cultured human keratinocytes**  Two other epidermal host defense proteins that were recently described (SKALP/elafin and SLPI) are known to be induced in differentiated cultures of epidermal keratinocytes (Wingens et al, 1998; Pfundt et al, 2000). We investigated the induction of cystatin M/E by FBS, as we have previously shown that SKALP/elafin and SLPI are both highly upregulated by FBS or normal human serum (Molhuizen et al, 1995). We used culture systems that we have previously described (van Ruissen et al, 1996) that allow the induction of regenerative differentiation of keratinocytes by the addition of serum. Northern blot analysis (Fig 7) showed that cystatin M/E mRNA is strongly upregulated in these cultures. This induction was also found at the protein level as detected by ELISA on the supernatant of cultured keratinocytes. In undifferentiated keratinocytes we found no detectable amounts of cystatin M/E in the supernatant, whereas the supernatant of differentiated keratinocytes contained significant levels of cystatin M/E (85 ng per ml).

**Cystatin M/E is a secreted protein in vitro and in vivo**  Based on the hydrophobic amino acid N-terminal sequence of cystatin M/E (Sotiropoulou et al, 1997), coding for a putative signal peptide, it was supposed that cystatin M/E could be secreted. As mentioned in the previous paragraph, cystatin M/E was found to be secreted in vitro in the supernatant of cultured keratinocytes. We also demonstrated by ELISA that cystatin M/E is secreted in vitro in human sweat and in low amounts in nasal mucus (respectively 88 and 7 ng per ml). These results were confirmed by western blot analysis, which showed a single ~14 kDa band of cystatin M/E in concentrated (5×) culture supernatant of differentiated keratinocytes and concentrated human sweat (Fig 8, lanes 2–3), whereas in concentrated culture supernatant of undifferentiated keratinocytes no cystatin M/E could be detected (Fig 8, lane 1). For comparison, recombinant cystatin M/E cleaved from the fusion protein was run on the gel (Fig 8, lane 4). We could also detect native cystatin M/E in psoriatic scale extracts, which was purified by affinity chromatography (Fig 8, lane 5).

**DISCUSSION**

SAGE is an efficient and comprehensive approach to examining cellular gene expression profiles, as we have recently done on cultured human keratinocytes (Jansen et al, 2001). Using this technique we have identified a number of new genes, and several genes not previously known to be expressed in epidermis. One of these, cystatin M/E, is characterized in this paper. We show by IHC that cystatin M/E protein expression in vivo is largely restricted to human skin. This observation is an apparent discrepancy with previous studies, which have reported expression of cystatin M/E at the mRNA level in several other human tissues (Ni et al, 1997; Sotiropoulou et al, 1997). These two papers, however, are also contradictory with respect to the observed expression in tissues such as brain, liver, and peripheral blood leukocytes. A possible explanation for the inconsistencies between these two papers is a difference between expression at the mRNA and protein level. We have found, however, that northern blotting and RT-PCR analysis on mRNA extracted from fresh autopsy and biopsy material confirms the specific expression of cystatin M/E in human skin. It is therefore likely that additional, hitherto unidentified transcripts
with high similarity to the cystatin M/E probe are present in specialized tissues (Ni et al., 1997). This assumption is plausible considering that transcripts larger in size than 0.6 kb were found by northern blot analysis (Sotiropoulou et al., 1997), and it was not clear whether these transcripts originate from cystatin M/E or from a closely related gene.

High cystatin M/E expression at the protein level was found only in the stratum granulosum of normal skin, in hair follicle, and in eccrine sweat glands. In psoriatic skin an increased number of cystatin M/E positive cells was found, possibly resulting from inflammatory cytokines that are known to induce abnormal activities present in mammalian epidermis. Four TGases (TGase 1, 2, 3, and X) are known to be expressed in the epidermis (Boehm et al., 1994). Further regulatory features of cystatin M/E and the possible involvement of inflammatory cytokines remain to be investigated, however.

Both in vivo and in vitro we found that cystatin M/E is a secreted protein. Using IHC, however, we could demonstrate high levels within the cells. In addition, the finding that cystatin M/E could be cross-linked to stratum corneum proteins, a process that occurs within the cell during terminal differentiation, opens the possibility that cystatin M/E also acts within the cell. Although these findings appear paradoxical, similar data have been reported for SKALP/ela®n, which is secreted into the extracellular space, was found paradoxical, similar data have been reported for SKALP/ela®n, which is secreted into the extracellular space, was found paradoxical, similar data have been reported for SKALP/ela®n, which is secreted into the extracellular space, was found paradoxical, similar data have been reported for SKALP/ela®n, which is secreted into the extracellular space, was found paradoxical, similar data have been reported for SKALP/ela®n, which is secreted into the extracellular space, was found paradoxical, similar data have been reported for SKALP/ela®n, which is secreted into the extracellular space, was found that cystatin M/E acts as an acyl acceptor but not as acyl donor. We could not detect oligomerization of recombinant cystatin M/E in a reaction with exogenous TGase 2, suggesting that the glutamine (Q) residues of cystatin M/E do not take part in the cross-linking reaction catalyzed by TGase 2.

The presence of cystatin M/E in the stratum granulosum opens the possibility that this molecule has a role in the physical barrier of human skin formed by the stratum corneum. The protective callus layer resulting from terminal differentiation of the squamous epithelium is thought to be cross-linked by different TGase activities present in mammalian epidermis. Four TGases (TGase 1, 2, 3, and X) are known to be expressed in the epidermis (Greenberg et al., 1991; Kim et al., 1991; 1994; Steinert and Marekov, 1995; Aeschlimann et al., 1998), but thus far only limited data are available with respect to their substrate specificities and relative contributions in CE assembly. In vitro, we have shown that cystatin M/E is an efficient substrate for tissue type 2 TGase in cross-linking reactions with biotinylated hexapeptides. Cross-linking of the biotinylated GQDPVK and GQDPVR hexapeptides to recombinant cystatin M/E was observed, but substitution of the acyl donor residue glutamine (Q) for asparagine (N) totally abolished cross-linking of the biotinylated GNDPVK and GNDPVR hexapeptides. From these results we can surmise that cystatin M/E has multiple functions, both intracellularly and extracellularly.

Figure 6. Cystatin M/E is a target for cross-linking by TGase. (A) Chemiluminescent detection of slot-blotted biotinylated proteins demonstrating TGase-mediated cross-linking to cystatin M/E. Baculovirus expressed cystatin M/E incubated with different biotinylated hexapeptides: GQDPVK, GQDPVR, GNDPVK, and GNDPVR. Amino acid residues that substitute for acyl acceptor (K) and acyl donor (Q) residues are underlined. (B) Western blot analysis of SDS-PAGE demonstrating stratum corneum protein cross-linking to recombinant cystatin M/E by endogenous TGases: 50 ng cystatin M/E (lane 1), and 50 ng cystatin M/E incubated with exogenous TGase type 2 (lane 2); stratum corneum protein extract of psoriatic scales (lane 3), incubated with cystatin M/E (lane 4), and with cystatin M/E in the presence of EDTA (lane 5). Staining was performed with an affinity purified polyclonal rabbit antisera against recombinant cystatin M/E.

Figure 7. Induction of cystatin M/E mRNA expression in cultured keratinocytes by FBS. Ten micrograms of total RNA was loaded and probed with 32P-labeled cystatin M/E cDNA. To check for equal RNA loading, the 18S and 28S ribosomal RNA signals are shown in adjacent lanes (lanes 1 and 3). Lane 2, undifferentiated keratinoctyes cultured in basal medium (KGM); lane 4, differentiated keratinoctyes cultured in KGM with FBS.

Figure 8. Detection of cystatin M/E by western blotting. Concentrated culture supernatants of undifferentiated keratinocytes and differentiated keratinocytes are shown in lanes 1 and 2, respectively, whereas concentrated human sweat is shown in lane 3. Lane 4, recombinant cystatin M/E (14 kDa) cleaved from the fusion protein; lane 5, affinity purified native cystatin M/E from psoriatic scale extract.
Desquamation is the final event in terminal differentiation of the epidermis, which occurs by the action of proteolytic enzymes. During desquamation there is proteinase-mediated breakdown of the desmosomal cohesive complexes that link the corneocytes (Lundström and Egelrud, 1990a, b; Suzuki et al, 1994). There is some evidence indicating that striatum corneum chymotryptic enzyme (SCCE), a serine proteinase, is responsible for the degradation of desmosomes leading to desquamation (Egelrud and Lundstrom, 1991; Hansson et al, 1994). Recently the inhibition of SCCE by SLPI was described, which may be of importance in the regulation of epidermal homeostasis (Franzke et al, 1996). Besides other striatum corneum serine proteinases that have been implicated in desquamation (Suzuki et al, 1993; Brattsand and Egelrud, 1999; Ekholm et al, 2000), recently an aspartic proteinase, cathepsin D, was found that appears to be associated with the final stage of desquamation (Horkoski et al, 1999). Lysosomal cysteine proteinases such as cathepsin B and L are known to be expressed in the basal and spinous layers of normal skin mainly as inactive precursors. These proteins were processed to activated mature enzymes in psoriatic epidermis (Kawada et al, 1997), where they are thought to be involved in the keratinization process. Cystatin M/E is a moderately active inhibitor of cathepsin B, which indicates that this cysteine proteinase inhibitor could have a possible function in the regulation of keratinization and desquamation. It is more likely, however, that the target protein for cystatin M/E is found in the extracellular space as it could be secreted. Interestingly, a new cysteine proteinase of late epidermal differentiation, striatum corneum thiol proteinase (SCTP), was recently described by Watkinson (1999), and could be a possible target enzyme for cystatin M/E. SCTP appears to be a specific product of differentiated keratinocytes, and furthermore it is apparently not stored within these cells but is actively secreted. A role for SCTP in degradation of extracellular stratum corneum substrates, which have been linked with the desquamatory process, and its possible regulation by cystatin M/E remain to be explored.

Finally, in addition to a role in regulation of endogenous proteinases, it is conceivable that cystatin M/E is involved in regulation of exogenous proteinases derived from the commensal skin flora or potential skin pathogens. Although we could not demonstrate a direct microbicidal effect on human skin flora or potential skin pathogens. Although we could demonstrate a direct microbicidal effect in vitro, it is very possible that microbial proteinases involved in skin colonization and invasion are targets for cystatin M/E. Clearly, identification of potential microbial target proteins or generation of cystatin M/E precursors. These proteins were processed to activated mature enzymes in psoriatic epidermis (Kawada et al, 1991; Hansson et al, 1993; Brattsand and Egelrud, 1999; Ekholm et al, 2000).


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