Expression Cloning of Human Corneodesmosin Proves Its Identity with the Product of the S Gene and Allows Improved Characterization of Its Processing during Keratinocyte Differentiation*

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In human epidermis and other cornified squamous epithelia, corneodesmosin is located in the desmosomes of the upper living layers, and in related structures of the cornified layers, the corneodesmosomes. During maturation of the cornified layers, the protein undergoes a series of cleavages, thought to be a prerequisite of desquamation. Partial amino acid sequencing of corneodesmosin fragments suggested that it is related to the product of the S gene, previously identified in the human major histocompatibility complex.

We report the expression cloning of corneodesmosin cDNA from a human epidermis library screened with monoclonal antibodies. Sequencing demonstrated that corneodesmosin is really the product of the S gene. However, analysis of 20 alleles of the gene revealed that its product is 27 amino acids longer than initially reported. Two additional polymorphic sites were described, and the position of the unique intron was ascertained.

Corneodesmosin cDNA expression in COS-7 cells led to secretion of the protein. Precise epitope mapping allowed further characterization of the molecular forms of corneodesmosin present in the most superficial cornified layers, where fragments corresponding to the central region of the protein were detected. This indicated a cleavage of the N- and C-terminal domains of corneodesmosin before desquamation. These serine- and glycine-rich domains are proposed to mediate an adhesive function.

The late stages of the terminal differentiation program in epidermis correspond to cornification, a particular cell death process transforming keratinocytes into anucleated, flattened corneocytes. Stacking of the corneocytes forms the most superficial epidermal layers, designated as the cornified layers or stratum corneum, and is responsible for the mechanical protection of the body. Corneocytes are continually shed from the epidermis surface in the tightly regulated process of desquamation (1, 2).

Corneocyte function is achieved by peculiar structures formed during cornification. The cornified cell envelope, a rigid pericellular shell, consists of covalently linked proteins. Its external side is covered by a monomolecular lipid layer, whereas its internal surface is linked to a cytokeratin-rich fibrous matrix that occupies the entire intracellular volume (3, 4). Intercellular structures derived from desmosomes have been described in the stratum corneum. Indeed, considerable morphological modifications of the desmosomes occur when keratinocytes reach the cornified layers. The desmosomal plaque, thought to be incorporated in the cornified cell envelope, is no longer observed, and the characteristic symmetrical trilamellar structure of the extracellular core becomes transformed into a homogeneous electron dense plug (5–9). These structures, called corneodesmosomes, play a major role in stratum corneum cohesion (9–15). Their degradation, performed at the skin surface, by the stratum corneum chymotryptic enzyme and other less characterized proteases, is required for desquamation (16–19). Although corneodesmosomes have been described for many years, the molecular changes associated with their formation remain unclear.

Using three murine monoclonal antibodies (mAbs)1 raised against homogenates of human plantar cornified layers, we identified a human epidermal protein referred to as corneodesmosin (9). In the upper spinous and granular layers (the last nucleated suprabasal keratinocytes), corneodesmosin is located in specialized secretory vesicles, called keratinosomes or lamellar bodies, that serve as delivery vehicles for intercellular lipids and enzymes. Then, in the upper granular layers, it is also associated to the desmosomal core soon before the transformation of desmosomes into corneodesmosomes. In the cornified layers, corneodesmosome plugs were also shown to contain the protein (9). Corneodesmosin is covalently linked to the cornified cell envelopes in discrete regions corresponding to corneodesmosomes (9, 20, 21). When extracted from the nucleated keratinocyte layers, corneodesmosin is detected as a glycosylated and phosphorylated basic protein with an apparent molecular mass of 52–56 KDa. During stratum corneum maturation, corneodesmosin is progressively proteolysed until desquamation occurs. In superficial corneocytes, the 52–56 KDa

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1 The abbreviations used are: mAb, monoclonal antibody; nt, nucleotide(s); bp, base pair(s); PCR, polymerase chain reaction.
form is no longer detected and immunoreactive fragments of 45 to 30 kDa are predominant (9, 21, 22). A close association exists also between corneodesmosin proteolysis and corneocyte shedding at the epidermal surface in an in vitro model of desquamation (22). Since location, biochemical characteristics and processing of corneodesmosin are similar in several mammals (23), the protein is probably essential for the function of corneodesmosomes and thus for corneocyte cohesion. Amino acid sequencing of two peptides, 10 and 15 amino acids long, obtained after proteolysis of purified human corneodesmosin showed that corneodesmosin is related to the product of the S gene (21), a gene previously identified by CpG island analysis in the class I region of the human major histocompatibility complex (24).

Here, we report the molecular cloning of human corneodesmosin cDNA and demonstrate that corneodesmosin is indeed encoded by the S gene. In addition, the predicted protein is shown to be longer than initially reported, and additional polymorphisms are described. In agreement with its location in keratinosomes, expression in COS-7 cells indicated that corneodesmosin is a secreted protein. Using in vitro synthesized truncated proteins, the epitopes of two anti-corneodesmosin antibodies were localized in the central domain of the protein. Fragments corresponding to this region were detected in extracts of the most superficial corneocytes. This shows that serine- and glycine-rich domains of corneodesmosin, probably folded as loops, are cleaved before desquamation and then could be responsible for putative adhesion properties of the protein.

**EXPERIMENTAL PROCEDURES**

**Materials—**An mRNA purification kit was purchased from Dynal (Oslo, Sweden). cDNA synthesis kit, Zap Express DNA, and Gigapack II packaging extracts were from Stratagene. Modification and restriction enzymes, used in subcloning experiments, and oligonucleotides were purchased from Eurogentec (Seraing, Belgium). DNA sequencing was performed with the ABI PRISM dye terminator cycle sequencing kit (Perkin-Elmer).

**Monoclonal Antibodies—**The anti-corneodesmosin mAbs G36-19, F28-17, and B17-21 are part of a series of antibodies produced and previously characterized in our laboratory (9, 23). The ascites fluid containing the mAb MOPC-21 (Sigma) was used as a negative control. In all experiments, G36-19 and F28-27 were used at 0.2 μg/ml; B17-21 and MOPC-21 were used at 2 μg/ml. The Western blotting method has been precisely described previously (23). Peroxidase-conjugated sheep peroxidase-conjugated sheep anti-mouse and anti-rabbit immunoglobulin G antibodies (Dako) were used at a 1:1,000 concentration.

**Construction and Screening of a Human Skin cDNA Library—**Normal skin (kindly provided by Prof. M. Costaglia, Rangueil University Hospital, Toulouse, France) was obtained from plastic surgery specimens and used immediately. Thin fragments obtained with a dermatomic were incubated for 2 h at 37 °C, in a 0.25% trypsin solution (solute A, Life Technologies, Inc., Cergy Pontoise, France, dermal side down. The epidermis was then peeled off with forces, rinsed gently in phosphate-buffered saline, pH 7.4, and homogenized in the lysis/binding buffer from the mRNA purification kit. Poly(A) RNA was extracted with oligo(dT)20-tagged magnetic microspheres, according to the supplier’s instructions, and quantified with the DNA DiStick kit (Invitrogen). cDNAs were synthesized by oligo(dT) priming of 2 μg of poly(A) RNA, size-fractionated (500 bp) and unidirectionally cloned in lambda ZAP Express vector, following the instructions of Stratagene. The library, consisting of approximately 2 × 1010 primary recombinants, was packaged with Gigapack II packaging extracts (Stratagene) and plated for immunoscreening without amplification.

The library was screened with a pool of the three mAbs G36-19, F28-27, and B17-21 essentially as described in the instruction manual of the picoBlue immunoscreening kit (Stratagene). Positive clones were plaque-purified and tested with each of the mAbs. In vitro excision of the pBK-CMV phagemid vectors from ZAP Express clones was performed as described by Stratagene. cDNA inserts were analyzed by restriction mapping and one-run sequencing, using T3 and M13 primers. Comparisons of the 3′ and 5′ sequences with the data bases were performed with BLAST software (25).

**Cloning of Full-length cDNA for Corneodesmosin—**To obtain the complete cDNA of corneodesmosin, poly(A) RNA (80 ng) was primed with random hexamers and reverse-transcribed using the SuperScript kit (Life Technologies, Inc.). PCR amplification was performed on 1/20th of the cDNA reaction mixture. The upper primer corresponded to S cDNA sequence from nucleotide (nt) 2 to nt 21 (GenBank L20851), with a SpeI linker. The lower primer was complementary to nt 998–1017 of the same sequence. The PCR product was digested with SpeI and EcoNI restriction enzymes and cloned in the pBK-CMV phagemid isolated from clone 1.1, leading to pS11. To generate an expression vector, the 2106-bp EcI136I/NotI fragment from plasmid pS11 was subcloned into pcDNA 1.1/amp (Invitrogen) digested with EcoRV and NotI, resulting in p14.9.

**DNA Sequencing and Genomic Analysis—**p14.9 was sequenced using various primers that were located from the T7 promoter priming site up to nt 1767 of S cDNA sequence. The Oligo software was used for primer design optimization, and sequencing reactions were repeated three times. Genomic analyses were performed on human DNA (obtained from Prof. M. Abbal, Rangueil University Hospital) extracted from blood samples of 10 healthy donors. Approximately 400 ng of genomic DNA or 0.1 ng of p14.9 were used for PCR amplification.

The nature of the nucleotides at positions 1118 and 1236 was assessed by restriction analyses after PCR amplification with primers 908–925 and 1573–1593 in standard conditions. Position 515 was analyzed in the same way with primers 1446–1465 and 1767–1786. To determine the nucleotide at position 66, allele-specific amplification was performed in two separate PCR mixtures, with forward specific primers 52–65 bearing A or T at position 66 and reverse unspecific primers 572–591 primer after optimization according to Sommer et al. (26). After 2 min of initial denaturation at 94 °C, 30 cycles were performed with 50 s of denaturation at 94 °C, 1 min of annealing at 56 °C, and primer extension at 72 °C for 1 min when p14.9 was used as template, or 2.5 min when genomic DNA was used.

**Expression in Mammalian Cells—**COS-7 cells (a kind gift from Dr. M. Corbani, CNRS, Toulouse) were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and transfected by electroporation with a gene pulser (BioRad), using 15 μg of plasmid per 2 × 106 cells (960 microfardas, 240 V in 400 μl of serum-free Dulbecco’s modified Eagle’s medium). After 24 h of culture, cells were rinsed three times with serum-free medium and grown in the absence of serum for an additional 30-h period. To purify secreted corneodesmosin, conditioned medium was centrifuged for 10 min at 3,000 rpm to eliminate cell debris. After addition of a protease inhibitor mixture (PharMingen), 15 ml of medium was purified by anion exchange and affinity chromatography, as described previously (21). To analyze intracellular corneodesmosin, cell monolayers were solubilized in lysis buffer (Tris-HCl, 40 mM, pH 7.5, 10 mM EDTA, 0.5% Nonidet P-40) containing the protease inhibitor mixture and cleared by 15 min of centrifugation at 10,000 × g. Samples were probed by Western blotting. Proteins from human epidermis extracted and analyzed with the same procedures were used as positive control for corneodesmosin.

**Epitope Mapping by PCR and in Vitro Cell-free Transcription/Translation—**To obtain serial C-terminal deletions of corneodesmosin, PCR was performed on p14.9 with an upper primer consisting of a promoter for T7 RNA polymerase linked to nt 15–34 (primer 14, Fig. 4) and a series of lower primers bearing an in-frame stop codon (primers 15–21, Fig. 4). PCR products were directly subjected to in vitro transcription/translation with the TNT-T7 coupled reticulocyte lysate system, according to the recommendations of Promega (0.5 μg of p14.9 or 3 μl of PCR reactions and 20 μl of the Quick Master Mix, 90 min at 30 °C), and analyzed by Western blotting.

**Immunodetection of Corneodesmosin Extracted from Human Stratum Corneum—**Superficial stratum corneum extracts were obtained as follows. Stratum corneum was collected with a nylon mesh (40 μm; Spectrum Medical Industries, Houston, TX) and painted with acetone-soluble varnish. After drying for a few minutes, the painted area was stripped. After a brief incubation in acetone, the tissue was recovered by centrifugation (500 × g, 1 min), washed in acetone, and air dried. The resulting powdery material (a kind gift from D. Bernard, L’Oreal, Clichy, France) was boiled for 10 min in 62.5 mM Tris-HCl, pH 6.8, containing 2% SDS and 0.2 μM DTT, and the suspension was clarified by centrifugation (10,000 × g, 10 min). Laemmli’s sample buffer was added to the supernatants, and the extracts were analyzed by Western blotting.
Corneodesmosin Is Encoded by the S Gene

RESULTS

Construction of a Human Epidermis Expression Library and Cloning of Corneodesmosin cDNA—To clone corneodesmosin cDNA with specific mAbs, poly(A)⁺ RNA was extracted from breast epidermis, and an expression cDNA library was constructed in a lambda-derived vector. The library, containing 2 × 10⁷ independent clones, was screened with a mixture of the three anti-corneodesmosin mAbs, G36-19, F28-27, and B17-21. Among 16 positive clones, five were plaque-purified and tested individually with each mAb (Fig. 1a). Three clones (1.1, 4.4, and 5.1) reacted with the three mAbs, one clone (5.5) reacted with G36-19 and F28-27, and one clone (1.2) was only positive for F28-27 (Fig. 1b). The inserts were sequenced from both extremities in one run. The sequences obtained were found to be 99% homologous to parts of the human keratinocyte S cDNA (GenBank file L20815; Ref. 24), as shown in Fig. 1c. The reactivity profile of each mAb toward the various clones, and the determination of the boundaries of the inserts, allowed us to delineate segments of the cDNA encoding each epitope. The epitopes recognized by B17-21 and G36-19, and F28-27 epitopes to be localized at nt 594/762, 762/1044, and 1044/stop, respectively.

Fig. 1. Immunoanalysis of recombinant phage clones. a, immunodetection of protein products from recombinant phages. After three rounds of phage isolation, nitrocellulose filters were cut into three parts that were immunodetected with the anti-corneodesmosin mAbs G36-19, F28-27, or B17-21, as indicated. Clone 5.1 was positive for the three mAbs, whereas clone 5.5 was positive for G36-19 and F28-27 but not for B17-21. b, immunoreactivity of five corneodesmosin clones toward the three mAbs in relation with the boundaries of the phage inserts as determined by sequence analysis. Nucleotide positions on the left schema are relative to the published sequence of the S gene cDNA. The data allow the sequences coding for the B17-21, G36-19, and F28-27 epitopes to be localized at nt 594/762, 762/1044, and 1044/stop, respectively.

Two S mRNAs of 2.2 and 2.6 kb, resulting from alternative polyadenylation splicing have been described (24). The 3′ end of clone 5.1 corresponded to the longer form, whereas the 3′ end of both clones 1.1 and 5.5 matched the shorter form, confirming the use of alternative polyadenylation signals in vivo. The 3′-untranslated region of both clones 4.4 and 1.2 was 9 nt longer than that of clones 1.1 and 5.5 (see Fig. 1b).

As none of the clones contained the ATG initiation codon of the S protein, the 5′ end of corneodesmosin cDNA was amplified by reverse transcriptase-PCR using poly(A)⁺ RNA extracted from human epidermis and primers derived from the GenBank sequence. The amplified product was cloned in phagemid 1.1 to reconstitute the entire coding sequence. The resulting insert was subcloned in pcDNA 1.1/amp leading to p14.9 and was sequenced from nt 2–1700. Four punctual differences were found between the corneodesmosin and S protein cDNA sequences. The insertion of G at position 1515 in the corneodesmosin sequence induced a frame shift resulting in the extension of the reading frame. The TAG codon at position 530 (the first ATG codon being designated as codon 1) replaces the stop codon TAG predicted in the S protein cDNA sequence at position 503. The two C-terminal amino acids (IS) of the predicted sequence of the S protein (s) are therefore replaced in the corneodesmosin (c) sequence by the 29 following amino acids: DILAQVRPLGQSLAQEAFPLPQGELLDPS. A silent mutation at codon 368 (GCA⁹→GCGc alanine) and two amino acid changes at codons 18 (TTO⁹→ATGc methionine) and 408 (TCC⁹→GCCc alanine) were also identified. Ten human genomic DNA samples (individuals 1–10) were analyzed by PCR to determine whether or not these sequence discrepancies originated from genetic polymorphism.

Analysis of the Polymorphism of Corneodesmosin Coding Sequence—We first analyzed a potential polymorphism at nt 1515 (Fig. 2a). The insertion of a G at this position in the corneodesmosin sequence created a NciI restriction site and suppressed a BsmI site present in the S protein sequence. The genomic fragment encompassing nt 1446–1796 was amplified by PCR from the 10 genomic DNA samples as well as from p14.9. In all cases, a fragment of the expected size (341 bp) was amplified and digested by NciI into fragments of 276 and 65 bp (Fig. 2a and data not shown). None of the amplified products were digested by BsmI. Consequently, all 10 genomic DNA samples matched the corneodesmosin, rather than the S protein, sequence. As all of the 20 alleles analyzed conformed to the corneodesmosin sequence, we speculate that the absence of a G at this position in the published S protein sequence results from a sequencing error, although the existence of a rare allele cannot be excluded. The corneodesmosin sequence has therefore been submitted to the EMBL/GenBank data base.

Sequence divergences at nt 1118 and 1236 were analyzed by a similar methodology (Fig. 2b). Sequence analysis predicted a BsmI restriction site at nt 1115 in the S protein cDNA but not in that of the corneodesmosin. Conversely, a HincII restriction
Corneodesmosin Is Encoded by the S Gene

Among the 20 alleles analyzed, 8 matched the protein gene sequence, suggesting homozygosity at this position. Conversely, among the 20 alleles analyzed, 8 matched the protein S sequence at nt 1118, whereas 3 matched the corneodesmosin sequence at nt 1236, establishing polymorphism at these two positions.

The position of the intron was analyzed by sequencing an amplification product obtained from genomic DNA sample 2 with primers 2–20 and 275–294. The 5′ splice site was AG<sub>ref</sub> GTAGGA and the 3′ splice site was ACAG/G½<sub>100</sub>GACCT.

Expression of Recombinant Corneodesmosin—To characterize the recombinant protein, COS-7 cells were transfected with p14.9. After 2 days of expression, the cells were lysed in a hypotonic buffer containing 0.5% Nonidet P-40 to analyze cellular corneodesmosin. A Western blotting analysis with G36-19 revealed an immunoreactive protein with apparent M<sub>r</sub> 62,000, which was not present in mock-transfected cells (Fig. 3, lanes 1 and 2). No additional immunoreactive proteins were detected when the cells were directly solubilized in Laemmli’s sample buffer (data not shown). Despite a transfection efficiency of approximately 30% (data not shown), corneodesmosin was only detected on overloaded gels after a 30-min exposure time, suggesting a low expression level. The presence of a site at nt 1234 was only predicted by the corneodesmosin sequence. The 908–1593 segment was amplified by PCR with the 10 genomic DNA samples, with p14.9 as a control, and submitted to enzymatic digestion by either BsmI or Hin6I. As expected, the PCR product from p14.9 was totally digested by Hin6I but was unaffected by BsmI digestion (Fig. 2b). Among the DNA genomic samples, six were partially digested by BsmI (see sample 1), one was totally digested with BsmI, whereas three were partially digested by Hin6I (see sample 4). Consequently, among the 20 alleles analyzed, 8 matched the protein S sequence at nt 1118, whereas 3 matched the corneodesmosin sequence at nt 1236, establishing polymorphism at these two positions.

At position 66, the sequences of the corneodesmosin and S genes contain A and T, respectively. To analyze a potential polymorphism at this position, the genomic DNA samples were amplified by PCR using allele-specific 5′ primes and a common 3′ primer. In a preliminary control experiment using p14.9 as a template, a band of the expected size (539 bp) was amplified with the 5′ primer corresponding to the corneodesmosin sequence, but not with that of the S protein (Fig. 2c). With one genomic DNA sample (individual 1), a fragment of 3.4 kb was amplified with both allele-specific 5′ primes (Fig. 2c). The size of this fragment was expected from the presence of a 2.9-kb intron between the primers. We therefore concluded that this genomic DNA was heterozygous at this position. Among the 10 DNA samples analyzed, 4 had the same profile. Six samples (including that from individual 2 shown in Fig. 2c) were only amplified with the 5′ primer corresponding to the corneodesmosin sequence, suggesting homozygosity at this position. Among the 20 alleles analyzed, 16 were of the corneodesmosin type and 4 of the S gene type. No sample homozygous for the S gene allele was found.

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Corneodesmosin Is Encoded by the S Gene

The results of representative analyses are shown in Fig. 4a. After in vitro transcription/translation, the PCR product generated using 3′ primer 20 gave a positive signal with G36-19, whereas no signal was detected with 3′ primer 21. F28-27 gave a positive signal with 3′ primer 19, but not with primers 20 or 21. Therefore, the data showed that the F28-27 epitope is encoded by the cDNA sequence located between primers 19 and 20 and the G36-19 epitope by the sequence between primers 20 and 21. None of the 3′ primers resulted in the detection of a specific signal with the unrelated mAb MOPC-21. Similar experiments were repeated with a new series of 3′ primers (Fig. 4b). The in vitro produced protein with the C-terminal sequence PISEGKYFSSM(353) was positive for F28-27, but the protein with C-terminal PISEG(343) was negative (data not shown). In addition, the N terminus of the protein encoded by the clone 1.2, which was only recognized by F28-27, was PI-SEGKYFSS. Therefore, the epitope recognized by F28-27 was localized around amino acids 349–353 (KYFSS). A similar analysis revealed that the G36-19 epitope was localized around amino acids 306–309 (YLVP).

Analysis of Corneodesmosin Extracted from Normal Superficial Corneocytes—Localization of the epitopes allowed us to characterize the molecular forms of corneodesmosin present at the epidermis surface more precisely. Proteins were extracted from corneocytes in the presence of SDS and dithiothreitol and analyzed by Western blotting (Fig. 5). After a brief exposure time, a complex pattern of molecular species with molecular mass extending from 45 to 30 kDa was revealed with all three anti-corneodesmosin mAbs, G36-19, F28-27, and B17-21, in agreement with our previously published data (22). After a longer exposure time, immunoreactive bands with higher molecular mass were observed. These bands correspond to corneodesmosin aggregates, or most probably to protein complexes including corneodesmosin. In addition, the mAbs G36-19 and F28-27 recognized corneodesmosin forms as small as 20 and 15 kDa, respectively. On the contrary, although B17-21 labeled additional bands, none of them were smaller than 30 kDa.

**DISCUSSION**

This report describes the cDNA cloning of corneodesmosin, a protein incorporated in preexisting desmosomes of cornified squamous epithelia that will undergo maturation into corneodesmosomes. Corneodesmosin is thought to play a key role in corneocyte cohesion, and its proteolysis seems to be a major event leading to desquamation.

First attempts in corneodesmosin cloning using a commercial human expression library from exponentially growing keratinocytes were unsuccessful, in agreement with corneodesmosin being a late differentiation antigen, undetectable in keratinocyte monolayer cultures (9). We therefore constructed a library with mRNA extracted from normal human epidermis. This library is, to our knowledge, the first reported human epidermis expression library and thus may be a relevant tool to clone cDNAs encoding proteins only expressed in the late stages of epidermal differentiation.

Partial amino acid sequencing of corneodesmosin proteolytic fragments (21) showed that the protein shares two peptides, 10 and 15 amino acids long, with the putative product of the S gene, isolated in the human and porcine major histocompatibility complex genomic region (24, 27). This indicated that corneodesmosin and the S gene product are related. Here, cloning of corneodesmosin clearly demonstrated that the protein is encoded by the S gene. The sequence of the coding region of the complete corneodesmosin cDNA differed from the previously published sequence only at four positions. Because the insertion of a G at nt 1515 was present in all 20 alleles examined, we believe that the protein is 27 amino acids longer than initially predicted. On the contrary, the three other discrepancies are substitutions corresponding to genetic polymorphisms.

Two of them were found to induce amino acid exchanges. The third, a synonymous substitution, had already been reported.
In fact, nine diallelic polymorphic sites within the coding region of the \( S \) gene have already been described in a Japanese population (28), three among them resulting in an amino acid substitution. It is not possible at the moment to know if any of these substitutions modify the protein function. However, it can be emphasized that among the five amino acid substitutions described within the coding region of the \( S \)/corneodesmosin gene, three are consecutive substitutions at amino acid positions 408, 409, and 410. Exchange of the three polar amino acids SGS(410) by the hydrophobic tripeptide AVL(410) could interfere with the secondary structure of the protein and possibly with its function. The substitution at nt 66 could also be of particular interest as it introduces a fourth AUG codon in the 5' end of the mRNA and modifies the context of the third AUG codon. It has been postulated that this third AUG codon (codon 17) defines the translational initiation site (24). However, in our point of view, and according to Kozak's model (29), the transcription is rather initiated at the first AUG codon at nt 15–17, which is located in a favorable context, at a distance from the cap site compatible with translation initiation.

The predicted amino acid sequence of corneodesmosin revealed its surprisingly very high content of serine (27.5%), glycine (16%), and proline (10%); these three amino acids represented more than half of the protein. When the Swiss Prot data base was scanned for human proteins containing more than 20% of serine and 12% of glycine, the request only evidenced three other epidermal proteins: (i) filaggrin, a cationic intermediate filament-associated protein that participates in the formation of the corneocyte fibrous matrix, (ii) a keratin only expressed in cuticle layers of differentiating hair follicles (ultra high sulfur matrix keratin), and (iii) loricrin, a major component of the cornified cell envelope.

Glycine-rich domains have been proposed as structural motives forming protein loops (30). They are widespread in epidermal cytokeratins, in loricrin, and in single-strand RNA binding proteins. The proposed function of these motives is to...
interact with similar glycine loops on the same or neighboring proteins. This inter- or intramolecular adhesion process, analogous to velcro adhesion, could be the basis for envelope-cytoskeleton interactions in the epidermis (30). The 65–175 region of corneodesmosin, which is serine- and glycine-rich but almost proline-free, may form such loops or related structures as it fulfills the criteria defined by Steinert et al. (30): (i) a high content of β-turns and a high degree of flexibility (data not shown), (ii) the general sequence form x(y)n, where x is an aromatic or an aliphatic residue, and y is a glycine or a serine or, less frequently, another polar residue, and (iii) at least two consecutive loops with an aromatic residue in the x position. However, in contrast to the glycine loop regions reported by Steinert et al., the most frequently encountered amino acid in the 65–175 corneodesmosin region is serine rather than glycine (Fig. 6). This difference could result in slight differences in loop function or affinity. Indeed, non-glycine substitutions seem to be a characteristic of a given glycine loop-containing domain. As an example, in the glycine loop domain of cytokeratins, glycine is frequently substituted by serine; some rare loops even contain no glycine (30). In the C terminus of corneodesmosin, another region (amino acids 370–450) is also serine-rich and may form glycine loop-related structures. Functional experiments will be necessary to test whether this region and the former domain are involved in putative adhesion properties of corneodesmosin. These velcro-related adhesion properties could be, in part, the basis for the observed reaggregation of mechanically dispersed corneocytes and in particular the reformation of corneodesmosomes (31).

We have previously shown that corneodesmosin is a phosphorylated and N-glycosylated basic protein (21). Consistently, the cDNA predicts numerous potential phosphorylation sites, one N-glycosylation site (at amino acid position 172), and a pI of 8.3.

We reported before that corneodesmosin is present in secretory vesicles of spinous and granular keratinocytes, i.e. keratinosomes (9). The cDNA sequence includes a hydrophobic domain at the N terminus of the protein (from M17 to A292), predicted, by the SignalP algorithm (32), to be a signal peptide with, most likely, a cleavage site at position A292-K33. Accordingly, we demonstrate now that corneodesmosin is secreted by COS-7 cells, transiently transfected with an expression plasmid. Whereas corneodesmosin has also previously been located in granular keratinocyte desmosomes (9), it was not detected by indirect immunofluorescence (even with a biotin/streptavidin amplification system) neither at the plasma membrane nor in the desmosomes of transfected HeLa cells. In sister cultures of HeLa cells, immunofluorescence detection of desmoglein with mAb DG3.10 (33) showed a punctuate labeling pattern indicative of the presence of desmosomes (data not shown). Corneodesmosin does not seem, therefore, to become localized in the desmosomes of monolayered cells. This could be due to already reported differences in the composition of HeLa cell and granular keratinocyte desmosomes. For example, HeLa cells are devoid of desmoglein 1, the major desmoglein isoform of granular keratinocyte desmosomes (33). In the epidermis, corneodesmosin is in a lipid environment all along its journey to the desmosomal core, both in the cytoplasmic keratinosomes and in the extracellular space. This particular environment could also be necessary for the correct localization of this hydrophilic protein. A direct interaction between corneodesmosin and any known glycine loop-containing protein, e.g. cytokeratins or loricrin, seems unlikely because the former is secreted whereas the latter are intracellular proteins. Therefore, we propose that corneodesmosin glycine loop-related domains are implicated in homophilic interactions. Corneodesmosin molecules of adjacent cells may interact, mediating cell-cell adhesion. Alternatively, its adhesion properties may contribute to the ultrastructural modifications that characterize the transformation of desmosomes into corneodesmosomes.

Corneodesmosin was proposed to be synthesized, in the living layers of epidermis, as a 52–56-kDa precursor that is progressively proteolysed in stratum corneum (21). When compared with recombinant corneodesmosin produced in vitro or in COS-7 cells, this epidermal form seemed not to comprise the entire amino acid sequence. This suggests that it already results from an early step of proteolysis occurring in the spinous and/or granular layers. To get more insight into the late corneodesmosin cleavage that occurs at the surface of the cornified layers, extracts of superficial corneocytes were analyzed by Western blotting after a precise localization of the anti-corneodesmosin mAb epitopes. The G36-19 and F28-27 epitopes, located in the central part of the molecule, were detected in fragments as small as 15 kDa. In contrast, the B17-21 epitope, located closer to the N terminus, was not detected in fragments smaller than 30 kDa. This clearly indicates that the smallest fragments do not contain the N terminus, at least the first 200 amino acids, of the protein. Moreover, the size of these fragments indicates that they probably do not contain the C terminus of the protein either. Cleavage of corneodesmosin seems therefore to occur at both ends of the molecule. We speculate that the cleavage progressively suppresses both the glycine loop-related domains. In that way, the adhesive parts of the molecule would be deleted, allowing desquamation. Antibodies raised against both termini of the molecule will be developed to analyze the fate of the corneodesmosin extremities.
An association between type I familial psoriasis vulgaris and the HLA-Cw6 allele has long been described (34) and was recently confirmed by genotyping (35). However, linkage disequilibrium is strongest for markers close to but other than HLA-C (36). Moreover, any demonstration of a direct role of this major histocompatibility complex polypeptide in psoriasis pathogenesis is lacking. A non-HLA gene seems therefore to be responsible for susceptibility to psoriasis. The identification of the S gene, exclusively expressed in epidermis, within the class I region of the HLA complex (160 kb telomeric of HLA-C) already raised the question of its responsibility in susceptibility to the disease (24). Because our results demonstrate that the product of this gene, corneodesmosin, is an extracellular protein integrated into the desmosomes soon before the formation of corneodesmosomes, they support this hypothesis. Indeed, in psoriasis epidermis, ultrastructural examination of the stratum corneum revealed that the abrupt transformation of desmosomes into corneodesmosomes at the stratum granulosum/stratum corneum interface does not occur. Instead, structures representing an intermediate stage of desmosome transformation are mainly present in the lower stratum corneum and can persist up to the upper stratum corneum (37, 38). These corneodesmosome abnormalities could be related to the impaired desquamation characteristic of the disease. We are therefore testing the hypothesis of the direct involvement of the corneodesmosin gene in primary predisposition to type I familial psoriasis vulgaris linked to HLA-Cw6.

Corneodesmosin seems to be a protein of major interest in several respects. Secreted by keratinocytes, corneodesmosin is secondarily located in the extracellular part of the desmosomes, differing in that from all desmosomal proteins previously described. Its putative adhesion function, as suggested by the glycan loop model, might be primordial in corneodesmosome formation or function. The high content of corneodesmosin in serine and glycine is only shared by other structural epidermal proteins. The meaning of this richness in proteins of some formation or function. The high content of corneodesmosin gene in primary predisposition to type I familial psoriasis vulgaris linked to HLA-Cw6.

REFERENCES

1. Holbrook, K. A. (1994) in The Keratinocyte Handbook (Leigh, I. M., Lane, E. B., and Watt, F. M., eds) pp. 3–39, Cambridge University Press, Cambridge

2. Roop, D. (1995) Science 267, 474–475

3. Simon, M. (1994) in The Keratinocyte Handbook (Leigh, I. M., Lane, E. B., and Watt, F. M., eds) pp. 275–292, Cambridge University Press, Cambridge

4. Reichert, U., Michel, S., and Schmidt, R. (1993) in Molecular Biology of the Skin: The Keratinocyte (Darmon, M., and Blumenberg, M., eds) pp. 107–130, Academic Press, San Diego

5. Mottet, D. N., and Eisen, A. Z. (1971) J. Ultrastruct. Res. 35, 247–264

6. Allen, T. D., and Potten, C. S. (1975) J. Ultrastruct. Res. 51, 94–105

7. Skerrow, C. J., Clelland, D. G., and Skerrow, D. (1989) J. Cell Sci. 92, 667–677

8. Chapman, S. J., and Walsh, A. (1996) Arch. Dermatol. Res. 282, 304–310

9. Serre, G., Mils, V., Haftek, M., Vincent, C., Croutte, F., Réano, A., Ouhayoun, J.-P., Bettinger, S., and Soleilhavoup, J.-P. (1991) J. Invest. Dermatol. 97, 1061–1072

10. King, I. A., Tabiowo, A., and Fryer, P. R. (1987) J. Cell Biol. 105, 3053–3063

11. Egelrud, T., and Lundström, A. (1989) Acta Derm. Venereol. (Stockh.) 69, 470–476

12. Egelrud, T., and Lundström, A. (1990) J. Invest. Dermatol. 95, 456–459

13. Lundström, A., and Egelrud, T. (1990) J. Invest. Dermatol. 94, 216–220

14. Chapman, S. J., Walsh, A., Jackson, S. M., and Friedmann, P. S. (1991) Arch. Dermatol. Res. 283, 167–173

15. Ghadially, R., Williams, M. L., Hoo, S. Y. E., and Elias, P. M. (1992) J. Invest. Dermatol. 99, 755–763

16. Walsh, A., and Chapman, S. J. (1991) Arch. Dermatol. Res. 283, 174–179

17. Suzuki, Y., Nomura, J., Koyama, J., and Horii, I. (1994) Arch. Dermatol. Res. 286, 249–253

18. Egelrud, T. (1993) J. Invest. Dermatol. 101, 200–204

19. Hansson, L., Strömqvist, M., Backman, A., Wallbrandt, P., Carlstein, A., and Egelrud, T. (1994) J. Biol. Chem. 269, 19420–19426

20. Haftek, M., Serre, G., Mils, V., and Thivolet, J. (1991) J. Histochem. Cytochem. 39, 1531–1538

21. Simon, M., Montézin, M., Guerrier, M., Durieux, J.-J., and Serre, G. (1997) J. Biol. Chem. 272, 31770–31776

22. Lundström, A., Serre, G., Haftek, M., and Egelrud, T. (1994) Arch. Dermatol. Res. 286, 369–375

23. Montézin, M., Simon, M., Guerrier, M., and Serre, G. (1997) Exp. Cell Res. 231, 132–140

24. Zhou, Y., and Chaplin, D. D. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 9470–9474

25. Aletsch, A., Stephen, F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990) J. Mol. Biol. 215, 403–410

26. Sommer, S. S., Guschbach, A. R., and Bottema, C. D. K. (1992) BioTechniques 13, 82–87

27. Velten, F., Rogel-Gaillard, C., Renard, C., Pontarotti, P., Tazi-Ahnini, R., Vaiman, M., and Chardon, P. (1998) Tissue Antigens 51, 183–194

28. Ishihara, M., Yamagata, O., Ohno, S., Naruse, T., Ando, A., Kawata, H., Ozawa, A., Okido, M., Minami, N., Shina, T., Ando, H., and Inoko, H. (1996) Tissue Antigens 48, 182–186

29. Kozak, M. (1989) J. Cell Biol. 108, 229–241

30. Steinert, P. M., Mack, J. W., Korge, B. P., and Gan, S.-Q. (1991) Int. J. Biol. Macromol. 13, 130–139

31. Brysk, M. M., Rajaraman, S., Penn, P., Barlow, E., and Bell, T. (1989) Exp. Cell Biol. 57, 60–66

32. Nielsen, H., Engelbrecht, J., Brunak, S., and von Heijne, G. (1997) Protein Eng. 10, 1–6

33. Schafer, S., Koch, P. J., and Franke, W. W. (1994) Exp. Cell Res. 211, 391–399

34. Tulkainen, A., Lassus, A., Karlsson J., and Vartiainen, P. (1980) Exp. Cell Res. 129, 105–119

35. Mallon, E., Bunce, M., Wójcikowska, F., and Welsh, K. (1997) J. Invest. Dermatol. 109, 185–186

36. Trembath, R. C., Clough, R. L., Rosbotham, J. L., Jones, A. B., Camp, R. D. R., Barker, J. N. W. N. (1997) Hum. Mol. Genet. 6, 813–820

37. Vicanova, J., Mommaas, A. M., Mulder, A. A., Koerten, H. K., and Ponec, M. (1996) Cell Tissue Res. 286, 115–122

38. Ghadially, R., Reed, J. T., and Elias, P. M. (1996) J. Invest. Dermatol. 107, 558–564