The cornified envelope (CE) is an insoluble sheath of e-(γ-glutamyl)lysine cross-linked protein, which is deposited beneath the plasma membrane during keratinocyte terminal differentiation. We have probed the structure of the CE by proteolytic cleavage of purified CE fragments isolated from CE rods formed spontaneously in cell culture. CNBr digestion, followed by trypsin and then proteinase K treatment released 25%, 42%, and 18%, respectively, of the CE protein. Purification and sequencing of released peptides has identified two novel CE precursors, S100A11 (100C, calgizarin) and S100A10 (calpactin light chain). We also sequenced peptides derived from annexin I and plasminogen activator inhibitor 2, two putative envelope precursors, as well as portions of the well-established CE precursor proteins SPRIA, SPRIB, and involucrin. Many desmosomal components were identified (desmoglein 3, desmocollin A/B, desmoplakin I, plakoglobin, and plakophilin), indicating that desmosomes become cross-linked into the CE. Fragments derived from envoplakin, the recently sequenced 210-kDa membrane CE precursor protein, which also appears to be a desmosomal component, were also identified. Analysis of the pattern of peptide release following the sequential digestion indicates that S100A11 is anchored to the envelope via Gln102 and/or Lys103 at the carboxyl terminus and at Lys8, Lys23, and/or Gln25 in the amino terminus. A similar type of analysis indicates that small proline-rich proteins 1A and 1B (SPRIA and SPRIB) become cross-linked at the amino terminus (residues 1–23) and the carboxyl terminus (residues 86–89). No loricrin, cystatin A, or elafin peptides were detected.

The cornified cell envelope (CE) is a 15-nm-thick cross-linked sheath of protein that forms beneath the plasma membrane during the final stages of epidermal keratinocyte differentiation (1–3). Transglutaminase enzymes catalyze the assembly of this structure via formation of e-(γ-glutamyl)lysine bonds between envelope precursors (4–7). The proteins that have been identified as constituents of the CE include loricrin (8–10), involucrin (11–14), the small proline-rich (SPR) family of proteins (15–18), cystatin A (19, 20), elafin (21–23), filaggrin (24–26), keratin (27, 28), desmosomal components (27), annexin I (29–31), plasminogen activator inhibitor-2 (PAI-2) (32), and the 195-kDa and 210-kDa proteins (33, 34). Cross-links have been identified within loricrin, elafin, filaggrin, keratin, SPR1, SPR2, and desmoplakin (35). Loricrin is present as a partner in most of these cross-links. It has been proposed that envelope formation is initiated with formation of an envelope scaffold that consists of soluble precursors, and that other precursors (both soluble and insoluble) are later deposited (3, 11, 35). The scaffold has been proposed to consist of involucrin, cystatin A, and possibly other proteins (25). In this model, other proteins, including loricrin, elafin, and SPRs, are deposited onto this scaffold.

In the present report, we study the composition of envelopes formed by cultured keratinocytes, as a model of the early stages in cornified envelope formation. We purify envelope fragments from CE rods formed in cell culture, digest them sequentially with CNBr, trypsin, and proteinase K, and determine the sequence of the released peptides. Using this procedure, we identify involucrin, PAI-2, keratins, SPRIA and SPRIB, annexin I, desmoplakin I, plakoglobin, envoplakin, plakophilin, desmocollin 3a/3b, desmoglein 3, S100A11 (100C, calgizarin), S100A10, and several unidentified proteins as components of the cornified envelope. The SPRs are, by far, the most abundant components released by the digestion protocol. Based on the pattern of fragments released by the enzymatic digestion, we identify the amino- and carboxyl-terminal regions of S100A11 and the SPRs, and the amino terminus of annexin I as sites of cross-link formation.

MATERIALS AND METHODS

Digestion of Cornified Envelopes—Cornified envelopes, formed spontaneously or induced by treatment with NaCl, were used to prepare highly purified cornified envelope fragments from human foreskin keratinocyte cultures (36). These envelope fragments were sequentially digested using the scheme shown in Fig. 1. CE fragments (10 mg) were resuspended in 16 ml of 70% formic acid containing 2 g of CNBr and digested, with continuous agitation, for 24 h at room temperature. The mixture was then centrifuged to yield soluble and pellet fractions. The soluble fraction was diluted 5-fold with water and lyophilized to remove CNBr. This was repeated twice. The resulting residue was dissolved in 0.4–1.0 ml of H2O, acidified with trifluoroacetic acid (for this and subsequent acidifications, the final trifluoroacetic acid concentration = 0.2%), and centrifuged to yield soluble and pellet fractions.

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1 The abbreviations used are: CE, cornified envelope; SPR, small proline-rich; PAI-2, plasminogen activator inhibitor-2; PVDF, polyvinylidene difluoride; RT, reverse transcription; PCR, polymerase chain reaction; HPLC, high performance liquid chromatography.

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The soluble fraction (fraction 2) was analyzed using a C-18 HPLC column, and the pellet fraction (fraction 1) was dissolved in Laemmli sample buffer for electrophoresis.

The CNBr pellet was washed three times with H2O and resuspended by sonication in 1–2 ml of 50 mM Tris-HCl, pH 8.0, containing 11.5 mM CaCl2, prior to proteinase K digestion for amino acid analysis. The remaining suspension was digested at 1% w/v (based on the initial concentration of CE fragments) with 1-λ-tosylamido-2-phenyl)ethyl chloromethyl ketone-treated trypsin (Worthington) for 24 h at 25 °C with agitation. The material was centrifuged to yield pellet and soluble fractions. The soluble fraction was acidified with trifluoroacetic acid and centrifuged, because the smeared peptides could not be resolved (data not shown). After acidification with trifluoroacetic acid and centrifugation, this mixture yielded a pellet and supernatant. The supernatant (fraction 6) was analyzed by HPLC. The pellet was not analyzed.

The pellet from the trypsin digestion was washed with proteinase K digestion buffer, suspended by sonication in 0.5 ml of the same buffer, and an aliquot was removed for amino acid analysis. The remaining sample was digested with 1 μg of proteinase K (Promega) for 30 h. Centrifugation of this reaction mixture yielded a pellet and supernatant. Acidification of the supernatant with trifluoroacetic acid and centrifugation yielded pellet and soluble fractions. The soluble fraction (fraction 8) was analyzed by HPLC and the pellet (fraction 7) was resuspended in Laemmli buffer for gel electrophoresis.

Determination of Protein Concentration—Protein concentration of CE fragments was determined using the solid-phase, dot blot assay as described previously (36). The protein content of the fractions released was determined using the Bio-Rad DC protein assay kit.

HPLC Purification of Peptide Fragments—Soluble peptides released by proteolytic digestion (Fig. 1, #1, #2, and #3) were filtered through a 0.2-μm Unifil-3 syringe filter (Schleicher & Schuell) and the samples were fractionated on a C18 reverse phase column (Advantage-100, 5 μm, 240 × 4.6 mm, Thermo Scientific Liquid Chromatography, Springfield, VA) at a flow rate of 1 ml/min. Peptides eluting during the acetonitrile gradient (gradient conditions indicated in Fig. 3 legend) were monitored at 220 nm using a Waters model 484 absorbance detector (Waters, Milford, MA). Peptides were collected, dried by rotary evaporation, and purified by re-chromatographing on the Advantage C18 column using a 0–100% acetonitrile gradient (0.1%/min). The purified peptides were concentrated by rotary evaporation and dissolved in 10 μl of 70% acetonitrile containing 1% trifluoroacetic acid for microsequencing.

Polyacrylamide Gel Electrophoresis—Samples of acid-insoluble, digested envelope fragments (Fig. 1, #1, #3, and #7) were electrophoresed on 10 or 12% polyacrylamide gels (37). Proteins were stained using Coomasie Blue or were transferred to PVDF (polyvinylidene difluoride) membranes and processed for microsequencing as described previously (36).

Microsequencing and Protein Assignment of Purified Peptides—Purified peptides were sequenced using a Perkin Elmer/Applied Biosystems Procise model 494 microsequenator. HPLC-purified peptides were applied to a BioBrench-Plus-treated (Applied Biosystems) glassfiber filter prior to sequencing. Polyacrylamide gel-purified peptides were sequenced directly on the excised PVDF membrane. In most cases, 10 sequencing cycles were performed for each peptide. Peptide sequences of >6 amino acids were matched to candidate proteins by searching the various proteolytic digests were measured using the Bio-Rad DC protein assay kit.

The term By defines a random vector with mean x and covariance $\Sigma_x$. The protein composition x is estimated by choosing B to minimize the trace covariance, $\Sigma_x B^T B$, subject to the constraints that $B A = I$ and (By), $\geq 0$ for $i = 1, \ldots, m$. The nonnegative constraint was imposed because large negative solutions, which are physiologically irrelevant, were generated without the constraint. Since the number of observations was fairly small, the estimates of sample covariance were unreliable and we used $\Sigma = I$. Therefore, the estimate for x is the positive part of the usual least squares estimate. The values calculated from the molar data were converted to percent mass.

The proteins included in this analysis were involucrin (38) (SWISS-PROT P07476), loricrin (8) (SWISS-PROT P23490), proelastin (21) (SWISS-PROT P19957), cystatin A (19) (SWISS-PROT P01040); filaggrin (39) (GenPept M24355); desmoplakin I (40) (GenPept M77830); plakoglobin (41) (EMB Z62228); annexin I (42) (SWISS-PROT P04083); envoplakin (34) (GenBank U53786); S100A11 (S100C) (43) (DNA Database of Japan D35833); S100A10 (44) (P08206); plakophilin (45) (EMB Z34974); the averaged composition of keratins K5, K6, K14, and K16 (46–49); keratin (50) (P02533); the averaged composition of desmoglein 3 (50) (SWISS-PROT P32926) and desmocollin 3a/3b (51) (SWISS-PROT Q02487). PAI-1 (52) (SWISS-PROT P05120) was not included in the analysis, because it was a minor envelope component and including it would exceed 15 proteins, the maximum number allowed. Methionine (Met) and tryptophan (Trp) are not considered in the

\[ y = Ax + E \]  

(Eq. 1)

\[ x = \begin{pmatrix} x_1 \\ x_2 \\ \vdots \\ x_n \end{pmatrix} \]  

Fig. 1. Sequential CE digestion scheme. Cornified envelope fragments were prepared as described previously (36) and then processed as outlined under "Materials and Methods." Fractions 1, 3, and 7 (#1, #3, and #7) were characterized by gel electrophoresis. Fractions 2, 4, 6, and 8 (#2, #4, #6, and #8) were characterized by HPLC. Since sodium dodecyl sulfate can effect the resolution of HPLC purification, CE fragments were washed with H2O prior to CNBr digestion for generation of fraction 2. Sample 5 was not characterized after gel fractionation, because the smeared peptides could not be resolved (data not shown).
analysis, since Met is destroyed by the CNBr digestion and Trp was not measured.

Polymerase Chain Reaction Cloning of Calgizzarin (S100A11)—Poly(A)^+ RNA, isolated from cultured human foreskin keratinocytes (55), was used as the template to clone a cDNA encoding the human S100A11 protein coding region by reverse transcription-polymerase chain reaction (RT-PCR) using 5'-CAT AGT GCA AAA ATC TTC AGC CC as the forward primer and 5'-GGA TCC TAG GTT GGT TAG TGT GCT CA (43) as the reverse primer. Poly(A)^+ RNA (1 μg) was reverse-transcribed in a standard reaction using a RT-PCR kit (Boehringer Mannheim, catalog no. 1483188) and 30 pmol of the reverse primer.

FIG. 2. Characterization of peptides present in fractions 1, 3, and 7. Cultured human foreskin keratinocytes were permitted to form cornified envelopes spontaneously, or envelopes were induced to form by addition of NaCl, according to our previously described protocol (36). Fractions 1, 3, and 7 (#1, #3, and #7) were prepared by digestion as outlined in Fig. 1, and then electrophoresed on a denaturing 12% polyacrylamide gel. Loading was normalized based on the initial concentration of undigested envelope protein as follows: spontaneous (S) envelopes (1, 120 μg; 3, 400 μg; and 7, 400 μg) and induced (I) envelopes (1, 120 μg; 3, 200 μg; and 7, 400 μg). The asterisks indicate lanes loaded with control reactions containing trypsin (#3) or proteinase K (#7), but no envelope fragments. Peptide fragments C1, C2, C3, and C4 from lane 1 (S) were sequenced after transfer to PVDF membrane. The molecular size standards are indicated to the right of the panel.

TABLE I

| Fraction^a | Treatment^b | mg protein/mg of CE |
|------------|-------------|---------------------|
| 1 and 2 CNBr | 0.25 ± 0.07 |
| 3 Trypsin (acid-insoluble) | 0.17 ± 0.05 |
| 4 Trypsin (acid-soluble) | 0.25 ± 0.07 |
| 7 Proteinase K (acid-insoluble) | 0.03 ± 0.03 |
| 8 Proteinase K (acid-soluble) | 0.15 ± 0.06 |

^a Fraction nos. refer to Fig. 1; fractions 5 and 6 were not assayed, as they are subsets of fraction 3.

^b CE envelopes (spontaneously formed) were digested sequentially with CNBr, trypsin, and proteinase K as outlined in Fig. 1 and described under "Materials and Methods."

^c Values are mean ± S.E. (n = 3) and were determined by protein assay (36).

TABLE II

| Peptide^a | Calculated M_r^b | Apparent M_r^c | Sequence^d | Protein | Residues^e | Quantity^e | pmol/mg |
|-----------|------------------|----------------|------------|---------|-----------|-----------|---------|
| C1        | 22,000           | 46,000         | LKSLEDLKLE | Desmoplakin I | 1021–1039 | 124       |
| C2        | 7,800            | 16,900         | VKGVEATI IDLTK | Annexin I | 57–71 | 117 |
| C3        | 13,900           | 12,100         | KGLTDEDT LIEILASTNKE | Annexin I | 128–148 | 58 |
| C4        | 13,900           | 8,200          | KGLTDEDT LIEILA | Annexin I | 128–142 | 956 |

^a Identification numbers correspond to peptides in Fig. 2.

^b Theoretical molecular weight of the peptide fragment based on known sequence.

^c Molecular weight determined from calibration of the SDS-polyacrylamide gel.

^d Residues indicate only the portion of the peptide that was sequenced.

^e Picomoles of peptide detected (by sequencing)/mg of starting CE fragments.

One half of this reaction was PCR-amplified using 2.5 units of Pwo polymerase (Boehringer Mannheim) in the presence of 0.5 mM MgCl_2, 3 mM MgSO_4, and 30 pmol of each primer (10 cycles of 94 °C for 15 s, 55 °C for 30 s, 72 °C for 45 s; and 15 additional cycles for which the elongation step was increased by 20 s/cycle). The PCR product was then end-cloned at the EcoRV site of pZERO1.1 (Invitrogen, San Diego, CA), and the DNA sequence of both strands was determined using the M13-reverse and T7 promoter primers and an Applied Biosystems model 377 sequencer.

RESULTS

Release of Peptide Fragments—Fig. 1 shows the scheme followed for digestion of the purified CE fragments. The strategy was to sequentially digest CE fragments with proteolytic agents and then to sequence the released peptides. At each stage, the residual pellet from the preceding step was digested with the next cleavage agent. The goal was to release smaller peptides at each step. We first cleaved with CNBr. CNBr cleaves at methionine residues, which occur relatively infrequently in proteins. The residual pellet from the CNBr step was treated with trypsin, which cleaves after lysines and arginines, and the pellet from this step was treated with the non-specific protease, proteinase K.

In preparation for HPLC separation of peptide fragments, the released material from each digestion was acidified with trifluoroacetic acid. Addition of trifluoroacetic acid resulted in the precipitation of larger peptides from each sample. The precipitates (fractions 1, 3, and 7) were characterized by gel electrophoresis, while the soluble fractions (fractions 2, 4, 6, and 8) were separated using a C18 HPLC column. Fig. 2 shows the Coomassie Blue-stained profile of a 12% polyacrylamide gel of fractions 1, 3, and 7. The CNBr digestion products, prepared from either spontaneous (S) or induced (I) envelope fragments, yielded a similar pattern of discrete peptide bands. The peptides released following trypsin digestion yielded a smear. In addition, a greater amount of material was released from the induced envelopes compared with the spontaneous envelope preparation (normalized based on the amount of starting CE protein). Proteinase K, in contrast, did not release large peptide fragments from induced envelope fragments; however, some high molecular weight material, which remained at the top of the separatory gel, was released from spontaneous envelope fragments.

Table I lists the quantity of protein released, from each major fraction, following sequential digestion of 1 mg of spontaneously formed CE fragments with CNBr, trypsin, and proteinase K. CNBr released 25% of the material, trypsin released 42%, and proteinase K released 18%. The total yield for all steps was 85% of the initial CE protein.

Sequence Analysis of CNBr-released Peptides—To identify proteins of the cornified envelope, selected CNBr-released pep-
tide fragments (Fig. 2, C1, C2, C3, and C4) were separated by gel electrophoresis, transferred to PVDF membrane, and microsequenced. The sequences were then used to search the nonredundant protein sequence database to identify each protein. One peptide matched desmoplakin I, and the other three matched segments of annexin I (Table II). A methionine (Met) residue always preceded the sequence, a result that is consistent with release by CNBr. The desmoplakin band (Fig. 2, C1) migrated at 46 kDa in the CNBr digest of spontaneous envelope fragments. It is interesting that this band is virtually absent in the CNBr digest of induced envelope fragments. This observation is in agreement with our previously published report that desmosomal remnants are present in electron micrographs of spontaneous CE fragments but are absent in CE fragments prepared from induced envelopes (36). The other three peptides (C2, C3, and C4) were all derived from human annexin I. Peptide C2 appears to be a partial digest, since the calculated size of the Val56–Met127 fragment, based on the known sequence (42), is 7.8 kDa and the molecular mass of C2 is 16.9 kDa. Likewise, peptides C3 and C4 have the same NH$_2$-terminal sequence, but different molecular masses. C4, as judged by Coomassie staining intensity, was the most abundant peptide released by CNBr.

The CNBr-released, trifluoroacetic acid-soluble fraction prepared from spontaneous envelope fragments (Fig. 1, #2) was analyzed by C18 HPLC. Since SDS adversely affects the resolution of the HPLC purification, CE fragments were extensively washed with H$_2$O and then CNBr-digested to yield fragment 2. HPLC fractionation of this material revealed a complex profile (Fig. 3A). To assure purity, the indicated peaks, C6–C20, were rechromatographed on the C18 column, using a shallower (0.1%/min) acetonitrile gradient. The samples were then concentrated and sequenced (Table III). In some cases, this second chromatography step resolved the sample into multiple peaks. The additional peaks are indicated by alphabetical extensions (a and b). Also, in some cases, sequencing of a single peak yielded two sequences, one predominant sequence and a second, less abundant sequence. The less abundant sequence is

**Fig. 3. HPLC separation of peptides present in fractions 2, 4, and 6.** Cornified envelope fragments, prepared from spontaneously formed envelopes, were digested as shown in Fig. 1. Peptide fragments present in fractions 2 (panel A), 4 (panel B), and 6 (panel C) were separated by HPLC. The injected sample was derived from digestion of 2 mg (panel A), 1 mg (panel B), or 3 mg (panel C) of initial envelope fragments. The C18 column was pre-equilibrated in 0.1% trifluoroacetic acid at a flow rate of 1 ml/min. The samples were injected at time zero and, after 15 min, an acetonitrile gradient was initiated. The percent acetonitrile was increased at a rate of 0.67%/min (panels A and C) or 0.33%/min (panel B). The left vertical axis indicates OD units at 220 nm. The horizontal axis is time in minutes, and the right vertical axis is % acetonitrile. The acetonitrile gradient is shown by the dashed line in each panel. Peaks that were collected for purification and sequence analysis are indicated as C5–C20 (panel A), T1–T9 (panel B), and P1–P13 (panel C). The asterisk denotes peaks that were present in control digestion reactions from which envelope fragments were omitted.
labeled “minor” (e.g. C9, C9-minor).

This analysis identified fragments corresponding to involucrin, annexin I, PAL-2, S100A10, S100A11, keratins 14 and/or 17, as well as the desmosomal components desmoplakin I, plakoglobin, envoplakin, desmoglein 3, plakophilin (band-6 protein), and desmocalin 3a/3b (Table III). One sequence, C7, was not matched in the non-redundant protein data base. All of the identified sequences were preceded by a Met residue. In the instances where the sequence ended before the 10th cycle (C5a, C5b, C6, C9, C9-minor, C15), the sequence in the identified protein was Met.

Sequence Analysis of Trypsin-released Peptides—We next characterized trypsin-released fraction 4 (Fig. 1). HPLC fractionation of this sample (Fig. 3B) yielded a simple profile, compared with the profile observed in Fig. 3A. Nine peaks (T1–T9) were purified and sequenced. As shown in Table IV, this fraction contained SPRs, S100A11, and annexin I. The SPRs were the most abundant proteins found in fraction 4. Due to the sequence similarity between SPR1A and SPR1B, it was not always possible to make definitive assignments of the protein from which the sequence was derived (e.g. peptide T5). Also, because of the repetitive nature of the amino acid sequence of the SPR family, it was not always possible to assign the sequence to a specific region of the protein (e.g. peptide T4). All of the peptide fragments, with the exception of T8, ended with the Lys or Arg residue characteristic of trypsin cleavage. T8 probability terminates with Pro-Lys (based on known sequence of the SPR family, it was not always possible to assign the sequence to a specific region of the protein (e.g. peptide T4). All of the peptide fragments, with the exception of T8, ended with the Lys or Arg residue characteristic of trypsin cleavage. T8 probability terminates with Pro-Lys (based on known sequence of the SPR family, it was not always possible to assign the sequence to a specific region of the protein (e.g. peptide T4). All of the peptide fragments, with the exception of T8, ended with the Lys or Arg residue characteristic of trypsin cleavage. T8 probability terminates with Pro-Lys (based on known sequence of the SPR family, it was not always possible to assign the sequence to a specific region of the protein (e.g. peptide T4).

Sequence Analysis of Proteinase K-released Peptides—Fig. 3C shows the HPLC profile of peptides present in fraction 6 (Fig. 1). The indicated peaks (P1–P13) were isolated and repurified. Upon repurification some were separated into multiple peptides which are designated by alphabetic extensions (Table V). Many of the peptides generated were ≤5 amino acids in length and were too short for definitive assignment of the protein from which they were derived. Several fractions contained only 1–3 amino acids (P1, P2, P5, P6, P11, and P12). Although 4-amino acid length sequences cannot be definitively assigned to a single protein sequence, they do identify possible proteins when screened against a data base that includes the known cornified envelope precursor proteins. Desmoglein (P3a) and involucrin (P3a-minor, P4a) were identified in this group. Three of the sequences (P3b, P8, and P8-minor) were not found in the CE precursor data base. Peptides that were 5 or 6 amino acids were screened using the PAM40 matrix of the blastp facility. Sequences P7 and P9a matched SPR1A protein and annexin I, respectively. P7-minor matched human desmoplakin I and II and one other human protein (Table V, legend). Peptides of ≥6 amino acids were screened using the Blosum-62 matrix. P4b matched S100A11, and P9b and P10 matched SPR proteins. P13, with the amino acid sequence GPAPCPAPAP, did not find a perfect match in the non-redundant protein data base. The closest matches were bovine β-crystallin B1 (GPAPCPAPAP) and myosin light chain 1 (PAPAPAPAP) from several species. Since the HPLC profile obtained from fraction 8 (Fig. 1) was virtually identical to that observed for fraction 6 (data not shown), these peptides were not sequenced.

Identification of Potential Cross-link Sites within S100A11—S100A11 (calgizzarin) is a newly identified precursor of the cornified envelope. To verify its presence in keratinocytes, we cloned and sequenced the S100A11 cDNA from cultured human keratinocytes. The sequence (Fig. 4) matches the previously published sequence of a S100A11 cDNA clone isolated from human colon carcinoma cells (43), indicating that the S100A11 protein detected in skin and in cultured keratinocytes (54, 55), and the colon carcinoma protein are identical. Our sequential proteolytic digestion results suggest that S100A11 is cross-linked in specific locations. Based on the S100A11 sequence,
that sequence could not be assigned. SPR3 appears to be the 22-kDa pancornulin (16).

SPR1A appear to be the 14.9-kDa and 16.9-kDa pancornulins, respectively (16).

The envelope structure. After this initial CNBr cleavage, the undigested residual was treated with trypsin. Trypsin digestion released peptide T9 (Ala90–Lys97). Peptide T9 (Table IV) was produced by CNBr cleavage downstream of Met89, followed by tryptic cleavage downstream of Lys97. The sequence of this peptide did not reveal any cross-links. These results strongly suggest that a cross-link at Gln102 and/or Lys103 is anchoring the carboxyl terminus of S100A11 to the envelope.

CNBr cleavage (at Met residues 1, 43, 63/64, and 89) is expected to release four peptide fragments (Fig. 4). CNBr cleavage of the envelope fragments releases peptide C12 (Table II) (Asn14–Arg62), suggesting that this region is not cross-linked to the envelope structure. After this initial CNBr cleavage, the undigested residual was treated with trypsin. Trypsin digestion released peptide T9 (Ala90–Lys97). Peptide T9 (Table IV) was produced by CNBr cleavage downstream of Met89, followed by tryptic cleavage downstream of Lys97. The sequence of this peptide did not reveal any cross-links. These results strongly suggest that a cross-link at Gln102 and/or Lys103 is anchoring the carboxyl terminus of S100A11 to the envelope.

Peptide T7 (Table IV) and peptide P4b (Table V) are positioned within the amino-terminal CNBr cleavage peptide (Ala2–Phe42) of S100A11. This segment was not released from the envelope following CNBr digestion, suggesting that this region contains cross-links. Lys3, Lys23, Lys27, Lys36, and Gln22 are possible cross-linking residues. However, trypsin cleaved immediately downstream of both Lys27 and Lys36, releasing peptide T7, suggesting that these sites are not cross-linked.

| Peptide | Rt | CH₃CN | Sequence | Protein | Residues | Quantity (pmol/mg) |
|---------|----|-------|----------|---------|----------|-----------------|
| T1      | 42.4 | 9.0  | EPCHPK  | SPR1B   | 39–44    | 922             |
| T2      | 42.9 | 9.2  | EFCQPQK | SPR1A   | 39–44    | 92              |
| T3      | 44.3 | 9.7  | TKECPHK | SPR1B   | 37–44    | 85              |
| T4      | 53.3 | 12.6 | VPECPHK | SPR1A   | 45–52    | 16,000          |
| T5      | 53.9 | 12.8 | VPECPQK | SPR1A and SPR1B | 53–60 | 21,740          |
| T6      | 58.2 | 14.2 | TPECPQK | SPR1A   | 61–68    | 366             |
| T7      | 60.0 | 14.8 | DGYNNTLSK | S100A11/calgizzarin | 28–36 | 115             |
| T7-minor| 60.0 | 14.8 | DITSDTGDF | Annexin I | 167–177 | 23              |
| T9      | 65.1 | 16.5 | QCPQPPPQPECI | SPR1A and SPR1B | 23–34 | 33              |
| T9      | 65.9 | 16.8 | ACHDPSFLK | S100A11/calgizzarin | 90–97 | 174             |

Table IV

| Peptide | Rt | CH₃CN | Sequence | Protein | Residues | Quantity (pmol/mg) |
|---------|----|-------|----------|---------|----------|-----------------|
| P1      | 24.3 | 6.2  | Y       | GLEPS   | Involucrin | 69–72, 24 |
| P1      | 27.9 | 8.6  | GYRK    | Involucrin | 8× | 29             |
| P3a     | 27.9 | 8.6  | GLPE     | S100A11/calgizzarin | 6–12 | 34             |
| P3a-minor| 27.9 | 8.6  | STQ      | Desmoglein 1 | 385–388 | 51             |
| P3b     | 27.9 | 8.6  | GYRK     | Involucrin | 8× | 19             |
| P4a     | 28.8 | 9.1  | HLEQ     | Desmoglein 3 | 199–201 | 2             |
| P4b     | 28.8 | 9.1  | SPPECTER | SPR1A   | 76–81    | 132            |
| P9      | 29.6 | 9.8  | FL       | Desmoplakin I | 2495–2499 | 19          |
| P10     | 34.0 | 12.8 | ELCEQ    | Desmoplakin II | 777–781 | 7          |
| P11     | 38.4 | 15.6 | SYTPV    | No match  | 5             |
| P12     | 41.4 | 17.8 | GEG      | No match  | 126          |
| P13     | 43.7 | 19.1 | GPAPC     | No match  | 70             |

Table V
The P9a peptide is contained within the Val4–Ile55 peptide. The ase K digestion of the trypsin acid-insoluble fraction 3 (Fig. 1). trypsin digestion, and peptide P9a was released after protein-recovered. CNBr digestion released peptides C2, C3, C4, C6, C9, C11, and C20; CNBr and trypsin (T7-minor, shown in brackets); or CNBr, trypsin, and proteinase K (P9a). The upward pointing short arrows indicate possible cross-linking sites, and the upward pointing long arrows indicate probably cross-linking sites (see “Results”). It is possible that residue Lys53 of annexin I is also a cross-linking site; however, this seems unlikely. Therefore, it is not indicated by an arrow. The methionine residues (M, CNBr cleavage sites) are shown in bold.

Thus, Lys3, Lys23, and Gln22 appear to be the best candidates as sites of cross-link formation within the amino-terminal segment of S100A11. The carboxyl residue of peptide P4b is Arg, a known tryptic cleavage site. However, P4b was not released from the envelope until a subsequent digestion with proteinase K, providing strong additional evidence that Lys23 is a cross-link site. We did not sequence any portion of fragment Lys65–Met89; thus, Lys9, Lys26, and Lys29, and Gln10, Gln19, and Gln23. Three of all were derived from the central portion of the protein (within residues 23–68). The region from Val69–Lys89 was not released by trypsin. As this region contains tryptic cleavage sites, this result suggests that one or more cross-links are present within the carboxyl terminus. Proteinase K digestion of the trypsinized, acid-insoluble pellet (Fig. 1, T7) released two peptides from within this region. The fact that the Val69–Lys89 region was not released by trypsin, but fragments located between Val69 and Ala82 were released by subsequent proteinase K digestion, confirms that it contains cross-links. This region contains Gln23, Gln24, Gln25, Lys26, Lys27, and Lys28 as potential cross-link sites.

Our data also suggest that the NH2 terminus (Met1–Lys22) of the protein may contain cross-links, as it was not recovered. This segment of the protein has 10 candidate cross-linking sites (Gln4, Gln5, Gln6, Lys7, Gln12, Gln17, Gln18, Gln19, Gln23, and Lys25) (Fig. 5A). Lys25 is not a likely site for cross-link formation, since tryptic cleaved at the carboxyl side of this residue, and cleavage would not be expected if an isodipeptide cross-link was present at Lys25. If we plot the picomoles of each SPR peptide released (per milligram of CE) versus fragment position within the protein, it is clear that the central region of the protein is preferentially released (Fig. 5B), and that peptides representing the amino and carboxyl termini are not detected.

**Prediction of Envelope Protein Composition**—Using mathematical modeling (26), we attempted to characterize the protein composition of the CE fragments. This method has been reported to accurately predict CE precursor content (25, 26). We performed amino acid composition analysis on undigested envelope fragments and on residual CE material (i.e. the ma-
terial that remained following CNBr, CNBr/trypsin, or CNBr/trypsin/proteinase K digestion). The amino acid composition data are shown in Table VI. This envelope composition data and the amino acid composition for each precursor protein (obtained from the literature) were used in a least squares analysis to estimate the percent of the total CE mass contributed by each protein. The precursor proteins included in this analysis are listed in the legend to Table VII. A nonnegative constraint was imposed upon the calculations (see “Materials and Methods”). In contrast to previous reports (25, 26), we obtained large negative values in the absence of this constraint. The results of the least squares analysis are given in Table VII. Keratins, plakoglobin, S100A11, desmoplakin, SPRs, and proelafin were predicted to be the major CE components. However, the values of the residuals (i.e. root-mean-square discrepancy and the median discrepancy, Table VII) were much greater than 1, indicating a poor fit. A good fit is indicated by residuals of ≤ 1.

A second, straightforward method was also employed to estimate the relative contributions of the precursor proteins. The pico moles of amino acid released during peptide sequencing was used to calculate the percentage of mass contributed by each precursor. When multiple fragments of the same protein were identified, the quantity of the most abundant peptide was used in the calculations. The estimates obtained using this method are given in Table VIII. This method is limited by several considerations. First, we know that losses occur during purification of each peptide. Second, there is no assurance that each peptide is quantitatively released by the digestion protocol. Last, this method does not give a comprehensive description of protein composition, since there may be precursors that are not released by our digestion protocols. These proteins would be missed in the analysis. Thus, this method yields the minimum amount of a protein present in the envelope. This method predicts that SPRs, desmoplakin I, and annexin I comprise a significant percentage of the CE mass.

DISCUSSION

In the present study, we use sequential proteolytic digestion to identify structural components of the cornified envelope of cultured keratinocytes. CNBr digestion, which cleaves after M residues, released fragments of involucrin, desmoplakin I, plakoglobin, envoplakin, desmoglein 3, plakophilin, PAI-2, desmocolin 3a/3b, annexin I, S100A11, and S100A10 (Tables II and III). Trypsin digestion of the CNBr-resistant pellet releases SPR1A and SPR1B protein fragments, as well as portions of S100A11 and annexin I (Table IV). Subsequent proteinase K digestion of the CNBr-digested and trypsin-digested, acid-insoluble residue releases peptide fragments of S100A11, desmoplakin I, involucrin, SPR1A, and SPR1B (Table V). These proteins can be divided into three functional groups including (i) keratins, plakoglobin, S100A11, desmoplakin, SPRs, and proelafin were predicted to be the major CE components. However, the values of the residuals (i.e. root-mean-square discrepancy and the median discrepancy, Table VII) were much greater than 1, indicating a poor fit. A good fit is indicated by residuals of ≤ 1.

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Precursors of the Cornified Envelope

The present report is the first to establish that S100 proteins are CE precursors. The S100 proteins are a family of small, acidic, Ca\(^{2+}\)-binding proteins that contain two Ca\(^{2+}\)-binding EF hands (67, 68). S100 proteins are thought to function like calmodulin, having an important role in calcium-dependent signaling (67, 68). However, unlike calmodulin, S100 proteins are expressed in a tissue-specific manner. It is thought that binding of calcium to the EF hands of the S100 proteins results in a conformation change that exposes protein-interaction sites (68). The activated S100 proteins then bind to and regulate the function of target proteins (67, 68). Many of the S100 genes, including S100A10 (69, 70) and S100A11 (54, 71), have been co-localized on the same chromosome as other epidermal structural protein genes, suggesting a possible function in skin. Thus, the S100 proteins join another keratinocyte envelope precursor, profilaggrin, as an EF hand-containing protein (72). Tryglycinin, a hair follicle protein, which may also be an envelope precursor, also contains an EF hand that may be functionally important (73).

S100A11 (S100C, calgizzarin) has been identified in gizzard, lung, heart (74, 75), skin (54), and cultured keratinocytes (55). Our cDNA sequence shows that epidermal S100A11 is identical to S100A11 isolated from human colon carcinoma cells (43). As mentioned above, the S100 proteins are thought to affect Ca\(^{2+}\)-signaling pathways by binding to and regulating the activity of target proteins. S100A11 interacts with annexin I (76, 77) in a calcium-dependent manner, an interaction that receives the first 12 amino acids of the annexin I amino terminus (77). The central hinge and carboxyl terminus are the least conserved regions among S100 proteins and are likely to provide the sites that co-evolve with other proteins (68). It has been suggested (77) that the COOH-terminal segment is not required for the interaction, implicating the hinge region as the annexin I interaction site. It is possible that annexin I, via its interaction with the plasma membrane, serves to anchor S100A11 near the plasma membrane, positioning S100A11 for cross-linking.

This study also identifies S100A10 as a component of the keratinocyte cornified envelope. S100A10 (also referred to as p10, p11, and calpactin light chain) binds to annexin II to form a tetramer, called calpactin 1, consisting of two annexin II and two S100A10 proteins (78). S100A10 binds tightly to the annexin II amino terminus via an interaction that does not require calcium. Annexin II differs from annexin I in that it is not a transglutaminase substrate (29, 30); however, annexin II does bind to phospholipids in a calcium-dependent manner and thus could be expected to be associated with plasma membranes. In vitro results suggest that S100A10 and annexin II form a high affinity, specific interaction that requires urea for dissociation (78) and that S100A10 cannot bind to membranes in the absence of annexin II (78). The fact that we find S100A10

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**Table VII**

| Precursor | PEI | Keratin | CysA | Fil | Annex I | SPR | Desmop | S100A11 | S100A10 | Plakog | Residuals
|-----------|-----|---------|------|-----|---------|-----|--------|---------|---------|--------|---------|
| Undigested | 6.0 | 42.4 | 0.6 | 3.8 | 0 | 6.8 | 7.7 | 8.2 | 0.4 | 23.9 | 30.6/24.5 |
| CNBr | 1.2 | 36.2 | 0.9 | 1.6 | 0 | 5.4 | 13.0 | 12.6 | 1.4 | 27.6 | 53.2/43.4 |
| Trypsin | 1.2 | 51.7 | 6.1 | 4.9 | 0 | 8.7 | 5.0 | 8.5 | 0 | 13.9 | 83.9/64.7 |
| Prot K | 2.0 | 68.9 | 2.9 | 4.2 | 3.8 | 8.6 | 0 | 0 | 0 | 9.6 | 45.0/35.1 |

**Notes:**
- Peptide and proteins correspond to those listed in Tables II–V. The most abundant peptide detected is shown.
- Peptides and proteins are not listed because they are not estimated to be present by the analysis.
- The CE amino acid composition data shown in Table VI, and the known amino acid composition of each protein (from the literature), were used to estimate, using least squares analysis, the protein composition of undigested CE fragments and of residual pellet following digestion with CNBr, CNBr, and trypsin (Trypsin), or CNBr, CNBr, and proteinase K (Prot K).
- The first value is the mean-square discrepancy, and the second is the median discrepancy. Both are expressed as % per amino acid residue.

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**Table VIII**

| Peptide | Protein | m\(_p\)/mg | Mass |
|---------|---------|------------|------|
| T5 | SPR1A/1B | 9,900 | 21.52 |
| C1 | Desmoplakin I | 331,771 | 124 |
| C4 | Annexin I | 35,723 | 34.1 |
| C8 | Plakoglobin | 81,744 | 64 |
| C12 | S100A11 | 11,740 | 287 |
| C17 | S100A10 | 11,072 | 200 |
| C13a | Plakophilin | 80,496 | 25 |
| C14 | Envolucrin | 230,899 | 8 |
| C5b | Keratin | 56,000 | 15 |
| C13 | Involutin | 68,476 | 0.06 |
| C11b | PAI-2 | 46,596 | 0.03 |
| C10 | Desmoglein 3 | 101,813 | 2 |
| C19 | Desmocollin 3A/3B | 84,727 | 1 |

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**Notes:**
- Peptides and proteins are not listed because they are not estimated to be present by the analysis.
- Picomoles detected (by sequencing)/mg of starting CE fragments.
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as a component of the keratinocyte cornified envelope, but not annexin II, suggests that annexin II may position S100A10 for cross-linking. In this model, annexin II could be released during envelope assembly, or it could remain associated with the CE, as a non-cross-linked or disulfide-linked precursor that is extracted by our CE purification procedures. This would suggest that annexin II may serve as an “envelope organizer protein” that never becomes covalently associated.

**Soluble Precursors**—Consistent with previous reports (13, 14, 16, 18, 35, 36), our study identifies involucrin and SPR1A and SPR1B as cytoplasmic, hydrophilic proteins that are incorporated into the cornified envelope. Our results, showing that the central portion of the SPR1A and SPR1B proteins is preferentially released by digestion of cornified envelopes, indicate that SPR1A and SPR1B are cross-linked via the amino- and carboxyl-terminal ends. This suggests that these SPRs may function as molecular cross-bridges to connect two proteins (16). A similar role has been proposed for involucrin (11). The yield of the SPR peptides suggests that SPR proteins are major components of the cornified envelope of cultured keratinocytes.

We also detected PAI-2. PAI-2, a serine protease inhibitor that inhibits urokinase-type plasminogen activator, has previously been suggested to be an envelope precursor (32). PAI-2 is one of a growing number of proteinase inhibitors, including elafin (21, 35) and cystatin A (20, 79), that are thought to be components of the CE. These inhibitors may have a role in regulating the process of envelope formation and/or protecting envelope integrity, by differentially inhibiting specific proteinases (1).

**Desmosomal Proteins**—Our studies identify desmocollin 3a/3b, desmoglein 3, desmoplakin I, plakoglobin, envoplakin, and plakophilin as envelope components. The desmosome, beginning on the intracellular side, consists of an inner plaque, outer plaque, and membrane-associated desmosome core. The desmosome core contains the extracellular domains of desmogleins and desmocollins (80). Plakoglobin, plakophilin, and the intracellular domains of the desmogleins and desmocollins are components of the outer plaque (80). Desmoplakin I, and presumably envoplakin, are components of the inner plaque (80). The structure of envoplakin, a 210-kDa membrane-associated protein, has recently been presented (34). This protein was originally shown to be a component of keratinocyte envelopes, following induced envelope formation in cultured keratinocytes (33). Thus, our studies show that desmosomal proteins become cross-linked components of the keratinocyte cornified envelope, confirming previous electron microscopic and immunohistological studies that identify desmosomes as part of purified CE fragments (13, 27, 36). Interestingly, the presence or absence of desmosome-like structures in CEs is affected by the cell culture conditions and method used to initiate envelope formation (36).

**Location of Cross-linking Sites**—Our results are the first to suggest that S100A11 is covalently modified; furthermore, although we did not directly sequence the cross-linking sites, our results suggest that Lys⁷, Gln¹⁰², and Lys¹⁰⁳ are likely sites of cross-link formation. Information regarding S100A11 cross-linking sites was derived from analysis of the release pattern of the envelope precursor peptides. In contrast to the central region, the carboxyl and amino termini of S100A11 are not readily released by CNBr treatment. However, pieces of these regions are released by subsequent trypsin or proteinase K digestion. These results suggest that the amino- and carboxyl-terminal ends are sites of attachment to the envelope. These results are consistent with the possibility that S100A11 specifically interacts with annexin I via the central region of S100A11. Annexin I binding to this region may prevent it from being available as a TG substrate.

SPR1A and SPR1B have been shown to be precursors of the cornified envelope in *in vivo* corneocytes (35). Cross-links have been identified at positions Lys⁴, Gln⁸⁸, and Lys⁸⁹ (35). Our results, indicating that the amino- and carboxyl-terminal ends are the sites of cross-link formation, are consistent with this result. In this previous report, loricrin was identified as the partner of SPR in cross-link formation. Although loricrin is likely to be expressed in our 3T3-dependent, retinoid-deficient culture system (81), proteolytic digestion did not release loricrin peptides. This is surprising as loricrin has been shown to be a frequent participant in *in vivo* cross-link formation (35). It is possible that loricrin is produced at low levels in our culture system, or that it is produced but is not efficiently delivered to the site of cross-link formation. It is also possible that our fractionation system does not favor retention of loricrin fragments. However, this appears unlikely, since our isolation and fractionation conditions are similar to those used to identify loricrin fragments from *in vivo* envelopes (35). Our results suggest that SPRs are cross-linked to other proteins in the cultured cells.

**Precursor Composition**—We used two methods to estimate the content of precursors in the cornified envelopes. The first method, using the experimentally determined amino acid composition of the CE fragments and the known amino acid composition of the precursor proteins, predicts envelope composition using a least squares best fit analysis (25, 26). Using this method, it has been reported that involucrin, cystatin A, and cysteine-rich protein (elafin) each comprise >25% (by mass) of cornified envelopes prepared from cultured cells (26). Mathematical modeling of our amino acid composition data predicts that keratins, plakoglobin, S100A11, desmoplakin, SPRs, and proelafin are the most abundant proteins (Table VII). Loricrin, cystatin A, involucrin, annexin I, desmoglein, desmocollin, plakoglobin, and envoplakin are predicted not to be present. To obtain these estimates, we were forced to apply the constraint that the composition values could not be negative. When this constraint was removed, large negative (non-physiologic) values were obtained. In addition, very large residuals were obtained, indicating that the method did not accurately predict the relative content of each CE precursor. Moreover, as described below, the estimates obtained using this method are not consistent with those obtained by peptide sequencing.

The second method we used estimates protein abundance by measuring the amount of each peptide fragment detected in sequencing experiments. By mass, SPRs, desmoplakin I, and annexin I are predicted to be the most abundant proteins. The estimation of a high content of annexin I is consistent with the gel shown in Fig. 2, which shows an abundance of annexin I peptide released by CNBr cleavage of envelopes. It seems unlikely that desmoplakin I is the second most abundant CE component. On a molar basis, S100A11, SPR1A, and SPR1B have been shown to be precursors of the cornified envelope in *in vivo* corneocytes (35). Cross-links have been identified at positions Lys⁷, Gln⁸⁸, and Lys⁸⁹ (35). Our results, indicating that the amino- and carboxyl-terminal ends are the sites of cross-link formation, are consistent with this result. In this previous report, loricrin was identified as the partner of SPR in cross-link formation. Although loricrin is likely to be expressed in our 3T3-dependent, retinoid-deficient culture system (81), proteolytic digestion did not release loricrin peptides. This is surprising as loricrin has been shown to be a frequent participant in *in vivo* cross-link formation (35). It is possible that loricrin is produced at low levels in our culture system, or that it is produced but is not efficiently delivered to the site of cross-link formation. It is also possible that our fractionation system does not favor retention of loricrin fragments. However, this appears unlikely, since our isolation and fractionation conditions are similar to those used to identify loricrin fragments from *in vivo* envelopes (35). Our results suggest that SPRs are cross-linked to other proteins in the cultured cells.

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been proposed for the insoluble envelope precursors, such as loricrin, involves delivery via a vesicle (10). Our present results, which identify S100A11, S100A10, and annexin I as envelope components, suggests a third mechanism, which uses what we call “envelope organizer proteins.” Annexin I is known to bind to membrane phospholipids in a calcium-dependent manner (56). It is also known that calcium levels rise as keratinocytes differentiate (82, 83). This suggests, that as cells differentiate and calcium levels rise, annexin I, as a complex with S100A11, moves to the plasma membrane and binds to the inner surface. In this manner, annexin I can be synthesized as a soluble precursor, and only later, when calcium levels rise, transferred to the site of cross-linking. A similar model can be envisaged for S100A10 and annexin II. In this model, the annexin functions as envelope organizer proteins (i.e. proteins that move precursors to the appropriate location, but are not themselves necessarily cross-linked).

The Precursor Availability Hypothesis—Based on previously published data (84) and our results, we suggest that the process of CE formation will utilize those reactive precursors (i.e. transglutaminase substrates) that are available at the time of cross-linked partner. This model predicts that there is a family of proteins, regardless of their role in other cellular processes, also function as envelope components (i.e. are not waste proteins), and that only specific cross-linking sites are utilized on each protein. Moreover, the site of cross-link formation within each protein does not depend upon the identity of the cross-linked partner. This model predicts that there is a family of proteins that function as CE precursors and that much of the difference in envelope composition results from difference in the abundance and availability of each precursor at the time of CE assembly.

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