Identification of Transglutaminase-reactive Residues in S100A11*

(Received for publication, August 15, 1997, and in revised form, November 14, 1997)

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The recent finding that S100A11 is a component of the keratinocyte cornified envelope (CE) (Robinson, N. A., Lapic, S., Welte, J. F., and Eckert, R. L. (1997) J. Biol. Chem. 272, 12035–12046) suggests that S100A11 is a transglutaminase (TG) substrate. In the present study we show that S100A11 forms multimers when cultured keratinocytes are challenged by increased levels of intracellular calcium and that multimer formation is inhibited by the TG inhibitor, cystamine. These S100A11 multimers appear to be incorporated into the CE, as immunoreactive S100A11 is detected in purified envelopes prepared from cultured and from foreskin epidermis. To study S100A11 as a transglutaminase substrate, recombinant human S100A11 (rhS100A11) was used in a cell-free cross-linking system. [14C]Putrescine, a primary amine, labels rhS100A11 in a TG-dependent manner. Trypsin digestion of [14C]putrescine-labeled rhS100A11 releases one radiolabeled peptide, Ala98-Lys102. The glutamine residue in this segment, Gln102, is the site of radiolabel incorporation indicating that Gln102 functions as an amine acceptor. The ability of S100A11 to form multimers indicates that it also has a reactive lysine residue that functions as an amine donor. To identify the reactive residue, we compared the high pressure liquid chromatography profile of trypsin-digested rhS100A11 monomer to that of cross-linked rhS100A11. A unique cross-linked peptide was purified and identified as Met-Ala-Lys7-Ile-Ser-Ser-Pro-Thr-Glu-Thr-Glu-Arg cross-linked via an Lys3-Gln102 isopeptide bond to Ala-Val-Pro-Ser-Gln102-Lys. These studies show that S100A11 is post-translationally modified by transglutaminase, that it can be cross-linked to form multimers, that it is present in CEs from cultured keratinocytes and in vivo epidermis, and that Lys3 and Gln102 are specific sites of cross-link formation.

Transglutaminases are calcium-dependent enzymes that catalyze the formation of interprotein epsilon-(γ-glutamyl)lysine bonds (i.e. covalent cross-links between the side groups of peptide-bound Gln and Lys residues) (1, 2). Keratinocyte transglutaminases, in response to an increase in intracellular calcium, covalently cross-link a variety of soluble and insoluble precursor proteins and form the cornified envelope (CE) (1) (3–5). The cornified envelope is deposited beneath the plasma membrane as keratinocytes differentiate into cornocytes (5, 6) and is necessary for proper maintenance of epidermal barrier function. Because of the importance of this structure, identifying the proteins that compose the envelope is an area of intense interest. We recently identified two S100 proteins, S100A11 (S100C, calzigazzin) and S100A10 (calpain light chain, p11), as components of cornified envelopes prepared from cultured keratinocytes (7).

S100 proteins are thought to be regulators of cell signaling, cell proliferation, and cell structure (8, 9). They comprise a family of 10–12-kilodalton acidic proteins that form homo- and heterodimers and bind calcium via two “EF hand motifs.” When intracellular calcium levels increase, calcium binding to the EF hand motifs triggers a conformational change that exposes hydrophobic domains promoting S100 complex formation with target proteins. As S100 proteins have no known enzymatic activity, they are thought to regulate the activity of specific target proteins in a calmodulin-like manner (8, 9). These target proteins, in turn, regulate cell signaling, cell shape, and other cell processes. Some of these target proteins are known (8). S100A11 and S100A10, for example, are known to complex, respectively, with annexin I (10–12) and annexin II (13).

Because of the proposed role that these proteins play in mediating calcium-dependent signal transduction events, it is important to identify post-translational modifications that may modulate their function. Only two reports describe covalent modification of S100 proteins (7, 14). Moreover, the physiological role of these proteins is poorly understood. In the present study we provide direct evidence that transglutaminase covalently modifies S100A11, and we also identify the residues that are modified. Our results suggest that one physiological role of S100A11 in keratinocytes is as a component of the keratinocyte-cornified envelope.

MATERIALS AND METHODS

Preparation of Cell Extracts, Conditioned Medium, and Cornified Envelopes from Keratinocytes—Normal human keratinocytes were grown on lethally irradiated 3T3 cells in 50-cm2 dishes as described previously (15). The feeder cells were removed and confluent keratinocyte cultures were incubated for 30 min in serum-free media with or without the addition of 20 μM cystamine and then cultured for 6 h in the presence or absence of 0.75 μM NaCl to induce cornified envelope formation (16). At the end of the incubation period, the culture medium (6 ml) was collected, transferred into Tris-buffered saline (TBS, 20 mM Tris-HCl, pH 7.4, containing 0.15 M NaCl), and concentrated to 1 ml (per 50-cm2 culture dish) using an UltraFree-15 centrifugal filter unit (Biomax-5K NMWL, Millipore Corp.). To prepare cell extracts, cells

* This work was supported by Grant GM43751 from the National Institutes of Health (to R. L. E.). The Skin Diseases Research Center of Northeast Ohio utilized by this study was supported by National Institutes of Health Grant P30 CA44370. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Supported as a trainee of the National Institutes of Health Training Program in Cardiovascular Research Grant HL07653.

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were washed with TBS and extracted with 1 ml of 125 mM Tris·HCl, pH 6.8, containing 3% sodium dodecyl sulfate (SDS) and 4% β-mercaptoethanol (BME) per 50-cm² culture dish. The samples were heated to 95°C for 5 min, and insoluble material was removed by centrifugation. Purified cornified envelope fragments were prepared from cultured keratinocytes. The Aβ₃₃ protein, a high molecular weight 164 amino acid species, was sonicated, and the samples were subjected to three cycles of sonication and freeze/thaw and centrifuged at 7,500 g; the cell pellet was suspended in 4 ml of binding buffer (20 mM Tris·HCl, pH 7.9, containing 0.5 mM NaCl and 0.6% BME) and electrophoresed on 16% polyacrylamide gels (18). The gels were allowed to polymerize overnight prior to use. The separated proteins were visualized using Coomassie Blue. To detect [14C]putrescine-labeled peptides, the gels were soaked in fluorographic agent (Fluro-Hance, RPI, Mount Prospect, IL), dried, and exposed on x-ray film. In some cases radioactivity was quantitated in gel slices. The slices were dissolved in alkaline H₂O₂ at 65°C and neutralized prior to scintillation counting (22).

RESULTS

S100A11 Is a Transglutaminase Substrate in Cultured Epidermal Keratinocytes—To study the process of S100A11 crosslinking in keratinocytes, we activated transglutaminase by promoting an increase in the level of intracellular free calcium using high extracellular NaCl (16). We then assayed for the formation of S100A11 multimers (Fig. 1A). In cells maintained under normal culture conditions, S100A11 monomer is detected in the absence (lane 1) or presence (lane 2) of 20 mM cysteine, a transglutaminase inhibitor. In the presence of elevated intracellular calcium (lane 3), a decrease in the level of S100A11 monomer (M) is associated with dimer (D), tetramer (T), and larger multimer (*) formation. The less intensely stained proteins at 45,000 daltons are not S100A11, as they are also detected in an identical blot probed with premuine serum (PI, lane 10). As expected, addition of cysteamine to this reaction inhibits protein cross-linking; however, the level of monomer was also unexpectedly reduced (lane 4). We reasoned that the reduced level of S100A11 monomer present in permeabilized cells under conditions where cross-linking is inhibited may result from leakage of S100A11 from the cells into the culture medium. As shown in Fig. 1A, S100A11 is not detected in the culture medium from non-permeabilized control cells in
the absence or presence of cystamine (lanes 6 and 7). In contrast, the S100A11 dimer and monomer, respectively, are detected in the cell culture medium from permeabilized cells (lane 8) and cystamine-treated permeabilized cells (lane 9). As a control, we measured involucrin distribution in a parallel experiment. As shown in Fig. 1B and previously reported (16, 23, 24), involucrin disappears from the extractable phase when cells were permeabilized in the absence of cystamine (lane 3). In cells that are permeabilized and treated with cystamine to inhibit cross-linking, involucrin monomer is also detected in the culture medium (lane 8).

S100A11 Is a Component of in Vitro and in Vivo Cornified Envelopes—To determine whether S100A11 is incorporated into cornified envelopes in cultured keratinocytes, envelopes that were formed spontaneously (25) or induced to form were sonicated, extensively washed, blotted onto nitrocellulose, and assayed for the presence of S100A11 by immuno-dot blot. As shown in Fig. 1C, the S100A11-specific antibody reacted with the envelope fragments. To assay for the presence of S100A11 in human in vivo envelopes, human foreskin envelopes were prepared and subjected to dot blot analysis. Immunoreactive S100A11 is detected in these envelopes. The blot prepared from neonatal foreskin envelopes and probed with anti-S100A11 were exposed seven times longer than those prepared from cultured keratinocytes. Thus, less S100A11 is immuno-detected in envelopes from neonatal foreskin compared with envelopes from cultured keratinocytes. No reactivity was detected for the preimmune serum (from S100A11 immunized rabbits) in in vivo or cell culture envelopes. As expected, involucrin was detected both in envelopes prepared from cultured cells and in envelopes prepared from human foreskin epidermis (15, 26). To ensure that the dot immunoblot was not detecting non-cross-linked S100A11, crude total extracts and purified envelopes prepared from human foreskin were electrophoresed on a 16% polyacrylamide gel and assayed for the presence of non-cross-linked S100A11 by immunoblot. Non-cross-linked S100A11 was detected in the crude extract; however, non-cross-linked S100A11 was not detected in the purified envelope preparation (not shown). These results provide additional evidence that the dot immunoblot is indeed detecting covalently cross-linked S100A11.

Purification of Recombinant S100A11 (rhS100A11) from Bacteria—As part of studies designed to identify the S100A11 cross-linking site, we purified rhS100A11 from bacteria transfected with pET28a–S100A11. Activation of pET28a–S100A11 transcription by isopropyl-1-thio-β-D-galactopyranoside addition results in production of S100A11 within 30 min. By 4 h, rhS100A11 is the predominant protein being produced by the bacteria (not shown). We used immobilized metal affinity chromatography (IMAC) to purify rhS100A11. Fig. 2 shows the crude bacterial lysate prepared from bacteria after a 4 h treatment with isopropyl-1-thio-β-D-galactopyranoside. In the presence of reducing agent, purified rhS100A11, recovered from the IMAC column bound fraction, migrates as a 13,400-dalton monomer (M) in the absence of reducing agent, it migrates as a 25,000-dalton disulfide-linked dimer (D). The protein migrating at 15,000 daltons in IMAC-purified material (BME) is a copurifying bacterial protein. After thrombin cleavage of the amino-terminal polyhistidine tract, the products were rechromatographed on the IMAC column. The contaminating bacterial protein and the cleaved polyhistidine tract were retained by the column, and rhS100A11, which contains a Gly-Ser-His amino-terminal extension, was recovered in the unbound fraction. As shown in the thrombin-cleaved sample, the protein migrates as a 10,000-dalton monomer (M*) in the absence of reducing agent. However, when electrophoresed in the absence

![Cross-linking of S100A11 Protein](http://www.jbc.org/)
of reducing agent, rhS100A11 migrates as disulfide-linked multimers. The even multimers predominate, with the dimer (D*) being the most abundant form.

Reduction followed by covalent modification of the cysteine residues with 4-vinylpyridine to prevent disulfide bond formation yields a band that migrates as a 10,000-dalton monomer in the absence of reducing agent. A small amount (5–10% of total protein) of what appears to be rhS100A11 dimer is resistant to this chemical modification. Such a reduction-resistant form has been previously noted (27). In general we obtained 24 mg of rhS100A11 per liter of bacteria.

**rhS100A11 Is a Transglutaminase Substrate**—To assay for S100A11 reactivity as a transglutaminase substrate, rhS100A11 was incubated with TG2 in a calcium-containing reaction. At various times, aliquots were removed and electrophoresed. As shown in Fig. 3A, the reaction produced dimers (D), trimers (T), tetramers (T4) and larger multimers (*, **). Multimer formation was inhibited by addition of the transglutaminase inhibitor, cystamine, or by addition of calcium chelator (not shown). To estimate the amount of multimer formed, we scanned the Coomassie Blue-stained gel and expressed the results as a percent of total rhS100A11 (Fig. 3B). At 180 min, 60% rhS100A11 is present as dimer, 5% as a trimer, and 4% as a tetramer. Varying the concentration of rhS100A11 from 0.25 to 3.5 mg/ml did not affect the pattern of multimer formation (i.e. dimer is the predominant form at all substrate concentrations, not shown). The finding that multimers are formed confirms that S100A11 is a TG substrate and contains at least one reactive glutamine and one reactive lysine residue.

Radiolabeled small primary amines, such as [14C]putrescine, can serve as amine donors in the transglutaminase-catalyzed cross-linking reaction (19) and can be used to label reactive glutamine residues. Addition of [14C]putrescine to the cross-linking reaction results in a time-dependent incorporation of label into rhS100A11 monomer (M), dimer (D), and higher multimers (*) (Fig. 4A). Prolonged exposure of the gel (Fig. 4B) reveals that radiolabeled protein is also present as trimers (T) and tetramers (T4). In addition, multimers are present at the stacking/separatory gel interface (*) and in the gel wells (**) (Fig. 4B). Little or no incorporation of [14C]putrescine is observed when rhS100A11 is omitted from the reaction (not shown) or cystamine (+cystamine) or EDTA (+EDTA) is included in the reaction. Quantitation of [14C]putrescine incorporation into rhS100A11 is shown in Fig. 4C. The percent of rhS100A11 labeled with [14C]putrescine ranged from 6.5 to 12% in three separate experiments. These results confirm that rhS100A11 is an in vitro TG substrate.

**Identification of Reactive Glutamine Residues in rhS100A11**—rhS100A11 contains 4 glutamine and 11 lysine residues. To identify the TG reactive glutamine(s), rhS100A11 was labeled with...
Mm cystamine or 100 mM EDTA was included in the reaction mixture.

Each of the four glutamine residues are contained within different S100A11 to produce 11 peptide fragments and 4 single amino acids. After digestion with trypsin, the sample was fractionated by HPLC using a C18 column. Trypsin cleaves cysteines were modified with 4-vinylpuridine to prevent reforming of disulfide bonds. The sample was digested with trypsin and analyzed by HPLC. Panel A shows the A220 absorbance profile. Each of the A220 peaks were collected to determine [14C]putrescine content. The three radioactive peaks (1, 2, and 3) are indicated. The dashed line indicates the progress of the acetonitrile gradient. The single asterisk indicates two peaks that were present in a control reaction lacking rhS100A11. The double asterisk indicates the elution position of undigested, intact rhS100A11. The radioactive peptide eluting at 30.2 min (peak 1) was purified and microsequenced. Two sequencing runs were performed as follows: the first to identify the cycle in which the radioactivity was released, and the second to identify the amino acid released at each cycle (panel B). The dashes in cycles 7–10 indicate an absence of amino acid released. The Glu residue (cycle 5) corresponds to Gln in the unmodified protein (see text).

Portions of this sample were used for two separate sequencing runs as follows: the first identified the cycle in which the radioactivity was released, and the second determined the amino acid sequence. The results of these analyses are shown in Fig. 5B. The radiolabel is released in sequence cycle five of the peptide Ala-Val-Pro-Ser-Glu-Lys. The amino acid sequence identifies a unique tryptic fragment, Ala16-Glu-Pro-Ser-Gln-Lys19, present at the carboxy end of S100A11. The fifth residue was originally [14C]putrescine-labeled Gln but is detected as Glu. This is either because [14C]putrescine-labeled Gln elutes with Glu or, more likely, the [14C]putrescine label is cleaved from the Gln residue yielding Glu due to acid treatment during the phenylthiohydantoin derivitization. Glu is known to be deaminated by treatment with strong acids (28). An alternate possibility is that the original S100A11 protein contained a Glu at position 102; however, sequencing the corresponding tryptic peptide from the non-cross-linked protein identified Gln at this position (see below). This analysis identifies Gln102 as the reactive amine acceptor.

Identification of Reactive Lysine Residues in rhS100A11—To identify reactive lysines, rhS100A11 was incubated with TG2 to form covalently cross-linked multimers. At various times after addition of TG2, aliquots containing 100 μg of rhS100A11 were reduced, modified with 4-vinylpyridine to prevent disulfide bond formation, and digested with trypsin. We identified peaks that change in amplitude or elution time with increased...
Peptides P1, P2, P3, and P4 were purified by C18 HPLC using a shallow acetonitrile gradient and sequenced. The three tryptic peptides that decreased were Gly-3-Lys3, Ala96-Lys103, and Ile4-Ile12; peptides P1, P2, and P3, respectively (Fig. 7A). Peptide P4 was the rhS100A11 amino-terminal peptide, Gly-7-Arg12, dipeptide cross-linked to the rhS100A11 Ala96-Lys103 peptide derived from near the carboxyl terminus (Fig. 7B). The amino acids were released from each cycle as follows: 1-Gly and Ala; 2-Ser and Val; 3-His and Pro; 4-Met and Ser; and 5-Ala. Cycle six released the e-γ-glutamyllysine dipeptide and Lys. Cycles 7–15 yielded one amino acid per cycle. Only one amino acid was released during cycle 5, as Glu102, which was cleaved from the AVPSQ102K peptide during this cycle, remained covalently attached to Lys3 of the GSHMK11SSPETER peptide (Fig. 7B). Lys and the e-γ-glutamyllysine dipeptide (Gln102/Lys3) are released in cycle six.

**DISCUSSION**

**S100 Protein Structure and Function**—The S100 proteins comprise a family of low molecular mass (10–12 kilodaltons) EF-hand-containing, calcium-binding proteins that are expressed in a tissue- and differentiation-specific manner. The S100 proteins are thought to function in a calmodulin-like manner to regulate calcium-dependent cell signaling, proliferation, and cell shape. S100 proteins appear to function in a wide variety of physiological processes, including regulation of phosphorylation by protein kinase C, regulation of energy metabolism by modulating the activity of metabolic enzymes, promotion of neurite extension, regulation of inflammation, regulation of cell cycle progression, and modulation of membrane-cytoskeletal interaction (for reviews see Refs. 8, 9, 29, 30). They also serve as markers of cell differentiation, are inappropriately regulated in some disease states, and function as precursors of the keratinocyte cornified envelope (7–9, 29, 30). The S100s are a family of proteins that share a common sequence and structure (8, 31). The structure of one of these proteins, S100A6, has been solved (32) and shows that S100 proteins possess a central ~55 amino acid globular core domain that contains two EF-hands (calcium binding loops), one in the basic amino terminus and a second in the acidic carboxyl ter-

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**FIG. 6.** Transglutaminase-dependent formation of a Lys3-Gln102 cross-link. rhS100A11 was incubated with TG2 and 50 μM calcium. Aliquots containing 100 μg of S100A11 were withdrawn, reduced, modified with 4-vinylpuridine, digested with trypsin, and analyzed by HPLC. Panels A and B show the A220 profile generated for the samples reacted with transglutaminase for 0 and 90 min, respectively. Peak P4, which elutes at 45 min, increases in a transglutaminase- and time-dependent manner. Peaks P1, P2, and P3 at 27.6, 28.7 and 37.5 min, respectively, decrease during the course of the transglutaminase cross-linking reaction. The area of the other peaks, including peak C (41.2 min), does not change. The dashed line indicates the proportion of the acetonitrile gradient. The asterisks are as defined in Fig. 5. Panel C compares the relative peak area of peaks P1, P2, P3, P4, and C at 0, 10, 40, and 95 min during the transglutaminase reaction.

**FIG. 7.** Amino acid sequence and trypsin cleavage products of rhS100A11. Panel A shows rhS100A11 divided into fragments based on the tryptic cleavage pattern. The S100A11 sequence begins with the underlined methionine residue, M1, and ends at Thr105. A three amino acid amino-terminal extension (Gly-3-Ser-2-His-1) remains attached to the rhS100A11 following thrombin cleavage of the amino-terminal polyhistidine tract. The shaded segments indicate the peptide fragments (P1, P2, and P3) that decrease during cross-link formation. Lys3 and Gln102 (arrows) participate in intraprotein cross-link formation. Panel B, shows the structure of the cross-linked peptide, P4, that is produced. The solid line indicates the e-γ-glutamyllysine cross-link between Lys3 and Gln102.
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minus (9). The globular domain is flanked by hydrophobic amino- and carboxyl-terminal domains, each of which is approximately 20 amino acids in length. The available evidence suggests that S100 proteins exist in cells as anti-parallel, non-covalent homo- and heterodimers (32) and that the interface formed by dimer interaction constructs a unique “homodimeric fold” that distinguishes S100 proteins from other EF-hand calcium-binding proteins. Potts et al. (32) have suggested that this interface may be required for calcium-dependent binding to target proteins. In cells, increased intracellular calcium triggers a conformation change in the dimeric unit to expose hydrophobic surfaces. This conformation change drives the interaction of the S100 protein with its target protein(s) (9).

Because of the role that these proteins play in regulating cell shape, proliferation, and differentiation (8, 9, 29, 30), it is important to identify covalent modifications that may affect function. To date, only one example of covalent post-translational modification of S100 proteins has been described, the phosphorylation of S100A9 (14).

S100A11 Is a Component of CEs from Epidermis—We recently demonstrated that S100A11 has a physiological function in cultured keratinocytes as a CE component (7). The cornified envelope is a protective structure that is formed during the final stages in keratinocyte differentiation. The envelope is assembled from a variety of precursor proteins via the action of transglutaminase enzymes that catalyze the formation of covalent interprotein ε(γ-glutamyl)lysine isopeptide bonds (5, 16, 33–35). We identified S100A11 in envelopes by sequencing proteolytic fragments of S100A11, as well as S100A10 and annexin I, prepared from purified CE preparations derived from cultured cells (7). In the present article, we produced purified rhS100A11 and used it as an immunogen to generate an S100A11-specific antibody. Using this antibody we show that purified envelopes isolated from foreskin epidermis contain S100A11. The staining is specific, and the intensity is proportional to envelope number. These results extend our previous results showing that S100A11 is a component of CEs from cultured cells (7) and provide the first evidence that S100A11 is a CE constituent in vivo.

In Vitro Cross-linking of S100A11 Identifies Lys³ and Gln¹⁰² as TG-Responsive Residues—Our previous study suggested that S100A11 was cross-linked to other envelope proteins in vivo via Lys³, Lys³², and/or Gln²³ at the amino terminus and Gln¹⁰² and/or Lys¹⁰³ at the carboxyl terminus (7); however, we could not identify the specific amino acids involved in cross-link formation. To identify these sites, we produced rhS100A11 and studied interprotein cross-link formation in vitro.

Incubation of rhS100A11 with transglutaminase results in the formation of dimers, trimers, tetramers, and larger multimers. These multimers are resistant to boiling in the presence of detergent and reducing agent, consistent with the presence of covalent cross-links. Cross-link formation is transglutaminase-dependent, as incubation in the absence of calcium, a cofactor required for transglutaminase activity, or in the presence of cystamine, a transglutaminase inhibitor, eliminates multimer formation. S100A11 contains 4 Gln residues and 10 Lys residues. Our results show that only Lys³ and Gln¹⁰² are sites of transglutaminase-dependent cross-link formation in rhS100A11. These residues are located at the extreme ends of the 105-amino acid protein (Fig. 7A). The three other glutamine residues are present within the central EF-hand-containing globular domain and are not likely to be accessible to transglutaminase. Of the 10 Lys residues, 7 are present within the globular domain. In contrast, Lys³, Lys⁹⁷, and Lys¹⁰³ are near the ends and are likely to be solvent-exposed. Despite this, only Lys³ is utilized as a site of cross-link formation. These results are consistent with previous studies indicating that TG may prefer solvent-exposed glutamine and lysine residues (2, 19, 36–38).

In vitro, covalently cross-linked S100A11 dimers, which we assume are anti-parallel, are the predominant product, with the formation of trimer and tetramer limited to 5 and 4% of total protein, respectively. As Lys³ and Gln¹⁰² are the only transglutaminase-reactive residues in S100A11, the formation of anti-parallel dimers that are cross-linked at both ends would produce a terminal product. In vivo, it appears unlikely that S100A11 forms only TG-catalyzed homodimers. If this were the case, S100A11 would not become a non-extractable component of the CE.

Cross-linking of S100A11 in Cultured Keratinocytes—Transglutaminase exists in three forms, TG1, TG2, and TG3 (2, 39–42). Steinert and coworkers (43) have shown that mRNAs encoding all three enzymes are expressed in human foreskin epidermis. Only TG1 and TG2 appear to be expressed in cultured epidermal keratinocytes and in cultured ectocervical epithelial cells (43–45). Expression of these enzymes is highly dependent upon the culture conditions. For example, TGFβ1 dramatically increases the level of TG2 produced in these cell types (44, 45), and TG1 is increased by cell confluence and increased differentiation (44, 46). TG2 may have a role in programmed cell death (apoptosis) in these cell types (45). However, the specific physiological function of the individual TG types is not well understood. TG1 is bound to the particulate (membrane) fraction, and TG2 and TG3 are cytosolic (47–49). In the present study, we characterize the ability of one of these enzymes, TG2, to cross-link rhS100A11 in vitro, and we show that only one Lys and one Gln are reactive. Our studies show that TG2 specifically catalyzes cross-link formation at two locations on the S100A11 protein, Lys³ and Gln¹⁰². However, various TG isoforms can display differing reactivity toward substrate proteins (50); thus, TG1 and TG3 may produce a different pattern of cross-link formation. Additional studies are underway to test this possibility.

There is at present little information available concerning the cross-link partner of S100A11 in vivo. We speculate that annexin I may become cross-linked to S100A11, since these two proteins interact with each other in a calcium-dependent manner (10–12), and annexin I is also a transglutaminase substrate (51–53). Our previous results (7), and the results of others (51), suggest that annexin I is cross-linked near its amino terminus, specifically at Gln³¹, Gln³², or Lys³⁸. It is this region, the first 13 amino acids of annexin I, including the acetylated amino terminus, that comprise the site where S100A11 interacts with annexin I (11, 12). Since annexin I possesses transglutaminase-reactive residues at its amino terminus, and annexin I and S100A11 form a complex, it is possible that S100A11 becomes cross-linked to annexin I via the annexin I amino terminus. Formation of an initial cross-link between annexin I and S100A11 would permit the S100A11 to multimerize and build a multiprotein complex. We are currently studying the interaction of S100A11 and recombinant annexin I.

The Annexin Shuttle Model, Moving Envelope Precursors to the Site of Cross-link Formation—The annexins are a family of proteins that bind to phospholipids in a calcium-dependent manner (54). Based on this fact and the known interaction of annexin I and S100A11, we proposed a model wherein annexin I serves as a shuttle protein to move envelope precursors to the internal surface of the plasma membrane (7). In this model, increased intracellular calcium causes the S100A11 dimer to undergo a conformational change that results in its interaction with annexin I. The S100A11-annexin I complex is then di-
rected to the plasma membrane by the annexin I phospholipid binding domain. The increased calcium also activates transglutaminases that catalyze the formation of covalent cross-links among S100A11 and annexin I. The net result of this process is that annexin I serves as a shuttle to move S100A11 into a position near the plasma membrane prior to cross-linking. We propose that annexin I, and possibly other members of the calcium-mobilized annexin family, may serve a general function as CE precursor shuttle proteins. In support of this idea, a recent study shows that, in addition to S100A11 (7), annexin I also interacts with another known envelope precursor, plasminogen activator inhibitor-2 (7, 55, 56).

Summary—Our studies show that S100A11, an S100 family protein, is a transglutaminase substrate that is incorporated into the keratinocyte-cornified envelope. Based upon the structural similarities among the S100 proteins, we predict that the cross-link sites will be localized at the extreme amino- and carboxyl-terminal ends of S100A10 and possibly other S100 proteins. The reactivity of these residues is consistent with the known tendency of transglutaminases to select solvent-exposed lysine and glutamine residues as reactive sites. Likely reactive sites in human S100A10 are Gln4 in the amino terminus and Lys92, Lys94, Lys96, Lys97, or Glu93 in the carboxyl terminus.

REFERENCES
1. Folk, J. E. (1980) Annu. Rev. Biochem. 49, 517–531
2. Greenberg, C. S., Birckbichler, P. J., and Rice, R. H. (1991) FASEB J. 5, 3071–3077
3. Roehnagel, J. A., and Rogers, G. E. (1984) Mol. Cell. Biochem. 58, 113–119
4. Goldsmith, L. A. (1983) J. Invest. Dermatol. 80, suppl. 39–41
5. Eckert, R. L., Crish, J. F., and Robinson, N. A. (1997) Physiol. Rev. 77, 377–424
6. Reichert, U., Michel, S., and Schmidt, R. (1993) in Molecular Biology of the Skin: The Keratinocyte (Darmon, M., and Blumenberg, M., eds) pp. 107–149, Academic Press, Inc., New York
7. Robinson, N. A., Lapic, S., Welter, J. F., and Eckert, R. L. (1997) J. Biol. Chem. 272, 12035–12046
8. Schafer, B. W., and Heizmann, C. W. (1996) Trends Biochem. Sci. 21, 134–140
9. Hilt, D. C., and Kligman, D. (1991) in Novel Calcium-binding Proteins: Fundamentals and Clinical Implications (Heizmann, C. W., ed) pp. 65–103, Springer-Verlag, Berlin
10. Naka, M., Qings, Z. X., Sasaki, T., Kise, H., Tawara, I., Hamaoichi, S., and Tanaka, T. (1994) Biochem. Biophys. Acta 1223, 348–353
11. Mailliard, W., Hainzy, H. T., and Schlaepfer, D. D. (1996) J. Biol. Chem. 271, 719–725
12. Seemann, J., Weber, K., and Gerke, V. (1996) Biochem. J. 319, 123–129
13. Gerke, V. (1991) in Novel Calcium-binding Proteins: Fundamentals and Clinical Implications (Heizmann, C. W., ed) pp. 139–155, Springer-Verlag, Berlin
14. van den Bos, C., Roth, J., Koch, H. G., Hartmann, M., and Sorg, C. (1996) J. Invest. Dermatol. 107, 101–107
15. Rice, R. H., and Green, H. (1979) Cell 18, 681–694
16. Sieffing, G. E., Jr., Apostoli, A. B., Velasco, P. T., and Lorand, L. (1978) Biochemistry 17, 2598–2604
17. Laemmli, U. K. (1970) Nature 227, 680–685
18. Simon, M., and Green, H. (1989) J. Biol. Chem. 263, 18093–18098
19. Henschen, A. (1986) in Advanced Methods in Protein Microsequence Analysis (Wittmann-Liebold, B., Salnikow, J., and Erdmann, V. A., eds) pp. 244–255, Springer-Verlag Inc., New York
20. Klotz, A. V., Thomas, B. A., Glazer, A. N., and Blacher, R. W. (1993) Anal. Biochem. 200, 3–9
21. Robinson, N. A., Matson, C. F., and Pace, J. G. (1991) J. Biochem. Toxicol. 6, 171–179
22. Blom, A. S., and Bennett, J. C. (1986) in Advanced Methods in Protein Microsequence Analysis (Wittmann-Liebold, B., Salnikow, J., and Erdmann, V. A., eds) pp. 208–215, Springer-Verlag Inc., New York
