Loricrin is the major protein of the cornified cell envelope of terminally differentiated epidermal keratinocytes which functions as a physical barrier. In order to understand its properties and role in cornified cell envelope, we have expressed human loricrin from a full-length cDNA clone in bacteria and purified it to homogeneity. We have also isolated loricrin from newborn mouse epidermis. By circular dichroism and fluorescence spectroscopy, the in vivo mouse and bacterially expressed human loricins possess no \( \alpha \) or \( \beta \) structure but have some organized structure in solution associated with their multiple tyrosines and can be reversibly denatured by either guanidine hydrochloride or temperature. The transglutaminase (TGase) 1, 2, and 3 enzymes expressed during epidermal differentiation utilized loricin in vitro as a complete substrate, but the types of cross-linking were different. The TGase 3 reaction favored certain lymenes and glutamates by forming mostly intrachain cross-links, whereas TGase 1 formed mostly large oligomeric complexes by interchain cross-links involving different lysines and glutamines. Together, the glutamines and lysines used in vitro are almost identical to those seen in vivo. The data support a hypothesis for the essential and complementary roles of both TGase 1 and TGase 3 in cross-linking of loricin in vivo. Failure to cross-link loricin by TGase 1 may explain the phenotype of lamellar ichthyosis, a disease caused by mutations in the TGase 1 gene.

Terminal differentiation in the epidermis involves the expression of a number of specific proteins that ultimately fulfill different structural roles in the cornified, dead stratum corneum cell. One set of proteins is the keratin intermediate filaments and the interfilamentous matrix protein filaggrin (1–3). A second set of proteins is used to construct the cornified cell envelope (CE), \(^1\) a 15-nm-thick layer of protein deposited on the inner surface of the cell periphery, which serves as a physical barrier for the epidermis (4, 5). The CE proteins are rendered insoluble by cross-linking by both disulfide bonds and the N*-\( \gamma \)-glutamyl)lysine isopeptide bond formed by the action of one or more of the three known epidermal transglutaminases (TGases) (4–6). Several proteins have now been documented as CE constituents by direct sequencing analyses of cross-linked peptides (7), including loricin, small proline-rich proteins 1 and 2 (SPR1 and SPR2), elafin, keratins, filaggrin, and desmoplakin. The proteins involucrin and cystatin \( \alpha \) are also likely constituents, but direct sequencing of cross-linked peptides involving these proteins has not yet been reported (reviewed in Ref. 8).

In particular, a variety of data have suggested that loricin comprises about 75% of the total CE protein mass (reviewed in Ref. 9), or 85–95% of the cytoplasmic two-thirds of the CE. In fact, amino acid sequencing of many peptides recovered by the proteolysis has now provided rigorous support for this idea (7). About 90% of the molar mass of peptides from the cytoplasmic two-thirds of the CE consisted of loricin-loricin cross-links, as well as smaller amounts of SPR1 and SPR2 proteins, which appear to serve as cross-bridging proteins among the loricin. Thus loricin appears to function as a major reinforcement protein for the CE on the cytoplasmic face of the structure. Presumably, loricin admixed with the SPRs, is deposited over a scaffold of elafin, cystatin \( \alpha \), involucrin, and possibly other as yet unidentified proteins (7–9).

Yet only anecdotal data are available on the structure of loricins and how they may be cross-linked by TGases. Loricins are unusual in their high contents of glycine, usually configured as tandem inexact peptide repeats (5, 10–12), that are predicted to have little organized structure (6). Based on their unusual flexibility properties, however, we have proposed that these sequences adopt a novel glycine loop motif (13). Whatever their structure, these sequences are flanked by lysoleucine and glutamine-rich terminal sequences and interrupted by glutamine-rich domains that recent sequencing analyses of CE peptides showed are involved in isodipeptide cross-linking (6, 7). Of these, the terminal lysine (Lys\(^{315} \)) and two internal glutamates (Gln\(^{215} \) and Gln\(^{236} \)) account for ~75% of the cross-links (7). The loricins of three species sequenced so far differ only in the sizes of the glycin e motifs; the flanking cross-linking sequences have been highly conserved (6, 11, 12, 14). Thus, it was postulated that loricins adopt a compact mesh-like array that provides insolubility yet flexibility to the CE of normal epidermis. Nor are data available on the nature of the TGase(s) responsible for cross-linking loricins. Extant models have suggested that TGase 2\(^1\) is involved in initial or scaffold assembly steps of the CE involving involucrin and perhaps cystatin \( \alpha \) and
elafin and that TGase 3 is responsible for the final reinforcement steps of loricrin accretion (4, 5, 8, 9). However, both TGase 1 (15–18) and TGase 3 (17, 19) are expressed in the epidermis essentially coincidently with loricrin, so this scheme awaits more rigorous study.

To date, direct biochemical and biophysical experiments on isolated native loricrins to explore their structure and these hypotheses have not been reported. An early study (20) described the isolation of granules from newborn rat epidermis that are likely to be the loricrin-containing L granules of that tissue (21), but no further data have been reported. One major problem is that loricrins are thought to be cross-linked into large oligomers immediately after synthesis (14). Accordingly, in this paper, we have expressed full-length human loricrin in bacteria and used it to study its structural and biochemical properties. We show here that while it can function as a complete substrate in vitro for the three TGases known to be active in the epidermis, the enzymes function differently, which is likely to have profound implications for the assembly of the CE.

MATERIALS AND METHODS

Expression of Loricin in pET-11a System—A full-length cDNA clone encoding human loricrin (6) was configured into the pET-11a bacterial expression vector (Novagen, Madison, WI) by the addition of suitable linkers to the existing RI ends of the cDNA clone (22). The 5' untranslated nucleotide sequence of the cDNA sequence TCCTCAG was modified to TCCTCAT in order to reduce the free energy to enable more efficient association with bacterial ribosomes (22–24). After transformation into the host Escherichia coli B strain BL2DE3 (Novagen), cultures (0.1–5-liter volume) (in LB broth supplemented with 50 µg/ml ampicillin) were grown to an A600 of 0.6, and protein expression was induced with 1.0 mM isopropyl-1-thio-β-D-galactopyranoside for 3 h. In some cultures, L-[35S]lysine (0.5 µCi/ml) was added immediately prior to induction.

Purification of Recombinant Loricin—Following induction, fresh or previously frozen (-70 °C) bacterial pellets (10,000 × g for 30 min) were lysed in a buffer of 50 mM Tris-HCl (pH 8.0), 0.1 M NaCl, 1 mM dithiothreitol, 1 mM EDTA, and the protease inhibitors leupeptin (1 mM), 4-(2-aminoethyl)benzene-sulfon fluoride (0.2 mM), calpain inhibitor (10 µM), and aprotonin (0.1 unit/ml) and pelleted at 10,000 × g for 30 min. The bacterial lysate was dialyzed against three changes of 100-fold volume of 25 mM citrate buffer, pH 3.6, containing 1 mM dithiothreitol and 1 mM EDTA. At each change of dialysis buffer, the precipitated bacterial proteins were removed by centrifugation at 10,000 × g for 10 min. Loricrin was then purified from this supernatant by chromatography on a 0.5 × 5-cm Mono-S column (Pharmacia Biotech Inc.) using a Pharmacia fast protein liquid chromatography system and a gradient of 0–100% NaCl in the citrate buffer. Loricrin eluted at about 0.2 M salt.

Loricrin purification was monitored on 8–16% or 4–12% SDS-polyacrylamide gradient gels (Novex) and by Western blotting using an established rabbit anti-human loricrin antibody (14), followed by staining with the horseradish peroxidase method (Bio-Rad). Loricrin was also easily monitored by autoradiography of [35S]-cysteine-labeled protein since bacterial proteins contain very little cysteine.

Isolation and Purification of Monomeric Mouse Loricin—Freshly prepared mouse newborn epidermis was extracted by homogenization (5 ml/tissue) in the above citrate buffer containing 0.2 M urea, freed of urea by dialysis, and chromatographed on the Mono-S column as above. Cross-linking of Loricin as a Complete Substrate by TGases in in Vitro Assays—Human full-length TGase 1 was expressed in bacteria and purified from the lysate (soluble) fraction as described previously (22). Guinea pig liver TGase 2 (Sigma) (25) and guinea pig epidermal TGase 3 (19) were also used.

For in vitro cross-linking studies using the recombinant human loricin and purified TGase substrates or [35S]loricin was equilibrated by dialysis into a buffer of 50 mM Tris-HCl, 50 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, pH 7.5. The solutions were made to 5 mM CaCl2 to initiate reaction at 37 °C. In analytical cross-linking experiments, 25 µg (1 nmd) of [35S]-labeled loricin (about 8 × 105 dpm) was utilized in a 100-µl reaction volume. In order to standardize the reactions for comparisons of the TGases, the same amount of enzyme activity was used for each enzyme. These activities were measured by [3H]putrescine incorporation into succinylated casein, and the amount of TGase 1, 2, or 3 that incorporated 0.45 pmol/min into the casen was used. This corresponds to 79 nM for TGase 1 and TGase 3 and 4 nM for TGase 2. Aliquots were stopped by the addition of EDTA (7 mM final concentration). The cross-linked products were separated on 4–12% polyacrylamide gels and analyzed by autoradiography. Selected bands were quantitated by scanning in a computing densitometer with ImageQuant version 3.0 (Molecular Dynamics). Reaction products with TGase 1 or 3, 4–10 nmol of loricrin were reacted in a volume of 250 μl for 2 h. In these cases, an excess of enzyme (equivalent to about 1 pmol/min of [3H]putrescine incorporation into casen) was used to drive the reaction to completion, as judged in control experiments.

Kinetic Studies of Putrescine Incorporation into Loricin—Kinetic reactions were carried out using full-length bacterial expressed TGase 1 and guinea pig TGase 2 and 3 in the same buffer used for in vitro assays. Five concentrations of unlabeled loricin (2.5, 3, 4, 5, and 7 µM), three different concentrations of [3H]putrescine (0.07, 0.10, and 0.14 µCi), and an appropriate amount of enzyme were used in a final volume of 100 µl for 10 min at 37 °C. For each enzyme, the amount of activity utilized was standardized with putrescine incorporation into casen as above. The reactions were stopped by spotting 25 μl of the initial mixture on 3MM filter paper (Whatman) and washing the filter sequentially, for 10 min, in 20, 10, and 5% cold trichloroacetic acid and finally 95% ethanol. Filters were dried, and the radioactivity was counted (26).

These reactions utilized a large molar excess of putrescine in order to monitor the linearity of reaction. Kinetics (22), so as to avoid measurement of only one amine group of the putrescine to loricin rather than TGase cross-linking of loricin by putrescine or cross-linking of loricin to itself. The data for initial velocity of [3H]putrescine incorporation into loricin conformed to a modified double displacement mechanism as described previously for TGase-catalyzed reactions (27). Kinetic constants were calculated as described (22).

Isolation and Quantitation of the Lysine-peptide Cross-link—Aliquots of loricin cross-linking reactions (200 pmol) were subjected to total proteolytic digestion with protease K (Promega), leucine aminopeptidase (Sigma), and carboxypeptidase Y (Boehringer Mannheim) (6, 7, 28). Control reactions consisted of enzymes only or of loricin before cross-linking. The N'-glycylglycine lysine isopeptide (Accurate Biological Corp.) elutes near methionine at 28.6 min on a Beckman 6300 amino acid analyzer.

Peptide Mapping and Amino Acid Microsequencing—The 2-h preparative cross-linking reactions of loricin with TGases 1 and 3 were digested with protease K (3% enzyme to loricin protein by weight) for 3 h at 37 °C (7). Peptides were resolved by HPLC using a reverse phase ultrasphere ODS C18 column (4.6 × 250 mm) with a gradient of 0–100% acetonitrile containing 0.08% trifluoroacetic acid. A control sample of uncross-linked loricin was digested and resolved similarly. New peaks that appeared in the former reactions with respect to the latter were collected, concentrated, and covalently attached to a polyvinylidene difluoride solid support (Sequelon-AA, Millipore Corp.). The peptides were sequenced to completion. In preparative experiments, spectra were corrected for possible Raman contributions. Monochromators were in the range of 2–4 nm. In all fluorescence experiments, spectra were corrected for possible Raman contributions by buffer-base line subtraction. Absorption and circular dichroism (CD) measurements were carried out using a Jasco Uvidec 650 spectrophotometer and a Jasco 600 spectropolarimeter, respectively. In both cases, 0.1-cm quartz cuvettes were used. The sample holders were thermostated at 22 °C and during measurements, using external circulation. In the CD stability studies, the loricin solutions were maintained at 50 °C for 1 h and reversed to 20 °C for 1 h before measurement.

RESULTS

Expression and Purification of Recombinant Human Loricin—The purpose of the present work was to undertake a detailed study of this major CE structural protein of the epidermis. In this study, we have expressed human loricin in

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bacteria using the pET11a system (22). The loricrin was very soluble in 25 mM pH 3.6 sodium citrate buffer, which enabled a simple two-step recovery from the bacterial proteins; >90% of bacterial lysate proteins was insoluble in this buffer and were removed by precipitation following dialysis, concentrating the loricrin by 2 orders of magnitude in one step without loss, enabling final purification by fast protein liquid chromatography (Fig. 1). The maximal yield was about 5 mg/liter. Several unsuccessful attempts were made to improve this yield, such as by growing the bacteria to higher density before induction of protein expression, making adjustments to the culture nutrients, changing the length of time of isopropyl-1-thio-β-D-galactopyranoside induction, and using an enriched culture medium (Terrific Broth, Life Technologies, Inc.). The lack of improved yields suggests that the loricrin is a toxic protein that kills the bacteria shortly after expression. Consistent with this idea, the loricrin was not retained within bacterial inclusion bodies but rather was entirely present in the bacterial lysate supernatant fraction, which, happily, simplified its purification.

By both Coomassie staining and Western blotting of SDS gels, human loricrin consisted of a single band of Mr ~30 kDa (Fig. 1, lanes 1 and 2), which is 15% high, based on its known amino acid sequence (6). Its amino acid composition, as determined following total enzymic digestion, was exactly as predicted from its deduced amino acid sequence, including high contents of Gly, Ser, and Cys (data not shown). In addition, we found that whereas the recombinant loricrin was very soluble at pH 3.6 (>20 mg/ml), its solubility at physiological pH was limited to about 0.4 mg/ml.

Isolation of Monomeric Loricrin from Newborn Mouse and Human Epidermis—Based on an early report (20) and on the ease with which we could purify recombinant human loricrin, we next tested the possibility of extraction of native loricrin with citrate buffers from mouse and human epidermis. By inclusion of 8 M urea to the citrate buffer used above, the living layers, but not stratum corneum layers, were dispersed as expected (29). Upon removal of the urea by dialysis, the keratins and profilaggrin were quantitatively precipitated (30), whereas the loricrin remained in solution. The supernatants from both newborn mouse and human foreskin epidermis were then resolved on the Mono-S column. A peak of in vivo mouse loricrin of ~65 kDa was eluted in exactly the same location as recombinant human loricrin (Fig. 1, lanes 3 and 4). This value is about 35% high (the size determined from its complete amino acid sequence is 48 kDa (14)), and as for human loricrin, this may be due to the anomalous binding of SDS due to the unusually glycine-rich sequences. The yield of ~4 μg of loricrin/epidermis was recovered in freshly excised tissue of 0–1-day-old pups only; no loricrin could be recovered in tissue from older mice or from tissue that had been stored for >2 h. The yield of in vivo human loricrin from foreskin epidermis in identical experiments, however, was <0.1 μg/tissue. As these yields represent only a trace of the total loricrin expressed in living epidermis, based on its abundance in CEs (7–9), loricrin is apparently rapidly cross-linked after synthesis, as proposed earlier (14).

Spectroscopic Properties Show That in Vivo Mouse and Recombinant Human Loricrins Have the Same Structures—The fluorescence spectra of in vivo mouse and recombinant human loricrins measured at pH 7.4 were found to be identical. The absorption spectrum (Fig. 2a) shows a maximum peak at 275 nm, indicating that the Tyr and Phe residues present play a relevant role in the absorption of light below 290 nm. The single Trp residue is responsible for the shoulder at 282 nm. Structured spectra are visible by steady state fluorescence excitation (Fig. 2b). The fluorescence signal observed through an emission monochromator at 303 nm (Fig. 2b, line 1) shows a maximum shifted to lower wavelengths, which still reveals absorption of the Tyr residues. This implies that the Tyr residues occupy a distant position with respect to the Trp environment, because the energy transfer between them is negligible.

In order to investigate whether the Tyr residues occupy a hydrophobic environment, the loricrins were denatured in 6 M guanidine hydrochloride (final concentration). The results showed only a 10% decrease in the 305–350-nm fluorescence emission ratio when excited at 280 nm (data not shown). Since it is highly unlikely that the distance between the Trp and Tyr residues should decrease with unfolding, the data indicate that denaturation shifts the bulk of the Tyr residues toward a more polar environment. These data are consistent with the glycine loop hypothesis (13), in which the aromatic residues are thought to associate in a hydrophobic core to anchor loops of Gly residues.

To explore the properties of the Trp residue alone, an emission monochromator at 350 nm yielded much broader unstructured spectra (Fig. 2b, line 3). The steady state emission spectra for both Tyr residues and Trp (excited at 280 nm) and for Trp alone (excited at 295 nm) are shown in Fig. 2c, lines 1 and 2, respectively. The selective excitation of Trp at 295 nm shows an emission spectrum centered at 356 nm, suggesting that this residue is exposed to the solvent and thus located on the external surface of the protein. These data are consistent with the cross-linking data (see below): Trp312 (human) (or Trp378 (mouse)) is located three residues from the terminal Lys, the most used Lys residue in vivo and in vitro cross-linking reactions (see Tables II and III and Fig. 6).

Measurements of the CD spectra of the loricrins (Fig. 3a) were done to evaluate their secondary structures. As predicted (6), they have little organized secondary structure in solution at 20 °C; there is essentially no α or β structure present. How-
ever, the spectra of the recombinant human and in vivo mouse loricrins were superimposable (Fig. 3a), establishing that the recombinant protein had refolded into the native configuration of mouse loricrin. Interestingly, these loricrin CD spectra are very similar to those of bovine pancreatic trypsin inhibitor (31) and filaggrins (32) which have unusually small amounts of secondary structure. In order to ascertain that the low degree of order was not due to denaturation during purification or to inappropriate folding following expression in bacteria, the stability of the overall protein structure was measured as a function of temperature and guanidine hydrochloride. Whereas the loricrins were unfolded by heating to 50 °C, the CD signals were normalized when returned to 20 °C, indicating refolding of the protein structure (Fig. 3b). Denaturation in 4 M guanidine hydrochloride increased the CD signal at 225 nm, implying some unusual secondary structure, but this was reversed on removal of the reagent, indicating protein refolding (Fig. 3c).

Recombinant Human Loricrin Is a Complete Substrate for Epidermal TGases—A variety of data have documented that loricrins are a major substrate for TGases in the epidermis (6, 7, 14), but there are no data on which TGase is responsible for the cross-linking. The reason is simply that TGases 1 and 3 are largely co-expressed in the epidermis (15–19), so that it has been difficult to dissect the individual roles of these enzymes. Therefore, we have used 35S-labeled recombinant human loricrin as a substrate for three TGases in in vitro reactions. Fig. 4 shows that all three TGases cross-link loricrin into oligomers. This means that the loricrin functions as a complete substrate in the sense that it provides both glutamines and lysines for cross-linking. However, reaction with equal amounts of TGase activities revealed differences in the amount of cross-links inserted and the nature of the products formed. For all three enzymes, the amount of activity used and the length of reaction (2 h) was determined in control reactions to allow complete reaction. The reactions resulted in the formation of 1.4, 0.2, and 2.3 mol of isopeptide cross-link/mol of loricrin for TGases 1, 2, and 3, respectively (Fig. 4, a, b, and c, respectively); use of more enzyme or increased incubation times did not alter the 2-h cross-link patterns shown nor the yields of cross-link/mol. In each reaction, a significant proportion of the loricrin in a time-dependent manner (25, 10, and 60%; TGases 1, 2, and 3, respectively) remained as a monomer that migrated somewhat faster than unreacted loricrin. Judging from Coomassie staining, Western blotting, and amino acid analyses, this shift was not likely due to degradation (data not shown). A more likely possibility is the insertion of intrachain cross-links; i.e. the monomeric loricrin had become more compact and migrated faster in the SDS field. Also, in each case, some of the loricrin remained uncross-linked (10, 80, and <5%; TGases 1, 2, and 3, respectively).
respectively), since it retained the same apparent molecular size as the control. In the cases of TGase 1 and 2, some loricrin (65 and 10%, respectively) was oligomerized into aggregates too large to enter the SDS gel (Fig. 4, a and b). In the case of TGase 3, however, most of the cross-linked products were only dimers, trimers, and tetramers, each of which themselves increased in migration rate suggestive of the formation of intrachain cross-links. No detectable protein formed large oligomers (Fig. 4c).

Thus the three enzymes treat the loricrin substrate differently. TGase 2 cross-links the loricrin relatively poorly, and about two-thirds of the cross-linking by TGase 1 (7 mol/mol) involves interchain cross-links to form very large oligomers; but most of the cross-linking by TGase 3 (2 mol/mol) involves intrachain cross-links. This pattern suggests that these two TGases may cross-link loricrin in a complementary manner. To test this idea, Fig. 4d shows that following an initial cross-linking by TGase 3 (2.4 mol of cross-link/mol of loricrin), subsequent reaction with TGase 1 resulted in further cross-linking (a total of 3.3 mol of cross-links/mol) into oligomers too large to enter the SDS gel.

Pilot experiments with the three TGases using the small amounts of available in vivo mouse loricrin gave almost identical results (data not shown).

Identification of Cross-links Formed in Recombinant Loricrin in Vitro by TGase 1 and 3—To characterize the nature of the cross-links formed in the above in vitro reactions, we performed preparative experiments involving 4–10 nmol of loricrin. After the 2-h reaction, the protein was digested with proteinase K in order to recover cross-linked peptides suitable for direct sequencing (7). The peptides were resolved by HPLC, and the profile was compared with a sample of unreacted loricrin (Fig. 5a) to identify shifted peaks likely to be cross-linked.

In the case of the TGase 1 reaction, 10 peptides were identified (Fig. 5b) and sequenced (Table I), with a total yield of cross-link of about 1.2 mol/mol. This means that >85% of the cross-link was recovered; the unfound 0.2 mol/mol (<15%) were peptides too small to be resolved in the HPLC. Peptides 1, 7, 9, and 10 involve likely interchain cross-links because adjacent Gln and Lys residues were used on the same sequences. Peptides 6 and 8 also may involve interchain cross-links between thebeginning of one chain and the end of another. Peptides 2–5 may involve either inter- or intrachain cross-links. Thus two-thirds (molar basis) involve interchain cross-links, in support of the pattern of oligomerization of loricrin by the TGase 1 enzyme (Fig. 4a).

In the case of the TGase 3 reaction, 16 peptides were identified (Fig. 5c) and sequenced (Table II), with a yield of 2.0 mol/mol (85% of cross-link recovered). Peptides 2, 3, 5, 7, 8, 14, 15, and 16 involved the same sequences as those formed by the TGase 1 enzyme. Peptides 14–16 involve obligatory interchain cross-links, and peptides 4, 5, 8, and 13 involve possible interchain cross-links through terminal sequences. However, together these amount to only 25% of the molar total. Thus ~75%
TABLE I
Peptides derived from T Gase 1 cross-linking of recombinant loricrin
The single letter code for amino acids is used for sequences. Amino acids in lowercase represent substoichiometric yield due to partial proteinase K digestion. The positions of cross-links were assigned as follows, using peptide 6 as an example. The sequence terminated after 10 cycles from the first cycle. It became clear that two peptide sequences were running concurrently adjacent by a cross-link from the 10 cycles, the nature of the two loricrin sequences involved was deduced. An important clue is that there is only one His residue in loricrin and few Val residues (6). Gln residues were released cleanly at cycles 1, 2, 4, 5, 7, and 8, the yield of Gln at cycle 4 was only about 50%, no Lys was released at cycle 2 as expected, but it was released at cycles 3 and 6. The phenylthiohydantoin-cross-link was released at cycle 4; therefore, the cross-link must involve the Gln at cycle 4 of one peptide arm, and the Lys at cycle 2 of the second arm.

| Peptide number | Sequence | Position | Yield |
|----------------|----------|----------|-------|
| 1              | HQTQq    | Gln305   | 0.15  |
|                | QKQA     | Lys307   |       |
| 2              | HQTQQ    | Gln305   | 0.02  |
|                | GSQVQ    | Lys308   |       |
| 3              | PTWPS     | Lys315   | 0.16  |
|                | SQVQTQ    | Gln319   |       |
| 4              | HQTQQA    | Lys325   | 0.03  |
|                | fSGQAVQCQ | Gln356   |       |
| 5              | KROPTPOPVPV | Lys5    | 0.08  |
|                | HQTQQA    | Gln305   |       |
| 6              | QKROPTPOPVPV | Lys4    | 0.16  |
|                | HQTQKAPTW | Gln305   |       |
| 7              | HQTQKAPTW | Lys307   | 0.13  |
|                | KOPTPOPVP | Gln40    |       |
| 8              | HQTQKAPTW | Lys307   | 0.03  |
|                | YQKQPTQ   | Gln305   |       |
| 9              | KOPTPOPVP | Gln40    |       |
| 10             | HQTQKAPTWSK | Lys307,Lys315 | 0.04 |
|                | PICHOTQq  | Gln305   |       |

(a) TGase 1 cross-links in vitro
(b) TGase 3 cross-links in vitro
(c) CEs in vivo

The single letter code for amino acids is used for sequences. Amino acids in lowercase represent substoichiometric yield due to partial proteinase K digestion. The positions of cross-links were assigned as follows, using peptide 6 as an example. The sequence terminated after 10 cycles from the first cycle. It became clear that two peptide sequences were running concurrently adjacent by a cross-link from the 10 cycles, the nature of the two loricrin sequences involved was deduced. An important clue is that there is only one His residue in loricrin and few Val residues (6). Gln residues were released cleanly at cycles 1, 2, 4, 5, 7, and 8, the yield of Gln at cycle 4 was only about 50%, no Lys was released at cycle 2 as expected, but it was released at cycles 3 and 6. The phenylthiohydantoin-cross-link was released at cycle 4; therefore, the cross-link must involve the Gln at cycle 4 of one peptide arm, and the Lys at cycle 2 of the second arm.

of the molar total involve intrachain cross-links, again in support of the pattern of cross-linking by the TGase 3 enzyme (Fig. 4c). In this case, the interchain cross-linking by TGase 3 involved predominantly residues Gln6, Gln10, Gln153, Gln216, and some Lys315, but the bulk of the likely intrachain cross-linking involved residues Gln307, Gln316, Lys88, and Lys315 (Fig. 6b).

Kinetics of TGase 1, 2, and 3 Cross-linking of Recombinant Loricrin in Vitro—Given that TGase 1 and TGase 3 can both utilize recombinant loricrin in vitro as a complete substrate, albeit with different behavior, we wanted to obtain more quantitative information on the rates of reaction. Many investigators have explored cross-linking reactions in vitro by various TGases of target substrate Gln(s), using model simple amines such as putrescine for example (25) or onto target substrate Lys(s) using Gln-containing peptides (33–35). However, to date, no kinetic data have been reported on the cross-linking of a complete TGase substrate protein in which both Gln and Lys are used simultaneously for intra- and/or interchain cross-linking. Thus a complexity arises because the TGases can use either the putrescine as the amine donor or the protein’s own Lys(s) as the amine donor(s). A second complexity arises in the present study because, unlike involucrin in which mainly only one Gln is used in in vitro cross-linking reactions (36), loricrin can provide multiple Gln and Lys residues for cross-linking simultaneously, and the preferred residues used differ between the TGase 1 and TGase 3 enzymes (Fig. 6; Tables I and II). To circumvent these complexities, we have used high concentrations of putrescine in the in vitro reactions, 1–2 orders of magnitude higher than the concentration of available Gln residues in the loricrin substrate. In this way, we have suppressed the propensity for intra-/interchain cross-linking of loricrin to itself and the likelihood of putrescine oligomerization of loricrin in order to measure only the incorporation of a single amine of putrescine onto one of the favored Gln residues of loricrin. Control experiments showed that no more than 5% of the substrate of the lowest concentrations was consumed, and there was no cross-linking of loricrin either to itself or by putrescine during the reaction. The data calculated are not true K_m values but represent “average” K_m values for the multiple Gln residues in loricrin.

Comparative kinetic constant data on the TGase 1, 2, and 3 enzymes are shown in Table III, and reveal that recombinant loricrin is a very efficient substrate for all three TGases. For example, by way of comparison with previous data, the kinetic efficiency (k cat/K_m) of TGase 1 for loricrin is 10-fold higher (0.29 min⁻¹µM⁻¹) than for succinylated casein (0.029 min⁻¹µM⁻¹) (21). While the average K_m value for TGase 1 (17 × 10⁻⁶ M) is similar to that for TGase 2 (16 × 10⁻⁶ M), it is 3 times higher than the average K_m value for TGase 3 (5 × 10⁻⁶ M).
of usage of Gln215 and Gln216 by TGase 3 than TGase 1 or of nant loricrin. Presumably, this reflects the greater proficiency means that TGase 3 is more efficient in cross-linking recombinant Gln10 and Gln305 by TGase 1. This is also supported by our 26388 for recombinant loricrin (1.7 mol/mol). Peptide derived from in vitro cross-linking of recombinant loricrin by

| Peptide number | Sequence | Position | Yield |
|----------------|----------|----------|-------|
| 1              | SGQ      | Gln153   | 0.02  |
| 2              | GSQVKY   | Lys88    | 0.12  |
| 3              | PTWPSK   | Lys315   | 0.43  |
| 4              | QKQPTPO  | Lys315   | 0.09  |
| 5              | OTOQQOQA | Lys307   | 0.20  |
| 6              | TSCAPQPSY| Gln225   |       |
| 7              | PTWPSK   | Lys315   | 0.04  |
| 8              | QKQPTPO  | Lys315   | 0.14  |
| 9              | OTOQQOQA | Lys307   | 0.04  |
| 10             | SGGSVYKSS | Lys6     | 0.09  |
| 11             | VSSQVTVQS| Lys5     | 0.06  |
| 12             | VSSQVTVQS| Lys5     | 0.17  |
| 13             | KKQPTPOPPV| Lys88    | 0.01  |
| 14             | PTWPSK   | Gln3     | 0.04  |
| 15             | YQKQPTPOPPV| Gln156  | 0.12  |
| 16             | PTWPSK   | Lys315   | 0.09  |

TABLE II Properties of Human Loricrin

Peptides derived from in vitro cross-linking of recombinant loricrin by TGase 3

Data analyses are as in the legend to Table I.

DISCUSSION

Recombinant Human and in Vivo Mouse Loricrins Have the Same Structures in Solution—By fluorescence spectroscopy (Fig. 2) and CD (Fig. 3) experiments, we show here that the structures of recombinant human and in vivo mouse loricrins are indistinguishable. The identical reversible structural changes induced by either temperature or guanidine hydrochloride denaturation support the notion that the recombinant human loricrin had refolded into a native conformation. These observations have allowed us to use the recombinant loricrin with confidence to explore its substrate properties by the three TGases active in the epidermis.

However, the loricrins have only a limited degree of structure in solution at physiological pH, as predicted by conventional algorithms (6). Based on an unusual motif present in loricrins and several other types of proteins, we proposed a glycan loop hypothesis (13), in which long sequences highly enriched in Gly residues were folded into loops by the interaction of occasional interspersed aromatic residues. Our present data now provide support for this hypothesis. We show that what little structure is present in loricrins involves the Tyr residues, which associate in a hydrophobic environment (Fig. 2).

Loricrin Is a Complete TGase Substrate in Vitro and in Vivo—The data of Figs. 4 and 5 show that loricrin can function in vitro as a complete substrate for the TGases 1 and 3 and 2 weakly; that is, Gln and Lys residues on the same protein chain are recruited for cross-linking. These data are in agreement with our earlier in vivo CE cross-linking data (7), which showed that loricrin, as well as SPR1, SPR2, and elafin, are also complete TGase substrates in vivo. Thus, our in vitro cross-linking paradigm can be used with confidence to ascertain the complex processes of cross-linking of these various CE substrates.

Interestingly, the TGases 1 and 3 utilized loricrin as a complete substrate in different ways. First, they used different Gln and Lys residues in quantitatively different amounts. TGase 1 used mostly Gln10, Gln219, Gln305, Lys4, Lys307, and Lys315; TGase 3 used mostly Gln6, Gln10, Gln156, Gln215, Gln216, Gln225, Lys88, Lys307, and Lys315 (Fig. 6). Second, the predominant reaction of TGase 1 was interchain cross-linking (−1 mol/mol) through the preferential use of Gln10, Gln219, Gln305, Lys315, with some intrachain cross-linking (−0.5 mol/mol) with Gln156, Lys88, and Lys315 (Table I, II). Conversely, the predominant reaction of TGase 3 was intrachain cross-linking (−2 mol/mol) through preferential use of Gln156, Gln215, Gln216, Gln225, Lys88, and Lys315 with some intrachain cross-linking (−0.5 mol/mol) with Gln6, Gln10, Lys307 (Tables I, II). This means there is a direct relationship between the types of cross-linking (inter- versus intrachain) and the specific residues utilized and the dual and complementary roles of these two TGases in this process.
The striking observation of the use of only certain residues for inter- and intrachain cross-linking in vitro prompted us to compare these data with the previous in vivo cross-linking data for loricrin obtained from CEs (Fig. 6c) (7). In the in vivo data set, most of the available Gln and Lys residues were utilized in both intra- and interchain cross-linking, although certain preferential residues, Gln\(^6\), Gln\(^{10}\), Gln\(^{156}\), Gln\(^{215}\), Gln\(^{216}\), Lys\(^4\), Lys\(^{88}\), Lys\(^{307}\), and Lys\(^{315}\) accounted for >90% of the total. Significantly, these same residues were used preferentially by TGase 1 and TGase 3 in the present study.

In the absence of more information, the in vivo data set cannot provide estimates of the percentage of intra- and interchain cross-linking, although both reactions had clearly occurred. However, we can now explore this question by estimation of the relative roles of the TGases 1 and 3 in the cross-linking of loricrin in vivo. In vitro, the molar usage rate of Lys\(^{315}\) seen in vivo would require 72% cross-linking by TGase 3 and 28% by TGase 1. Likewise, the in vivo Lys\(^{307}\) data would require 71% cross-linking by TGase 3 and 29% by TGase 3. Thus, least squares fitting (9) of the summed fractions for each of the residue positions Lys\(^4\), Lys\(^{88}\), Lys\(^{307}\), Lys\(^{315}\), Lys\(^{316}\), Lys\(^{317}\), Lys\(^{318}\), Gln\(^{303}\), and Gln\(^{306}\) were not used at all in vitro, but since these five residue positions were used infrequently in vivo, their weighted contributions to the mean are low.

Accordingly, these considerations indicate a hitherto unrecognized important role for the TGase 1 enzyme in the cross-linking of loricrin to the CE in vivo.

A Model for the Complementary Cross-linking of Loricrin in Vivo—Based on protein expression patterns, extant models on CE assembly (4, 5, 7–9) suggest that the TGase 1 enzyme cross-links such soluble proteins as involucrin and cystatin \(\alpha\) to form a scaffold, onto which the TGase 3 enzyme deposits loricrin in a final stabilization event. These events must be very complex because the TGase 3 enzyme is a soluble (cytosolic) enzyme (18, 19), whereas the TGase 1 enzyme system consists of membrane-associated (15, 16, 18) and multiple soluble forms (18). Our new data strongly suggest that both TGase 1 and TGase 3 are involved in loricrin deposition in vivo and in different ways. We propose that the TGase 1 enzyme cross-links the loricrin to the CE scaffold to form a large polymeric structure through interchain cross-linking with the preferential use of certain Gln and Lys residues. Subsequently, or possibly simultaneously, the TGase 3 enzyme further cross-links the attached loricrin by mostly intrachain cross-links using primarily different Gln and Lys residues to form a more compact structure. We cannot exclude an alternative model in which the soluble TGase 3 enzyme first cross-links the loricrin into small compact oligomers, which are then glued together and to the CE by interchain cross-links with the soluble and/or membrane-associated forms of the TGase 1 enzyme. Both models are consistent with our present recovery of trace amounts of soluble monomeric loricrin from mouse epidermis and with pulse-chase experiments in which it was shown that newly synthesized loricrin in organ cultured epidermis is rapidly cross-linked into a form too large to enter an SDS gel (14).

Implications for Lamellar Ichthyosis—This realization for the first time of the probable in vivo roles of both TGases 1 and 3 in loricrin assembly onto the CE has important implications for the autosomal recessive disease lamellar ichthyosis. This disorder of cornification of the epidermis manifests as generalized plate-like scales with pathology showing disruption in the uppermost differentiating and stratum corneum layers of the epidermis (37). Two papers have reported reduced expression of the TGase 1 enzyme and abnormal loricrin deposition (38, 39). More recent reports (40–42) have shown that mutations in the TGM1 gene resulting in a defective or inactive TGase 1 enzyme are the cause of this disease. Our new data suggesting an essential role for the TGase 1 enzyme in cross-linking loricrin and CE formation offers a new insight into the mechanism of pathogenesis of this disease.

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TABLE III

| Kcat | kcat / Km | Kcat / km | Vmax | Km of putrescine |
|------|----------|----------|------|----------------|
| min\(^{-1}\) | \(\mu\) | min\(^{-1}\) | \(\mu\) | mol min\(^{-1}\) | \(\mu\) |
| TGase 1 | 5.02 ± 0.75 | 16.9 ± 3.28 | 0.29 ± 0.058 | 39.8 ± 7.9 | 188.8 ± 37.0 |
| TGase 2 | 4.47 ± 0.89 | 15.0 ± 2.20 | 0.28 ± 0.056 | 1.99 ± 0.4 | 26.4 ± 5.2 |
| TGase 3 | 5.73 ± 1.14 | 5.04 ± 1.01 | 1.14 ± 0.22 | 45.6 ± 9.1 | 847.0 ± 169.4 |
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