Trichohyalin (THH) is a major structural protein of the inner root sheath cells and medulla layer of the hair follicle and, to a lesser extent, of other specialized epithelia. THH is a high molecular weight insoluble α-helix-rich protein that forms rigid structures as a result of post-synthetic modifications by two Ca²⁺-dependent enzymes, transglutaminases (TGases) (protein cross-linking) and peptidyl-arginine deiminase (conversion of arginines to citrullines with loss of organized structure). The modified THH is thought to serve as a keratin intermediate filament matrix protein and/or as a constituent of the cell envelope. In this paper, we have explored *in vitro* the order of processing of THH to fulfill these functions, using an expressed truncated, more soluble form THH-8. THH-8 is a complete substrate for three known TGases expressed in epithelia, but the kinetic efficiency with TGase 3 is by far the greatest. Following maximal conversion of its arginines to citrullines, THH-8 is cross-linked even more efficiently by TGase 3, using most glutamines partially and all lysines. In addition, we show that insoluble aggregates of THH-8 or native pig tongue THH can be solubilized following peptidyl-arginine deiminase modification. Together, these data suggest an *in vivo* model in which THH located in insoluble cytoplasmic droplets is first modified by peptidyl-arginine deiminase which denatures it and makes it more soluble. This renders it available for efficient cross-linking by TGase 3 to form highly cross-linked rigid structures in the cells. This temporal order of reaction is supported by the observation that THH is expressed in hair follicle cells before the TGase 3 enzyme.

Trichohyalin droplets were first described by Vörner (1) as non-membrane-bound inclusions in the cytoplasm of the inner root sheath (IRS)¹ cells of the hair follicle and in the medullar cells of the developing hair shaft. Subsequently, it was shown that the major if not only component of these droplets is a protein termed trichohyalin (THH) (2). THH has been isolated from sheep hair follicles (2) and porcine tongue epithelium (3) and is now known to be expressed in a number of specialized epithelial tissues including human foreskin epidermis, hard palate, and nail bed, as well as in a variety of pathological conditions of human skin (4–8). THHs from sheep (9) and human (10) have been cloned and sequenced. The THHs are high molecular mass (≥200 kDa) proteins that are insoluble under physiological conditions. They have been predicted to adopt an elongated (>200 nm) single-stranded α-helical conformation based on their unusually high content of charged residues (10). Their sequences suggested multiple roles in tissues such as calcium binding, structural roles, an interfilamentous matrix, and cornified cell envelope precursors (10).

Although the role of THH in the palate, tongue, epidermis, or other tissues is not yet clear, its role in hair follicle biology is better understood. IRS cells are first identifiable at the base of the follicle by their content of THH droplets. A few cell layers higher, KIF are expressed. As terminal differentiation proceeds, the THH droplets disperse, and the contents become intermixed with the KIF (2, 6, 11, 12). The IRS cells harden to form a rigid insoluble multi-layer sheath that plays an essential role in shape determination of the hair fiber cortical cells internal to the sheath structure (11–13). The mature IRS cells are packed with KIF that become highly aligned along the long axis of the cell and are separated by 1–2 filament diameters by a less densely staining matrix of THH (2, 6, 11–13). The medulla, however, contains few if any KIF (11, 14). In the terminally differentiated medulla cells within the mature hair fiber, the THH protein becomes dispersed as amorphous vacuolated deposits before the fiber emerges from the skin surface (12). By entrapment of air, these vacuoles play an essential role in the maintenance of body temperature in mammals.

Some of the biochemical events that modify the α-helical THH protein to an interfilamentous matrix (IRS) or unstructured (medulla) protein are now emerging. Early histochemical analyses revealed that while the THH droplets contain an arginine-rich protein, the mature IRS and medulla cells stain for citrulline (15). Subsequently it was shown that the Ca²⁺-dependent enzyme peptidyl-arginine deiminase (PAD) is responsible for this modification reaction (16–19). Three related cytosolic PAD enzymes are now known in mammalian tissues. These include the following: the type 1 enzyme that is expressed in the epidermis and uterus, type 2 enzyme that is expressed ubiquitously, and type 3 enzyme that is expressed in the hair follicle (20–22). A large body of indirect data have implicated THH as a major target for PAD activity in these tissues (16, 19, 23–27). Recently, we have shown that a bacterially expressed shorter form of human THH (THH domain 8, THH-8) is a substrate for the type 2 PAD enzyme (28).
substrate specificity of which has been shown to be very similar to that of the type 3 enzyme (20). Interestingly, this reaction converts the highly α-helical THH-8 to a disorganized structure (28).

In addition, it was found that the proteins of the medulla were cross-linked by Nε-(γ-glutamyl)lysine isopeptide bonds (29), formed by the action of one or more Ca2+−dependent transglutaminase (TGase) enzymes present in these tissues (30–34). Again, it was deduced that THH was a major substrate, based on limited sequencing analyses of isolated peptides from medulla (and IRS) digests (16, 24), although characterization of peptides from THH containing citrulline and cross-linked by isopeptide bonds has not been reported yet.

KIF can be isolated from mature IRS cells following limited proteolysis (35). Apparently, proteolysis cleaves sequences on the end domains of the constituent KIF chains that are joined by cross-links between KIF and the interfilamentous THH (26). This releases morphologically intact KIF (although with somewhat pruned chains) and solubilized peptides highly enriched in cross-link and citrulline with a THH-like amino acid composition (26).

However, several questions remain to be elucidated. For example, the TGase enzymes used for cross-linking, and the glutamine and lysine residues of THH utilized, are not known. It has been speculated that the cytosolic so-called hair follicle or TGase 3 enzyme is involved (30–34), but cytosolic and membrane-associated forms of the TGase 1 enzyme, and the ubiquitously expressed TGase 2 enzyme, are also likely to be present in these cells (35–37). In this study, we have examined the substrate properties of THH-8 for TGases, established that TGase 3 enzyme is indeed favored to cross-link THH, and identified the glutamine and lysine residues used. Another fundamental question concerns the temporal order of the multiple postsynthetic modification processing events of THH. We demonstrate here that THH is most likely modified by PAD prior to cross-linking, in order for it to be dispersed from the droplets and fulfill its roles in forming rigid structures in a variety of advanced differentiated epithelial tissues.

EXPERIMENTAL PROCEDURES

Materials—The following were purchased: type 2 peptidyl-arginine deiminase (PanVera Corp., Madison, WI); [1,4-14C]putrescine dihydrochloride (specific activity, 109 mCi/mmol) (Amersham Corp.); the serine protease inhibitor 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (ICN Biomedicals, Aurora, OH); endoproteinase Asp-N (sequencing grade, Boehringer Mannheim, Mannheim, Germany); precast polyacrylamide gels (Novex, San Diego, CA); and anti-TGase 2 antibody (clone TG100, NeoMarkers, Freemont, CA). An anti-TGase 3 antibody was raised in goats against bacterially expressed human TGase 3,2 and (clone TG100, NeoMarkers, Freemont, CA). An anti-TGase 3 antibody was raised in goats against bacterially expressed human TGase 3,2 and the cross-reactivity against TGase 1 was pre-absorbed on an affinity column prepared from bacterially expressed human TGase 1 (39, 40).

Purification of THH and Modification by PAD—Domain 8 of human THH (THH-8, residues 1250–1849) was expressed and purified from Escherichia coli as reported previously (28). Intact pig tongue THH was purified as described (3), but after every step aliquots were frozen for further investigation. The protein concentration of the samples was determined by amino acid analysis or by the method of Bradford (41). Purified THH-8 (60 μg/ml) was incubated with PAD (2 μg/ml) in a buffer of 20 mM Tris-HCl (pH 8.8), 0.3 mM NaCl, 1 mM EDTA, 1 mM DTT, 10 mM CaCl2 for 18 h at 37 °C. Under these conditions, THH-8 becomes maximally modified (~65% of arginines converted to citrullines (28)). Reactions were terminated by the addition of EDTA (10 mM final concentration) and subsequent heating to 70 °C for 30 min. Aliquots were hydrolyzed (5.7 n HCl, 110 °C, 20 h) and analyzed (Beckman 6300 Analyzer). Corrections were made for hydrolytic losses of citrulline to ornithine (28).

Generation of THH-8 Polyclonal Antibody—A specific polyclonal antibody was raised in rabbits (Lofstrand, Gaithersburg, MD) against recombinant human THH-8, which had been purified and refolded from bacterial inclusion bodies as described previously (28). The antibody was affinity purified by chromatography on a column of THH-8 covalently bound to ReactiGel (6 ×) (Pierce). The specificity and cross-reactivity of the antibody were determined by Western blotting using THH-8 bacterial cultures and mouse hair follicle lysates.

Cross-linking of THH-8 and PAD-modified THH-8 with TGases—Cross-linking reactions were carried out using full-length baculovirus expressed TGase 1,3 guinea pig liver TGase 2 (Sigma), or guinea pig hair follicle TGase 3 purified as described previously (34). The enzyme activities were measured by [14C]putrescine incorporation into succinylated casein, and an equal amount of activity (4 pmol of putrescine/min) was used for all TGases to compare their cross-linking efficiencies (43). THH-8 and PAD-modified THH-8 (50 μg) were incubated in a 1-ml reaction volume at 37 °C with the TGases in the presence of 1 mM DTT and 10 mM CaCl2. Aliquots were removed at different time points and terminated by the addition of EDTA and immediate boiling in SDS gel loading buffer. The reaction products were separated on 6% SDS-PAGE, blotted overnight onto polyvinylidene difluoride membrane, and detected with anti-THH-8 antibody. Lanes 1–5, 0 time, 0.5, 1, 3, and 5 h, respectively; C, control reaction, 5-h cross-linking carried out in the presence of 10 mM EDTA.

**FIG. 1.** Cross-linking of THH-8 and PAD-modified THH-8 by TGases in vitro. THH-8 (A, C, and E) and PAD-modified THH-8 (B, D, and F) were cross-linked with equivalent activities of TGase 1 (A and B), TGase 2 (C and D), and TGase 3 (E and F). Aliquots were withdrawn, and the reaction was stopped by EDTA and boiling. The products were separated on 6% SDS-PAGE, blotted onto polyvinylidene difluoride membrane, and detected with anti-THH-8 antibody. Lanes 1–5, 0 time, 0.5, 1, 3, and 5 h, respectively; C, control reaction, 5-h cross-linking carried out in the presence of 10 mM EDTA.
points by spotting 25 μl of the mixture on 3MM filter paper (Whatman) as described previously (43). The incorporated radioactivity was determined with liquid scintillation counting. The kinetic constants were calculated based on the modified double displacement mechanism as described previously (39). In these studies putrescine was used in a large molar excess to avoid the cross-linking of THH-8 through putrescine bridges and, more importantly, to avoid the possible competition between the lysines of the THH-8 molecule and the putrescine for the acyl donor glutamines.

Identification of the Glutamines Used in Cross-linking of THH-8—[14C]Putrescine was incorporated into THH-8 or PAD-modified THH-8 (80 μg) using 4 pmol of putrescine/min of TGase 3 for 30 min. The reaction was stopped by heat (20 min, 70 °C), and the protein was digested with Asp-N (1:200 w/w for 18 h at 37 °C). The peptides were separated by HPLC on a 2.1 × 250-mm μBore column (Vydac, Hesperia, CA) with a flow rate of 0.24 ml/min and a linear 5–60% acetonitrile gradient in 60 min. The fractions containing radioactivity were sequenced (LPI300 sequencer, Beckman Instruments, Fullerton, CA), and the radioactivity in each cycle was measured.

The amount of isodipeptide cross-link inserted into THH-8 was measured following total enzymatic digestion followed by amino acid analysis, as described previously (44). Amounts could also be estimated from the loss of lysine and glutamine residues.

Indirect Immunofluorescence Procedures—Frozen serial sections (6 μm) of 5-day-old mouse skin were hydrated in phosphate-buffered saline for 2 min and fixed in 4% paraformaldehyde for 10 min at 23 °C. Sections were incubated in 10% bovine serum albumin in saline for 2 h at 23 °C to reduce nonspecific binding. The antibodies used were as follows: polyclonal anti-human TGase 1 (dilution 1:500); mouse monoclonal anti-guinea pig TGase 2 (dilution 1:10); polyclonal rabbit anti-human TGase 3 (dilution 1:5); and polyclonal rabbit anti-human TGase 1 (dilution 1:50) (40); mouse monoclonal anti-guinea pig TGase 2 (dilution 1:50); mouse monoclonal anti-guinea pig TGase 3 (dilution 1:5); and polyclonal rabbit anti-human TGase 3 (dilution 1:50). The antigens were separated by HPLC as described above, and the shifted peaks were sequenced.

The amount of isodipeptide cross-link inserted into THH-8 was measured following total enzymatic digestion followed by amino acid analysis, as described previously (44). Amounts could also be estimated from the loss of lysine and glutamine residues.

A summary of the kinetic parameters of cross-linking by TGases 1, 2, and 3 of recombinant THH-8 before and after maximal modification by PAD is presented in Table I.

### Table I

|                 | $k_{cat}$ | $K_M$ THH-8 | $k_{cat}/K_M$ | $V_{max}$ | $K_M$ putrescine |
|-----------------|-----------|--------------|---------------|-----------|-----------------|
|                 | min⁻¹ μM | μM | min⁻¹ | pmol/ min⁻¹ | μM |
| Intact THH-8    | 0.2 ± 0.07 | 0.4 ± 0.1 | 0.5 ± 0.15 | 0.6 ± 0.18 | 78 ± 21 |
| TGase 1         | 2.4 ± 0.7 | 2.9 ± 0.9 | 0.8 ± 0.25 | 1.5 ± 0.5 | 86 ± 26 |
| TGase 2         | 16.1 ± 0.3 | 1.4 ± 0.12 | 11.2 ± 3.8 | 3.2 ± 0.2 | 205 ± 50 |
| THH-8 after PAD modification | | | | | |
| TGase 1         | 21.0 ± 0.6 | 4.4 ± 1.3 | 0.5 ± 0.2 | 6.3 ± 0.2 | 653 ± 210 |
| TGase 2         | 8.7 ± 2.6 | 10.1 ± 3.0 | 0.9 ± 0.3 | 2.8 ± 0.8 | 105 ± 32 |
| TGase 3         | 118.3 ± 35 | 2.5 ± 0.18 | 35.9 ± 11 | 8.8 ± 0.2 | 39 ± 5 |

The purpose of this study was to understand better the complex series of posttranslational modifications of THH, a major differentiation product of the IRS and medulla cells of the hair follicle/hair fiber, and a component of other epithelial tissues. However, full-length pig THH for example is only slightly soluble in buffers of physiologic ionic strength and pH values (~3 μg/ml); indeed, the method of its isolation is based in part on this unusual property (2, 3). This also explains why the protein is initially deposited in cells in large insoluble droplets (reminiscent of inclusion bodies). Moreover, this property poses an important question: how does this insoluble protein fulfill its major presumed functions? Furthermore, THH in the fully differentiated cells is cross-linked by isopeptide bonds inserted by TGases, which cannot be cleaved specifically without also breaking peptide bonds; this imposes severe limitations on biochemical characterization of THH processing in vivo. To circumvent these technical difficulties, we have expressed the domain 8 portion of human THH (THH-8) that constitutes about 40% of the full-length protein (10) and is sufficiently soluble (~60 μg/ml) for in vitro biochemical assays (28). We have also demonstrated that THH-8 is a substrate for the PAD enzyme (28).

### FIG. 2

PAD modification of THH-8 increases the rate of cross-linking by the TGase 3 enzyme. O, before; ●, after PAD cross-linking.

**TABLE I**

| Kinetic parameters of cross-linking by TGases 1, 2, and 3 of recombinant THH-8 before and after maximal modification by PAD |
|---------------------------------------------------------------|
| $k_{cat}$ | $K_M$ THH-8 | $k_{cat}/K_M$ | $V_{max}$ | $K_M$ putrescine |
|-----------|--------------|---------------|-----------|-----------------|
| Intact THH-8 | 0.2 ± 0.07 | 0.4 ± 0.1 | 0.5 ± 0.15 | 0.6 ± 0.18 | 78 ± 21 |
| TGase 1 | 2.4 ± 0.7 | 2.9 ± 0.9 | 0.8 ± 0.25 | 1.5 ± 0.5 | 86 ± 26 |
| TGase 2 | 16.1 ± 0.3 | 1.4 ± 0.12 | 11.2 ± 3.8 | 3.2 ± 0.2 | 205 ± 50 |
| THH-8 after PAD modification | | | | | |
| TGase 1 | 21.0 ± 0.6 | 4.4 ± 1.3 | 0.5 ± 0.2 | 6.3 ± 0.2 | 653 ± 210 |
| TGase 2 | 8.7 ± 2.6 | 10.1 ± 3.0 | 0.9 ± 0.3 | 2.8 ± 0.8 | 105 ± 32 |
| TGase 3 | 118.3 ± 35 | 2.5 ± 0.18 | 35.9 ± 11 | 8.8 ± 0.2 | 39 ± 5 |

**RESULTS AND DISCUSSION**

The purpose of this study was to understand better the complex series of posttranslational modifications of THH, a major differentiation product of the IRS and medulla cells of the hair follicle/hair fiber, and a component of other epithelial tissues. However, full-length pig THH for example is only slightly soluble in buffers of physiologic ionic strength and pH values (~3 μg/ml); indeed, the method of its isolation is based in part on this unusual property (2, 3). This also explains why the protein is initially deposited in cells in large insoluble droplets (reminiscent of inclusion bodies). Moreover, this property poses an important question: how does this insoluble protein fulfill its major presumed functions? Furthermore, THH in the fully differentiated cells is cross-linked by isopeptide bonds inserted by TGases, which cannot be cleaved specifically without also breaking peptide bonds; this imposes severe limitations on biochemical characterization of THH processing in vivo. To circumvent these technical difficulties, we have expressed the domain 8 portion of human THH (THH-8) that constitutes about 40% of the full-length protein (10) and is sufficiently soluble (~60 μg/ml) for in vitro biochemical assays (28). We have also demonstrated that THH-8 is a substrate for the PAD enzyme (28).

**THH-8 Is a Complete Substrate for TGases in Vitro—**Early studies identified N⁶-(γ-glutamyl)lysine cross-links in the citrulline-containing proteins derived from the medulla (29), most likely having derived from THH, but sequences of peptides containing citrulline and cross-links have not been reported. Nor is it known which of several TGase enzymes expressed in epithelial tissues is responsible for the cross-linking. We have used THH-8 as well as THH-8 that had been maximally modified by PAD (~65% of arginines converted to citrullines, with a total citrulline content of 15%), prepared as described previously (28), as substrates for cross-linking in vitro by equal amounts of activity of the TGase 1, 2, and 3 enzymes. The reaction products were separated on SDS-PAGE, Western blotted, and detected with the new antibody. Unmodified THH-8
produced multiple cross-linked products with all three enzymes (Fig. 1, A, C, and E, respectively). Thus THH-8 (and by deduction full-length THH) serves as a complete TGase substrate in the sense that it provides both donor glutamines and acceptor lysines to form cross-links. In this regard it is similar to loricrin (43). However, the degree of reaction was significantly different for the three enzymes (Fig. 1); TGases 1 and 2 could cross-link only 20–40% of the THH-8, whereas TGase 3 efficiently cross-linked all of the THH-8, producing high molecular weight products that were unable to enter the gel. Cross-linking of PAD-modified THH-8 was somewhat more complicated (Fig. 1, B, D, and F). Due to the irregular migration of modified THH-8 on SDS-PAGE (28), the starting material migrated as \( 160,000 \). Identification of oligomers of this were difficult because of inefficient electroblotting transfer. However, based on the intensity of the original band of 160,000 (at zero cross-linking time, and EDTA control), it is clear that the TGase 1 and 2 enzymes did not significantly cross-link the PAD-modified protein, whereas the TGase 3 enzyme oligomerized this band to high molecular weight species at least as quickly as the unmodified THH-8 (compare Fig. 1, E with F).

**Kinetics Studies with \([^{14}C]\)Putrescine Incorporation into THH-8 and PAD-modified THH-8 by TGase 1, 2, and 3**—To obtain more quantitative information on cross-linking of the modified and non-modified THH-8 with the three TGases, kinetic constants were measured. The apparent \( K_m \) and \( k_{cat} \) values for THH-8 (Table I) were very low in comparison to the incorporation of putrescine into succinylated casein (30) and more similar to those of putrescine incorporation into loricrin (43). However, comparisons showed that there are striking differences in the kinetic efficiency (\( k_{cat}/K_m \)) of the reactions. As predicted from the SDS-PAGE data of Fig. 1E, the TGase 3 enzyme cross-linked THH-8 15–20 times more efficiently than TGases 1 or 2. Moreover, following maximal modification of THH-8 by PAD, the efficiency of the cross-linking reaction showed a further 3-fold increase for TGase 3 but did not change significantly with TGases 1 and 2 (Table I). Together, these data suggest that THH-8, and by deduction intact THH, is preferably cross-linked by TGase 3 in vivo.

**Isolation of Isodipeptide Indicates That PAD Modification Increases the Rate of Cross-linking Reactions**—One important implication of the above data is that PAD modification makes THH-8 a better substrate for the TGase 3 reaction. To test this, we measured the amount and rate of insertion of cross-links using the standardized amount of TGase 3 enzyme. Following complete protease digestions, the amount of isodipeptide was measured directly by amino acid analysis, as well as indirectly...
by estimation of the disappearance of lysines and glutamines. The data of Fig. 2 show that the rate of cross-linking was similar to that of Fig. 1 and was markedly increased following loss of organized structure by PAD modification, as predicted from the kinetic analyses. This result is reminiscent of the increased in vitro cross-linking of denatured involucrin by TGase 1 (45). In addition, the data show that the maximal amount of cross-link that can be formed is about 6 residues/100 residues. By way of comparison, the measured amount of cross-link formed in vivo in guinea pig IRS and medulla tissues is at least 3.3/100 residues (29). Moreover, THH and THH-8 contain about 6% lysine. Thus, the maximal extent of formation of isopeptide cross-links within THH in vitro and in vivo is limited by the total amount of lysines.

The TGase 3 Enzyme May Utilize Other Acyl Acceptors—From the double displacement kinetic mechanisms involved, we also calculated the $K_M$ values for putrescine (Table I). These data for TGase 3 show a relatively higher value, suggesting that this enzyme prefers to recruit lysines as amines from the THH-8 substrate for cross-linking. After PAD modification, however, this value was reduced by 18-fold, demonstrating that the modification makes THH-8 more accessible for cross-linking to external amines. This may have implications in vivo for the possible cross-linking of THH to other proteins, such as KIF. In addition, if all lysines are exhausted in protein-protein cross-linking (Fig. 2), then it may be possible for some of the many additional glutamines to become cross-linked by the TGases to other acyl acceptors, such as polyamines.

**Confirmation That PAD Modification Increases the Numbers of Glutamines Accessible for Cross-linking by the TGase 3 Enzyme**—To determine which glutamine residues participate in cross-links, radioactive putrescine was incorporated by TGase 3 and used to prepare an inter/intra-chain cross-links formed within THH-8 by the TGase 3 enzyme before and after PAD modification

| Before PAD modification | After PAD modification |
|-------------------------|-----------------------|
| 1. DRKFREELL (not unique) | 18 DRKSQEEKQLLREE  |
| 18 DRKSQEEKQLLREE | Gln-22 |
| 66 DRKFREELLQEQGKRFLLE | Lys-82 |
| 134 DRKFREEEQVRQRERKLF | Lys-68 |
| 327 DEQLQEREQMLHQLRER | Lys-307 |
| 500 DRKFREEQGQQLRERQERQ | Gln-516 |
| 548 DRQYRAEEFQATQGEEQ | Gln-550/Gln-556 |
| 500 DRKFREEEQLRERQERQER | Gln-684 |
| 524 DEQLQEREQMLHQLRER | Gln-338 |
| 304 GKYREDLQEREEQLQLE | Lys-256 |
| 445 ERYKMLEEQQEQ | Lys-256 |
| 282 DRKFLEEQKVRQEQ | Gln-550/Gln-556 |
| 18 DRKSQEEKLQLLREE | Gln-22 |
| 205 DRKFREQQSLQEP | Lys-68 |
| 66 DRKFREELLQEQGQR | Gln-211 |
| 118 DRKFREEQSLRQER | Gln-307 |
| 304 DEQLQEREQMLHQLRQ | Gln-120 |
| 500 DRKFREEQSLRQERQERQ | Gln-516 |
| 500 DRKFREEEQLQEREQMLRER | Lys-826 |
| 524 DGKYREDLQEREEQLLQERQERQ | Gln-550/Gln-556 |
| 345 DRKFREEEQVRQRERKLF | Gln-68 |
| 18 DRKFREELQQQER | Lys-25 |
| 42 DRKFREELQQQER | Lys-44/Gln-50 |
| 500 DRKFREELQQQER | Gln-502 |

**Table III: Examples of inter/intra-chain cross-links formed within THH-8 by the TGase 3 enzyme before and after PAD modification**

Note that in peptides 7, 8, and 14, there was not a unique assignment of which peptide arms were attached. For example, in peptide 7, it is not known whether Gln-531 is cross-linked to Lys-307 or Lys-221. Note that in peptides 9–14, most arginines were sequenced as citrullines.
3 into THH-8 before and after PAD modification, using conditions that limited the degree of THH cross-linking with itself. The proteins were then digested with Asp-N, and the fragments were separated on reverse-phase HPLC and sequenced, and the label was measured at each Edman degradation cycle. In general peptides were not well resolved, in part because of overlapping sequence repeats, and especially in the case of PAD modification, due to variable arginine modification (28). However, the peptides sequenced accounted for ~80% (before modification) and ~60% (after modification) of the total labeled putrescine incorporated, which represented 65% of all the glutamines of THH-8. Before PAD modification, we found that only a limited number of glutamines, corresponding to about one in five, was targeted for reaction (Table II, upper numbers). Following PAD modification, we identified and sequenced most of the same set of peptides as seen before modification, as well as several others that were not labeled before modification. In this case, we found that almost all glutamines were partially labeled, so that most had become roughly equally targeted for reaction (Table II, lower numbers). These data are consistent with the known loss of structure of THH after PAD modification (28). Similar data have been obtained for involucrin; although only a few glutamines could be labeled in the native protein, many more became available after denaturation (45).

Recovery and Identification of Cross-links in THH-8—In a similar experiment, we isolated and sequenced isopeptide cross-linked peptides of THH-8 from before and after modification with PAD. Following cross-linking with the TGase 3 enzyme, the reactions were digested with Asp-N, and peptides were separated by HPLC for sequencing. In THH-8 (Fig. 3A), about half the total protein was recovered as well as resolved peptide peaks, of which six were sequenced. The other half eluted as a broad peak of highly cross-linked material, from which two 1-min fractions were removed for sequencing. In PAD-modified THH-8 (Fig. 3B), all of the peptides eluted in the unresolved peak from which several 1-min fractions were removed for sequencing. Table III lists eight THH sequences from before PAD modification (>80% of the total inserted isopeptide bonds), and six peptides from after PAD modification (25% of inserted cross-link). Although none of these was exactly the same, useful information was nevertheless obtained. First, the usage of glutamines followed the patterns summarized in Table II in that several additional glutamines were used only in PAD-modified THH-8 (e.g., Gln-50, -211, -307, -354, -531, -516, -539, -550, and -556). Second, the most commonly used lysine residue was that in the repeating Asp-Arg-Lys motif, and there was no apparent change before or after PAD modification. There are two possible reasons for this as follows: (i) 81% of the lysines of THH-8 reside in this motif; and (ii) the specificity of TGase cross-linking is determined by the availability of glutamine residues for the first part of the enzyme reaction (46, 47). Finally, some Asp-N peptides were found multiple times but were usually cross-linked to different partners through different glutamines (Table III). To some extent this may reflect ascertainment bias of recovery of less cross-linked peptides.

Localization of THH and TGases in Hair Follicles—The above in vitro kinetic and cross-link data suggest that TGase 3 is the major enzyme involved in the cross-linking of THH. This can only be true if THH and TGase 3 are co-expressed. Accordingly, we have explored the expression of the three TGase enzymes and THH in the hair follicles of mouse skin, using indirect immunofluorescence methods on frozen sections with polyclonal antibodies that cross-react with mouse and human.

Our new antibody made against THH-8 recognized the presence of THH in the column of IRS cells from the papillary bulb all the way to the point at which the mature IRS structure is
dispersed high in the follicle canal. It was also expressed in the developing medullary cells of the living hair follicle and retained in the mature cells of the hair shaft (Fig. 4), as expected from earlier reports (2–7). The polyclonal antibody revealed that TГase 1 was widely expressed in most if not all cell types of the hair follicle, from the extreme base of the papillary bulb to the surface of the epidermis (Fig. 4A). This is somewhat different from previous studies that used a monoclonal antibody in which expression was thought to be more restricted to the outer and inner root sheaths (36–38). Double staining with TГH and TГase 1 antibodies confirmed significant co-expression only in the IRS and medulla (Fig. 4A). The expression of TГase 2, however, was much weaker in all parts of the follicle (Fig. 4B). TГase 3 expression was different from TГase 1 in that it was restricted to the IRS and medulla cells of the follicle, but beginning from a higher level corresponding to above the bulb region, and was precisely co-localized with TГH from this point (Figs. 4C and 5, B and C). Together, these data reveal that when TГase 3 expression was observed, it co-localized precisely with TГH, although TГH expression often occurred one or more cell layers earlier than TГase 3. These data correlate well with the in vitro cross-linking data and together strongly suggest that the TГase 3 enzyme is primarily responsible for cross-linking of TГH in vivo.

Modification by the PAD Enzyme Significantly Increases the Solubility of TГH—We performed experiments in vitro to explore how insoluble TГH could become soluble for cross-linking by the TГase 3 enzyme in vivo. Methanol precipitates of TГH-8 were resuspended at 1 mg/ml and equilibrated in buffer, under which conditions an equilibrium solubility concentration of about 60 μg/ml was established. Most of the protein remained as aggregates in suspension with particle sizes of ≥1 μm. These particles are thus of a size comparable to TГH droplets in vivo. We then added PAD enzyme to see if more protein became solubilized as a result of modification. These experimental conditions were designed to mimic in vivo conditions. The data of Fig. 6 show that the TГH-8 indeed became progressively more soluble with increasing modification, to >200 μg/ml at maximal conversion. A similar experiment was done with intact pig TГH. In this case, its solubility increased from ~3 to 25 μg/ml with maximal PAD modification of ~85% (Fig. 6). In both cases, the rates of solubilization seemed to accelerate following >40% conversion of arginines to citrullines. These data show that progressive PAD modification can disperse insoluble TГH from large particles in suspension into a soluble form. By analogy with keratinocytes in vivo, we propose that progressive modification of PAD promotes TГH droplet dissolution.

In this regard, during the isolation of pig tongue TГH, which is based on the insolubility of the protein (2, 3), we retained the soluble washing fractions. As determined by Western blotting, each of these contained TГH of about 200 kDa (data not shown), as well as other soluble proteins. Amino acid analyses of cut out TГH bands revealed the presence of significant amounts (5–10%) of citrulline. These data indicate that TГH in vivo is modified by PAD and becomes soluble without degradation and before cross-linking.

Summary—A variety of earlier data has demonstrated that TГH undergoes a number of modifications including conversion of many arginines to citrullines (16, 19, 23, 27), morphological changes recognizable with specific monoclonal antibodies (6), and extensive cross-linking by TGases (24–26, 29). The present data address questions on the molecular details, the likely sequence of processing events, and the consequences of the post-translational modifications of TГH. The variety of data presented here indicates that TГase 3 is the preferred enzyme for cross-linking of TГH. However, following PAD modification, TГH becomes a much better substrate for TГase 3, primarily because most glutamines become available for cross-linking, favor a significantly faster rate of reaction, and perhaps transfer to other acyl acceptors as well.

An Hypothesis for the Sequential Modification of TГH by PAD and TGases in Vivo—These data afford the following model on the sequence of postsynthetic modification events to
THH in hair follicle cells (Fig. 7). First, in the IRS and medulla, THH is synthesized as a large insoluble protein and deposited into droplets and constitutes a major protein of the cells. The insolubility is likely due to the high content of charged residues that favor the formation of a single-stranded α-helical conformation that is stabilized by frequent ionic salt bridges between alternate turns of the α-helix (10). In the IRS, the THH droplets become enmeshed with KIF (2, 6, 11, 12) (Fig. 7A), perhaps due to formation of favorable ionic interactions with parts of the THH sequence (10). Second, several cell layers later in the hair follicle, we propose that the cytosolic PAD enzyme(s) begin to attack THH protein at the periphery of the droplets (Fig. 7B). Our analyses indicate this process converts the THH to a more soluble but denatured form. This transformation from an insoluble highly α-helical protein to a more soluble structurally unordered protein would seem essential for its subsequent role as an interfilamentous matrix protein. Accordingly, it would be of interest to explore the temporal expression of the PAD enzyme in hair follicle cells; however, no specific antibodies are available. Third, coincidental with these events, the cytosolic TGase 3 enzyme begins to cross-link the solubilized THH dispersed on the periphery of the droplets to itself and to the admixed KIF in the IRS (Fig. 7C), and perhaps with a regular periodicity (6). In the absence of KIF in the medulla, the cross-linked THH forms aggregates of denatured protein leaving a highly vacuolated structure within the cells.

Fourth, in the IRS, THH become highly aligned, perhaps with a regular periodicity (6). Completion of these events results in a rigid highly insoluble structure. In the absence of KIF in the medulla, the cross-linked THH forms aggregates of denatured protein leaving a highly vacuolated structure within the cells.

The effective removal of solubilized THH by cross-linking thus shifts the equilibrium to facilitate more rapid THH droplet dissolution (6). Fourth, in the IRS, KIF become highly aligned along the long axes of the cells, forming a rigid insoluble composite material (Fig. 7D). Using an established mathematical modeling algorithm (48) with amino acid composition data of IRS tryptic peptides (23, 26), this composite consists of an approximately 1:2 ratio of THH:KIF. The ease with which morphologically intact KIF can be harvested from IRS cells (35) implies that most cross-links occur between THH molecules. The relatively fewer cross-links between KIF and THH probably involve end domain sequences of the keratin chains (26), and in this regard, we have found that in vitro cross-linking of KIF with the TGase 3 enzyme primarily involves a single lysine located in the head domain of the type II keratin chain. Likewise, most PAD modification occurs on THH, although some may also occur on the KIF chains (26, 50). Fifth, THH is also expressed in trace or modest amounts in other epithelial tissues (4–8) and rodent forestomach, each of which contain KIF as the major differentiation product. It is possible therefore that processing events similar to those of the IRS may also occur in some of these tissues. Sixth, in the special case of the medulla which expresses few if any KIF (14), dispersal of the THH droplets leaves interconnected disordered aggregates of protein and large vacuolated spaces in the cells (Fig. 7D), which are then suggested to fulfill a critical function in thermal regulation in mammals (12). Last, studies with specific monoclonal antibodies (6), and our present work with pig THH, suggest that the protein chain of THH remains intact during these modification events and, therefore, leave open the possibility that THH is incompletely processed by PAD before the entire structure becomes insolubilized by extensive cross-linking (Fig. 7D).

Finally, the formation of a cross-linked THH structure has important consequences for barrier function in the epithelial tissues. Based on its sequence homology to involucrin, it was proposed that THH may also serve as a component of the protein CE of epithelia (10). Recent data have shown that THH is co-expressed in a variety of epithelia with several known CE protein precursors including involucrin (51) and small proline-rich proteins (42, 49). Preliminary data have now demonstrated cross-links between citrulline-containing modified THH and such CE components as loricrin and small proline-rich proteins in mouse forestomach CE preparations. Thus, cross-linking of modified THH into the CE would be expected to form a more rigid structure that would have important barrier functions in hardened epithelia.

4 E. Candi, E. Tarcsa, J. J. DiGiovanna, J. G. Compton, P. M. Elias, L. N. Marekov, and P. M. Steinert, submitted for publication.
5 P. M. Steinert, T. Kartasova, and L. Marekov, unpublished observations.
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