Transglutaminase 1 Mutations in Lamellar Ichthyosis

LOSS OF ACTIVITY DUE TO FAILURE OF ACTIVATION BY PROTEOLYTIC PROCESSING*

(Received for publication, December 17, 1997, and in revised form, March 12, 1998)

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Lamellar ichthyosis is a congenital recessive skin disorder characterized by generalized scaling and hyperkeratosis. It is caused by mutations in the TGM1 gene that encodes the transglutaminase 1 (TGase 1) enzyme, which is critical for the assembly of the cornified cell envelope in terminally differentiating keratinocytes. TGase 1 is a complex enzyme existing as both cytosolic and membrane-bound forms. Moreover, TGase 1 is proteolytically processed, and the major functionally active form consists of a membrane-bound 67/33-kDa complex with a myristoylated and palmitoylated amino-terminal 10-kDa membrane anchorage fragment. To understand better how point mutations, deletions, and truncations found in lamellar ichthyosis disease affect the structure and function of TGase 1, we have expressed in baculovirus and keratinocytes a number of reported TGase 1 mutants. The structural implications of these mutations were examined using a homology-derived three-dimensional model of TGase 1 generated from the known x-ray structure of the related coagulation factor XIIIa enzyme. The present studies demonstrate that loss of TGase 1 activity is not restricted to mutations that directly affect the enzymatic activity. We report a new class of mutations that impair the subsequent post-synthetic processing of the protein into its highly active functional forms.

* The work was supported in part by Grant E413 from Telethon and the Neuroblastoma Association (to G. M.). The costs of publication of this article were defrayed in part by the payment of page charges. This advertisement is for information only and should not be considered as an endorsement or approval by the National Institutes of Health of any particular material or service. The views and opinions expressed do not necessarily represent those of the National Institutes of Health. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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† The abbreviations used are: CRI, congenital recessive ichthyosis; CRI, congenital recessive ichthyosis; CRI, congenital recessive ichthyosis; NHEK, normal human epidermal keratinocytes; TGase 1, transglutaminase 1; PAGE, polyacrylamide gel electrophoresis.
examined the likely structural consequences of the mutations by comparison with the known three-dimensional structure of the related factor XIIIa (fXIIIa) enzyme.

**MATERIALS AND METHODS**

**Construction of the Recombinant Transfer Vectors and Recombinant Baculoviruses in Insect Cells**—All recombinant DNA technology was done according to standard procedures (28, 29). A full-length wild type human TGase 1 cDNA (30) was cloned into the baculovirus vector pVL1392 (PharMingen) by insertion at the EcoRI sites. This was then used as a template for the eight mutant forms (see Table 1) that correspond to mutations seen in LI patients selected in this study. The TransforMART Site-directed Mutagenesis Kit (CLONTECH) based on the method described by Deng and Nickoloff (31) was used for introduction of mutations.

The TGase 1 cDNAs in the pVL1392 vector were under the transcription control of the strong baculovirus polyhedrin promoter. The nine TGase 1 recombinant virus clones (wild type and eight mutant forms) were obtained by co-transfection of each vector with the modified Autographa californica nuclear polyhedrosis virus DNA (BaculGold DNA, PharMingen). BaculGold DNA carries a lethal deletion and does not code for viable virus particles by itself. Co-transfection of this DNA with a complementing plasmid construct rescues the lethal deletion of this virus (32). Insect cells (Sf9, Bombyx mori) were obtained by co-transfection of each vector with the modified baculovirus vector pVL1392 (PharMingen) and the expression vector pCMV for transfection (Promega). Cells were labeled with [35S]cysteine/methionine as described previously (28, 33). The cultures were labeled with 2 × 10^6cpm [35S]cysteine/methionine (100 Ci/mmol). The labels were added 4 h prior to transfection. Transfection efficiency was always monitored by use of a cytomegalo virus β-galactosidase construct (pCMV-β-galactosidase) (Promega). Cells were infected with 200–250 multiplicity of infection (MOI) of virus, and the bacterial cell lysate was then added to 200 MOI of baculovirus. The cultures were immediately following transfection (25–27 h) for 6 h, and then medium was replaced with fresh complete medium for 48 h. At the end of the transfection, the medium was replaced with fresh fresh Grace’s insect medium, and the cells were kept for 5 days at 27 °C. The medium containing the nine recombinant virus clones was amplified to production, cells were maintained and infected, either as monolayer or as monolayer and filaggrin that are expressed in cultured NHEK cells in differentiating conditions; these proteins do not contain cysteine or methionine (35).

**Cell Culture, Transfections, β-Galactosidase Activity, and Protein Assay in Keratinocytes**—Cryopreserved normal human epidermal keratinocytes (NHEK) were obtained from CLONTECH (San Diego, CA) and grown in calf skin collagen (Sigma)-coated dishes in serum-free keratinocyte growth medium (KGM, Clonetics) at 0.05 mM Ca2+ supplemented with 60 μg/ml bovine pituitary extract. Third passage cells were used for transfection experiments. Transient transfections were performed in triplicate using Lipofectin reagent (Life Technologies, Inc.) as described previously (28) and incubated at 37 °C in 5% CO2/95% air. The medium was replaced every 2–3 days and the cells were harvested 48–72 h after transfection. Transfections were done when cultures had reached 70% confluency. Transfection efficiencies were always monitored by use of a cytomegalo virus β-galactosidase construct (pCMV-β-galactosidase) (Promega). Cells were added with 0.5 Ci/ml of [methyl-3H]thymidine for 16–20 h and then were harvested. Transfections were done when cultures had reached 70% confluency. Transfection efficiencies were always monitored by use of a cytomegalo virus β-galactosidase construct (pCMV-β-galactosidase) (Promega). Cells were added with 0.5 Ci/ml of [methyl-3H]thymidine for 16–20 h and then were harvested. Transfections were done when cultures had reached 70% confluency. Transfection efficiencies were always monitored by use of a cytomegalo virus β-galactosidase construct (pCMV-β-galactosidase) (Promega). Cells were added with 0.5 Ci/ml of [methyl-3H]thymidine for 16–20 h and then were harvested. Transfections were done when cultures had reached 70% confluency. Transfection efficiencies were always monitored by use of a cytomegalo virus β-galactosidase construct (pCMV-β-galactosidase) (Promega). Cells were added with 0.5 Ci/ml of [methyl-3H]thymidine for 16–20 h and then were harvested. Transfections were done when cultures had reached 70% confluency. Transfection efficiencies were always monitored by use of a cytomegalo virus β-galactosidase construct (pCMV-β-galactosidase) (Promega). Cells were added with 0.5 Ci/ml of [methyl-3H]thymidine for 16–20 h and then were harvested.
RESULTS

Mutant TGase 1 Forms Studied—The purpose of the present work was to explore the structural-functional consequences of some of the known mutations of the TGM1 gene seen in LI. Data from protein expression and transfection studies were combined with structural information from a predicted three-dimensional model of the TGase 1 enzyme based on the known crystal structure of the related factor XIIIa enzyme. The TGase 1 mutations studied in the present work are listed in Table I, and as our data show, they offer a spectrum of alterations affecting the enzyme and afford new insights into the molecular basis of LI disease.

Expression and Biochemical Characterization of Recombinant Human Wild Type and Mutant Forms of TGase 1—We expressed in the baculovirus system the human wild type TGase 1 cDNA and the eight mutant forms that have been reported in LI which are listed in Table I. Protein expression was evident from 48 h postinfection and reached a maximum at 72 h (Fig. 1, A and B). Western blot analyses using a specific polyclonal anti-human TGase 1 antibody revealed that the recombinant enzymes were not proteolytically processed in this system and remained as the full-length protein with an apparent molecular mass of 97 kDa (Fig. 1B). The mutation M442stop generated a truncated protein of size about 55 kDa (Fig. 1, A and B). As has been observed in NHEK cells, the baculovirus-expressed TGase 1 (wild type and mutant forms) was present in both the cytosol and membrane-bound fractions. About 90% of each of eight recombinant proteins was retained in the latter (data not shown), which is very similar to the distribution of the TGase 1 enzymes in proliferating basal epidermal keratinocytes or stationary NHEK cells grown in low Ca²⁺ submerged liquid cultures (26). The one exception was mutant form S41Y, in which about half of the protein was present in the cytosol (see Fig. 4F). The recombinant baculovirus-expressed wild type TGase 1 was also modified by lipids (Fig. 1C). The labeling of the cytosolic and membrane-bound enzyme forms with myristate indicates the protein was constitutively N-myristoylated as in keratinocytes (27). The weak incorporation of palmitate also implies partial N-myristoylation and palmitoylation.

Nucleotide and amino acid positions correspond to GenBank™ accession numbers M55183, M57623, and for the major variant of M86360. Protein sequence numbers were counted from the first amino acid following the initiation codon.

Specific Activities of Recombinant Wild Type and Mutant Forms of Human TGase 1—Table II lists the specific activities of the cytosolic and membrane-bound forms of the wild type and eight mutant recombinant enzymes. The very low specific activities of the wild type enzyme were almost identical to those of the native intact (106-kDa form) enzyme expressed in NHEK cells or isolated foreskin keratinocytes (26). As expected, most analyzed mutants showed reduced enzymatic activities. Both membrane-bound and cytosolic TGase 1 activities were reduced to 0–20% of the wild type control for mutations R141H, R142H, R141C, and L268S. The specific activity of R332Q was also reduced. The truncated enzyme generated by the premature stop codon (M442stop) was inactive, as expected from earlier deletion cloning experiments (34). Unexpectedly, two mutant forms (S41Y and R314L) showed 2–4-fold increases in specific activities in comparison to the wild type enzyme (Table II).

Transfections of Wild Type and Mutant Forms into NHEK Cells—The full-length wild type and four mutant cDNAs (two of low or zero activity, R141H and R332Q, and two of higher activity, S41Y and R314L) were transfected into NHEK cells. Transfection efficiencies within the range of 15–30% were obtained in these experiments as estimated by co-transfection with a β-galactosidase control vector (data not shown; see Fig. 3C). Each activity value was standardized for units of β-galactosidase in order to compare the different transfection experiments with the different constructs. These experiments were performed under proliferating (in low Ca²⁺, 0.05 mM) and differentiating (in high Ca²⁺, 1.2 mM) conditions (Fig. 2, A and B, respectively). As expected for NHEK cells (26, 27), in both cases most of the transfected TGase 1 enzyme was directed to the membrane fraction. In low Ca²⁺ media (Fig. 2A), while the activity level of the wild type transfected constructs increased commensurate with the transfection efficiency, the activities of the R141H and R332Q mutants were similar to those of the untransfected or sham-transfected NHEK cells, indicating they are not functional, as observed in baculovirus (Table II). Likewise, the two active mutants (S41Y and R314L) generated total levels of enzyme activity that were higher than for the wild type construct and commensurate with the transfection efficiencies, indicating they were also as functional in NHEK cells as in baculovirus. However, in high Ca²⁺ media, the level of activity of each of the mutant constructs was at background level and lower than for the wild type construct (p < 0.01) (Fig. 2B). Similar results were obtained from the measurement of the formation of cross-linked cell bodies in the same cells in that the data paralleled the activity levels (data not shown).

The S41Y and R314L Mutant TGase 1 Forms Are Not Pro-

<table>
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<tr>
<th>Name</th>
<th>Exon</th>
<th>Nucleotide(s) changed</th>
<th>Amino acid changed</th>
<th>Ref.</th>
</tr>
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<tbody>
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<td>S41Y</td>
<td>2</td>
<td>TCC → TAC (+1044)</td>
<td>Ser¹¹ → Tyr</td>
<td>14</td>
</tr>
<tr>
<td>R141C</td>
<td>3</td>
<td>CGG → TGC (+1488)</td>
<td>Arg¹¹ → Cys</td>
<td>14</td>
</tr>
<tr>
<td>R141H</td>
<td>3</td>
<td>CGG → CAC (+1489)</td>
<td>Arg¹² → His</td>
<td>15</td>
</tr>
<tr>
<td>R142H</td>
<td>3</td>
<td>CGG → CAC (+1492)</td>
<td>Arg¹¹ → His</td>
<td>15</td>
</tr>
<tr>
<td>R314L</td>
<td>6</td>
<td>CTT → CTT (+5316)</td>
<td>Arg¹¹ → Leu</td>
<td>43</td>
</tr>
<tr>
<td>R322Q</td>
<td>6</td>
<td>CCG → CAG (+3540)</td>
<td>Arg²² → Gln</td>
<td>14</td>
</tr>
<tr>
<td>M442stop</td>
<td>8</td>
<td>T deletion (+4724)</td>
<td>Met⁴ ⁄₂ → Stop</td>
<td>14</td>
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<tr>
<td>L268S</td>
<td>5</td>
<td>TCTT → C (+3249)</td>
<td>Deletion Leu³⁸⁸</td>
<td>43</td>
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</tbody>
</table>

* Nucleotide and amino acid positions correspond to GenBank™ accession numbers M55183, M57623, and for the major variant of M86360.

[^bp]: base pair

[^Ref.]:
Fig. 1. Expression and characterization of wild type and mutant forms of TGase 1. A, Coomassie staining of the 30 μg of total protein from Sf9 cells infected with wild type baculovirus (CTRL) and recombinant baculoviruses bearing wild type TGase 1 and the eight mutants cDNAs as shown. B, Western blot using a specific polyclonal antibody for TGase 1; the asterisks denote the TGase 1 protein forms of about 97 or 55 kDa. C, autoradiography of Sf9 cells infected with wild type baculovirus (ctrl) or recombinant baculoviruses bearing wild type TGase 1 labeled with [14C]palmitic and [14C]myristic acid in the cytosolic and membrane-bound fractions. D, in vitro cross-linking of 35S-labeled human loricin by membrane-bound recombinant baculovirus TGase 1, in the presence of EDTA (ctrl) of after reaction for the times shown. As for the bacterially expressed enzyme (24), both oligomerization and intrachain cross-linking occurs.

<table>
<thead>
<tr>
<th>Wild type TGase 1</th>
<th>S41Y</th>
<th>R141C</th>
<th>R141H</th>
<th>R142H</th>
<th>R314L</th>
<th>R322Q</th>
<th>L268D</th>
<th>M442stop</th>
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<tr>
<td>Cytosolic %</td>
<td>16.8±6.7</td>
<td>74.4±12</td>
<td>&lt;0.01</td>
<td>0.5±0.2</td>
<td>3.4±0.1</td>
<td>33.6±10.8</td>
<td>12.2±2.4</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Membrane-bound %</td>
<td>5.9±1.6</td>
<td>11.4±3.3</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>13.9±4.5</td>
<td>1.5±1.2</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

Specific activities of wild type and mutant TGase 1 forms expressed in baculovirus

Specific activities are expressed as picomoles of [14C]putrescine incorporated into succinylated casein/h/pmol of TGase 1 protein. TGase 1 amounts are estimated by active site titration with [14C]iodoacetamide (34). The results are averages ± S.D. of 3–5 measurements.

Structural Consequences of Transglutaminase 1 Mutations

To explore further these differences, new constructs of the wild type and S41Y and R314L mutant TGase 1 forms were assembled in a mammalian expression vector system in which a tag of six histidine residues had been attached to the carboxyl-terminal end. This was done in order to distinguish between the endogenous and mutant TGases following transfection into NHEK cells.

In initial experiments, we tested whether the presence of this tag interferes with the expression and/or processing of the wild type TGase 1 protein in differentiating NHEK cells grown in high calcium conditions as above, chromatography (Fig. 3, A–F) of the immunoprecipitates of both antibodies were resolved on gradient SDS-PAGE gels (Fig. 4, B and E). The results obtained were the same and demonstrated that in both cases about half of the total TGase 1 protein was processed into the 67-33-, and 10-kDa bands. Thus the presence of the histidine tag on the carboxyl terminus does not interfere with TGase 1 processing or activity.

These experiments were repeated with NHEK cells transfected with the constructs containing the His-tagged S41Y and R314L mutant TGase 1 forms. By using identical culture conditions as above, chromatography (Fig. 3, E–H, respectively) and SDS-PAGE gels (Fig. 4, F and G, respectively) revealed that the products immunoprecipitated by the polyhistidine antibody correspond to the intact membrane-bound 106-kDa form. Therefore, for both mutants, only trace amounts had been...
proteolytically processed under these terminally differentiating conditions. In pulse-chase experiments we estimated the half-lives of the intact wild type and S41Y and R314L mutant forms. Whereas the half-life of the membrane-bound wild type protein was about 30 h as expected (27), the half-lives of the S41Y and R314L mutants were clearly greater (50 and 60 h, respectively) (Fig. 5A). Similarly, the cytosolic forms are more stable (40 and 60 h, respectively, versus 20 h for the wild type) (Fig. 5B).

Together, these data demonstrate that although these two mutants are stably expressed and retained in the NHEK cells, they remain as the intact very low specific activity (zymogen) proteins. They are not processed into the highly active functional enzymes as for the wild type protein.

**Predicted Structural Model of TGase 1**—In order to explore the structural consequences of the mutant forms studied in this paper, we constructed a three-dimensional model of human TGase 1 (Fig. 6) using as the basis the known crystal structure of the related human fXIIIa (39). The high sequence conservation and few deletions provide a realistic model of the TGase 1 molecule. This includes four main domains that have been designated the amino-terminal \(\beta\)-sandwich, the central core domain containing the active site (catalytic core domain), and \(\beta\)-barrels 1 and 2 (39). In the case of fXIIIa, an activation peptide precedes the \(\beta\)-sandwich domain. In the case of TGase 1, a \(\sim\)90-residue sequence that is involved in membrane anchorage and substrate recognition or specificity (zymogen) proteins. They are not processed into the highly active functional enzymes as for the wild type protein.

(Fig. 7). Since both the full-length 106-kDa TGase 1 and the 67/33/10-kDa complex normally purify in monomeric form (Refs. 25–27; see Fig. 3), only one monomer of the dimeric fXIIIa structure was used in the modeling study, omitting the part of the fXIIIa activation peptide preceding Asn18, the part mediating the intermolecular inhibition (Figs. 6 and 7) (39).

Following proteolytic activation at Arg 92, most of the membrane-bound TGase 1 remains associated with the 10-kDa membrane anchorage domain (26, 27) to form a 67/33/10-kDa complex. In the crystal structure of Ca\(^{2+}\)-activated and thrombin-cleaved fXIIIa, the activation peptide remains associated with the body of the enzyme and adopts a practically identical conformation as compared with the fXIIIa zymogen (39, 40). In particular, we note a high homology between two hexapeptide segments of this domain of TGase 1, \(\sim\)NAAADDD and \(\sim\)NAAGDG (Fig. 7), with a region of the fXIIIa activation peptide, \(\sim\)NAEDD, that tightly interacts with the \(\beta\)-sand-
wich domain and the catalytic core domain (39). This interaction is mediated by FXIIIa Asp\textsuperscript{243} and Arg\textsuperscript{252}, both conserved in TGase 1 (Asp\textsuperscript{305} and Arg\textsuperscript{314}). This suggests that the high overall structural similarity between these two enzymes also includes parts of the activation peptide of FXIIIa and the membrane anchorage domain of TGase 1. Based on the observed phenotype for the R314L mutant, we predict the second 97NAAGDG\textsuperscript{102} hexapeptide motif is associated with the amino-terminal \(\beta\)-barrel domain in the unprocessed full-length 106-kDa form of the enzyme. The second motif, 55NAADD\textsuperscript{60}, might represent an alternative mode of binding, for example to modulate substrate specificity after cleavage at Arg\textsuperscript{92} (26, 34). Only the part of the FXIIIa activation peptide starting from Asn\textsuperscript{18} (corresponding to TGase 1 Gly\textsuperscript{95}) was therefore included in the TGase 1 model. In the loop region following the 97NAAGDG\textsuperscript{102} segment, small local re-arrangements centered around 106GG\textsuperscript{107} were sufficient to close the loop leading to the first \(\beta\)-strand of the \(\beta\)-sandwich domain.  

**FIG. 4.** SDS-PAGE gels reveal absence of proteolytic processing of S41Y and R314L mutant forms of TGase 1. Cultures similar to above were harvested at the times indicated, processed as in Fig. 3, but analyzed by SDS-PAGE on 10–20\% gradient gels and autoradiographed for 4–16 days. Immunoprecipitated with the following: A–C, TGase 1 antibody; D–G, polyhistidine antibody. A and D, non-transfected NHEK cultures; B and E, transfected with the wild type TGase 1 construct; C and F, transfected with the S41Y construct; and G, transfected with the R314L construct. The sizes of the processed TGase 1 protein components are shown. Note that in these experiments, much of the 10-kDa band (arrowhead) was lost by apparent degradation. 

![SDS-PAGE gel images](image-url)
Asp253 in the linker region to the catalytic core domain (Fig. 8B). Since both mutants showed expression levels and lifetimes comparable to that of the wild type enzyme in the baculovirus expression system, overall protein stability seems not to be affected. A conformational change affecting the relative orientation between the amino-terminal domain and the catalytic core domain is therefore the most likely cause for their enzymatic inactivity, as the amino-terminal domain is indispensable for substrate recognition and processing (26).

Deletion Mutants L268D and M442stop—The absence of structural integrity, a prerequisite for most enzyme activity, readily explains the complete absence of catalytic activity in case of the M442stop mutation. Truncation of the polypeptide chain at this position removes a considerable part of the catalytic core domain (residues 443–572), presumably creating a misfolded central domain unable to assemble a functionally active site. Previous deletion cloning experiments documented loss of activity when these sequences were removed (34). As before for the R141H and R142H mutations, proper function of the enzyme will be destroyed due to the absence of a correctly oriented amino-terminal β-sandwich domain.

Active Mutants S41Y and R314L—Both of these mutants exhibited an increased basal activity of the unprocessed 106-kDa polypeptide and do not undergo activation by proteolytic cleavage at Arg92 in differentiating keratinocytes (Figs. 3 and 4). The relationship between the two phenotypes seems at first sight to be unrelated. Arg314 is located in the interface between the amino-terminal β-sandwich domain and the catalytic core domain and mediates (through Asp305) specific interactions to the initial part of the hexapeptide motif of the 10-kDa fragment (Fig. 8B). Substitution of Arg314 into Leu would abolish these interactions and potentially destabilize the preceding loop region, as found for an analogous mutation in fXIIIa (R252I), which resulted in undetectable protein levels presumably due to reduced protein stability (45). However, the presence of Pro310 in the preceding loop region is likely to significantly increase the local conformational stability in TGase 1. At the corresponding position of fXIIIa, an aspartic acid (Asp248) is instead present. Presumably also, this increased stability, as reflected by a very long half-life (Fig. 5), prevents or interferes with proteolytic activation at the nearby Arg92.

For the S41Y mutation, no direct prediction can be derived from our model, since this residue is located in the membrane anchorage segment which could not be modeled. We suggest that this substitution promotes a conformational change in the 10-kDa fragment, which as for R314L, results in a net reduction of the inhibitory capacity of the 10-kDa fragment domain.

DISCUSSION

The TGase 1 enzyme exists in keratinocytes in multiple forms due to post-translational proteolytic processing (25–27). In proliferating or stationary cells, most protein exists as the intact form of low specific activity (zymogen) and is tightly
bound to cellular membranes. (This form retains some activity and is not “inactive” as previously thought (26)). Only minor (5–10%) amounts cycle into the cytosol. However, in terminally differentiating keratinocytes, up to 50% of the membrane-bound TGase 1 is proteolytically processed by cleavage at sites that correspond very closely to inter-domain junctions of its predicted structure. The resulting 67/33/10-kDa complex shows a 200-fold increase in specific activity and is responsible for most of the TGase 1 activity in keratinocytes during terminal differentiation (26).

In the present paper, we have explored the structure-function relationship for several mutants of the TGase 1 enzyme observed in LI disease. We have combined biochemical data obtained for expressed forms of the mutants with structural information from a homology-derived model of TGase 1. The mutant form M442stop results in large deletions of sequences known to be essential for activity. Apart from mutant R322Q, which has only a modestly reduced activity, most other mutants caused by point mutations (R141C, R141H, R142H, and L268D) resulted in loss of function due to the failure to establish key ionic and hydrogen bond interactions (Fig. 8, A and B). These changes are likely to cause partial protein misfolding and/or domain rearrangements with resultant loss of activity, that is LI disease was caused by the greatly reduced enzyme activity in the keratinocytes.

Identification of a Novel Type of Mutant: Interference with Postsynthetic Proteolytic Activation—For the last two mutants, S41Y and R314L, a more detailed characterization was necessary in order to understand why they should cause LI disease. At first glance they appear to involve gain of function; both mutant enzymes were significantly more active than wild type TGase 1 when expressed in baculovirus or transfected keratinocytes grown under proliferating conditions, in situations where only the unprocessed 106-kDa form of the enzyme is observed (Fig. 2 and Table II). Further experiments in terminally differentiating NHEK cells showed, however, that both variants could not be proteolytically processed into the functional highly active form (Figs. 3 and 4). In view of the large differences in specific activities between the unprocessed 106-kDa and the proteolytically activated 67/33/10-kDa complex
Detailed view of the predicted interaction between the \textsuperscript{97}NAAGDG\textsuperscript{102} hexapeptide following the 10-kDa membrane anchorage domain and the body of the TGase 1 enzyme. Asp\textsuperscript{305} and Arg\textsuperscript{314} take the role of fXIIIa Asp\textsuperscript{243} and Arg\textsuperscript{252} in linking the fragment to the catalytic core domain. Mutation of R314 into Leu (L314, shown in green) will disrupt the chain of electrostatic and hydrogen bond interactions between the catalytic domain and functional backbone groups around Asn\textsuperscript{97}. As a result, we predict that local structural changes occur affecting the conformation of residues preceding Asn\textsuperscript{97}, including also Arg\textsuperscript{92}, which is the site of proteolytic cleavage. For illustrative purposes a tentative position for Arg\textsuperscript{92} has been included in the figure.

FIG. 9. Stereo images illustrating the effect of the R314L mutation on the interaction between Arg\textsuperscript{314} and the \textsuperscript{97}NAAGDG\textsuperscript{102} hexapeptide motif. Detailed view of the predicted interaction between the \textsuperscript{97}NAAGDG\textsuperscript{102} hexapeptide following the 10-kDa membrane anchorage domain and the body of the TGase 1 enzyme. Asp\textsuperscript{305} and Arg\textsuperscript{314} take the role of fXIIIa Asp\textsuperscript{243} and Arg\textsuperscript{252} in linking the fragment to the catalytic core domain. Mutation of R314 into Leu (L314, shown in green) will disrupt the chain of electrostatic and hydrogen bond interactions between the catalytic domain and functional backbone groups around Asn\textsuperscript{97}. As a result, we predict that local structural changes occur affecting the conformation of residues preceding Asn\textsuperscript{97}, including also Arg\textsuperscript{92}, which is the site of proteolytic cleavage. For illustrative purposes a tentative position for Arg\textsuperscript{92} has been included in the figure.

(26), the net consequence of the mutant forms was therefore the same as for the loss-of-function mutants: insufficient enzyme activity for terminal differentiation.

We have shown in earlier deletion cloning experiments in bacteria that the 10-kDa membrane anchorage fragment dictates the catalytic state of the TGase 1 enzyme (26, 34). There are at least two possible ways how this could occur. One is the modulation of substrate specificity controlling the type of substrate that may approach the active site. Although no direct structural data are yet available, we propose a second possibility where at least parts of the membrane anchorage domain fold back onto the body of the TGase 1 enzyme so as to partially obstruct access to its active site. This model is reminiscent of what is observed to occur for the fXIIIA activation peptide, albeit intra-molecular instead of inter-molecular (Fig. 6). Our hypothesis is based on two findings. In the first, in vitro overlay experiments on the binding of different post-translationally modified 10-kDa domains showed that S-myristoyl and S-palmitoyl modifications of the cysteine-rich cluster (\textsuperscript{57}CCGCCSC\textsuperscript{59}) are required for interactions with the 67-kDa and 67/33-kDa components to form the highly active 67/33/10-kDa complex form of the TGase 1 enzyme (27). Second, in this study, we have recognized regions of high sequence homology of the membrane anchorage domain of TGase 1 (residues 97–102, and perhaps residues 55–60) with the region encompassing residues 20–25 of the fXIIIA activation sequence that is responsible for the inhibition of the fXIIIA enzyme. The contact points of this interaction involve specifically the catalytic domain residue Arg\textsuperscript{202} (fXIIIA) and Arg\textsuperscript{314} (TGase 1). Together, these data indicate the existence of rather specific interactions between the membrane anchorage and amino-terminal \beta-sandwich/catalytic core domains of the TGase 1 enzyme. Thus in the R314L mutant, the substitution of Arg\textsuperscript{314} and failure to make key interactions (Figs. 6 and 9) partially releases the inhibitory effect of the membrane anchorage domain, resulting in a molecule of increased specific activity. Given the structural context around Arg\textsuperscript{314} in our TGase 1 model (Fig. 9), we furthermore conclude that this molecular event also increases the stability of the protein and interferes with proteolytic activation. Mutation of Arg\textsuperscript{314} into Leu is likely to affect Arg\textsuperscript{92} directly preceding the \textsuperscript{97}NAAGDG\textsuperscript{102} hexapeptide motif and, as a consequence, alter the accessibility to the cleavage site.

In the case of the S41Y mutant form, a similar scenario has to be assumed, although in this case our model does not allow a direct correlation with structural information. However, we note the presence of an Arg-Arg dipeptide (\textsuperscript{35}RR\textsuperscript{36}) immediately upstream Ser\textsuperscript{41} that could in principle assume a function similar to fXIIIA Arg\textsuperscript{11}/Arg\textsuperscript{12}, which contact acidic residues near the active site thus blocking enzymatic activity (39). Conformational alterations caused by the presence of the substituted tyrosyl side chain could prevent the 10-kDa fragment to exhibit its full inhibitory effect and may also explain why the S41Y mutant form was less efficiently bound to membranes (Fig. 4).

Our data confirm the key role of the 10-kDa membrane anchorage domain for TGase 1 activity and highlight the sensitivity of the enzyme toward alterations affecting this region. Any mutation that disturbs the proper conformation of the 10-kDa membrane anchorage could therefore have drastic consequences for enzymatic activity or substrate specificity. More detailed structural information on this domain therefore seems warranted.

Altogether, the present studies demonstrate that loss of TGase 1 enzyme activity, which results in LI disease, can arise either from mutations that affect directly the correct folding of the protein or which instead affect the subsequent post-synthetic processing of the protein into its highly active functional forms. Accordingly, it is to be expected that LI disease can be caused by deficient TGase 1 activity due to adverse effects on other processing events, such as N-myristoylation, membrane anchorage due to failed lipid S-myristoylation, or S-palmitoylation, phosphorylation, etc. Furthermore, it is conceivable that mutations that affect directly those enzyme systems responsible for these post-synthetic modifications should result in a TGase 1 enzyme of greatly reduced specific activity or potential for proteolytic processing, with consequences of LI disease. In this regard, it is noteworthy that cases of CRI have been identified that do not involve mutations in the TGM1 gene (11–13). Further work must be done now to explore whether mutations in these ancillary gene systems, directly or indirectly affecting the TGase 1 enzyme, are the cause of CRI or LI disease.

During the preparation of this manuscript, another paper was published describing several new mutations of the TGM1 gene in LI, and structural modeling was performed to explain the diminished TGase 1 activity of the mutant forms (46). Many of the data and conclusions conform to our analyses described here. Two common mutant forms were analyzed. As in the present study, the mutant form S41Y was demonstrated to be less efficiently bound to membranes and to have a higher activity than the wild type (when assayed following transfection into keratinocytes derived from LI patients with no background TGase 1 activity). However, that study did not identify the observation discovered here that under differentiating con-
ditions, the S41Y mutant form is likely to be disease-causing since it could not be processed into a highly active functional enzyme. Our study offers a possible explanation for this in the association of the membrane anchorage region of TGase 1 to the β-sandwich domain. Similarly, that study described a mutant form R314C, which possessed low specific activity, presumably due to protein misfolding. In the present work, we show that the Leu substitution instead leads to an excessively stable protein that cannot be processed. Thus, biochemical analyses as performed in this study, coupled with activity and structural analyses, have provided more profound information about the properties of the TGase 1 enzyme system.

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Transglutaminase 1 Mutations in Lamellar Ichthyosis: LOSS OF ACTIVITY DUE TO FAILURE OF ACTIVATION BY PROTEOLYTIC PROCESSING
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doi: 10.1074/jbc.273.22.13693

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