Novel Mutations of the Transglutaminase 1 Gene in Lamellar Ichthyosis

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Lamellar ichthyosis, one form of congenital autosomal recessive ichthyosis, is caused by mutations in the gene (*TGM1*) encoding the transglutaminase 1 enzyme. Mutations, deletions, or insertion of *TGM1* have been reported so far. Here we report that three novel mutations of *TGM1*, D101V, N288T, and R306W, cause lamellar ichthyosis in two different families. The patient in family LI-KD has N288T and R306W mutations, and the patient in family LI-LK has D101V and R306W mutations. The activity of the transglutaminase 1 enzyme of the patient in family LI-LK was only about 15% of normal. Also, three-dimensional structural prediction analyses revealed that the N288T and R306W mutations, and possibly the D101V mutation, cause misfolding in the central catalytic core domain of the transglutaminase 1 enzyme that would probably result in reduced enzyme activity. Our data suggest that the greatly reduced transglutaminase 1 activities are due to disruptions of the native folding of transglutaminase 1, and that these mutations may play a critical role in the pathology of lamellar ichthyosis. ***Key words: lamellar ichthyosis/mutations/transglutaminase 1 gene. J Invest Dermatol 117:214–218, 2001***

Congenital autosomal recessive ichthyoses are a heterogeneous group of disorders that present at birth with generalized involvement of skin and lack of manifestations in other organ systems. There is no consensus on the classification of the autosomal recessive ichthyoses subclasses, but depending on the findings of erythema two clinical subsets, lamellar ichthyosis (LI) and nonbullous congenital ichthyosiform erythroderma, were distinguished by Williams and Elias (1985). LI is apparent at birth, and the newborn often presents encased in a collodion membrane that desquamates during the first 10–14 d of life. Over time, the skin forms large, dark, plate-like scales. Many patients also exhibit ectropion and eclabian due to tautness of the facial skin. Scarring alopecia on the scalp and eyebrow is a common sequela. Histopathologic findings show orthokeratotic hyperkeratosis and mild to moderate acanthosis. The estimated incidence of LI is 1:200,000–300,000.

Transglutaminases (TGases) constitute a family of Ca^{2+} and sulfhydryl-dependent enzymes that catalyze the formation of isopeptide cross-links in proteins between the γ-amide of a donor glutamine residue and an ε-NH_2 group of an acceptor lysine residue (Folk, 1980; Greenberg *et al*, 1991; Melino *et al*, 2000). This cross-link thus contributes to the formation of a highly insoluble macromolecular structure. Of the nine known different members of the TGase family in human, at least five are expressed in the epidermis, and two have proven functions in the formation of the cell envelope barrier structure (Steinert, 2000): the membrane-associated TGase 1, which is expressed throughout the epidermis and is involved in both cross-link formation and ester-linkage of ceramides to cell envelope proteins; and the cytosolic TGase 3, known to be expressed in the granular layer of the epidermis and hair follicle. Both enzymes require proteolytic activation at conserved sites to achieve optimal enzyme activity.

Russell *et al* (1994) reported that the likely causative gene defect in LI in some families mapped to chromosome 14q11 near the *TGM1* locus encoding the TGase 1 enzyme, and subsequently mutations in *TGM1* were identified (Huber *et al*, 1995; Russell *et al*, 1995). Since then, several different mutations of *TGM1* have been identified (Parmentier *et al*, 1995; Huber *et al*, 1997; Laiho *et al*, 1997; Petit *et al*, 1997; Schorderet *et al*, 1997; Bichakjian *et al*, 1998; Hennies *et al*, 1998a; 1998b; Shevchenko *et al*, 2000). Later, a second locus, at chromosome 2q33–35, was identified in families from Morocco (Parmentier *et al*, 1996). The existence of additional families apparently unlinked to either *TGM1* or chromosome 2q suggests that at least one additional gene can cause LI (Bale *et al*, 1996; Parmentier *et al*, 1996). These findings suggest that LI is genetically heterogeneous.

In this paper, we report three novel mutations in *TGM1* in two LI patients, N288T (exon 5) and R306W (exon 6) in one patient, and D101V (exon 2) and R306W in the other patient.

MATERIALS AND METHODS

Family LI-KD The proband (II-1) is a Japanese girl who was born prematurely (38 wk) in 1996 from unaffected nonconsanguineous parents. The newborn was encased in a translucent collodion membrane.

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Abbreviations: LI, lamellar ichthyosis; TGase, transglutaminase.

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At 24 h after birth, the collodion membrane began to dry and ectropion and eclabium appeared. At 7 d after birth, the collodion membrane began to peel off and the skin was slightly erythrodermic. At 1 mo of age, the dry collodion membrane peeled off and large, slightly brownish scales appeared, mainly on her trunk. Other body sites appeared near normal, although the skin was dry. Ectropion and eclabium had reduced by this time. At 6 mo, the brownish scales turned into fine, white scales. Since then, the girl has been successfully controlled with petroleum.

Family LI-LK The clinical history of the patient (II-2) in this family is very similar to that of patient LI-KD. The proband is a Korean boy who was born in 1999 from unaffected nonconsanguineous parents. The newborn was encased in a translucent collodion membrane, and was placed in an incubator at the local hospital to control the skin problem. At 24 h after birth, the collodion membrane began to dry and ectropion and eclabium appeared. At about 1 mo of age, the dry collodion membrane peeled off and large, slightly brownish scales appeared on his trunk, buttocks, and face. Both extremities appeared near normal, although the skin was dry. Ectropion and eclabium had reduced by this time. The brownish large thick scales improved slowly with age and are now confined to forehead, buttocks, and flank.

Source of DNA Genomic DNA was isolated as described earlier (Yang *et al*, 1994) from freshly drawn blood from both affected and unaffected family members. Genomic DNA was also extracted from 50 unrelated individuals from the normal Korean population for use as controls.

Direct polymerase chain reaction (PCR) amplification of *TGM1* and DNA sequencing The oligonucleotide primers and the conditions of PCR used for amplifying all *TGM1* exons, and subsequent DNA sequencing, have been described previously (Russell *et al*, 1995). A second reamplification reaction was required for exons 2, 5, and 6.

Screening assay for *TGM1* mutations We used the assays to facilitate the detection of each identified mutation of the patient and to screen for the occurrence of that mutation in the normal population. The R306W substitution destroys an Msp I restriction enzyme site (C/CGG) in DNA amplified from exon 6. Completed PCR products were purified from the gel and digested with 2 units of Msp I for 2–4 h, and analyzed on 6% polyacrylamide gels. As the D101V and N288T substitutions did not destroy or create restriction enzyme sites, a PCR amplification of specific alleles (PASA) assay was developed for each using two sets of specific wild-type and mutant primers. The mutant primers differ only at the extreme 3′-end and have the nucleotide change complementary to the identified mutation in *TGM1*. Amplified products were then characterized as before (Yang *et al*, 1994).

Molecular modeling A three-dimensional model of human TGase 1 was generated on the basis of the known atomic structure of human factor XIIIa (fXIIIa) as search model as present in the Protein Data Bank with accession code 1GGT (Bernstein *et al*, 1997). The modeling packages INSIGHT-II and the Program O (Jones *et al*, 1991) were used to build the model of TGase 1. The structurally aligned sequence of TGase 1 residues was inscribed onto the structural frame provided by fXIIIa residues from 231 to 319 for the R306W mutation, and residues 206–295 and 663–680 for the N288T mutation. The resulting model of TGase 1 was then optimized at the positions where mutation or nonconservative substitutions occurred. The stereo representation was made with the program Molscript (Kraulis, 1991) and rendered using Raster 3D (Merritt and Murphy, 1994). The D101V mutation cannot be modeled as the equivalent region in fXIIIa has not been solved.

TGase 1 activity from cultured keratinocytes from family LI-LK Keratinocytes were cultured from skin biopsies obtained from father, mother, and patient of the LI-LK family as described by Rheinwald and Green (1975). TGase 1 activity was measured from cultured keratinocytes of LI-LK family members using a method adapted from Lichti *et al* (1985). In brief, cells were lyzed by sonication in 20 mM sodium phosphate (pH 7.2), 0.5 mM ethylenediamine tetraacetic acid, 10 mM dithiothreitol, and 50 μg per ml phenylmethylsulfonyl fluoride. After centrifugation at 25,000 × *g* at 4°C for 30 min, the supernatant was used as the cytosolic fraction. The pellet was re-extracted by sonication with the same buffer plus 1% Triton X-100. After incubation for 10 min at 37°C, the lysate was centrifuged again and the supernatant was taken as the membrane fraction. TGase 1 activity was expressed as picomoles ^{3}H-putrescine incorporated into dimethylcaseine per hour and per milligram of protein. Results are indicated as the mean ± SE in cell extracts from at least two different cell passages, each measured in triplicate.

Northern blot analysis Total RNA was extracted from cultured keratinocytes using TRIzol (Gibco BRL) according to the manufacturer's instructions. Ten micrograms of total RNA from each sample were separated through a 1% agarose gel containing 2 M formaldehyde, transferred to an H-bond nitrocellulose membrane (Amersham), and hybridized for 24 h at 42°C to cDNA probe of TGase 1 radiolabeled with α^{32}P-dCTP. The membrane was washed twice for 30 min at 23°C in a solution containing 2 × sodium citrate/chloride buffer (SSC) and 0.1% sodium dodecyl sulfate (SDS) and then for 30 min at 60°C with 0.2 × SSC and 0.1% SDS. The blots were exposed for 48 h at –70°C. Rehybridization with α^{32}P-dCTP labeled glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe was used to normalize the relative amount of mRNA transcripts.

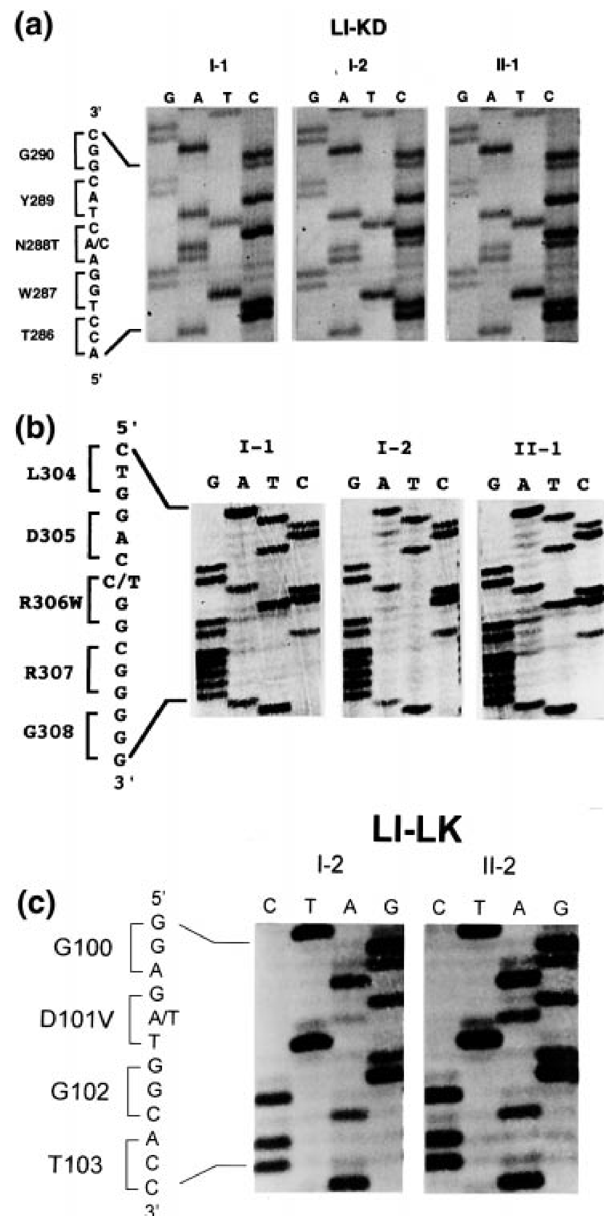

Figure 1. DNA sequences of *TGM1*. (*a*) The carrier mother (I-2) and the patient (II-1) of family LI-KD have an A to C change, which results in N288T substitution in exon 5. (*b*) The carrier father (I-1) and the patient (II-1) of family LI-KD have a C to T substitution, which results in R306W substitution in exon 6. (*c*) The mother (I-2) and patient (II-2) of family LI-LK have an A to T change, which results in D101V substitution in exon 2.

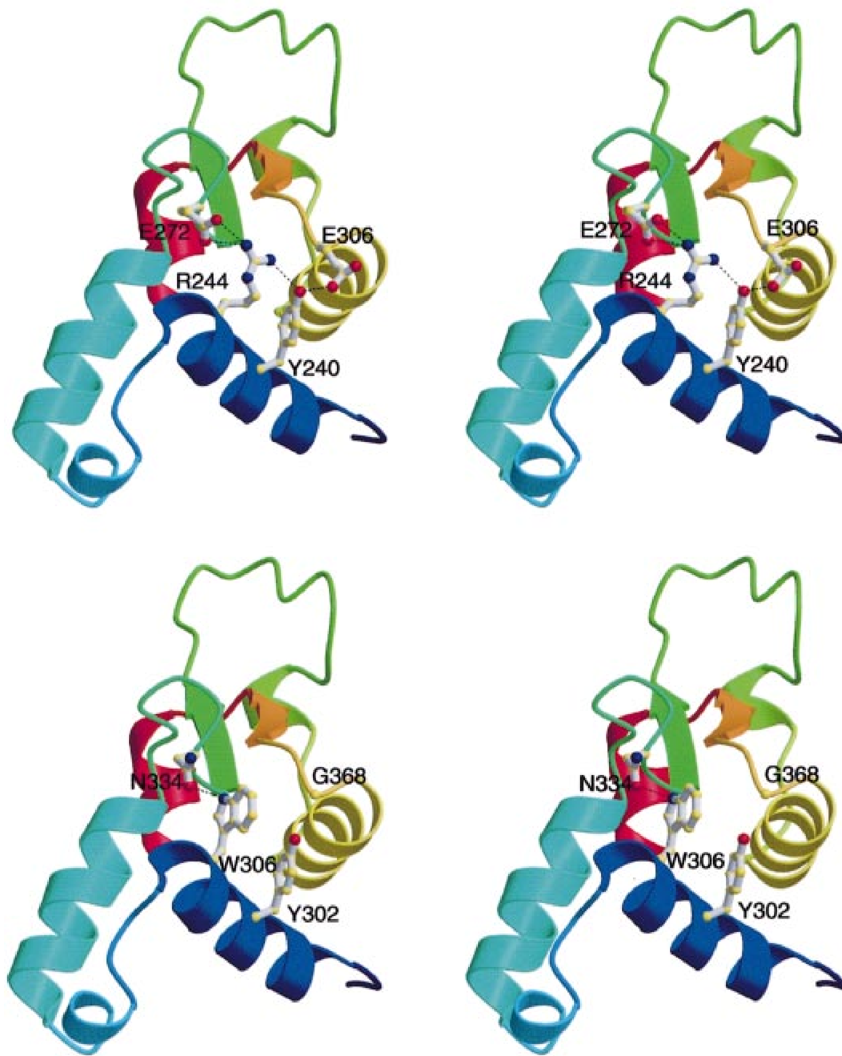

Figure 2. Stereo modeling images of the R306W substitution. *Upper panels*: Wild-type fXIIIa showing that the conserved R244 (R306 in TGase 1) forms a network of H-bonds with E272 (isosteric with N334 in TGase 1), Y240 (Y302 in TGase 1), and E306 (replaced by G368 in TGase 1). *Lower panels*: In the mutant TGase 1, the W residue can make only one H-bond, resulting in a probable substantial reduction of stability at this point in the catalytic core domain.

RESULTS AND DISCUSSION

Identification of *TGM1* mutations To identify the mutations within *TGM1*, all 15 exons and flanking intron sequences were amplified by PCR and were directly sequenced. The numbering of basepairs and amino acids is according to Kim *et al* (1992).

Family LI-KD We found two missense mutations in the patient of this family: an A to C transition in exon 5, resulting in the substitution of threonine for asparagine (AAC to A**C**C) at the amino acid position 288 (N288T), and a C to T transition in exon 6, resulting in the substitution of tryptophan for arginine (CGG to **T**GG) at the amino acid position 306 (R306W). The first mutation is from her mother, and the second mutation is from her father (**Fig 1*a*, *b***). The R306W substitution destroys an Msp I restriction enzyme site (C/CGG), but there are two Msp I enzyme sites in exon 6 of *TGM1*. Three different sized bands (119 bp, 112 bp, and 48 bp) can be seen after enzyme digestion of the PCR products of the wild-type allele. The mutation destroys one enzyme site, so that a 167 bp (119 bp + 48 bp) band is seen in persons who have the mutant allele (data not shown). More than 50 other normal individuals did not have this band. As the N288T substitution does not create or destroy any restriction enzyme site, we performed the PASA assay to selectively amplify the wild-type allele or allele carrying the mutation. Only the persons who have the mutant allele (II-1 and I-2) give a 186 bp PCR product with both the mutant and wild-type specific primers (data not shown). Because the father and 50 other unrelated unaffected persons do not yield this PCR product with mutant primer, the A to C substitution represents a new mutation.

Family LI-LK We again found two missense mutations: the same R306W in exon 6, and an A to T transition in exon 2, resulting in the substitution of valine for aspartic acid (GAT to G**T**T) at the amino acid position 101 (D101V). The first mutation is from his father, and the second mutation is from his mother (**Fig 1*c***). As the D101V substitution does not create or destroy any restriction enzyme site, we performed the PASA assay to selectively amplify the wild-type allele or allele carrying the mutation. Only the persons who have the mutant allele (I-2 and II-2) give a 374 bp PCR product with both the mutant and wild-type specific primers (data not shown). Because the father and 50 other unrelated unaffected persons do not yield this PCR product with mutant primer, the A to T substitution represents a new mutation.

Modeling of the mutations Using the solved structure of the related fXIIIa TGase enzyme, we have modeled the three mutations reported here to explore their structural/functional consequences.

R306W substitution This residue is conserved in the core domain of all TGase enzymes and is R244 in fXIIIa. The guanidinium side chains are involved in a network of H-bonds with E272 and Y240, which in turn are connected to E306 (**Fig 2**, *upper pair of stereo images*). These are structurally equivalent to isosteric N334 and Y302 in TGase 1; in addition, E306 in fXIIIa is replaced by G368 in TGase 1. Thus in the R306W substitution of TGase 1, the

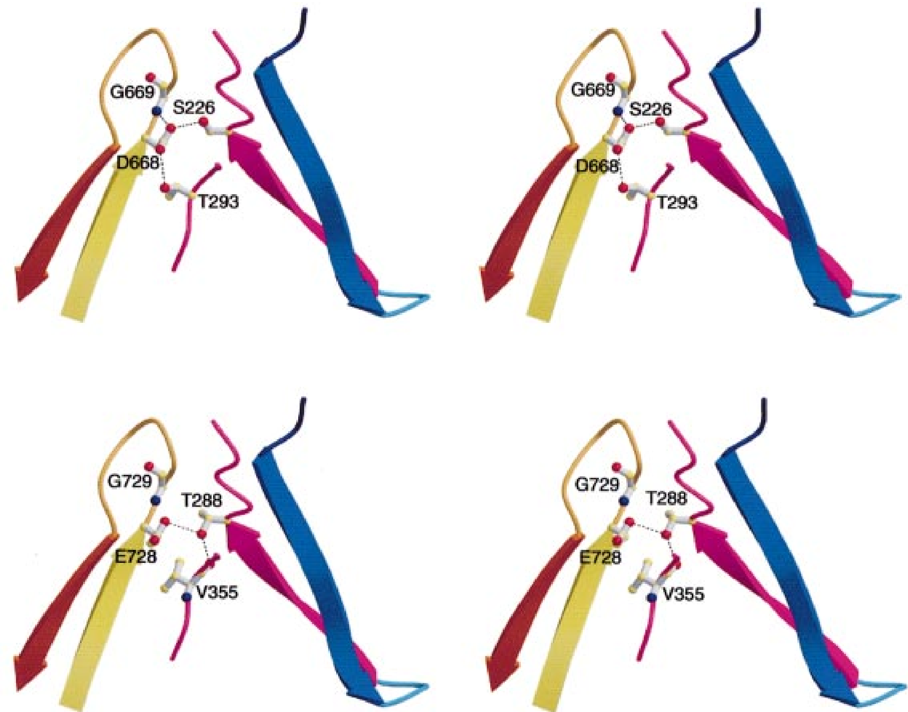

Figure 3. Stereo modeling images of the N288T substitution. *Upper panels*: Wild-type fXIIIa showing that S226 (isosteric with T288 in TGase 1) forms a network of H-bonds with D668 (isosteric with E728 in TGase 1) and G669 (G729 in TGase 1) to stabilize interactions between the catalytic core domain and the β-barrel 2 domain. *Lower panels*: In the mutant TGase 1, only one H-bond can be formed, resulting in a probable reduced molecular stability between the two protein domains.

W306 is more bulky and only one H-bond can be made with N334 (**Fig 2**, *lower pair*). Together with the wild-type G368, it is to be expected that this region will exhibit considerably more flexibility, which might loosen the structural integrity of the catalytic core domain at this point, with probably significantly reduced activity.

N288T substitution In fXIIIa, the two residues S226 (structurally isosteric with N288 in TGase 1) and T293 (replaced by V355 in wild-type TGase 1) in the catalytic core domain are involved in forming an H-bonding bridge with D668 (isosteric with E728 in TGase 1) of the β-barrel 2 domain (**Fig 3**, *upper pair of stereo images*). Also, H-bonding occurs between D668 and the main chain nitrogen of G669 (G729 in TGase 1) of the β-barrel 2 domain. These interactions from separate antiparallel β-strands form a strong network with the twisted β sheet core domain. The N288T substitution will allow only one H-bonding interaction between the core and β-barrel 2 domain (**Fig 3**, *lower pair*). Again, this is likely to cause local destabilization of the structure that would manifest as reduced enzyme activity.

D101V substitution The equivalent position does not exist in fXIIIa and so cannot be modeled directly. We have noticed previously, however (Candi *et al*, 1998), two sequence motifs NAADDD (residues 54–59) and NAAGDG (residues 97–102) on the membrane anchorage domain of TGase 1. These are closely similar to NAAEDD (residues 20–25) on the activation peptide of fXIIIa, which controls access to the active site on the catalytic domain. In TGase 1 these regions have been predicted to fold back onto the catalytic domain so that N97 and D101, for example, could form a cluster of H-bonds with R314 (Candi *et al*, 1998), and thereby facilitate subsequent proteolytic activation of TGase 1. Therefore, it seems plausible that the D101V mutant might inhibit proteolytic activation of TGase 1, resulting in severely diminished activity.

TGase 1 activity from skin samples of family LI-LK We obtained skin samples from members of the LI-LK family. Keratinocytes were cultured and TGase 1 activity was measured. As expected, most enzyme activity resides in the membrane fraction (Kim *et al*, 1994) (**Table I**). Both the cytosolic and membrane bound forms of the TGase 1 activity were reduced in comparison to the normal control. As shown the TGase 1 activities of the father (R306W), mother (D101V), and the patient (R306W/D101V) were reduced to around 50%, 50%, and 5%–15%, respectively, of controls from the skin of normal individuals.

Table I. Specific activities of TGase 1 in cultured keratinocytes from family LI-LK[a]

	Cytosolic activity	Membrane activity
I-1 (R306W)	305.96 ± 110.18 (56%)	1007.52 ± 104.29 (52%)
I-2 (D101V)	284.07 ± 92.63 (52%)	939.12 ± 205.91 (48%)
II-2 (R306W/D101V)	82.32 ± 42.12 (15%)	102.41 ± 27.28 (5%)

[a]The data are averages ± SE of triplicate analyses.

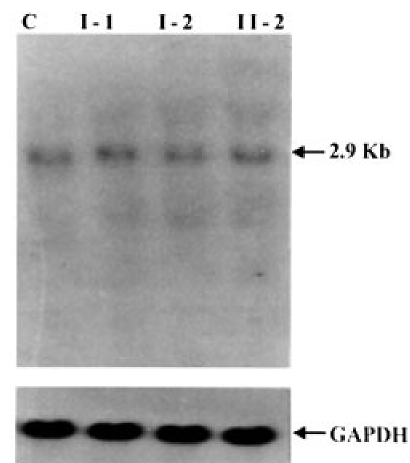

Figure 4. Northern blot analysis. Ten micrograms of total RNA hybridized with an α^{32}P-dCTP labeled cDNA probe. TGase 1 transcripts in similar amounts were clearly detectable in mRNAs from father (I-1), mother (I-2), and patient (II-2) of the LI-LK family, and in a control (c).

Northern blot analysis Northern blot analysis of total RNA from cultured keratinocytes was performed for father (I-1), mother (I-2), and patient (II-2) of the LI-LK family and a normal control. Both parents and the patient demonstrated mRNA of normal size (2.9 kb) and in amounts comparable to the control (**Fig 4**). These results were obtained after standardization by GAPDH hybridization of the quantity of RNA present in each lane.

In summary, we have documented three novel mutations of the *TGM1* gene in two different LI families: N288T and R306W in family LI-KD, and R306W and D101V in family LI-LK. According to our molecular modeling analyses, each mutation is likely to cause significant destabilization or prevent activation of TGase 1, leading to reduced activity of the enzyme. In family LI-LK, both heterozygous parents, assuming both the wild-type and mutant alleles are equally expressed, apparently retain sufficient activity to present a normal phenotype, and retain about 50% of normal activity, suggesting that the TGase 1 enzyme produced from each abnormal allele was mostly inactive. Indeed, the simultaneous occurrence of both mutations in the same patient reduced the net TGase 1 enzyme activity to about 15%, apparently below a threshold level, resulting in an LI presentation. A similar conclusion seems likely for the LI-KD family involving the novel N288T mutation.

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