Consequences of Seven Novel Mutations on the Expression and Structure of Keratinocyte Transglutaminase*

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We report the molecular characterization of seven new keratinocyte transglutaminase mutations (R315C, S358R, V379L, G473S, R687C, deletion Δ679–696, R127Stop) found in lamellar ichthyosis patients. Arg-315, Ser-358, Val-379, and Gly-473 are located in the catalytic core domain, and Arg-687 and the deletion are in the β-barrel domains. The side chains of amino acids Arg-315, Ser-358, and Gly-473 make ionic and hydrogen bonds important for folding and structural stability of the enzyme but are not directly involved in catalysis. Val-379 is two amino acids away from the active site cysteine, and its change into leucine disturbs the active site structure. The decreased activity and protein level after expression of the R687C and Δ679–696 TGK cDNA in TGK negative keratinocytes excluded that they are polymorphisms. These results identify important amino acids in the core domain of transglutaminases and show that the C-terminal end influences the structural and functional integrity of TGK.

Transglutaminases (EC.2.3.2.13, protein-glutamine: amine γ-glutamyl-transferase) are a superfamily of enzymes which catalyze the formation of intra- and intermolecular γ-glutamyl-ε-lysine isodipeptide bonds (1, 2). They are calcium-dependent enzymes that contain an active site consisting of a catalytic triad (Cys, His, Asp) (3–5). The six different classes of transglutaminases are participating in a wide variety of physiological processes (3, 6, 7). One member of this family, keratinocyte transglutaminase (TGK), is involved in cross-linkage during formation of the cornified cell envelope (CE), a highly insoluble 8–15-nm wide structure replacing the plasma membrane in terminal differentiating epidermis (8, 9). During this process, CE precursor proteins such as loricrin, involucrin, and small proline-rich proteins are sequentially cross-linked on the inner side of the plasma membrane (10–13). TGK is localized mainly to the cell periphery in the granular layer. The enzyme consists of 815 amino acids, and it is post-translationally modified by fatty acid acylation and phosphorylation (14–17). Several complexes consisting of the full-length protein and polypeptides proteolytically cleaved from it have been identified in the cytosolic and membrane fractions (18, 19). Most of the enzyme complexes are attached to the membrane through myristate and palmitate chains (20, 21). About 5–10% of TGK activity is found in the cytoplasmic fraction, which might be involved in the final steps of CE assembly. Deletion analysis showed that a molecule in which the first 109 and the last 240 amino acids have been removed retains a specific activity comparable with the full-length enzyme (22). The human TGK gene consists of 15 exons and is located on chromosome 14q11 (23–27). At least two different allelic variants have been detected in the human population (24).

Autosomal recessive lamellar ichthyosis (LI) (Mendelian Inheritance in Man No. 242100, 242300) is a severe congenital scaling skin disorder with a frequency of about 1:250,000 (28, 29). The clinical phenotype is heterogeneous and can range from generalized large brownish plate-like scales with no erythroderma to fine white scales with underlying erythroderma. Moreover, patients may have palmoplantar hyperkeratosis, scarring alopecia, ectropion, eclairbium, and decreased sweating. Patients are often born encased in a shiny, thick parchment-like membrane (collodion baby). By electron microscopy, five types of lamellar ichthyosis (ichthyosis congenita type I-V) have been distinguished (30). Deleterious mutations in the TGK gene have been reported in lamellar ichthyosis patients providing compelling evidence for the importance of the cornified cell envelope for epidermal homeostasis and the barrier function of the skin (31–33). However, biochemical data clearly showed that about 50–60% of LI patients have normal TG activity (34). Genetic heterogeneity is further supported by genetic mapping studies identifying two other disease-causing genes, one on chromosome 2q33–35 and another at a currently unknown location (35).

We report seven novel TGK mutations found in LI patients. The consequences of these mutations (five missense mutations, one premature stop codon, and a deletion of 18 amino acids) on chain reaction; RT, reverse transcriptase; TG, transglutaminase; bp, base pair(s); SDS, sodium dodecyl sulfate.
the biosynthesis of TGK mRNA and protein are described. The effects of these mutations on folding and structure are analyzed using the three-dimensional structure of factor XIII A-subunit as model. This study identifies structurally and functionally important amino acids of TGK and provides new insight into the structure-function relationship in transglutaminases.

EXPERIMENTAL PROCEDURES

Patients—Families from Switzerland (LI-8), Holland (LI-11), Sweden (LI-20), and the United States (LI-22) were investigated (see Fig. 1). The proband of family LI-8 was born as a colloidion baby and died shortly after birth due to bacterial infection. The affected individual of family LI-11 was not born as a colloidion baby; his trunk and neck are covered with large plate-like yellow-brown hyperkeratotic scales, and he has very extensive palmar-plantar keratoderma with large fissures. The face is not involved and there is no alopecia or ectropion. Palms and soles show moderate hyperkeratosis. No blistering or significant erythroderma are apparent. Palms and soles show moderate hyperkeratosis. No blistering or significant erythroderma are apparent.

Cell Culture—Punch biopsies obtained from the probands were used to establish primary cultures on lethally irradiated murine 3T3 fibroblasts as described earlier (37–39). Secondary cultures were grown in high calcium keratinocyte medium, 10% fetal calf serum until confluence. After an additional 5 days in culture, genomic DNA, RNA, and proteins for measuring transglutaminase activity and immunoblotting were extracted as described below.

Isolation of DNA and RNA—Genomic DNA was purified from cultured cells by phenol/chloroform extraction as described earlier (40) or from blood using Nucleospin columns (Macherey-Nagel). Total RNA was isolated using the guanidine-thiocyanate method (41).

Northern Blot Analysis—Denaturing RNA gels and transfer to Zeta-probe membrane (Bio-Rad, Richmond, CA) were performed as described earlier using 15 μg of total RNA/lane (42). Membranes were hybridized with 32P-labeled TGK probes (DH42, 3′-GTTTATTAGCATCTGTTCCCCCAGT-3′, nt -79 to -59 (16) and DH9 5′-GTTTATTAGCATCTGTTCCCCCAGT-3′, nt +2580 to +2604 (16)) and then washed with 4× SSC at 65 °C for 2 h. Autoradiographs were exposed for 1 day. Densitometry was performed using the NIH Image software.

Transglutaminase Assay—Cells were lysed by sonication in 20 mM sodium phosphate, pH 7.2, 0.5 mM EDTA, 10 mM dithiothreitol, 50 mM sodium phosphate, pH 7.2, 0.5 mM EDTA, 10 mM dithiothreitol, 50 μg/ml phenylmethylsulfonyl fluoride. The supernatant, after centrifugation at 25,000 × g for 30 min, was used as cytosolic fraction. The cell pellet was re-extracted by sonication with the same buffer supplemented with 1% Triton X-100 and centrifugation. 40 μg of protein was size-fractionated by SDS-polyacrylamide gel electrophoresis through a 10% separation and 4% concentration gel (containing 4 M urea) and, after partial renaturation, electroblotted to nitrocellulose (9). TGK protein was visualized with antibody B.C1 (8) and the ECL detection kit (Amersham, Switzerland).

Protein Concentration Determination—Protein concentration was determined with the Bradford assay (Bio-Rad) using bovine serum albumin as standard (44).

DNA Sequencing and Family Analysis of Mutations—The 15 exons of the TGK gene were amplified by PCR as described (33). Forward primers were biotinylated. PCR products were purified by QiAquick PCR purification kit (Qiagen), and single-stranded DNA was isolated with streptavidin-coated magnetic beads (Dynal) and sequenced with the reverse primers using the Sequenase sequencing kit (Amersham). Nucleotides have been numbered according to Phillips et al. (26). To number amino acids, the first methionine of the open reading frame (15) was designated as number 1. For inheritance analysis in families, DNA was amplified by PCR, digested with restriction enzymes, and separated on agarose or polyacrylamide gels.

Expression of Mutant Proteins—Full-length TGK cDNA was obtained by RT-PCR using patients keratinocyte RNA and primers DH5′-CATCCATCCTGACGTGTTCA-3′ (nt -79 to -59 (16) and DH9 5′-GTTTATTAGCATCTGTTCCCCCAGT-3′, nt +2580 to +2604 (16)) and cloned into the NotI site of pCI (Promega). The sequence was verified by sequencing. β-galactosidase cDNA was obtained from plasmid pUC19(67,70)low2 (45) and cloned into the NotI site of pCI. Plasmids were purified over Qiagen columns and by Triton X-114 extraction (46). Secondary keratinocytes from a TGK negative LI patient cultured on irradiated 3T3 fibroblast feeder layer were transfected at 80–90% confluency with 4 μg of the TGK-expressing plasmid and 2 μg of the β-galactosidase expressing plasmid (47). Two days later, β-galactosidase and transglutaminase activities were determined (43, 48).

Modeling of the Protein Defects—The three-dimensional structure of the human factor XIII A-subunit zymogen dimer, experimentally determined by single crystal x-ray diffraction (5), was used as a template for constructing a homology model of the human keratinocyte transglutaminase enzyme using the Biosym InsightII software package. Atomic coordinates for the factor XIII structure were obtained by refining the model of the x-ray diffraction data from 10.0 to 2.65 Å resolution using the program X-PLOR (49) to give a crystallographic R factor of 21.7%. The final model exhibits good geometry (root mean square deviation from ideality of 0.012 Å for bond lengths, 1.8° for bond angles, 25.6° for torsion angles, and 1.5° for improper torsion angles); the average value of the individually refined atomic temperature factors is 26.7 Å2. Re- fined coordinates for the factor XIII structure have been deposited with the Protein Data Bank (identifying code: 1ggt). Models of the keratinocyte transglutaminase mutant structures were generated by modifying the homology model using the computer program O (50), and figures were drawn with the program MOLSCRIPT (51).

RESULTS

Biochemical Characterization of the Patients—The membrane TG activities in cultured cells from the probands ranged from 2.2 (LI-20 III.1) to 175.4 pmol/h mg (LI-20 II.1), significantly different from that in normal and heterozygotic (LI-8 I, LI-22 III.3) individuals (Table 1). Northern blots showed miss-sense mutations in exons 3, 13, and 15 TGK exons in individuals with low TG activities. LI-8 II.2 and LI-22 III.3) individuals (Table 1). Northern blots showed miss- sense mutations in exons 3, 13, and 15 TGK exons in individuals with low TG activities.

Sequence Analysis of Patients TGK Gene—The mutations shown in Fig. 4 were detected by direct DNA sequencing of all 15 TGK exons in individuals with low TG activities. LI-8 II.2 had a homozygous C to T mutation at position +1354 in exon 3, changing R127 to a stop codon. This mutation creates a new DdeI site in exon 3 giving rise to a new band of 181 bp. DdeI digestion of amplified exon 3 from the patient and his parents showed that the patient was homozygous for the 181-bp band, whereas the parents were heterozygous for the 181- and 207-bp bands.

Fig. 1. Pedigrees of the LI families LI-8 (a), LI-11 (b), LI-20 (c), and LI-22 (d). Genomic DNA samples were analyzed from the individuals marked with underlined numbers. Cell cultures were established from individuals marked with bold numbers.
### Table I: TG activity in keratinocytes from LI families and normal individuals

| Family | Proband | Activitya | Mutationsb |
|--------|---------|-----------|------------|
| LI-8   | II.1    | 248.8 ± 46.0 | 294.0 ± 711.0 |
| LI-8   | II.2    | 5.8 ± 1.1   | 13.9 ± 1.8  |
| LI-11  | II.1    | 10.9 ± 2.2  | 24.3 ± 7.3  |
| LI-20  | II.1    | 76.3 ± 5.5  | 17.4 ± 4.4  |
| LI-20  | III.1   | 2.0 ± 1.1   | 2.2 ± 0.3   |
| LI-20  | III.2   | 5.6 ± 1.1   | 4.0 ± 1.9   |
| LI-22  | II.2    | 497.8 ± 52.0| 6194.0 ± 210.0|
| LI-22  | III.3   | 205.1 ± 81.0| 2600.0 ± 414.0|
| LI-22  | IV.4    | 4.0 ± 2.4   | 52.0 ± 4.9  |
| Normal |         | 351.9 ± 125.7| 4200.0 ± 200.0|

a Activity is presented as pmol/h mg of putrescine incorporated. Results are given as mean ± S.E. from at least two cell passages in duplicate.
b SA means the splice acceptor site of the intron; PTC means premature stop codon.
c Results are derived from 7 unaffected individuals and are presented as mean ± S.E.

### Fig. 2. Northern blot analysis of total RNA from cultured keratinocytes.

LANES: Lane 1, LI-11 II.1; lane 2, LI-8 II.2; lane 3, LI-20 I.1; lane 4, LI-20 III.1; lane 5, LI-20 III.2; lane 6, LI-22 IV.4; lane 7, LI-22 II.2; and lane 8, unaffected individual. The cDNA probes are from the 5’- (DH42) and 3’-ends (3’ NC) of the TGK mRNA and the repetitive region of involucrin (INV), see also under “Experimental Procedures.”

Involucrin is an epidermal differentiation marker that was included to verify the differentiation status of keratinocyte cultures.

### Fig. 3. a, TGK protein level is strongly decreased in LI patients.

Shown are immunobLOTS of cytosolic (lanes 2, 4, 6, 8, 10, 12, 14) and membrane (lanes 1, 3, 5, 7, 9, 11, 13, and 15) extracts from cultured keratinocytes. Lane 1, unaffected individual; lanes 2 and 3, LI-8 II.2; lanes 4 and 5, LI-11 II.1; lanes 6 and 7, LI-20 II.1; lanes 8 and 9, LI-20 III.1; lanes 10 and 11, LI-20 III.2; lanes 12 and 13, LI-22 III.3; lanes 14 and 15, LI-22 IV.4. Note abundant protein in LI-22 III.3 who is a heterozygous carrier. b, transfected mutant TGK molecules are proteolytically degraded. Immunoblot of cytosolic (lanes 2, 4, 6, 8, 10, 12, 14, and 16) and membrane (lanes 1, 3, 5, 7, 9, 11, 13, and 15) extracts from transfected TGK negative keratinocytes. Nontransfected (lanes 1 and 2), normal TGK (lanes 3 and 4), R687C (lanes 5 and 6), D679–696 (lanes 7 and 8), S42Y (lanes 9 and 10), R142C (lanes 11 and 12). S42Y/R142C (lanes 13 and 14), R315C/SA intron 5. Molecular sizes (kilodaltons) are indicated on the left.

The second mutation at +3434 destroys the single HaeIII site in exon 6. The restriction enzyme analysis showed that only the patient has this mutant band (data not shown). Whether this mutation was inherited from the father or represents a new mutation could not be tested since DNA from the father was not available.

CpG dinucleotides have on the average a much higher rate of mutations than other dinucleotides (54). In four of the presented 7 mutations (R127Stop, R315C, G473S, and R687C), C nucleotides in CpG are mutated into a T either on the sense or on the antisense DNA strand (G473S). Therefore, these sites could constitute mutational hot spots.

### Protein Modeling of the Mutants—The extensive conservation of amino acid residues of 42% between keratinocyte transglutaminase and factor XIII A-subunit indicates that their folding is conserved. Therefore, the factor XIII A-subunit crystal structure served as a reliable scaffold to construct a homology model of keratinocyte transglutaminase (Fig. 5) to better understand the molecular basis for the decreased enzymatic activity caused by the keratinocyte transglutaminase mutations. Factor XIII A-subunit is composed of four domains, which, from the N-terminal end, have been designated as β-sandwich, central core domain, and β-barrels 1 and 2 (5). For additional indications of the structural and functional importance of the mutation sites, a structure-guided alignment of 19
known transglutaminase sequences was additionally used (data not shown).

R315C—Residue Arg-315, in the catalytic core domain, is located in a surface loop between two helices (Fig. 5). The arginine side chain is buried in the structure and forms a salt bridge with Asp-306 as well as a hydrogen bond to the main chain carbonyl group of Met-310 (Fig. 6). These bonds serve to stabilize the conformation of this surface loop. The Arg-315–Asp-306 salt bridge is conserved in a total of 13 of the TG sequences including factor XIII A-subunit and TGK; in one of the other sequences, the equivalent arginine residue interacts with a glutamic acid side chain, and in the remaining molecules, the size of this loop is altered by either amino acid insertions or deletions. The equivalent arginine in factor XIII A-subunit is the site of the deficiency mutation R252I (55), underlining the structural importance of this residue. The TGK
R315C mutation has three effects. First, the described bonds formed by the arginine side chain are removed, rendering the surface loop more mobile and susceptible to proteolytic cleavage. Second, the introduction of the smaller cysteine side chain leaves a void in the molecule that would destabilize the structure; a possible consequence is interference with proper folding and the generation of an altered structure. Third, the introduction of an additional cysteine residue can interfere with proper folding by allowing the formation of an unwanted disulfide bond. Thus, the most likely result of the R315C mutation is the altered conformation of the surface loop yielding a modified structure that is less stable and more susceptible to proteolytic cleavage.

S358R—The Ser-358 residue is absolutely conserved in all transglutaminase sequences, which suggests structural and/or functional importance. This residue is located in the catalytic core domain (Fig. 5), and its side chain group is buried in the molecule. The Ser-358 side chain hydrogen bonds to the side chains of residues Thr-386 and Trp-288 and to the main chain carbonyl of Gly-382. Replacement of Ser-358 with an arginine residue (white) leads to the disruption of hydrogen-bonding interactions and the larger arginine side chain introduces a number of short contacts that are relieved by a conformational rearrangement of the protein. The likely result is a dramatic misfolding of the catalytic core domain.

V379L—Residue Val-379, which is absolutely conserved, is located 2 positions C-terminal to the catalytic Cys-377 in the active site helix (shown as a coil), two residues C-terminal to the catalytic Cys-377 residue. The conserved Val-379 side chain, shown in dark gray bead and stick, is buried in a closely packed hydrophobic pocket in the catalytic core domain. The V379L mutation (white ball and stick) introduces a larger side chain, resulting in a number of sterically unfavorable short contacts that are relieved by distortion of the protein fold. This in turn leads to a shift of the catalytic Cys-377 residue affecting the enzymatic activity of TGK.

FIG. 7. Stereo view of the modelled S358R mutation site. The Ser-358 residue (dark gray) is absolutely conserved in all transglutaminase sequences. Its side chain group is buried in the catalytic core domain and involved in a number of hydrogen-bonding interactions (dashed lines) with the side chain groups of conserved residues Trp-288 and Thr-386 and to the main chain carbonyl of Gly-382. Replacement of Ser-358 with an arginine residue (white) leads to the disruption of hydrogen-bonding interactions and the larger arginine side chain introduces a number of short contacts that are relieved by a conformational rearrangement of the protein. The likely result is a dramatic misfolding of the catalytic core domain.

FIG. 8. Stereo view of the modelled V379L mutation site. The Val-379 residue is located in the active site helix (shown as a coil), two residues C-terminal to the catalytic Cys-377 residue. The conserved Val-379 side chain, shown in dark gray ball and stick, is buried in a closely packed hydrophobic pocket in the catalytic core domain. The V379L mutation (white ball and stick) introduces a larger side chain, resulting in a number of sterically unfavorable short contacts that are relieved by distortion of the protein fold. This in turn leads to a shift of the catalytic Cys-377 residue affecting the enzymatic activity of TGK.
active site helix that contains a number of conserved amino acids (Fig. 8). The Val-379 side chain is buried in a tightly packed hydrophobic environment formed by predominantly conserved residues and that cannot accommodate the larger leucine side chain of the V379L mutation without distortion of the local conformation of the protein. As a result of the ordinarily conservative V379L mutation, the position and orientation of the catalytic Cys-377 are likely to be altered, and the catalytic activity of the enzyme compromised.

G473S—Residue Gly-473 is conserved in all TG sequences except for the two band 4.2 proteins. This glycine residue is found on the surface of the catalytic core domain (Fig. 5) and, along with Pro-474 (conserved in all but the band 4.2 sequences), forms the only cis-peptide bond in the factor XIII A-subunit. The main chain atoms of Gly-473 form hydrogen bonds with the side chains of the highly conserved Arg-323 and Asp-490 residues. The pattern of conservation of Gly-473 is unsuitable for any other amino acid with its larger side chain; substitution of Gly-473 with a serine residue (white ball and stick) would interfere with proper folding of the protein.

G473S—Residue Gly-473 is conserved in all TG sequences except for the two band 4.2 proteins. This glycine residue is found on the surface of the catalytic core domain (Fig. 5) and, along with Pro-474 (conserved in all but the band 4.2 sequences), forms the only cis-peptide bond in the factor XIII A-subunit. The main chain atoms of Gly-473 form hydrogen bonds with the side chains of the highly conserved Arg-323 and Asp-490 residues. The main chain conformation of Gly-473 is unsuitable for any other amino acid with its larger side chain; substitution of Gly-473 with a serine residue (white ball and stick) would interfere with proper folding of the protein.

G473S—Residue Gly-473 is conserved in all TG sequences except for the two band 4.2 proteins. This glycine residue is found on the surface of the catalytic core domain (Fig. 5) and, along with Pro-474 (conserved in all but the band 4.2 sequences), forms the only cis-peptide bond in the factor XIII A-subunit. The main chain atoms of Gly-473 form hydrogen bonds with the side chains of the highly conserved Arg-323 and Asp-490 residues. The main chain conformation of Gly-473 is unsuitable for any other amino acid with its larger side chain; substitution of Gly-473 with a serine residue (white ball and stick) would interfere with proper folding of the protein.
would influence TG activity. Therefore, these mutant cDNAs
Since the mutations R687C and D679–696 lost.
ificity, specificity of enzyme cleavage, and activation) will be
tion served by the barrel domains (substrate binding and spec-
have an altered structure but will also not be packed against
scenario, the C-terminal portion (barrels 1 and 2) folds into an
stable and degraded as shown by Western blot analysis (Fig.
the barrel-core domain interface, the barrel domains are ex-
evident as for the other four missense mutations. The variabil-
the barrel A-subunit deficiency mutant R260C (33, 56). The G473S
will lead to a misfolded structure that is conforma-
tionally distorted since the glycine main-chain torsion angles
cannot accommodate any other amino acid with its larger side
chain; the serine side chain would introduce sterically unfavor-
short contacts with main-chain atoms. The serine substi-
tution will interfere with the folding process and yield an
altered structure that is less stable or more susceptible to
proteolytic cleavage.

R687C—The Arg-687 residue is buried at the interface be-
tween the C-terminal β-barrels and the catalytic core domains
(Figs. 5 and 10) and is found only in the keratinocyte
sequences. The consequence of the R687C mutation is not as
evident as for the other four missense mutations. The variabil-
ity of this residue among the transglutaminases indicates that
this residue is not likely to be important during the protein
folding process. Although there are few conserved residues at
the barrel-core domain interface, the barrel domains are ex-
pected to be packed well against the catalytic core domains in
all transglutaminase structures. Substitution of the large, posi-
tively-charged buried Arg-687 side chain with the much
smaller cysteine residue is likely to interfere with the interdo-
main interface, and to yield a modified quaternary structure.
The result is a globally altered molecule that is less stable or
more easily degraded by proteases (see Fig. 3a, lanes 4 and
and Fig. 3b, lanes 5 and 6) but also might have altered sub-
strate binding and specificity.

Δ679–696—The 18 residue deletion in the region corre-
spanding to residues 618–635 in factor XIII A subunit forms
one long strand that starts in barrel 1 and continues to barrel
2 (Fig. 5). The first part of the peptide forms most of a β-strand
in barrel 1 at its interface with the core domain. The second
part of the peptide forms half of a β-strand in barrel 2. Deletion
of these 18 residues has dramatic structural effects. In a worst-
case scenario, the C-terminal portion of the protein is unable to
fold into a globular structure, and the entire molecule is un-
stable and degraded as shown by Western blot analysis (Fig.
3a, lanes 8–11 and Fig. 3b, lanes 7 and 8). In a best-case scenario,
the C-terminal portion (barrels 1 and 2) folds into an
altered globular structure, and the modified protein is stable.
However, in this case, the new C-terminal domain will not only
have an altered structure but will also not be packed against
the catalytic core in the same manner, thus any putative func-
tion served by the barrel domains (substrate binding and spec-
ificity, specificity of enzyme cleavage, and activation) will be
lost.

Transient Expression of the Mutants R687C and Δ679–696—Since the mutations R687C and Δ679–696 do not con-
cern highly conserved residues, it was less evident if they would
influence TG activity. Therefore, these mutant cDNAs were
transiently expressed by cotransfections with a β-galactosi-
dase expression plasmid into TGK negative keratinocytes
derived from a LI patient. The mutation R687C reduces mem-
brane TG activity to about 5% of the normal level (Table II). An
even stronger reduction was observed for the Δ679–696 pro-
tein molecule (Table II). The three mutations S42Y, R142C,
and R323Q were reported earlier in a LI family (33) and were
included in Table II to demonstrate the ability of this transient
expression assay to detect deleterious mutations. The data
show also that the S42Y change, located close to the membrane
attachment site of the molecule, is not a disease-causing mu-
tation but does lead to increased cytosolic accumulation of TGK
as previously postulated (33). Furthermore, Western blot anal-
ysis of cell extracts from transfected cells demonstrated an
excellent correlation between the levels of TGK protein and TG
activity (Fig. 3b). These results prove that the mutations
R687C and Δ679–696 are indeed disease-causing mutations.

DISCUSSION

In this study, we have investigated structure-function rela-
tionships in TGK by analyzing mutants found in LI patients.
Using biochemical techniques and direct sequencing, we have
identified 7 novel mutations in the gene of keratinocyte tran-
glutaminase. Five of the mutations were one-nucleotide
changes resulting in single amino acid alterations (R315C,
S358R, V379L, G473S, R687C), one point mutation led to a
premature termination codon (R127Stop), and one mutation
affected the splice donor site of intron 13 leading to an in-frame
deletion of 18 amino acids (Δ679–696). One mutation changing
the splice acceptor site of intron 5 has already been reported in
a family (33) and, in fact, the aberrant RNA banding pattern
(Fig. 2) gave an important clue to identify the mutation. In the
case of the nonsense mutation, R127Stop, the steady-state
transcript level was very low (Fig. 2). The association of stop
mutations with reduced mRNA levels has been reported in
other genes and is due to low efficiency in transcript processing
and/or mRNA transport from the nucleolus (57). In con-
trast, the mRNA levels of all missense mutations are expressed
in comparable amounts as in normal probands, in accordance
with observations for other genes.

The three-dimensional structure of TGK is currently not
known. Thus, the structural effects of the reported missense
and deletion mutations were analyzed using the factor XIII
A subunit structure (Fig. 5) (5). The central core domain,
containing the active site cysteine, displays the highest homology
between factor XIII A subunit and TGK, whereas the other
domains are less conserved. Sequence alignment of the two
proteins shows that most of the mutations (R315C, S358R,
V379L, G473S) are located in a region corresponding to
the central core domain. Amino acid changes can decrease enzyme
activity either by interfering directly with the catalytic mech-
anism, by introducing gross structural alteration, or by block-
ing the binding of essential cofactors. With the exception
of V379L, these mutations do not concern residues close to the
catalytic site. Rather, these mutations are predicted to inter-
fere with proper formation of hydrogen bonds and salt bridges
and introduce spatial constraints due to differing side chain
sizes that alter protein structure. These mutants are unstable
and/or more susceptible to proteolytic degradation (58, 59). Our
predictions are supported by the results of immunoblotting
experiments showing strongly decreased levels of TGK proteins
in cultured cells from these patients. Thus, these missense
mutations lead to protein instability and premature degrada-
tion but do not interfere directly with the catalytic mechanism.
Congenital factor XIII deficiency, a rare bleeding disorder, can
be caused by mutations in the gene for the factor XIII A sub-
unit. Arg-252, which corresponds to Arg-315 in TGK, was
altered to Ile in a patient affected by this disorder (55). In agree-
ment with our results, low TG activity and protein level were

| Mutant | Cytoplasm | Membrane |
|--------|-----------|-----------|
| Wild-type | 100.0 | 100.0 |
| R687C | 7.4 ± 1.9 | 5.4 ± 1.7 |
| Δ679–696 | 2.3 ± 1.0 | 0.1 ± 0.02 |
| S42Y | 159.2 ± 14.3 | 93.0 ± 3.2 |
| R142C | 4.7 ± 1.2 | 0.5 ± 0.1 |
| S42YR142C | 6.2 ± 4.9 | 0.2 ± 0.02 |
| R323Q | 18.3 ± 0.4 | 2.2 ± 0.5 |

Relative TG activities have been normalized for transfection effi-
ciency by cotransfection with a β-galactosidase expression plasmid (see
“Experimental Procedures”) and are presented as percent of the activity
of the wild-type molecule set as 100%. Results are presented as mean ±
S.E. from two independent experiments measured in duplicate.

| Relative TG activity of transfected mutant TGK cDNA |
|---------------------|---------------------|
| Mutant | Cytoplasm | Membrane |
| Wild-type | 100.0 | 100.0 |
| R687C | 7.4 ± 1.9 | 5.4 ± 1.7 |
| Δ679–696 | 2.3 ± 1.0 | 0.1 ± 0.02 |
| S42Y | 159.2 ± 14.3 | 93.0 ± 3.2 |
| R142C | 4.7 ± 1.2 | 0.5 ± 0.1 |
| S42YR142C | 6.2 ± 4.9 | 0.2 ± 0.02 |
| R323Q | 18.3 ± 0.4 | 2.2 ± 0.5 |

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A-subunit structure (Fig. 5) (5). The central core domain,
containing the active site cysteine, displays the highest homology
between factor XIII A-subunit and TGK, whereas the other
domains are less conserved. Sequence alignment of the two
proteins shows that most of the mutations (R315C, S358R,
V379L, G473S) are located in a region corresponding to
the central core domain. Amino acid changes can decrease enzyme
activity either by interfering directly with the catalytic mech-
anism, by introducing gross structural alteration, or by block-
ing the binding of essential cofactors. With the exception
of V379L, these mutations do not concern residues close to the
catalytic site. Rather, these mutations are predicted to inter-
fere with proper formation of hydrogen bonds and salt bridges
and introduce spatial constraints due to differing side chain
sizes that alter protein structure. These mutants are unstable
and/or more susceptible to proteolytic degradation (58, 59). Our
predictions are supported by the results of immunoblotting
experiments showing strongly decreased levels of TGK proteins
in cultured cells from these patients. Thus, these missense
mutations lead to protein instability and premature degrada-
tion but do not interfere directly with the catalytic mechanism.
Congenital factor XIII deficiency, a rare bleeding disorder, can
be caused by mutations in the gene for the factor XIII A sub-
unit. Arg-252, which corresponds to Arg-315 in TGK, was
altered to Ile in a patient affected by this disorder (55). In agree-
ment with our results, low TG activity and protein level were
reported in this patient. The qualitative agreement between these results underlines that the factor XIII A-subunit subunit can serve as valuable model for predicting the structural and functional effects of TGK mutants. Val-379 is located two amino acids C-terminal of the active site Cys-377. Although Val-379 does not belong to the catalytic triad (Cys, His, Asp), it is located in the same α-helix as Cys-377. Replacing valine with leucine changes the conformation of this α-helix and therefore the spatial position of Cys-377 relative to the unmutated enzyme (60). The difference in reduction between the enzymatic activities of the two mutants is most likely due to the different sizes of the side chain (Ala versus Leu) replacing valine. Furthermore, we found in another LI patient a homozygotic V379M substitution. These results indicate that the TG activity is very sensitive to changes in the valine two amino acids C-terminal of the active site cysteine.

The mutations R687C and Δ679–696 are located at the C terminus, which corresponds to the β-barrel domains of factor XIII A-subunit. These domains are not highly conserved; Arg-687 is found only in the keratinocyte sequences, and 11 of 18 amino acids from the deletion mutant are different between TGK and factor XIII A-subunit. Since this precluded analysis of these mutations using the factor XIII A-subunit model, the corresponding mutant cDNAs were expressed in TGK negative keratinocytes. This showed that both mutations strongly decreased the enzymatic activities and led to premature degradation of the enzyme in a manner comparable with mutations in the highly conserved central core or β-sandwich domains (Fig. 3b and Table I). Three mutations, two missense and a premature stop codon, in the β-barrel 2 of factor XIII A subunit were also reported to diminish enzyme activity and protein levels (61–63). Previous experiments in which deletion constructs of TGK were expressed in bacteria showed that removal of amino acids 675–816 resulted in a substantially reduced specific activity (22). Interestingly, further deletion of 100 amino acids restored the activity nearly to the level of the full-length protein (22). In a series of experiments, it was demonstrated that TGK exists in keratinocytes as complexes of polypeptides derived from the full-length enzyme by proteolysis (18, 19). Depending on the differentiation status and cellular localization, enzymatically active 67 kDa, 67/33 kDa, and 10/67/33 kDa complexes were found in which the 67 kDa, the 33 kDa, and 10 kDa molecules correspond to the β-sandwich plus central core domains, C-terminal β-barrel domains, and the first 92 amino acids of the N terminus, respectively. Elimination of the two β-barrels in bacterial-expressed factor XIII A-chain molecules only slightly diminished enzymatic activity, and the shortened molecules conserved the ability to be activated by thrombin and calcium and the binding and cross-linking of fibrin (64). Interestingly, C-terminal deletions of human tissue transglutaminase were reported to enhance its intrinsic GTP/ATPase activity concomitant with a lowering in TG activity (65). In summary, these data indicate that the β-barrel domains are not absolutely required for transglutaminase activity, but they augment activity possibly due to better substrate interaction and enzyme activation. However, our results and those from others suggest that mutations within the β-barrel domains have in most cases a profound influence on the whole molecule because they promote premature degradation and/or interfere with the proper functioning of the active site. Additional structural investigations are needed to elucidate further the function of the C-terminal domains of transglutaminases.

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