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Transglutaminase 1 Mutations in Autosomal Recessive Congenital Ichthyosis: Private and Recurrent Mutations in an Isolated Population

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Summary

Autosomal recessive congenital ichthyosis (ARCI) is a rare, heterogenous keratinization disorder of the skin, classically divided into two clinical subtypes, lamellar ichthyosis (LI) and nonbullous congenital ichthyosiformis erythroderma (CIE). Recently, strong evidence for the involvement of the transglutaminase 1 gene (TGM1) in LI has evolved. We have studied ARCI in the isolated Finnish population, in which recessive disorders are often caused by single mutations enriched by a founder effect. Surprisingly, five different mutations of TGM1 (Arg141His, Arg142Cys, Gly217Ser, Val378Leu, and Arg395Leu) were found in Finnish ARCI patients. In addition to affected LI patients, we also identified TGM1 mutations in CIE patients. Moreover, haplotype analysis of the chromosomes carrying the most common mutation, a C→T transition changing Arg142 to Cys, revealed that the same mutation has been introduced twice in the Finnish population. In addition to this Arg142Cys mutation, three other mutations, in Arg141 and Arg142, have been described elsewhere, in other populations. These findings suggest that this region of TGM1 is more susceptible to mutation. The corresponding amino acid sequence is conserved in other transglutaminases, but, for example, coagulation factor XIII (FXIII) mutations do not cluster in this region. Protein modeling of the Arg142Cys mutation suggested disruption or destabilization of the protein. In transfection studies, the closely related transglutaminase FXIII protein with the corresponding mutation was shown to be susceptible to degradation in COS cells, further supporting evidence of the destabilizing effect of the Arg142Cys mutation in TGM1.

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Introduction

Autosomal recessive ichthyosis congenita (ARCI) is a clinically heterogenous hereditary keratinization disorder of the skin (MIM 242300 [ichthyosis congenita] and MIM 242100 [ichthyosiform erythroderma]) (McKusick 1994). The estimated incidence of ARCI is 1:150,000–300,000 (Bale and Doyle 1994). As a group, ARCI disorders have a great variation in severity. The skin may show mild or heavy hyperkeratosis with or without general or localized inflammation.

There is no consensus on the classification of the ARCI subclasses, because clear-cut biological, biochemical, or molecular markers have been lacking (Ghadially et al. 1992). Two clinical subsets, lamellar ichthyosis (LI) and nonbullous congenital ichthyosiformis erythroderma (CIE), were distinguished by Williams and Elias (1985). At present, there is evidence that at least three different genetic loci are linked to LI (Russell et al. 1995; ParmEntier et al. 1996).

Recently, the pathogenesis of LI was associated with mutations in the transglutaminase 1 (also called "keratinocyte transglutaminase") gene (TGM1) (Russell et al. 1994, 1995; Huber et al. 1995; ParmEntier et al. 1995). Transglutaminases are calcium-dependent thiol enzymes that catalyze covalent cross-linking of proteins, thus enhancing the stability of biological structures. These enzymes are present in various tissues and body fluids, such as coagulation factor XIII (FXIII) in the blood stream, tissue transglutaminase in red blood cells, liver cells and chondrocytes and keratinocyte and epidermal transglutaminases in the skin (Aeschlimann and Paulsson 1994). TGM1 catalyzes the formation of ε-(γ-glutamyl)lysine cross-links between the precursor proteins of the cornified cell envelope (CE). The CE is a 15–20 nm–thick, highly insoluble structure that is formed, during the late phases of epidermal differentiation, on the inner side of the plasma membrane (Rice and Green 1977). Several proteins, such as involucrin, loricrin, and the small proline-rich proteins, have been implicated as precursor proteins to the CE (Rice and Green 1979; Mehrel et al. 1990; Hohl et al. 1995). Simultaneously with cross-linking of these precursor proteins, Ω-hydroxy-

acyl-sphingosine lipids covalently bonded to the outer surface of the protein CE and the plasma membrane are replaced by the CE.

Finland provides an exceptional environment in which to study autosomal recessive diseases such as ARCI, because of its long-lasting genetic isolation, which is due to the peculiar population history of the country (de la Chapelle 1993). Finland was populated by a relatively small number of settlers ~2,000 years ago and has since remained genetically isolated, for linguistic, cultural, religious, political, and geographic reasons. This has led to the enrichment of certain recessive disorders, whereas some disorders common in other Caucasian populations, such as cystic fibrosis, are almost missing. This phenomenon has created a concept known as the Finnish disease heritage, referring to ~30 recessive diseases occurring in Finland at rates higher than those elsewhere. Typically, one mutation causes most of the cases of a recessive disease, reflecting a founder effect. Because of this population history, we hypothesized that ARCI in Finland might be caused by one major mutation originating from a common ancestor.

We identified five different point mutations in Finnish ARCI patients, of which four—Arg142Cys, Gly217Ser, Val378Leu, and Arg395Leu—had not been described previously. Two of the five mutations, Arg141His and Arg142Cys, are clustered within two adjacent arginines (Arg141 and Arg142). It was demonstrated further that the Arg142Cys mutation associated with two different haplotypes, suggesting two independent origins of the single nucleotide alteration. The mutation of exactly the same nucleotide twice in a small isolated population and the description, so far, of a total of four mutations, in Arg141 and Arg142 (Huber et al. 1995; ParmEntier et al. 1995; Russell et al. 1995), support the idea that this area of TGM1 is susceptible to nucleotide changes. Furthermore, the results in this study suggest that mutations in this region may result in destabilization of the mutated protein product and can occur in two clinically distinctive subtypes of ARCI, LI and CIE.

Patients and Methods

Patients

Forty-nine patients and 117 unaffected family members from 38 families, including all the known living cases in Finland of severe ichthyosis, were studied. The diagnosis of ARCI, on the basis of clinical and histologic features, was made at the Department of Dermatology of the Helsinki University Central Hospital. The clinical phenotypes of the patients with TGM1 mutations are listed in table 1. The patients do not show any involvement of other organ systems. The study was approved by the ethical committee of the Department of Dermatology of the Helsinki University Central Hospital.

Isolation of DNA

Genomic DNA was extracted from leukocytes of peripheral venous EDTA blood (10 ml), by standard procedures (Sambrook et al. 1987).

DNA Amplification

To search for mutations, 15 exons of TGM1 were amplified by PCR on 13 fragments, by use of the primers shown in table 2, most of which have been described elsewhere (Russell et al. 1995). PCR reactions were performed in 50-μl volumes containing 50 ng of DNA, according to the methods described by Saiki et al. (1988). The reaction conditions were optimized for each individual region.

SSCP Analysis

The radioactive PCR products were analyzed by SSCP analysis, as described elsewhere (Orita et al. 1989; Mikkola et al. 1994).

Sequence Analysis

A solid-phase sequencing strategy using biotinylated primers was used for the regions where mobility shifts were detected by SSCP analysis, as described elsewhere (Sanger et al. 1977; Syvänen et al. 1989).

Solid-Phase Minisequencing

After detection of the nucleotide alterations, the samples from family members and from healthy controls were screened for each mutation, by solid-phase minisequencing, which unequivocally identifies the genotype (the minisequencing primers are shown in table 2) (Syvänen et al. 1990; Mikkola et al. 1994). Also, the affected individuals were screened, to ascertain the SSCP data. To evaluate the frequency of the identified point mutations, in the general population, DNA samples from 400 blood donors were screened, also by use of the solid-phase minisequencing method.

Haplotype Analysis

All the 14 affected individuals with TGM1 mutations were analyzed by use of an intragenic polymorphism in intron 14 of TGM1 and by use of four other markers—D14S972, D14S64, D14S264, and D14S1032—flanking TGM1. The genetic order of the markers and the distances between them were based on a published genetic map (Dib et al. 1996). TGM1 was mapped physically in relation to the markers, and their order was verified by PCR assays on radiation hybrids (Stanford G3 panel; Research Genetics). Markers D14S972 and D14S1032 and the intragenic TGM1 marker were detected by 5′ end-labeling with [γ-^{32}P]ATP (Amersham), and markers D14S264 and D14S64 were detected by silver staining of the gels. The amplified fragments were

Table 1

Patients and Clinical Phenotypes

Mutation(s) and[a] Family/Patient	Sex	Age[b] (years)	Skin at Birth	Scales	Clinical Diagnosis	Accentuation of Ichthyosis[c]			
						Ectropion	Alopecia	Flexures	Palms/Soles
Arg142Cys/Arg142Cys:									
1/6	Female	31	Collodion	Thick	LI	+	+	+	+
2/2	Female	28	Collodion	Thick	LI	+	+	+	+
Arg142Cys/Val378Leu:									
3/II-4	Male	49	Collodion	Thick	LI	+	+	+	+
3/III-1	Male	29	Collodion	Thick	LI	+	−	+	+
3/IV-1	Female	1	Collodion	Thick	LI	+	−	+	+
4/2	Male	62	Collodion	Thick	LI	+	+	+	+
5/2	Male	2	Collodion	Thick	LI	+	−	+	+
6/2	Female	17	Collodion	Thin	CIE	+	+	+	+
6/3	Male	10	Collodion	Thin	CIE	+	+	+	+
Arg142Cys/Arg141His:									
7/2	Male	1	Erythematous	Thin	CIE	+	−	+	+
Arg142Cys/unknown:									
8/1	Female	1	Ichthyotic	Locally thick	CIE	−	−	+	−
Arg141His/Val378Leu:									
9/2	Male	11	Collodion	Thick	LI	+	−	+	+
Val378Leu/unknown:									
10/1	Female	25	Ichthyotic	Thin	CIE	+	+	+	+
Val378Leu/Arg395Leu:									
11/2	Male	10	Collodion	Thick	LI	+	−	+	+
Gly217Ser/Arg395Leu:									
12/1	Female	17	Collodion	Thick	LI	+	−	+	+
Arg395Leu/unknown:									
13/2	Male	24	Collodion	Thin	CIE	+	−	−	+
13/3	Male	20	Collodion	Thin	CIE	+	−	−	+

[a] Family and patient numbering refer to figure 1, except for that of families 9–13 (pedigrees not shown). Mutations represent the two alleles of TGM1.

[b] At time of diagnosis.

[c] A plus sign (+) indicates the presence of the condition, and a minus sign (−) indicates the absence of the condition.

separated by electrophoresis (58 W for 2–5 h) on 6% denaturing polyacrylamide sequencing gels.

To study the geographical distribution of the different mutations and to look for consanguinity, as well as for interfamiliar relationships, the great-grandparents and their birthplaces were traced, for each of the sibships, through church registers. For some families, their ancestors were traced back even further. The birthplaces of the grandparents (the parents of obligate carriers) were used to depict the geographical distribution.

Modeling

The three-dimensional structure of the FXIII A-subunit zymogen dimer, as determined by single-crystal x-ray diffraction (Yee et al. 1994), was used as a template for creation of a homology model of human keratinocyte transglutaminase, by use of the Biosym Insight II software package (version 2.3.0.; Biosym Technologies). Coordinates for the FXIII structure were obtained by refinement against x-ray diffraction data within the range of 10.0–2.65 Å of resolution, by use of the program X-PLOR (Brunger 1992), giving a crystallographic *R* factor of 21.7%. The final model had good geometry (root-mean-square deviation from ideality of 0.012 Å for bonds, 1.8° for bond angles, 25.6° for torsion angles, and 1.5° for improper torsion angles), and the average value of the individually refined atomic temperature factors was 26.7 Å^2. The coordinates for this experimentally determined FXIII structure have been deposited with the Protein Data Bank (identification code 1ggt). Models of the keratinocyte-transglutaminase mutant structures were generated by use of the computer program O (Jones et al. 1991), and figures were drawn with the program MOLSCRIPT (Kraulis 1991).

Creation of the Expression Construct and Mutagenesis

A FXIII A-subunit cDNA clone was obtained from Dr. Dominic Chung (University of Washington, Seattle) (Ichinose et al. 1986). An *XbaI*/*Hind*III fragment containing the coding regions was subcloned into the SV40poly expression vector (Stacey and Schnieke 1990). The mutagenesis for the Arg78Cys mutation in FXIII,

Table 2

Sequences of Oligonucleotides

Method and Exon(s)	Forward Primer	Reverse Primer	Size (bp)
Genomic PCR:			
1	ACTTGGGCTGCAACAGAACTCGG	GCACGGCCTCTGATAGTGTGG	406
2	ACTGGCTGGACTACCTGGTTA	AGTCTCTGGTCCCATTAAAGC	430
3	AATGATAAGGGCCTGGGCACC	GCCTCTCCCCACCAAACATAG	302
4	GTCCCAGGCTCCATCCCCTCTCCT	TCCCTGTCTTTCCCTCCCATCTAC	361
5, 6	AACTGGCCAGAAGTAGGTGAG	AGTATCCTTTACAGGGCAGGG	558
7	TTTAGGGGTAAGGGGTGGTTG	CTGTAGGGCCCGGGCCACTCCT	328
8, 9	ATACTCCTGACACGATGCCTC	GGACTGTGTTAATCAGGTGGG	525
10	TCGCATCCCTCTCCGCCTTCTCAG	CCATGACTGAAGCCCAAGAAGGC	197
11	CCTCAGACCCTCTGGCTCAC	CACTTGGCAGGAACACTTGTTGTG	265
12	AGTGTTCCTGCCAAGTGGTTG	TCCATGTCCACAGCCCTGAG	415
13	GGGAAGCCTCATGTAGGGAAG	GTCCTTATCCGGCCTTCACTC	274
14	TACGGTCCCCGTGTGTTTGG	TGGGAAGGCCAGAGTGGAAG	247
15	AGCTCTTACTCCCCACTCCAC	ACTGACTCCCTCTCCGGGAG	422

	Primer	Mutation	Wild-Type/ Mutated Nucleotide
Minisequencing:			
3	ACGACGAGCTGATAGTGCGC	Arg142Cys	C/T
3	GTACGACGAGCTGATAGTGC	Arg141His	G/A
4	CTTCCCCCAACGCCATCATC	Gly217Ser	G/A
7	GTCCCCTATGGCCAGTGCTGG	Val378Leu	G/C
8	CTGCCTGGGTCTGGCCACCC	Arg395Leu	G/T

corresponding to the mutation site Arg142Cys in TGM1, was performed by the Chameleon mutagenesis kit (Stratagene), by use of a mutation-specific oligonucleotide (5′CTGATTGTCCGCTGCGGGCAGTCTTTC3′) and an oligonucleotide (5′CGCATGCGATGTCGAGCTCTC3′) that abolishes the *Eco*RV restriction site at the polylinker of the expression vector. Positive clones containing the mutation were identified by solid-phase minisequencing. The entire reading frame of the mutant clone was screened for possible artifactual mutations, by SSCP analysis and by direct sequencing.

COS-Cell Culture and Transfection

COS1 cells were maintained in culture in Dulbeccos's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, penicillin, and streptomycin. One day before transfection the cells were seeded at 70% confluency. The cells were transfected with the 1 μg SV40poly/ml cDNA constructs, by the diethylaminoethyl-dextran-chloroquine method (Sigma Chemicals), and were analyzed 48–94 h later.

Analysis of Transfection Efficiency and Steady-State mRNA Levels

For mRNA quantitation, the cells were double transfected with an equal amount of wild-type and mutant constructs. Two days after transfection the cells were harvested in ice-cold PBS, were lysed in a lysis buffer, and were centrifuged to separate the nuclear and the cytoplasmic fractions, as described elsewhere (Jalanko et al. 1995). One microliter of the cytoplasmic fraction was used for reverse-transcription PCR (Mikkola et al. 1994). The nuclear fraction containing transfected cDNA was diluted in 100 μl of PCR buffer, was lysed for 5 min at 100°C, and was used as a template for PCR. The ratios of the two clones in cDNA (nuclear fraction) and in RNA (cytoplasmic fraction) were analyzed after PCR, by solid-phase minisequencing, which provides relative allele-specific quantitation of the product (Mikkola et al. 1996).

Western Blot

Seventy-two hours after transfection, the cells were harvested and lysed by freeze-thawing. One hundred micrograms of protein of the cell homogenates were analyzed by use of 10%-SDS PAGE and polyclonal anti-FXIII A-subunit antibody (Clotimmun; Behringwerke AG). Visualization was performed by use of enhanced chemiluminescence (Paunio et al. 1994). The correct migration of the expressed FXIII polypeptides was verified by use of platelet FXIII A-subunit and of prestained kaleidoscopic molecular-weight markers (Bio-Rad) as standards.

Metabolic Labeling and Immunoprecipitation

Transfected cells were cultured for 72 h and were starved for 30 min in a methionine-free and serum-free DMEM, followed by a 1-h radioactive pulse with 100 mCi of ^{35}S-methionine/dish (Amersham). Cells were chased for 4 h, 8 h, and 24 h in a medium lacking ^{35}S-methionine and fetal calf serum and were harvested by rapid trypsinization and the addition of leupeptin (Sigma Chemicals). Cells were lysed by freeze-thawing in PBS that contained 1% Triton-X 100 and leupeptin. Cell lysates as well as the concentrated culture media (Sentricon; Amicon) were immunoprecipitated with a 1:500 dilution of FXIII A-subunit antibody and *Staphylococcus aureus* (Immunoprecipitin; Gibco BRL) (Proia et al. 1984). The precipitated proteins were analyzed in 10%-SDS PAGE gels and were visualized by autoradiography for 1–5 d.

Transglutaminase-Activity Measurement by Ammonia-Release Assay

Transglutaminase activity was analyzed by the Berichrom activity assay (Behrichrom; Behringwerke AG). The cells were lysed by freeze-thawing in 50 mM of Tris buffer (pH 7.4) and 150 mM of NaCl, and 100 μg of cell homogenate were used in the activity measurement. FXIII-free fibrinogen was added at a concentration of 1 mg/ml.

Results

Screening for Mutations, by SSCP Analysis

To screen for mutations of TGM1, at least one member from each of the 38 families was analyzed by SSCP. A total of five different mobility shifts were detected in the amplification products of exons 3, 4, 7, and 8.

Identification of Mutations

Sequence analysis of the five abnormally migrating SSCP fragments showed five different missense mutations: a C→T transition in exon 3, resulting in the substitution of cysteine for arginine at amino acid position 142; a G→C transversion in exon 7, changing the valine to leucine at position 378; a G→A transition in exon 4, changing glycine to serine at position 217; a G→T transversion in exon 8, resulting in the substitution of leucine for arginine at position 395; and a G→A transition in exon 3, causing a previously described (Russell et al. 1995) substitution of histidine for arginine at position 141. No other mutations were detected. By use of sequence analysis and solid-phase minisequencing analysis, 17 patients from 13 families were found to carry the TGM1 defect. In 3 patients from 3 families, only one mutated TGM1 allele was identified, whereas the other allele remains unknown. Most of the patients were compound heterozygotes: only 2 patients were homozygous for the Arg142Cys mutation. Of the 17 patients with a TGM1 mutation, 10 patients (from 8 families) were classified clinically as LI and 7 patients (from 5 families) as CIE. The clinical picture of the patients carrying TGM1 mutations is presented more precisely in table 1.

Determination of the Prevalence of the Mutations, by Solid-Phase Minisequencing

To evaluate the frequency of the observed nucleotide alterations, in the general population, and to exclude the possibility of a common polymorphism, leukocyte pools of 400 unrelated blood donors were analyzed for each specific nucleotide alteration, by solid-phase minisequencing. Among 400 controls, one heterozygote individual carrying the Arg142Cys mutation was found, which is in agreement with the calculated carrier frequency. No Arg141His, Gly217Ser, Val378Leu, or Arg395Leu mutations were detected among the 400 individuals analyzed by the study of the pooled samples.

Haplotype Analysis

To study whether a specific haplotype was associated with a specific mutation, four markers flanking the TGM1 gene (D14S972, D14S64, D14S264, and D14S1032) and one intragenic marker were analyzed in the 13 families with a TGM1 mutation. The order and distances between the markers and the full haplotype of affected individuals carrying the Arg142Cys mutation and of their family members are shown in figure 1. We defined two different haplotypes for the Arg142Cys mutation. In the case of other mutations only one haplotype was found. When the birthplaces of the ancestors of the affected individuals carrying the Arg142Cys mutation were studied, it was found that different haplotypes were clustered in different geographical areas in Finland (fig. 2). Two different haplotypes that seem to originate from separate areas suggest two separately occurring Arg142Cys mutations.

Modeling

The experimentally determined three-dimensional x-ray crystal structure of the FXIII A-subunit zymogen dimer was used to generate a homology model of the TGM1 protein, which in turn was used as a template to model the possible structural consequences of the novel Arg142Cys, Gly217Ser, Val378Leu, and Arg395Leu mutations identified in this work. Both the Arg142 and Gly217 residues are located far from the active site (>20 Å). The Arg142 side chain is involved in a number of hydrogen-bonding interactions with other residues in the same domain and across a domain interface; a mutation of Arg142 is expected to affect protein folding and/or stability. The Gly217Ser mutation would require a local change in the protein main chain, which

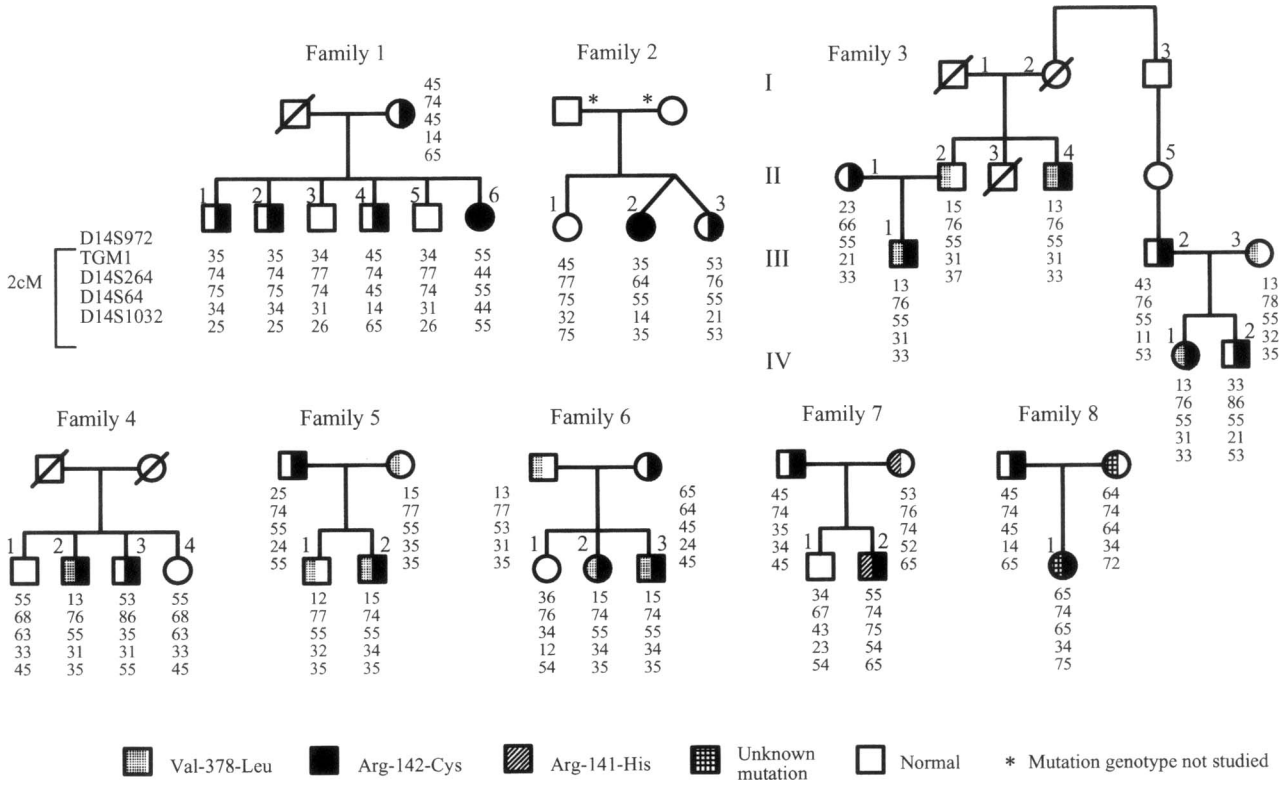

Figure 1 Pedigrees and haplotypes of the families in which the affected persons carried the Arg142Cys mutation. The order of the microsatellite markers is shown. Only two patients (in families 1 and 2) were homozygous for the Arg142Cys mutation. Two different haplotypes associated with the Arg142Cys mutation were found. One patient homozygous for the Arg142Cys mutation (person 2 in family 2) carried those two haplotypes in her chromosomes. Affected individuals in families 3 and 4 carried different haplotypes than those in families 1, 5, 6, 7, and 8. These two different haplotypes suggest two different nucleotide alterations in Arg142.

would yield a misfolded protein. The Val378Leu substitution is a conservative replacement that is likely to result in a limited local conformational change. However, the proximity of the Val378 site to the catalytic Cys376 residue means that any local disruption of protein structure is likely to dramatically affect the catalytic behavior of the enzyme. The fourth residue identified as a mutation site, Arg395, is at one end of a conserved string of hydrogen-bonding interactions that extend to the active site. Thus the Arg395Leu mutation is expected to have structural consequences that are transmitted to the active site of the enzyme and that affect catalysis.

In Vitro Expression

To further explore the mutation-cluster region, the Arg142Cys mutation of TGM1 was created in the corresponding position of the FXIII A-subunit cDNA (Arg78-Cys). The relative steady-state mRNA levels of wild-type and mutant FXIII, in the transient-expression system, were studied by double transfection of COS cells with an equal amount of wild-type FXIII and the mutant clone corresponding to the Arg142Cys mutation of TGM1. The ratio (r) of mutant and wild-type cDNA in the nuclear fraction, as measured by the incorporation of the ^{3}H-test nucleotides in the PCR products, was close to 1 in four parallel measurements, confirming the equal transfection efficiency of the two clones ($r = cpm_{mut}/cpm_{wt} = 1.05$; SD = 0.17). Furthermore, the ratio of the mRNAs of the two clones in the cytoplasmic fraction was also close to 1 ($r = cpm_{mut}/cpm_{wt} = 1.11$; SD = 0.20) and was similar to the ratio of the two clones in the nuclear fraction, indicating that the steady-state mRNA levels of the wild-type and the mutant clones were equal in the transiently transfected COS cells.

In western blots of individually transfected cells, the amount of mutant FXIII polypeptide that accumulated inside the cell was reduced drastically, compared with the wild-type protein, in three repeated experiments (fig. 3). Furthermore, no transglutaminase activity was detected by the ammonia-release assay, in the case of the mutant FXIII protein (data not shown). In three repeated pulse-chase experiments, the wild-type protein demonstrated no significant degradation, in a 24-h chase, but some of the protein had leaked into the medium. Even though the antigen level of the mutant protein, after a 1-h pulse, was about one-half that of the wild-type pro-

Figure 2 Birthplaces of the parents of Arg142Cys-carrier parents are shown. The unblackened circles indicate the birthplaces of ancestors carrying the Arg142Cys mutation associated with the haplotype 3-6-5-1-3, and the blackened circles indicate the birthplaces of ancestors also carrying the Arg142Cys mutation, with the haplotype 5-4-5-4-5. The distribution of the birthplaces to two geographically different areas, according to haplotype, suggests two different origins for the Arg142Cys mutation.

tein, the antigen level gradually disappeared, as was detected on time points (fig. 4). These results indicate the instability of the mutant polypeptide and the increased susceptibility for intracellular proteolytic degradation.

Discussion

In this study, we identified a mutational cluster region in TGM1 and five point mutations, of which four are novel. For many autosomal recessive Finnish diseases

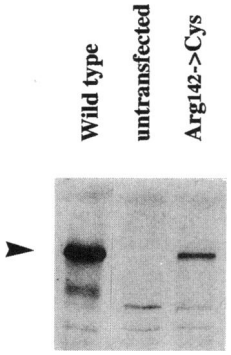

Figure 3 Results of immunoblotting of 100 μg of protein of transfected COS-cell homogenates electrophoresed on 10%-SDS PAGE gels. Decreased intracellular accumulation of the FXIII protein containing the mutation corresponding to the Arg142Cys mutation in TGM1 is demonstrated.

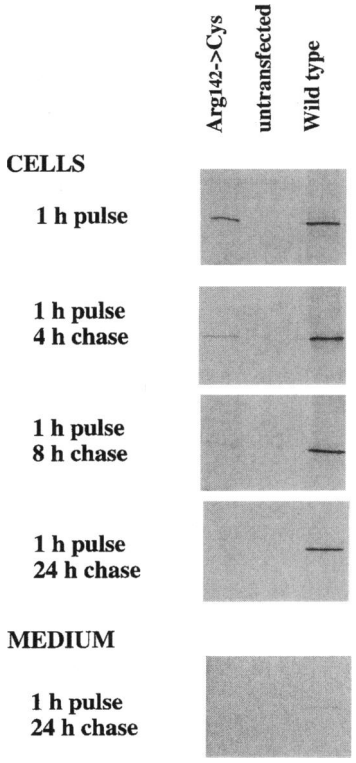

Figure 4 Metabolically labeled, transfected COS cells and the corresponding media subjected to immunoprecipitation and 10%-SDS PAGE demonstrate intracellular degradation of the FXIII protein with the mutation corresponding to the Arg142Cys mutation in TGM1.

for which the molecular defect has been uncovered, >90% of the disease alleles carry the same causative mutation. Aspartylglucosaminuria and infantile neuronal ceroid lipofuscinosis are representative examples. For both of these lysosomal accumulation diseases, one major point mutation is carried in 98% of disease alleles in Finland (Ikonen et al. 1991; Syvänen et al. 1992; Vesa et al. 1995). In this respect, ARCI seems to differ from a typical autosomal recessive disease in Finland and resembles more the situation observed for cystic fibrosis in Finland, where three founder mutations have been found (Kere et al. 1994). Five different point mutations causing ARCI in Finland were detected, suggesting that numerous, relatively new mutations contributed to the pathogenesis of the disease within the population. Only 2 patients were homozygous; all the other 14 patients were compound heterozygotes.

Four of 12 TGM1 mutations published to date have occurred in the arginine residues at positions 141 and 142, indirectly suggesting a mutation-prone area of the gene (fig. 5*A* and *B*). These mutations have been found to be distributed to different populations all over the world. Two mutations, Arg141His and Arg142His, have been described independently by two research groups, in different populations: the G→A transition

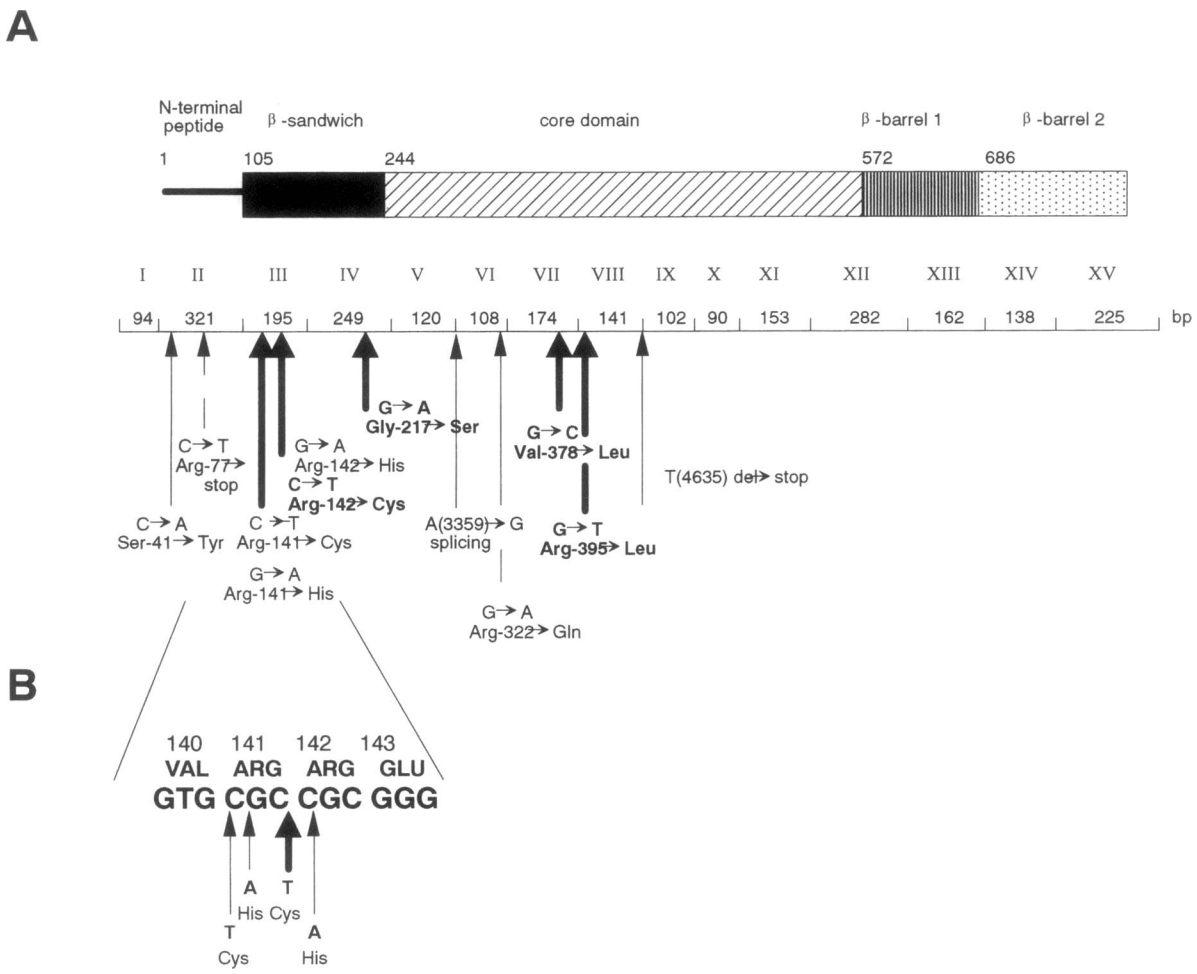

Figure 5 *A*, Sequential arrangement of the domain structure of transglutaminases adjusted from the FXIII A-subunit for TGM1 and from the corresponding cDNA of TGM1. The locations of the mutations reported thus far are shown (the N-terminal peptide is not conserved among transglutaminases). The positions of the exons are indicated by roman numerals, and the sizes of the exons are marked. The five mutations found in the Finnish population are indicated by the boldface arrows. For further details regarding the mutations, see Huber et al. 1995 (Ser41Tyr, Arg141Cys, A[3359]G splicing, Arg322Gln, and delT[4635]-stop), ParmEntier et al. 1995 (Arg77-stop), and Russell et al. 1995 (Arg141His and Arg-142-His). *B*, A close-up view of the residues 140–143 of TGM1 and the corresponding sequence. Four of the 10 mutations published so far are located in this area, and these mutations are indicated by arrows; the boldface arrow points to cytosine that was mutated twice. All the 4 mutations are located to CpG dinucleotides, whether in coding or noncoding strands of DNA.

causes a substitution of histidine for arginine at position 141 (Russell et al. 1995; this study), and the G→A transition changes arginine to histidine at position 142 (ParmEntier et al. 1995; Russell et al. 1995).

Closer analysis of the nucleotide sequence adjacent to the Arg141 and Arg142 mutations revealed a symmetric element, G CGC cGC G (fig. *5B*). All the four different mutations occurred in the underlined CpG dinucleotides and were C→T transitions in either of the DNA strands (the C that, in our study, was altered twice to T, causing two Arg142Cys mutations, is lowercase). A corresponding mutation-prone area has not been observed in another transglutaminase, FXIII, although the amino acid sequence is identical in this area. However, the nucleotide-sequence alignment between FXIII and TGM1 revealed differences; the corresponding sequence of FXIII is C CGC AGA G. So far, no sequence alteration has been described in this area of the FXIII A-subunit gene.

The three-dimensional structure of TGM1 has yet to be determined experimentally; the x-ray crystallographic model of the human FXIII A-subunit is the only experimentally determined structure of any member of the calcium-dependent transglutaminase family. The extensive amino acid conservation (42%) between TGM1 and FXIII allows the FXIII x-ray structure to serve as a reliable scaffold for the construction of a homology model of TGM1. This homology model, in turn, serves as the basis of the modeling of the Arg142Cys, Gly217Ser, Val378Leu, and Arg395Leu mutants.

From structure-guided alignment of 19 transglutami-

nase sequences (data not shown), all four residues, Arg142, Gly217, Val378, and Arg395, were shown to be conserved absolutely. In addition, the Arg141 residue adjacent to Arg142 also is conserved absolutely, and both of these amino acids are sites of keratinocyte-defect mutations (Huber et al. 1995; ParmEntier et al. 1995; Russell et al. 1995). Residues that surround these four amino acids are almost all conserved in TGM1 and in FXIII, and, thus, it is very likely that the local protein structure is conserved in these regions as well. The major structural difference between the two enzymes is in the aminoterminal region, which in TGM1 consists of a membrane-anchorage domain and in FXIII of an activation peptide that is cleaved by thrombin. However, it has been shown that terminally differentiated cells also contain soluble, active forms of TGM1, where the membrane-anchorage domain is cleaved at a site corresponding to the thrombin activation site of FXIII (Kim et al. 1995).

Evidence for instability caused by the Arg142Cys mutation was obtained, on one hand, from modeling studies that suggested either an altered quaternary or a misfolded structure and, on the other hand, from in vitro expression studies. The FXIII protein with the corresponding mutation accumulated in the cells in reduced quantities, in spite of normal mRNA levels, and demonstrated rapid degradation in pulse-chase experiments. Thus, on the basis of both the structural homology of the FXIII and the TGM1 enzymes and the conservation of the polypeptide sequence around the mutation area, it is very likely that instability and susceptibility for proteolytic degradation is at least one of the consequences of the Arg142Cys mutation. The corresponding mutation in FXIII also was devoid of transglutaminase activity. However, since the two enzymes have different substrates, it is not relevant to study the catalytic activity of the TGM1 mutants in a different enzyme.

The consequence of the third mutation in the β-sandwich domain, residue Gly217Ser, was not approached by use of expression studies, but modeling studies suggested an altered protein folding, which can affect protein stability. Two of the novel mutations, Val378Leu and Arg395Leu, were located in the catalytic core domain. Definitive interpretations of the effects of these mutations on the catalytic functions of the TGM1 protein cannot be provided, owing to lack of good models. However, modeling studies suggest altered protein structures extending to the active site of the enzyme protein.

In previous studies, the defects of the TGM1 gene were found only in LI patients. However, in this study, it seemed that mutations in the TGM1 gene could result in both LI and CIE. Although in most of the CIE families the other mutation remains unknown, it seems evident that patients carrying identical mutations might exhibit different phenotypes.

In conclusion, four private mutations and one recurrent mutation of the TGM1 gene were found in Finnish ARCI patients. The TGM1 gene seems to have a mutational cluster in the area coding for Arg141 and Arg142, which is due to deamination of methylated cytosines in a CpG island. Structural predictions and expression studies suggest that replacement of either of these arginines most likely results in destabilization of the protein structure and renders it susceptible to proteolytic degradation.

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