When the peptide sequences were aligned and analyzed with respect to the potential reactive glutamine residue, the following significant tendencies among the sequences were observed: (a) a hydrophobic amino acid was commonly observed at position +3 (relative to the glutamine residue); (b) lysine or arginine mainly was found at position +2; and (c) in most sequences, an aromatic amino acid and a serine/threonine residue were frequently located at positions −1 and +1, respectively. Figure 1 shows the representative sequences that were further analyzed for their reactivity as substrates.

Evaluation of selected sequences as recombinant peptide-fused glutathione *S*-transferase (GST) proteins

To evaluate the ability of the screened peptide sequences as a glutamine-donor substrate, we measured the amount of enzymatic incorporation of the primary amine, monodansylcadaverine (Dansyl-Cd), in recombinant peptide-GST(QN) fusion proteins, in which all the glutamine residues in glutathione *S*-transferase (GST) had been substituted by asparagines. The peptide sequences shown in Fig. 1 were expressed as

*E12			Y	D	Y	W	P	M	Q	T	R	T	R	T					
*E50					W	G	P	Q	Q	T	R	I	P	S	Y	R			
E524				I	K	F	P	E	Q	T	R	I	W	H	A				
*E8							W	T	Q	T	R	F	T	T	P	F	P	E	
E47					E	W	F	G	Q	V	R	I	H	P	M	S			
*E51	P	P	P	Y	S	F	Y	Q	S	R	W	V							
*E10				W	N	F	A	E	Q	T	R	L	F	K	A				
*E21						L	M	P	Q	T	R	L	E	P	H	M	L		
E33								F	Q	M	R	L	P	L	A	G	P	S	F
E9								Y	Q	Q	R	L	Y	M	P	T	W	P	P
*E1								Y	Q	T	K	W	P	M	E	F	S	S	R
*E18								F	Q	L	K	V	P	A	A	V	W	S	D
*E6								Y	Q	L	K	Y	T	H	W	A	H	T	P
E53								F	Q	Y	K	L	N	F	G	Q	Y	V	Y
*E125	A	E	R	P		N	I	M	Q	V	K	L	V						
*E42						W	T	T	Q	M	K	M	P	H	H	A	F		
E110							F	Y	Q	R	P	L	P	A	H	L	L	G	
*E4						F	N	Y	Q	A	Y	L	D	I	P	R	Y		
E55						F	P	Y	Q	T	L	F	N	P	T	P	M		
E68						W	P	Y	Q	I	M	M	G	H	A	R	A		
*E46	N	Y	W	S		W	P	G	Q	I	S	Y	H						
E17						N	P	Y	Q	Y	Q	I	F	N	W	A	W		
E510						W	E	T	Q	Q	S	W	L	F	S	N	L		
E45							T	Y	Q	H	I	W	H	P	S	L	A	L	
E5								Y	Q	I	T	L	P	Y	R	Y	E	M	P
*E111								W	Q	S	P	I	T	F	P	L	T	L	A
*E115								W	Q	T	Q	V	V	L	H	E	E	P	L
E70							Y	S	Q	S	T	H	A	L	F	S	A	R	
*E2					K	M	P	E	D	T	R	L	H	N	F	A			

Fig. 1. Selected sequences and alignment of candidate substrate peptide. Selected 12-mer peptide sequences were aligned based on the putative reactive glutamine residue. The glutamine residue, hydrophobic amino acids at position +3 (relative to the glutamine), and the arginine and the lysine residues at position +2 are shaded. Phage ID (E) is shown at the left of the amino acid sequence. The asterisk indicates the sequences of which reactivities were subsequently shown in Fig. 2.

fusion proteins and the time-course products formed by the catalytic reaction of TGase 3 were subjected to SDS/PAGE and visualized by UV illumination. Using this procedure, 16 of the 29 sequences, including a no-glutamine-containing sequence as a negative control, were selected based on their reactivities (Fig. 1, marked with asterisk). The enzymatic products, with dimethylcasein as a positive control, were aligned as shown in Fig. 2A. Among the sequences that exhibited incorporation of Dansyl-Cd, seven peptides (underlined sequences: E8, E12, E18, E115, E46, E10 and E51) were selected based on their reactivities and further analyzed for their isozyme specificity with respect to human TGase 1, guinea-pig liver TGase (as TGase 2) and human Factor XIII (Fig. 2B). All the sequences showed negligible reactivity in the reaction catalyzed by Factor XIII. However, five sequences (E8, E12, E115, E46 and E10) exhibited cross-reactivities to guinea-pig liver TGase and one (E18) to TGase 1. Among the seven peptide–GST(QN) fusion proteins evaluated in this study, only the E51 sequence showed less cross-reactivity to other isozymes although displaying prominent reactivity to TGase 3. Thus, this peptide sequence was selected for further analysis.

Substitution-mutant analysis for each amino acid to alanine in the E51 peptide sequence

To examine the contribution of each amino acid residue of the E51 sequence (PPPYSFYQSRWV) in the catalytic reaction of TGase 3, alanine substitution mutants for every residue of the 12-amino acid peptide were generated as GST(QN)-fusion proteins. Wild-type and a mutant in which the reactive glutamine was substituted by asparagine were also subjected to the analysis (Fig. 3). According to the catalytic reaction of TGase 3, substitutions at positions −2 (F), +1 (S), +2 (R) and +3 (W) significantly affected the reactivity (colored as darker gray: < 50% intensity of that in wild-type). In addition, substitution at positions −5 (P) and +4 (V) resulted in a moderate decrease in reactivity. These results suggest that these amino acids contribute to the interaction of E51 with the enzyme to form an intermediate between the glutamine residue in the substrate and the cysteine residue in the active site pocket of TGase 3.

Assessment of reactivity and specificity of E51 sequence in the peptide form (pepE51)

To assess the reactivity and isozyme-specificity of E51 (PPPYSFYQSRWV) in peptide form, biotinylated peptides for E51 (pepE51) or E51 in which the reactive

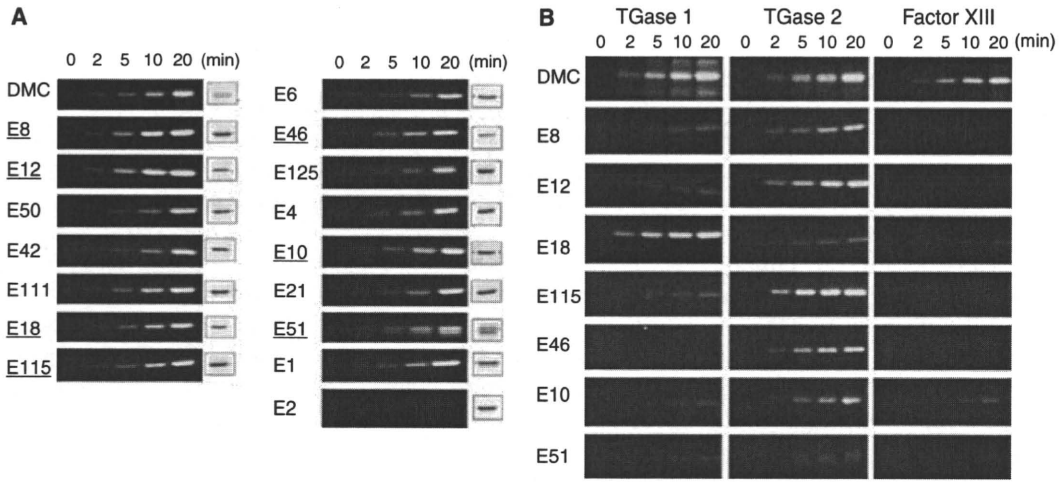

Fig. 2. Evaluation of the reactivities of the selected peptides as GST(QN) fusion proteins. (A) Incorporation of Dansyl-Cd into the purified recombinant GST(QN) fusion proteins with peptide that were selected by phage display screening, in the presence of activated TGase 3 (1 ng·μL^{-1}). At the times indicated, the reaction products were separated on 12.5% SDS/PAGE and illuminated by UV light. Unreacted fusion proteins were stained with Coomassie Brilliant Blue and are shown on the right. The underlined sequences are subjected to further analysis for cross-reactivities. (B) Cross-reactivities to three isozymes regarding the selected seven GST(QN)-fusion proteins. Each protein reacted at the indicated times in the presence of TGase 1 (1.5 ng·μL^{-1}), guinea-pig liver TGase (TGase 2) (2.5 ng·μL^{-1}) and activated Factor XIII (5 ng·μL^{-1}) were analyzed by SDS/PAGE and UV illumination. All the enzymatic activities were normalized based on the incorporation of Dansyl-Cd into dimethylcasein.

glutamine residue was substituted by asparagine (pepE51QN: PPPYSFYNSRWV) were synthesized for examination. Both peptides were subjected to a TGase 3-catalyzed cross-reaction with a primary amine (spermine), covalently immobilized to a microtiter well, in the presence of activated TGase 3 [32,33]. As shown in Fig. 4A, a time-course-dependent incorporation of pepE51 was observed, whereas pepE51QN did not show any reactivity. Moreover, in contrast to pepE51QN, increasing concentrations of pepE51 enzymatically cross-linked with the coated spermine (Fig. 4B). In addition, β-casein as a glutamine-acceptor substrate appeared to accept pepE51 (data not shown). These results demonstrate that pepE51 acts as a good substrate, similarly to the fusion protein.

To further investigate whether the isozyme specificity was preserved, the reactivity of pepE51 at various concentrations was evaluated in the presence of other TGase isozymes including TGase 1, TGase 2 and activated Factor XIII (Fig. 5). A negative control was paralleled using pepE51QN. In each case, pepE51 showed less cross-reactivity with the isozymes at the examined peptide concentrations, except for a weak reaction with guinea-pig liver TGase at a higher concentration (> 2.5 μM). This result suggests that pepE51 at a concentration below 1 μM can be used as a specific peptide in this reaction.

Detection of *in situ* activities of TGase in the skin and hair follicles

Previously, we found that a fluorescent-labeled substrate peptide for TGase 1 [fluorescein isothiocyanate (FITC)–pepK5] could be used as a prominent probe for detecting *in situ* activity of TGase 1 in both mouse and human skin [16,19]. Therefore, using a similar procedure, fluorescent-labeled E51 peptide (FITC–pepE51) was prepared and evaluated for detecting *in situ* activity of TGase 3 in a frozen mouse skin section.

As shown in Fig. 6, in the presence of $CaCl_2$, specific incorporation of FITC–pepE51 (1 μM) in endogenous glutamine-acceptor substrate proteins was observed in the epidermis. Reaction using FITC–pepE51QN, or in the presence of EDTA resulted in no signal, indicating that the signal was specific for TGase 3 activity.

Moreover, we inspected enlarged images of the skin section (Fig. 7A). In the epidermis, positive signals were observed around the granular and spinous layers and not in the outermost cornified layers, judging from the merged image with differential interference images. When compared with signals obtained using FITC–pepK5, the slightly weak and more limited regions in the layers were stained with FITC–pepE51. This result suggested that TGase 3 was active in more differentiating keratinocytes.

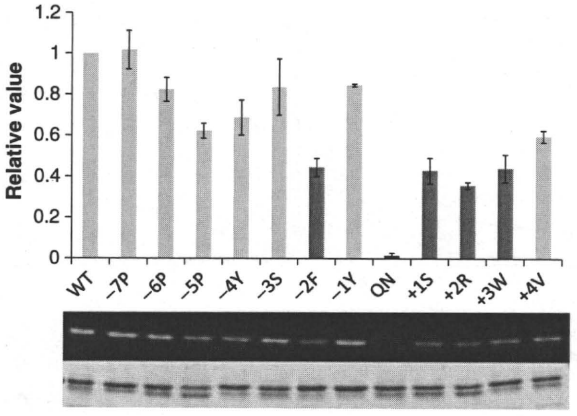

Fig. 3. Assessment of contribution of each amino acid residue of E51 sequence to substrate recognition. Alanine substitution mutants in the E51 sequence were produced as GST(QN) fusion proteins, and then incubated with Dansyl-Cd for 10 min in the presence of activated TGase 3 (1 ng·μL^{-1}). The reaction products were subjected to SDS/PAGE, followed by UV illumination. The fluorescence intensity was analyzed by Fuji multigauge quantification system. The relative values are normalized to the intensity for the reaction of wild-type. Data represent the means ± SD of duplicate samples. Numbers (−7P to +4V) with amino acid residue indicate the position of substitution; WT, peptide in which there were no amino acid substitution; QN, peptide in which the glutamine residue was changed to asparagine. The mutations that resulted in decrease in the reactivity at < 50% of that in wild type are shaded in darker gray.

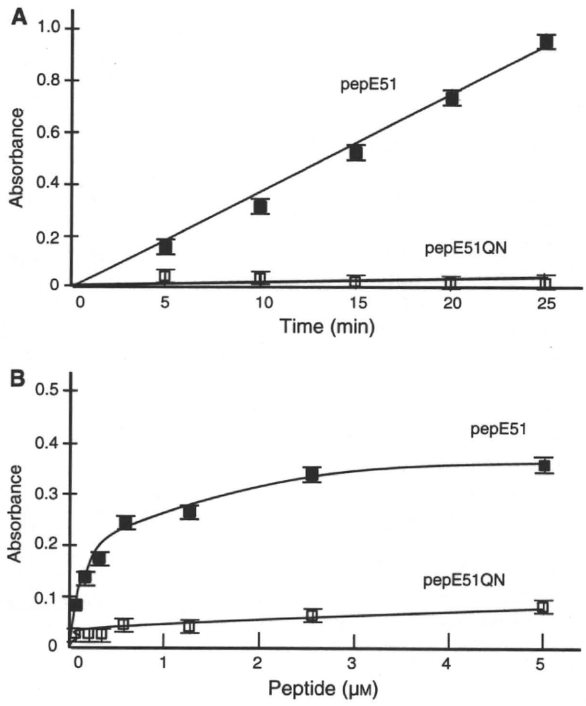

Fig. 4. Analysis of the reactivity of E51 sequence in the peptide form. (A) The time-dependent incorporation of 5 μM biotinylated peptide E51 (pepE51) into spermine, that covalently attached to microtiter well, was examined in the presence of activated TGase 3 (0.5 ng·μL^{-1}). The mutant peptide in which the glutamine was changed to asparagine (pepE51QN) was paralleled. (B) On the various concentrations of biotin-labeled peptides, incorporation into coated-spermine was measured in the same reaction condition at incubation time of 10 min. The closed and open symbols represent the reactions for pepE51 and pepE51QN, respectively. Data represent the means ± SD of triplicate samples.

Next, the staining pattern of the hair follicles was investigated (Fig. 7B,C). The distribution of signals was different when FITC–pepK5 and FITC–pepE51 were used. According to the FITC–pepE51 pattern, the activated TGase 3 was mainly located in the medulla and the hair cortex. However, according to the FITC–pepK5 pattern, TGase 1 activity was observed around the outer root sheath and cuticle and in differentiated inner root sheath cells. Thus, TGase 1 and TGase 3 appeared active in distinct regions of the hair follicle cells.

Discussion

During differentiation of keratinocytes and hair formation, isopeptide cross-linking of several structural proteins is essential for the formation of the insoluble proteinaceous layers, the CE, which contribute to effective physical and water barrier formation. Upon CE formation in keratinocytes and hair follicle cells, TGase 3 cross-links various substrate proteins such as SPRs, involucrin, loricrin and trichohyalin [7–9,23,24]. In addition to the endogenous substrates, some proteins of human papillomavirus have been described as possible substrates for inducing an abnormality in CE formation [34]. Previous studies have determined the cross-linking sites of these proteins and suggest that they display a pattern distinct from that obtained with TGase 1 [35–38]. In these reports, for example, the sequences QLQQQQVK (SPR1, Q19), SQQVTQT (loricrin, Q219), HQTQQK (loricrin, Q305), SSQQQKQ (SPR1, Q5 and Q7) and SQQVTQT (loricrin, Q215 and Q216) were determined as cross-linking sites by TGase 1 and TGase 3, respectively. However, differences in reaction specificities between these two isozymes are not fully understood. A better understanding of the preferred substrate sequences for TGase 3 will provide useful information for clarifying the process of cross-linking.

To date, with respect to the major members of the TGases family such as TGase 1, TGase 2 and Factor XIII, we have investigated the preferred substrate

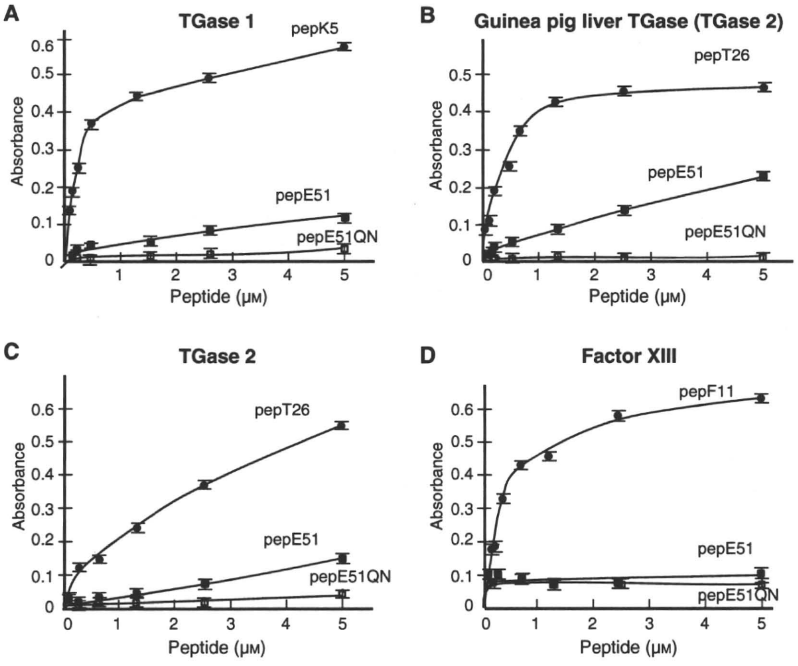

Fig. 5. Cross-reactivities of pepE51 with other major isozymes. On the various concentrations of pepE51 and pepE51QN as well as three specific biotin-labeled peptides (pepK5; TGase 1, pepT26; TGase 2, pepF11; Factor XIII), incorporation into coated-spermine was measured in the presence of each isozyme, TGase 1 (0.075 ng·μL^{-1}) (A), guinea-pig liver TGase (0.12 ng·μL^{-1}) (B), TGase 2 (0.06 ng·μL^{-1}) (C), and activated Factor XIII (0.24 ng·μL^{-1}) (D) for 15 min. All the enzymatic activities were normalized based on the incorporation of Dansyl-Cd into dimethylcasein. The closed circles represent the reactions for pepK5, pepT26 and pepF11 in each isozyme reaction. The closed and open rectangles indicate the reaction for pepE51 and pepE51QN, respectively. Data represent the means ± SD of triplicate samples.

sequences around the reactive glutamine residue from a phage-displayed peptide library [15,16]. In these previous studies, the identified preferred substrate sequences displayed a unique tendency for each isozyme, Q-x-R/K-Ψ-x-x-x-W-P to TGase 1, Q-x-P-Ψ-D-P to TGase 2 and Q-x-x-Ψ-x-W-P to Factor XIII (x and Ψ are any amino acid and hydrophobic amino acid residues, respectively). We applied a similar approach to obtain information regarding the preferred substrate sequence for TGase 3, with particular interest in a highly reactive substrate peptide suitable for the detection of *in situ* enzymatic activity.

In this study, the preferred sequences for TGase 3 selected from the phage-displayed peptide library exhibited different tendencies compared with other TGase isozymes. With respect to the peptides that exhibited higher reactivities to TGase 3, the Q-S/T-K/R-Ψ consensus primary sequence was identified. The sequence motif, Q-x-K/R is frequently observed in several skin substrate sequences and also contained in the preferred substrate sequence that we previously identified for TGase 1 [16]. In the case of TGase 3, serine or threonine residues are frequently observed at position +1. Interestingly, the amino acid located at this position is not important for the reaction in other Tgases, including TGase 1. In addition, at the N-terminal side of the glutamine residue including position -1, bulk amino acid residues such as tyrosine, proline and phenylalanine are located in the case of the selected sequence for TGase 3. This tendency is specific to TGase 3 and is not observed in TGase 1 and other isozymes.

Among the selected sequences, E51 (PPPYSFYQSRWV) was the most prominent substrate with respect to TGase 3 reactivity and isozyme specificity. This sequence also satisfied the amino acid residue tendency, described previously. Alanine substitutions at positions −2, +1, +2 and +3 of the selected E51 sequence significantly affected reactivity. The results suggest that these residues are essential for interaction with activated TGase 3.

Recently, we established a rapid and sensitive assay system using biotinylated preferred substrate peptide and spermine-coated microtiter plates. The reactivity and specificity of the E51 sequence was maintained in a biotinylated peptide form (pepE51) when the primary amine was used as a glutamine-acceptor substrate. At low concentrations, pepE51 exhibits high reactivity with TG3 and very low reactivity with other isozymes under these enzymatic activities (Fig. 5). In the case of guinea-pig liver TGase, used as TGase 2, a weak cross-reactivity was observed possibly resulting from the co-purification of activated TGase 3. Thus, the synthesized peptide for the E51 sequence represents a valuable tool for studying TGase 3 substrate recognition and enzymatic activity.

Therefore, we examined the ability of the E51 sequence to detect endogenous TGase activity in the

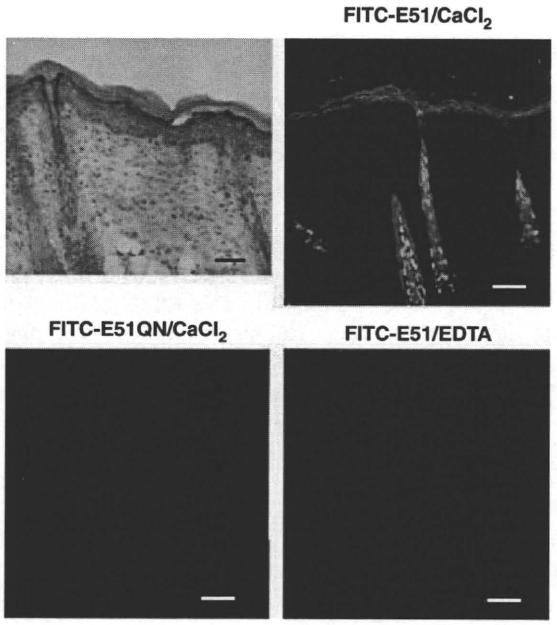

Fig. 6. Detection of *in situ* TGase 3 activities in the mouse skin section. Hematoxylin and eosin staining is shown at the left. FITC–pepE51 (1 μM) was reacted with frozen mouse skin section in the presence of $CaCl_2$. As a negative control, incubation with FITC–pepE51QN and co-presence of EDTA in the reaction of pepE51 were carried out under the same reaction condition. Bar represents 50 μm.

skin, as previously established for TGase 1-preferred substrate peptide, K5 [16]. Using a similar approach, calcium-dependent incorporation of FITC–pepE51 through its glutamine residue into lysine residues of endogenous substrate proteins was observed (Figs 6 and 7). TGase 3 has been observed in both differentiating keratinocytes and hair follicles of the epidermis by immunochemical analyses [38–40]. However, in this study, we present the first direct evidence for the detection of activated TGase 3 in the epidermis. Therefore, this finding provides more precise information on the physiological significance of TGase 3 because this enzyme is synthesized as an inactive zymogen form.

In the epidermis, endogenous TGase 3 activity was observed mostly in the granular and spinous layers. However, the activity was detected within a more limited region when compared with the staining results obtained with FITC–pepK5, a preferred substrate for TGase 1. In addition, in hair follicle cells, the staining pattern of TGase 3 was distinct from that of TGase 1. *In situ* activity of the enzyme was observed mainly around the inner root sheath, which is consistent with results obtained previously using immunostaining analyses [39,40]. By contrast, TGase 3 activity was found around the medulla and hair cortex. These results for TGase 3 in the epidermis and hair follicles are convincing; however, in cells with higher TGase 1 activity, there might be the possibility of a slight cross-reaction with TGase 1.

In a recent study that used immunochemical analysis and *in situ* detection of the activity by FITC-labeled cadaverine, Thibaut *et al.* [40] reported that TGase 3 was mainly present in hair fibers. This is mostly consistent with our results. However, in their study, the

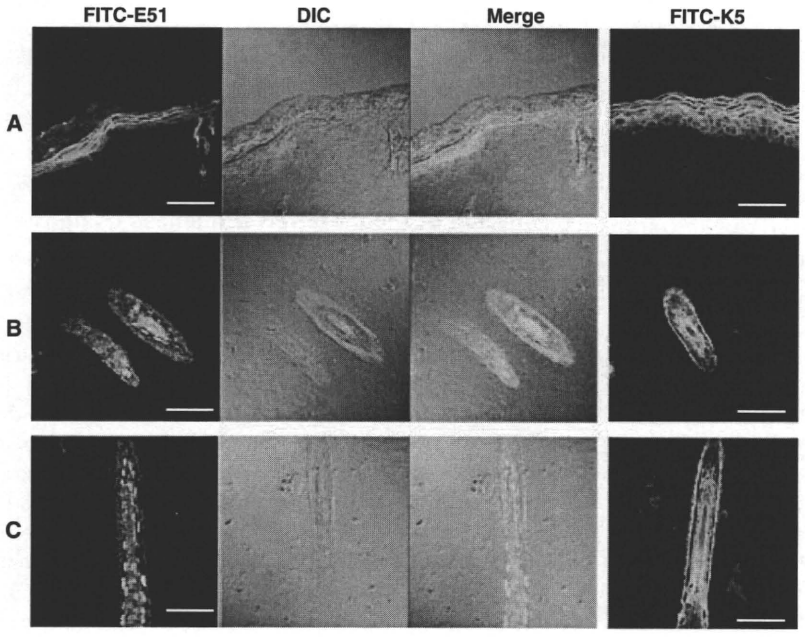

Fig. 7. *In situ* TGase activities detected with FITC-labeled peptides in the mouse skin epidermis and hair follicles. *In situ* activity of TGase 3 was detected under the observation at enlarged scale in the same reaction condition as described in the legend to Fig. 6. From left, FITC–pepE51 (1 μM), differential interference images and their merged images are aligned. FITC–pepK5 (1 μM) was paralleled in each experiment (right). (A) Skin epidermis, (B) transversal and (C) longitudinal sections of hair follicles. Bar represents 50 μm.

detection procedure for TGase *in situ* activity was not specific for TGase 3 in principle, because cadaverine is an amine substrate known to react with any active TGase.

Although aberrant TGase 1 activity has been reported in several skin diseases, as a consequence of genetic mutation [41,42], nothing has been reported regarding a TGase 3 defect in specific pathologies. Investigation of *in situ* activity of TGase 3 is a valuable method for elucidating the precise role of this isozyme in a variety of tissues and cells. Recently, detection of altered enzymatic activities in patients with TGase 1 mutation was successfully achieved using FITC–pepK5 [19]. Because this method is applicable for monitoring aberrant expression of TGase 3 activity, it will assist in the investigation unknown diseases which may be caused by TGase 3 mutations.

In conclusion, we have identified several preferred substrate sequences for TGase 3. The most reactive peptide sequence, E51, permitted the detection of *in vitro* and *in situ* activities of the active enzyme. In addition to pepK5, a specific preferred substrate peptide for TGase 1, pepE51 could become a useful tool to further characterize TGase activity and identify endogenous substrates in the skin and hair follicles.

Experimental procedures

Transglutaminases

For screening, human recombinant TGase 3 obtained by expression and purification from baculovirus-infected insect cells was used, as described previously [27]. For evaluation of the obtained sequences, recombinant human TGases 1, -2 and -3 and purified guinea-pig liver TGase were purchased from Zedira (Darmstadt, Germany) and Sigma (St. Louis, MO, USA). For the activation of TGase 3, the zymogen was proteolyzed by treatment with dispase (Roche, Mannheim, Germany). Human Factor XIII (Fibrogammin^{R}P; ZLB Behring, Marburg, Mannheim, Germany) was activated (Factor XIIIa) by treatment with bovine thrombin (Sigma).

Screening of preferred sequences from a phage-displayed peptide library

Screening was carried out as described previously, using an M13 PhD-12 phage-display system (New England Biolabs Inc., Ipswich, MA, USA) [15]. Briefly, $\sim 1.5 \times 10^{11}$ (first-round panning) phage clones were incubated at 37 °C with dispase-activated TGase 3 (1 ng·μL^{-1}) in 10 mM Tris/HCl (pH 8.0), 150 mM NaCl (TBS buffer) containing 1 mM dithiothreitol, 5 mM $CaCl_2$ and 5 mM bio-Cd [EZ-link™ 5-(biotinamido)pentylamine; Pierce Biotechnology, Rockford, IL, USA]. The catalytic reaction was stopped by the addition of EDTA. The phage particles were precipitated in the presence of poly-(ethylene glycol) and NaCl with salmon sperm DNA as a carrier. Next, phage clones that covalently incorporated bio-Cd were selected by affinity chromatography using mono-avidin gel (SoftLink™ Soft Release Avidin Resin; Promega Corp., Madison, WI, USA). After washing with TBS containing 0.1 or 0.5% Tween 20 and 2 mM EDTA and then with TBS, the bound phage particles were eluted by competition using 5 mM biotin in TBS buffer. The entire eluate was used to infect ER2738 host bacteria to amplify the phages. The phage particles were concentrated by precipitation with poly-(ethylene glygol)–NaCl and then used for subsequent rounds. After panning five times in all, DNA sequences of the displayed peptides of the selected phage clones were determined.

Construction of the expression vector for GST fusion proteins

The vector plasmid pET24d–GST(QN) was used to express modified GST, in which all the glutamine residues were substituted by asparagine residues, and fused with a peptide at the N-terminus and hexahistidine at the C-terminus [15]. The DNA of each phage was isolated and the sequences of the displayed 12-mer peptides were amplified by PCR. The amplified PCR products were digested and inserted into pET24d–GST(QN). To generate peptide mutants in which each amino acid was substituted to alanine, PCR-based mutagenesis was carried out.

Escherichia coli BL21(DE3)LysS or BL21(DE3)LysE was transformed with the plasmids and expression was induced by the addition of isopropyl β-D-thiogalactoside. Recombinant proteins were purified using TALON Metal Affinity Resin according to the manufacture's instructions (BD Bioscience, San Jose, CA, USA). The concentration of the purified protein was determined by quantification of the intensity for the separated bands in SDS/PAGE analysis using imaging software (MULTIGAUGE software; Fujifilm, Tokyo, Japan).

Evaluation of the preferred sequences using the recombinant proteins

The reactivities of recombinant GST(QN)-fusion proteins were evaluated by the incorporation of Dansyl-Cd (Sigma), a fluorescence-labeled pentylamine. Recombinant protein (200 ng·μL^{-1}) and 0.5 mM Dansyl-Cd were incubated in TBS containing 5 mM $CaCl_2$ and 1 mM dithiothreitol in the presence of activated TGase 3 (1 ng·μL^{-1}). Dimethylcasein (Sigma) was used as a positive control at a final concentration of 200 ng·μL^{-1}. The reaction mixture was incubated at 37 °C and then separated by 12.5% SDS/PAGE. A fluorograph of the gel was obtained by UV irradiation (254 nm) to visualize the amount of incorporated Dansyl-Cd. To quantify the results, the fluorescence intensity of each

product was analyzed using imaging software (MULTIGAUGE software).

Evaluation of synthetic peptides as a substrate

The 12-amino acid peptide corresponding to the E51 sequence (PPPYSFYQSRWV) was synthesized and biotinylated at the N-terminus (pepE51). A mutant peptide in which glutamine was substituted to asparagine was also synthesized (PPPYSFYNSRWV) and biotinylated as pepE51QN. TGase 1-, TGase 2- and Factor XIII-preferred substrate biotinylated peptides, being pepK5 (YEQHKLPSSWPF), pepT26 (HQSYVDPWMLDH) and pepF11 (DQMMLPWPAVAL), respectively, were used for comparison.

To evaluate the activity and specificity of the peptides, a microtiter plate assay was performed as described previously [32,33]. Spermine, as a primary amine, was immobilized covalently onto microplates. The enzyme reaction mixture, in a total volume of 100 μL, contained biotinylated peptide in the presence of the enzymes in an appropriate buffer (final concentration: 20 mM Tris/HCl, pH 8.3, 140 mM NaCl, 2.5 mM dithiothreitol, 15 mM $CaCl_2$). The microtiter plates were incubated at 37 °C for the indicated time intervals and the reaction was stopped by the addition of EDTA (50 mM at final concentration). The wells were then washed with a Tris-based buffer (10 mM Tris/HCl, pH 8.0, 150 mM NaCl, 0.1% Tween-20). The incorporated biotinylated peptides were detected using streptavidin-peroxidase (Rockland Immunochemicals Inc., Gilbertsville, PA, USA) and the peroxidase substrate 3,3′,5,5′-tetramethylbenzidine (Sigma).

Detection of *in situ* TGase activities in the mouse skin sections

Animal care and experiments were conducted according to the Regulations for Animal Experiments in Nagoya University.

Immediately after the mice had been killed by diethylether anesthetization, the skin was dissected and embedded in medium (Sakura Finetek, Tokyo, Japan) as a standard method. Frozen sections were dissected into 4–8 μm slices and kept frozen until use. Fluorescence-labeled peptides (FITC–pepE51, FITC–pepE51QN and FITC–pepK5) were synthesized.

For the reaction, sections were dried and then blocked by incubation in NaCl/P_i containing 1% BSA (Sigma) for 30 min at room temperature. Sections were incubated for 90 min with a solution containing 100 mM Tris/HCl (pH 8.0), 5 mM $CaCl_2$ or 5 mM EDTA and 1 mM dithiothreitol, in the presence of FITC-labeled peptide at 37 °C. After washing with NaCl/P_i three times, anti-fading solution was mounted onto the section with a cover-glass. Differential interference images and fluorescence were analyzed with a confocal laser-scanning microscope, LSM5 PASCAL (Zeiss, Göttingen, Germany). For hematoxylin and eosin staining, the tissue section was fixed, then stained using standard methods and analyzed with a microscope, BZ-8100 (Keyence, Osaka, Japan).

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Supporting information

The following supplementary material is available:
Fig. S1. Screening procedure for substrate sequences preferred by TGase 3 using a phage-displayed random peptide library.

This supplementary material can be found in the online version of this article.

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REVIEW ARTICLE

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FLG mutations in ichthyosis vulgaris and atopic eczema: spectrum of mutations and population genetics

M. Akiyama

Department of Dermatology, Hokkaido University Graduate School of Medicine, Sapporo, Japan

Summary

Filaggrin is a key protein involved in skin barrier function. Mutations in the gene encoding filaggrin (*FLG*) have been identified as the cause of ichthyosis vulgaris and have been shown to be major predisposing factors for atopic eczema (AE), initially in European populations. Subsequently, *FLG* mutations were identified in Japanese, Chinese, Taiwanese and Korean populations. It was demonstrated that *FLG* mutations are closely associated with AE in the Japanese population. Notably, the same *FLG* mutations identified in the European population were rarely found in Asians. These results exemplify differences in filaggrin population genetics between Europe and Asia. For mutation screening, background information needs to be obtained on prevalent *FLG* mutations for each geographical population. It is therefore important to establish the global population genetics maps for *FLG* mutations. Mutations at any site within *FLG*, even mutations in C-terminal imperfect filaggrin repeats, cause significant reductions in amounts of profilaggrin/filaggrin peptide in patient epidermis as the C-terminal region is essential for proper processing of profilaggrin into filaggrin. Thus, no genotype–phenotype correlation has been observed in patients with *FLG* mutations. A restoration of the barrier function seems a feasible and promising strategy for treatment and prevention in individuals with filaggrin deficiency.

Correspondence
Masashi Akiyama.
E-mail: akiyama@med.hokudai.ac.jp

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Mutations in FLG, the gene encoding profilaggrin/filaggrin, have been identified as the underlying cause of ichthyosis vulgaris (IV; OMIM 146700)[1] and have also been shown to predispose patients to atopic eczema (AE).[2] Although FLG is very difficult to analyse because of its large size (> 12 kb) and highly repetitive nature, a polymerase chain reaction (PCR) strategy that permits routine and comprehensive sequencing of the entire coding region has recently been developed.[3] Using this method and the information from other identified mutant alleles, filaggrin mutation searches have been carried out in a variety of geographical populations including European and Asian populations. Based on the information of population-specific *FLG* mutations, many cohort studies of AE for *FLG* mutations have been performed and approximately 25–50% of patients with AE were revealed to harbour *FLG* mutations as a predisposing factor.

Skin barrier defects caused by *FLG* mutations are thought to play a crucial role in the pathogenesis of atopic disorders including AE, asthma and allergic rhinitis. Recently, it was demonstrated that mice deficient in filaggrin expression show enhanced transfer of antigens through the epidermis, thus providing compelling experimental proof for the barrier hypothesis in AE pathogenesis.[4] This review provides an overview of *FLG* population genetics because the information is essential for global *FLG* mutation screening in patients with AE.

Filaggrin is an indispensable component for the skin barrier

Filaggrin is initially synthesized as profilaggrin, a > 400-kDa, highly phosphorylated, histidine-rich polypeptide, which comprises a S100 calcium-binding domain, a B-domain and two imperfect filaggrin-repeat domains flanking 10–12 essentially identical filaggrin repeats, as well as a C-terminal domain.[3,5] During the keratinization of epidermal keratinocytes, keratohyaline granule degradation products subsequently occupy the cytoplasm of keratinized cells in the stratum corneum and play important roles in skin barrier function.[6] Keratohyaline granules in the granular layer of the epidermis are predominantly composed of profilaggrin polyproteins.[7–9]

Upon keratinocyte terminal differentiation, profilaggrin is dephosphorylated and cleaved into 10–12 essentially identical 37-kDa filaggrin peptide units. The liberated filaggrin subsequently and highly efficiently aggregates the keratin filament cytoskeleton,[7] causing the collapse of the granular cells into

flattened squames. The collapsed cytoskeleton is crosslinked by transglutaminases to bind it to the cornified cell envelope. Filaggrin degradation products also contribute to moisture retention in the cornified layers as a natural moisturizing factor.[6,10] Thus, filaggrin is a key epidermal protein essential for the formation of a normal, intact, protective and correctly moisturized skin barrier.[6,11]

Filaggrin deficiency caused by *FLG* mutations results in ichthyosis vulgaris

IV is a common inherited skin disorder exhibiting scaling and dry skin typically on the flexor limbs and lower abdomen, associated with palmoplantar hyperlinearity.[1,11,12] Histologically, IV is characterized by a decrease in the size and number or complete absence of keratohyaline granules in the upper epidermis.[12] Marked reduction in epidermal keratinocyte filaggrin due to *FLG* loss-of-function mutations was identified as the cause of IV.[1] Loss or reduction of filaggrin expression correlates with excessively dry skin and impaired barrier function, which variously manifests as IV.

Filaggrin mutations are a major predisposing factor for atopic eczema in Europe

AE is among the most common diseases in children from developed countries. Despite considerable efforts to elucidate AE susceptibility genes and to clarify the genetic background of atopic disorders, until recently no strong and reproducible genetic factor has been identified.[13] It has long been proposed that a permeability barrier abnormality in AE is not just an epiphenomenon but is rather an important driver of disease activity[14] because the level of the permeability barrier abnormality precisely parallels AE severity[15,16] and both clinically uninvolved skin regions and skin sites cleared of inflammation for as long as 5 years continue to show significant barrier abnormalities.[17]

As mentioned above, filaggrin is a major epidermal moisturizing factor and significantly contributes to the skin barrier function. For a long time, we as dermatologists have realized that AE often occurs in patients with IV,[18–20] although the pathophysiological mechanisms of this co-occurrence have not been fully clarified. Linkage of AE to the chromosome locus 1q21, containing the epidermal differentiation complex where *FLG* resides, has also been reported.[21] In addition, decreased filaggrin expression has been reported in the skin of patients with AE at both mRNA and protein levels.[22,23] Palmer *et al.*[2] initially reported that decreased or absent *FLG* expression due to loss-of-function mutations leads to impaired barrier function which manifests as AE.

Subsequently, it was confirmed that the strong effect of *FLG* mutations on AE risk exceeds that of any other candidate predisposing gene for AE identified so far.[24] A correlation between *FLG* mutations and eczema is one of the most robust genotype–phenotype linkages in complex trait genetics and several case–control association studies have been reported to date.[3,24–30] These studies have established *FLG* as a major genetic factor predisposing for AE, although they showed considerable differences in study design and strength of the genetic effect.

Henderson *et al.*[31] sought to determine the natural history and course of atopic diseases conferred by the two most common *FLG* mutations in a large, population-based birth cohort study in the U.K. and reported that eczema associated with these *FLG* mutations presents in early life and is more persistent. The risk of asthma was remarkably high in the context of eczema and firm associations were confirmed with sensitization to multiple allergens including grass, house dust mite and cat dander.

Prevalent filaggrin mutations are distinct in each population

To date, it has generally been considered that *FLG* mutations are a significant predisposing factor for AE in Europeans, Asians and quite possibly most other races worldwide to differing degrees.

Mutations in *FLG* were initially identified in European families. After the establishment of sequencing methods for the entire *FLG* coding region,[1–3] to date approximately 40 loss-of-function *FLG* mutations have been identified in IV and/or AE.[32,33]

Major differences exist in the spectra of *FLG* mutations observed between certain globally distinct ancestral groups. In the European population, the genetic spectrum of *FLG* mutations is complicated, with up to six recurrent mutations and several other family-specific mutations, and the two mutations R501X and 2282del4 are the most prevalent in the U.K. population (Fig. 1).[3]

From 2006 to date, to establish baseline *FLG* mutation data in the Japanese population, we performed *FLG* mutation searches in more than 30 Japanese families with IV. We carried out comprehensive sequencing of the entire *FLG* coding region using an overlapping PCR strategy and identified four Japanese population-specific mutations in *FLG*, c.3321delA, p.Ser2554X, p.Ser2889X and p.Ser3296X.[34,35] Two *FLG* mutations among them, p.Ser2889X and p.Ser3296X, were reported later by another Japanese group independently using shotgun methods.[36] In 2009, we reported two additional novel *FLG* mutations, p.Ser1695X and p.Gln1701X, in the Japanese population.[37] Furthermore, we studied 19 newly recruited Japanese patients with AE and identified a novel *FLG* nonsense mutation c.12069A>T (p.Lys4021X) in one patient with AE without any other known Japanese *FLG* mutation (Fig. 1).[33] In addition, one of the common European mutations p.Arg501X was reported in a Japanese family, although the mutant allele with p.Arg501X reported in the Japanese family was shown to be on a different haplotype from the common European variant of the same residue.[38] Thus, the Japanese p.Arg501X mutation was thought to arise separately.[38] This p.Arg501X mutation is a CpG mutation and can arise commonly as well as being present in Europeans as an

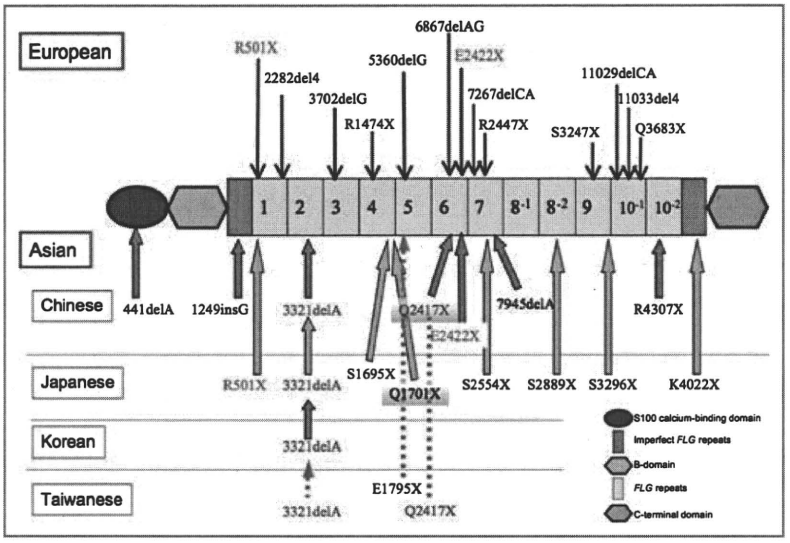

Fig 1. Reported *FLG* mutations shown in a scheme of profilaggrin peptide. Profilaggrin contains 10–12 highly homologous filaggrin-repeat domains. Note that *FLG* mutations in the European and the Asian populations appear to be unique in each population. Only two mutations shown in green (R501X and E2422X) were reported in both European and Asian populations. 3321delA, shown in red, was found in all the four East Asian populations. Q2417X, shown in blue, was reported in both Chinese and Taiwanese populations. Mutations are distributed widely in the profilaggrin sequence and the mutation p.Lys4022X (K4022X) we reported recently[29] is the most distal mutation located in the C-terminal incomplete filaggrin repeat. Some individuals have duplication of the 8th and/or 10th filaggrin repeat(s). Duplicated filaggrin repeats are represented as 8^{-1}, 8^{-2}, 10^{-1} and 10^{-2}.

ancient, ancestral mutation. In total, there are at least eight *FLG* variants in the Japanese population.

A Japanese AE case–control study for the eight *FLG* mutations demonstrated that about 27% of the patients in our Japanese AE case series carry one or more of the eight *FLG* mutations and that these variants are also carried by 3·7% of Japanese control individuals.[33] It was thus confirmed that *FLG* mutations are significantly associated with AE in the Japanese population (Fig. 2).

In other Asian populations, for example the Singaporean Chinese population, it was reported that *FLG* mutations are again different from those found in Europeans and Japanese.[39] In total, six *FLG* mutations, five previously unreported mutations and one known mutation, were found in eight Singaporean Chinese patients with IV.[39] The known mutation was previously identified in a single patient with IV from the Netherlands[3] and, in fact, the patient had Chinese ancestry.[39]

Examining the Taiwanese population, we examined 12 individuals from four unrelated Taiwanese families with IV and identified three *FLG* mutations.[40] One mutation, E1795X, was a previously unidentified *FLG* mutation which might be Taiwanese specific. Interestingly, another *FLG* mutation, 3321delA, is prevalent both in the Japanese population[34] and the Chinese population.[3] This mutation 3321delA was also reported in a Korean patient with IV.[41] The other mutation, Q2417X, was found in the Singaporean Chinese population. No *FLG* mutation identified in the European population was found in the Taiwanese population. The present findings suggest that the Taiwanese population, as an East Asian group, shares *FLG* mutations with both the Japanese and the Chinese populations. These results exemplify differences in filaggrin population genetics between Europe and Asia (Fig. 1).

As mentioned above, most *FLG* mutations are specific to each population, such as European,[3] Japanese,[33,35,37] Singaporean Chinese[39] and Taiwanese.[40] Major differences exist in the spectra of *FLG* mutations observed between different ancestral groups. Prevalent *FLG* mutations are distinct in both the European and the Asian populations. In addition, there is a need for assessing the ancestral admixture in geographical regions in order to know precisely the spectrum and preferential occurrence of *FLG* mutations in different populations.

Every population is likely to have a unique set of *FLG* mutations. Population differences highlighted by *FLG* mutations make it difficult to perform world-wide screening for *FLG* mutations in patients with AE. We cannot perform *FLG* mutation screening in one population using the *FLG* mutations reported in other populations. For example, we cannot use the prevalent European *FLG* mutations when we perform screening of Asian patients with AE. For the mutation screening, we have to obtain information on prevalent *FLG* mutations in each population. It is therefore important to establish global population genetic maps for patient *FLG* mutations.

No genotype–phenotype correlation in *FLG* mutations so far

Genotype–phenotype correlation in *FLG* mutations is lacking. *FLG* truncation mutations at any site within the profilaggrin

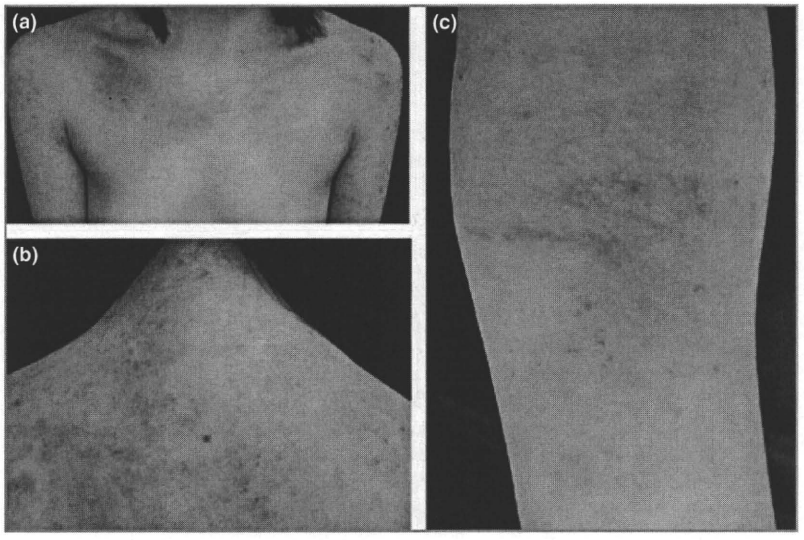

Fig 2. Clinical features of a patient with atopic eczema with compound heterozygous *FLG* mutations. (a) Erythematous lesions and reddish papules with scratch marks and lichenification are seen on the chest (a), back (b) and arm (c). Mutation screening revealed that the patient is a compound heterozygote for *FLG* mutations c.3321delA and p.Ser2554X.

peptide were reported uniformly to result in severe deficiency of profilaggrin/filaggrin processing.[3] It has been hypothesized that the C-terminal region of profilaggrin is essential for proper processing of profilaggrin to filaggrin and, in due course, truncation at any site of profilaggrin results in abolishment of filaggrin/profilaggrin peptides.[3] The nonsense mutation p.Lys4022X that we identified most recently[33] is in the C-terminal incomplete filaggrin repeat and is the most distal mutation among those previously reported. In the epidermis of the patients carrying this mutation, *FLG* mRNA expression including messages derived from the mutant alleles was not significantly reduced. However, histopathologically the size of keratohyaline granules in the granular layers decreased and immunohistochemically profilaggrin/filaggrin peptides were remarkably reduced in the patients' epidermis.[33] These observations further support the hypothesis that the profilaggrin C-terminal region is essential for proper profilaggrin processing. In this context, it is now generally considered that all the truncation mutations lead to serious loss of profilaggrin/filaggrin peptides, resulting in a lack of genotype–phenotype correlations as regards *FLG* mutations in IV or AE.

Novel skin barrier-oriented care and prevention approach to atopic eczema

The concept of epidermal barrier dysfunction caused by *FLG* mutations as a major contributor to the pathogenesis of AE has opened up a new era over the past few years. It is now believed that, at least in a subset of patients with AE, the skin barrier defect is the primary event that initiates disease pathogenesis, allowing the entrance of numerous antigens into the epidermis.[42] Thus, restoration of barrier function seems a feasible and promising strategy for prophylactic treatment of AE in an individual with a filaggrin deficiency.

The range of clinically valuable methods to restore skin barrier function in individuals harbouring *FLG* mutations includes general moisturization measures, or specific lipid replacement therapy. Moisturizers have already been widely used in AE[43] and have been shown to reduce topical steroid use by a specialist dermatology nurse.[44] Lipid replacement therapy is well under development as a triple-lipid, ceramide-dominant, barrier repair therapy for AE, that is provided in an acidic formulation.[43]

One clinical study supports the efficacy of targeted, ceramide-dominant lipid replacement therapy in AE.[15] In the study, topical application of a ceramide-dominant, physiological lipid-based emollient improved skin barrier defects and reduced AE severity significantly in the majority of patients.

Regarding the association between filaggrin deficiency and sensitization to specific antigens: during early life allergen exposure may increase the risks of AE, but the protective effect of reduction in allergen exposure remains uncertain. According to the population-based, longitudinal birth cohort study by Henderson *et al.*,[31] eczema associated with *FLG* mutations presents in early life and is more persistent. In addition, a strong association of *FLG* mutations was identified with sensitization to grass, house dust mite and cat dander. Our study revealed that AE disease severity and specific IgE for house dust, mite allergen and cat dander were significantly correlated in *FLG* mutation-related patients with AE.[45]

In this context, if we select patients with *FLG* mutations and perform early intervention to reinforce/improve their skin barrier function and reduce sensitization to allergens, we may achieve a significant prophylactic effect against AE development. Further studies are required to clarify the preventive effect of early intervention to AE in filaggrin-deficient, high-risk children.

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MUTATION UPDATE

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ABCA12 Mutations and Autosomal Recessive Congenital Ichthyosis: A Review of Genotype/Phenotype Correlations and of Pathogenetic Concepts

Masashi Akiyama*

Department of Dermatology, Hokkaido University Graduate School of Medicine, Sapporo, Japan

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ABSTRACT: Mutations in *ABCA12* have been described in autosomal recessive congenital ichthyoses (ARCI) including harlequin ichthyosis (HI), congenital ichthyosiform erythroderma (CIE), and lamellar ichthyosis (LI). HI shows the most severe phenotype. CIE and LI are clinically characterized by fine, whitish scales on a background of erythematous skin, and large, thick, dark scales over the entire body without serious background erythroderma, respectively. To date, a total of 56 *ABCA12* mutations have been reported in 66 ARCI families including 48 HI, 10 LI, and 8 CIE families of African, European, Pakistani/Indian, and Japanese origin (online database: http://www.derm-hokudai.jp/ABCA12/). A total of 62.5% of reported *ABCA12* mutations are expected to lead to truncated proteins. Most mutations in HI are truncation mutations and homozygous or compound heterozygous truncation mutations always results in HI phenotype. In CIE families, at least one mutation on each allele is typically a missense mutation. Combinations of missense mutations in the first ATP-binding cassette of ABCA12 underlie the LI phenotype. ABCA12 is a keratinocyte lipid transporter associated with lipid transport in lamellar granules, and loss of ABCA12 function leads to a defective lipid barrier in the stratum corneum, resulting in an ichthyotic phenotype. Recent work using mouse models confirmed ABCA12 roles in skin barrier formation.
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KEY WORDS: ABCA12; congenital ichthyosiform erythroderma; harlequin ichthyosis; lamellar ichthyosis

Introduction

Severe autosomal recessive congenital ichthyoses (ARCI) can be devastating to patients' quality of life in those seriously affected, even though other organs are uninvolved. ARCI comprises three major subtypes, harlequin ichthyosis (HI; MIM# 242500), congenital ichthyosiform erythroderma (CIE; MIM# 242100), and lamellar ichthyosis (LI; MIM#s 242300, 604777, 601277, 606545) [Akiyama and Shimizu, 2008]. HI is the most devastating congenital ichthyosis, and affected newborns show large, thick, plate-like scales over the whole body with severe ectropion, eclabium, and flattened ears [Akiyama, 2006a]. Patients with CIE demonstrate fine, whitish scales on a background of erythematous skin over the whole body. Conversely, LI is clinically characterized by large, thick, dark scales over the entire body surface without a serious background erythroderma [Akiyama et al., 2003].

Because transglutaminase 1 gene (*TGM1*; MIM# 190195) mutations were identified as the cause in LI in 1995 [Huber et al., 1995; Russell et al., 1995], significant progress has recently been made in the understanding of the molecular basis of the human epidermal keratinization processes, and mutations in several other genes have also been identified in ARCI. In HI cases, only ABCA12 mutations have been reported as underlying genetic defects [Akiyama and Shimizu, 2008]. In contrast, CIE and LI are both heterogeneous genetic disorders and several causative or underlying molecules including ABCA12 have been identified [Jobard et al., 2002; Lefèvre et al., 2003, 2004; Lefèvre, 2006]. Mutations in six genes have been described in non-HI ARCI to date, including *TGM1* [Huber et al., 1995; Russell et al., 1995], *ABCA12* [Lefèvre et al., 2003; Natsuga et al., 2007], *NIPAL4* (also known as *ICHTHYIN*) [Lefèvre et al., 2004], *CYP4F22* [Lefèvre, 2006], *ALOX12B* and *ALOXE3* [Jobard et al., 2002]. Among them, *TGM1* is thought to be the most prevalent causative gene [Fischer, 2009; Herman et al., 2009]. *TGM1* encodes transglutaminase 1, which is expressed in the upper epidermis and catalyzes crosslinking of cornified cell envelope precursor proteins to form a cornified cell envelope in the stratum corneum [Herman et al., 2009]. *NIPAL4* (or *ICHTHYIN*) encodes a protein of unknown function. ALOX12B and ALOXE3 encode for arachidonate 12(R)-lipoxygenase and arachidonate lipoxygenase-3, respectively. The protein product of CYP4F22, a cytochrome P450 protein, and the two lipoxygenases arachidonate 12(R)-lipoxygenase and arachidonate lipoxygenase-3 are part of the lipid metabolism pathway involved in the formation of ω-hydroxyceramides from arachidonic acid [Brash et al., 2007]. ABCA12 (MIM# 607800) missense mutations leading to defects in the ATP-binding cassette were reported in LI cases (type 2 LI [MIM# 601277]) [Lefèvre et al., 2003] and ABCA12 truncation mutations were reported underlying HI patients [Akiyama et al., 2005; Kelsell et al., 2005]. Recently, we reported that *ABCA12* missense mutations are a major cause of CIE cases in the Japanese population [Akiyama et al., 2008; Natsuga et al., 2007; Sakai et al., 2009]. Thus, *ABCA12* mutations lead to all three ARCI clinical phenotypes including HI, LI and CIE and *ABCA12* mutations are highlighted as one of the major causes of ARCI.

Additional Supporting Information may be found in the online version of this article.

*Correspondence to: Masashi Akiyama, Department of Dermatology, Hokkaido University Graduate School of Medicine, North 15 West 7, Sapporo 060-8638, Japan. E-mail: akiyama@med.hokudai.ac.jp

ABCA12 is a member of the large superfamily of the ATP-binding cassette (ABC) transporters [Annilo et al., 2002], which bind and hydrolyze ATP to transport various molecules across a limiting membrane or into a vesicle [Borst and Elferink, 2002]. The ABCA subfamily members are thought to be lipid transporters [Peelman et al., 2003]. ABCA12 was recognized as a key molecule in keratinocyte lipid transport [Akiyama et al., 2005; Sakai et al., 2007]. ABCA12 is a keratinocyte transmembrane lipid transporter protein associated with lipid transport in lamellar granules to the apical surface of granular layer keratinocytes [Akiyama et al., 2005]. In this article, the importance of *ABCA12* mutations as a cause for ARCI is reviewed and a genotype/phenotype correlation of ARCI with *ABCA12* mutations is discussed.

ABCA12 Mutations

A review of the literature was performed to identify all of the known *ABCA12* mutations. To date, 56 *ABCA12* mutations have been described (online database: http://www.derm-hokudai.jp/ABCA12/) in 66 unrelated families including 48 HI, 10 LI and 8 CIE families (Supp. Table S1 and Fig. 1). Nucleotide numbering reflects cDNA numbering with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence (GenBank NM_173076.2), according to journal guidelines (www.hgvs.org/mutnomen). The initiation codon is codon 1. Mutations have been reported among ARCI patients with African, European, Pakistani/Indian, and Japanese backgrounds, from almost all over the world. Of the 56 mutations, 36% (20) are nonsense, 25% (14) are missense, 20% (11) comprise small deletions, 11% (6) are splice site, 5% (3) are large deletions, and 4% (2) are insertion mutations. At least, 62.5% (35) of the total reported mutations are predicted to result in truncated proteins. There is no apparent mutation hot spot in *ABCA12*, although mutations underlying LI phenotype are clustered in the region of the first ATP-binding cassette [Lefèvre et al., 2003].

The most common reported mutation in *ABCA12* is c.7322delC (p.Val2442SerfsTer28) in exon 49, which has been reported in seven HI families with Pakistani background [Kelsell et al., 2005; Thomas et al., 2006, 2008]. This mutation has been identified only in the Pakistani population. Thomas et al. [2008] reported that 80% of HI patients and parents (10 screened) originated from the Pakistani/Indian area had the mutation 7322delC. Microsatellite-based haplotype analysis of the genomic region harboring *ABCA12* in three patients homozygous for the mutation c.7322delC suggested that c.7322delC is a possible founder mutation in the Pakistani population [Thomas et al., 2008]. The second most common reported *ABCA12* mutation is a missense mutation p.Asn1380Ser in Walker A motif of the first ATP-binding cassette, which is essential for the transporter function of ABCA12 [Lefèvre et al., 2003]. This missense mutation p.Asn1380Ser has been identified in five LI families from Africa (three families from Morocco and two families from Algeria) [Lefèvre et al., 2003]. Haplotype analysis confirmed that p.Asn1380Ser is a founder mutation in the patients from Morocco/Algeria region [Lefèvre et al., 2003]. Out of further 10 different *ABCA12* mutations, each mutation has been identified in two unrelated families from certain geographic regions. Among these 10 mutations, 5 *ABCA12* mutations, c.2021_2022del2, c.3295−2A>G, p.Thr1387del, p.Arg1950Ter, and p.Arg2482Ter, were found in two independent patients from Japan [Akiyama et al., 2005, 2006a, 2007a; Sakai et al., 2009]. As for the other five mutations, p.Trp1294Ter, p.Gly1651Ser, p.Tyr1090Ter, c.2025delG, and p.Trp1744Ter were found in two independent families with Pakistani [Rajpar et al., 2006; Thomas et al., 2006], Algeria [Lefèvre et al., 2003], Albanian/Bosnian [Thomas et al., 2008], Anglo-Saxon [Thomas et al., 2006], and native American [Kelsell et al., 2005] origins, respectively. These data suggest the presence of founder mutations in patients in Pakistani/Indian, African, European, and Japanese origins.

Clinical Significance; Prevalence of *ABCA12* Mutations as a Causative Gene for ARCI Patients

ARCI is a basket diagnosis, and HI, CIE, and LI are the major subtypes comprising the ARCI group. Among the 48 HI families in whom *ABCA12* mutation analysis has been reported (Supp. Table S1), ABCA12 mutations have been identified in all HI families; the *ABCA12* mutation detection rate is 100% (48/48) in the HI families. Kelsell et al. [2005] reported one HI patient in whom *ABCA12* mutation was not detected by direct sequencing analysis. However, multiplex PCR and oligonucleotide array analysis subsequently revealed deletion of exon 8 in this patient [Thomas et al., 2006]. In this context, HI is thought to be genetically homogeneous for causal *ABCA12* mutations.

In contrast, CIE and LI, the other two ARCI subtypes, to date, six genes, *ABCA12* [Lefèvre et al., 2003], *TGM1* [Huber et al., 1995; Russell et al., 1995], *ALOX12B* (MIM# 603741) [Jobard et al., 2002], *ALOXE3* (MIM# 607206) [Jobard et al., 2002], *ICHTHYIN* (MIM# 609383) [Lefèvre et al., 2004] and *FLJ39501* (MIM# 611495) [Lefèvre, 2006], have been reported to cause CIE; and four out of these six, *ABCA12* [Lefèvre et al., 2003], *TGM1* [Huber et al., 1995; Russell et al., 1995], *ALOX12B* [Jobard et al., 2002], and *ICHTHYIN* [Lefèvre et al., 2004], are also known to underlie LI. Recently, Fischer [2009] reported that in her cohort of 520 patients from 520 independent families with LI and CIE, causative mutations were detected by direct sequencing analysis in 78% of the patients. Only 5% of the patients were harbored *ABCA12* mutations although only exons 28–32 of *ABCA12* were sequenced for the majority of the patients in this study [Fischer, 2009]. The results suggest that ABCA12 is rather a minor cause for ARCI probably in the European and African populations, although the exact ethnic background of the 520 families was not provided in the report. Different from the situation in Europe, from the results of our mutation search, *ABCA12* mutations were frequently found in CIE families, but not in LI families, at least in the Japanese population [Sakai et al., 2009]. Thus, there might be a difference in the prevalence of causative genes for CIE and LI between the global subpopulations.

Genotype–Phenotype Correlation in *ABCA12* Mutations

Several genotype/phenotype correlations with *ABCA12* mutations have now come to light.

In HI (Supp. Fig. S1A), 44 *ABCA12* mutations were reported to date. Among them, most mutations are truncation mutations including nonsense mutations, frameshift mutations (deletion/insertion mutations), and splice site mutations (Table 1). Other mutations reported in HI families are missense mutations, exon deletions, and single amino acid deletions.

Most truncation or deletion mutations underlying HI are thought to lead to severe loss of ABCA12 protein function affecting important nucleotide-binding fold domains and/or transmembrane domains. Thus far, in HI patients, at least one mutation on each allele must be a truncation or deletion mutation

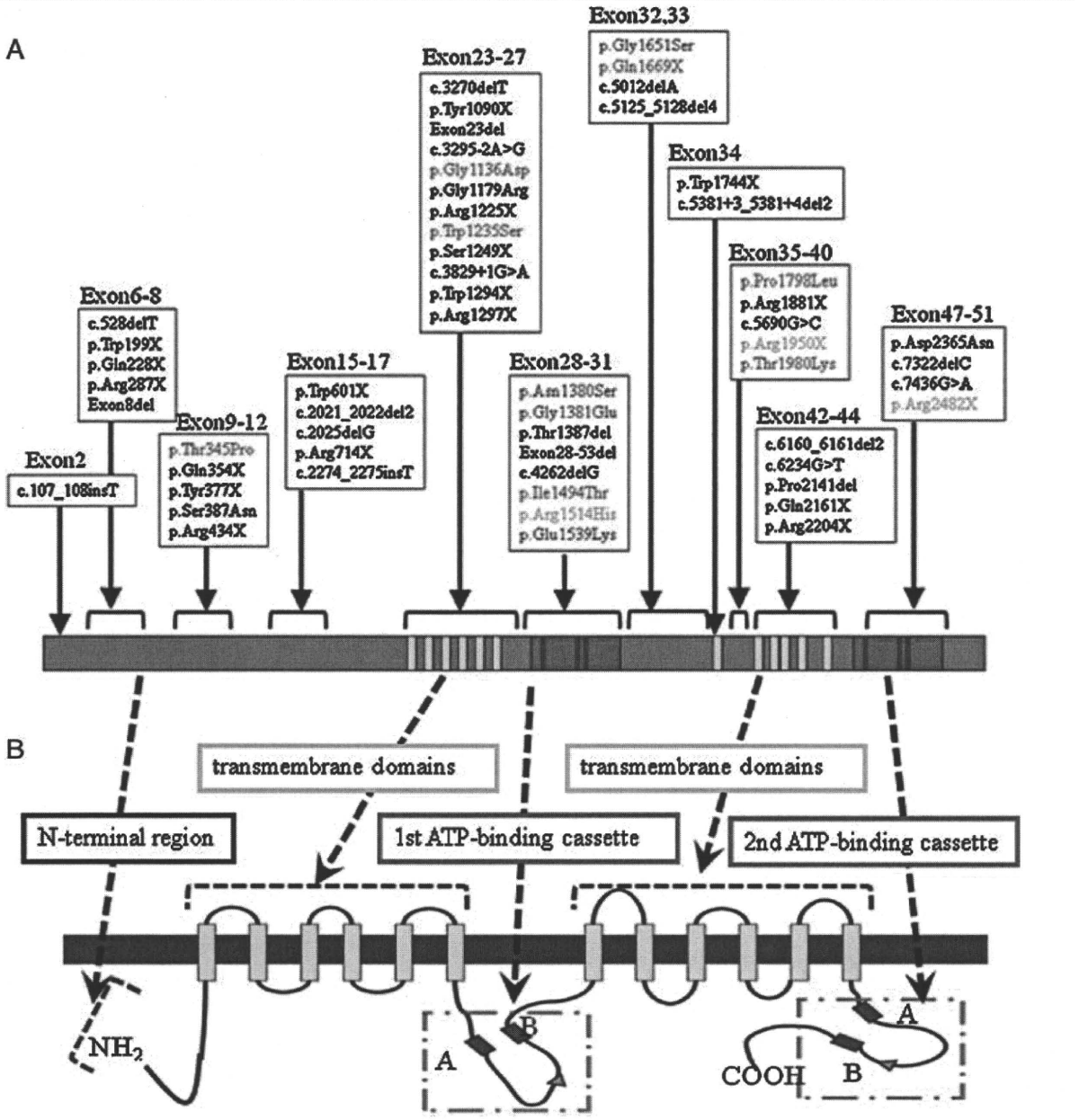

Figure 1. *ABCA12* mutations associated with autosomal recessive congenital ichthyosis. **A:** Reported *ABCA12* mutations and their localization within the *ABCA12* cDNA sequence. Mutations in black, red, and blue characters underlie HI, CIE, and LI, respectively. Mutations in green letters lead to two distinct phenotypes, p.Arg1950Ter and p.Arg2482Ter both result in CIE and HI phenotypes; p.Arg1514His underlies both CIE and LI phenotypes. Nucleotide numbering reflects cDNA numbering with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence (GenBank NM_173076.2), according to journal guidelines (www.hgvs.org/mutnomen). The initiation codon is codon 1. **B:** ABCA12 protein structure and domains. Analysis of the ABCA12 predicted protein disclosed features that are typical of ABCA transporters, and the position of the conserved ATP-binding cassettes as well as that of the two transmembrane domains, each composed of six well-defined hydrophobic helices [Annilo et al., 2002]. See Supp. Table S1 for a complete list of mutations with both DNA and protein names.

within a conserved region to cause serious loss of ABCA12 function [Akiyama et al., 2005, 2006a,b, 2007a, b; Castiglia et al., 2009; Kelsell et al., 2005; Rajpar et al., 2006; Thomas et al., 2006, 2008]. Complete loss of ABCA12 function due to homozygous or compound heterozygous truncation mutations always results in the HI patient phenotype (Table 1).

In contrast, most mutations underlying LI and CIE are missense mutations and are expected to affect ABCA12 function more modestly.

In LI, five *ABCA12* mutations were reported in nine families and all the patients were homozygotes or compound heterozygotes for the mutations [Lefèvre et al., 2003]. None of the LI mutations was demonstrated to cause HI phenotype, although one mutation p.Arg1514His was identified to result in both LI and CIE phenotypes (Supp. Table S1). All the five mutations were missense mutations resulting in only one amino acid alteration in the first ATP-binding cassette of the ABCA12 peptide [Lefèvre et al., 2003] (Table 1). All the families were from Africa [Lefèvre

Table 1. Genotype/Phenotype Correlation in *ABCA12* Mutations in Harlequin Ichthyosis (HI), Congenital Ichthyosiform Erythroderma (CIE), and Lamellar Ichthyosis (LI)

Genotype →	Phenotype
[truncation] + [truncation]	HI
[truncation] + [exon or conserved amino acid deletion]	HI
[exon or conserved amino acid deletion] + [exon or conserved amino acid deletion]	HI
[truncation] + [missense]	HI, CIE
[exon or conserved amino acid deletion] + [missense mutation]	HI, CIE
[missense] + [missense]	LI, CIE
Phenotype →	**Genotype**
HI	[truncation] + [truncation]
	[truncation] + [exon or conserved amino acid deletion]
	[exon or conserved amino acid deletion] + [exon or conserved amino acid deletion]
	[truncation] + [missense mutation]
	[exon or conserved amino acid deletion] + [missense mutation]
LI	[missense] + [missense]
CIE	[missense] + [missense]
	[missense] + [truncation]
	[missense mutation] + [exon or conserved amino acid deletion]

et al., 2003]. These LI patients showed clinically generalized LI with large dark pigmented scales, ectropion, palmoplantar keratoderma and no erythema. They were born as collodion babies.

CIE patients (Supp. Fig. S1B) were also reported to harbor *ABCA12* mutations as the causative genetic defect [Akiyama et al., 2008; Natsuga et al., 2007; Sakai et al., 2009]. To date, 10 *ABCA12* mutations have been reported in eight CIE families. Two mutations, p.Arg1950Ter and p.Arg2482Ter, were identified to cause both CIE and HI disease phenotypes (Supp. Table S1). Only one mutation p.Arg1514His was reported to underlie both CIE and LI phenotypes (Supp. Table S1). In CIE, most underlying mutations are missense mutations. At least one mutation on each allele is a missense mutation in CIE (Table 1). In the CIE cases with *ABCA12* mutations, the scales are slightly larger than those in typical CIE cases and are classified as "medium sized" rather than "fine" scales.

Intrafamilial variation, for example, of CIE and HI cases from one family, has never as yet been reported. Thus, there is no evidence that any other gene(s) in these patients play a noticeable role affecting the phenotypes.

Further accumulation of patients and their *ABCA12* mutation data is needed to better elucidate genotype/phenotype correlations and will aid in predicting patients' prognosis.

Biological Significance; Pathomechanisms of Ichthyosis Involving *ABCA12* Mutations

In HI affected epidermis, several morphologic abnormalities including abnormal lamellar granules in the keratinocyte granular layer and a lack of extracellular lipid lamellae within the stratum corneum had been reported [Akiyama et al., 1994, 1998; Dale et al., 1990; Milner et al., 1992]. Lack of ABCA12 function subsequently leads to disruption of lamellar granule lipid transport in the upper keratinizing epidermal cells resulting in malformation of the intercellular lipid layers of the stratum corneum in HI [Akiyama et al., 2005] (Fig. 2). Cultured epidermal keratinocytes from an HI patient carrying *ABCA12* mutations demonstrated defective glucosylceramide transport, and this phenotype was recoverable by in vitro *ABCA12* corrective gene transfer [Akiyama et al., 2005]. To date, intracytoplasmic glucosylceramide transport has been studied using cultured keratinocytes from a total of three patients harboring *ABCA12* mutations. One patient was a homozygote for a splice site mutation c.3295−2A>G [Akiyama et al., 2005] and another patient was a compound heterozygote for p.Ser387Asn and p.Thr1387del [Akiyama et al., 2006a]. Only one heterozygous mutation p.Ile1494Thr was identified in the other patient [Natsuga et al., 2007]. Cultured keratinocytes from all the three patients showed apparently disturbed glucosylceramide transport, although this assay is not quantitative.

Interestingly, ABCA3, a member of the same protein superfamily as ABCA12, functions in pulmonary surfactant lipid secretion again via the production of similar lamellar-type granules within lung alveolar type II cells [Shulenin et al., 2004; Yamano et al., 2001].

In addition, defective lamellar granule formation was observed in the skin of two CIE patients with *ABCA12* mutations [Natsuga et al., 2007]. Electron microscopic observation revealed that, in the cytoplasm of granular layer keratinocytes, abnormal, defective lamellar granules were assembled together with some normal-appearing lamellar granules [Natsuga et al., 2007].

Formation of the intercellular lipid layers is essential for epidermal barrier function. In ichthyotic skin with ABCA12 deficiency, defective formation of the lipid layers is thought to result in a serious loss of barrier function and a likely extensive compensatory hyperkeratosis [Akiyama, 2006b].

A study in one *Abca12* disrupted *Abca12*$^{-/-}$ HI model mouse indicated that a lack of desquamation of skin cells, rather than enhanced proliferation of basal layer keratinocytes, accounts for the fivefold thickening of the *Abca12*$^{-/-}$ stratum corneum using in vivo skin proliferation measurements [Zuo et al., 2008]. It was suggested that this lack of desquamation was associated with a profound reduction in skin linoleic esters of long-chain ω-hydroxyceramides and a corresponding increase in their glucosylceramide precursors. ω-hydroxyceramides are required for correct skin barrier function, and these results from the HI model mice establish that ABCA12 activity is required for the generation of long-chain ceramide esters that are essential for the development of normal skin structure and function [Zuo et al., 2008].

One hypothetical pathomechanism for ABCA12 deficient in ARCI is the differentiation defect theory (Fig. 2), derived from the clinical features of HI patients. Fetuses affected with HI start developing their ichthyotic phenotype while they are in the

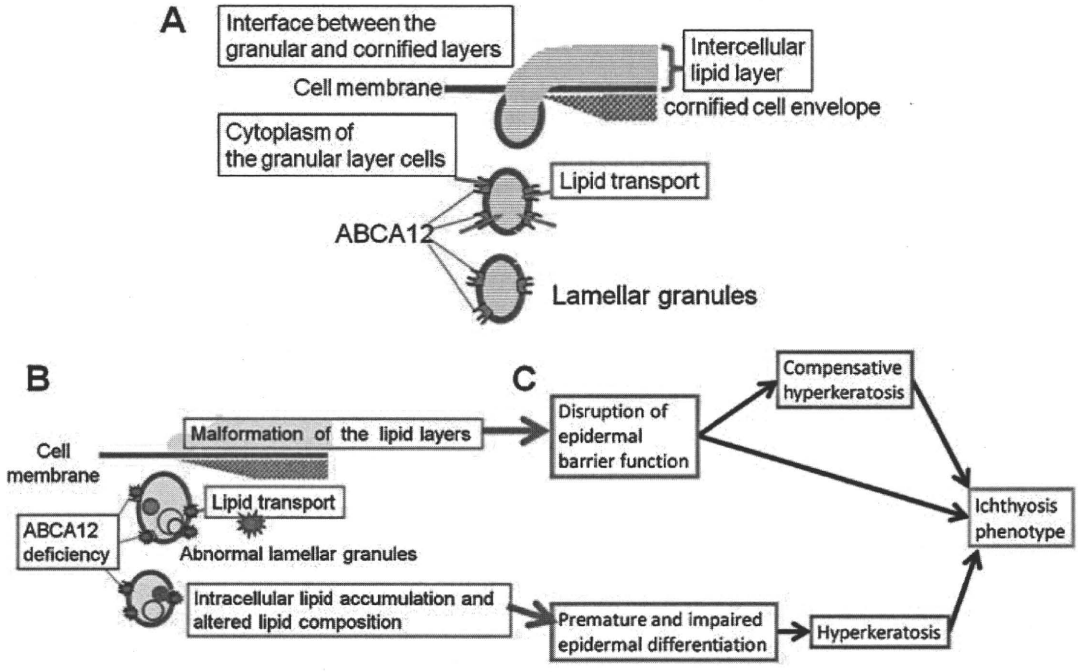

Figure 2. Physiological role(s) of ABCA12 in lipid trafficking of epidermal keratinocytes and the model of ichthyotic pathogenetic mechanisms underlying ABCA12 deficiency. **A:** Model of how ABCA12 transports lipids for keratinocyte differentiation and epidermal barrier function. ABCA12 in the limiting membrane of lamellar granules transports lipid into the lamellar granules. Accumulated lipid contents in the lamellar granules are secreted to the intercellular space forming the intercellular lipid layers, which are important for epidermal barrier function. **B:** Model of how loss of ABCA12 function leads to lipid abnormality and lipid barrier malformation in the upper epidermis. Loss-of-function mutations in ABCA12 disrupts lipid accumulation into the lamellar granules and normal lamellar granule formation, resulting in disturbed lipid transport and secretion to the extracellular space and abnormal lipid deposit in the cytoplasm. **C:** Disruption of epidermal barrier function and epidermal differentiation defects result from malformation of the stratum corneum lipid layers and abnormal intracellular lipid accumulation, respectively. It is hypothesized that lipid barrier defects and disturbed keratinocyte differentiation coordinately cause hyperkeratosis and the ichthyosis phenotype.

amniotic fluid where stratum corneum barrier function is not required. Thus, barrier defects cannot be involved directly in the pathogenesis of HI phenotype, at least during the in utero fetal period. In this context, disturbed keratinocyte differentiation is speculated to play an important role in the pathogenesis of HI phenotype. In fact, three dimensional culture studies revealed that HI keratinocytes differentiate poorly using morphologic criteria, and show reduced expression of keratin 1 and defective conversion from profilaggrin to filaggrin [Fleckman et al., 1997].

In an *ABCA12* ablated organotypic coculture system, an in vitro model of HI skin, expression of keratinocyte late differentiation-specific molecules was dysregulated [Thomas et al., 2009]. Expression of specific proteases associated with desquamation, kallikrein 5 and cathepsin D, was dramatically reduced in the ABCA12 ablated organotypic coculture system [Thomas et al., 2009]. In the model system, *ABCA12* ablation resulted in a premature terminal differentiation phenotype [Thomas et al., 2009]. Furthermore, in the mutant mice carrying a homozygous spontaneous missense mutation, loss of Abca12 function led to premature differentiation of basal keratinocytes [Smyth et al., 2008]. In contrast, in our *Abca12*$^{-/-}$ HI model mice, immunofluorescence and immunoblotting of *Abca12*$^{-/-}$ neonatal epidermis revealed defective profilaggrin/filaggrin conversion and reduced expression of the differentiation-specific molecules, loricrin, kallikrein 5, and transglutaminase 1, although their mRNA expression was upregulated [Yanagi et al., 2010]. These data suggest that ABCA12 deficiency may lead to disturbed keratinocyte differentiation during fetal development, resulting in an ichthyotic phenotype at birth. From these observations, ABCA12 deficiency might have global effects on keratinocyte differentiation, resulting in both impaired terminal differentiation and premature differentiation of the epidermis.

Animal Models

Recently, bioengineered disease models were established to investigate ichthyotic pathomechanisms due to ABCA12 defective function and to aid development of innovative treatments for ichthyosis with ABCA12 deficiency.

We transplanted cultured keratinocytes from patients with HI and succeeded in reconstituting HI skin lesions in immunodeficient mice [Yamanaka et al., 2007]. These reconstructed HI lesions showed similar changes to those observed in HI patients' skin. In addition, we generated *Abca12* disrupted (*Abca12*$^{-/-}$) mice and our *Abca12*$^{-/-}$ mice closely reproduced the human HI phenotype, showing marked hyperkeratosis with eclabium and skin fissure [Yanagi et al., 2008a]. Lamellar granule abnormalities and defective ceramide distribution were remarkable in the epidermis. Skin permeability assays of *Abca12*$^{-/-}$ mouse fetuses revealed severe skin barrier dysfunction after the initiation of keratinization. Surprisingly, *Abca12*$^{-/-}$ mice also demonstrated lung alveolar collapse immediately after birth. Lamellar bodies in alveolar type II cells from *Abca12*$^{-/-}$ mice lacked normal lamellar structures [Yanagi et al., 2008a]. The level of surfactant protein B, an essential component of alveolar surfactant, was reduced in the *Abca12*$^{-/-}$ mice [Yanagi et al., 2008a]. Another group independently