Truncation of CGI-58 Protein Causes Malformation of Lamellar Granules Resulting in Ichthyosis in Dorfman-Chanarin Syndrome

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Dorfman–Chanarin syndrome is a rare autosomal recessive inherited lipid storage disease characterized by ichthyosis, leukocyte lipid vacuoles, and involvement of several internal organs. Recently, CGI-58 mutations were identified as the cause of Dorfman–Chanarin syndrome. The physiologic roles of the CGI-58 protein and the pathomechanisms of Dorfman–Chanarin syndrome still remain to be clarified, however. The patient, a 16-y-old male, demonstrated ichthyosis, small ears, lipid vacuoles in his leukocytes, liver dysfunction, and mental retardation. Sequencing of CGI-58 revealed that the patient was homozygous for a novel nonsense mutation R184X, in exon 4. The putative truncated protein was 52.4% of the length of the normal CGI-58 polypeptide and lacked approximately 60% of the lipid binding region, 66.4% of the α/β hydrolase folding segment of the polypeptide, and two of the CGI-58 catalytic triads, resulting in a significant loss of lipase/esterase/thioesterase activity. Electron microscopy revealed a large number of abnormal lamellar granules, a disturbed intercellular lamellar structure, and lipid vacuoles in the epidermis. These results suggested that CGI-58 protein is involved in the lipid metabolism of lamellar granules and that defective lipid production in lamellar granules caused by a CGI-58 protein deficiency is involved in the pathogenesis of ichthyosis in Dorfman–Chanarin syndrome.
***Key words:** esterase/ichthyosis/lamellar body/lipase/neutral lipid storage disease. J Invest Dermatol 121:1029–1034, 2003*

Dorfman–Chanarin syndrome (DCS) (MIM 275630), also referred to as neutral lipid storage disease with ichthyosis, is a rare autosomal recessively inherited lipid storage disease (Dorfman *et al*, 1974; Chanarin *et al*, 1975). This entity is characterized by nonbullous congenital ichthyosiform erythroderma (NBCIE), leukocyte lipid vacuoles, and by the involvement of several internal organs. All cases present with skin manifestations of moderate to severe NBCIE (Williams and Lynch, 1996; Griffiths *et al*, 1998; Pena-Penabad *et al*, 2001). Extracutaneous manifestations variably include fatty liver, myopathy, cataracts, and a variety of neurologic symptoms.

Amounts of triacylglycerol in lymphocytes, macrophages, and fibroblasts cultured from patients were remarkably larger than those of normal controls (Williams *et al*, 1988; Hilaire *et al*, 1995). Activities of various enzymes for lipid metabolism, including lipase and esterase, have been studied and found to be normal (Williams *et al*, 1988; Hilaire *et al*, 1995; Igal and Coleman, 1996; 1998). Thus, the causative defects in lipid metabolism are expected to involve triacylglycerol metabolism (Williams *et al*, 1991), especially in the catabolism of long-chain fatty acids (Hilaire *et al*, 1994; 1995) or in the recycling pathway of triglycerol-derived monoacylglycerols or diacylglycerols into phospholipids (Igal and Coleman, 1996; 1998)

In 2001, CGI-58 mutations were identified in DCS families from the Mediterranean region (Lefèvre *et al*, 2001), although no following report of CGI-58 mutations in DCS has been published. The physiologic roles of the protein coded by CGI-58 in various tissues including the epidermis have not yet been clarified (Lai *et al*, 2000), and the pathomechanisms of NBCIE caused by CGI-58 mutations remain unsolved.

In this study, in order to elucidate whether CGI-58 mutations lead to abnormal granules, epidermal lamellar granules were studied ultrastructurally in a typical DCS patient carrying a novel homozygous CGI-58 mutation R184X. In addition, to clarify whether CGI-58 mutations cause other abnormalities in keratinization, ultrastructural observation and immunofluorescent staining of other keratinization-associated molecules and *in situ* transglutaminase (TGase) activity assays were performed on epidermal samples from the patient with defective CGI-58. Institutional approval was obtained for all work performed in this study, along with adherence to the Helsinki Principles. Informed guardian's consent was provided regarding our work with patient's tissue and cells.

MATERIALS AND METHODS

Mutation detection The strategy for mutation detection was as previously reported (Lefèvre *et al*, 2001). Briefly, genomic DNA isolated from peripheral blood was subjected to PCR amplification, followed by direct automated sequencing. Oligonucleotide primers used for amplification of all exons 1–7 of CGI-58 are shown in **Table I**. These

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Abbreviations: DCS, Dorfman–Chanarin syndrome; NBCIE, nonbullous congenital ichthyosiform erythroderma; TGase, transglutaminase.

Table I. Primer sequences for CGI-58

	Sequence (5′-3′) Forward	Reverse
Exon 1	GGCCGTGCTAGTGCGCGGAAGACGCATGCG	GGTGGCTTATACAACAACGGGGCGGACCCTCC
Exon 2	CCATGCTTTGTGCATGTTAG	AAACAAATCTCCTTGGGGTC
Exon 3	TGAGGTAGGTCTTCCCCTTT	AGAGAATGTCTGCCTTGTGG
Exon 4	CGTGAAGGTTTTTGAAGGTG	GGGTTCAGGGTTTTCTTGTT
Exon 5	AATGTGTGCTTTTTCCCACC	GACCTGGGGTCAGAAGTTCA
Exon 6	CTTAGGTGCTGGAAAAGCTA	GTAGTTCACGGTTTGGACAT
Exon 7	TTTAAATACAGTGGCTCTCACTT	TCAGAAATCACTTCCTAAATTGG

primer sequences were derived from the report by Lefèvre *et al* (2001) and were partially modified according to the subsequently reported mRNA sequence (Genbank Accession No. AL606839) for this study. Detailed PCR conditions were previously described (Lefèvre *et al*, 2001).

Morphologic observations Skin biopsy samples were fixed in one-half strength Karnovsky's fixative or 2% glutaraldehyde solution, postfixed in 1% OsO_4, dehydrated, and embedded in Epon 812 (Perry *et al*, 1987). A peripheral blood sample from the patient was centrifuged and blood cell pellets were obtained. The cell pellets were fixed in glutaraldehyde, OsO_4, and embedded in epoxy resin. The samples were sectioned at 1 μm thick for light microscopy and thin sectioned for electron microscopy (70 nm thick). The histologic sections were stained by the method of Richardson *et al* (1960). The thin sections were stained with uranyl acetate and lead citrate (Reynolds, 1963) and examined in a transmission electron microscope.

Antibodies The primary antibodies to keratinization-associated proteins used in this study were mouse monoclonal antikeratin 1 antibody, 34βB4 (Novocastra Laboratory, Newcastle upon Tyne, UK), mouse monoclonal antikeratin 10 antibody, DE-K10 (Dako, Glostrup, Denmark), rabbit polyclonal antihuman involucrin antibody (Biomedical Technologies, Stoughton, MA), and rabbit polyclonal antimouse loricrin antibody, which cross-reacts with human loricrin (Berkeley Antibody, Richmond, CA). Goat polyclonal antibody against human recombinant TGase 1 (major variant) (Kim *et al*, 1992), whose epitope(s) is located in the central core of TGase 1 (Kim *et al*, 1995), was also used.

Immunofluorescent labeling Immunofluorescent labeling was performed as described previously (Akiyama *et al*, 1998a). Briefly, sections of fresh patient's skin 6 μm thick were cut using a cryostat. The sections were incubated in primary antibody solution for 1 h at 37°C. Antibody dilutions were as follows: 1:1 for anti-involucrin, 1:20 for antikeratin 1 antibody, 1:100 for antikeratin 10 antibody, 1:50 for antiloricrin antibody, and 1:10 for anti-TGase 1 antibody. The sections were then incubated in fluorescein isothiocyanate conjugated to rabbit antimouse immunoglobulins, goat antirabbit immunoglobulins, or rabbit antigoat immunoglobulins diluted 1:100 (Dako) for 30 min at room temperature, followed by 10 μg per ml propidium iodide (Sigma Chemical, St Louis, MO) to counterstain nuclei for 10 s. The sections were extensively washed with phosphate-buffered saline (PBS) between incubations. The stained sections were then mounted with a cover slip in 50% glycerol mounting medium and observed by conventional epifluorescence microscope or using a confocal laser scanning microscope.

***In situ* TGase activity assay** In order to study the enzyme activity of TGase in the skin specimens, an *in situ* TGase activity assay was performed as follows. Frozen sections were incubated in 100 mM Tris–HCl pH 7.4, 1% normal mouse serum for 30 min to block nonspecific binding, and then in 100 mM Tris–HCl pH 7.4, 5 mM $CaCl_2$, 12 μM monodansylcadaverine (Sigma Chemical, St Louis, MO) for 1 h to detect TGase activity (Hohl *et al*, 1998; Raghunath *et al*, 1998; Akiyama *et al*, 2001). At PH 7.4 buffer conditions for the detection of TGase 1 activity, but not TGase 3 activity were selected. (Raghunath *et al*, 1998; Akiyama *et al*, 2001). For a negative control, ethylenediaminetetraacetic acid (EDTA) was added to the monodansylcadaverine solution to a final concentration of 20 mM. After stopping the TGase reaction with 10 mM EDTA in PBS, sections were incubated with rabbit antidansyl antibody (1:100) (Molecular Probes, Eugene, OR) in 12% bovine serum albumin (BSA)/PBS for 3 h. Sections were then incubated with fluorescein isothiocyanate (FITC) labeled mouse antirabbit antibody (1:40) in 12% BSA/PBS for 30 min. Nuclei were counterstained by incubating the sections in 10 μg per ml propidium iodide (Sigma Chemical) for 10 s. Localization of TGase activity was observed under a fluorescence microscope.

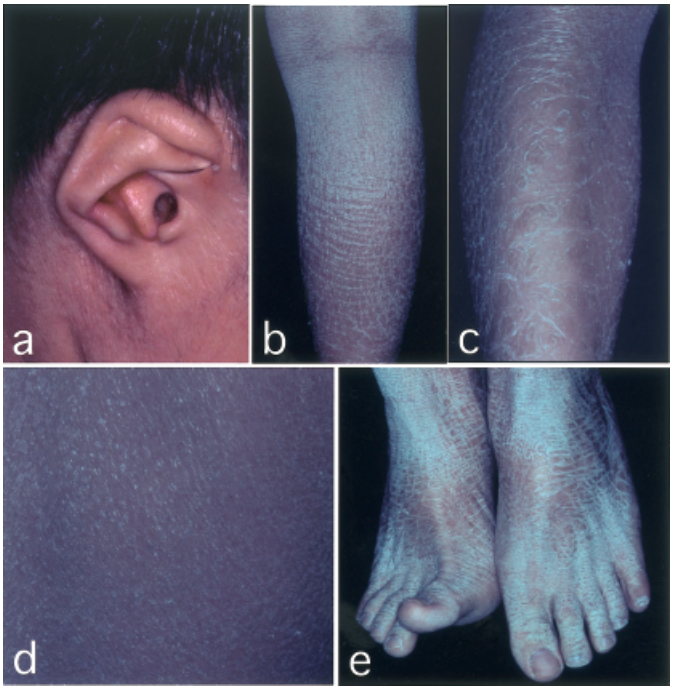

Figure 1. The patient showed a typical clinical phenotype of DCS. The auricle was small and malformed (*a*). Fine, light-gray to gray scales were seen on the flexor aspect (*b*) and extensor aspect (*c*) of erythrodermic skin of the patient's lower leg and back (*d*). Scales were seen on the dorsa of the feet although the nails were normal (*e*).

RESULTS

Clinical presentation and routine morphologic observations The patient was a 16-y-old Japanese male. He was the third child of nonconsanguineous healthy parents. There was no family history of congenital ichthyosis or abnormal lipid metabolism. He has two unaffected elder brothers. The patient had presented with severe ichthyosis since birth. Physical examination of the patient at 16 y of age revealed fine, gray, or light-brownish scales over the entire body surface including the face, palms, and the soles (**Fig 1**). The skin was slightly erythrodermic over his entire body surface. The patient's skin manifestation was categorized as NBCIE (Akiyama *et al*, 2003). His auricles were abnormally small (**Fig 1*a***) although hair, teeth, and nails appeared normal. He had demonstrated

liver dysfunction from infancy and a liver biopsy showed cirrhosis with severe fatty degeneration. He also had slight mental retardation, although myopathy, cataracts, hearing loss, or neurologic symptoms were not seen. Steroid sulfatase activity in the peripheral blood sample was normal. Light microscopy of the lesional skin sample from the patient's trunk showed marked hyperkeratosis with only a small number of parakeratotic cells (**Fig 2*a***). There were large cytoplasmic vacuoles containing amorphous material in the basal cells and the lowermost spinous cells (**Fig 2*b***). The large vacuoles were similar to those in the white blood cells and were presumably lipid vacuoles. In the white blood cells in the peripheral blood, large cytoplasmic lipid vacuoles characteristic of DCS were observed (data not shown).

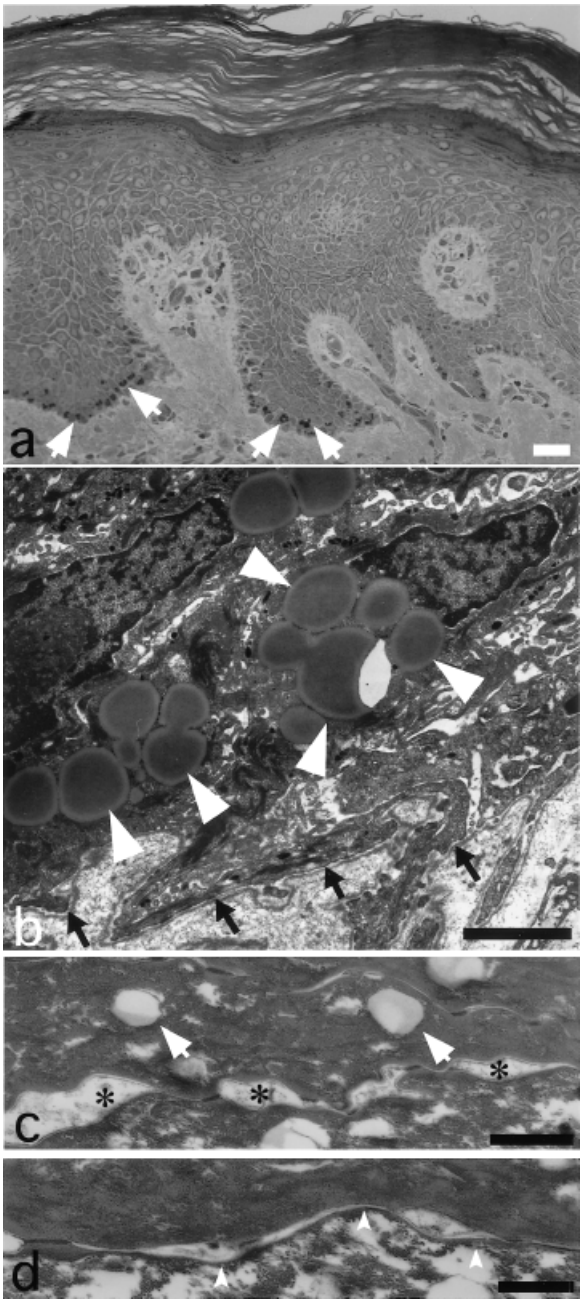

Figure 2. Lipid vacuoles characteristic of DCS were observed in the basal layer of the patient's epidermis. (*a*) A light microscopic view of a semithin section of the patient's hyperkeratotic skin showed lipid droplets (*arrows*) mainly in the basal layer. (*b*) Electron microscopy revealed large intracellular lipid vacuoles (*arrowheads*) within basal keratinocytes. *Arrows*, basement membrane. (*c*) In the lower cornified layers, intracytoplasmic vacuoles (*arrows*) and electron-lucent intercellular clefts (*) were seen. (*d*) An intact cornified cell envelope (*arrowheads*) was formed at the periphery of the cornified layer cells. *Bars*: (*a*) 50 μm; (*b*) 5 μm; (*c*), (*d*), 2 μm.

CGI-58 mutation analysis Direct sequencing of the patient's PCR products revealed that the patient was a homozygote for a novel nonsense mutation 550C→T transition in exon 4 (sequence according to Lefèvre *et al*, 2001) (GenBank Accession No. AL606839) that changed an arginine residue to a stop codon (R184X). This mutation was not found in 50 normal unrelated Japanese alleles (25 normal unrelated Japanese individuals) by sequence analysis, and was unlikely to be a polymorphism (data not shown). Direct sequencing of all seven exons and exon–intron borders of CGI-58 failed to detect any other pathogenic mutation in the patient's DNA. One polymorphism in exon 7 (amino acid 363, arginine→isoleucine) was found in the patient's DNA and some control DNA samples.

Ultrastructure of the upper epidermis in the ichthyotic skin lesion In the upper spinous and granular layer cells, small lipid droplets were seen in the cytoplasm and abnormal lamellar granules were observed (**Figs 2*c*, 3**). The features that abnormal lamellar granules were assembled and forming lipid droplets in

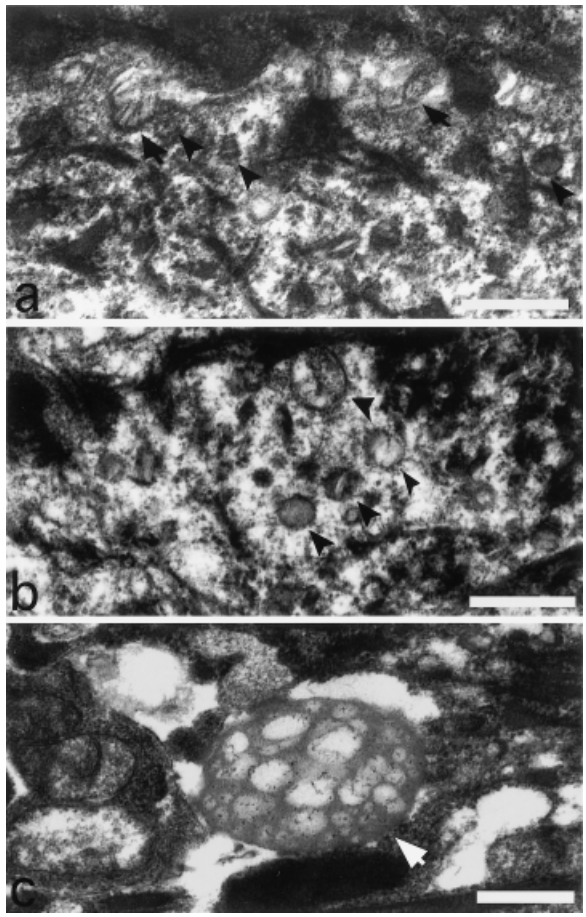

Figure 3. A large number of abnormal lamellar granules in keratinizing cells of the patient's epidermis. (*a*) Numerous, large, and irregularly shaped abnormal lamellar granules (*arrowheads*) lacking the normal lamellar structure were seen at the periphery of granular cells and some of them had been secreted into the intercellular space. A disturbed intercellular lamellar structure was also seen between the granular and cornified cells (*arrows*). (*b*) Abundant abnormal lamellar granules (*arrowheads*), irregular in size and shape, were apparent in the upper spinous cell layers. (*c*) Abnormal lamellar granules had accumulated and formed an intermediate structure (*arrow*) between abnormal lamellar granules and a lipid vacuole. *Bars*: 1 μm.

the cytoplasm were seen in the upper spinous and granular layers. Intercellular lipid lamellae were disturbed between the granular cells and the cornified cells. Lipid droplets were also found in the cornified cells. The cornified cell envelope was normally formed at the cell periphery of the cornified layer cells (**Fig 2*d***). In some fibroblasts and nerve cells in the dermis, abnormal cytoplasmic lipid inclusions were seen (data not shown).

Immunofluorescent labeling of keratinization-associated proteins Immunofluorescence studies revealed normal expression of keratin 1 and keratin 10 in the suprabasal layers of the patient's hyperkeratotic epidermis (data not shown). Normal membranous involucrin staining was observed in the upper spinous and granular layer cells (data not shown). Normal distribution of loricrin in the uppermost spinous and the granular layers was also seen in the patient's epidermis (data not shown). These staining patterns were similar to those in normal control skin. Immunofluorescence demonstrated a normal expression of the TGase 1 molecule mainly in the upper spinous and the granular layer of the patient's epidermis (data not shown).

In situ* TGase activity assay** From the middle to upper spinous layers, normal membrane localized TGase 1 activity was detected (**Fig 4*a). In the upper spinous and the granular layers, the membrane-associated activity became reduced and an abnormal cytoplasmic granular distribution of TGase 1 activity was seen.

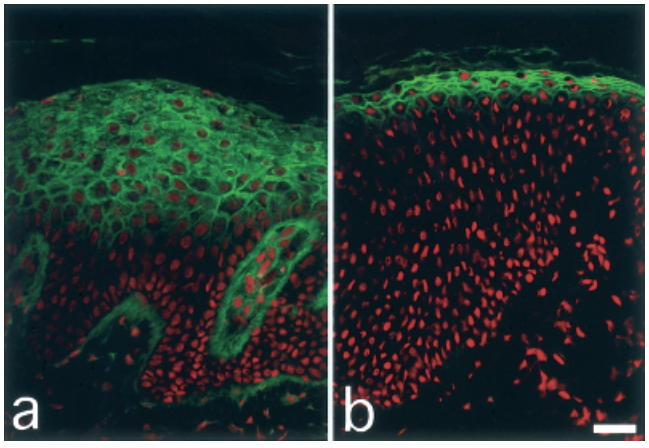

Figure 4. Disturbed distribution of *in situ* TGase 1 activity in the patient's epidermis. An *in situ* TGase activity assay under pH 7.4 buffer conditions showed granular and vacuolated TGase activity in the patient's upper spinous and granular layers (*a*). In control epidermis, membranous TGase activity is seen in the granular layers (*b*). (*a*) The patient's skin; (*b*) control normal human skin. *In situ* TGase activity was labeled using FITC (*green*). Nuclear staining was done with propidium iodide (*red*). *Bar*: 50 μm.

DISCUSSION

Based on the data from clinical, ultrastructural, immunohistologic, and enzyme-histologic studies, this patient should be diagnosed as a typical DCS. The mutation analysis revealed that the patient was a homozygote for a novel nonsense mutation, R184X, in exon 4 of CGI-58.

CGI-58 is one of the genes recently identified using the comparative proteomic approach between *Caenorhabditis elegans* and human (Lai *et al*, 2000). CGI was named after comparative gene identification. Neither the tissue expression profile nor the physiologic roles of CGI-58 have been clarified yet. CGI-58 belongs to a large family of proteins characterized by an α/β-fold domain (Ollis *et al*, 1992; Heikinheimo *et al*, 1999) and a catalytic triad composed of a nucleophile, an acid, and histidine (Zhang *et al*, 1998; Nardini and Dijkstra, 1999). From the analysis of the reported amino acid sequences, CGI-58 has a putative catalytic triad as do other members of the esterase/lipase/thioesterase subfamily (Lefèvre *et al*, 2001). The first element of the triad is a motif close to a lipase consensus sequence, included in amino acid residues 149–156 (Lefèvre *et al*, 2001). The third element of the triad is around the histidine at residue 327 in the conserved motif, between amino acid residues 324 and 328. The putative second element of the triad could be either the aspartate residue at 301, inside the motif GARSCIDG (amino acid residues 295–302), or the glutamic acid residue at 260, inside the motif PSGETA (amino acid residues 257–262) (Cygler *et al*, 1993; Schrag and Cygler, 1997; Lefèvre *et al*, 2001). From these facts, CGI-58 is postulated to be a member of the esterase/lipase/thioesterase subfamily of proteins.

According to the CGI-58 protein modeling, based on the reported amino acids sequence (Lefèvre *et al*, 2001), the novel mutation R184X in this patient leads to a premature termination codon in the central portion of the CGI-58 polypeptide (**Fig 5**). This mutation eliminates approximately 60% of the lipid binding region and 66% of the α/β hydrolase fold of the polypeptide. The putative truncated protein from the mutated allele is 52.4% of the length of the normal CGI-58 polypeptide and lacks two of the catalytic triads that make CGI-58 a member of the lipase/esterase/thioesterase subfamily. This results in a significant loss of lipase/esterase/thioesterase activity, and consequently CGI-58 activity was predicted to be seriously impaired or completely lost in this case.

The pathomechanisms of the CGI-58 mutations causing the multiorgan symptoms in DCS including ichthyosis remain to be clarified, and the physiologic function of the CGI-58 protein is still unknown. In the case reported here, in the upper spinous and granular layers a large number of abnormal lamellar granules were seen. Some lamellar granules were secreted into the intercellular space between the granular and cornified cells, but the content secreted from the abnormal lamellar granules formed a disturbed intercellular lamellar structure and intercellular clefts. Other abnormal lamellar granules remained in the cytoplasm

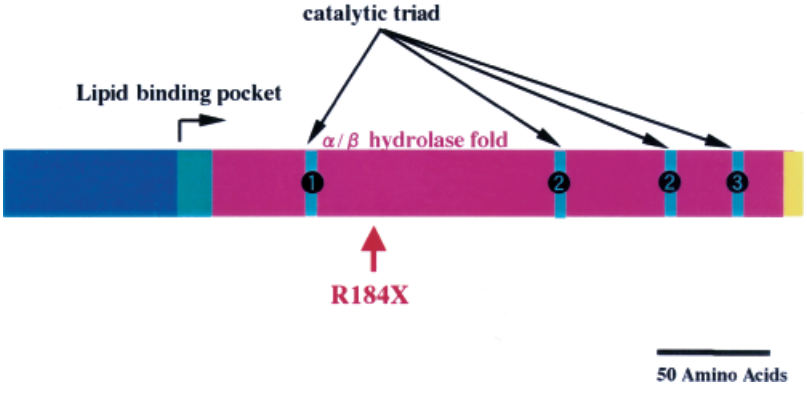

Figure 5. Schematic sequential arrangement of the domain structures of CGI-58 protein. Mutations in the DCS patient are marked by an *ascending arrow*. Note that the mutation is just at the N-terminal side (*left*) of the middle portion of the molecule. The genetic defect causes a premature termination codon at the mutation site, resulting in the deletion of more than half of the amino acid sequence of the protein including the second and third elements of the catalytic triad.

and developed an intermediate structure midway between lamellar granules and lipid vacuoles. Similarly structured granules resembling both lipid vacuoles and lamellar granules have previously been reported in different neurocutaneous syndromes associated with ichthyosis and abnormal lipid metabolism (Koone *et al*, 1990). Finally, these intermediate structured granules underwent transformation into lipid vacuoles within the granular cells and cornified cells. These observations suggested that a defective synthesis of lipid in lamellar granules caused by a CGI-58 protein deficiency is involved in the pathogenesis of ichthyosis in DCS.

Formation of a normal epidermal permeability barrier and normal desquamation require the secretion of lamellar granule contents into the intercellular spaces between the granular and cornified cells. Lamellar granules contain both polar lipids and a family of hydrolytic enzymes that catalyze the extracellular processing of the secreted polar lipids into more hydrophobic reaction products (Rassner *et al*, 1999). Abnormal lamellar granules and an abnormal secretion of lamellar granule contents were reported as the likely pathomechanisms for other congenital ichthyoses including harlequin ichthyosis and NBCIE (Milner *et al*, 1992; Akiyama, 1999; Fartasch *et al*, 1999). In addition, decreased quantities of secreted lamellar granule contents in the intercellular space were linked to abnormal barrier function in epidermolytic hyperkeratosis (Schmuth *et al*, 2001). Elias and Williams (1985) also reported an abnormality in the lipid contents of lamellar granules in multiple DCS cases from a single family, although underlying mutations from that family had not been detected.

In harlequin ichthyosis, lamellar granules are absent or, if present, they show an empty or vesiculated content without any lamellar structure. These abnormal lamellar granules are not secreted to the intercellular space and they form a large number of lipid droplets within the cornified cells (Dale *et al*, 1990; Hashimoto and Khan, 1992; Milner *et al*, 1992; Akiyama *et al*, 1994, 1998b). In contrast, the case with DCS reported here showed that most of the lamellar granules do have a lamellar structure although they are incompletely formed and some granules are secreted to the intercellular space. These findings suggest that harlequin ichthyosis has a different pathomechanism from the defective CGI-58 protein, although both DCS and harlequin ichthyosis do show abnormal lamellar granules.

After the first report by Lefèvre *et al* (2001), no subsequent CGI-58 mutations in DCS have appeared. Thus, we cannot completely exclude the possibility that the cause of DCS may be heterogeneous, especially in patients from outside the Mediterranean region. The case reported is important because the DCS patient is from a non-Mediterranean region but still harbors CGI-58 mutation defects.

In the report of Lefèvre *et al* (2001), eight CGI-58 mutations were found as the causative mutations out of 13 DCS patients from nine families. All the patients were homozygous for their CGI-58 mutations and the CGI-58 mutations were thought to cause a partial or total loss of CGI-58 activity and lead to various clinical features belonging to DCS (Lefèvre *et al*, 2001). In the patients carrying the milder, missense mutations, no poorly formed ears were observed and, morphologically, no lipid droplets were seen in the basal layer keratinocytes. In contrast, not all, but most, of the patients with nonsense mutations that might result in the production of truncated proteins that lack the entire lipid binding domain or more than half of the lipid binding domain including two elements of the catalytic triad showed small ears and abnormal lipid vacuoles in the basal cells of the epidermis. The mutation R184X also causes a truncated CGI-58 protein and the patient showed small ears and a large number of lipid vacuoles in the basal and lowermost spinous layers of the epidermis. These facts support the hypothesis that a serious loss of CGI-58 activity may lead to small ears and the presence of lipid droplets in the basal epidermal layer.

In this case, large cytoplasmic vacuoles characteristic of DCS were observed in the basal and lowermost spinous layers and the vacuoles gradually became obscured in the upper layers. Considering that the most important site for the pathogenesis of cornification disorders is not the basal layer but the upper spinous and the granular layers of the epidermis, we hypothesized that lipid vacuoles in the basal cells are not important in the pathogenesis of ichthyosis in DCS with CGI-58 mutations. In all previous reports, DCS patients showed ichthyosis, but the lipid vacuoles in the basal epidermal layer were not always seen (Dorfman *et al*, 1974; Chanarin *et al*, 1975; Miranda *et al*, 1979; Elias and Williams, 1985; Srebrnik *et al*, 1987; 1998; Venencie *et al*, 1988; Williams *et al*, 1988; Banuls *et al*, 1994; Kaassis *et al*, 1998; Wollenberg *et al*, 2000; Pena-Penabad *et al*, 2001). Srebrnik *et al* (1998) suggested that large lipid vacuoles were not correlated with DCS clinical severity. In addition, a series of DCS patients with CGI-58 mutations reported by Lefèvre *et al* (2001) did not always show lipid vacuoles in the basal cells, although patients with DCS always showed ichthyosis. These facts also support the hypothesis that large lipid vacuoles in the lower epidermis are not pathogenic.

Immunofluorescent labeling for keratin 1, keratin 10, involucrin, and loricrin showed a normal distribution of keratinization-associated structural molecules. These results together with the ultrastructural findings clearly indicate that the cornified cell envelope and keratin network were not significantly disturbed in the patient's epidermis. The *in situ* TGase activity assay, however, revealed an aberrant distribution pattern of TGase 1 activity in the patient's upper spinous and granular layers. It is known that TGase 1 not only participates in the cross-linking of the cornified cell envelope structural proteins but also helps form the lipid bound envelope by esterification of long-chain ω-hydroxyceramides with cornified cell envelope proteins (Nemes *et al*, 1999). In X-linked ichthyosis, supraphysiologic levels of cholesterol sulfate in keratinocyte membranes distort the conformational molecular structure of TGase 1 and inhibit TGase 1 activity (Nemes *et al*, 2000). In this context, the abnormal distribution of TGase 1 activity in our patient may be caused by the accumulation of abnormal lipid droplets and malformed lamellar granules close to the cell membrane.

In conclusion, this study has revealed that a seriously truncated CGI-58 protein leads to the defective formation of lamellar granules in human epidermis and that the CGI-58 protein is likely to play an important role in the metabolism of lamellar granule lipid contents.

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