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A novel in situ method for the detection of deficient transglutaminase activity in the skin

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Abstract **Autosomal recessive congenital ichthyoses are disorders of epidermal cornification, but are clinically and etiologically heterogeneous. Some cases, known as lamellar ichthyosis, are caused by mutations in the *TGM1* gene encoding transglutaminase 1, which result in markedly diminished or lost enzyme activity and/or protein. In some cases, this enzyme is present but there is little detectable activity, and in other clinically similar cases, transglutaminase 1 levels appear to be normal. Since conventional enzyme assays and mutational analyses are tedious, we developed a novel assay for the rapid screening of transglutaminase 1 activity using covalent incorporation of biotinylated substrate peptides into skin cryostat sections. Coupled with immunohistochemical assays using transglutaminase 1 antibodies, our method allows rapid identification of those cases caused by alterations in this enzyme.**

Key words Transglutaminase · Epidermis · Lamellar ichthyosis · Skin · Histochemistry

Introduction

Transglutaminases (TGases) constitute a family of enzymes that stabilize protein assemblies by γ-glutamyl-ε-lysine crosslinks [7, 21]. Six different gene products are so far known in the human genome [1], of which at least three play important roles in the skin. TGase 2 (tissue TGase) is likely to be involved in apoptosis and also crosslinks anchoring fibrils of the dermoepidermal junction [30]. TGases 1 and 3 participate cooperatively in the crosslinking of structural proteins such as involucrin, small proline-rich proteins and loricrin to form the cornified cell envelope in terminally differentiating epidermis [1, 31]. Thus, TGases are prime candidates as causes of genetic disorders of cornification, which would likely manifest as congenital recessive ichthyoses (CRI). One such disease is lamellar ichthyosis (LI) which is always nonbullous and shows nonspecific epidermal hyperplasia, and thus is distinct from the epidermolytic ichthyoses [41]. The established dermatology literature lists LI as a heterogeneous disorder with variable presentation ranging from fine translucent scaling (often combined with marked erythema) to large dark-brown plate-like scales (often without erythema), and moreover, may be transmitted both as an autosomal dominant or autosomal recessive disorder [40].

An initial study of scales derived from patients with CRI disease has revealed normal or even increased epidermal TGase activity [11]. However, in a second study, the amount of TGase 1 protein evident by immunhistochemical staining in three CRI patients has been shown to be diminished [10]. Subsequently, linkage of some families to chromosome 14q11 was established [32], which is the locus of the *TGM1* gene encoding TGase 1 [16, 43], and indeed, several *TGM1* mutations have been reported to date [12, 14, 27, 33]. In most cases where *TGM1* mutations have been recorded, the disease presented as large brown scales [3]. Biochemical assays using culture of keratinocytes from these patients generally have shown drastically reduced TGase 1 activity, although in some cases TGase protein is still detectable [12, 14]. However, other cases of CRI are heterogeneous, since they do not link to the *TGM1* gene [3, 13]; some link to chromosome 2q33–35, and others to another undefined locus instead [28], yet deficiencies in TGase activity have not been demonstrated here [13].

These studies raise a number of important questions as to the role of TGases in CRI, yet the functional assess-

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ment of TGases in the epidermis of CRI patients is necessary in order to set the stage for appropriate molecular analyses. However, the biochemical and molecular biological assays to examine the separate TGase activities present, and mutations in the *TGM1* gene, are tedious, expensive, time consuming and dependent on the cultivation of keratinocytes, which can be difficult. In order to clarify these issues, we have devised a simple screening method to assess the in situ TGase 1 activity in skin cryostat sections. Coupled with immunohistochemical methods employing TGase 1 antibodies, we can rapidly ascertain which patients have TGase 1 deficiency either at the protein level (stability) and/or in activity.

Material and methods

Reagents

Two biotinylated donor substrates for TGase were used. The amine donor substrate monodansylcadaverine (biotMDC) was purchased from Molecular Probes Europe, Leiden, The Netherlands. A solution of 2 mg biotMDC in 50 µl 0.1 *N* HCl was prepared and then mixed with 394 µl distilled water. This rendered a 10 m*M* stock solution which was stored frozen at –20° C and could be repeatedly thawed without notable loss of activity. TGase substrate buffer was prepared by adding 10 µl of the biotMDC stock and 25 µl 200 m*M* CaCl2 solution to 965 µl 100 m*M* Tris/HCl, pH 8.4 or 7.4, respectively. For control experiments CaCl2 was replaced by 200 m*M* EDTA. A second substrate was the hexapeptide (biotinyl-Ala-Pro-Gln-Gln-Glu-Ala-OH) which was custom-synthesized for us (Bachem, Basel, Switzerland). This peptide represents the aminoterminus of osteonectin (biotNON) and was expected to serve as amine acceptor substrate since osteonectin is a known TGase substrate [2, 9]. Both substrates were used at a final concentration of 100 µ*M*.

Detection of TGase in skin sections

Punch biopsies were taken with informed consent from six CRI patients. To gain further information we also studied a control group of 15 volunteers with normal skin and the involved skin of two individuals with psoriasis vulgaris. Cryostat sections (5 µm) were air-dried for 10 min at room temperature (RT), preincubated with 1% BSA in 0.1 *M* Tris/HCl, pH 8.4 or pH 7.4, for 30 min at RT and then incubated for 2 h at RT with substrate buffer at the respective pH. The TGase reaction was stopped with PBS/25 m*M* EDTA for 5 min and two further washes in PBS. The sections were then incubated for 30 min at RT with streptavidin-DTAF (Jackson Immunoresearch, West Grove, PA, USA) 1:100 in PBS, washed three times in PBS and mounted in MOWIOL (Hoechst, Frankfurt-Hoechst, Germany) prepared in PBS. The complete assay including handling time of slides took 4.5 h.

Immunohistochemical procedures

A goat antiserum against TGase 1 [18] was applied diluted 1:200 for 16 h at RT and was visualized using a secondary rabbit antigoat DTAF conjugate (Jackson Immunoresearch) 1:100 for 30 min at RT. The monoclonal involucrin antibody (Sigma, St. Louis, Mo.) was applied at a dilution of 1:50 for 16 h and was detected with goat antimouse Texas Red conjugate (Jackson Immunoresearch) 1:50 for 30 min at RT. Photographs were taken on Tmax 3200 ASA (Kodak) using Axiophot (Zeiss, Jena, Germany) epifluorescence equipment.

Mutational analysis in the *TGM1* gene by SSCP analysis and sequencing

DNA was prepared by standard methods from blood samples. Exons 2 through 15 of the *TGM1* gene were amplified by PCR with intronic primers designed according to the published *TGM1* sequence [16, 43]. DNA was amplified using Taq DNA polymerase (Perkin Elmer, Norwalk, CT, USA) with 0.5 µ*M* of each primer and 0.1 µ*M* dNTPs. Conditions were 30 cycles of 10 s at 94° C, 10 s annealing at appropriate temperature and 10 s at 72° C for extension. For SSCP analysis, approximately one-tenth of the volume of the PCR sample was denatured and separated on 6% or 8% polyacrylamide gels [25]. Electrophoresis was done at 40 W for 1.5 h to 6 h at 4° C or 15° C depending on the length of the PCR product. In order to further analyse any changes in migration or number of bands detected by SSCP, corresponding PCR products were directly sequenced either using a cycle sequencing protocol (Amersham, Little Chalfont, UK) or by solid phase sequencing with magnetic beads (Dynal, Oslo, Norway) and T7 DNA polymerase (Pharmacia, Uppsala, Sweden). Where sequencing of some of the exons was difficult to perform, PCR products were in addition directly cloned using the TA cloning kit (Invitrogen, Leek, The Netherlands). For each clone sample, DNA of several colonies was prepared, amplified by PCR and again analysed by SSCP. A number of clones from each group of clones detected in this way were then sequenced with T7 DNA polymerase (Pharmacia).

Results

Mutation and linkage analyses of patients

We identified six probands with CRI, two of whom were found by linkage and sequencing analyses to have mutations in the *TGM1* gene. Patient DB was a compound heterozygote for a missense mutation R142H in exon 3 (equivalent to R141H identified in reference 33 due to differences in the published sequences) and a splice site mutation of nt A3447G which destroys the 3′ splice site of intron 5 (identical to A3366G in reference 3). The substitution R142H was not identified in 100 chromosomes of unaffected control individuals. Patient HR was also homozygous for the splice mutation nt A3447G. Both of these mutations have been recorded previously [8, 12, 27, 33], and are expected to interfere with the proper folding of the protein with resultant complete loss of enzyme activity. In the remaining four probands, however, linkage analyses excluded the *TGM1* locus on chromosome 14q11.

TGase activity in normal human skin

Human skin contains at least three TGases, TGase 1, 2, and 3 [7, 19, 21]. Of these, TGase 2 is only very weakly expressed in the epidermis and is confined mostly to the dermis and basement membrane zone of the skin (Fig. 1) [30]. The many hitherto reported biochemical assays for these TGases have been performed under both neutral (pH 7.5) and basic (pH 8.5) conditions [7, 20, 21]. However, more recent data have indicated that in in vitro assays the optimal pH for TGase 3 is 8–8.4, using both natural substrates and short peptide substrates [4–6], while TGases 1 and 2 operate efficiently at both pH ranges.

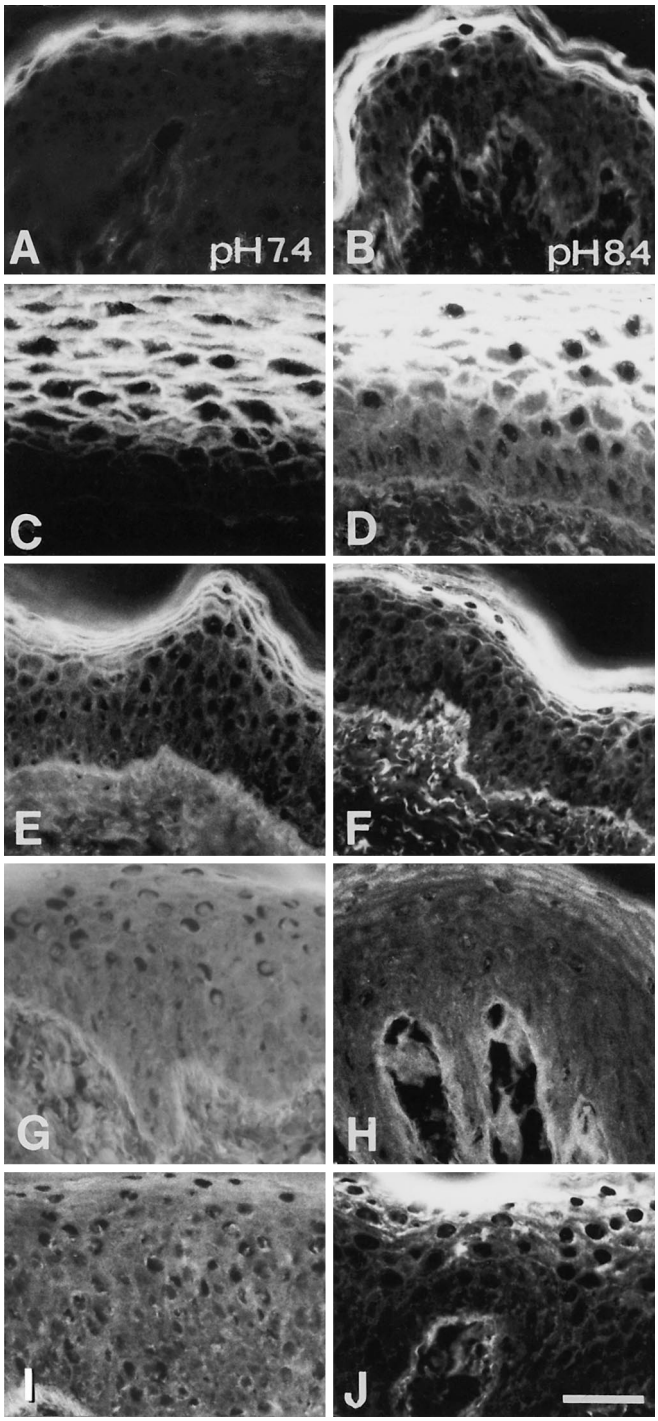

Fig. 1 A–J Skin cryostat sections allow convenient localization of TGase activity using a biotinylated amine donor substrate. TGase activity was detected in skin cryostat sections by incubation with the biotinylated amine donor substrate monodansylcadaverine (biotMDC) in the presence of Ca^{2+} at two different pH values (**A–G** and **B–H**). Incorporation of biotMDC was visualized using fluorochrome-coupled streptavidin. In normal epidermis (**A, B**), epidermal TGase activity is confined to the cell membranes of keratinocytes of the granular layer. TGase 2 activity in the dermis is very faint at pH 7.4 but enhanced at pH 8.4 in normal skin. In psoriasis (**C, D**), the zone of epidermal TGase activity is broadened extending to the suprabasal layer. Skin of a patient with LI not linked to the *TGM1* locus shows a broadened zone of otherwise normal pericellular activity (**E, F**). Faint to absent epidermal activity at pH 7.4 is apparent in skin from patients HR (**G**), and DB (**I**). At basic pH epidermal activity is present in the granular layer of skin from these two patients showing intracellular accumulation of activity which is emphasized by the dark intercellular spaces and consistent with the distribution of TGase 3 (**H, J**). The dermal TGase 2 activity served as a useful internal comparison (*bar* 10 μm)

Therefore, by the use of the pH 7.4 buffer conditions, we can selectively assay for the TGase 1 activity in the epidermis, whereas at pH 8.4, we assay for both TGases 1 and 3.

Cryostat sections of human skin were incubated with two biotinylated peptides, the amine donor substrate biotMDC (Fig. 1) and the novel amino acceptor substrate biotNON (Fig. 2). The incorporation of these peptides into the tissue was subsequently identified by streptavidin, which thereby allows an in situ localization of endogenous TGase activity. We found that these two biotinylated substrates were readily incorporated into normal skin sections using both pH values (Figs. 1, 2). In normal human skin (Fig. 1A,B), biotMDC incorporation was detected in the stratum granulosum, along the basement membrane zone and in the cutaneous microfibrillar appparatus and surrounding papillary capillaries. The peptide biotNON gave similar results after 16 h of incubation, but was only weakly incorporated into the basement membrane zone and dermal structures. Incubation of tissue sections with peptides and purified TGase 2 led to peferential incorporation of biotMDC into the basement membrane zone and connective tissue of the papillary dermis, and to almost exclusive incorporation of biotNON into the basement membrane (data not shown). Together, these data indicate that the biotMDC peptide was efficiently incorporated by all three TGases present in the skin, whereas the biotNON peptide was more efficiently incorporated by TGases 1 and 3. In control experiments, incubations in the presence of EDTA completely inhibited incorporation of both biotinylated peptides. In addition, involucrin colocalized well with the high TGase activities in the granular layer (data not shown).

TGase activity in pathologically altered skin

Skin with an increased epidermal turnover (psoriasis) showed increased activity which was concentrated in the inter/pericellular space and involved almost the complete epidermis just sparing the basal layer (Fig. 1C,D; Fig. 2C). Four skin samples from patients with ARLI in whom linkage to *TGM1* had been excluded showed findings comparable with normal skin or skin with increased epidermal turnover (Fig. 1E,F). In contrast, the skin biopsies from two patients with the confirmed *TGM1* mutations showed an absence of TGase activity when measured at pH 7.4; that is, TGase 1 activity was clearly abnormally dimished (Fig. 1G and I for patients HR and DB, respectively). In contrast, when measured at pH 8.4, TGase activity was present in the granular layer but confined to the cytoplasm of

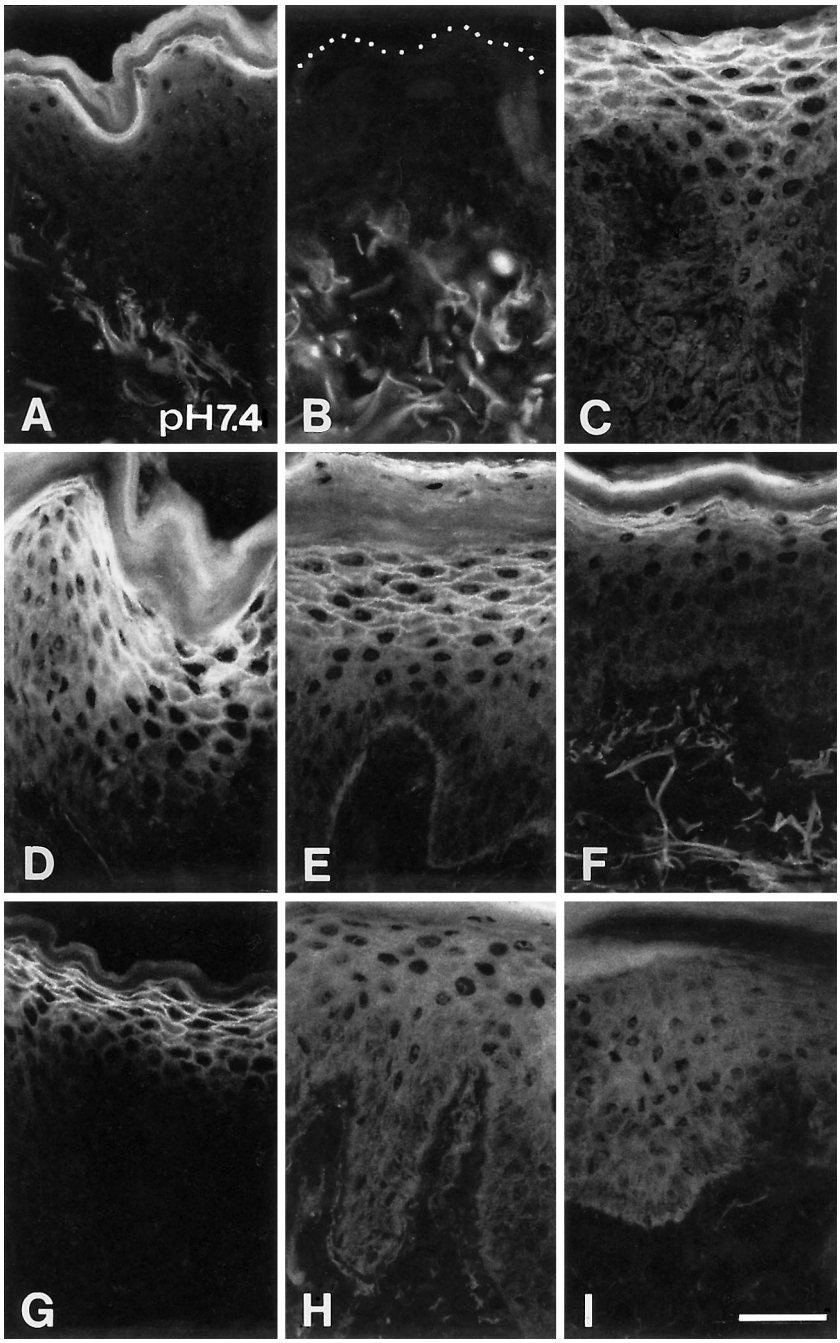

Fig. 2 A–J A peptide resembling the aminoterminus of osteonectin can be used as novel amine acceptor substrate for epidermal TGase activity. The biotinylated hexapeptide Ala-Pro-Gln-Gln-Glu-Ala-OH resembling the aminoterminus of osteonectin was applied as outlined in Fig. 1 at physiological pH. The incorporation pattern in normal skin was essentially the same as with biotMDC (**A**). The addition of EDTA to the reaction inhibited the incorporation of the substrate. The dotted line marks the margin of the cornified layer (**B**). Psoriatic skin (**C**) shows a broadened TGase acitivity zone in a reticular fashion (compare with Fig. 1C). Skin from LI patients with exluded *TGM1* gene linkage shows a reticular membrane-associated epidermal TGase activity partially in an extended fashion (**D–G**). Skins from LI patients HR (**H**) and DB (**I**) with confirmed *TGM1* mutations shows complete absence of activity (*bar* 10 μm)

the keratinocytes which was evident by the absent activity in the intercellular spaces (Fig. 1H and J for patients HR and DB, respectively). This pattern is identical to that previously determined for TGase 3 distribution in the epidermis [26, 29]. The results obtained with the amine acceptor biotNON substrate at pH 7.4 were identical: four patients with disease and excluded linkage to *TGM1* showed a pericellular TGase activity in the granular layer (Fig. 2D–G), but the two patients with confirmed *TGM1* mutations showed complete absence of staining (Fig. 2H,J), thus confirming the absence of TGase 1 activity.

Absence of TGase 1 activitiy does not necessarily mean the absence of TGase 1 protein

By use of a goat polyclonal antibody against human TGase 1 [18] we were able to could detect the presence of TGase 1 in all skin sections, including those lacking TGase 1 activity (Fig. 3). The distribution of TGase1 protein in skin from the normal individual, psoriatic patients and the four patients without mutations in the *TGM1* gene was identical to that of the TGase 1 activities shown in Fig. 2. However, skin from the two patients with confirmed *TGM1* mutations and absent TGase 1 activity showed a normal distribution of TGase 1 protein.

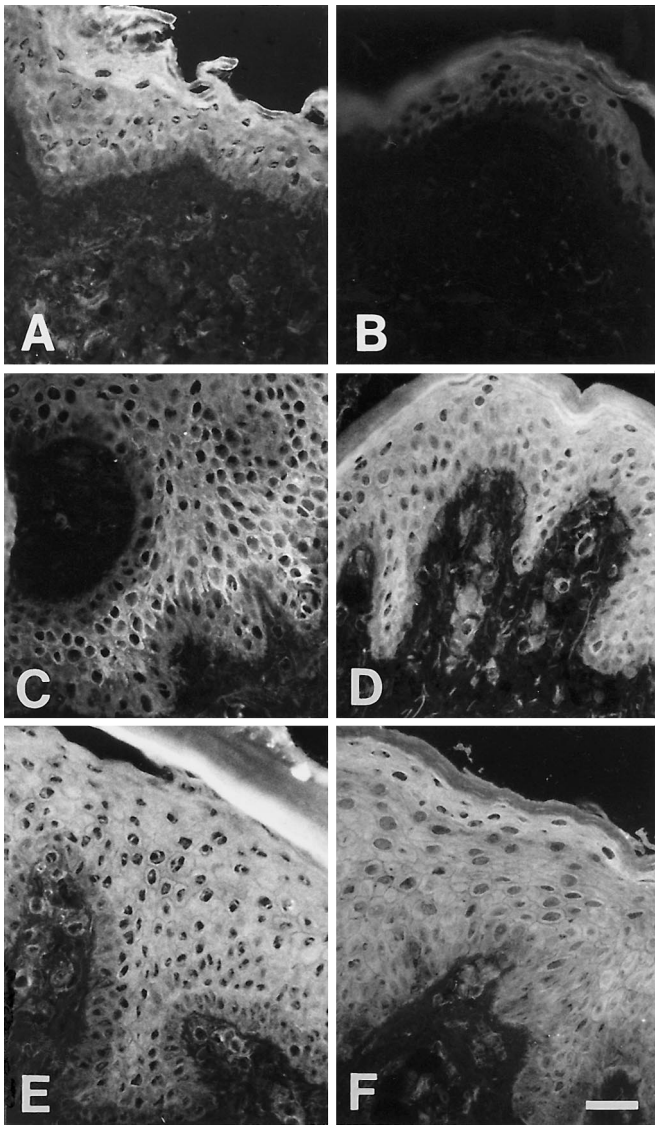

Fig. 3 A–F Immunohistochemical demonstration of TGase 1 protein in skin lacking TGase 1 activity. TGase 1 was visualized by indirect immunofluorescence using a goat antibody. In normal skin (**A**), the distribution of the enzyme is not only confined to the stratum granulosum but also extends to the spinous layer where it gives pericellular staining. No staining is obtained with a nonspecific goat serum (**B**). Tgase 1 protein is present in a similar distribution in psoriatic skin (**C**) and in skin from a patient (**D**) with LI not linked to the *TGM1* gene (same patient as in Fig. 1E,F and Fig. 2D). Skin from patients HR (**E**) and DB (**F**) with LI with confirmed *TGM1* mutations and lack of TGase1 activity shows cytoplasmic immunostaining for TGase 1 (*bar* 10 μm)

Discussion

The role of TGases in congenital recessive ichthyoses is complicated by several factors. Because there are at least three different TGases present in human skin, examination of total TGase activity in skin specimens or explant cultures of keratinocytes should not be performed without distinction between the separate enzymes. To date, this has involved complex and tedious experiments using column chromatography or immunoprecipitation methods. Yet the use of specific antibodies alone provides at best an incomplete view of the distribution of these enzymes, since as we show here in Fig. 3, TGase 1 protein may be present in the absence of TGase 1 enzyme activity, apparently because simple point mutations of the *TGM1* gene can result in an expressed but inactive protein that nevertheless still retains epitopes of the normal active protein. The extensively characterized goat polyclonal antibody we used was elicited against truncated recombinant TGase 1 protein missing the first 109 and the last 241 amino acids [18]. The homozygous splice site mutation in patient HR predicts a truncated protein ending before the active domain but containing the amino acid sequence our antibody was raised against. This explains the positive immunostaining. In contrast, others could not detect TGase 1 protein in skin cryosections and keratinocyte extracts from another patient with the same mutation with the commercially available monoclonal antibody B.C1 [12]. In light of the uncertain specificity of B.C1 [18], we only can speculate that its epitopes on TGase 1 reside C-terminal to the putative truncation.

Furthermore, TGases 1 and 3 show overlapping expression patterns in the epidermis. Membrane-associated TGase 1 is abundantly expressed in cultured epithelial cells and in intact epidermis [18, 38, 39]. TGase 1 mRNA is detectable in the basal (weakly), spinous and granular layers of the epidermis by in situ hybridization [24, 34], and concordantly, TGase 1 protein is detectable immunhistochemically in all layers (this communication; [26]). In contrast, TGase 3 is a cytosolic proenzyme requiring specific proteolysis for activation [15, 17, 26]. Protein expression occurs in the granular layer of intact epidermis [26, 29], and it is not significantly expressed in cultured keratinocytes [17]. TGase 2 expression in the skin is largely restricted to the dermoepidermal junction and dermal structures [30].

In an attempt to overcome some of these technical difficulties, we developed a rapid screening assay for monitoring endogenous TGase activity in the skin which is based on the in situ incorporation of biotinylated amine acceptor or donor peptides onto endogenous substrates, which are then detected by streptavidin. This procedure can be perfomed within one working day. The nonbiotinylated form of biotMDC has been applied and visualized previously on skin cryosections by exploiting the autofluorescence of this compound [23]. However, the use of antibodies against biotMDC [30] or the introduction of the biotinylated form greatly enhances the sensitivity of the method. In addition, the pH of the reaction in this previous work was not precisely specified. This is an important issue, since we were able in the present work to distinguish the activities of TGases 1 and 3 by manipulation of the pH of the reactions.

Traditionally, TGases have been assayed in vitro by incorporation of putrescine into succinylated casein at pH values in the ranges 7.5 [4, 7, 8, 21, 22], 8.1–8.8 [4, 5, 37] and 9–9.5 [20]. In other more recent work utilizing bacterially expressed natural TGase substrates of the epidermis,

TGase 3 operates most efficiently in vitro at pH 8.5–8.8 with trichohyalin [37] and small proline-rich proteins [6], although pH 7.5 is required for loricrin due to its insolubility at alkaline pH [4]. Similarly, TGase 3 crosslinks short peptides into natural substrates in vitro efficiently only at pH 8.5 [5]. On the other hand, TGase 1 operates with equivalent efficiency in the pH range 7.5–8.8 [4–7, 21, 22, 24, 37]. Likewise, TGase 2 works well in the pH range 7–9 (D. Aeschlimann, personal communication), is typically assayed at pH 8.3 [2, 30], but has diminished efficiency at physiological pH (this communication; [4]). In particular, by performing the present experiments at pH 7.4, we could selectively visualize the activity of TGase 1, whereas at pH 8.4, we could measure the activities of both TGases 1 and 3. That the activities of the two enzymes can in fact be separately demonstrated at the different pH values was independently confirmed. Our biotinylated peptides detected TGase 1 activity essentially throughout normal epidermis at pH 7.4 (Figs. 1A, 2A, 3A) and 8.4 (Fig. 1B). In patients with demonstrated mutations in the *TGM1* gene that results in loss of TGase 1 activity, there was no staining in the epidermis at pH 7.4 (Figs. 1G,I, 2H,I), but at pH 8.4, staining typical of the expression of TGase 3 in normal epidermis was observed (Fig. 1H,J) [26, 29].

This work raises the question as to why TGase 3 cannot compensate for the missing TGase 1 activity in patients with *TGM1* mutations. It is probable that crosslinking by TGase 1 precedes crosslinking by TGase 3. This idea is consistent with current views that TGase 1 is required for the initial stages of cornified cell envelope assembly by crosslinking such proteins as involucrin, to form a scaffold, to which other components such as the small proline-rich proteins and loricrin are added later by TGase 3 [36, 42]. Likewise it has been demonstrated that crosslinking of loricrin requires both TGases 1 and 3 by utilization of different glutamines and lysines [4], so that apparently the two enzymes cannot compensate for each other.

We should like to emphasize that in two patients with the *TGM1* mutations the distribution of TGase 1 protein appeared to by essentially normal (Fig. 3H,I). Thus, when coupled with conventional immunohistochemical methods, our procedure provides a powerful new method to identify those individuals with disease that involves relatively simple *TGM1* mutations. Given the spectrum of mutations reported so far [3, 12–14, 27, 28, 33], it is to be expected that mutations that result in a severely truncated or an unstable protein will test negative for both TGase 1 activity and protein. Finally, this method should easily detect mutations in the *TGM3* gene that result in loss of activity of TGase 3, if they should exist.

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