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Inactive Zymogen and Highly Active Proteolytically Processed Membrane-Bound Forms of the Transglutaminase 1 Enzyme in Human Epidermal Keratinocytes

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The transglutaminase 1 enzyme is important for the formation of a cornified cell envelope in terminally differentiating keratinocytes. We show here that it is present in low levels in proliferating foreskin or cultured epidermal cells as an inactive zymogen full length form of 106 kDa, of which >95% is attached to membranes. In terminally differentiating keratinocytes, there is a ≥100-fold induction of mRNA and protein. In addition to some cytosolic protein, most of the newly expressed protein is attached to membranes, of which about half exists in the zymogen form. Other protein consists of a 67/33/10 kDa complex formed by proteolytic processing at specific sites, and is anchored by way of the 10 kDa fragment. This processed form is very highly active and thus accounts for almost all transglutaminase 1 activity in keratinocytes.

Terminally differentiating epidermal keratinocytes construct a specialized structure termed the cornified cell envelope (CE) which is composed of a series of defined structural proteins crosslinked by transglutaminases (TGases) (1–5). Of the three enzymes, TGases 1, 2 and 3, active in the epidermis, TGase 1 is the most complex, since it can exist in both membrane-bound (6–11) and cytosolic (soluble) (7–11) forms. A variety of data have indicated that TGase 1 is an important marker for terminal differentiation in keratinocytes, since it is especially prevalent in the granular layers of the epidermis, consistent with its role in CE assembly (12–15). However, some data have suggested that TGase 1 is also present in smaller amounts in proliferative basal keratinocytes (7,11,14,16,17). Recently, we have shown that the cytosolic TGase 1 enzyme consists of three active forms (11): a full-length 106 kDa form; and two forms produced by proteolysis at specific sequence sites that have been conserved in the family of TGase proteins, including a 67 kDa fragment, and a complex consisting of associated 67/33 kDa fragments. These cytosolic forms represent up to a third of the total TGase 1 activity in cultured or foreskin epidermal keratinocytes (11). While it has been known for many years that the bulk of the TGase 1 enzyme in keratinocytes is bound to membranes, and is highly active (1–5), its molecular form is not yet known. In this paper, we report that the membrane-bound enzyme consists of two major forms in proliferating, stationary or terminally differentiating keratinocytes: an inactive zymogen full-length form, and a highly active complex of 67/33/10 kDa fragments activated by proteolysis at conserved sequence sites. Whereas in proliferating cells most is retained as the zymogen form, up to half is proteolytically processed in differentiating cells.

MATERIALS AND METHODS

Standard procedures. Procedures for growth and ^{35}S-cysteine/methionine labeling of normal human epidermal keratinocytes (NHEK) in cell culture or from human foreskins, cell lysis and fractionation, immunoprecipitation of TGase 1 antigens, gradient SDS-PAGE gels, fractionation by monoQ FPLC chromatography, assays for TGase activities, and amino acid sequencing, were done as described (11).

Release of membrane-bound TGase 1 forms. Pellets of cell lysates were washed at 4°C by gentle sonication in lysis buffer containing either 0.1% Triton X-100 for 15–30 min, or with 1 M NH_2OH-HCl for 1–2 h (9), and then washed to remove these reagents before further use (14).

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Semi-quantitative RT-PCR methods and primers. RNA was isolated from epidermal cells with Trizol (Gibco-BRL). Thermostable rTth DNA polymerase (Perkin Elmer) was used to transcribe 50 ng of total RNA per 10 μl reactions as recommended and with 2 mM $MgCl_2$ for 29 cycles with denaturing at 95°C for 1 min, annealing at 60°C and extension at 72°C for 90 sec each (18). cDNA clones (1 ng) were amplified separately as controls. To monitor reactions, α-[^{32}P]-dCTP (5 μCi, 3000 Ci/mmol, New England Nuclear) (one-eighth of the standard molar amount of dCTP) was included. The PCR products were resolved on 6% polyacrylamide gels, dried, and scanned on a PhosphoImager. By similar PCR amplification of 0.1 ng of plasmid cDNA clones, the RT-PCR data could be expressed as pg of specific mRNA/μg of total RNA. The primers designed crossed over introns, are are: keratin 1 (18) (+): 5′-TCCTGGAGCAGCAGA-ACCAGGTAC-3′; (−): 5′-CTTGATGGTCACAAATTCATTCTCT-3′; TGase 1 (19) (+): 5′-GCCAACACTG-CTGGCAGGCATGATGGA-TGG-3′; (−) 5′-GTCGAGGGTTCAGGTCCCCAGTCGT-CAT-3′; Involucrin (20) (+) 5′-CTTTACAGCAGTCAGGT-GCTTTTCCTCTTG-3′; (−) 5′-GCACTGGACAATAATTCCCTCTCTCCC-CCA-3′; and glyceraldehyde phosphate dehydrogenase (GAPDH) (GenBank #M33197): (+) 5′-TGAAGGTCGGAGTCAACGGATTG-3′; (−) 5′-GCCATGGAATTTGCCATGGGTGGA-3′.

RESULTS AND DISCUSSION

TGase 1 mRNA is expressed in proliferating epidermal keratinocytes. By use of semi-quantitative RT-PCR we have explored the expression in keratinocytes of mRNAs for three markers of epidermal differentiation. Foreskin epidermal cells were recovered (11) and plated for 1, 2, 4 or 12 h. Other data have shown that the cells which attach within 4–6 h are proliferating basal cells (21) and those which attach within 1–2 hours are enriched for epidermal stem cells (22). We calculate that 1, 2 or 4 h attached cells contain no detectable mRNAs for keratin 1 or involucrin, but contain 1–2 pg of TGase 1 mRNA/μg of total RNA (Fig. 1), which is significant in comparison to levels measured in certain keratinocyte cell lines (17). NHEK cells grown in low Ca^{2+} (0.05 mM) proliferating conditions contained ~10 pg of TGase 1 mRNA/μg, and in older confluent stationary cultures, the levels were ~20 pg/μg, in agreement with earlier data (17,23). In cells grown in high Ca^{2+} (1.2 mM) differentiating conditions, expression of mRNAs for all markers was greatly increased, as expected, and within 5 days, was >100 times higher for TGase 1 mRNA. These data confirm previous suggestions of modest expression of TGase 1 mRNA in proliferating or stationary keratinocytes (11,24), and moreover, reaffirm the established view that TGase 1 is a valid marker for terminal differentiation in keratinocytes (7,11,14,16,17).

Membrane-bound and cytosolic forms of the TGase 1 enzyme in foreskin epidermal cells. Lysates of 4 h attached (basal) and 4 h unattached (suprabasal) ^{35}S-labeled foreskin epidermal cells were separated into cytosolic and membrane-bound fractions. The latter were treated with Triton X-100 (Fig. 2, lanes 2,3,7) or with 1.0 M NH_2OH-HCl (lanes 4,5,8,9), and immunoprecipitated with the TGase 1 antibody. Release of antigens was essentially complete within 15 min with Triton X-100

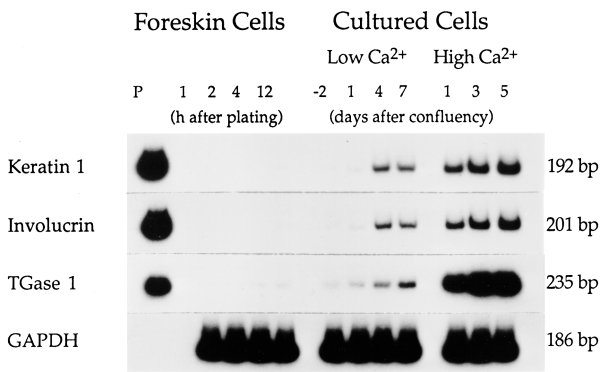

FIG. 1. Semi-quantitative RT-PCR of mRNAs encoding proteins as shown. Loadings were adjusted to the same amount of label as for GAPDH. Templates were from: Lane P: plasmid DNAs; lanes marked 1, 2, 4 and 12 h were attached foreskin epidermal cells; cultured cells were plated for the numbers of days shown after achieving confluency (~4 days after initial plating) in low Ca^{2+}.

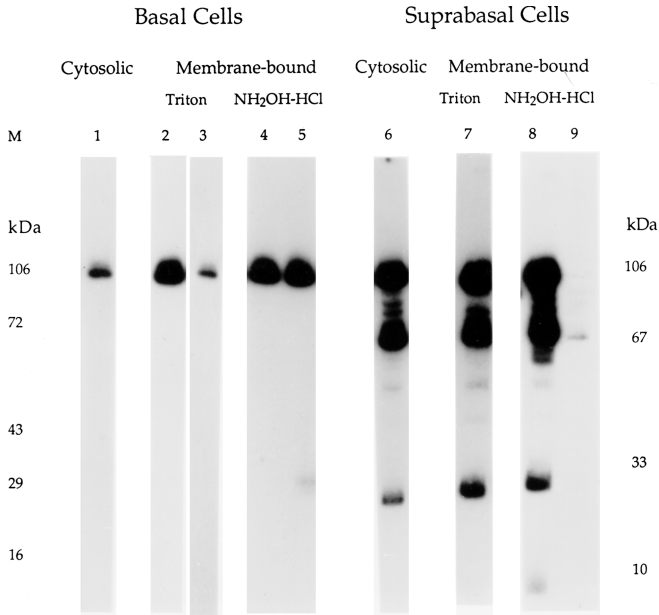

FIG. 2. Cytosolic and membrane-bound foreskin epidermal TGase 1 forms. Membrane-bound TGase 1 antigens from ^{35}S-methionine/cysteine labeled 4 h attached (lanes 1–5), or 4 h unattached (lanes 6–9) foreskin cells were released with 0.1% Triton X-100 for 15 min (lanes 2,7), or 1 M NH_2OH-HCl for 1 (lanes 4,8) or 2 hour (lane 5), immunoprecipitated, and the products resolved on 10–20% gradient SDS-PAGE gels. Lanes 3,9: the residues remaining after reaction as in lanes 2 or 8 were repelleted and treated with Triton X-100 for another 15 min (lane 3), or NH_2OH-HCl for a second 1 hour (lane 9). Sizes of TGase 1 forms are as shown (right) based on standard markers (left).

(cf lanes 2,3), and 1 hour with NH_2OH-HCl (cf lanes 8,9), although some chemical cleavage occurred within 2 hour with the latter (cf lanes 4,5) (24). In basal cells, both the cytosolic and membrane-bound fractions of the TGase 1 were present entirely as the 106 kDa full length protein, of which ~95% was membrane-bound (lanes 1–5). In suprabasal cells, the cytosolic (~25% of total) and membrane-bound (~75%) proteins each consisted of major bands of 106, 67 and 33 kDa (lanes 6–8), but the latter also contained a novel band of 10 kDa (lanes 7,8). This 10 kDa species was not recognised or precipitated by the polyclonal antibody and was not seen in the soluble fractions (11). About 50% of the cytosolic and membrane-bound protein was processed.

Membrane-bound and soluble forms of the TGase 1 enzyme in NHEK cells. Similar experiments were done with NHEK cells grown in low (Fig. 3A, 3B) or high (Fig. 3C, 3D) Ca^{2+} media. The 67 and 33 kDa TGase 1 forms were major components of both the soluble and membrane-bound fractions in differentiating cultures, and were present in smaller amounts in proliferating cultures as well (11). The 10 kDa band appeared only in the membrane-bound fractions. Based on the amounts of ^{35}S-labeled bands, we can estimate that 5 day old high Ca^{2+} cultures express ~100 times more TGase 1 protein than proliferating or stationary cultures grown in low Ca^{2+}, a value similar to the observed increases in TGase 1 mRNA levels.

The 67, 33 and 10 kDa TGase 1 forms arise from specific cleavage at conserved sites of the full-length 106 kDa protein. Gels of Fig. 2 lanes 1,2,4,6–8 were transferred to PVDF membranes and the 106, 67, 33 and 10 kDa bands were cut out for amino acid sequencing for 8–15 Edman degradation cycles (11). The amino termini of the 106 and 10 kDa bands corresponded to residue position 3 of the TGase 1 protein. Thus the 10 kDa band, seen only following release of the membrane-bound TGase 1, harbors the membrane anchorage region, as expected (9,10). Likewise, the amino termini of the 67 and 33 kDa bands from each of the cytosolic and membrane-bound fractions tested resided at residue positions 93 and 573, respectively, as found before (11).

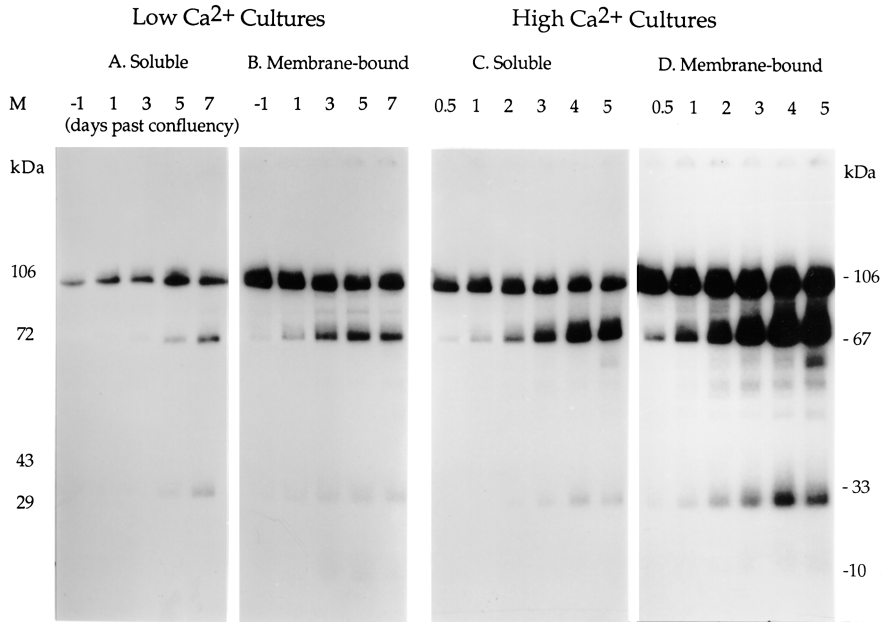

FIG. 3. Multiple forms of immunoprecipitated TGase 1 proteins in cytosolic (soluble) (A, C) or membrane-bound (B, D) fractions of cultured NHEK cells grown in low Ca^{2+} (A, B) or high (C, D) Ca^{2+} cultures. Lane numbers refer to days of culturing past confluency. M, standard protein markers as shown. Sizes of TGase 1 forms are as shown (right) based on standard markers (left).

Separation of multiple TGase 1 enzyme forms. The membrane-bound 106 and 67/33/10 kDa TGase 1 forms of 3 day high Ca^{2+} cultures were released with Triton X-100 and resolved by FPLC chromatography (Fig. 4). Whereas there were two peaks of ^{35}S-labeled protein, which contained the 106 kDa and 67,33,10 kDa bands (Fig. 4A), only the latter contained enzymic activity (Fig. 4B). Thus the 67/33/10 kDa bands are held together by secondary forces as a chromatographically stable, active complex.

The membrane-bound 67/33/10 kDa complex is the most highly active form of TGase 1 in keratinocytes. These two membrane-bound as well as the three cytosolic TGase 1 forms were

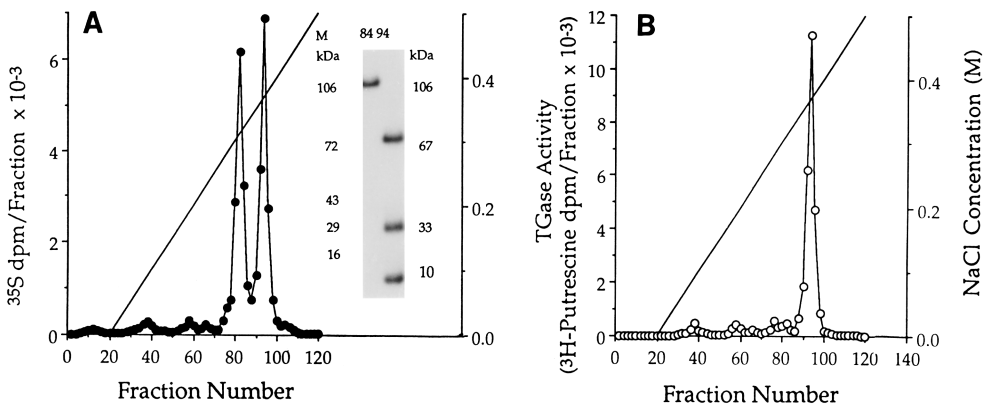

FIG. 4. Fractionation of membrane-bound forms of TGase 1 from 3 day high Ca^{2+} cultures. Following release of proteins from ^{35}S-labeled (A) or unlabeled (B) cultures with Triton X-100, immunoprecipitated products were resolved on a 0.5×5 cm monoQ FPLC column exactly as described (11). Inset in A shows fluorograph of SDS gel from peaks 84 and 94; M, standard markers of sizes shown; sizes of TGase 1 bands are shown on right. In B, fractions were assayed for TGase activity.

TABLE 1
Specific Activities of Various TGase 1 Forms, Expressed as pmol of ^{3}H-Putrescine Incorporated into Succinylated Casein/h/pmol of TGase 1 Protein Form, and Are the Averages ± S.D. of 3-5 Measurements

	Cytosolic			Membrane-bound	
Size (kDa)	106	67	67/33	106	67/33/10
Foreskin cells:					
2 h attached	52 ± 27			5 ± 4	
4 h attached	60 ± 28			6 ± 4	
4 h unattached	43 ± 25	237 ± 71	615 ± 43	7 ± 5	1003 ± 217
Low Ca^{2+} NHEK Cultures:					
2 day	51 ± 32	281 ± 82	586 ± 63	5 ± 4	
4 day	65 ± 28	269 ± 56	601 ± 78	4 ± 3	959 ± 176
6 day	48 ± 13	238 ± 36	568 ± 42	6 ± 3	1028 ± 253
High Ca^{2+} NHEK Cultures:					
2 day	62 ± 14	259 ± 33	543 ± 45	5 ± 3	1091 ± 173
5 day	63 ± 11	263 ± 17	572 ± 39	4 ± 4	995 ± 165

Protein forms were recovered by FPLC chromatography of unlabeled samples (Fig. 4 and reference 11).

assayed for activity as before (11). The data show (Table I) that the membrane-bound 106 kDa form from several sources was inactive; that is, it exists in cells as a zymogen form. However, the specific activity of the membrane-bound 67/33/10 complex was >100 times higher, and is significantly greater than that of the cytosolic 67/33 and 67 kDa forms (Table I). This suggests that most of the TGase 1 activity in keratinocytes exists as the membrane-bound 67/33/10 kDa complex form.

The role of the 10 kDa fragment in TGase 1. The findings of lower specific activities of the cytosolic enzymes lacking the 10 kDa fragment are consistent with our earlier studies on a series of deletion constructs of recombinant human TGase 1 (25). Cleavage of it at residue position 93 and 593 with dispase resulted in a two-fold increase in specific activity, but if the first 10 kDa were deleted, there was no activation (11). That is, cleavage at residue 93 to form the 10 kDa fragment is likely responsible for activation of TGase 1. Together, these data suggest that the 10 kDa fragment not only provides the dual role of membrane anchorage and high affinity attachment of the 67/33 complex, but also dictates the catalytic state of TGase 1.

Conclusions. Our new data indicate that (1) the membrane-bound intact 106 kDa form serves as an inactive or zymogen form of the TGase 1 enzyme in keratinocytes; (2) some is reversibly cycled on or off membranes, so that in proliferating cells, there is a small amount of TGase 1 activity in the cytosol; (3) upon commitment to terminal differentiation, ≥100 times more mRNA and protein are expressed; (4) during differentiation, up to 50% is proteolytically processed into 67, 33 and 10 kDa fragments at conserved sequence sites, which are retained as a 67/33/10 kDa complex held together by secondary interactions while still bound to membranes; (5) the extent of processing directly correlates with the degree of differentiation, both in foreskin epidermis *in vivo* and in NHEK cells grown *in vitro;* (6) this form is very highly active and constitutes the bulk of TGase 1 activity in keratinocytes; and (7) some active processed TGase 1 protein as a 67/33 kDa complex, or free 67 kDa form, is cycled into the cytosol, presumably by detachment from the 10 kDa anchorage fragment.

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