The transglutaminase 1 (TGase 1) enzyme is involved in the formation of a cornified cell envelope in terminally differentiating epidermal keratinocytes. The enzyme is present in proliferating cells but is more abundantly expressed in differentiating cells and exists in several intact or proteolytically processed cytosolic or membrane-anchored forms. We show here that the equilibrium partitioning of TGase 1 between the cytosol and membranes is controlled by variable modification by myristate and palmitate. During synthesis, it is constitutively N-myristoylated. Later, it is modified by an average of two S-myristoyl adducts in proliferating cells or one S-palmitoyl adduct in differentiating cells. The three myristoyl adducts of the former provide more robust anchorage to membranes than the one myristoyl and one palmitoyl adduct of the latter. The half-lives of the S-myristoyl and especially the S-palmitoyl adducts are less than that of the TGase 1 protein, suggesting a mechanism for cycling off membranes. In in vitro overlay assays, the S-acylated 10-kDa anchorage fragment facilitates binding of TGase 1 forms, supporting a mechanism of cycling back onto membranes in vivo. We conclude that differential acylation increases the repertoire of functional TGase 1 forms, depending on the differentiation state of epidermal keratinocytes.

As far as is known, the transglutaminase 1 (TGase 1) enzyme is involved in the formation of a cornified cell envelope in terminally differentiating stratified squamous epithelia by cross-linking a series of defined structural proteins (1–10). However, in the epidermis, the enzyme is first expressed in minor amounts in the proliferative basal layer (11–14), in modest amounts in the suprabasal cells committed to differentiation, and then in much larger amounts in the granular layer as terminal differentiation in the epidermis and cornified cell envelope assembly proceed (5, 11–16). Recently, we have demonstrated that this enzyme system exists in epidermal keratinocytes in several forms that are differentially partitioned between the cytosol and membranes. The cytosolic enzyme exists as a full-length form of 106 kDa of low specific activity and two proteolytically processed forms that have 5–10-fold higher specific activities, one of 67 kDa and a second consisting of 67 and 33 kDa fragments that are held together by noncovalent bonds. Together, these three forms constitute from about 5%, up to 35%, of the total TGase 1 enzyme activity in proliferating or terminally differentiating cells, respectively (10). The membrane-bound enzyme exists in two forms: a full-length essentially inactive zymogen, or a complex of 67/33/10 kDa chains that is very highly active (11). The membrane-bound zymogen form constitutes the bulk of TGase 1 protein in keratinocytes. During terminal differentiation, much more TGase 1 protein is expressed than is present in proliferating basal cells. Most of this remains as the full-length inactive membrane-bound zymogen form, but up to one-half of it may be proteolytically processed while still anchored to the membranes into the highly active 67/33/10 kDa form, in this way providing the bulk of the TGase 1 enzyme activity in differentiating keratinocytes (11).

Earlier studies have shown that the enzyme is acylated by palmitate, and to a lesser extent by myristate, at a cluster of five cysteine residues located on the amino-terminal extension peptide, which is unique to the TGase 1 enzyme. The lipid adducts were thought to be most likely attached by thiol-ester bonds since they could be efficiently cleaved by concentrated NH$_2$OH-HCl (17). Mutational analyses of these cysteines showed that several are involved in attachment of the enzyme to membranes (18). In addition, recombinant experiments showed that when this cysteine cluster motif is added to another protein, involucrin, it becomes attached to membranes. Thus, these thio-ester linkages adequately explain how TGase 1 is anchored to membranes (17, 18). However, the roles of these different lipid adducts remain unknown.

In this report, we have explored the mechanism by which enzyme activity is partitioned between the cellular membrane and cytosol compartments during proliferation and differentiation by way of the lipid acyl adducts. We show here that all membrane-bound or cytosolic forms of intact TGase 1 are constitutively labeled by an N-acyl myristate linkage on glycine residue 3. We show that in proliferating and differentiating cells, the TGase 1 is differentially S-myristoylated or S-palmitoylated, respectively, which seem to confer differing degrees of attachment. Since these adducts have different half-lives, the differential labeling affords a variable mechanism for partitioning and cycling TGase 1 on or off membranes.
mmol), or 9,10-2H-myristic acid (11.2 Ci/mmol) (DuPont NEN). The labeled purines or hydroxy-lipids were added for 4 h and then chased with unlabeled medium for up to 7 days (10). Some cultures also contained 100 µg/ml of cycloheximide. Harvested cells were lysed in buffer containing the protease inhibitor set as before and pelleted at 10,000 x g to obtain the cytosolic and membrane fractions (10).

**Release of Membrane-bound TGase 1 Forms**—The release of membrane-bound TGase 1 forms was accomplished by using either 0.1% Triton X-100 for 15 min or 1 mM NH₄OH-HCl for 1 h (11). In some experiments, 0.4 mM NH₄OH-HCl was used for up to 8 h. The supernatant fractions were used directly for either immunoprecipitation with the TGase 1 polyclonal antibody or Mono Q FPLC chromatography (10, 11, 19).

**Biochemical Assays**—Immunoprecipitation pellets were resuspended in SDS sample buffer, and the proteins were resolved on 10–20% gradient or 15% linear SDS-polyacrylamide gel electrophoresis gels (10), dried, and visualized after 3–15 days of autoradiography. In the case of the 0.5–4 M NH₄OH-HCl was used for up to 8 h. The supernatant fractions were used directly for either immunoprecipitation with the TGase 1 polyclonal antibody or Mono Q FPLC chromatography (10, 11, 19).

**Reverse-phase Thin Layer Chromatography**—This was used to resolve myristate and palmitate. Samples of immunoprecipitated proteins from column fractions were hydrolyzed in 50 µL of 5.7 N HCl at 106 °C in vacuo for 2–22 h, cooled, and extracted with 100 µl of CHCL₃. Control experiments showed that ~99% of the labeled lipids were extracted into the CHCl₃ phase. The CHCl₃ was evaporated in a stream of N₂. The residue was redissolved in 3 µl of isopropyl alcohol, spotted onto C₁₈ reverse-phase thin layer chromatography plates (Altrich), developed in a solvent of methanol:water:acetic acid (89:11:0.2, v/v/v), and autoradiographed for 6 days (21).

**Fractionation of Peptide-Lipid Adducts and Mass Spectroscopy**—Peaks of TGase 1 proteins from FPLC columns that had been labeled with [³⁵S]cysteine and either [³H]myristate or [³H]palmitate were described (22). Briefly, 10 pmol (quantitated by amino acid analysis) of myristate-labeled proteins, gels were soaked in Enlightening (DuPont NEN) for fluorography. Selected bands were quantitated in a Molecular Dynamics). Assays for TGase 1 activities were done as before (10, 20).

**In Vitro Overlap Experiments**—These were done essentially as described (22). Briefly, 10 pmol (quantitated by amino acid analysis) of unlabeled 10-kDa TGase 1 fragments were dot-blotted onto nitrocellulose (Schleicher & Schuell; pore size, 0.45 µm), and washed in phosphate-buffered saline containing 0.1% (w/v) Tween 20, 1 mM dithiothreitol, 5% (w/v) bovine serum albumin, and the protease inhibitor set (20). Dried dots were overlaid with 20–50 pmol amounts of [³⁵S]labeled intact TGase 1 protein or fragment forms, reacted for 2 h at 4 °C, washed with the same buffer, dried, and exposed to X-ray film.

**RESULTS**

**Metabolic Labeling by Myristate and Palmitate of the 10-kDa Portion of TGase 1 in Cultured NHEK Cells**—We have shown previously (11) that membrane-bound TGase 1 antigens can be solubilized from the membrane fraction of cultured NHEK cells in one of two ways: incubation with 0.1% Triton X-100 within 15 min at 4 °C; or with 1 mM NH₄OH-HCl within 1–2 h at 23 °C. The former method dissolves membranes, leaving the lipid adducts attached to proteins; the latter method hydrolyzes the thio-ester bond by which the lipids are attached (17, 23, 24).

In this study, low (Fig. 1, A and B) or high (Fig. 1, C and D) Ca²⁺ cultures of NHEK cells were labeled with either [¹⁴C]myristate (Fig. 1, A and C) or [¹⁴C]palmitate (Fig. 1, B and D) for 4 h at various days of culturing and resolved into cytosolic and membrane fractions. The latter were solubilized with either Triton X-100 or 1 mM NH₄OH-HCl for immunoprecipitation with the specific TGase 1 polyclonal antibody; then the products were resolved on gradient gels, which were developed by Fluorography. In all cases where labeling occurred, only the 106- and 10-kDa TGase 1 species were labeled, which confirms that the lipids were attached to the 10-kDa portion of the TGase 1 protein (17). Comparisons between Triton- and NH₄OH-HCl-solubilized membranes from low Ca²⁺ cultures (Fig. 1A) showed that the former was reproducibly two times more labeled than the latter, suggesting that about ~65% of the adduct was attached through a thio-ester linkage and ~35% was not. In high Ca²⁺ cultures, the Triton-X-100 and NH₄OH-HCl-solubilized membrane fractions were approximately equally labeled with myristate (Fig. 1C), which means that only a small amount of the label was attached through the thio-ester linkage and most was not. In high Ca²⁺ cultures, the membrane-bound TGase 1 fraction was labeled with [¹⁴C]palmitate, and since ~98% could be removed by NH₄OH-HCl (Fig. 1D), the linkage was most likely through thio-ester bonds. The cytosolic TGase 1 forms in myristate-labeled cultures were much less labeled than the membrane-bound TGase 1 fractions (Fig. 1, A and C). This myristate label was refractory to NH₄OH-HCl treatment (data not shown), and thus most was not due to thio esterification. Based on comparisons of [³⁵S]-labeling in parallel cultures, we estimate the stoichiometry of myristate label on the cytosolic 106-kDa TGase 1 was ~0.1 mol/mol. The cytosolic fractions were not labeled by palmitate (Fig. 1, B and D).

These observations were confirmed by Mono Q FPLC chromatography of membrane-bound TGase 1 antigens immunoprecipitated from 5-day high Ca²⁺ cultures. In this case, when Triton X-100 was used for release, two peaks were labeled with [¹⁴C]myristate (Fig. 2A) or [¹⁴C]palmitate (Fig. 2C). By [³⁵S]cysteine labeling in separate parallel experiments, we estimate the stoichiometry of myristate label on the 106-kDa TGase 1 was ~0.1 mol/mol. The cytosolic fractions were not labeled by palmitate (Fig. 1, A and C). This myristate label was refractory to NH₄OH-HCl (Fig. 2D).

The labeling by myristate and palmitate was confirmed by reverse-phase thin layer chromatography of the FPLC peak following hydrolysis in 5.7 N HCl for 2 h and extraction into CHCl₃ (data not shown). We also noted that ~3% of the myristate label migrated in the position of palmitate, presumably due to metabolic elongation during the 4-h incubation of the NHEK cells.

**Evidence That TGase 1 Is Cotranslationally N-Acylated by Myristate**—The above data suggest that a hitherto unrecognized portion of the myristate label was attached through non-thio-ester bonds. We have shown previously that the amino terminus of the 106- and 10-kDa fragments of TGase 1 are blocked but could be freed after 2 h hydrolysis in 5.7 N HCl, exposing residue 3 glycine (10, 11). Therefore, one possibility is that the amino terminus of the TGase 1 protein is labeled by N-acyl myristate, as has been found for the subplasma membrane protein band 4.2 (25).

Cultures of NHEK cells grown for 5 days in high Ca²⁺ medium were pulse-labeled for 4 h with either [³⁵S]cysteine/methionine, [¹⁴C]myristate, or [¹⁴C]palmitate in the absence or presence of cycloheximide. TGase 1 antigens were immunoprecipitated from the cytosolic and membrane fractions following treatment with either Triton X-100 or NH₄OH-HCl. In the cytosolic fraction, only the 106-kDa form was labeled with myristate, which was resistant to release with NH₄OH-HCl (Fig. 3; compare lanes 3 and 4 with lanes 9 and 10). Only traces of palmitate label were present in the cytosolic TGase 1 anti-
gens (Fig. 3, compare lanes 5 and 6 with lanes 11 and 12). In the membrane-bound fractions, the 106- and 10-kDa bands were labeled with myristate, of which about 50% was resistant to release by NH₂OH-HCl (Fig. 3, compare lanes 15 and 16 with lanes 21 and 22).

In addition, both [³⁵S]cysteine and [³H]myristate labeling of TGase 1 antigens was greatly reduced in the presence of cycloheximide, which suggests that myristate labeling occurs cotranslationally with protein synthesis. However, in high Ca²⁺ cultures, significant labeling by palmitate still occurred in the presence of cycloheximide (Fig. 3, compare lane 17 with lane 18), all of which was removed by NH₂OH-HCl (Fig. 3, lanes 23 and 24), suggesting that some S-palmitoylation may occur independently of protein synthesis.

N-Myristoylation and S-Myristoylation or S-Palmitoylation Labeling of TGase 1—We next examined the nature of the lipid adducts on the amino terminus and membrane-anchorage cysteine cluster on the TGase 1 protein. The 106- and 67/33/10-kDa forms were recovered by FPLC from cultured NHEK cells labeled with [³⁵S]cysteine and either [⁹,¹⁰-³H]myristate or [⁹,¹⁰-³H]palmitate. Each sample was then digested to completion with trypsin and proteinase K, and the peptides were resolved by HPLC on a C₄ column. Under the conditions used, free cysteine-labeled peptides from throughout the protein should not be retarded, but peptides containing lipid adducts should be significantly retarded. This procedure should release from TGase 1 retarded peptide species containing the cysteine cluster (CCGCCSCR, residues 46–53) with attached myristate or palmitate. Another retarded species containing the amino terminus (GPR, from residue 3) should be found if it is N-myristoylated. Each of these peaks should be separable from the free lipids.

In 5-day high Ca²⁺ palmitate-labeled cultures, ³⁵S and ³H labels were present in resolved peaks at fractions 45 (major), 80, and 110 with Triton X-100 extraction (Fig. 4A). Following treatment with NH₂OH-HCl, the [³H]palmitate label shifted to fraction 60, coeluting with free palmitic acid. There was a concomitant increase in the size of a non-retarded ³⁵S-labeled peptide peak at the void volume of the HPLC column (Fig. 4B). These data are expected for hydrolysis of [S]palmitate to the free peptide and palmitic acid.

In 5-day high Ca²⁺ myristate-labeled cultures, a major ³H-labeled peak eluted at fraction 50 with Triton X-100 (Fig. 4C), which was resistant to change by NH₂OH-HCl (Fig. 4D). This was consistent with the N-acylated amino-terminal tripeptide. Other double-labeled peaks were obtained at fractions 45, 80, and 110, which on treatment with NH₂OH-HCl, the [³H]palmitate label shifted to fraction 60, co-eluting with free palmitic acid. As described above, these double-labeled peaks were most probably due to the elongation during culture of some myristate label to palmitate, which was then used for this esterification.

In confluent cultures grown in high Ca²⁺ for only 1 day, the profiles were somewhat more complicated. With palmitate labeling, in addition to a major peak at fraction 45, minor double-labeled peaks were found at fractions 80 and 110, as well as 38, 66, and 80 (Fig. 4E). On treatment with NH₂OH-HCl, these...
shifted to major peaks at fractions 60 and 53, coeluting with free palmitic and myristic acids, respectively (Fig. 4F). Similar observations in converse were seen with myristate labeling in 1-day cultures; minor double-labeled peaks were found at fractions 38, 45, 66, 80, 95, and 110 (Fig. 4G), which shifted to peaks coeluting with palmitic and myristic acids on treatment with NH₂OH-HCl (Fig. 4H). In addition, in this case, the peak at fraction 50 was unchanged. These data are consistent with the concept that in cultures recently shifted to high Ca²⁺ medium, the TGase 1 newly expressed in the differentiating condi-
tions was modified by palmitate instead of myristate. Likewise, stationary confluent NHEK cells maintained in low Ca$^{2+}$ for 6 days contained both myristate and palmitate peaks (Fig. 4, I and J), perhaps because some cells “escaped” to begin terminal differentiation in these conditions (26), and some myristate had been elongated to palmitate.

Use of Sequencing and Mass Spectroscopy to Confirm the Nature of the N-Myristoyl, S-Myristoyl, and S-Palmitoyl Adducts—The chemical nature of the peaks eluted in fractions 45, 80, and 110 (palmitate labeled), and 38, 50, 66, and 94 (myristate labeled) from the C$_4$ HPLC was determined. Following a 2-h acid hydrolysis, peak 50 was confirmed by sequencing to contain the tripeptide Gly-Pro-Arg, and thus it corresponds to residues 3–5 near the amino terminus of TGase 1. The amino acid compositions of peaks 38, 45, 66, 80, 94, and 110 were identical and contained Cys$_x$, Gly, Ser, and Arg, consistent with the cysteine-rich anchorage peptide of residues 46–53 of TGase 1. However, this could not be confirmed by direct sequencing because the phenylthiohydantoin derivative of Cys is destroyed during the sequencing reactions.

Therefore, mass spectrometric analyses were performed on 10–50-pmol aliquots of each of the retarded peaks. The material of fraction 50 of Fig. 4D had a mass of 539.4, was unchanged on treatment with NH$_2$OH-HCl, but was reduced to 328.3 after acid hydrolysis (Fig. 5A). These data correspond well to calculated values of 539.8 for N-myristoylated-Gly-Pro-

**Fig. 4. Identification of palmitate and myristate adducts.** Confluent cultures of NHEK cells grown in the presence of $[^{35}S]$cysteine/methionine in high Ca$^{2+}$ medium for 5 days (A–D), or 1 day (E–H), or in low Ca$^{2+}$ medium for 6 days (I and J) were labeled with 9,10-$^3$H-palmitate (A, B, E, and F) or 9,10-$^3$H-myristate (C, D, and G–J). The membrane fractions were treated with Triton X-100 (A, C, E, G, and I) or NH$_2$OH-HCl (B, D, F, H, and J), immunoprecipitated, and digested with trypsin and proteinase K; the products were resolved on a C$_4$ HPLC column. The peaks were identified following detailed characterization by amino acid sequencing, mass spectroscopy (see Fig. 5), and calibration with free myristic and palmitic acids. NM-GP, N-myristoylated-Gly-Pro-Arg peptide; SP$_1$-cys, SP$_2$-cys, and SP$_3$-cys, cysteine cluster peptide containing one, two, or three thio-esterified palmitates; SM$_1$-cys, SM$_2$-cys, and SM$_3$-cys, the same, but with one, two, or three thio-esterified myristates.

**Table:**

| Fraction | NM-GP | Palmitic Acid |
|----------|-------|---------------|
| A        | NM-GP | Palmitic Acid |
| B        | NM-GP | Palmitic Acid |
| C        | NM-GP | Palmitic Acid |
| D        | NM-GP | Palmitic Acid |
| E        | NM-GP | Palmitic Acid |
| F        | NM-GP | Palmitic Acid |
| G        | NM-GP | Palmitic Acid |
| H        | NM-GP | Palmitic Acid |
| I        | NM-GP | Palmitic Acid |
| J        | NM-GP | Palmitic Acid |
Arg, and of Gly-Pro-Arg, respectively. However, the expected mass species at 228.4, corresponding to free myristic acid, was below the level of detection of the assay. Peaks 38, 66 (Fig. 5B), and 94 had masses of 1045.1, 1255.3, and 1465.6, all of which were reduced to 833.5–834.1 on treatment with NH₂OH-HCl. These coincide very closely to species containing one, two, or three myristates attached to the cysteine cluster peptide Cys-Cys-Gly-Cys-Cys-Ser-Arg of mass 834.0. Similarly, peaks 45, 80 (Fig. 5C), and 110 had masses of 1072.1, 1311.9, and 1548.8, which were reduced to 833.9–834.3 on treatment with NH₂OH-HCl. These are consistent with species containing one, two, or three palmitates attached to the cysteine cluster peptide.

Together, these data show that up to three lipid molecules are attached to the cysteine cluster peptide, although the predominant stoichiometry was two for myristate and one for palmitate. The exact cysteine residues acylated could not be determined.

Anchorage of TGase 1 to Membranes by S-Myristoylation Is More Robust Than S-Palmitoylation—The above data have shown that in proliferating or stationary NHEK cells grown in low Ca²⁺ medium, the membrane-bound forms of TGase 1 possess one N-myristoyl and two S-myristoyl adducts. In terminally differentiating cells grown in high Ca²⁺ media for up to 5 days, the larger amounts of TGase 1 protein present possess the one N-myristoyl but predominantly only one S-palmitoyl adduct. We then tested whether these differences affect attachment to membranes. Membrane-bound TGase 1 antigens present in 6-day low Ca²⁺ cultures or in 5-day high Ca²⁺ cultures could be released by 1 M NH₂OH-HCl (Fig. 6), as described previously (11, 17, 18). However, using 0.4 M NH₂OH-HCl instead, we found that release of TGase 1 antigens from membranes of low Ca²⁺ cultures required 5–6 h, which was reproducibly much longer than the 2–3 h required for release from membranes of high Ca²⁺ (Fig. 6).

Half-lives of the N-Myristate, S-Myristate, and S-Palmitate Adducts Are Shorter Than the TGase 1 Protein—We showed previously (10) that the soluble 106-, 67-, and 33-kDa forms from high Ca²⁺ cultures have half-lives in cultured NHEK cells of about 20, 7, and 6–7 h, respectively. Similar experiments were done on the membrane-bound TGase 1 proteins from 1–4- and 3–7-day low Ca²⁺ cultures, or 1–4- and 2–5-day high Ca²⁺ cultures (Fig. 7). The soluble 106-, 67-, and 33-kDa TGase 1 forms from both low and high Ca²⁺ cultures had the same half-lives as measured before (10). In early (1–4-day) low Ca²⁺ cultures, the half-life of the 106-kDa form was ~40 h, but in 3–7-day cultures, the line was not linear, with slopes varying between 30–40 h (Fig. 7A). In 1–4-day high Ca²⁺ cultures, its half-life was ~26 h, but in 2–5-day cultures, it was ~20 h (Fig. 7C). These changing values must be due to the appearance of increasing amounts of S-palmitoylated TGase 1, the half-life of which is, therefore, <20 h. In all cases, the half-lives of the 67- and 33-kDa forms were ~7 h, as seen before (11), and that of the ~10-kDa form was ~5 h. These data suggest that the stability of the 106-kDa form depends on the program of the cells, that is, whether they are proliferating or stationary (TGase 1 is more stable) or terminally differentiating (less stable). Once the 106-kDa has been proteolytically cleaved into the 67/33/10-kDa complex form and is still attached to the membranes, the fragments become much less stable, or about
as stable as the membrane-detached soluble 67- and 33-kDa forms (10). The very short half-life of the ~10-kDa band and its lower specific activity (as visualized by its weaker intensity on gels) suggests that it may be partly lost during the isolation and analysis procedures.

However, somewhat different values were obtained for the half-lives of the [14C]myristate (Fig. 7, B and D) or [14C]palmitate (Fig. 7E). With [14C]myristate in low Ca²⁺ cultures, the half-life of the 106-kDa species varied from 40–28 h, depending on the age of the culture (Fig. 7B), which thus affords a range of the summed half-lives of the N-myristate and S-myristate adducts. In high Ca²⁺ cultures, the half-life was 18–20 h (Fig. 7D). These values are similar to the half-life of the protein. Since by 3–5 days in high Ca²⁺ medium, the TGase 1 protein is almost completely S-palmitoylated (Figs. 1 and 3–5), these data afford estimates of the half-lives of the N-myristate and S-myristate adducts of >30 h and about 18 h, respectively. With [14C]palmitate, the half-life was reproducibly much lower, 10–11 h in high Ca²⁺ cultures (Fig. 7E).

Thus, whereas the half-life of the N-myristate adduct was similar to that of the protein, the half-lives of the S-acyl adducts were much less than for the protein. Therefore, the non-linear half-lives indicate that the S-myristate adduct, and to a much greater extent the S-palmitate adduct, turn over rapidly.

The Thio-esterified 10-kDa Membrane-Anchorage Fragments Facilitate Re-association of the 67- and 33-kDa Fragments of TGase 1—The rapid turnover rates of the S-acyl adducts provides a mechanism by which the highly active 67/33/10-kDa complex of TGase 1 may cycle off membranes. We next explored the question of whether the 10-kDa fragment may augment the recycling of the 67-, 33-, or 67/33-kDa forms back onto membranes. We used in vitro overlay binding experiments with separated TGase 1 fragments. The fragments were harvested from the 67/33/10-kDa complexes eluted in Fig. 2, A-C, following dissociation in 2 M urea and then rechromatography on the FPLC column equilibrated in 1 M urea (Fig. 8A). In addition to the 67-kDa fragments that retained activity and the inactive 33-kDa fragment (Fig. 8A), this provided separate 10-kDa fragments, respectively, containing: N-myristoyl, two S-myristoyl (NM-2SM) adducts; N-myristoyl (NM) adduct only; and N-myristoyl, one S-palmitoyl (NM-1SP) adduct. Also, soluble 106-, 67-, and 67/33-kDa complexes were obtained by FPLC chromatography from cytosolic fractions of 5-day NHEK cells cultured in low Ca²⁺ (10). In each overlay experiment, 10 pmol of the three unlabeled 10-kDa forms were bound to nitrocellulose membranes and were then overlayed with 20–50 pmol of 33S-labeled TGase 1 fragment. The results show (Fig. 8B) that the 33-kDa fragment or each of the three 10-kDa fragments did not bind significantly. The 67-kDa forms alone, or in combination with the 33-kDa form added separately, or as the soluble 67/33-kDa complex, bound to the NM-1SP and NM-2SM forms efficiently, with reproducible potentiation with the latter, but did not bind to the NM form. In addition, in control reactions, the full-length TGase 1 forms that had not been exposed to 1–2 M urea bound in the same way but with less efficiency. Together, these data show that the presence of the thio-esterified adducts on the 10-kDa fragment facilitates re-association of the TGase 1 enzyme. Alternatively, the adducts may stabilize the 10-kDa fragment in a conformation that favors recombination with the 67- or 67/33-kDa forms.

Similar solution recombination experiments were performed. The addition of the separate 67- and 33-kDa fragments to the NM-2SM and NM-1SP 10-kDa fragments but in equimolar amounts resulted in 4-fold increases of TGase activity in comparison to the 67-kDa fragment alone but no increase with the NM 10-kDa fragment. Reproducibly significant potentiations of TGase activities were also found in combinations with the soluble 67/33 complex and 106-kDa forms (data not shown).
DISCUSSION

The present studies document that the TGase 1 system is more complicated than previously thought. In addition to its multiple membrane-bound and soluble forms that are variably expressed, and which have widely varying specific activities (10, 11) and presumed substrate specificities (20), we show here that these forms are also N-acylated by myristate and variably thio-esterified by either myristate or palmitate during proliferation or differentiation, respectively. Our studies confirm and extend the initial elegant studies on this enzyme (17) and now correlate these modifications with the differentiation status of the keratinocytes. These data allow a new model for the properties and processing of the TGase 1 enzyme in keratinocytes (Fig. 9).

TGase 1 Is Constitutively N-Myristoylated—We show here that the TGase 1 enzyme is constitutively N-myristoylated, probably during or immediately after translation (Fig. 3). In this way, this enzyme is remarkably similar to the band 4.2 TGase-like protein constituent of the subplasma membrane of eukaryotic cells (25). Presumably, this modification contributes to the partitioning of TGase 1 to membranes in keratinocytes, since ≈10% of the full-length TGase 1 enzyme found in the cytosol is labeled (Fig. 1, A and C; Fig. 2). However, these data differ from initial studies that suggested that TGase 1 is only thio-esterified (17, 18). One plausible explanation for this difference is the type of cell culture systems used. For example, we showed here that <5% of myristate is elongated to palmitate during the 4-h labeling experiment in serum-free KGM medium, and this palmitate is rapidly used for thio esterification. However, in serum-containing medium, we have seen that this rate of elongation is ~25% (data not shown), an observation which may explain the different results.

TGase 1 Is Largely S-Myristoylated in Proliferating or Stationary Cultured NHEK Cells—In proliferating or stationary confluent cultures, the TGase 1 protein is also thio-esterified by an average of 2 mol of myristate/mol, apparently shortly after synthesis. This “triple-barreled” labeling thereby affords a more robust anchorage to the membranes, during the 4-h labeling experiment in serum-free KGM medium, and this palmitate is rapidly used for thio esterification. However, in serum-containing medium, we have seen that this rate of elongation is ~25% (data not shown), an observation which may explain the different results.

TGase 1 Is S-Palmitoylated in Terminally Differentiating Cells—In NHEK cells induced to terminally differentiate in high Ca²⁺ medium, much larger amounts of protein become expressed by about 5 days (11), all of which becomes N-myristoylated (Figs. 1 and 2). Interestingly, this newly synthesized protein is S-palmitoylated. The switch from myristate to palmitate seems to occur almost immediately after initiation of the differentiation signal, since both N-myristate and S-palmitate storage form of thezymogen. The half-life of the S-myristoylated label is about 18 h, which is less than for the protein itself (Fig. 7). This provides a novel mechanism by which TGase 1 can cycle off the membranes. Since this soluble full-length enzyme has some activity (10), this can account for the soluble TGase 1 activity present in proliferating or stationary keratinocytes. However, the half-life of the cytosolic 67-kDa form is much shorter, which may mean that most of it is degraded before it can reassemble onto the 10 kDa to form a highly active TGase complex.
Variable Lipid Acylation of Transglutaminase 1

The Role of Differential Myristoylation and Palmitoylation of the TGase 1 Enzyme—Acylation by myristate and palmitate is a commonly used mechanism for the posttranslational control of partition proteins to membranes (23, 24), including the band 4.2 (25). Current views suggest that the shorter N-myristoylated lipid adduct is used because it may confer a less permanent anchor of the protein to membranes as opposed to longer acyl chains, which could serve as more permanent anchors (23). S-Palmitoylation is also used widely for titratable reversible membrane anchorage. For example, protein kinase p56, which possesses both myristate and palmitate anchors near its amino terminus, like the TGase 1 system described here, uses the reversible palmitoylation for differential reversible interactions with cellular membranes (28). Many other protein systems also use variable acylation by both myristate and palmitate for differential anchorage to membranes or for partitioning onto specific membranes for specific purposes (29–33). Thus, proliferating keratinocytes may use multiple myristoylations for stable storage of the enzyme, whereas predominantly single palmitoylation may be used as a less stable anchorage to facilitate partitioning into the cytosol. In the case of the nitric oxide synthetase enzyme in endothelial cells, depalmitoylation is regulated by the agonist bradykinin (34). By analogy, we can speculate that in the case of TGase 1, a terminal differentiation signal such as Ca2+ might act in a similar way to initiate both proteolytic processing and depalmitoylation leading to membrane detachment.

Thus, we suggest that the differential acylation of the TGase 1 enzyme by lipids during proliferation and differentiation is used specifically to increase the repertoire of partitioned membrane-bound and cytosolic TGase 1 forms, each of which may fulfill different functions in keratinocytes. Further work will now be required to determine these functions. Preliminary studies have suggested that recombinant TGase 1 proteins cross-link synthetic mimetic substrate peptides with different efficiencies (20), and different TGases cross-link the known substrate loricrin in different ways (35). The complexity of the TGase 1 system has been foreshadowed by the disease lamellar ichthyosis, some cases of which are caused by deficient levels of TGase 1 activity due to mutations in the TGM1 gene (36–39); pathology typically involves only the epidermis and not the numerous other internal epithelia (40) that normally express abundant levels of the enzyme (1–3).

Acknowledgments—We thank Drs. C. Chipev, T. Kartasova, and E. Taresa for their comments, advice, and assistance and Drs. D. Downing and I. Marekov for advice on the lipid fractionation methods.

REFERENCES

1. Greenberg, C. S., Birckbichler, P. J., and Rice, R. H. (1991) FASEB J. 5, 3071–3077
2. Reichert, U., Michel, S., and Schmidt, R. (1993) in Molecular Biology of the Skin (Darmon, M., and Blumenberg, M., eds) pp. 107–150, Academic Press, New York
3. Aeschlimann, D., and Paulson, M. (1994) Thromb. Haemost. 71, 402–415
4. Rice, R. H., and Green, H. (1977) Cell 11, 417–422
5. Thacher, S. M., and Rice, R. H. (1985) Cell 40, 685–695
6. Thacher, S. M. (1989) J. Invest. Dermatol. 92, 578–584
7. Eckert, R. E., Yale, M. F., Lykkesfeldt, J., and Peck, K. (1993) J. Invest. Dermatol. 100, 613–617
8. Steinert, P. M. (1995) Cell Death Differ. 2, 31–40
9. Steinert, P. M., and Marekov, L. N. (1995) J. Biol. Chem. 270, 17702–17711
10. Kim, S.-Y., Chung, S.-I., and Steinert, P. M. (1995) J. Biol. Chem. 270, 18026–18035
11. Steinert, P. M., Chung, S.-I., and Kim, S.-Y. (1996) Biochem. Biophys. Res. Commun. 231, 101–106
12. Duve, M., Nelson, D. C., Annarella, M., Cho, M., Esgleyes-Ribot, T., Remenyik, E., Ulmer, R., Rapini, R. P., Sack, P. G., Clayman, G. L., Davies, P. J. A., and Thacher, S. (1994) J. Invest. Dermatol. 102, 462–469
13. Parenteau, N. L., Pilato, A., and Rice, R. H. (1986) Differentiation 33, 130–141
14. Shroeder, W. T., Thacher, S. M., Stewart-Galeka, S., Annarella, M., Chema, D., Siciliano, M. J., Davies, P. J. A., Tang, H.-S., Sowa, B. A., and Duve, M. (1992) J. Invest. Dermatol. 99, 27–34
15. Michel, S., Bernard, P. J., Setten, A. M. F., Shroot, B., and Reichert, U. (1992) J. Invest. Dermatol. 98, 364–368
16. Kim, S.-Y., Chung, S.-I., Yoneda, K., and Steinert, P. M. (1995) J. Invest. Dermatol. 104, 211–217
17. Chakravarty, R., and Rice, R. H. (1989) J. Biol. Chem. 264, 625–629
18. Phillips, M. A., Qin, M., Mehrpouray, M., and Rice, R. H. (1993) Biochemistry 32, 11057–11063
19. Schmidt, R., Michel, S., Shroot, B., and Reichert, U. (1988) J. Invest. Dermatol. 90, 475–479
20. Kim, S.-Y., Chung, S.-I., and Steinert, P. M. (1994) J. Biol. Chem. 269, 27979–27986
21. Steffen, W., and Bierwirth, E. (1962) Angewandte Chemie 74, 905–907
22. Merdes, A. M., Brunkener, H., Horstmann, H., and Georgatos, D. (1991) J. Cell Biol. 115, 397–410
23. Johnson, D. R., Bhattacharyya, R., Knoll, L. J., and Gordon, J. I. (1994) Annu. Rev. Biochem. 63, 869–914
24. Ross, E. M. (1995) Curr. Biol. 1, 107–109
25. Rübsamer, M. A., Dotzmar, E. M., and Cohen, C. M. (1992) J. Biol. Chem. 267, 5603–5605
26. Poumay, Y., and Pittelkow, M. (1995) J. Invest. Dermatol. 104, 271–276
27. Nakao, H., Perez, D. M., Baek, K. J., Tan, H., Husain, R., Misono, K., Im, M. J., and Graham, R. H. (1994) Science 264, 1593–1596
28. Shroyer, S. A., and Timson, G. K. L., Kwong, J., Shaw, A. S., and Lublin, D. M. (1993) Mol. Cell. Biol. 13, 6385–6392
29. Robbins, S. M., Quinotrell, N. A., and Bishop, J. M. (1995) Mol. Cell. Biol. 15, 3557–3561
30. Kwong, J., and Lublin, D. M. (1995) Biochem. Biophys. Res. Commun. 207, 686–687
31. Galiberti, F., Guzzi, F., Magee, A. I., Milligan, G., and Parenti, M. (1994) Biochem. J. 303, 697–701
32. Degtyarev, M. Y., Spiegel, A. M., and Jones, T. L. Z. (1994) J. Biol. Chem. 269, 30988–30993
33. Neubert, T. A., Johnson, R. S., Hurley, J. B., and Walsh, K. A. (1992) J. Biol. Chem. 267, 18267–18277
34. Robinson, L. J., Busconi, L., and Michel, T. (1995) J. Biol. Chem. 270, 995–998
35. Candi, E., Melino, G., Mei, G., Taresa, E., Chung, S.-I., Marekov, L. N., and Steinert, P. M. (1995) J. Biol. Chem. 270, 26382–26390
36. Russell, L. J., DiGiovanna, J. J., Hashem, N., Compton, J. G., and Bale, S. J. (1994) Am. J. Hum. Genet. 55, 1146–1152
37. Huber, M., Rettler, I., Bernasconi, K., Frenk, E., Lavrijsen, S. P., Pomaec, M., Beeke, A., Lautenschlager, S., Schorderet, D. F., and Hohl, D. (1998) Science 276, 525–527
38. Russell, L. J., DiGiovanna, J. J., Rogers, G. R., Steinert, P. M., Hashem, N., Compton, J. G., and Bale, S. J. (1995) Nat. Genet. 9, 279–283
39. Parmenier, L., Blanche-Brard, C., Nguyen, S., Prudhomme, J.-F., Dubertret, L., and Weissenbach, J. (1995) Hum. Mol. Genet. 4, 1391–1395
40. Traupe, H. (1989) The Ichthyoses: A Guide to Clinical Diagnosis, Genetic Counseling, and Therapy, pp. 111–134, Springer-Verlag, Berlin
The Transglutaminase 1 Enzyme Is Variably Acylated by Myristate and Palmitate during Differentiation in Epidermal Keratinocytes
Peter M. Steinert, Soo-Youl Kim, Soo-Il Chung and Lyuben N. Marekov

J. Biol. Chem. 1996, 271:26242-26250.
doi: 10.1074/jbc.271.42.26242

Access the most updated version of this article at http://www.jbc.org/content/271/42/26242

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 38 references, 14 of which can be accessed free at http://www.jbc.org/content/271/42/26242.full.html#ref-list-1