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CALCIUM INDUCTION OF TRANSGLUTAMINASE AND THE FORMATION OF ε(γ-GLUTAMYL)LYSINE CROSS-LINKS IN CULTURED MOUSE EPIDERMAL CELLS

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SUMMARY

Terminal differentiation can be induced in cultured mouse epidermal cells by increasing the extracellular calcium concentration from 0.07 mM to 1.2 mM. An ultrastructural characteristic of this process is the formation of a cornified envelope in which membrane proteins are cross-linked by ε(γ-glutamyl) lysine bonds. This process is catalyzed by the calcium-requiring enzyme transglutaminase. Elevation of medium calcium levels increases both the activity of transglutaminase and the fraction of lysine residues involved in ε(γ-glutamyl)lysine cross-links.

Terminal differentiation of cultured epidermal cells can be regulated by the level of calcium in the culture medium (1,2). Low calcium (0.07 mM Ca^{++}) growth is characterized by a rapid rate of proliferation, the absence of desmosomes, and growth as a monolayer rather than as a stratified epithelium. After raising the calcium level of the medium to 1.2 mM, desmosomes form rapidly and cell proliferation is inhibited. The cells stratify and differentiate morphologically, and mature epidermal cells are shed from the culture dish. However, the rate of synthesis of the major keratin proteins is unaffected by calcium elevation. The initial events in the sequence of terminal differentiation induced by calcium are not affected by inhibitors of RNA or protein synthesis (Hennings, H., & Holbrook, K., unpublished results). Thus, post-

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translational processing of proteins is likely to be involved in calcium-induced differentiation. A characteristic morphological feature of epidermal differentiation in vivo is the formation of a cornified envelope, a 120 Å electron-dense thickening inside the plasma membrane of cells in the most superficial epidermal layer, the stratum corneum (3). Biochemically, cornified envelope formation involves the cross-linking of membrane proteins by the formation of a bond between the ε-amino group of protein-bound lysine and the γ-carboxyl group of protein-bound glutamine (4). This process is catalysed by transglutaminase, a calcium-requiring enzyme (4). We report here that the induction of terminal differentiation by calcium elevates epidermal transglutaminase activity and subsequently the formation of ε(γ-glutamyl) lysine cross-links in cultured mouse epidermal cells.

METHODS

Cell Culture. Epidermal cells were prepared from newborn BALB/c mouse skin by a trypsin flotation method (5). Cell growth was altered by varying the calcium concentration in the media as previously described (2).

ε(γ-Glutamyl)lysine Cross-link Determination after [^{3}H]Lysine Pre-labeling. Epidermal cells were plated at $4x10^6$ cells per 60 mm dish and maintained for 6 days in medium 199 with 2% chelated fetal bovine serum containing 0.07 mM calcium. Proteins were labeled by incubation with 5μ Ci/ml [^{3}H]lysine beginning at Day 3. Six days after plating, the [^{3}H]lysine was removed and the cells were maintained in medium with 0.07 mM calcium or shifted to medium with 1.2 mM calcium. The time of this medium change is designated Zero Time. At this time and at 6, 24, 48 and 72 hours later, the proteins insoluble in 8 M urea-0.1 M 2-mercaptoethanol were totally digested enzymatically (6,7) and the percent of total [^{3}H]lysine involved in ε(γ-glutamyl-[^{3}H]lysine bonds was calculated.

ε(γ-Glutamyl)lysine Determination in Newborn Mouse Stratum Corneum. After separation of the newborn mouse epidermis from the dermis by overnight flotation on cold trypsin the epidermis was minced and filtered through 157 mesh/inch Dacron gauze (5). The material retained on the gauze, a crude stratum corneum preparation, was extracted twice with 0.1% SDS-0.1 M 2-mercaptoethanol-0.05 M tris-HCl (pH 9) at 37°, yielding an insoluble pellet containing the cross-linked cornified envelopes. This material was hydrolyzed in 5.7 N HCl at 105° for 22 hr and the total lysine content determined after amino acid analysis. To estimate the extent of lysines involved in crosslinks, an aliquot of the envelope preparation was totally digested enzymatically by successive incubations with trypsin, pronase, carboxypeptidase A, carboxypeptidase B and aminopeptidase M (6,7). From the values for total lysine and cross-linked lysine, the percent of total lysine present as ε(γ-glutamyl)lysine was calculated.

Epidermal Transglutaminase Assay. Transglutaminase activity was estimated in supernatants after centrifugation of epidermal cell homogenates for 1 hr at 100,000 x g (8).

RESULTS AND DISCUSSION

Cross-linked cornified envelope proteins are characterized by their insolubility in solutions containing sodium dodecylsulfate and a reducing agent. In a preparation of epidermal cell proteins insoluble in 8 M urea -0.1% sodium dodecylsulfate - 0.1 M mercaptoethanol, the level of cross-link was quantified by determining the fraction of protein-bound lysine involved in ε (γ-glutamyl)lysine bonds. In cells grown in low calcium medium in the presence of [^{3}H]lysine for 3 days, 2% of the lysine residues in attached cells were involved in the cross-link (Fig. 1). Continued growth in low calcium medium without [^{3}H]lysine for an additional 3 days resulted in only a slight decrease in cross-link level (Fig. 1). The addition of calcium to the medium of [^{3}H]lysine pre-labeled low calcium cells did not affect the fraction of [^{3}H]lysine involved in ε(γ-glutamyl)lysine cross links at 6 hours, but by 22 hours a 2-fold increase was seen (Fig. 1). This increase continued, with cross-link levels of between 6 and 7% found at 2 and 3 days. Thus, calcium-induced terminal differentiation is characterized by a substantial increase in the level of ε(γ-glutamyl)lysine cross-links in the proteins insoluble in 8M urea-0.1% SDS-0.1M mercaptoethanol.

In parallel experiments, the activity of transglutaminase in extracts of cultured epidermal cells was determined. In 0.07 mM Ca^{++}, transglutaminase levels were relatively constant 6-8 days after plating. Addition of calcium to the culture medium resulted in a rapid 1.5 - 2 fold increase in the level of transglutaminase at 6 hours, 1 and 2 days (Fig. 2). This calcium-induced increase in activity, shown here by a fluorescent technique (8), has been verified using a more sensitive [^{3}H]putrescine labeling technique (9).

The apparent level of cross-link in mouse epidermal cultures (about 7% of total lysine in Fig. 1) was somewhat lower than the 17-18% reported for guinea pig hair in vivo (10) and human epidermal cells in culture (6). However,

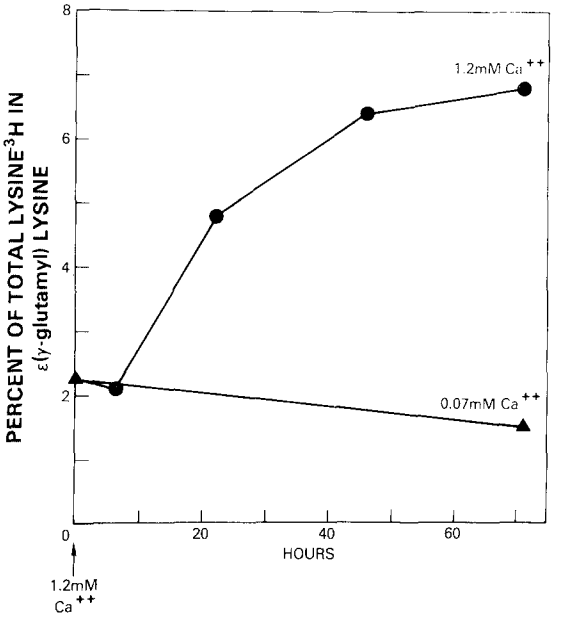

Figure 1. Effect of Extracellular Calcium on ε(γ-Glutamyl)lysine Cross-links. Cells grown in medium with 0.07 mM calcium for 6 days were labeled with [^{3}H]lysine from Day 3 to Day 6, then shifted to [3H]lysine-free medium with 0.07 mM or 1.2 mM Ca^{++}. The percent of total lysine in cornified envelope cross-links is plotted versus time in 0.07 mM Ca^{++} (▲) or 1.2 mM Ca^{++} (●).

exhaustive enzymatic hydrolysis of cornified envelope proteins from newborn mouse stratum corneum revealed levels of 15-18% of the lysine residues involved in ε(γ-glutamyl)lysine cross-links. The lower extent of lysine cross-linking found in the differentiating cells in our in vitro experiments (Fig. 1) may reflect differences between the process of terminal differentiation in vitro and in vivo (11,12, Holbrook, K. & Hennings, H., unpublished results). Alternatively these apparent differences could be a result of the experimental protocol Pre-labeling with [^{3}H]lysine for three days prior to calcium addition labels only those lysine residues in the proteins synthesized during that period. If the lysine-containing proteins involved in cornified envelope formation are stable structural proteins which were present prior to the 3-day labeling with [^{3}H]lysine, then many of the lysine residues involved in calcium-induced cross-linking would go undetected in our assay of tritium (See Methods and Legend to

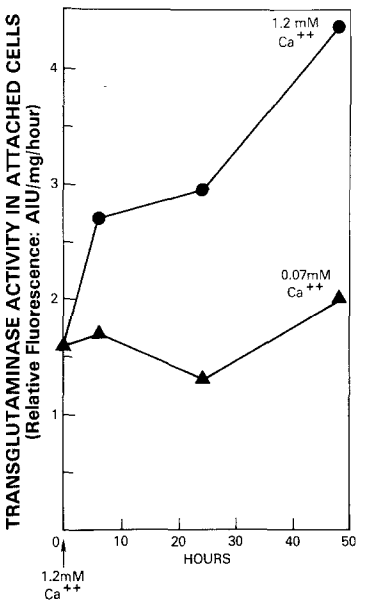

Figure 2. Effect of Extracellular Calcium on Epidermal Transglutaminase Activity. Epidermal cells were plated and maintained in 0.07 mM Ca^{++} medium for six days, then either continued in this low calcium medium or shifted to medium with 1.2 mM Ca^{++}. At Zero Time and 6, 24 and 48 hours later, transglutaminase activity was estimated in supernatants after centrifugation of epidermal homogenates for 1 hr at 100,000 x g. Transglutaminase activity, expressed as amine-incorporating units (AIU)/mg soluble protein/hour (5), is plotted versus hours after Zero Time in 0.07 mM (▲) or 1.2 mM (●) calcium.

Fig. 1). That is, the percent of total lysine involved in ε(γ-glutamyl)lysine cross-links shown in Figure 1 is likely to be artificially low. Nevertheless, elevation of extracellular calcium induces a clear-cut increase in cross-linking From our experimental design, one can also conclude that cells grown in low calcium medium, which possess many of the characteristics of basal cells (13), synthesize the lysine-containing proteins which act as a substrate for transglutaminase.

The increase in transglutaminase activity in response to elevation of calcium in the culture medium is apparently due to synthesis of the enzyme since the increase can be blocked by treatment with cycloheximide at a dose which inhibits protein synthesis by more than 90% (data not shown). The reported increase in transglutaminase activity in chick embryonic skin

treated with hydrocortisone also appears to involve enzyme synthesis rather than activation (14). These results are in contrast to the findings of Rice and Green (15,16) in cultured human epidermal cells in which increased transglutaminase activity and subsequent cornified envelope formation apparently result from an increase in the calcium which is available to endogenous transglutaminase. In human epidermal cells, this increase in cross-linking occurs in the absence of protein synthesis and is greatly enhanced by high doses of cycloheximide. In mouse epidermal cells, cross-linking plateaus at a time when protein synthesis has been reduced by only about 50%. Thus, calcium is critical for cornified envelope formation in both mouse and human epidermal cells in culture, but the mechanisms of action in the two species appear to be quite different.

Possible changes in intracellular calcium concentrations and alterations in calcium at the cell surface in response to elevation of extracellular calcium remain to be evaluated. Alterations in the intracellular levels of K^+ or Na^+ may be more important since ouabain blocks Ca^{++}-induced terminal differentiation (17). Alternatively, the rapid establishment of desmosomal contacts after elevation of extracellular calcium (1,2) may allow new channels of cell communication which could be crucial to the control of ε(γ-glutamyl)lysine bond formation as well as other aspects of the terminal differentiation program.

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