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CHARACTERIZATION OF THE CALCIUM SENSITIVITY OF DIFFERENTIATION IN SCC-13 HUMAN SQUAMOUS CARCINOMA CELLS

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SUMMARY

The sensitivity to calcium of the human squamous carcinoma cell line, SCC-13, was demonstrated and characterized. Cultures grown to confluence in the presence of 0.2 to 2 m*M* calcium had approximately 10-fold higher levels of particulate transglutaminase activity and envelope competence than those grown in low calcium (0.025 to 0.05 m*M*) medium. Raising the calcium from 0.025 to 1.8 m*M* induced expression of this enzyme and of competence over the course of a week. Conversely, for cultures grown to confluence in 1.8 m*M* calcium, subsequent reduction of calcium to 0.025 m*M* resulted in a substantial decline in transglutaminase over a similar time period. Immunoprecipitable transglutaminase was clearly identifiable in cultures grown in 1.8 m*M* calcium-containing medium but not in those grown in low calcium medium or in the presence of retinoic acid, suggestive of regulation at the level of mRNA accumulation or translation rather than posttranslational modification.

Key words: keratinocyte; transglutaminase; cross-linked envelope; retinoic acid.

INTRODUCTION

Mouse epidermal cells in primary culture show a striking sensitivity to calcium ion. Concentrations typically found in mammalian cell culture medium (1 to 2 m*M*) induce cell death and biochemical properties associated with terminal differentiation, including cornified envelope formation, isopeptide cross-linking, and expression of a particulate transglutaminase (7,8,12). Certain morphologic features characteristic of the mouse cells in low-calcium medium, i.e. lack of stratification and absence of desmosomes (6), are evident in normal human epidermal cells under similar conditions (2,15). In the human cells calcium regulates the organization of intracellular structural components such as desmosomes and keratins (26,28). However, at least some cellular maturation occurs in low-calcium medium because a substantial fraction of the cells enlarge and produce the human envelope precursor, involucrin (27). Because normal human epidermal cells have a requirement for calcium to sustain growth at low cell densities, it is difficult to employ them in quantitative kinetic studies of the role of calcium in their differentiation. Use of the squamous carcinoma line SCC-13, derived from a lesion of facial epidermis (17), circumvents this problem. These cells retain a significant ability to differentiate (4) and they will proliferate even under low-calcium conditions (19).

Diagnostic markers of keratinocyte differentiation include the ability to form cross-linked envelope structures and expression of a particulate transglutaminase responsible for cross-linking at the cell periphery (21,24). Both markers have been demonstrated in SCC-13 cells and their regulation by retinoids examined (19,23). The present demonstration of calcium sensitivity in their expression, which in SCC-13 is reversible, will assist in the detailed analysis of the molecular basis of this phenomenon.

MATERIALS AND METHODS

Cell culture. SCC-13 keratinocytes (Passages 30 to 41) were inoculated at a density of 2 to 5 $\times$ 10^5 cells/6-cm dish and grown with irradiated 3T3 feeder layer support (17) in Dulbecco-Vogt Eagle's medium supplemented with 5% fetal bovine serum. One day after inoculation, cultures were rinsed twice in phosphate buffered saline (PBS) (137 m*M* NaCl, 3 m*M* KCl, 8 m*M* Na_2HPO_4, 2 m*M* KH_2PO_4, pH 7.2) and given fresh medium, which was changed twice weekly. The fresh medium contained calcium concentrations of 0.025 m*M* (determined by atomic absorption spectrophotometry) or higher as specified and fetal bovine serum depleted of steroids with charcoal-dextran (1) and of calcium with Chelex-100 (BioRad Laboratories, Richmond, CA) ion-exchange resin (3). SCC-13 and 3T3 cells were tested and found negative for mycoplasma contamination before the start of this work.

Envelope competence. The SCC-13 cells were disaggregated in trypsin and EDTA and suspended at 8 $\times$ 10^5/ml in serum-free medium containing 1.8 m*M* calcium and the ionophore X537A (Hoffman LaRoche, Nutley, NJ) at a

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concentration of 50 μg/ml. After 2 h at 37° C, the suspension was adjusted to 1% in sodium dodecyl sulfate (SDS) and 20 m*M* in dithioerythritol (DTE), held at room temperature for at least 10 min and scored for envelopes by phase contrast microscopy (4). Envelope competence was defined as the percentage of ionophore-treated cells that formed visible envelope structures.

Transglutaminase assay. Cultures were rinsed 3 times in PBS, scraped into 0.3 to 0.5 ml of ice-cold buffer (10 m*M* Tris-HCl (pH 7.4), 1 m*M* EDTA, 1% Emulgen 911) and stored frozen at −70° C. Samples were thawed, briefly sonicated, and assayed directly for enzyme activity. Aliquots (25 to 50 μl containing 20 to 150 μg of protein) were incubated for 30 min at 37° C in 0.26 ml final volume containing 0.5 mg of dimethyl casein (14), 0.1 *M* Tris-HCl (pH 8.2), 4 m*M* $CaCl_2$, 0 - 0.4 m*M* EDTA, 5 m*M* DTE, and 0.5 μCi (4 nmol) of [^{3}H]putrescine. Trichloroacetic acid-precipitable radioactivity was recovered on glass fiber filters, rinsed, and scintillation counted.

Transglutaminase immunoprecipitation. One week after reaching confluence, SCC-13 cultures were scraped singly from 10-cm dishes into 1 ml of 10 m*M* Tris-Cl (pH 7.4), 1 m*M* EDTA, 1 m*M* DTE, and stored frozen at −70° C. Upon thawing, 3 ml of ice-cold buffer (50 m*M* Tris-Cl, 10 m*M* EDTA) was added and the cultures were Dounce homogenized. The particulate fraction was isolated by high speed centrifugation (100 000 ×*g* for 40 min), and the transglutaminase solubilized by stirring at 4° C for 2 h in 5 ml of 20 m*M* Tris-Cl (pH 7.4), 1 m*M* EDTA, and 0.3% Emulgen. After removal of insoluble material by high speed centrifugation, the extracts were adjusted to 0.2 *M* in NaCl, and 5 μg of B.C1 monoclonal antibody were added. (Experiments employing B.D4 antibody in addition gave the same results.) The immunocomplexes were precipitated with rabbit anti-mouse IgG and protein A-Sepharose, washed extensively, and heated 2 min in a boiling water bath in the presence of 2% SDS and 10 m*M* DTE. The samples were electrophoresed in 10% polyacrylamide slab gels (10), and bands were visualized by silver staining (29). Analysis by Western blotting, which sometimes results in cleaner electrophoretic patterns, was not feasible with these antibodies because they do not bind to the denatured enzyme.

Protein concentration. Measurements were made using the bicinchoninic acid (Pierce Chem Co., Rockford, IL) method of Smith et al. (22).

RESULTS

Calcium dependence of transglutaminase expression and envelope competence. The degree to which SCC-13 cells exhibited certain keratinocyte properties was highly dependent on the concentration of calcium ion in the growth medium. Parallel cultures were raised to confluence and held for 1 wk in medium containing calcium concentrations from 0.02 to 2 m*M*. In several experiments under these conditions, the specific activity of particulate transglutaminase in the cells was nearly 10-fold higher at 0.5 to 2 m*M* than at 0.025 to 0.05 m*M* calcium (Fig. 1 *A*). The ability of the cells to form cross-linked envelopes (assayed in 1.8 m*M* calcium by ionophore permeabilization) was also strongly dependent on the calcium concentration in the growth medium. As seen in Fig. 1 *B*, the magnitude of the difference between the low- and high-calcium conditions was essentially the same as found for transglutaminase expression. It was also apparent that the cell density in the highest calcium concentrations employed was approximately twice that in low-calcium medium (Fig. 1 *C*). For each parameter measured (transglutaminase, competence, density), half-maximal effects were observed between 0.1 and 0.2 m*M* Ca^{++}.

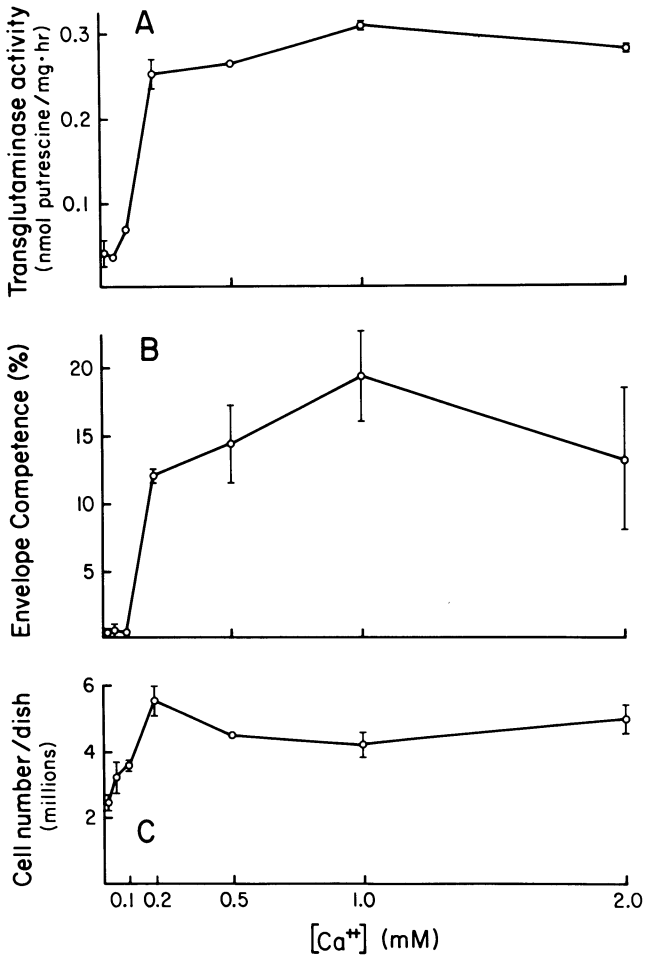

FIG. 1. Dependence of SCC-13 properties on calcium concentration. In this representative experiment, cultures were held at confluence for 1 wk before measurement of (*A*) transglutaminase activity, (*B*) envelope competence, and (*C*) cell number. Calcium concentrations were varied by addition of 1 *M* $CaCl_2$ to 0.025 m*M* calcium-containing medium. *Error bars* = range of values in duplicate cultures.

In subsequent experiments we observed that cells grown to confluence in low-calcium medium developed abundant transglutaminase and high envelope competence after the calcium concentration in the medium was adjusted to 1.8 m*M*. As shown in Fig. 2, transglutaminase activity (*A*) and envelope competence (*B*) were stimulated in parallel over the course of several days, reaching 10- to 20-fold higher values after 1 to 2 wk in high-calcium medium supplemented with serum (or, in preliminary

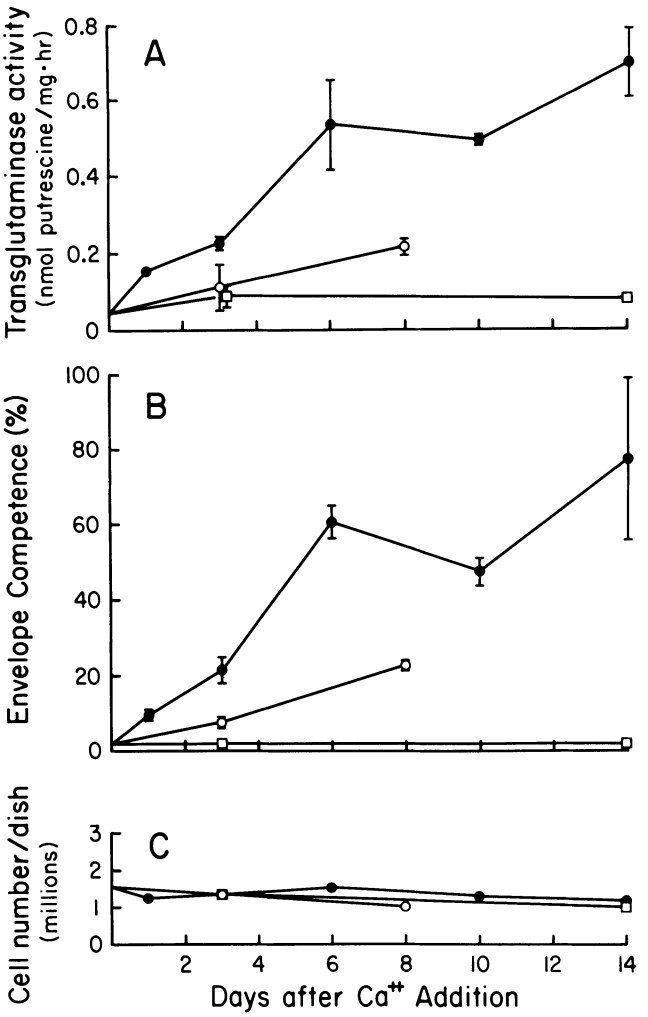

FIG. 2. Time course of calcium effects. Cultures were held for 1 wk after confluence in 0.025 *M* calcium-containing medium supplemented with 5% charcoal and Chelex-treated fetal bovine serum, and either maintained under these conditions (□) or switched at Day 0 to medium containing 1.8 m*M* calcium with (●) or without (○) serum. *A*, transglutaminase activity; *B*, envelope competence; *C*, cell number; *error bars* = range of values in duplicate cultures.

experiments, platelet-poor plasma). As shown in Fig. 2 *A*, *B*, transglutaminase activity and envelope competence were stimulated by the calcium increase even in medium without added serum. The rate of increase was slower, however, perhaps due to the more stringent nutritional environment. The cell viability in all cases was high (94% after 8 d without serum and 95% after 10 d with serum) as measured by trypan blue dye exclusion (16). Measurement of cell number showed little difference among the various treatment conditions (Fig. 2 *C*), indicating that the stimulatory effect of calcium was not mediated by an increase in cell density.

Morphologic differences in the cultures grown in low- and high-calcium conditions were quite distinct, as reported for mouse epidermal cultures (7). In low-calcium medium, the cells seemed detached from each other and the cell borders were easily distinguishable. Upon addition of $CaCl_2$ to 1.8 m*M*, the tightly packed and more flattened appearance ordinarily observed under this condition became obvious within several hours. Similarly, when the calcium was adjusted from high to low concentration, the characteristic low-calcium morphology was evident within an hour.

In calcium withdrawal experiments, cultures grown and held for a week at confluence in 1.8 m*M* calcium-containing medium were switched to 0.025 m*M* calcium-containing medium. As shown in Fig. 3 this calcium deprivation resulted in a substantial decline in transglutaminase content during the 7 d after the switch. In four experiments the activity at the end of this period was 8 to 20% of the original level. In two of these experiments, envelope competence was measured and found to decline in parallel to 6 to 12% of the initial values with little or no change in cell number per dish during that week. This phenomenon seems not to be due to selective loss of competent cells, because the floating

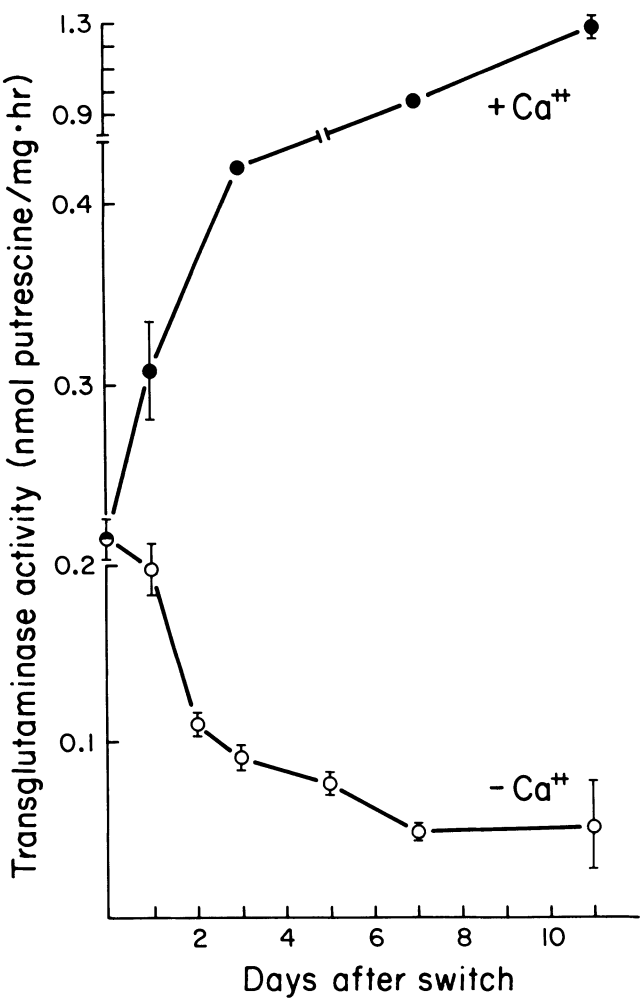

FIG. 3. Effect of calcium withdrawal on transglutaminase activity. A week after reaching confluence, cultures grown in 1.8 m*M* calcium-containing medium were rinsed in serum-free 0.025 m*M* calcium-containing medium, incubated 30 min in this medium, and switched to this medium with serum supplementation (○). Other cultures were treated in parallel with 1.8 m*M* calcium-containing medium and maintained in the high calcium medium with serum (●). *Errors bars* = range of values in duplicate cultures.

cells removed during medium changes gave competence values similar to those remaining attached to the dish, but more likely reflects cessation of synthesis of new enzyme and degradation of preexisting enzyme.

Calcium and retinoid regulation of transglutaminase accumulation. Like retinoic acid (19,23), calcium ion clearly affects the specific activity of the keratinocyte particulate transglutaminase. It is unclear, however, whether such modulation reflects levels of enzyme accumulation in the cells. Figure 4 shows the relative amounts of immunoprecipitable transglutaminase in SCC-13 cultures grown under different conditions. As shown in Fig. 4, *lane d*, a substantial band of the enzyme protein is visible by silver staining in the particulate extract of cells grown in 1.8 m*M* calcium-containing medium. Such samples immunoprecipitated without addition of antitransglutaminase monoclonal antibody (24) lacked this prominent band of 92 kDalton (*lane c*). Similarly, little if any of this protein was visible in the particulate fraction of cells grown in 0.025 m*M* calcium-containing medium (*lane a*). Particulate (*lane b*) and soluble fractions (the latter containing tissue transglutaminase) of cells grown with 1 μg/ml retinoic acid were also negative. These results suggest strongly that modulation of particulate transglutaminase activity by calcium and retinoic acid reflects accumulation of the enzyme protein rather than activation or inactivation by posttranslational modification.

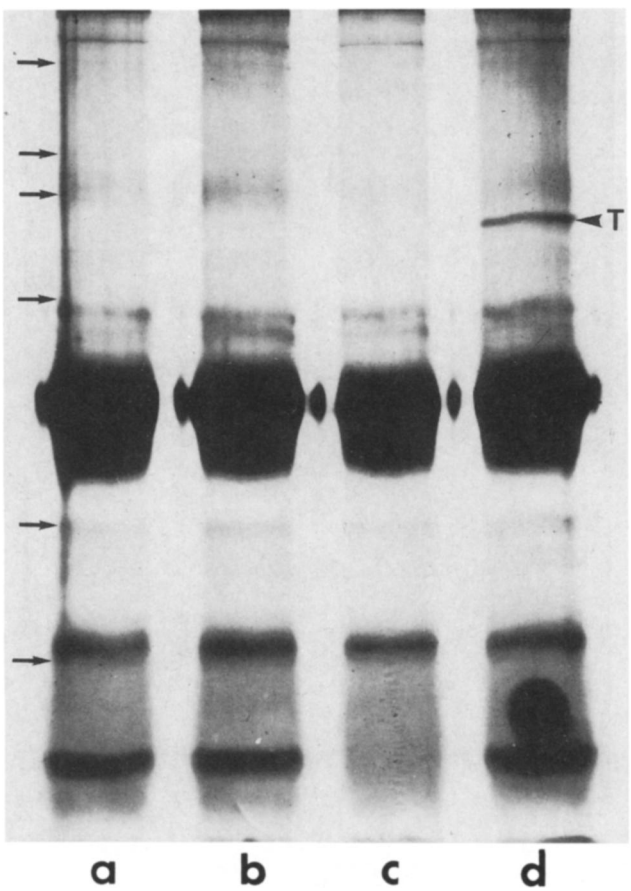

FIG. 4. Modulation of immunoprecipitable particulate transglutaminase by calcium and retinoic acid. Cultures were grown in medium containing 0.025 m*M* calcium (*lane a*) or 1.8 m*M* calcium with (*lane b*) or without (*lanes c, d*) 3.3 μ*M* retinoic acid, and harvested 1 wk after confluence. Each lane represents the material immunoprecipitated from half a 10-cm culture with antitransglutaminase monoclonal antibodies (*a, b, d*) or a control without added monoclonal antibody (*c*). *Arrowheads* in the left margin of the gel show the migration of molecular weight standard proteins run in a neighboring lane (*top* to *bottom*): myosin (205 kDalton), β-galactosidase (116 kDalton), phosphorylase b (97 kDalton), bovine serum albumin (66 kDalton), ovalbumin (45 kDalton), and carbonic anhydrase (29 kDalton). The band of transglutaminase in *lane d* is indicated by *T*.

DISCUSSION

Upon exposure to high-calcium medium, mouse epidermal cells in primary culture quickly cease synthesizing DNA and replicating (7). Thus it is not clear how directly transglutaminase or other markers are induced by elevated calcium, because they may appear as a consequence of growth arrest. On the other hand, SCC-13 cells express differentiated functions only under conditions of slower growth as at confluence, but they remain viable and even retain germinative capability (4). The present experiments reveal that although confluence is permissive for expression of the particulate transglutaminase, high calcium is still needed for its induction and maintenance. Although SCC-13 cells can display substantial differentiated character, they do not readily enter a state of *terminal* differentiation, judging by their inability to form cross-linked envelopes spontaneously (18). The reversibility of calcium-induced differentiation in these cells reflects their escape from the final stages of maturation. Inasmuch as the rate of cell shedding was low in these experiments, changes in the degree of differentiation were more direct than simply from selective loss of differentiated (calcium withdrawal) or undifferentiated cells (calcium addition). The absence of immunoprecipitable transglutaminase in low-calcium or in retinoic acid-containing cultures argues against the possibility that these agents regulate the activation of a previously synthesized inactive enzyme. Instead, the regulation probably occurs either at transcriptional or translational levels, similar to that of keratins by vitamin A in normal epidermal cells (5,25), and may even involve a fundamental change in keratinocyte programming. Distinguishing among these possibilities will be aided by development of the appropriate molecular probes.

This work indicates that a period of several days is required for the substantial increase in activity upon addition of calcium to cells grown to confluence in low-calcium medium. The rate-limiting step(s) in this slow response remain to be identified, but a role for cell growth cannot be ruled out. Although our measurements of cell number indicate that the action of calcium addition or withdrawal is not mediated by alteration in cell density, the possibility remains that a low level of DNA synthesis and cell turnover promotes such a change in programming. Studies in a variety of systems,

including rabbit trachea (11) and cultured mouse mammary gland (20) are compatible with the view that growth in the presence of an inductive stimulus is an essential permissive condition for expression of keratinocyte character during squamous metaplasia. Among possible stimuli, serum has been reported effective in producing squamous differentiation of epithelial cells cultured from the upper airway of human (13) and rabbit (9), for example. In our experiments, serum supplementation stimulated maximal transglutaminase activity and envelope competence in calcium-repleted cultures, but was evidently not required for the calcium induction of more modest increases.

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