Involucrin Synthesis Is Correlated with Cell Size in Human Epidermal Cultures

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ABSTRACT  Late in terminal differentiation, human epidermal keratinocytes form an insoluble protein envelope on the cytoplasmic side of the plasma membrane. Involucrin, a soluble protein precursor of the envelope, is synthesized at an earlier stage of differentiation, both in the natural epithelium and in cultured keratinocytes. Because keratinocytes are known to enlarge during differentiation, we looked for a correlation between involucrin synthesis and cell size, using antisera raised against the purified protein. We found that virtually no cultured epidermal keratinocytes with a diameter ≤14 μm contained involucrin, but most cells >17 μm did.

Using density gradient centrifugation, we were able to isolate a population of small cells containing almost no involucrin, as judged by immunodiffusion, PAGE, and immunoprecipitation. Large cells possessed translatable mRNA for involucrin, whereas small cells did not. We conclude that when cultured keratinocytes reach a certain size (~14 μm in diameter) the specific mRNA for involucrin begins to accumulate and synthesis of the protein begins.

In stratified squamous epithelia, whether natural or cultured, cell division takes place in the basal layer. When cells leave this layer they begin terminal differentiation and no longer divide. They enlarge progressively, and at the site of stratum corneum formation (the granular layer) there forms beneath the plasma membrane an insoluble envelope consisting of protein cross-linked by cellular transglutaminase (17, 18, 24). Involucrin (from the Latin, involucrum—envelope), a soluble protein precursor of the cross-linked envelope, has been isolated from cultured human epidermal cells (19). Antiserum raised against involucrin reacts with the outer living layers of epidermis but not with the inner layers (1, 19). Evidently synthesis of involucrin begins only after the keratinocyte has migrated appreciably beyond the basal layer.

The size of cultured keratinocytes has already been related to some aspects of their differentiation. In surface culture, the ability to replicate DNA is confined to small cells (8); on the other hand, cross-linked envelopes are found only in the largest cells (24), presumably because it is in these cells that transglutaminase becomes active (19). We therefore investigated whether the onset of involucrin synthesis also bears a relation to cell size and, if so, by what means synthesis is initiated.

MATERIALS AND METHODS

Cell Culture

Human keratinocytes (strain N, third to sixth passage), derived from newborn foreskin, were grown in the presence of lethally irradiated 3T3 cells (15). In early experiments the medium was supplemented with 20% fetal calf serum (15). However, using the data of Barnes and Sato (2), S. Banks-Schlegel and H. Green (unpublished observations) found that in the presence of transferrin (5 μg/ml), insulin (5 μg/ml), and triiodothyronine (2 x 10^{-10} M), the concentration of fetal calf serum required for optimal cell growth could be reduced to 10%, and these conditions were used for later experiments. The keratinocytes grew equally well in either medium and the experimental results were the same. The medium also contained hydrocortisone at 0.4 μg/ml (15), and 10^{-10} M cholera toxin (7). Epidermal growth factor (EGF) prepared by the method of Savage and Cohen (21) was added to 5–10 ng/ml of medium beginning 2–3 d after subculture (16). The medium was usually changed every 2–3 d and the day before cells were harvested. Before keratinocytes were harvested, any remaining 3T3 cells were removed by vigorous pipetting with an isotonic solution of EDTA (24). The keratinocytes were then dislodged with a mixture of trypsin and EDTA. Cultures were usually harvested when three-quarters confluent.

Labeling of Cell Protein with [35S]Methionine

The medium of keratinocyte cultures was replaced with medium containing a methionine concentration reduced from 30 mg/L to 3 mg/L. Approximately 100 μCi of [35S]methionine (Amersham Corp., Arlington Heights, Ill.; specific
activity of 600–1300 Ci/mmol in 8 ml of medium were added per 100-mm dish containing ~4 × 10^6 cells. The cells were harvested after incubation at 37°C for 24 h.

Preparation of Cell Extracts

Harvested cells were washed once and resuspended in isotonic phosphate buffer containing 10 mM EDTA. The cells were sonicated and insoluble material was removed by centrifugation for 2 min at 12,800 g in a Brinkmann Eppendorf Centrifuge 3200 (Brinkmann Instruments, Westbury, N. Y.).

Separation of Keratinocytes of Different Size

Keratinocytes were separated by density gradient centrifugation according to the method of Prewlow et al. (14, 24). After harvesting, cells were washed once in isotonic phosphate buffer and resuspended in the buffer at 4°C. 5–15 × 10^6 cells were then layered on a 90-ml linear gradient of 25–65% (vol/vol) Percoll (Pharmacia Fine Chemicals, Piscataway, N. J.) in isotonic phosphate buffer. The gradients were centrifuged at 4°C in an International refrigerated centrifuge with a swinging bucket rotor. Each centrifugation was carried out for 20 min at 800 g, with 4 min slow manual acceleration and deceleration. 5-ml fractions were collected by upward displacement. Samples were electrophoresed in 8.5% acrylamide slab gels according to the method of Laemmli (10). Gels were stained in a solution containing 0.1% Coomassie Brilliant Blue R250, 50% methanol, and 10% acetic acid, and destained in a solution of 5% methanol and 10% acetic acid. Gels were fluorographed on Kodak RP2X-Omat film using the procedure of Bonner and Laskey (3). For quantitative immunoprecipitation studies, the film was preflaged (11).

RESULTS

Cell Size and the Presence of Involucrin

Epidermal cells were disaggregated, collected by centrifugation, and resuspended in a small amount of medium containing serum. The cells were allowed to attach to cover slips, then were fixed and examined for the presence of involucrin by immunofluorescence. Cells containing involucrin fluoresced brightly (Fig. 1 a), whereas cells lacking it had the same level of background fluorescence as cells stained with preimmune serum (Fig. 1 b).

The size distribution of cells possessing or lacking detectable involucrin is shown in Fig. 2. Virtually no cells with a diameter of <14 μm contained the protein, but most cells ≥18 μm did. About 30% of the entire population contained involucrin.

Isolation of Poly A+ mRNA and Translation In Vitro

After density gradient centrifugation, total RNA was extracted from pellets of fractionated keratinocytes, using the guanidine procedure of Strohman et al. (23), as modified by Fuchs and Green (5). Poly A+ mRNA was then isolated by affinity chromatography with oligo(dT)-cellulose (Type 3; Collaborative Research Inc., Waltham, Mass.) (4).

Rabbit reticulocyte lysates were prepared by a method based on that of Schimke et al. (22) and treated with micrococcal nuclease (Worthington Biochemicals Corp., Freehold, N. J.) (13) before use. The mRNA was translated at a final concentration of 9–18 μg/ml, in a total volume of 11 μL. The reaction mixture, containing 1 μM [35S]methionine (Amersham), was incubated for 1 h at 30°C.

Methylmercury hydroxide (Alfa Div., Ventron Corp., Danvers, Mass.) was added to some mRNA solutions, to a final concentration of 2.5 mM (12). Control samples were diluted with an equal volume of water. After 3–5 min, translation was initiated by addition of reticulocyte lysate and reaction mixture.

Immunoprecipitation of Involucrin

Involucrin was immunoprecipitated either from cell extracts or from translation products, using a method based on that of Kessler (9). Briefly, undiluted antiserum was added to the sample to an optimal ratio (usually 2 μl/10 μl of cell extract) containing 10 μM phenylmethylsulfonyl fluoride (PMSF). After incubation at 4°C overnight, 70 μL of a 10% (wt/vol) suspension of formalin-fixed protein A-rich Cowan I strain Staphylococcus aureus (The Enzyme Center, Inc., Boston, Mass.) were added. The S. aureus had been washed three times in a buffer solution consisting of 50 mM Tris, pH 7.4, 0.05% Nonidet P-40, 1 mg/ml of ovalbumin, 150 mM of NaCl, and 5 mM of EDTA. After 30 min at 4°C, the antibody-antigen complex bound to S. aureus was centrifuged and washed four times in buffer. Finally, the precipitate was resuspended in gel sample buffer and prepared for electrophoresis.

Scoring of Involucrin-containing Cells by Immunofluorescence and Determination of Cell Size

Cells suspended in medium containing serum were either allowed to attach to collagen-coated glass cover slips for 5 min or air-dried onto uncoated cover slips. The cells were fixed in 3.7% formaldehyde in isotonic phosphate buffer at room temperature for 8 min, in methanol (~20°C) for 4 min, and in acetone (~20°C) for 2 min. After a brief rinse in buffer, the cells were stained for immunofluorescence, using the procedure of Weber et al. (26). Involucrin antiserum (19) or preimmune serum was used at a dilution of 1:20 in isotonic phosphate buffer, and fluorescein-conjugated goat antiserum to rabbit IgG (Miles Laboratories Inc., Miles Research Products, Elkhart, Ind.) at a dilution of 1:16. Preparations were examined with a Zeiss photomicroscope III, and several randomly selected fields were photographed. Cell diameters were measured from photographs, using a Bausch and Lomb measuring magnifier.

Separation of Keratinocytes of Different Size

Keratinocytes were separated by density gradient centrifugation according to the method of Pretlow et al. (14, 24). After harvesting, cells were washed once in isotonic phosphate buffer and resuspended in the buffer at 4°C. 5–15 × 10^6 cells were then layered on a 90-ml linear gradient of 25–65% (vol/vol) Percoll (Pharmacia Fine Chemicals, Piscataway, N. J.) in isotonic phosphate buffer. The gradients were centrifuged at 4°C in an International refrigerated centrifuge with a swinging bucket rotor. Each centrifugation was carried out for 20 min at 800 g, with 4 min slow manual acceleration and deceleration. 5-ml fractions were collected by upward displacement with 60% (wt/vol) sucrose using a specially designed tapping device (14) (Halpro, Inc., Rockville, Md.). Fractions were diluted with buffer and the cells recovered by low speed centrifugation at 500 g for 5 min.

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PAGE

Samples were electrophoresed in 8.5% acrylamide slab gels according to the method of Laemmli (10). Gels were stained in a solution containing 0.1% Coomassie Brilliant Blue R250, 50% methanol, and 10% acetic acid, and destained in a solution of 5% methanol and 10% acetic acid. Gels were fluorographed on Kodak RP2X-Omat film using the procedure of Bonner and Laskey (3). For quantitative immunoprecipitation studies, the film was preflaged (11).
Isolation of Keratinocytes Lacking Involucrin

Keratinocytes of different sizes can be separated by density gradient centrifugation, because the small cells have a greater buoyant density than the large ones (24). This method was used to confirm the relation between cell size and the presence of involucrin and to obtain a population of cells substantially free of involucrin.

Epidermal cultures were disaggregated and the cells were washed in isotonic phosphate buffer, resuspended in buffer, and applied to Percoll gradients. After centrifugation, fractions collected from the gradients were examined microscopically and those containing cells of similar size were pooled. Typically, the largest cells, which had a buoyant density of ~1.04 g/ml, were found in the top six fractions, and the smallest, most dense cells (1.07 g/ml) in the bottom third of the gradient.

The large-cell fraction was appreciably contaminated with small cells (Fig. 3a), but there were very few large cells in the small-cell fraction (Fig. 3b). Thus, although only ~40% of the large-cell fraction showed positive staining, >96% of the cells in the small-cell fraction did not contain involucrin. For cells of a given size in either fraction, the proportion containing involucrin was in agreement with that found in the unfractionated population shown in Fig. 2.

Although immunofluorescence gives a reliable indication of the presence of involucrin in cells, it provides little information about the abundance of the protein. A quantitative measure of the amount of involucrin in different cell extracts was obtained by immunodiffusion, gel electrophoresis, and immunoprecipitation techniques. When serial dilutions of cell extracts were studied by Ouchterlony immunodiffusion, it was found that involucrin could be detected at a 1:16 or 1:32 dilution of large-cell extracts, but only faint precipitin bands formed against small-cell extracts, even when undiluted.

Extracts of large and small cells were subjected to gel electrophoresis in the presence of SDS and stained with Coomassie Brilliant Blue. The molecular weight of involucrin has been estimated previously to be 92,000 daltons (19) but, under the conditions of electrophoresis used here, the mobility of involucrin was somewhat less than that of rabbit muscle phosphorylase b (Sigma Chemical Co., St. Louis, Mo.). The extract of large cells contained an abundant protein with the same mobility as purified involucrin, whereas the small-cell extract showed only a very faint band at the same position.

Immunoprecipitated extracts of cells labeled with [35S]methionine for 24 h before harvesting were subjected to gel electrophoresis and fluorographed. The results were similar to those obtained on stained gels. Preimmune serum did not precipitate involucrin from extracts of either large or small cells (Fig. 4, tracks 3 and 6). The large-cell extract gave a strong band corresponding to involucrin, and this protein was selectively enriched by immunoprecipitation (Fig. 4, tracks 1 and 2). Densitometry of these tracks indicated that involucrin comprised, on the average, 5.5% of the soluble cell protein. Extracts of small cells produced no definite band corresponding to involucrin, either before or after immunoprecipitation (Fig. 4, tracks 4 and 5), and by densitometry we calculated that involucrin was <0.3% of the soluble protein. Because the small-cell fraction isolated by density gradient centrifugation contained up to 4% of large cells, the observed difference in involucrin content of at least 18-fold between the two cell populations suggests that the protein is totally absent from small cells.

Control of Involucrin Synthesis

Having established that a population of keratinocytes which had not yet begun to synthesize involucrin could be separated from cells which had, we investigated whether these small cells also lacked translatable mRNA for involucrin. Poly A+ mRNA was prepared from fractionated cells and translated in vitro.
using a rabbit reticulocyte lysate. The translated proteins were precipitated with antiserum to involucrin and subjected to electrophoresis. Fig. 5 shows clearly that there was a considerable amount of mRNA for involucrin in the large cells (tracks 1 and 2) but virtually none in the small cells (tracks 4 and 5). By densitometry, we calculated that any involucrin mRNA present in small cells must be <6% of that in large cells. This is in reasonable agreement with the amount expected from contaminating large cells in the small cell fractions (Fig. 2). Pretreatment of mRNA with methylmercury hydroxide (12) did not improve the efficiency of translation of involucrin mRNA, suggesting that secondary structure is not a factor limiting translation. It may be concluded that involucrin synthesis is controlled by the amount of specific mRNA available for translation. Fig. 5 also shows that there is no substantial difference in the size of involucrin synthesized in vivo and in vitro; this appears to rule out substantial post-translational alteration of the size of the protein.

**DISCUSSION**

We have found a correlation between cell size and involucrin synthesis in cultured human keratinocytes. Using density gradient centrifugation, we have separated a population of small cells lacking involucrin from larger, less dense cells containing the protein. By preparing mRNA from the two cell fractions and translating it in vitro, we have shown that involucrin synthesis is controlled by the cellular content of specific mRNA.

The timing of some of the changes known to occur during

![Figure 5 In vitro translation of poly A+ mRNA from large and small cell fractions. Tracks 1–3: mRNA from large-cell fraction. Tracks 4–6: mRNA from small-cell fraction. Tracks 1 and 4: complete translation products. 2 and 5: products precipitated by anti-serum to involucrin. 3 and 6: products precipitated by preimmune serum. 7: involucrin from large-cell extract precipitated with anti-serum as described in Materials and Methods. 8: Rabbit muscle phosphorylase b (M, = 92,000), labeled with [3H]formaldehyde (New England Nuclear, Boston, Ma.) (20). Arrow indicates position of involucrin.](image)

| Process | Cell Size |
|---------|-----------|
| Synthesis of DNA and cell division (basal layer of epidermis; basal layer of cultured epithelium) | Smallest |
| Onset of involucrin synthesis (variable distance through spinous layer; immediately suprabasal in culture) |
| Onset of synthesis of large keratins (in spinous layer of epidermis; still to be demonstrated in culture) |
| Onset of cross-linking (granular layer of epidermis; upper layers of cultured epithelium) | Largest |

![Figure 6 Sequence of biosynthetic changes during terminal differentiation of keratinocytes.](image)
terminal differentiation of keratinocytes is summarized in Fig. 6. As previously described, the maturation of keratinocytes in epidermis is associated with a switch in synthesis from small to large keratins (6). It is clear that the onset of involucrin synthesis is controlled independently of large keratin synthesis: under the culture conditions used here, the switch to large keratin synthesis does not take place (6), whereas involucrin synthesis probably begins even earlier (with respect to the position of the cell) than in intact epidermis (1). In considering what signals lead to the appearance of mRNA for involucrin, it must be noted that the size of a cell is correlated with its position in the stratified epithelium and perhaps with the length of time since it ceased to divide. To analyze this problem further, it is necessary to have a means of evaluating the relation of cell size to biosynthetic properties independent of other variables.

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