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Involucrin Acts as a Transglutaminase Substrate at Multiple Sites

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SUMMARY Involucrin is a keratinocyte protein with a specialized function in terminal differentiation. Synthesized initially as a soluble protein, it later becomes a preferred substrate for a membrane-bound transglutaminase and becomes cross-linked into an insoluble envelope.

When a crude keratinocyte extract containing about 2% involucrin is heated to 95°, most proteins precipitate, but all of the involucrin remains in solution, where it is over 90% pure. This step has been incorporated into a simplified procedure for purification of the protein.

Like intact involucrin, polypeptide fragments formed by the tryptic hydrolysis of involucrin are good substrates for the keratinocyte transglutaminase. Evidently amino acid residues participating in the enzyme-catalyzed cross-linking are distributed at numerous sites along the involucrin molecule.

INTRODUCTION During the terminal differentiation of the keratinocyte, an insoluble envelope made of cross-linked protein forms beneath the plasma membrane (1,2). The cross-linking is catalyzed by a transglutaminase that is cell-type specific and attached to the cell membrane (3,4). Soluble protein substrates for transglutaminase are present in keratinocytes (5,6). One of these, involucrin, is incorporated into the cross-linked envelope (6). This protein is enzymatically cross-linked to other proteins *in vitro* by a particulate cell fraction containing membranes (4).

Involucrin is an acidic protein, glutamine and glutamic acid residues accounting for nearly 50% of the molecule. From its behavior in sedimentation and gel filtration, the molecule appears rod-like (6). It is not clear how the involucrin molecule is adapted to its specialized function in the keratinocyte.

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We describe here some properties of involucrin, and the preparation of tryptic fragments active as substrates in the transglutaminase-catalyzed crosslinking system.

MATERIALS AND METHODS

Cell Cultivation Human epidermal keratinocytes (strain N) were used in their 3rd to 6th subculture. Stock cultures were grown in 75 or 150 cm^2 flasks as described earlier (4,7). The medium was changed every 3 days.

Preparation of crude cell extracts The stratified cell layer of cultures confluent for 10-12 days was washed 3 times with 15-30 ml of isotonic phosphate buffer containing 20 mM EDTA, and the tissue removed with a rubber policeman. The cells were then disrupted with 50 strokes in a Dounce-type glass homogenizer. The homogenate was clarified by centrifugation at 100,000 x g for 30 min at 4°.

Gel Electrophoresis Acrylamide was mixed with bis-acrylamide at a ratio 30:0.8 and poured between two glass plates separated by 1.5 mm. Samples were heated at 95°C for 5 min in 62.5 mM Tris-HCl buffer (pH 6.8), containing 2% SDS, 5% mercaptoethanol and 10% glycerol. Samples were applied to a stacking gel of 3% acrylamide and electrophoresis was carried out at a constant current of 25 mA for 5 hr at room temperature. The gel was stained with 0.2% Commassie brilliant blue in a solution containing 50% methanol and 10% acetic acid for 2 hr and destained with 10% methanol and 10% acetic acid. Alternatively, silver-staining was used (8).

Extraction of involucrin from gels Pieces of gel were placed in a solution containing 8 mM Tris, pH 7.8, 0.2 mM EDTA and 1 mM sodium acetate. The protein was electroeluted in the ISCO apparatus at a constant current of 12 mA/cm^2 for 3 hr at 4°, using a spectropore #3 membrane. The protein solution was recovered from the chamber, dialyzed against 2L of 2 mM dithiothreitol and 2L of distilled water for 12 hr each, and lyophilized.

Involucrin solutions already containing over 0.5 mg/ml were further concentrated by the addition of 10 volumes of cold methanol. After 30 minutes on dry ice, the suspension was centrifuged, and the precipitate was dried.

Preparation of fragments 300 ug of dried involucrin were dissolved in 0.15 ml sterile water (HPLC grade) and any nondissolved material was removed by centrifugation at 15,000 x g for 5 min. The solution was then made 1% in sodium bicarbonate, 3 ug (2 BAEE units) of diphenyl carbamyl chloride-treated trypsin were added, and the proteolysis carried out at 37° for 16 hrs. The enzyme was inactivated by adding SDS to 2% and immediately heating the solution to 100° C. The proteolytic fragments were separated on a 13% polyacrylamide separating gel (9). The stacking gel and sample buffer contained a tris-phosphate buffer made according to Maizel (10). To obtain purified fragments, the gel lanes were cut into 1 mm slices and the protein from each slice was eluted by overnight incubation in 0.5 ml water.

For amino acid analysis or cross-linking assay, fractions containing a given fragment were pooled, lyophilized, redissolved in 200 ul of water and dialyzed for 6 hrs against 3 x 1 liter of water (HPLC grade).

Crosslinking Assays The activity of each tryptic fragment as a transglutaminase co-substrate was assayed using [^{3}H] putrescine (11). The transglutaminase was present in a particulate fraction prepared by sonication of epidermal keratinocytes and centrifugation at 100,000 x g for 30 min. to remove all cytosol from the pellet. The pellet was washed once, recentrifuged at 15,000 x g for 15 min and the particulate fraction was collected. Reaction mixtures containing 200 ug of particulate protein, 12.5 ug of tryptic fragments and 10 uCi of [^{3}H] putrescine (18.6 Ci/mmole) were incubated at 37°C for 90 min in the presence of 10 mM $CaCl_2$. The reaction was terminated by adding EDTA to 20 mM, and nonlabelled putrescine to 2mM and the particulates were removed by centrifugation at 15,000 x g for 15 min. The supernatants containing the labelled fragments were then dialyzed, using Spectropore #6 dialysis

membranes, against 2 x 1 liter of 10 mM HEPES pH 7.4 to remove free labelled putrescine and electrophoresed. The gel lanes were cut into 1 mm slices and the protein was eluted as described above. Aquasol was added for counting.

RESULTS

Electrophoretic migration of involucrin as a discrete band in the absence of SDS In ordinary gel electrophoresis in the presence of SDS, involucrin, like other proteins, migrates as a discrete band (Fig. 1A). The behavior of the involucrin is anomalous in that its mobility relative to other proteins depends on the acrylamide concentration (12), but in a 6.5% gel, the Rf is 0.44.

When SDS was used to dissolve the sample but was omitted from the gel and running buffer, most proteins did not resolve into discrete bands (Fig. 1B), but involucrin did migrate as a discrete band, with Rf of 0.34. When SDS was omitted not only from the running buffer but also from the poured gel and sample as well, the mobility of involucrin was the same as when SDS was omitted only from the running buffer. Involucrin therefore migrated as a discrete band even when the protein had never been in contact with SDS.

Under non-denaturing conditions, most cellular proteins evidently do not remain as discrete molecular species during electrophoresis but probably undergo aggregation and dissociation as they move through the gel. The dis-

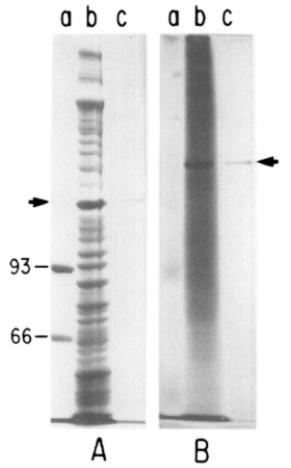

Figure 1. Distinctive electrophoretic behavior of involucrin
A. Electrophoresis through 6.5% acrylamide in the presence of SDS. a) Molecular weight standards. b) Crude extract of keratinocytes. c) Purified involucrin. B. Electrophoresis under the same conditions, except that SDS was omitted from the gel and running buffer. a,b and c have the same meaning as in A. Involucrin is the only protein that migrates as a discrete band. Like the molecular weight standards, most keratinocyte proteins are distributed in a continuous smear.

crete nature of the involucrin band implies that it does not participate in such reactions. Since the mobility of involucrin was little affected by omission of SDS, involucrin must carry a negative charge nearly as great as when the molecule has bound SDS.

Purification of involucrin by heating When a crude extract containing keratinocyte cytosolic proteins was heated in isotonic phosphate buffer supplemented with 20 mM EDTA, 62.5 mM Tris-HCl (pH 6.8) and 10% glycerin at 95°C for 10 min, most cytosolic proteins precipitated and could be removed by centrifugation. When precipitate and supernatant were subjected to electrophoresis it was found that all of the involucrin was recovered in the supernatant, where it was estimated to be over 90% pure. The purification factor was over 40 fold (Fig. 2).

These effects permitted a new and much simpler method of involucrin purification. Cytosolic extracts were heated as just described, and the precipitate was removed by centrifugation. SDS and β-mercaptoethanol were then added to final concentrations of 2% and 5% respectively and the solution was heated for 5 min in a boiling water bath. Subsequently an amount of solution containing about 1 mg of protein was introduced to the sample chamber of a 6.5% acrylamide separating gel. The stacking gel contained 3% acrylamide and was made with a slot 12.5 cm wide, 6 mm deep and 1.5 mm thick. SDS was omitted from the running buffer. Electrophoresis was carried out at 20 mA

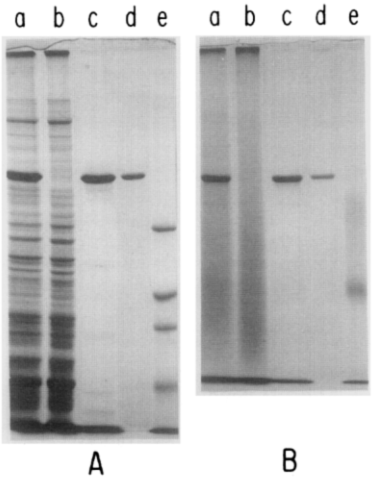

Figure 2. The use of heat in the purification of involucrin

Electrophoresis of cytosolic fractions. A. with SDS in the running buffer and in a gel concentration of 6,5%; B. without SDS in the running buffer and in a gel concentration of 5.0%. a) Crude cytosolic extract of keratinocytes. b) Proteins precipitated by heating (involucrin is absent). c) Supernatant proteins after heating (involucrin is the principal protein). d) Purified involucrin cut from gels and reelectrophoresed. e) Marker proteins (phosphorylase B 92.5 Kd, bovine serum albumin 66.2 Kd; ovalbumin, 45 Kd; carbonic anhydrase 31 Kd. In the absence of SDS from running buffer (B), only involucrin gave a discrete band.

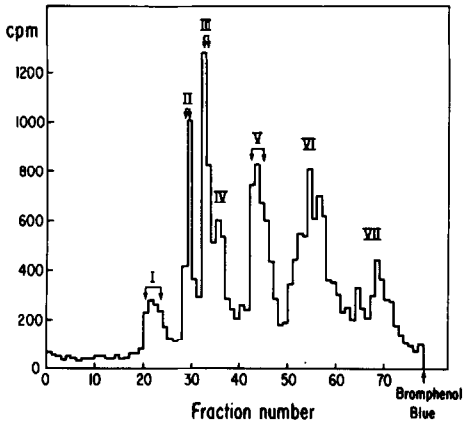

Figure 3. Tryptic Peptides of Involucrin
Involucrin was labelled by growth of keratinocytes in the presence of ^{3}H-Leucine, and isolated. The labelled involucrin was hydrolyzed to completion with trypsin and the products were electrophoresed through 9 cm of a 13% polyacrylamide gel. Slices 1 mm in width were cut and soaked in 0.5 ml dH_2O overnight to elute the peptides, and the radioactivity of each fraction was counted. The arrows delineate the fractions pooled for analysis.

until the marker dye, bromphenol blue, migrated about 10 cm. The gel was then removed and the involucrin band located by the altered reflection of incident light. If the protein content of the involucrin band was too low, a strip was cut out of the side and stained for protein. The protein band was cut out of the gel and the involucrin was electroeluted, dialyzed and lyophilized. On subsequent electrophoresis in the presence of SDS this material appeared essentially pure. The overall yield of pure involucrin was not less than 50 micrograms per 150 cm^2 culture.

Tryptic Fragments of Involucrin Involucrin contains about 7.6% lysyl residues and about 0.5% arginine. When leucine-labelled protein was exposed to trypsin at a ratio of 1:100, and the products were analyzed electrophoretically, it was found that within seconds the involucrin began to hydrolyze and within 15 minutes, digestion was virtually complete. The fragments were electrophoretically resolved into numerous peaks accounting for about 92% of the radioactivity present in the original involucrin (Fig. 3). The gel regions containing the better resolved peaks (I, II, III, and V) were cut out, the proteins were eluted and dialyzed and their amino acid composition was determined. All had a high glx content, in some cases (peaks II and III) even higher than that of the whole involucrin (Table 1).

Crosslinking ability of peptide fragments The presence of a high concentration of glx in tryptic fragments suggested that these fragments, like the involucrin molecule itself, might be active in the cross-linking reaction. Accordingly we tested the ability of the fragments to accept [^{3}H] putrescine, a reaction in which involucrin is about 80 times more effective than the

Table I. Activity of tryptic peptides as substrates of transglutaminase

	% Glx	H^3 putrescine crosslinked per ug of protein or peptide (cpm x 10^{-3})
Involucrin	45.6	44.8
I	28.6	26.9
II	55.0	9.4
III	56.1	10.4
V	40.0	15.6
Bovine Serum Albumin	9.1	0.07

average cytosolic protein (4). 12.5 ug of limit tryptic digest of involucrin were incubated with keratinocyte particulates (200 ug of protein) and 10 uCi of [^{3}H] putrescine. The fragments were then isolated electrophoretically and counted. As seen in Table 1, all fragments tested incorporated abundant putrescine, although somewhat less than the whole involucrin molecule. Bovine serum albumin, present in the reaction mixture, incorporated less than 0.2% as much radioactivity as involucrin. The crosslinking of putrescine to involucrin and its peptide fragments was calcium dependent and inhibited by cystamine. Since all the tryptic fragments tested (including some not reported here) were good substrates of the transglutaminase, the reactive groups of involucrin must be numerous and widely distributed over the molecule. This should facilitate the polymerization necessary to form the insoluble cell envelope.

ACKNOWLEDGMENT

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