ISOLATION OF TWO IMMUNOLOGICALLY RELATED TRANSGLUTAMINASE SUBSTRATES FROM CULTURED HUMAN KERATINOCYTES

J. KUBILUS AND H. P. BADEN

Department of Dermatology, Massachusetts General Hospital, Harvard Medical School, Boston, Massachusetts 02114

(Received July 13, 1981; accepted November 18, 1981)

SUMMARY

Transglutaminase substrates A and B were identified in soluble extracts of cultured keratinocytes and human epidermis by their reactions with dansyl cadaverine in the presence of Ca++ ion. Substrate B was present in substantial amounts in both extracts whereas A, easily seen in cell extracts, was decreased and sometimes not detected in tissue extracts. Substrates A and B from cultured cells were separated by Sephadex G-75 chromatography and isolated by preparative sodium dodecyl sulfate (SDS) gel electrophoresis by which A had a mol wt of 125,000 and B had a mol wt of 12,000. Amino acid analysis of A, B, and cornified envelope were similar but not identical. The isopeptide bond is not a significant structural feature of A inasmuch as its content is less than 0.25 bonds/molecule. Antibodies raised to A cross-reacted with B and vice versa and A showed partial identity to B when reacted with anti-B. Anti-A reacted with epidermis being adsorbed by the edges of cornifying cells but only weakly by cells of the Malpighian layer. Anti-B also reacted with cornifying, but its reaction was more intense with the cytoplasm of Malpighian cells. Substrate A appears to be incorporated into cornified envelope immediately after its appearance in cells of the granular layer and seems similar to a protein isolated by a different method. Substrate B, convertible by transglutaminase to higher molecular weight species, may also participate in cornified envelope assembly and shares some structural similarities to A.

Key words: keratinocyte; cornification; transglutaminase; cross-linking; epidermis; electrophoresis.

INTRODUCTION

Keratinized cells of epidermis are contained by a cornified envelope that consists largely of protein cross-linked by ε-(γ-glutamyl) lysine isopeptide bonds (1-4).

Recent evidence suggests that the envelope is formed from cytoplasmic precursor proteins by the action of epidermal transglutaminase. Antibodies raised to a soluble protein isolated from cultured human keratinocytes cross-react with both in vitro produced isolated cornified envelopes and with those present in normal epidermal cross sections (5). In bovine snout epidermis, a 150,000 dalton protein reacted with antibodies raised to cyanogen bromide fragments of bovine cornified envelope (6). This 150,000 dalton protein also reacted with antibodies raised to a cross-linked derivative of a 36,000 dalton component, and it was suggested that the cornified envelope was assembled by successive cross-linking of this smaller protein (7,8). Endogenous protein substrates of epidermal transglutaminase also have been detected in newborn rat epidermis and in human stratum corneum (9) but the relationship of these proteins to the cornified envelope has not been elucidated.

This paper describes the isolation of two soluble proteins from cultured human keratinocytes, their immunologic relationship both to cornified envelope and to each other, and their possible roles in cornified envelope assembly.

447
MATERIALS AND METHODS

Cell growth. Epidermal cells were prepared from human infant foreskins as previously reported (10). Swiss 3T3 cells were treated with 10 μg/ml mitomycin C, washed extensively with media, and trypsinized. Primary cultures were initiated in 35-mm petri dishes with 50,000 epidermal cells and 140,000 3T3 cells. The medium was Dulbecco's modified Eagle's medium supplemented with 20% fetal bovine serum, 0.4 μg/ml hydrocortisone, and 10^{-4} M cholera toxin. At the first feeding epidermal growth factor was added at a concentration of 10 ng/ml as recommended previously (11). Epidermal cells were subcultured at 200,000 cells/100 mm petri dish together with 900,000 mitomycin C treated 3T3 cells. Labeling of cell proteins with [guanido-^{14}C]-L-arginine was done as described earlier (10).

Reaction of cell extracts with dansyl cadaverine. Subcultured epidermal cells that were confluent from 2 to 7 d were homogenized with 50 mM Tris, 0.5 mg/ml dansyl cadaverine, pH 7.5, at a ratio of about 1.0 ml/100 mm dish. After centrifugation at 10,000 x g, the extract that was about 10 mg/ml in protein was made 1 mg/ml in dithiothreitol (DTT) and 6 mM in calcium chloride. After incubation for 3 h at 37 °C EDTA was added to 10 mM and the samples were centrifuged. The clear supernatant solution contained the dansylated products.

Undansylated proteins were prepared by homogenizing the cultures in 50 mM Tris, 2 mM EDTA, pH 7.5, followed by centrifugation. In some experiments, the Tris-EDTA extracts were made 8 mM in CaCl_2 and 0.5 mg/ml in DTT and incubated at 37 °C for 3 h followed by centrifugation.

Sephadex G-75 chromatography. Dansylated extracts were made 0.1 M in NaCl and 2.0 ml were passed down a calibrated 0.9 x 90 cm Sephadex G-75 column that had been equilibrated in 50 mM Tris, 1 mM EDTA, and 0.1 M NaCl, pH 7.3. Proteins were determined by their absorption at 280 nm. Fluorescence of individual fractions was determined at 355 nm using an excitation of 525 nm on an Aminco-Bowman spectrophotofluorometer.

After the pooling of individual tubes, Areas I and II (Fig. 1) were dia lyzed exhaustively in 0.1 mM EDTA, pH 7.5 and lyophilized.

Electrophoresis. Samples were electrophoresed in SDS by the method of Neville (12) or Weber and Osborn (13) in 10% polyacrylamide cast in 6 x 60 mm cylindrical gels, or by the method of Laemmli (14) in 10% polyacrylamide cast in 1.5 x 100 mm slabs. Isoelectric focusing and two dimensional electrophoresis were done according to O'Farrell (15).

Conversion to high molecular weight products. Tris EDTA extracts of cells labeled with [guanido-^{14}C]-L-arginine were separated into pools corresponding to Areas I and II by G-75 chromatography. Specific activity of each pool was 100,000 cpm/mg of protein. Reaction mixtures containing 7 mg of fresh extract and 200,000 cpm of Area I or II were incubated for 3 h in the presence of CaCl_2 and DTT. Aliquots of the reaction mixtures as well as unincubated control samples were electrophoresed in 7% gels. Gels were sliced and individual 1 mm slices were incubated at 55 °C for 4 h in a mixture of 0.4 ml 30% H_2O_2 and 0.2 ml 70% HClO_4. Digested gel slices were counted in a liquid scintillation counter after addition of 10 ml of Aquasol II (New England Nuclear, Boston, MA).

Isolation of dansylated substrate proteins. Chromatography on Sephadex G-75 gave two peaks of fluorescence (Fig. 1), which are designated Areas I and II. The two pools were made and each was dia lyzed exhaustively against 0.1 mM EDTA, pH 7.5, and lyophilized. Samples were redissolved in water to give protein concentrations of 10 mg/ml for Area I and 3 mg/ml for Area II. These solutions were prepared for SDS electrophoresis according to Neville (12). Area I was electrophoresed in 7% polyacrylamide gels (5 x 60 mm) at a load of 70 μg/gel. Area II was electrophoresed in 15% polyacrylamide gels at a protein load of about 30 μg/gel. About 100 gels were run and the sharp fluorescent bands visible in ultraviolet light were excised. Slices were placed on the surface of a 15 x