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A new class of soluble basic protein precursors of the cornified envelope of mammalian epidermis

Howard P. Baden, Joseph Kubilus, Scott B. Phillips, Joseph C. Kvedar and Steven R. Tahan

Harvard Medical School, Department of Dermatology, Massachusetts General Hospital, Boston, MA (U.S.A.)

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The cornified envelope has been shown to be formed beneath the plasma membrane as a result of the cross-linking of soluble and membrane-associated precursor proteins by transglutaminase. We have obtained a monoclonal antibody which reacts with the periphery of cells in the upper layers of human epidermis by indirect immunofluorescence (IIF) following immunization of mice with cornified envelopes of cultured human keratinocytes. The antibody also stained the cell peripheries of bovine, rat and mouse epidermis as well as stratified epithelium. Neutral buffer extracts of human cultured keratinocytes and epidermis examined under denaturing conditions contained polypeptides of molecular weight 14900 and 16800 which reacted with the antibody, and an additional component of molecular weight 24800 was found in cultured cells. The polypeptides were shown to have a p*I* of about 9.0. Under non-denaturing conditions the two lower-molecular-weight polypeptides had an apparent molecular weight of 30000, while the 24800 protein had one of 60000. Incubation of the polypeptides under conditions that activate transglutaminase resulted in a disappearance of the polypeptides or the formation of cross-linked products. Basic polypeptides with somewhat different p*I* values and molecular weights were identified in neutral buffer extracts of bovine and rat epidermis. The HCE-2 antibody appears to identify a new class of basic protein precursors of mammalian cornified envelope.

Introduction

The cornified envelope of keratinocytes is a highly insoluble subcellular structure that is formed beneath the plasma membrane during the late stages of differentiation. [1,2]. This results from the ε-(γ-glutamyl)lysine cross-linking of precursor molecules by the enzyme transglutaminase [3,4]. It has been shown in cultured human keratinocytes that there is a particulate and two cytoplasmic forms of the enzyme [5]. One of the soluble forms is similar to the particulate type in its immunologic properties [5] and it associates with the plasma membrane when cultured cells are induced to cornify, suggesting these may be the enzymes essential for the cross-linking of the envelope precursor proteins [6]. The second type of cytoplasmic transglutaminase likely corresponds to the guinea-pig liver or tissue type which has been described in a number of guinea-pig tissues [7].

Abbreviations: DTT, dithiothreitol; EDTA, ethylene diamine tetracetate; ELISA, enzyme linked immunoabsorbent assay; IEF, isoelectric focusing; IIF, indirect immunofluorescence; NEPHG, non-equilibrium pH gradient electrophoresis; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate.

Correspondence: H.P. Baden, Harvard Medical School Department of Dermatology, Massachusetts General Hospital, Boston, MA 02114, U.S.A.

Involucrin was identified as the preferred transglutaminase substrate in human cultured keratinocytes and shown to be a putative precursor of the cornified envelope [8]. It was also demonstrated to be present in human epidermis and to have the same function. However, recent immuno-ultrastructural studies have suggested that involucrin may also become cross-linked to keratohyalin granules and cytokeratins [9].

Two membrane-associated proteins of molecular weight 195 000 and 210 000 were also reported to be cross-linked in intact cultured human keratinocytes and presumably incorporated into the envelope [10]. More recently, it has been shown that the 195 000 protein is not an integral part of the membrane but rather has both cytoplasmic and submembranous distribution [11]. The latter progressively increases in amount during epidermal differentiation at the expense of the former.

A 36 000 molecular weight cytoplasmic protein was identified in bovine epidermis as the major substrate for cytoplasmic transglutaminase [12]. It could be reduced to M_r 8000–10 000 subunits by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Antibodies raised to the protein were reported to stain the cytoplasm of malphigian cells and the periphery of stratum corneum cells. More recently it was reported that a similar protein was present in human epidermis, but it was found that the bovine and human proteins were immunologically distinct [13].

In an earlier report we described proteins of M_r 125 000 and 12 000 which were substrates for transglutaminase in cultured human keratinocytes and human epidermis [14]. The former is now known to be identical to involucrin [15], while the latter was more similar in molecular weight to the protein reported above for bovine and human epidermis. We report here a monoclonal antibody prepared to purified cornified envelopes of cultured human epidermis which stains the periphery of cells of the upper layers of human epidermis similarly to the antibody prepared against involucrin and reacts with low-molecular-weight cytoplasmic proteins of human cultured keratinocytes and epidermis. This antibody also reacts with rat, bovine and mouse epidermis and likely identifies a class of mammalian proteins which are precursors of the cornified envelope and share at least one common epitope.

Materials and Methods

Tissue

Calf snouts were obtained from a local slaughterhouse, newborn Sprague-Dawley rats and BALB/c mice from Charles River Breeding Laboratories, and normal human skin from surgical specimens that would otherwise be discarded. To obtain epidermal tissue, the skin slices were soaked in NH_4Cl (pH 9.5) for 30 min, and the epidermis was removed with forceps [16]. In some cases the epidermis was separated by heating the skin to 55 °C for 1 min and scraping with a scalpel.

Human foreskin keratinocytes were cultured using the method of Rheinwald and Green [17] with some modification [18]. Stratified cultures were rinsed extensively with serum-free media and then harvested with a plastic policeman.

Electrophoretic and immunologic techniques

Single-dimension sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis was done in 7% or 15% polyacrylamide slab gels according to Laemmli [19]. Two-dimensional electrophoresis using either isoelectric focusing (IEF) or non-equilibrium pH gradient electrophoresis (NEPHG) in the first dimension was done according to O'Farrell [20] or O'Farrell et al. [21]. The second dimension used the Laemmli system with 15% polyacrylamide separating gels and a 4% polyacrylamide stacking gel. In gels using IEF in the first dimension, isoelectric points were measured utilizing an extra gel which was sliced into 0.5 cm lengths; the individual segments were incubated in 1 ml of deaerated water for 30 min and the pH of each determined. Isoelectric points were determined by comparison to this 'pH map'. In gels using NEPHG in the first dimension, isoelectric points were determined by position relative to trypsinogen (Sigma) ($pI = 9.3$) and lactic acid dehydrogenase (Sigma) ($pI = 8.5$). Transfer of separated polypeptides from polyacrylamide gels to nitrocellulose was accomplished by the method of Towbin et al. [22] using the Bio-Rad Trans Blot apparatus. The blots were stained with Fast Green.

Frozen tissue sections were stained by indirect immunofluorescence (IIF) as previously described [23] and formalin-fixed tissue sections by the im-

munoperoxidase technique [24]. Secondary antibodies for IIF were fluorescein isothiocyanate-(FITC-) conjugated goat IgG to mouse IgG (Cooper Biomedical) diluted 1 : 30. For immunoperoxidase staining of sections or immunoblotting, horseradish peroxidase-conjugated goat antibodies to mouse IgG (Bio-Rad) were used at dilutions of 1 : 2000. Column fractions were analyzed by electrophoresis in 15% SDS gels followed by immunoblotting as described above.

Isolation of an M_r 14000 dansylated polypeptide

Cultured human keratinocytes were homogenized at a ratio of 1 ml of buffer to one confluent 60 mm petri dish in a glass homogenizer using 50 mM Tris (pH 7.6)/1 mM EDTA (Tris/EDTA). The homogenate was made 10 mM in $CaCl_2$, 2 mM in dithiothreitol, and 1 mg/ml in dansyl cadaverine. After incubation for 2 h at 37 °C the reaction mixture was centrifuged at 50000 × *g* for 30 min to obtain a clear supernatant. A dansylated band of molecular weight about 14000 was identified in 15% polyacrylamide gels by viewing with ultraviolet A radiation, excised from the slab, and eluted from the gel using an Isco model 1750 sample concentrator.

Isolation of cornified envelope

Human cultured keratinocytes were homogenized at a ratio of 2.0 ml buffer to one 60 mm petri dish in a glass homogenizer in 100 mM Tris (pH 9.0)/1% SDS/10 mM dithiothreitol (Tris/SDS/DTT) and the homogenate was heated with stirring for 2 h at 50 °C. The suspension was clarified by centrifugation at 50000 × *g* for 30 min and the pellet was reextracted as above five additional times. Protein was undetectable in the final supernatant by the method of Lowry et al. [25]. The insoluble residue was freed of SDS by washing with 95% ethanol, and then resuspended in 100 mM sodium bicarbonate buffer. To quantitate the amount of cornified envelope protein an aliquot was hydrolyzed for 24 h in 6 M HCl, and the free amino acids were measured by the ninhydrin method using L-leucine as a standard [26]. The weight of amino acid (μg) was assumed to equal the weight of cornified envelope material.

Preparation of cornified envelope monoclonal antibodies

A suspension of 50 μg of purified cornified envelopes from cultured human keratinocytes in 0.2 ml of Freund's incomplete adjuvant was injected intraperitoneally into BALB/c mice. The injections were repeated every other week until the mouse serum showed a reaction to sections of human epidermis by IIF. After a 2 month rest period the mice were immunized on three successive days. On the 4th day, animals were killed, spleens removed and the cells from the spleens were fused with NS-1 mouse myeloma cells in the presence of 37% poly(ethylene glycol) (M_r 1000). Monoclonal antibodies were prepared as described previously [27,28]. Hybridoma cultures were screened for antibodies which gave a peripheral reaction with human epidermis by IIF and these clones were recloned and expanded. The resulting panel of monoclonal antibodies were then tested for their reactivity by immunoblotting to the M_r 14000 dansylated polypeptide of human cultured keratinocytes. One such monoclonal antibody was identified and designated HCE-2. The antibody was purified from culture supernatant using the Bio-Rad Monoclonal Antibody Purification System. 1 ml of medium yielded 0.028 mg of purified antibody as measured by the Bio-Rad reagent. The antibody is an IgG1 as determined by the Ouchterlony technique using a goat anti-mouse IgG1 (Tago).

Chromatography techniques for isolation of the polypeptides reacting with HCE-2 antibody

Cultured human keratinocytes 10 days post-confluence were rinsed with phosphate buffered saline and harvested with a plastic policeman. They were homogenized at 0 °C in a glass homogenizer at a ratio of 10 : 1 (v/w) in Tris/EDTA with 0.1 mM PMSF and centrifuged at 100000 × *g* for 30 min. The supernatant containing 32 mg/ml of protein was made 0.15 M in NaCl and 10 ml were put down a 100 × 2.5 cm column of Sephadex G-100. An 8 ml aliquot of a Tris/EDTA cell extract at 4.5 mg protein per ml adjusted to pH 7.4 was also put down a 4 × 0.9 cm Whatman DE-52 column. In addition, an 8 ml aliquot of the same extract adjusted to pH 8.3 was applied to a 4 × 0.9 cm column of Blue Agarose (Bethesda

Research Laboratories).

An affinity column was prepared by reacting 24 ml of purified HCE-2 antibody at 0.25 mg/ml diluted 1:1 with 0.1 M $NaHCO_3$ (pH 8.4) with 0.5 g of CNBr-activated Sepharose 4B for 4 h at 25°C. The mixture was made 0.2 M in glycine, allowed to stand for 2 h and washed extensively with 0.1 M $NaHCO_3$ and 0.1 M sodium acetate. A 4×0.9 cm column was packed with the gel in 10 mM Tris (pH 7.4)/0.5 M NaCl.

Cross-linking of the HCE-2-reactive polypeptides

Cross-linking of the HCE-2-reactive polypeptides was attempted by homogenizing cultured human keratinocytes at a ratio of 1 ml of buffer per 1 confluent 60 mm petri dish in a glass homogenizer in Tris/EDTA; the homogenate was made 10 mM in $CaCl_2$ and 2 mM in DDT and incubated at 37°C for 1 h and 2 h. Suspensions of 10^6 keratinocytes in 0.5 ml Dulbecco's medium with 0.04% Triton X-100 and 1 cm^2 pieces of facial skin in 5 ml of Dulbecco's medium with 0.04% Triton X-100 were incubated in a similar fashion. The homogenates and cell suspension were made 1% in SDS, 100 mM in Tris (pH 9.0) and 10 mM in DTT, heated at 90°C for 5 min and centrifuged at $50000 \times g$ for 30 min to obtain a clear supernatant. The skin samples were heated at 50°C for 1 min to separate the epidermis, which was extracted with 0.5 ml of the Tris/SDS/DTT buffer as above. SDS-polyacrylamide gel electrophoresis was done using 7% gels where the non-cross-linked HCE-2-reactive peptides ran close to the front.

Results

The reaction of HCE-2 with frozen sections of human leg skin is shown in Fig. 1a and reveals peripheral staining of the granular and upper spinous cell layers. When plantar skin was used, the periphery of the flattened cells of the lower layers of the stratum corneum were also stained (Fig. 1b). Formalin-fixed sections showed a reaction at the same level of the epidermis, but the staining was cytoplasmic (data not shown). These reactions are identical to those which we and others have observed with the antibody to involucrin [29]. HCE-2 reacted with frozen sections of bovine (Fig. 1c), rat and mouse epidermis and showed the same peripheral type of staining. It also reacted with the stratified epithelium of rat tongue (Fig. 1d), buccal mucosa, and esophagus. The antibody did not react with fish (carp), frog, tortoise or chicken leg epidermis [30].

Three bands were observed when HCE-2 was reacted with immunoblots of Tris/EDTA extracts of human cultured cells separated by SDS-polyacrylamide gel electrophoresis (Fig. 2). Their respective molecular weights were 14900, 16800 and 24800. The same mobility was observed when the reducing agent was omitted from the equilibrating buffer for the electrophoresis. Only trace amounts of the three bands and no new bands were observed when the Tris/EDTA-insoluble pellet was reextracted with Tris/SDS/DTT and studied by immunoblotting.

When a homogenate of cultured human keratinocytes was incubated with dansyl cadaverine and the supernatant (see Methods) was run by SDS-polyacrylamide gel electrophoresis (15% gels), fluorescent bands were observed that corresponded closely in mobility to those described above. Immunoblots of the dansylated preparation were reacted with HCE-2 and showed that the fluorescent bands were stained by the antibody (Fig. 3). However, when immunoblots of the dansylated and non-dansylated preparations were directly compared using HCE-2, it was found that the dansylated bands had an altered mobility (Fig. 3). The band of the lowest molecular weight was the one used to screen for HCE-2.

In order to determine the molecular weights of the native proteins which gave rise to the polypeptides observed by SDS-polyacrylamide gel electrophoresis, the Tris/EDTA extract was passed down a calibrated Sephadex-G 100 column and the fractions tested by SDS-polyacrylamide gel electrophoresis followed by immunoblotting using the HCE-2 antibody. Immunoblotting showed that the M_r 14900 and 16800 bands were at peak intensity in the fraction of apparent molecular weight 30000. The tube showing the most intense straining with the 24800 band had an apparent molecular weight of 60000. The disparity between molecular weights determined in SDS and under native conditions could result from the polypeptides existing as monomers in

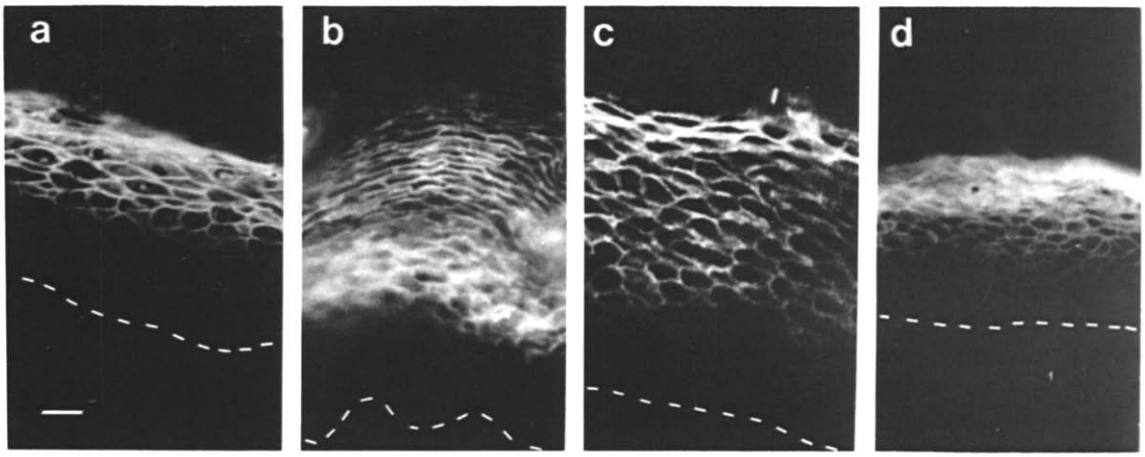


Fig. 1. Indirect immunofluorescence of human leg (a), human plantar (b), and bovine (c) epidermis and rat tongue epithelium (d) using HCE-2. The dashed line indicates the base of the epidermis. There is peripheral staining of keratinocytes in the upper living layers in a–d, while in b there is also peripheral staining of the flattened stratum corneum cells. The magnification is the same for all tissues and the bar equals 30 μm.

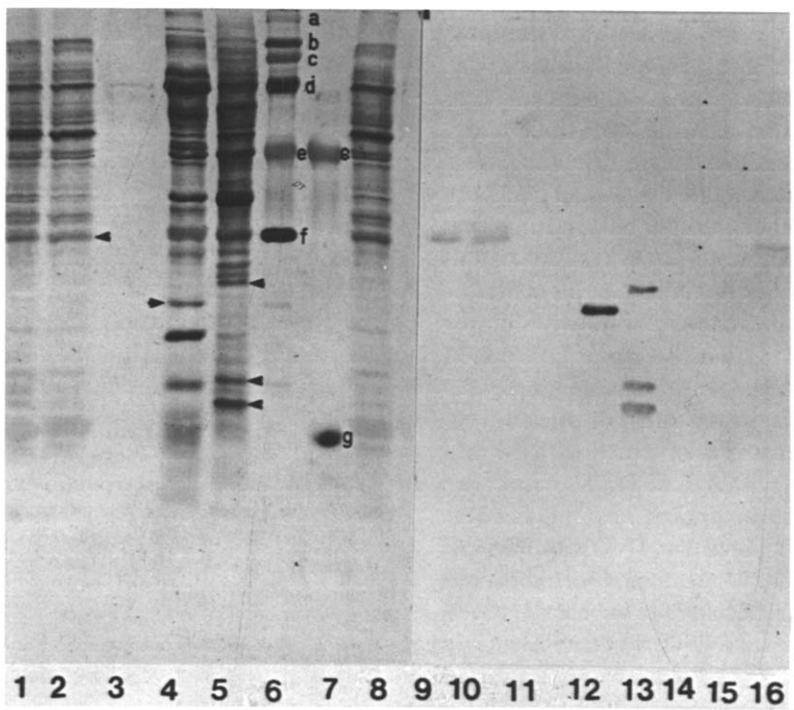


Fig. 2. Western blot analysis of the Tris/EDTA extracts of cultured human keratinocytes, bovine epidermis and rat epidermis separated by 15% SDS polyacrylamide gels. 1 to 8 are stained with Fast Green and 9 to 16 are the same lines reacted with HCE-2. Lanes 1, 2, 8, 9, 10 and 16 are rat extracts, 3 and 11 are blank, 4 and 12 are bovine extracts, 5 and 13 are human cultured keratinocyte extracts and 6, 7, 14 and 15 are standards (a is myosin (M_r 205000), b is β-galactoside (116000), c is phosphorylase *b* (97400), d is bovine serum albumin (66000), e is ovalbumin (45000), f is carbonic anhydrase (29000), and g is cytochrome *c* (12800). The arrowheads indicate the Fast Green bands in the various extracts which reacted with HCE-2; this was determined by placing pencil marks at the position of the various bands on the reverse side of the Fast-Green-stained blot.

SDS and aggregates under native conditions. Alternatively, the higher molecular weight may be a consequence of a molecular shape which is more rod-like than spherical when undenatured [31].

A blot of the two-dimensional pattern of the Tris/EDTA extract of cultured cells obtained by performing IEF in the first direction and SDS-polyacrylamide gel electrophoresis in the second showed no stained bands when reacted with HCE-2. The highest pH measured on the IEF gel was 7.2. When this procedure was done using NEPHG in the first direction the M_r 14900, 16800 and 24800 bands were found at the basic end (Fig. 4). These corresponded to the most basic spots seen on the two-dimensional blot stained with Fast Green prior to reaction with the antibody. These could be aligned with two bands seen on a stained NEPHG gel run at the same time as the one used for the two-dimensional pattern (Fig. 4). These correspond very closely with trypsinogen, which has a p*I* of 9.3.

Purification of the polypeptides was attempted using the affinity column prepared with HCE-2. When the Tris/EDTA extract of cultured cells was passed down the column the three polypeptides passed directly through. The tube from the Sephadex G-100 column which had the peak amount of the 14900 and 16800 polypeptides and one tube on each side were pooled. 28 ml of this pool were made 0.5 M in NaCl, applied to the affinity column and washed with two column volumes of 10 mM Tris buffer (pH 7.4)/0.5 M NaCl. No HCE-2-reactive protein could be detected in the protein which did not stick to the column. The column was then eluted with 10 mM Tris buffer (pH 7.4)/4 M KSCN. The first two tubes showing a rise in optical absorbance were combined, dialyzed against 1 mM Tris buffer (pH 7.4) and lyophilized. An aliquot of this was analyzed by SDS-polyacrylamide gel electrophoresis and showed the two lower-molecular-weight

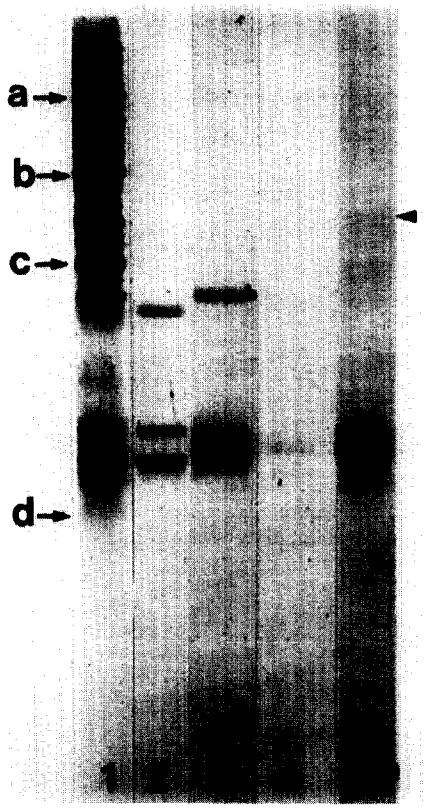
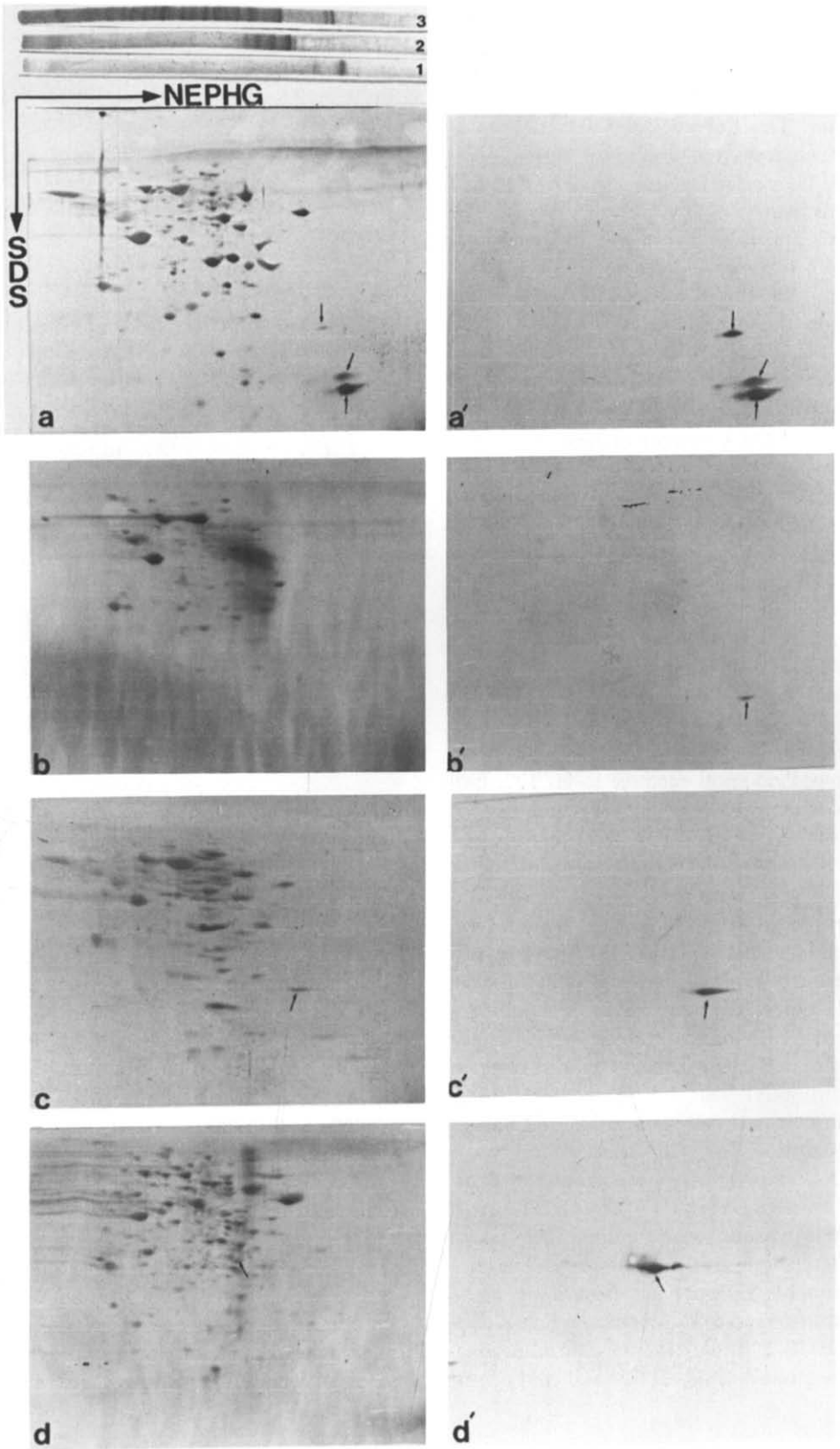


Fig. 3. Western blot analysis of Tris-buffer-soluble proteins of cultured human keratinocytes separated by 15% SDS polyacrylamide gels. Lane 1 is stained with Fast Green and 2, 3, 4 and 5 are reacted with HCE-2. a, b, c, and d mark the mobilities of bovine serum albumin, ovalbumin, carbonic anhydrase, and cytochrome *c*, respectively. Lanes 1 and 2 show an unincubated Tris/EDTA extract, lane 3 shows the extract incubated with $CaCl_2$, DTT, and dansyl cadaverine for 2 h. The black dots in 3 indicate the fluorescent bands observed with ultraviolet A radiation. The fluorescent bands are stained by the antibody indicating the immunoreactive bands are substrates for transglutaminase. Comparison of lanes 2 and 3 shows that dansylation alters the mobility of the immunoreactive bands. Lanes 4 and 5 show the extracts incubated with $CaCl_2$ and DTT for 2 h and 1 h, respectively. In lane 5 the M_r 14900, 16800 and 24800 bands are decreased in intensity and immunoreactive bands of higher molecular weight are seen. The most evident of these is indicated by an arrowhead. In lane 4, these bands are now absent and the initial bands are further decreased.

Fig. 4. Immunoblots of two-dimensional electrophoresis patterns of Tris/EDTA extracts of cultured human keratinocytes (a), human epidermis (b), bovine epidermis (c) and rat epidermis (d) using NEPHG in the first direction and 15% SDS-polyacrylamide gel electrophoresis in the second. The blots on the left were stained with Fast Green and those on the right the same blots subsequently stained with HCE-2. The arrows point to the immunoreactive bands. 1 is the stained NEPHG gel of trypsinogen, 2 lactate dehydrogenase, and 3 the extract of cultured human keratinocytes. Lactate dehydrogenase runs as several bands in this system according to the manufacturer. All first dimension gels were from the same NEPHG run.


3
2
1
NEPHG
SDS
a
a'
b
b'
c
c'
d
d'

polypeptides, which had molecular weights of 14400 and 15700 (Fig. 5).

As expected from the p*I* of the three polypeptides, they passed directly though a Whatman DE-52 column. The polypeptides did stick to a Blue agarose column and could be eluted with 0.15 M NaCl. The active fractions from both these columns were run by SDS-polyacrylamide gel electrophoresis and then transferred to nitrocellulose paper. When they were reacted with HCE-2, some smearing of the bands was observed, so purification on the affinity column was not attempted.

Normal human epidermis was examined for its content of polypeptides reactive with HCE-2 antibody. The Tris/EDTA extract of heat separated epidermis revealed the M_r 14900 and 16800 polypeptides (Fig. 6), although in the five specimens tested, the M_r 14900 one was invariably present in greater amounts. No M_r 24800 band was observed in any of the samples, but a weak band of high molecular weight (over 100000) near the top of the gel was seen in two (Fig. 6). Extraction of the two Tris/EDTA-insoluble pellets with Tris/SDS/DTT did not solubilize any protein which reacted with HCE-2. Two-dimensional analysis of the human epidermal extract using IEF in the first direction showed no spots reactive to HCE-2, but when the two-dimensional analysis was done using NEPHG, a definite stained spot was seen corresponding to the M_r 14900 band of cultured cells (Fig. 4) and a very weak one at 16800 when a heavy protein load was applied. The amount of these polypeptides in the Tris/EDTA extract of human epidermis was small, as bands could not be unequivocally detected in the Fast-Green-stained two-dimensional blot or the stained NEPHG gel which corresponded in position to those detected by HCE-2 antibody. This is different from the situation with cultured cells, where stained bands were clearly visible.

Since HCE-2 showed peripheral staining of rat and bovine epidermis by IIF, Tris/EDTA extracts of these tissues were studied for polypeptides that reacted with the antibody. The extracts were run by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose paper and stained with HCE-2. The extract of cow epidermis showed a strong band at M_r 22000 and that of rat

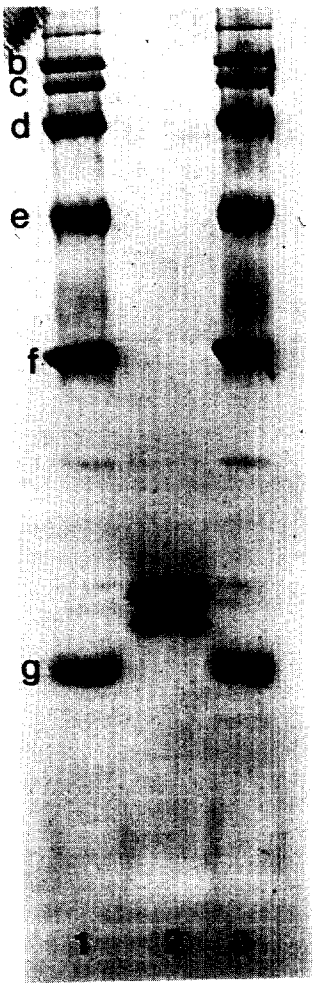


Fig. 5. Western blot analysis of the affinity column purified 14900 and 16800 polypeptides run on 15% SDS polyacrylamide gels. Lanes 1 and 3 are standards and 2 the polypeptides. a is myosin, b is β-galactosidase, c is phosphorylase *b*, d is bovine serum albumin, e is ovalbumin, f is carbonic anhydrase, and g is cytochrome *c*. When purified the bands gave molecular weights of 14400 and 15700.

epidermis at 29000 (Fig. 2). When these extracts were run by two-dimensional electrophoresis using IEF in the first direction and immunoblots were done with HCE-2, no spots could be seen, indicating they had a p*I* > 7.2. Repeat of the two-dimensional electrophoresis and immunoblots using NEPHG in the first direction showed the bovine protein had a p*I* between pH 8.5 and pH 9.0, while the rat protein had a number of isoelectric variants with a major spot between pH 7.0 and

pH 8.5 (Fig. 4). The bovine and rat proteins could be identified on the Fast-Green-stained two-dimensional pattern as shown in Fig. 4.

Cross-linking of the HCE-2-reactive polypeptides in homogenates of cultured human keratinocytes was demonstrated by the loss of the HCE-2 antigens and the generation of immunoreactive material of higher molecular weight after 1 h of incubation. After a 2nd hour, both starting material and the generated products were further decreased, presumably due to continued cross-linking (Fig. 3, lanes 4 and 5). In epidermis there was a disappearance of the low-molecular-weight peptides without the appearance of high-molecular-weight ones, suggesting the formation of products that were too large to enter the gel (data not shown). In suspensions of cultured human keratinocytes induced to cornify, there was also a generation of higher-molecular-weight immunoreactive products (Fig. 7).

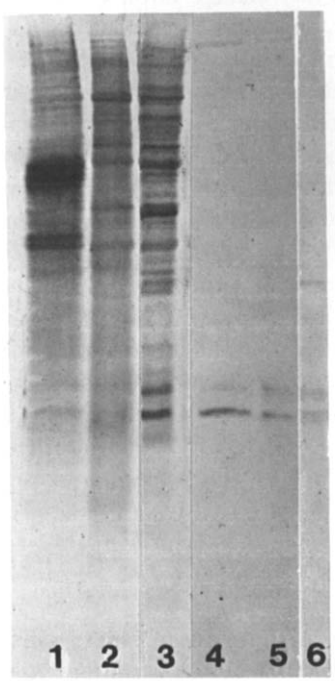


Fig. 6. Western blot analysis of Tris/EDTA extracts of human epidermis separated by 15% SDS polyacrylamide gels. Lanes 1, 2 and 3 were stained with Fast Green and 4, 5 and 6 were reacted with HCE-2. Lanes 1 and 2 are separate extracts of human epidermis and their immunoblots in 4 and 5 reveal the M_r 14900 and 168000 bands as well as a weak high-molecular-weight band (over 100000) near the top of the gel. Lanes 3 and 6 show the Tris/EDTA extract of cultured human keratinocytes for comparison.

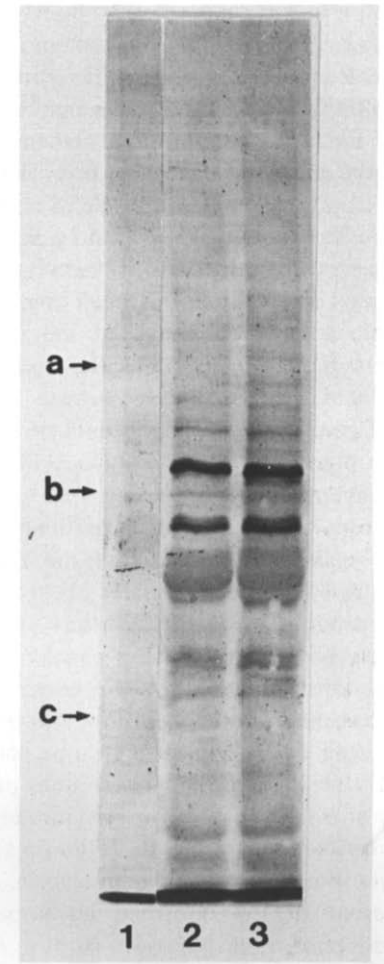


Fig. 7. Western blot analysis using 7% SDS polyacrylamide gels of the Tris/SDS/DTT extracts of cultured human keratinocyte suspensions which were incubated under transglutaminase-activating conditions. All the lanes were reacted with HCE-2. Lane 1 is the zero time, lane 2 is after 1 h and lane 3 is after 2 h. Discrete cross-linked reactive bands are seen at 1 and 2 h. Arrows denoting molecular weights of 97000 (phosphorylase *b*), 67000 (bovine serum albumin), and 43000 (ovalbumin) are indicated by a, b and c respectively.

Discussion

Involucrin has been considered a major constituent of the cornified envelope of human epidermis and has also been noted to be present in the epidermis of other primates [32]. The structure

of the involucrin gene has recently been reported [33] and it has confirmed that the protein is rich in glutamic acid and glutamine and has a molecular weight of 68 000, a value much less than reported by physical methods. Membranous associated precursors of the envelope have also been described [10,34]. An opposing view to the role of involucrin in epidermis has been expressed, and a protein of molecular weight 6000 by SDS-polyacrylamide gel electrophoresis, keratolinin, has been described as the principle soluble precursor of the envelope [13]. A protein of similar molecular weight has been postulated as a precursor of bovine cornified envelope. However, convincing data that keratolinin is a precursor of the envelope in human epidermis have not been presented.

In this study we have more definitively identified a new class of precursors of the cornified envelope which had been initially observed as a dansylated product in an earlier study [14]. This was accomplished by screening a panel of monoclonal antibodies prepared to the cornified envelope of cultured human keratinocytes and the characterization of HCE-2. The epitope to which the antibody reacts is present in soluble proteins of a variety of mammalian species not just primates and based on the IIF studies this epitope appears to have distribution similar to involucrin and is clearly present in the cornified envelope. The dansyl cadaverine incorporation studies further identify these proteins as substrates of epidermal transglutaminase. Furthermore, incubation of homogenates of cultured human cells or whole cultured cells under transglutaminase-activating conditions results in the cross-linking of the subunits to higher-molecular-weight species.

The proteins of cultured human keratinocytes have a basic p*I* and aggregate under non-denaturing conditions. The former is also true for the polypeptides of human epidermis. In rat and bovine epidermis, the proteins while still basic, have a p*I* closer to neutrality. These proteins must contain glutamine residues based on their ability to incorporate dansyl cadaverine, and the presence of lysine residues is very likely in proteins this basic. Amino-acid analysis of the purified proteins will be necessary to establish this and demonstration of cross-linking of the purified polypeptides by transglutaminase is needed to establish that the polypeptides cross-react with each other.

The M_r 24 800 polypeptide could not be identified in human epidermis but has been consistently observed in cultured keratinocytes even at different degrees of confluence. Furthermore, we have obtained the same results with five different lines of normal human keratinocytes. Although the staining intensity by immunologic methods is similar for the M_r 24 800 and two smaller polypeptides the protein staining is weaker for the former, indicating that it is present at a lower concentration in the cytoplasm. Since we do not know the kinetics of turnover for these proteins, this can not be used as an absolute indication of their relative contribution to envelope formation. This may also apply to human epidermis.

The relationship of the M_r 24 800 and lower-molecular-weight polypeptides has not been established. In cross-linking experiments we have not observed any change in the proportion of the polypeptides. However, in order to answer this question satisfactorily we shall have to isolate messenger RNA and determine the products that are synthesized. This is particularly important in view of the finding that filaggrin, the keratohyalin basic protein, is derived from a high-molecular-weight precursor [35] by proteolytic digestion, a fact which was not recognized for many years.

Involucrin is a rather acidic polypeptide, with a p*I* in the 4.5 to 5.0 range, and it is tempting to speculate that the basic polypeptides may aggregate with it during the formation of the cornified envelope. This could explain why peripheral staining by IIF is observed in the upper layers of the epidermis prior to the apparent presence of a cornified envelope by morphologic study.

We think it unlikely that the polypeptides we have described are identical to keratolinin. They are almost double the molecular weight seen in denaturing (SDS) conditions, and their isoelectric points are basic, while the keratolinins are acidic. In addition, unlike HCE-2, an antibody prepared to cow keratolinin does not react with human keratolinin.

Like involucrin, the basic polypeptide is an excellent marker of stratified epithelium and envelope formation. It has the added advantage of sharing epitopes with proteins in other species so that HCE-2 can be used as a probe for envelope

formation in laboratory animals. For example, it has been suggested that involucrin may have a different distribution in benign and malignant human epidermal tumors [29] and HCE-2 could be used in carcinogenesis studies in animals to examine this question more carefully.

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