Differentiation-dependent Changes in the Solubility of a 195-kD Protein in Human Epidermal Keratinocytes

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Abstract. We have prepared a monoclonal antibody, AE11, that recognizes specifically a 195-kD protein (pI 5.4) of human keratinocytes. This antigen constitutes ~0.01–0.1% of total protein in keratinocytes of skin, esophagus, and cornea, and is readily detectable in these cells by immunofluorescent staining and immunoblotting. However, it is barely detectable in MCF mammary carcinoma cells and HeLa cells, and is undetectable in nonepithelial cell types. Results from serial extraction experiments have shown that this protein exists in two distinct pools: a Tris-soluble, and a Tris-insoluble but urea- or SDS-soluble one. The distribution of the 195-kD protein between these two pools appears to be differentiation-related, since relatively undifferentiated cells selected by a low-calcium medium contain primarily the soluble form, while highly differentiated cells contain mainly the insoluble form. Data from immunofluorescent staining and trypsin-sensitivity experiments suggest that the soluble form is cytoplasmic, whereas the insoluble form is submembranously located at the cell periphery of upper, differentiated cells. The insoluble, cell peripheral form of the 195-kD antigen increases progressively during epidermal differentiation; its insolubility appears to be related to the formation of disulfide-bond(s). These results indicate that the 195-kD protein, which has recently been suggested to be involved in cornified envelope formation (Simon, M., and H. Green, 1985, *Cell*, 36:827–834), undergoes significant changes in its solubility characteristics and intracellular location during keratinocyte maturation.

During terminal differentiation, epidermal cells undergo major biochemical changes. The molecules that undergo such alterations can be divided into two broad categories according to their intracellular location. (*a*) Cytoplasmic proteins such as keratins. Immunolocalization and cell fractionation experiments have established that basal cells of normal epidermis synthesize two major keratin polypeptides, a 50/50'-kD acidic keratin and a 58-kD basic keratin, whereas cells of the suprabasal layers possess an additional 56.5-kD acidic and a 65–67-kD basic keratin (3, 5, 20, 23, 26, 27, 30, 31, 33). (*b*) Cell peripheral proteins, including involucrin (1, 18), kerotolinin (34), and several other high molecular weight proteins (21, 22). Serving as the substrates for the Ca^{++}-dependent transglutaminase (8, 28), these proteins are covalently cross-linked, forming a cornified envelope which is a submembranous structure characteristic of the cornified cells (17, 24).

In a series of experiments designed to generate monoclonal antibodies to the keratinocyte cell surface and its related components, we fused the spleen cells from Balb/c mice previously immunized with intact, human epidermal keratinocytes with X63.Ag8.653 mouse myeloma cells. Supernatants from wells containing hybridoma cells were screened for their ability to stain the periphery of keratinocytes in frozen sections of human epidermis by indirect immunofluorescent staining. We report here that the antibody secreted by one of these hybridoma lines, AE11, defines a 195-kD keratinocyte component that undergoes novel differentiation-related changes in its solubility property and its intracellular location (from cytoplasmic to peripheral).

Materials and Methods

Human Tissues

Specimens of human tissues were obtained from autopsies performed within 12–24 h after death. Human cornea and newborn foreskin were obtained from the Manhattan Eye, Ear, and Throat Hospital in New York, and from the nursery at the New York University Hospital, New York, respectively. Tissues were frozen in OCT-embedding medium, stored at −20°C, and sectioned within 2 wk.

Cell Culture

Human epidermal cells were grown from newborn foreskin using mitomycin-treated 3T3 fibroblasts as a feeder layer (15, 16). Melanoma cell line RPMI-72 and cultured human endothelial cells were kindly supplied by Drs. Ruth Crowe and Daniel Rifkin of New York University. Neuroblastoma cell lines T17-SY5Y were kindly supplied by Dr. Douglas Ishii of Columbia University, New York. Epidermal cell "monolayer" were obtained by an overnight treatment of a confluent epidermal culture with a low-calcium (0.2 mM) medium (7).

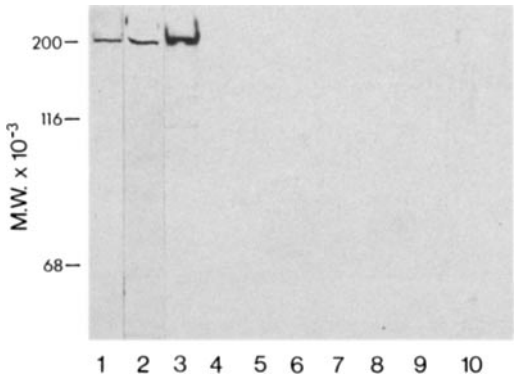

Figure 1. Immunoblot analysis of total proteins (50 μg) from various human cells using AE11 antibody. Lane *1*, abdominal epidermis. Lane *2*, cultured newborn foreskin epidermal cells. Lane *3*, esophageal epithelium. Lane *4*, HeLa cells. Lane *5*, mammary carcinoma cells (MCF-7). Lane *6*, cultured melanoma cells (RPMI-72). Lane *7*, cultured foreskin capillary endothelial cells (courtesy of R. Crowe and D. Rifkin). Lane *8*, cultured neuroblastoma cells (CRL-1420). Lane *9*, dermal fibroblasts. Lane *10*, CRL-1420 pancreatic carcinoma cells. Note that AE11 antibody detects a 195-kD protein in the epidermis, cultured epidermal cells, and in vivo esophageal epithelium, with little or no reaction in other cell types.

Monoclonal Antibody Production

Human epidermal cells were obtained by trypsinization of in vivo epidermis, suspended in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum and 10% glycerol, and stored in liquid nitrogen. Approximately 10^6 thawed cells were washed and resuspended in phosphate buffered saline (PBS), mixed with an equal volume of Freund's complete adjuvant, and injected subcutaneously into female Balb/c mice. Subsequent immunizations were given intraperitoneally at 2–4-wk intervals until the mouse sera gave positive immunofluorescent staining on frozen skin sections at 1:100 dilution. Mouse spleen cells were fused with X63.Ag8.653 mouse myeloma cells in polyethylene glycol, and selected in hypoxanthine/aminopterin/thymidine medium (9). The supernatants were screened by immunofluorescence or peroxidase-antiperoxidase (PAP)[1] staining of human skin sections (33). The hybridoma cells that gave peripheral staining of epidermal cells were cloned three times by serial dilution.

Sequential Extraction of Proteins

Tissues or cultured cells were first extracted at 4°C in a buffer containing 50 mM Tris.HCl (pH 7.4), 1 mM phenylmethyl sulfonylfluoride, 1 mM EGTA, 1 mM EDTA, 5 μg/ml antipain, and 5 μg/ml pepstatin A (Buffer A, [33]), centrifuged at 15,000 *g* for 30 min. The pellet was then extracted with 1% Triton X-100 in Buffer A, and finally with either 2% SDS or 8 M urea in Buffer A.

Total proteins were prepared by heating the cells in Buffer A containing 2% SDS, with or without 1% β-mercaptoethanol, at 95°C for 5 min.

Gel Electrophoretic and Immunoblot Techniques

Proteins were separated by SDS gel electrophoresis according to Laemmli using a 12.5% gel (acrylamide to bis-acrylamide ratio: 120/1; [10]). Two-dimensional, nonequilibrium gel electrophoresis was performed as described previously (12). The separated proteins were electrophoretically transferred onto nitrocellulose paper and stained with monoclonal antibody, using the PAP method (29, 33).

Immunocytochemical Staining and Antibodies

Unfixed, frozen tissue sections were stained by the indirect immunofluoresence, PAP, or avidin-biotin complex techniques (Vector Laboratories, Inc., Burlingame, CA). Other antibodies used include a supernatant from P3 (secretor) myeloma cells, a rabbit antikeratin antiserum (25, 26), AE3 monoclonal antikeratin antibody (33), a rabbit antidesmosomal antiserum from Dr. Malcolm Steinberg of Princeton University, Princeton, NJ, (6), and a mouse polyclonal antiserum specific for a 195-kD protein from Drs. Marcia Simon and Howard Green of Harvard Medical School, Boston, MA. (21).

1. *Abbreviation used in this paper*: PAP, peroxidase-antiperoxidase.

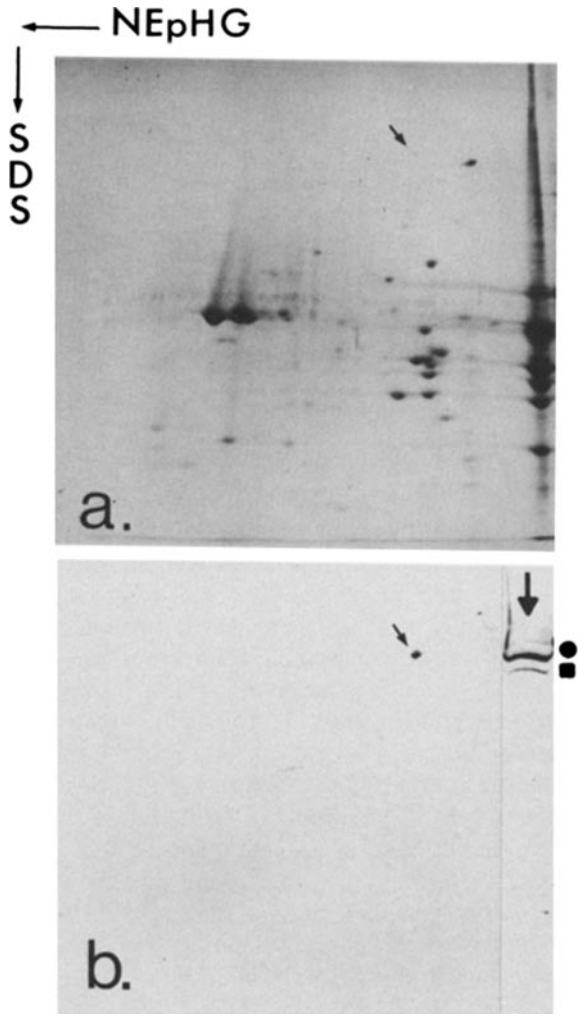

Figure 2. Identification of the AE11-antigen in cultured human epidermal keratinocytes by two-dimensional immunoblotting. Total proteins (200 μg) of cultured human epidermal cells were separated by nonequilibrium pH-gradient (*NEpHG*) electrophoresis in the first dimension, and SDS gel in the second dimension. (*a*) Fast green staining of protein spots transferred electrophoretically to nitrocellulose paper. Arrows in this gel and in *b* denote the single spot recognized by AE11 antibody. (*b*) The same blot as shown in *a* was stained with AE11 by the PAP technique. Downward arrow on the right denotes a side lane of one-dimensional blotting included to facilitate the comparison of one- and two-dimensional results. In the side lane the additional AE11-reactive band (■) that is slightly below the major 195-kD band (●) is variable in intensity and is probably a degradative product.

Results

Antigenic Specificity of AE11 Antibody

In the course of characterizing a number of monoclonal antibodies prepared against human epidermal keratinocytes, we found that one of these antibodies, AE11, produced strong peripheral staining in cells of the suprabasal layers of normal

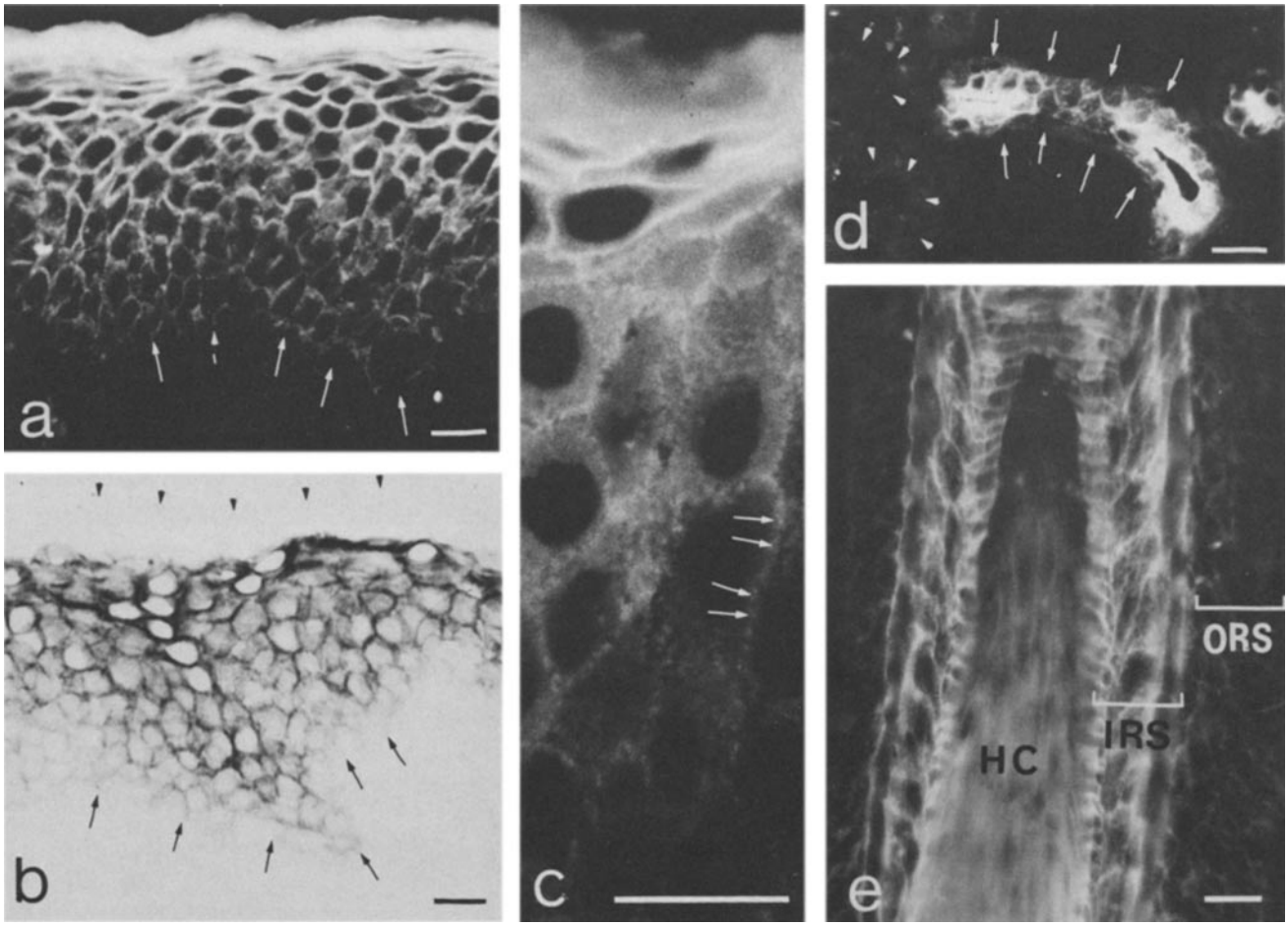

Figure 3. Immunohistochemical staining of various human skin-related epithelial cells using AE11 antibody. (*a*, *c–e*) By immunofluorescence. (*b*) By the PAP technique. (*a* and *b*) Epidermis. Note the strong cell periphery staining of the upper cell layers. Arrows denote the epidermal–dermal junction. Arrowheads in *b* denote the superficial surface of stratum corneum. The immunofluorescent staining of stratum corneum (*a*) may be nonspecific, as no stratum corneum staining was produced by the more specific PAP staining (*b*). (*c*) Epidermis at a higher magnification. Note the punctate or granular AE11-staining pattern in some areas (*arrows*). (*d*) Sweat glands. Note the preferential staining of upper cells in the ducts (*arrows*, epithelial–mesenchymal junction) and the lack of AE11-staining in the glandular portion (*arrowheads*). (*e*) Hair follicle. Note the positive staining of inner root sheath (*IRS*), and the weak or no staining of the outer root sheath (*ORS*). Hair cortex (*HC*) exhibits weak, possibly nonspecific, staining. Bars, 20 μm.

human epidermis, suggesting that the antigen defined by this antibody undergoes differentiation-related changes. Immunoblotting experiments show that AE11 recognizes a single 195-kD component in keratinocytes of normal abdominal epidermis (Fig. 1, lane *1*), cultured newborn foreskin epidermal cells (lane *2*), and in vivo esophageal epithelium (lane *3*). This AE11-reactive, 195-kD antigen is barely detectable in MCF mammary carcinoma cells (lane *4*) and HeLa cervical carcinoma cells (lane *5*; also data not shown); but is undetectable in melanoma cells (lane *6*), foreskin-derived capillary endothelial cells (lane *7*), neuroblastoma cells (lane *8*), skin fibroblasts (lane *9*), or pancreatic carcinoma cells (lane *10*).

When total proteins of cultured human epidermal keratinocytes were separated by two-dimensional, nonequilibrium gel electrophoresis, and immunoblotted, the AE11-antigen was detected as a single spot (pI ~5.4; Fig. 2). This spot is at least partly proteinaceous in nature because it is protease-sensitive (see below) and can be labeled biosynthetically with [^{35}S]methionine (data not shown). It was barely detectable by Coomassie Blue R-250 or Fast green staining when 200 μg of total proteins from cultured keratinocytes was loaded on a two-dimensional gel. This suggests it is a relatively minor component constituting <0.1% of total cellular proteins. Co-electrophoresis experiments have established that this same 195-kD, pI 5.4 spot exists in normal human epidermis (data not shown).

Immunolocalization of the 195-kD Protein

Immunohistochemical staining of various human cells and tissues shows that AE11 decorates primarily the cell periphery of upper cells in various stratified squamous epithelia including epidermis (Fig. 3, *a* and *b*), sweat gland ducts (Fig. 3 *d*), hair follicle (Fig. 3 *e*), esophageal epithelium (Fig. 4 *a*), corneal epithelium (Fig. 4 *b*), exocervical epithelium (Fig. 4 *c*), and cultured human epidermal colonies (Fig. 5). In most of the above-mentioned AE11-positive cells, we also noticed a variable degree of cytoplasmic staining (see below). However, only extremely weak to negligible staining was observed in kidney (Fig. 4 *d*), liver (Fig. 4 *e*), and in various

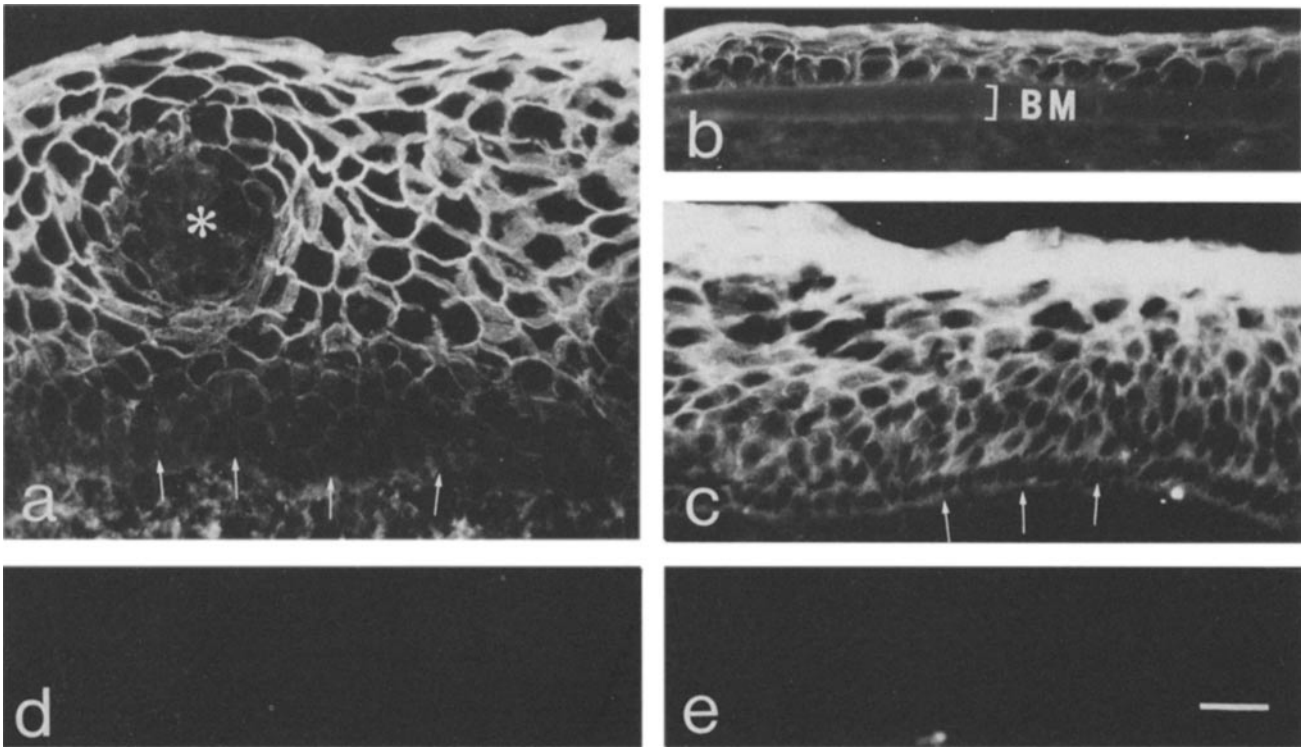

Figure 4. AE11-immunofluorescent staining of various human (nonepidermal) epithelial tissue. (*a*) Esophageal epithelium. Note the cell periphery staining of upper layers. Arrows denote the epithelial–stromal junction, and * represents the lumen of an esophageal duct coursing through the epithelium. (*b*) Corneal epithelium. *BM*, Bowman's membrane. (*c*) Exocervical epithelium. Arrows point to epithelial–stromal junction. (*d*) Liver. (*e*) Kidney. Bar, 20 μm.

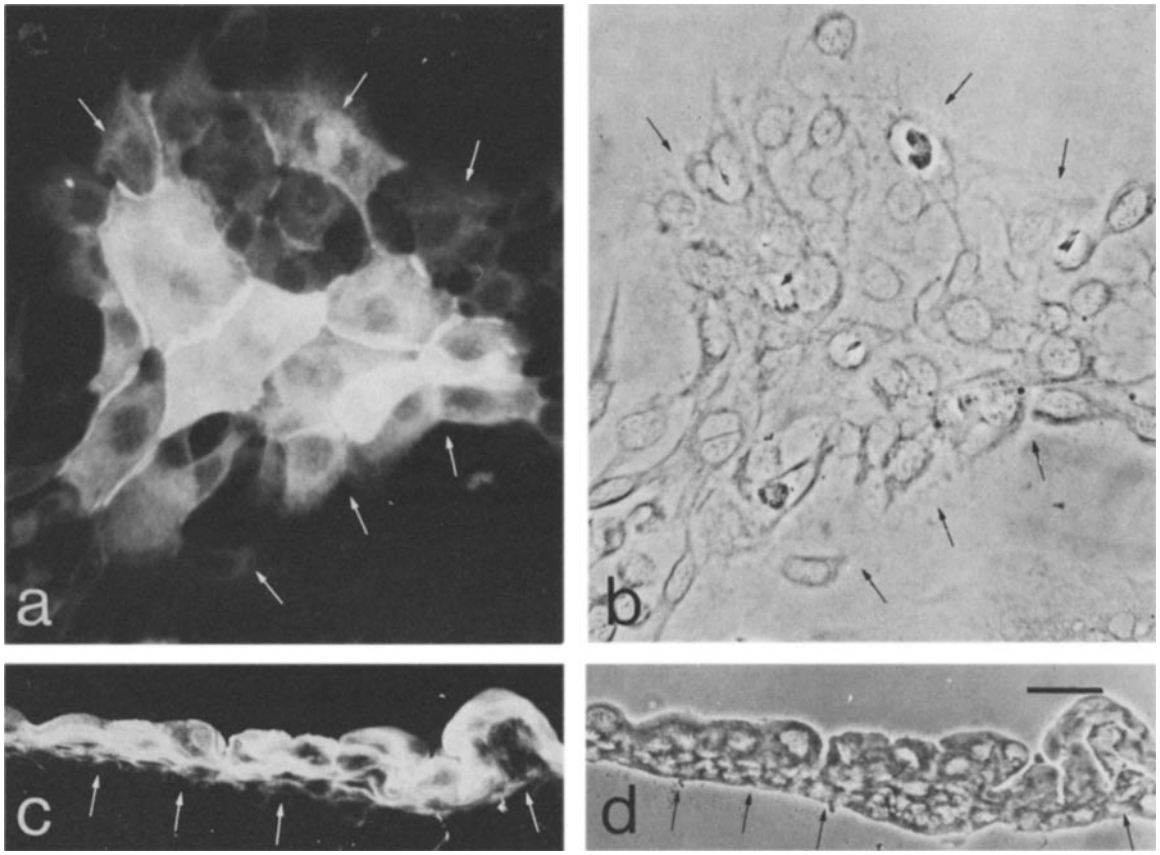

Figure 5. Immunofluorescent staining of cultured human epidermal colonies with AE11 antibody. (*a*) Top view. Human epidermal colonies were grown on glass coverslips, fixed with methanol, and stained with AE11. Arrows denote the edge of the colony. Note the uniform,

nonepithelial components (Figs. 3 and 4), confirming the predominantly keratinocyte-specific distribution of the 195-kD molecule.

An interesting feature of AE11-staining is that sometimes reaction products appear as punctate spots located at the cell periphery (Fig. 3 *c*). To determine whether such a staining pattern may be due to desmosomes, we stained (methanol-permealized) cultured human epidermal colonies with a rabbit antiserum against desmosomal proteins (kindly provided by Dr. Malcolm Steinberg of Princeton University; Fig. 6 *c*). A staining pattern clearly different from that produced by AE11 was obtained. While AE11 stains all upper, squamous cells in a more or less uniform fashion (Figs. 5 and 6 *a*) with occasional microvilli-like surface extensions (Fig. 6 *b*), anti-desmosome antibody stains intercellular punctates, some of which form rows presumably existing between the superficial and the underlying cells (Fig. 6 *c*). Thus, although we cannot rule out the possibility that some of the 195-kD protein may be present in desmosomes, it is certainly not limited to such sites.

The 195-kD Protein is a Submembranous Protein

To determine whether the 195-kD molecule is exposed on the cell surface, we stained living, cultured human epidermal keratinocytes with AE11. With the exception of weak, punctate staining in the cell–cell junctional zone of some cells, no specific staining was observed (data not shown). In another experiment, we found that the 195-kD antigen remains intact in trypsinized human epidermal cells that are viable (Fig. 7, lane *1*), but it can be readily degraded by trypsin in detergent-permeabilized cells (Fig. 7, lane *4*). Taken together, these results indicate that the 195-kD antigen is primarily an intracellular protein that becomes susceptible to AE11 staining (Fig. 5) and to proteolytic degradation only when the cellular membrane is disrupted.

The 195-kD Protein Is Not an Integral Membrane Protein, and Exists in Two Separate (Tris-soluble vs. -insoluble) Pools

To investigate the solubility property of the 195-kD protein, we serially extracted cultured human epidermal keratinocytes with Tris buffer (to release the "cytosolic" proteins), Triton X-100 ("membrane" proteins), and finally SDS ("insoluble" proteins). When these three fractions were heated in SDS sample buffer containing a reducing agent and then analyzed by immunoblotting using AE11, the 195-kD protein was detected in both the cytosolic and insoluble fractions, but not in the membrane fraction (Fig. 8, *a* and *a'*). That the Tris-extraction step is complete is established by the observation that the 195-kD antigen is not detectable in the Triton-fraction even in grossly overloaded (>5 times) gels. These results strongly suggest that the 195-kD protein is not an integral membrane protein, and that it exists in two separate pools as defined by their solubility in Tris-buffer.

The mechanism by which the 195-kD protein assumes different solubility characteristics (Tris-soluble vs. -insoluble) was investigated. Fig. 8 *b* shows the SDS gel electrophoretic patterns of the three keratinocyte fractions that have been heated in SDS sample buffer without a reducing agent. While the overall protein patterns of the nonreduced samples (Fig. 8 *b*) are very similar to those of the reduced samples (Fig. 8 *a*), individual bands of the former tend to be

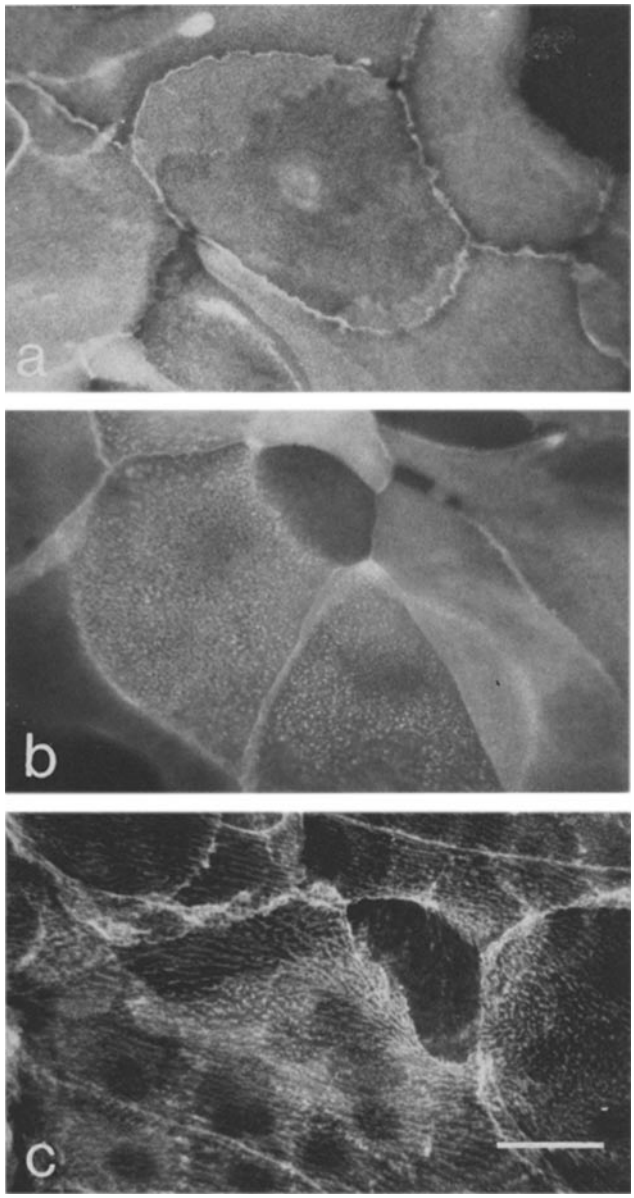

Figure 6. Comparison of the AE11 and antidesmosome antibody staining patterns of cultured human epidermal keratinocytes. Keratinocytes were MEOH-treated and stained with (*a* and *b*) AE11, or (*c*) antidesmosome. Note in *a* that the staining is somewhat concentrated at the cell periphery. Occasionally, the staining appears to be associated with microvilli (*b*), suggesting a close proximity of the AE11-antigen and cell surface structures. (*c*) Antidesmosome staining. Note strong, punctate staining at cell–cell junctions; many of the stained spots form "streaks" connecting the superficial squamous cells and the underlying cells. Bar, 20 μm.

strong AE11 staining of many superficial, squamous cells. (*b*) Phase contrast picture of the same field as in *a*. (*c*) Side view. A confluent sheet of epidermal keratinocytes was detached from the dish with dispase, embedded in OCT medium, sectioned vertically, and stained with AE11. Arrows denote the bottom of the colony originally in contact with the plastic surface. Note the preferential AE11-staining of superficial squamous cells. (*d*) Phase contrast picture of the same field as *c*. Bar, 20 μm.

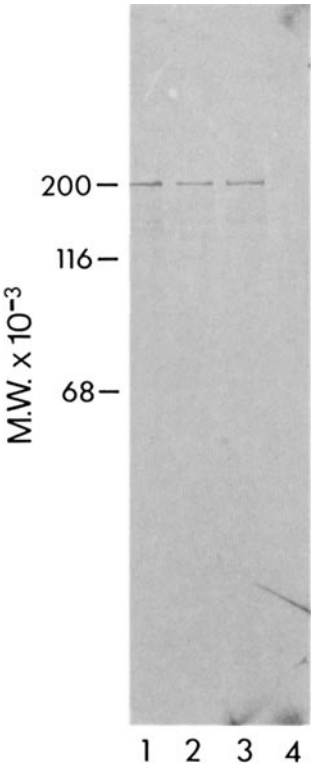

Figure 7. Trypsin sensitivity of the 195-kD antigen. Total proteins were extracted from cultured human epidermal keratinocytes after various treatments, and analyzed with AE11 by immunoblotting. Lane *1*, intact keratinocytes isolated by scraping with a rubber policeman. Lane *2*, viable keratinocytes isolated by trypsinization. The cells were then incubated as a single-cell suspension in serum-free medium at 37°C for 25 min to serve as a control. Lane *3*, same as in lane *2*, but cells were treated with 0.1% Triton X-100 before the 37°C incubation starts. Lane *4*, same as in lane *3*, except that the Triton-treated cells were digested with 0.125% trypsin in serum-free medium during incubation. Note that the 195-kD protein is present in lanes *1–3*, but not in lane *4*.

more diffuse. This diffusion is particularly pronounced in the region of the gel containing the 56-, 58-, and 59-kD keratins (Fig. 8 *b*, vertical bar next to lane *6*). Immunoblotting revealed a small but reproducible difference in the mobilities of the soluble and the insoluble 195-kD band, with the insoluble species displaying a slightly higher apparent molecular weight. Since this difference in electrophoretic mobility can be abolished by treating the samples with a reducing agent (Fig. 8 *a'*), it is probably related to the presence of a disulfide bond(s) in the nonreduced 195-kD molecule. In a preliminary experiment designed to test whether this disulfide bond may be directly responsible for the insolubility of a subpopulation of the 195-kD antigen, we extracted the Tris-insoluble fraction of cultured human epidermal keratinocytes with 50 mM Tris/HCl (pH 7.4) containing 5% β-mercaptoethanol. Immunoblotting results indicate that the insoluble fraction was not released. This negative finding does not exclude the possibility that disulfide bond formation may play a role in the insolubilization of the 195-kD antigen, however, since it is possible that disulfide bonds buried in the interior, hydrophobic domain of a protein may be protected from reducing agents unless the protein is first unfolded by a denaturant.

Solubility of 195-kD Protein in "Basal" vs. "Differentiated" Cells

To determine whether the solubility of the 195-kD molecule may be differentiation-related, we subjected confluent human epidermal cultures to an overnight treatment with a medium containing a low concentration of calcium (7). This allowed us to dislodge the suprabasal cell layers, leaving behind a monolayer highly enriched in undifferentiated, basal cells. The 195-kD molecules of such a basal culture were found to be highly soluble (Fig. 9, lanes *1* and *2*), as compared with the control culture (Fig. 9, lanes *3* and *4*).

Discussion

Using the AE11 monoclonal antibody, we have identified and characterized a 195-kD antigen in human epidermal keratinocytes. This antigen has a tissue distribution different from that of several other previously studied molecules of the same molecular weight range including myosin heavy chain (200 kD [13]), a 210-kD microtubule-associated protein (4), fibronectin (220 kD [19, 34]), alpha-spectrin (240 kD [14]), and ankyrin (200 kD [2]). It is also immunologically (Fig. 4) and electrophoretically distinguishable from desmoplakin I (230 kD) and II (205 kD) which are structural proteins of the desmosomes (6, 11).

The AE11-reactive 195-kD protein exists in two different pools in keratinocytes: a Tris-soluble and a Tris-insoluble one. The insoluble 195-kD protein is also distinguishable from the soluble form in that the former exhibits a slightly higher apparent molecular weight and is most likely disulfide-cross-linked. There are two possible mechanisms by which this disulfide bond can potentially affect the solubility of a protein. An intramolecular bond may "fix" the protein in a configuration that is less soluble. Preliminary experiments indicate that such a bond, if it exists, may not be readily accessible to a reducing agent without first denaturing the protein. Alternatively, an intermolecular bond may cross-link the protein, which in itself may be quite soluble, to another highly insoluble molecule. The latter possibility seems less likely, however, since the difference in the apparent molecular weights of the insoluble vs. soluble forms of the 195-kD antigen is only 1–2 kD, which would be surprisingly small to account for a molecule that can cause a significant shift in the solubility of a protein as large as 195-kD.

An obvious question with regard to the two pools of 195-kD antigen concerns their possible precursor–product relationship. Because our AE11 antibody does not work well in immunoprecipitation, we have not yet been able to perform the definitive pulse–chase experiment. However, existing evidence strongly suggests that the soluble pool may represent a precursor of the insoluble pool, and that the conversion from soluble to insoluble is related to keratinocyte-differentiation. First, human epidermal keratinocyte cultures enriched in relatively undifferentiated cells contain predominantly the soluble 195-kD protein, whereas highly differentiated cultures and in vivo epidermis contain mainly the insoluble form (Fig. 9). Second, immunohistochemical staining of frozen sections of normal human epidermis showed that the AE11-staining of the lower layers can be largely eliminated by prolonged washing of the tissue sections with an aqueous buffer. In contrast, cells of the upper layers exhibit strong cell–peripheral staining, in addition to some soluble, cytoplasmic AE11-reactivity. Significantly, this cell-peripheral staining of the differentiated cells is resistant to Tris-extraction and therefore probably corresponds to the Tris-insoluble pool of the 195-kD antigen.

Although it is quite clear that the expression of the 195-kD protein increases as the keratinocytes become more differentiated, immunohistochemical-staining data suggest that it may already be present in small quantities (both cytoplasmic and peripheral) in basal cells (Figs. 3 *a* and 4 *b* and *c*). The

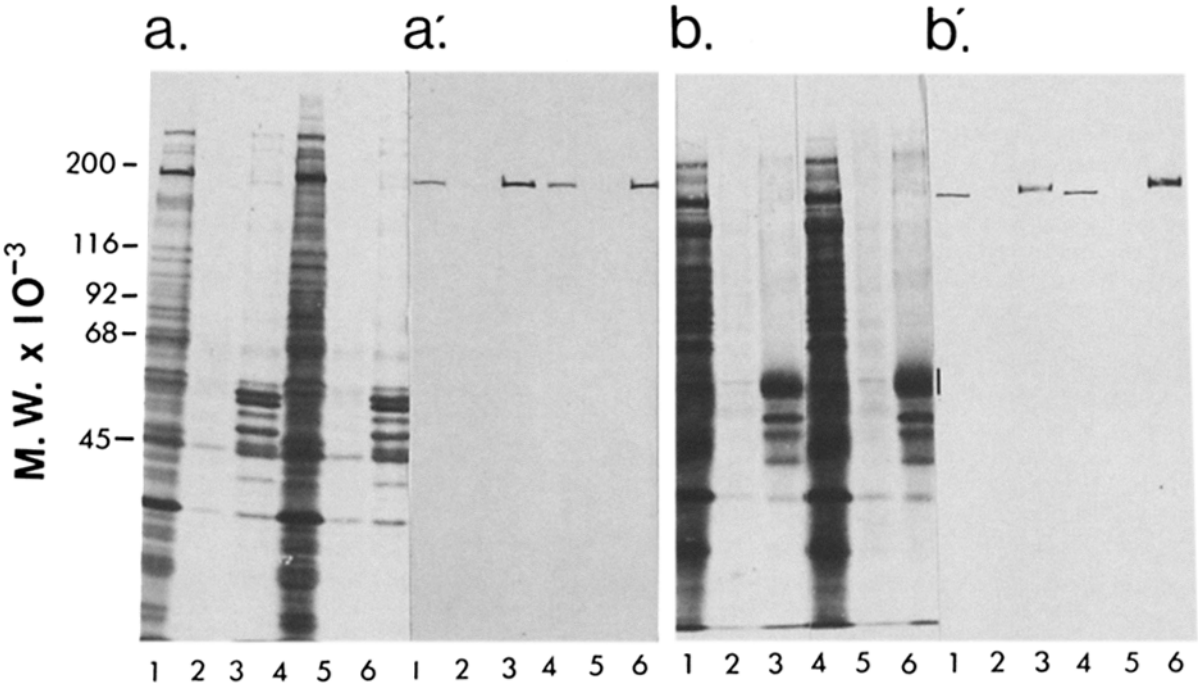

Figure 8. Solubility and electrophoretic mobility of the 195-kD protein. Human epidermal keratinocytes were serially extracted with equal volumes of 50 mM Tris-HCl (pH 7.4) (lanes *1* and *4*), 50 mM Tris plus 0.1% Triton X-100 (lanes *2* and *5*), and 50 mM Tris plus 9 M urea (lanes *3* and *6*). Equal proportions of the samples were then treated with SDS sample buffer with (*a* and *a'*) or without (*b* and *b'*) 1% B-mercaptoethanol, and analyzed by 12.5% polyacrylamide gel electrophoresis (acrylamide/bisacrylamide ratio: 120:1). (*a* and *b*) Fast green staining; (*a'* and *b'*) AE11 blotting patterns. Lanes *4–6* are slightly overloaded repeats of lanes *1–3*; they better illustrate the small difference in the mobilities of the soluble vs. insoluble forms of the 195-kD band. Note that the AE11-reactive 195-kD antigen is present in ~1:2 ratio in the Tris- vs. urea-fraction, but is not detectable in the Triton-fraction. Also note that under the gel electrophoresis conditions of *b* the urea-soluble 195-kD protein migrates slightly behind the Tris-soluble counterpart. The vertical bar next to lane *6* of *b* denotes the 56–59 kD keratin region.

observation that the 195-kD molecule is present in a Tris-soluble form in a low-calcium–induced monolayer culture is also consistent with its being present in basal cells (Fig. 9). This latter observation must be interpreted with caution, however, because monolayers prepared by the low-calcium medium most likely contain some differentiated cells in addition to the undifferentiated, true basal cells (32).

Based on the observations that the 195-kD antigen is not present in the Triton-fraction, and that it is protected from trypsin digestion in intact cells, we conclude that the antigen cannot be an integral membrane protein, and must be located intracellularly. These observations, coupled with the peripheral AE11-staining of the upper, differentiated cells, suggest that the insoluble form of the 195-kD antigen is located in the submembranous zone. The precise immunolocalization of the antigen at the electron microscopic level has not yet been achieved, because none of the four anti-195-kD antibodies (one conventional and three monoclonals) that we have tested so far worked satisfactorily on fixed tissues (Warhol, M., M. Simon, and A. Ma, unpublished observations).

Recently, Simon and Green described several membrane-associated proteins that are expressed late in keratinocyte differentiation, and showed that these proteins are substrates for transglutaminase-mediated cross-linking (21, 22). Our 195-kD antigen appears to be identical to one of these proteins in its molecular weight (195-kD), charge (pI 5.4), immunofluorescent staining pattern in epidermis, and immunoreactivity.[2] The solubility of this previously described 195-kD molecule seems to differ from ours, however, in that it was found only in the particulate membrane fraction during sucrose–density-flotation, whereas in our experiments a significant proportion of the 195-kD antigen is detected in a soluble pool (Figs. 8 and 9). The reason for this dis-

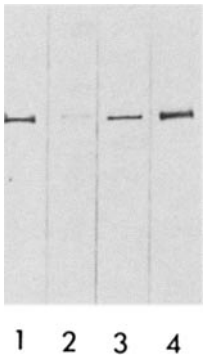

Figure 9. Solubility of the 195 kD protein in "basal" keratinocytes (lanes *1* and *2*) and in intact, stratified colonies (lanes *3* and *4*). Lanes *1* and *3* are Tris-soluble, and lanes *2* and *4* are urea-soluble proteins. Human epidermal keratinocytes were treated overnight with a medium containing a low calcium concentration (see Materials and Methods) in order to loosen the suprabasal cell layers, leaving a monolayer highly enriched in undifferentiated, basal cells (lanes *1* and *2*). Immunoblotting of equal proportions of samples with AE11 (similar to Fig. 8) shows that >70% of the 195-kD protein is soluble in basal keratinocytes (lanes *1* and *2*), but only <30% is soluble in intact, stratified colonies (lanes *3* and *4*).

2. Two-dimensional immunoblotting experiments have established that the same 195-kD protein was recognized by both our AE11 antibody and a mouse polyclonal antiserum previously made by Simon and Green (21) against a 195-kD transglutaminase substrate (Simon, M., and A. Ma, data not shown).

crepancy is not clear, although it could be related to different methods of detection. Immunoblotting with a monoclonal antibody as described here may be more sensitive an assay than surveying the protein spots by two-dimensional gel electrophoresis, as was used in the earlier study (21, 22). If this interpretation is correct, results accumulated to date may be incorporated into a unifying scheme in which the 195-kD protein may be first synthesized as a cytosolic component. In the submembranous zone, particularly that of the differentiated cells, some of this protein may become disulfide-cross-linked, perhaps enzymatically, and become Tris-insoluble. Finally, this membrane-associated 195-kD molecule is cross-linked through isopeptide-bonds, either among themselves or to other molecules such as involucrin, by transglutaminase which is known to be largely membrane-bound (8, 22, 28), and becomes covalently incorporated into the cornified envelope. More experiments are needed to test the validity and to elucidate the detailed mechanisms of this scheme.

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