

Suprabasal change and subsequent formation of disulfide-stabilized homo- and hetero-dimers of keratins during esophageal epithelial differentiation

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SUMMARY

Rabbit esophageal epithelium, a parakeratinized stratified epithelium, synthesizes as one of its major differentiation products a keratin pair consisting of a basic K4 (59 kDa) and an acidic K13 (41 kDa) keratin. Although immunohistochemical staining data suggest that in esophageal epithelia of some other species these two keratins are suprabasally located, antigenic masking of the epitopes in the basal cells has not been ruled out. Using several well-characterized monoclonal antibodies including AE8, which specifically recognizes K13, coupled with biochemical analysis of keratins of basal and suprabasal cells isolated from confluent rabbit esophageal epithelial culture, we have obtained direct evidence that K4 and K13 keratins are largely absent in the undifferentiated basal cells, but are present in large amounts in suprabasal cells. We also show that in the cornified cell layers that are formed during the terminal stage of esophageal epithelial differentiation, K4 and K13 keratins become disulfide-crosslinked to form three different dimers. Two of them (110 kDa and 100 kDa) are heterodimers and consist of equimolar amounts of K4 and K13; they presumably represent isomers crosslinked via different cysteine residues. The

third dimer (90 kDa) was found to be a homodimer of the acidic K13 keratin. Trypsinization experiment established that at least some of the disulfide crosslinks in the K4/K13 heterodimer must involve cysteine residues residing in the trypsin-resistant rod domains of keratins. Air-oxidation of *in vitro* reconstituted filaments reproduced the two heterodimers, which most likely involve the crosslinking between type I and type II keratins of different coiled coils. The formation of these disulfide-crosslinked keratin dimers, instead of higher molecular mass oligomers or polymers as occurring in the epidermis and hair, may contribute to the formation of cornified cells with a physical stability and rigidity that are optimal for esophageal function. Our data also suggest that interactions involved in the formation of homodimers, thought to be metastable and unimportant during the initial step of filament assembly (i.e. tetramer formation), may actually play an important role in stabilizing a higher order structure in mature keratin filaments.

Key words: keratin, esophageal epithelium, differentiation

INTRODUCTION

Keratin is a group of about 30 cytoskeletal proteins that form intermediate filaments in almost all epithelial cells (Moll et al., 1982; Lynch et al., 1986; Heid et al., 1986). Detailed studies of keratin expression in epidermis both *in vivo* and in cultured cells showed previously that the basal cells of this stratified squamous epithelium synthesize a basic keratin K5 and an acidic keratin K14 (forming a keratin "pair"; Eichner et al., 1984; Sun et al., 1984), while the suprabasal cells synthesize mainly K1 and K10 keratins (Woodcock-Mitchell et al., 1982; Skerrow and Skerrow, 1983; Schweizer et al., 1984; Stoler et al., 1988). During wound healing, cell culture and other conditions which

somehow suppress the expression of K1 and K10 keratins, the suprabasal cells synthesize instead K6 and K16 (and/or K17) keratins (Sun and Green, 1978; Fuchs and Green, 1978; Stoler et al., 1988). Similar studies on corneal epithelium showed that, as in the epidermis, its basal cells make K5/K14 keratins, and that under nonpermissive conditions its suprabasal cells synthesize K6/K16. However, under permissive conditions the more differentiated corneal epithelial cells make K3/K12 (rather than the epidermal K1/K10; Schermer et al., 1986, 1989). Fewer studies were conducted, however, on the *in vivo* and *in vitro* differentiation-related changes in keratin expression of esophageal epithelium - a third important keratinocyte prototype - which is "parakeratinized" in rabbit, human and bovine

(Doran et al., 1980; Tseng et al., 1984). This epithelium expresses as its main differentiation products another keratin pair K4/K13 (Moll et al., 1982; Sun et al., 1984). Immunohistochemical staining data generated using monoclonal antibodies specific for these keratins have previously shown that these two keratins are mainly detected in the suprabasal cell layers of esophageal epithelium (Van Muijen et al., 1986). A similar suprabasal expression pattern was observed from *in situ* hybridization experiments (Knapp et al., 1986; Rentrop et al., 1986; cf., Leub et al., 1988). Although these data imply that K4/K13 keratin proteins are localized mainly suprabasally, immunohistochemical staining results can be complicated by false negatives due to antigenic masking (Woodcock-Mitchell et al., 1982; Franke et al., 1983). *In situ* hybridization generates more reliable RNA localization data, which, however, do not always correspond to the protein data. Therefore, it remains to be established on a protein level that K4 and K13 keratins are indeed localized specifically in the suprabasal compartment.

An important feature of keratinocytes is that they undergo terminal differentiation, forming superficial squamous cells containing disulfide-crosslinked keratins. Although it is well known that such crosslinking serves to improve the physical stability of the keratin network, virtually nothing is known about the biochemical details of this process. For example, it is unclear whether the crosslinking occurs between the acidic and basic keratin members of a pair, thus forming a stabilized heterodimer (or oligomers), or between keratins of the same kind forming homodimers (or oligomers). It is difficult to address this kind of question by studying the skin differentiation-related keratin pair K1/K10 or the hair differentiation-related keratin pairs, because our preliminary experiments indicate that under conditions favoring disulfide bond formation these keratins rapidly form crosslinked products far beyond the dimer stage, making them extremely difficult to analyze (Sun and Green, 1978; Pang and Sun, unpublished observation). In this regard, esophageal epithelial keratins provide an optimal model system for tackling this particular problem.

In this paper, we describe: (i) the characterization of keratins synthesized by rabbit esophageal epithelium both *in vivo* and in culture by one- and two-dimensional immunoblotting; (ii) a direct, biochemical analysis of keratins associated with the basal and suprabasal compartments of rabbit esophageal epithelial culture; and finally (iii) the characterization of two heterodimer (K4/K13) and one homodimer (K13-K13) that become disulfide-crosslinked during an advanced stage of *in vivo* esophageal epithelial differentiation. These results provide strong evidence that K4 and K13 keratins are associated mainly with the suprabasal compartment of esophageal epithelium, and clearly indicate that the formation of disulfide-crosslinked homo- and heterodimers of K4 and K13 keratins is an important step during an advanced stage of *in vivo* esophageal epithelial differentiation.

MATERIALS AND METHODS

Extraction of keratins from epithelial tissues

Water-insoluble extracts from a variety of human, bovine and

rabbit epithelial tissues and cultures were prepared. Human tissues, except corneas, were acquired from autopsy; bovine tissues from freshly slaughtered cows; and rabbit tissues from freshly killed adult New Zealand White (NZW) rabbits. The human corneas were excised from eyes kindly provided by New York Eye Bank. *In vivo* epithelial tissues were harvested by (i) scraping either at room temperature (cornea, esophagus, intestine, bladder) or over solid CO₂ (epidermis) or (ii) incubating the tissues in 2.5 mg/ml dispase (Boehringer Mannheim) in Dulbecco's Modified Eagle's Medium (DMEM) for 16 h at 4°C to separate the epithelium from its underlying mesenchymal tissue. Confluent cell cultures of rabbit corneal epithelium, rabbit epidermis, rabbit esophageal epithelium, human epidermis, human mesothelium, cow snout epithelium and MDBK cells were rinsed with phosphate-buffered saline (PBS) and harvested with a plastic scraper (Costar).

All tissues were immediately transferred to cold 25 mM Tris-HCl (pH 7.4), 0.6 M KCl, 1% Triton X-100, plus a mixture of five protease inhibitors to remove the water-soluble proteins (Woodcock-Mitchell et al., 1982; Eichner et al., 1984, 1986). The remaining cytoskeletal preparation containing mainly keratins ("native" intermediate filament) was extracted with 9.5 M urea, 10 mM Tris-HCl (pH 7.4) and stored frozen at -70°C.

In studies of *in vivo* disulfide-crosslinking, a mid-esophagus was isolated from a freshly killed NZW rabbit and its epithelium was scraped off and extracted as above in the presence or absence of 10 mM *N*-ethylmaleimide (NEM). The remaining cytoskeletal preparation was then solubilized directly with either 9.5 M urea or 1% SDS buffer.

Antibodies to keratins

AE8 is a mouse monoclonal antibody raised against SDS-denatured rabbit esophageal keratin extract, and was selected for its specificity to acidic K13 keratin (see Results). AE1, AE3, AE14 and aIF monoclonal antibodies were prepared as described previously. AE1 recognizes acidic K10, K11, K14, K15, K16 and K19 keratins (Sun et al., 1984; Cooper et al., 1985); AE3 recognizes all known basic keratins (Sun et al., 1984; Cooper et al., 1985); AE14 recognizes the basic K5 keratin characteristic of basal cells of almost all stratified squamous epithelia (Lynch et al., 1986); and a-IFA recognizes most intermediate filament proteins (Pruss et al., 1981; Cooper et al., 1985).

Cell culture

Cultures of human epidermal cells, rabbit epidermal cells and rabbit esophageal epithelial cells were grown in the presence of lethally irradiated 3T3 feeder cells in DMEM containing 17% fetal bovine serum, 0.5 µg/ml hydrocortisone and 15 ng/ml epidermal growth factor. Cell cultures were maintained at 37°C in a humidified atmosphere of 5% CO₂ in air (Rheinwald and Green, 1975, 1977). MDBK cells (a bovine kidney-derived simple epithelial cell line) were obtained from the American Type Culture Collection (ATCC CCL22) and were grown in DMEM containing 10% calf serum.

Immunohistochemical staining

Tissues were embedded in OCT medium, and snap frozen in isopentane near its freezing point. Frozen sections (6-8 µm) of rabbit esophageal epithelium were stained by indirect immunofluorescence as described (O'Guin et al., 1985).

Isolation of basal and suprabasal cells from stratified keratinocyte cell cultures

Postconfluent, stratified cultures of rabbit esophageal and corneal epithelial cells were treated with 2 mM EGTA in calcium-free DMEM (MA Bioproducts) containing 17% fetal calf serum for 5

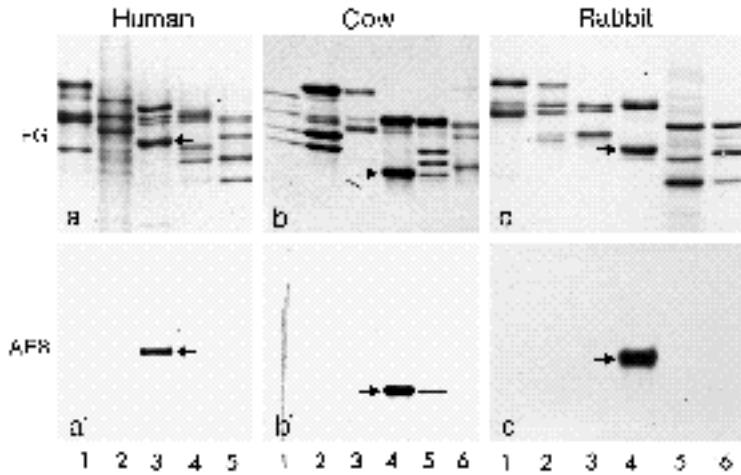


Fig. 1. Keratin chain specificity of AE8 monoclonal antibody. Water-insoluble cytoskeletal proteins were isolated from human (a and a'), cow (b and b') and rabbit (c and c') epithelial tissues, and analyzed by SDS-PAGE. The protein bands were transferred electrophoretically to nitrocellulose paper and stained with (a-c) Fast Green (FG). The same samples were also stained immunohistochemically with AE8 antibody by the peroxidase-antiperoxidase (PAP) technique (a-c). Human keratins were from (lane 1) epidermis, (2) cornea, (3) esophagus, (4) cultured epidermal cells and (5) cultured mesothelial cells. Bovine keratins were from (lane 1) epidermis, (2) snout, (3) cornea, (4) esophagus, (5) cultured esophageal keratinocytes and (6) cultured MDBK cells. Rabbit keratins were from (lane 1) cornea, (2) epidermis, (3) cultured epidermal cells, (4) esophagus, (5) bladder and (6) intestine. Note that AE8 detected a 51 kDa esophageal keratin in human, a 41 kDa esophageal keratin in cow,

and a 46 kDa esophageal keratin in rabbit (arrows). Although these three keratins differ significantly in size, they all represent the predominant acidic keratin in normal esophageal epithelium, they all diminish in cultured cells and are thus differentiation-dependent, and they all share an AE8 epitope; these results strongly suggest that they are all K13-equivalents. For two-dimensional gel keratin maps of rabbit and bovine keratins see Schermer et al. (1986) and Cooper and Sun (1986).

hours at 37°C. This was followed by vigorous pipetting of the supernatant medium over the colonies. Cells that dislodged into the supernatant were washed with PBS, collected by centrifugation and processed for SDS-PAGE (Schermer et al., 1986).

Localization of free sulfhydryl and disulfide bonds

Frozen sections (7 μm thick) of rabbit esophageal epithelium from a freshly killed animal were stained with DACM (N-[7-dimethyl-amino-4-methyl-3-coumarin]-maleimide), which is specific for SH groups (Ogawa et al., 1979). A Zeiss fluorescence microscope equipped with an ultraviolet filter was used to detect the fluorescence of DACM.

Isolation of keratins from different layers of esophageal epithelium

Rabbit esophageal epithelium from a freshly sacrificed animal was frozen and horizontal sections of 12 μm were cut (Fuchs and Green, 1978). Total proteins were extracted immediately from the sections with sample buffer containing 1% SDS with or without 1% beta-mercaptoethanol. Every third section was loaded on three separate gels and proteins were separated by SDS-PAGE.

Air-oxidation of reconstituted keratin filaments

Keratin extracts were incubated in 50 mM Tris-HCl (pH 8.5), 5 mM dithiothreitol, and 9.5 M urea for 30 min at 37°C, centrifuged

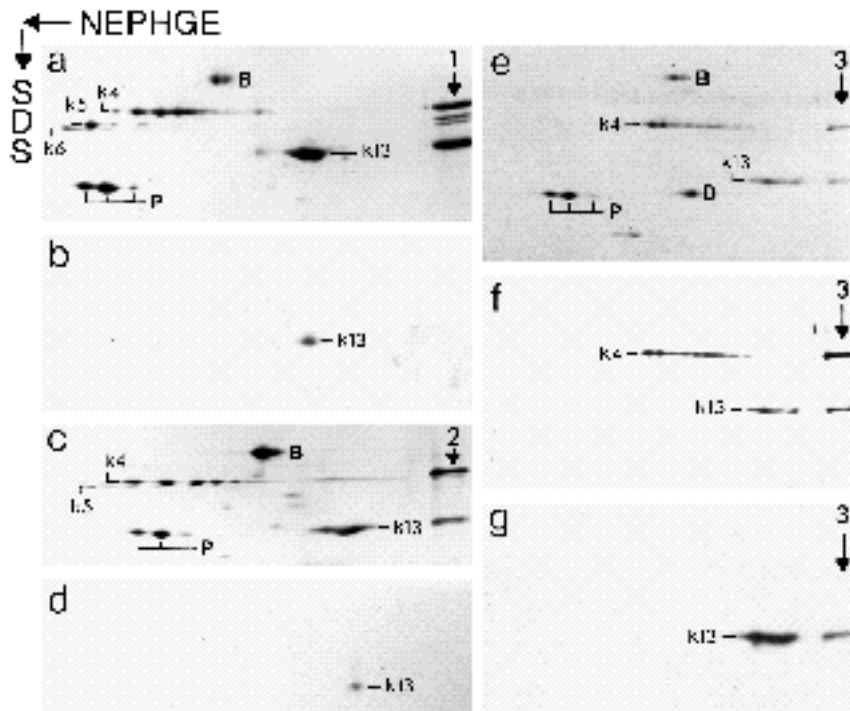


Fig. 2. Identification of the AE8-reactive keratin chain as K13 by two-dimensional immunoblot analysis. Esophageal keratins from human (a, b), cow (c, d) and rabbit (e, f, g) were separated by two-dimensional gel electrophoresis, and the proteins were transferred to nitrocellulose paper and stained with Fast Green (a, c, e) or AE8 (b, d, g). (f) Shows the reaction of rabbit esophageal keratins with MaK, a mixture of AE1, AE3, AE8 and aIF, which in combination recognized all keratins. The side lanes show the one-dimensional SDS-PAGE pattern of (1) human, (2) cow and (3) rabbit esophageal keratins; these are included to facilitate the correlation between one- and two-dimensional patterns. B, P and D mark the positions of bovine serum albumin, 3-phosphoglycerate kinase and DNase, respectively. Arrows marked with NEPHGE and SDS denote the directions of the first- and second-dimensional separation, respectively. Note that AE8 specifically detected K13 in all three species.

for 10 min at 50,000 *g*, and the supernatants dialyzed against 10 mM Tris-HCl (pH 7.5) for 16 h at 4°C (Steinert et al., 1976; Sun and Green, 1978; Aebi et al., 1983; Hatzfeld and Franke, 1985; Eichner et al., 1986).

Partial trypsin digestion of reconstituted keratin filaments

Air-oxidized, reconstituted keratin filaments were digested with 0.001 to 0.01 mg/ml of trypsin (Sigma) in 10 mM Tris-HCl (pH 8.7) at room temperature for 30 min. The reaction was stopped by adding excess amount of trypsin inhibitor (Sigma).

RESULTS

AE8 monoclonal antibody recognizes K13

To facilitate the analysis of esophageal keratins, we immunized Balb/c mice with rabbit esophageal keratins, generated hybridomas, and screened by Elisa for antibodies that reacted with esophageal keratins without cross-reacting with human epidermal keratins. A panel of such esophageal-specific antibodies were cloned and characterized, and the properties of one of them, AE8, is described here because of its superior stability, broad species reactivity and immunostaining properties.

To establish the keratin chain-specificity of this antibody, we used it to perform one- and two-dimensional immunoblots against the keratins of a selected panel of human, cow and rabbit epithelia. These studies indicated that AE8 recognized the major acidic keratin of esophageal epithelia of all three animal species (Fig. 1). The tissue-distribution and two-dimensional gel electrophoretic positions of this keratin, which had an apparent molecular mass of 51 kDa in man, 41 kDa in cow, and 46 kDa in rabbit, clearly established that it was keratin 13 (Fig. 2).

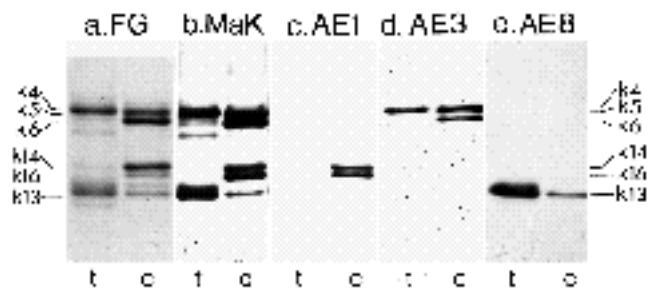


Fig. 3. Modulation of rabbit esophageal epithelial synthesis of keratins (*in vivo* versus in culture). Water-insoluble cytoskeletal preparations were prepared from normal rabbit esophageal epithelial tissue (t) and confluent esophageal epithelial culture (c), separated by SDS-PAGE, and transferred onto nitrocellulose. Samples were then stained with: (a) Fast Green to visualize all proteins; (b) MaK, a mixture of AE1, AE3, AE8 and aIF monoclonal antibodies to visualize all keratins; (c) AE1 to visualize acidic keratins including K14 and K16; (d) AE3 to visualize all basic keratins including K4, K5 and K6; and (e) AE8 to visualize K13. Note that cultured cells contain greatly increased amounts of K5/K14, K6/K16, and a decreased but still significant amount of K13.

Keratin pattern of esophageal epithelium: *in vivo* versus in culture

Although it is well known that keratins of cultured epidermal keratinocyte differ from their *in vivo* counterparts (Fuchs and Green, 1978; Sun and Green, 1978; Eichner et

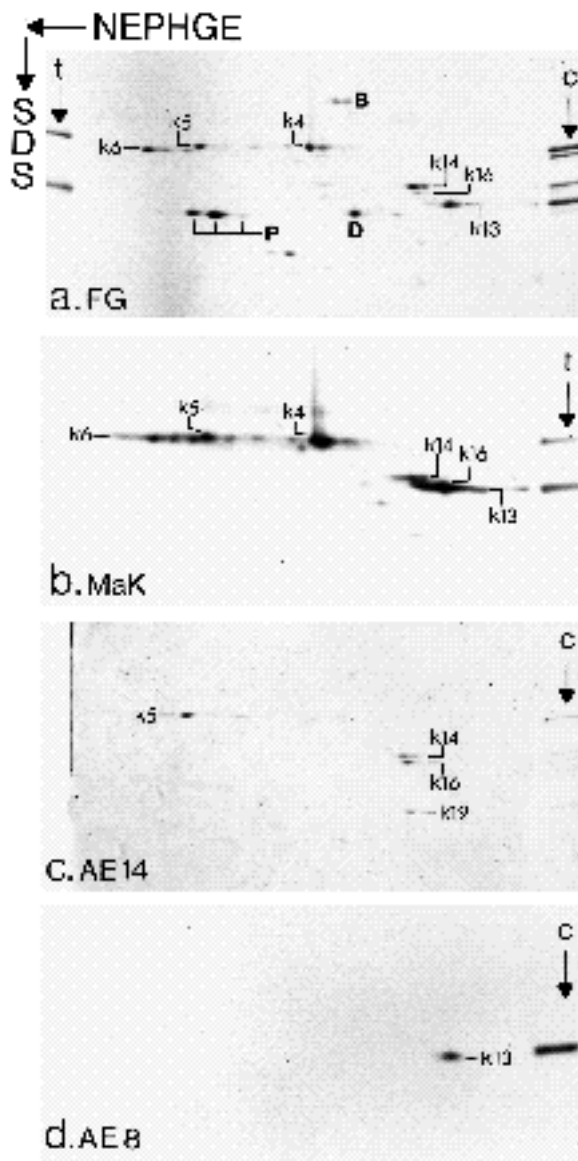


Fig. 4. Keratins of cultured rabbit esophageal keratinocytes as analyzed by two-dimensional immunoblotting. Water-insoluble cytoskeletal proteins were prepared from a confluent culture of rabbit esophageal epithelial cells, separated by two-dimensional gel electrophoresis, and transferred onto nitrocellulose. Identical samples were stained with: (a) Fast Green, (b) MaK, (c) AE14, and (d) AE8. Side lanes show the SDS-PAGE pattern of keratin of cultured cells (marked c) or *in vivo* tissue (t). The two arrows on the upper left corner marked with NEPHGE and SDS denote the directions of the first- and second-dimensional separations, respectively. B, P and D are protein markers (see Fig. 2). Note that although rabbit K4 has the same molecular mass as K5, they are immunologically distinct as only the latter reacted with AE14.

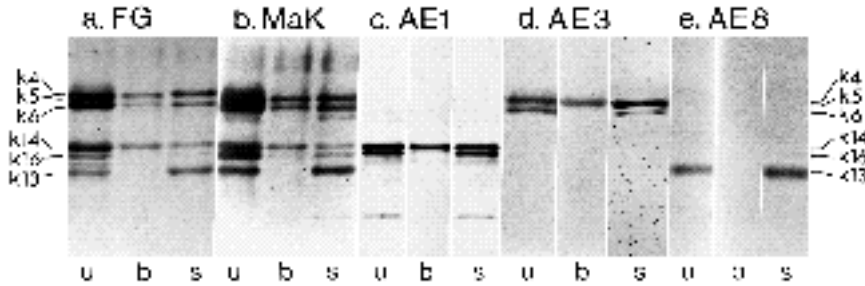


Fig. 5. Keratins of the basal (b) and suprabasal (s) compartments of a rabbit esophageal keratinocyte culture. A confluent, heavily stratified culture of rabbit esophageal epithelial cells was separated into basal and suprabasal compartments (see Materials and Methods). Water-insoluble cytoskeletal proteins from such isolated basal cells (marked b), suprabasal cells (s), and intact unfractionated culture (u) were separated by SDS-PAGE, and transferred

to nitrocellulose. Identical samples were then stained with: (a) Fast Green, (b) MaK, (c) AE1, (d) AE3 and (e) AE8. Note that basal (b) cells contain predominantly K5/K14, whereas suprabasal (s) cells contain in addition K13 and K6/K16. Although not evident from this figure, suprabasal cells also contain large amounts of K4 (see Fig. 6).

al., 1984), the difference between the keratin pattern of cultured rabbit esophageal epithelial cells and that of *in vivo* rabbit esophageal epithelium is even more striking in that the former contained large amounts of K5/K14 “basal” keratins, K6/K16 “hyperproliferation” keratins, plus the K4/K13 differentiation type keratins, while the latter contained almost exclusively K4/K13 (Figs 3 and 4). Such a difference could be explained by several considerations: (i) the *in vivo* esophageal epithelium (with >30 cell layers) is much thicker than the cultured epithelial colonies (only <5-6 layers); hence the basal K5/K14 keratins contribute quantitatively much less to the overall *in vivo* keratin pattern. (ii) Even though under normal conditions K5/K14 synthesis is turned off in the suprabasal layers, under hyperproliferative conditions this synthesis may continue even in the suprabasal compartment (Stoler et al., 1988; Leube et al., 1988). (iii) Keratinocytes cultured under standard, submerged conditions tend to synthesize relatively little differ-

entiation-related keratins (in this case the K4/K13 keratins), and to synthesize an elevated level of the so-called hyperproliferation-related K6/K16 keratins (Weiss et al., 1984; Cooper et al., 1985; Sun et al., 1985).

Direct analysis of keratins in the basal versus suprabasal compartments

Direct analysis of the keratin proteins in different compartments of a stratified epithelium requires cell fractionation. Although we have not been able to adequately separate the basal vs suprabasal cells of normal rabbit esophageal epithelium, we succeeded in separating these two cell populations from cultured rabbit esophageal epithelial cells using an EGTA-protocol. In this protocol (which we originally developed to separate the basal and suprabasal cells of rabbit corneal epithelial culture; Schermer et al., 1986), confluent cultures of heavily stratified esophageal epithelial cells were treated with a solution of EGTA, which

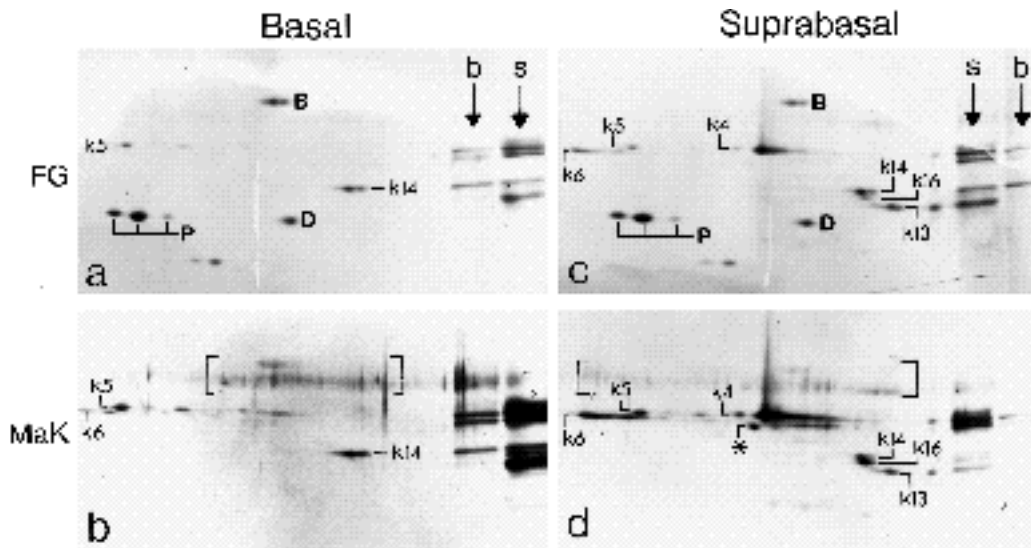


Fig. 6. Keratins synthesized by basal (a,b) and suprabasal (c,d) cells of a stratified rabbit esophageal keratinocyte culture as analyzed by two-dimensional immunoblotting. Cytoskeletal proteins were separated by two-dimensional gel electrophoresis, transferred to nitrocellulose, and stained with (a,c) Fast Green, and (b,d) MaK. Asterisk denotes complex formation between some acidic and basic keratins. Brackets mark a small amount of human K1 originating from human stratum corneum contamination. Side lanes contain keratins from basal (b) or suprabasal (s) compartments, separated by one-dimensional SDS-PAGE. B, P and D are protein markers (see Fig. 2). Note that basal cells contain only K5/K14, and that suprabasal cells contain, in addition, K4/K13 and K6/K16.

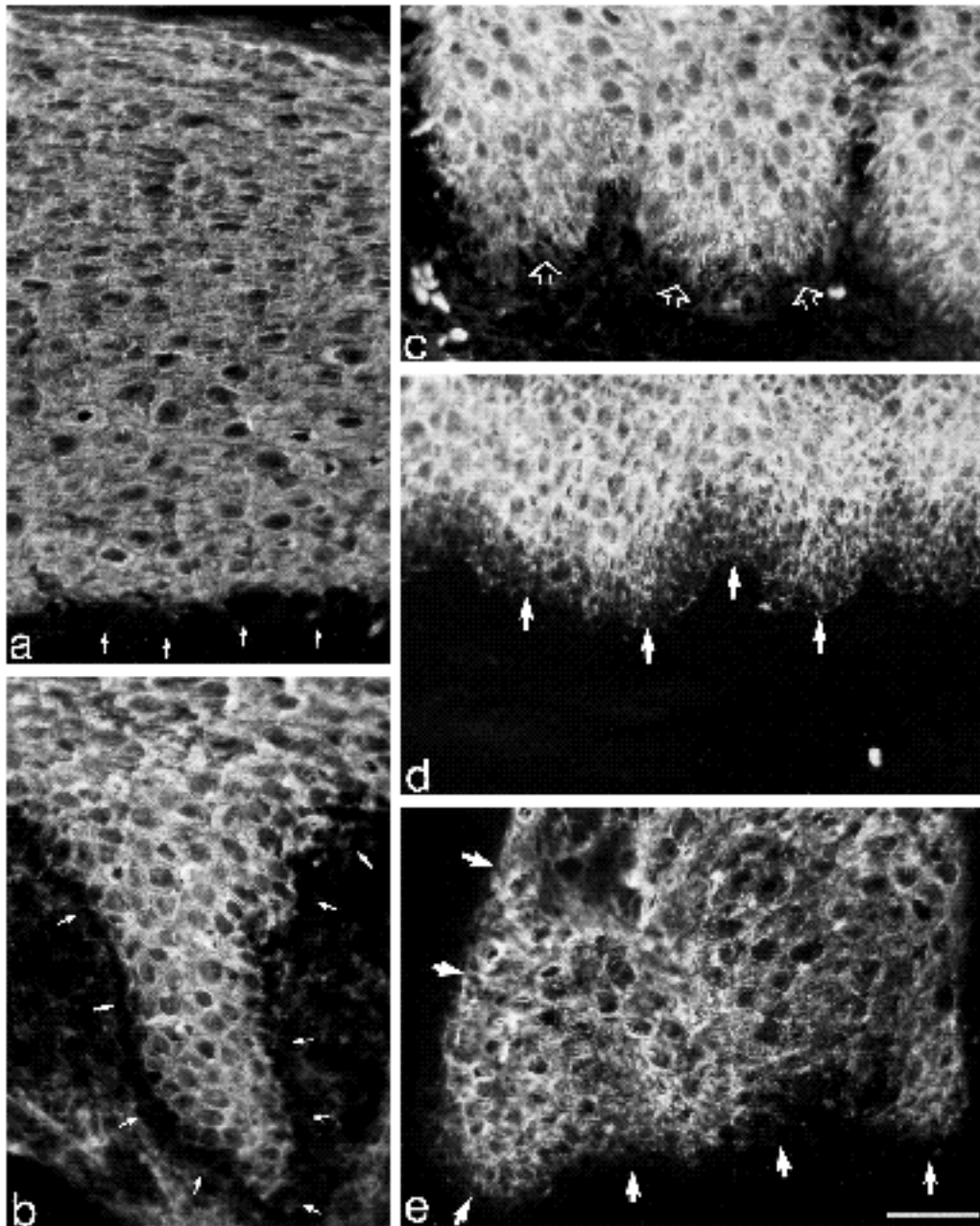


Fig. 7. Immunolocalization of K13 *in vivo*. AE8 antibody was used to stain by indirect immunofluorescence the frozen sections of (a) human esophageal epithelium, (b) human dorsal tongue epithelium, (c) cow esophageal epithelium, (d) rabbit esophageal epithelium, and (e) rabbit dorsal tongue epithelium. Arrows demarcate the basement membrane. Note in (d) the weak but discernibly filamentous AE8 staining in the basal cells of rabbit esophageal epithelium, and in (e) the strong staining of basal cells at the shoulder of rabbit dorsal tongue epithelium. Bar 50 μ m.

resulted in the selective detachment of all suprabasal cells (Schermer et al., 1986). We then analyzed the keratins of the basal (lanes labeled b in Fig. 5) and suprabasal (s) cells, compared with those of an unfractionated cell population (u), by one- and two-dimensional immunoblotting (Figs 5 and 6). The results indicated that the isolated basal cells contained mainly K5/K14 (Figs 5 and 6); there was also a small amount of a 55 kDa band (Fig. 5a, lane b), which was AE3-negative and most likely represented vimentin of the contaminating feeder/fibroblasts. The suprabasal cells

contained the same basal K5/K14 keratins, but in addition they contained K6/K16 and K4/K13 keratins (Figs 5 and 6).

Consistent with its K13 specificity, AE8 stained positively the frozen sections of all human, bovine and rabbit epithelial tissues known to contain K13 (Fig. 7 and data not shown). In esophageal epithelium it stained strongly all the upper cell layers (Fig. 7d). The basal cells showed only weak but discernibly filamentous staining (Fig. 7d). The staining pattern of rabbit dorsal

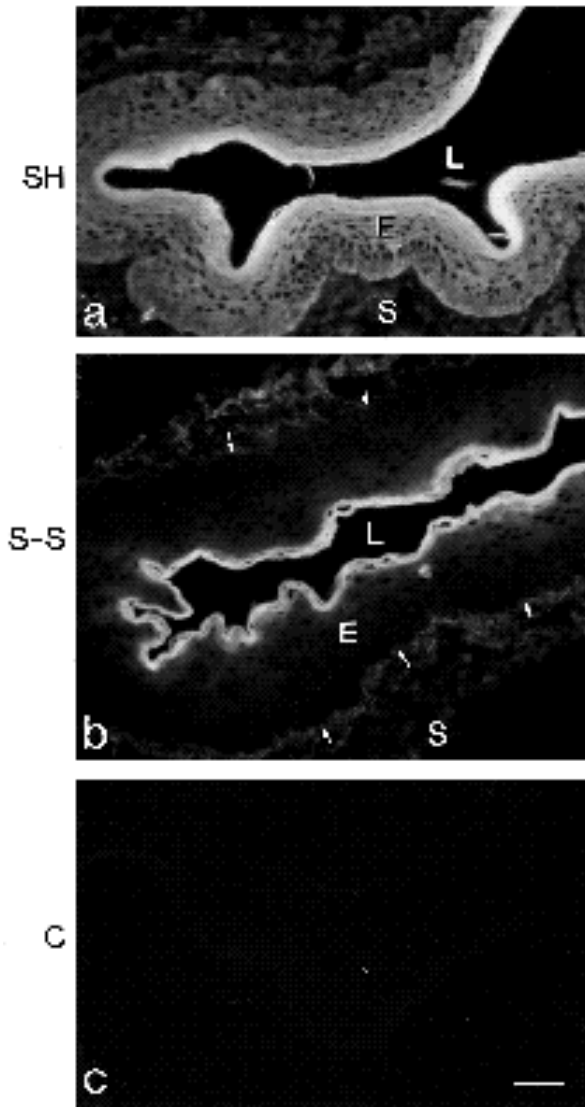


Fig. 8. Formation of disulfide bonds in the cornified layers of rabbit esophageal epithelium. (a) Localization of sulfhydryl group. A frozen section of mid-esophagus from a freshly killed rabbit was stained with DACM, which binds to free sulfhydryl group (see Materials and Methods). Note the presence of DACM fluorescence throughout the epithelium. (b) Localization of the disulfide group. A similar frozen section was first treated with *N*-ethylmaleimide to block all the free sulfhydryl groups, followed by the reduction of pre-existing disulfide bonds. The SH groups newly "released" from cystine were then stained with DACM. Note the selective staining of cornified cells. (c) A control section which had been treated as in (b) except that the "released" free sulfhydryl groups were blocked by NEM before DACM staining. L, E and S denote lumen, epithelium and submucosa, respectively. Arrows mark the basement membrane. Bar, 200 μm .

tongue epithelium is interesting in that, although the bottom of deep rete ridges showed "suprabasal" staining, the shoulders of these rete ridges exhibited almost "uniform" staining (basal cell also stained positively; Fig. 7e). Taken together, these *in vivo* and cultured cell results provided strong support to the notion that in

esophageal epithelium K13 was present in relatively small amounts in basal cells, but its level increased significantly in suprabasal cell layers (Van Muijen et al., 1986; Knapp et al., 1986; see Discussion).

Disulfide-crosslinking of esophageal keratins

As mentioned earlier, esophageal epithelial keratins are uniquely suitable for studying disulfide bond-mediated crosslinking of keratins. Using a histochemical staining agent, DACM (Ogawa et al., 1979), we showed that although the entire thickness of esophageal epithelium contained free sulfhydryl group (Fig. 8a), only the superficially located, terminally differentiated squamous cells were disulfide-rich (Fig. 8b). To determine whether this increase in disulfide content parallels the covalent crosslinking of keratins, we horizontally sectioned rabbit esophageal epithelium, extracted their total proteins with 1% SDS, and analyzed these proteins by SDS-PAGE (with or without mercaptoethanol; Fig. 9). The keratins in these fractions were then visualized using a mixture of several mouse monoclonal anti-keratin antibodies (Fig. 9b). Most of the viable esophageal epithelial cell layers contained, as expected, the K4 and K13 monomers. However, the superficial cell layers contained three additional high molecular mass (110 kDa, 100 kDa and a weak 90 kDa) keratin bands which were sensitive to reduction (Fig. 9c). Although in these fractions the crosslinked bands accounted for only 20-30% of the total keratin, the true value must be significantly higher, since these superficial cell fractions were heavily contaminated by lower viable cell layers (see Discussion).

We determined the composition of these crosslinked keratins by immunoblotting using two monoclonal antibodies - the AE3 antibody, which has been shown previously to react with all known basic keratins including the major esophageal basic keratin K4 (Sun et al., 1984; Lynch et al., 1986), and AE8 antibody, which (as shown in Figs 1 and 2) recognizes K13. The results indicated that the 110 kDa and 100 kDa bands (labelled I and II, respectively, in Figs 9 and 10) were heterodimers of the 59 kDa K4 and the 41 kDa K13, while the 90 kDa band (III) was a homodimer of K13. Additional analyses of these samples by two-dimensional "diagonal" gel electrophoresis (Hatzfeld and Weber, 1990), in which the sample was reduced after the first-dimensional separation, established clearly that bands I and II were indeed heterodimers made up of about equimolar amounts of K4 and K13, whereas the 90 kDa band III was a homodimer of K13 (Fig. 11).

To rule out the possibility that these crosslinked bands were formed artifactually during the extraction procedure, we solubilized total proteins from intact rabbit esophageal epithelium under a number of conditions including 9.5 M urea or by heating the tissue directly in 1% SDS (Fig. 9b, lane 4). We also extracted the tissue in the presence of 10 mM *N*-ethylmaleimide in order to block free sulfhydryl groups that are known to be required for disulfide exchange to occur (Fig. 9b, lane 4). Similar results were obtained under all these conditions, suggesting that the three high molecular mass bands as shown in Figs 10 and 11 must represent genuine crosslinked keratin species existing *in vivo*.

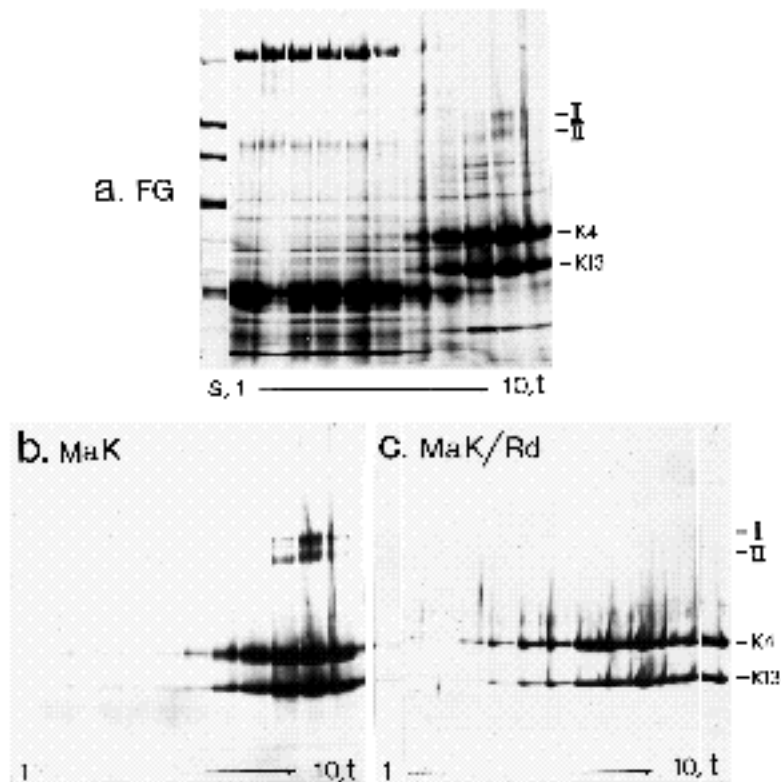


Fig. 9. Formation of disulfide-crosslinked keratin species during *in vivo* esophageal epithelial differentiation. A piece of mid-esophagus tissue was obtained from a freshly killed rabbit, snap-frozen, and embedded in OCT medium. Consecutive frozen sections (12 μ m thick) were cut at a plane parallel to the luminal surface. Proteins were extracted with 1% SDS, separated by SDS-PAGE, and transferred to nitrocellulose. (a) Fast Green staining to visualize total proteins. Lane S contains molecular mass markers including carbonic anhydrase (29 kDa), egg ovalbumin (45 kDa), bovine serum albumin (66 kDa), rabbit muscle phosphorylase B (97.4 kDa), galactosidase (116 kDa) and rabbit muscle myosin (205 kDa). Lane t shows total proteins of intact esophageal epithelium. Lanes 1-10 are samples from different depths, fraction 1 being the deep connective tissue and 10 the apical esophageal epithelial layer. (b) Immunochemical staining using MaK to visualize all esophageal keratins. Note the appearance of two high molecular mass keratins (I and II) in the most superficial sections of esophageal epithelium. (c) Same as (b) except the samples were reduced with 1% beta-mercaptoethanol before electrophoresis. Note the disappearance of bands I and II, indicating that they are disulfide-crosslinked.

Formation of disulfide-crosslinked dimers on *in vitro* reconstituted keratin filaments

To determine whether one can reproduce the formation of these disulfide-crosslinked dimers under well-defined *in vitro* conditions, we reconstituted esophageal keratin filaments by dialyzing urea-solubilized rabbit esophageal keratins into 10 mM Tris-HCl (pH 7.8) in the absence of a reducing agent (Lee and Baden, 1976; Steinert et al., 1976). Analysis of such air-oxidized filaments by SDS-PAGE showed the formation of bands I and II, with a yield of about 20-30% (Fig. 12). Diagonal gel analyses showed that, like the *in vivo* bands I and II, these two bands represented heterodimers of K4 and K13 (Fig. 13). Small amounts of tetramers consisting of K4 and K13 in a 1:1 molar ratio were also detected (Fig. 13).

Although under mild oxidative conditions esophageal keratins can be crosslinked *in vitro* to form heterotypic dimers and tetramers, no homodimer of K13 (band III) was formed. To determine whether reconstitution in the absence of a reducing agent might have resulted in precocious disulfide crosslinking (giving rise to bands I and II), which somehow hindered band III formation, we first reconstituted filaments in the presence of a reducing agent, which was then removed during a second step of dialysis. Although the two heterodimers were again generated, K13 homodimer still did not form, indicating that under *in vitro* conditions we can reproduce only partially the disulfide crosslinking reactions occurring *in vivo*.

Partial trypsin digestion of air-oxidized, reconstituted esophageal filaments

To determine whether the disulfide crosslinking of het-

erodimers involved cysteine residues located in the alpha-helical rod domains or the non-helical end domains, we digested air-oxidized, reconstituted filaments with trypsin (Fig. 14). As the trypsin concentrations increased from 0.001 mg/ml to 0.01 mg/ml, the non-helical regions of the keratins were progressively digested, leaving behind a trypsin-resistant helical core 40-45 kDa in size (see the reduced samples in Fig. 14a, lane 4; Steinert et al., 1983). Analyses of samples in the absence of a reducing agent showed that the crosslinked heterodimers (bands I and II) were gradually degraded to a 80-90 kDa trypsin-resistant species (Fig. 14a, lane 4) containing both basic K4 (Fig. 14b) and acidic K13 keratins (Fig. 14c). These results suggest that at least some of the disulfide crosslinks must involve cysteine residues residing in the trypsin-resistant rod domains of K4 and K13 keratins.

DISCUSSION

Keratins of the basal versus suprabasal compartments

By analyzing the keratins of the basal and suprabasal compartments of cultured rabbit esophageal epithelial cells, we have been able to provide for the first time direct evidence that: (i) esophageal basal cells contain primarily K5/K14 keratins (Figs 5 and 6). We and others have previously shown by immunohistochemical and *in situ* hybridization techniques that basal cells of several stratified squamous epithelia contain K5 and K14 (Woodcock-Mitchell et al., 1982; Stoler et al., 1988; Leube et al., 1988). Biochemical analysis of the keratins of highly purified basal cells of the

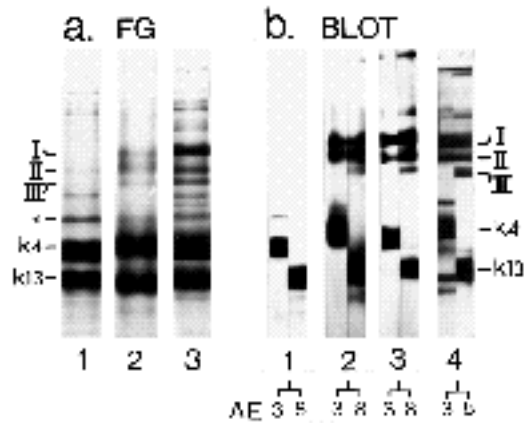


Fig. 10. Identification of two disulfide-crosslinked heterodimers and one homodimer. The epithelium was scraped from the mid-esophagus of a freshly killed rabbit, and extracted to remove the soluble proteins. The residual cytoskeleton was solubilized in 9.5 M urea (lanes 1-3) or 1% SDS plus *N*-ethylmaleimide (lane 4), separated by SDS-PAGE, and transferred to nitrocellulose. (a) Fast Green staining. Lane (1) urea-solubilized esophageal keratins (reduced) showing no high molecular mass crosslinked keratins. Asterisk marks a minor, uncharacterized keratin-related component. Lane (2) the same sample analyzed without reduction. Lane (3) esophageal keratins solubilized in the presence of 10 mM *N*-ethylmaleimide; analyzed without reduction. Note the presence of several high molecular mass bands. (b) Immunochemical staining. Lanes (1) to (3) were immunoblots of samples corresponding to lanes (1) to (3) of (a). Lane (4) showed keratins solubilized in SDS/NEM. The blots of each lane were divided longitudinally into two halves, which were stained with AE3 and AE8 antibodies to identify the basic (59 kDa) K4 keratin and the acidic (46 kDa) K13 keratin, respectively. Note that bands I and II reacted with both AE3 and AE8, while band III reacted only with AE8.

epidermis and corneal epithelium provided direct proof that these two keratins indeed represent the major keratins of the basal layer (Skerrow and Skerrow, 1983; Bowden et al., 1984; Schermer et al., 1986, 1989). We have now shown that this is also the case for esophageal epithelium, a third important keratinocyte prototype, thus extending the generality of this concept. (ii) K4 and K13 keratins are associated exclusively with the suprabasal compartment of cultured rabbit esophageal epithelial cells (Figs 5 and 6). This result confirms and extends the findings of Van Muijen et al. (1986), who showed that their IC7 and 2D7 monoclonal antibodies selectively stained the suprabasal cells of human esophageal epithelium, and those of Rentrop et al. (1986), who showed by *in situ* hybridization that K13 mRNA is localized mainly in suprabasal cells of mouse tongue (for conditions allowing the expression of K13 in basal cells, see below). Our results are also fully compatible with those of Schweizer et al. (1988), who showed that mouse K4/K13 and K1/K10 are associated with the isolated suprabasal cells of mouse forestomach epithelium. (iii) The K6/K16 keratins are located in the suprabasal compartment (Figs 5 and 6). By analyzing keratins of basal and suprabasal cells isolated from cultured rabbit corneal epithelium, we have previously provided the

first direct evidence that K6/K16 keratins are associated with the suprabasal compartment (Schermer et al., 1986, 1989). A similar conclusion was reached by Stoler et al. (1988), who demonstrated immunohistochemically that an antibody to K6 keratin stains the suprabasal cells of human psoriatic epidermis. We have now proved that, in cultured rabbit esophageal epithelial colonies, K6/K16 are also associated exclusively with the suprabasal compartment (Figs 5 and 6). Taken together, these data provide strong evidence that K6/K16 keratins are markers for the suprabasal compartment of all three major prototypes of stratified squamous epithelium, i.e. epidermis, corneal epithelium and esophageal epithelium.

We described in 1984 that K6 and K16 keratins may be regarded as markers for "hyperproliferation" (Weiss et al., 1984). This was based on our finding that these keratins are present in all hyperproliferative human epidermal diseases that we have analyzed so far including psoriasis, actinic keratosis and squamous cell carcinoma, and that these two keratins are synthesized in large quantities in all kinds of cultured keratinocytes (McGuire et al., 1984; Weiss et al., 1984). However, it is important to note that although all hyperproliferative keratinocytes seem to express K6/K16, the reverse is not true, i.e. not all K6/K16-positive stratified epithelia are hyperproliferative. For example, outer root sheath cells (of hair follicle), which are by no means "hyperproliferative", express K6/K16 perhaps as their normal differentiation products (Stark et al., 1987). Cultured human foreskin epidermal cells continue to synthesize K6/K16 even when the cells become senescent and apparently stop growing (Schermer et al., 1989). Cultured rabbit corneal epithelial cells continue to synthesize K6/K16 even when cell proliferation is completely inhibited by DNA synthesis inhibitors (Schermer et al., 1989). On the basis of these data and the suprabasal location of K6/K16, we think that their expression is perhaps more related to "non-permissive" conditions under which the expression of the differentiation-related suprabasal keratins (i.e. K1/K10 of the epidermis, K3/K12 of cornea and K4/K13 of esophagus) is suppressed. Under such conditions, K6/K16 appears to be turned on by a default mechanism. If so, then these two keratins may be regarded more appropriately as markers for an advanced stage of an "alternative pathway" of keratinocyte differentiation (Galvin et al., 1989; Schermer et al., 1989).

A corollary of the above idea is that the synthesis of K6/K16 should be inversely related to that of the differentiation-related keratins, which is precisely what we have observed in both human epidermis (Weiss et al., 1984) and cultured rabbit corneal epithelial cells (Schermer et al., 1986, 1989). The biochemical basis for this "mutually exclusive" expression of these two major classes of suprabasal keratins (the differentiation type versus the hyperproliferation type) is not understood. However, these two types of keratins most likely can be co-expressed in the same suprabasal cells, since almost 100% of the suprabasal cells in cultures of rabbit esophageal and corneal epithelium are K13- and K3-positive, respectively (Schermer et al., 1986, and data not shown).

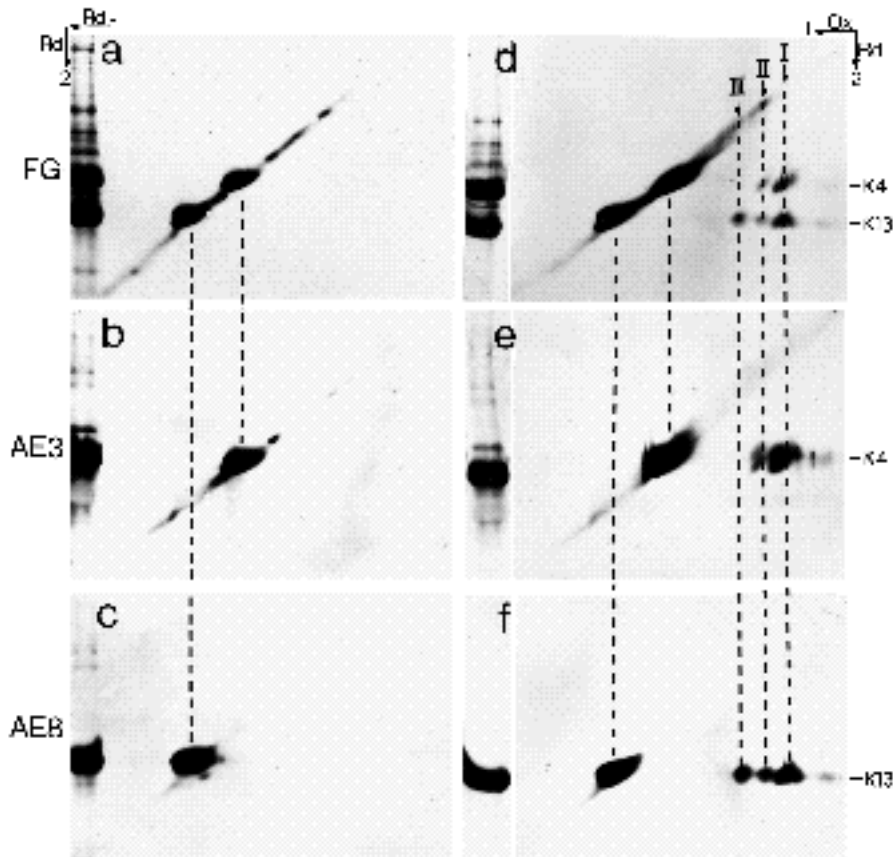


Fig. 11. Characterization of the disulfide-crosslinked rabbit esophageal keratin dimers by diagonal gel electrophoresis. Esophageal keratins were solubilized in 9.5 M urea and 10 mM *N*-ethylmaleimide and analyzed by the first-dimensional slab SDS-PAGE with (a-c) or without (d-f) beta-mercaptoethanol. All the gel lanes were then reduced before they were "loaded" horizontally on another slab gel for second-dimensional separation. (a) to (c) Controls in which both dimensions were run under reduced condition (Rd). Blots were stained with: (a) Fast Green showing that all protein spots align well on the "diagonal"; (b) AE3 staining allowing the identification of K4; and (c) AE8 staining allowing the identification of K13. (d) to (f) Samples that were not reduced in the first-dimensional SDS-PAGE (Ox). The blots were stained with (d) Fast Green showing that the high molecular mass bands I, II and III gave rise to reduced proteins migrating to new positions vertically below; (e) AE3 staining allowing the identification of the two 59 kDa protein spots (under I and II) as K4; and (f) AE8 staining allowing the identification of all three 46 kDa protein spots (under I, II and III) as K13. These results established I and II as heterodimers of K4 and K13, and III as a homodimer of K13.

Occasional expression of K13 in basal keratinocytes

Although in confluent cultures of rabbit esophageal cells the K13 keratin is found exclusively in the suprabasal cell layer and thus may be regarded as a marker for an advanced stage of "esophageal-type" differentiation, under several *in vivo* and cell culture conditions this keratin can be seen to be associated with the basal cells: (i) although there is no doubt that the suprabasal cells of normal rabbit esophageal epithelia are stained much more intensely by AE8 than the basal cells, these *in vivo* basal cells consistently show weak but discernibly filamentous AE8 staining (Fig. 7d), suggesting that they do contain small amounts of K13. (ii) In rete ridges of rabbit dorsal tongue epithelium K13 is clearly present in the basal cells that line the "shoulder" of the rete ridges, suggesting that, as far as K13 marker is concerned, these basal cells may have attained a more differentiated state than the basal cells located at the bottom of the rete ridges (Fig. 7e). This expression pattern is reminiscent of our previous finding on corneal/limbal epithelium where we found that central corneal epithelial basal cells, unlike limbal basal cells, are K3-positive; this and several other observations have led us to develop the concept that corneal epithelial stem cells reside in the basal layer of limbal zone (Schermer et al., 1986; Cotsarelis et al., 1989; Tsai et al., 1990). The K13 expression pattern may suggest a similar situation; i.e. the K13-negative basal cells located at the bottom of the rete ridges may represent the stem cells of dorsal tongue epithelium (Hume and Potten, 1976). (iii)

K13 keratin can be readily detected in the basal-like A431 cells (data not shown). These results indicate that K13 expression is not incompatible with cell proliferation (for related data on other differentiation-related keratins, K1, K3 and K4, see Blessing et al., 1989; Schermer et al., 1986; and Leube et al., 1988; respectively). They also raise the interesting possibility that the molecular mechanisms underlying the suprabasal expression of K4/K13 and the corneal K3/K12 keratins (all of which have been seen to express in specific populations of basal cells) may be more "flexible" than that of epidermal K1/K10, which are rarely seen to be expressed in the basal layer.

Nature of the cystine-stabilized keratin dimers

Our data indicate that the formation of cystine-stabilized keratin dimers is a major event that occurs during the terminal stage of esophageal epithelial differentiation (Figs 8 and 9). It is interesting to note that, unlike epidermal and hair keratins, which form high molecular mass disulfide-crosslinked polymers (Sun and Green, 1978; Woodcock-Mitchell et al., 1982; Heid et al., 1986; Lynch et al., 1986; Pang and Sun, unpublished observation), the crosslinking of esophageal epithelial keratins is largely limited to the formation of dimers (Figs 10-13). This particular feature of esophageal keratin has important functional implications (see below).

The three crosslinked dimers that we identified in esophageal epithelium are unlikely to be artifacts produced during sample preparation. First, the formation of these

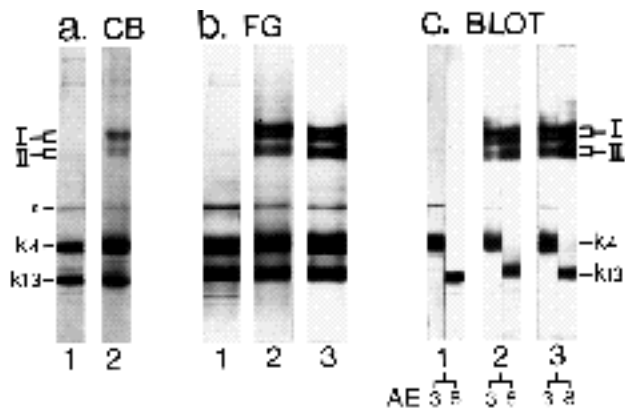


Fig. 12. *In vitro* formation of disulfide-crosslinked keratin dimers using reconstituted rabbit esophageal keratin filaments. Esophageal keratins solubilized in 9.5 M urea and 5 mM dithiothreitol were dialyzed against 10 mM Tris-HCl (pH 7.5) with or without 10 mM dithiothreitol in order to reconstitute 10 nm filaments (Steinert et al., 1976). The sample was then air-oxidized by dialysis against 10 mM Tris-HCl (pH 7.5) at 4°C for 16 hours, followed by SDS-PAGE analysis. (a) Coomassie Blue (CB) staining patterns of the air-oxidized keratin filaments solubilized in SDS sample buffer with (lane 1) or without (lane 2) 1% beta-mercaptoethanol. Note the formation of disulfide-crosslinked dimers I and II in lane 2. Asterisk denotes a minor unknown esophageal component. (b) Fast Green staining of proteins transferred to nitrocellulose. Lanes 1 and 2 are air-oxidized filaments originally reconstituted in the absence of dithiothreitol; while filaments shown in lane 3 were reconstituted in the presence of a reducing agent before air-oxidation. Sample in lane 1 was reduced before SDS-PAGE. Note that the same two disulfide-crosslinked bands were formed in filaments reconstituted in the absence (lane 2) or presence (lane 3) of a reducing agent. (c) The same blots as shown in (b) were stained with AE3 and AE8 monoclonal antibodies by the PAP technique. Note that the dimers were stained by both AE3 and AE8.

dimers parallels closely the disulfide abundance *in vivo* as assayed with DACM (Figs 8 and 9). Second, the same three crosslinked dimers can be extracted not only with 9.5 M urea but also by solubilizing the tissue in 1% SDS (Fig. 10b, lane 4). Inclusion of EDTA, or 10 mM NEM, which blocks sulfhydryl groups needed for disulfide exchange, also did not alter the pattern of these crosslinked products (Fig. 10b and data not shown). Finally, at least the two heterodimers can be faithfully reproduced on reconstituted keratin filaments (Figs 12 and 13).

The two heterodimers have several implications. First, they have the same protein composition (Figs 10 and 11) and are therefore isoforms possibly generated through the crosslinking of different sulfhydryl groups. Second, as mentioned above, they can be reproduced on reconstituted filaments, suggesting that soluble proteins or enzymes removed during the preparation of cytoskeleton are not necessary for their formation (Figs 12 and 13). Third, since such heterodimers can survive trypsinization, which removes the head and tail domains of the keratin molecule (Fig. 14), the crosslinking must involve at least some of the cysteine residues of the helical cores. Fourth, the yield of the heterodimer crosslinking is relatively high *in vivo* (certainly greater than 50%; Fig. 9), but is relatively low on

reconstituted filaments (<20%; Fig. 12). This may reflect an imperfection or polymorphism in certain structural aspects of the reassembled filaments (see below). Alternatively, this may be due to subtle differences between keratins of the lower, viable cell layers and those of the superficial cell layers in terms of secondary modifications such as phosphorylation and minor proteolytic digestion. Finally, these heterodimers are formed during the terminal stage of esophageal epithelial differentiation (Fig. 9). Their formation is therefore a late event, most likely designed to stabilize the final filament network/bundle. In this regard, it is important to distinguish the *in vivo* cystine-stabilized heterodimers that we describe here from the heterodimer involved in the initial step of filament formation. For example, Hatzfeld and Weber (1990) and Steinert (1990) recently reported that the formation of a heterodimer is an obligatory first step during keratin filament formation. In the former study, Hatzfeld and Weber (1990) introduced by site-directed mutagenesis a cysteine residue in exactly the same relative positions of the alpha-helical domains of two simple epithelial keratins K8 and K18, both of which lack cysteine. Since these two cysteine residues are introduced into the "d" position of the heptad repeat pattern, they face each other within a coiled coil. Thus air-oxidation of a mixture of these two keratins results in the efficient formation of a cystine-stabilized heterodimer, which, interestingly, is fully capable of forming 10 nm filaments. The heterodimers that we describe here (Figs 9-11) are distinct from this type of artificially constructed, cystine-stabilized heterodimer, in that the former most likely involve the crosslinking between type I and type II keratins of *different* coiled coils. We make this speculation because cysteines of type I and II keratins of all coexpressed keratin "pairs", including the mouse K4 and K13 (Rentrop et al., 1986; Knapp et al., 1986), reside in very different locations of the helical domains; this makes it impossible for the two keratin helices, lying parallel and in perfect register in an (initial) heterodimer (Ip et al., 1985; Parry et al., 1985; Steinert and Roop, 1988), to form disulfide bonds. Another reason is that a great majority of cysteines of the coiled coil domains are *not* in the "a" and "d" positions of the heptad, and are therefore pointing outwards away from the area of coiled coil interactions. For these reasons, we strongly suspect that the cystine-stabilized heterodimers that we describe here are primarily designed to stabilize the longer-range interactions between neighboring coiled coils (Aebi et al., 1983).

The disulfide-stabilized homodimer of K13 has two interesting implications. First, recent data showed quite clearly that interactions between the two members of a homodimer are less stable and are therefore thermodynamically less important than those of heterodimers during filament initiation (i.e. the formation of the initial dimer; Hatzfeld and Weber, 1990; Steinert, 1990). However, the fact that some K13 keratins are cystine-stabilized as a homodimer in terminally differentiated esophageal epithelial cells raises the possibility that interactions involved in forming certain homodimers may be involved in stabilizing the overall filament structure during a late step of filament assembly. Second, the fact that we could not generate cystine-stabilized K13 homodimers using reconstituted filaments again raises questions about the normalcy of such filaments

(Steinert, 1990). On one hand, the *in vitro* formation of the two heterodimers indicates that these reconstituted filaments must be quite normal in terms of the precise spatial

alignment of the several pairs of sulfhydryl groups that are involved in heterodimer formation (Figs 12 and 13). On the other hand, the fact that no homodimer is formed at all

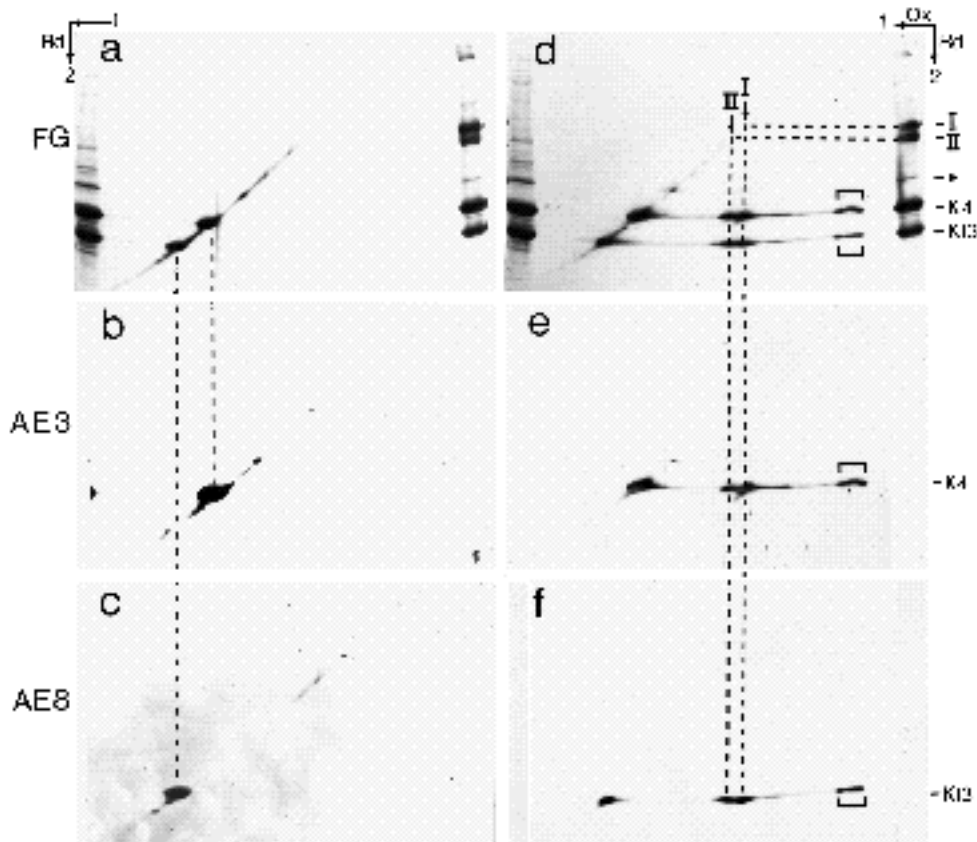


Fig. 13. Characterization of the *in vitro* reconstituted, disulfide-crosslinked keratins by diagonal gel electrophoresis. Esophageal keratins were reconstituted, air-oxidized (Ox), and analyzed by diagonal gel electrophoresis. (a) to (c) Controls in which both dimensions were run under reduced condition (Rd). (d) to (f) Samples in which the first-dimensional separation was done without reduction. (a) and (d) Fast Green staining; (b) and (e) AE3 staining; and (c) and (f) AE8 staining. Side lanes in (a) and (d) show samples separated by one-dimensional SDS-PAGE with and without beta-mercaptoethanol in the sample buffer, respectively.

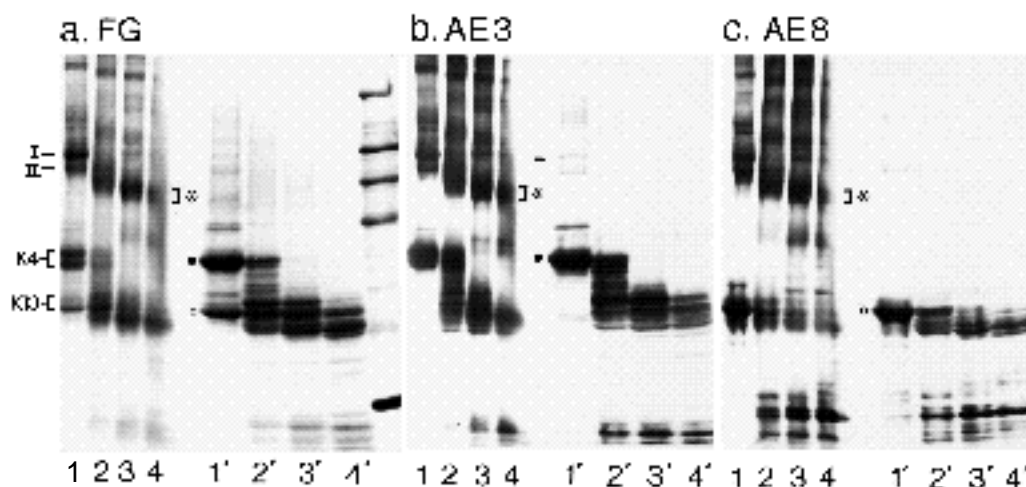


Fig. 14. Partial trypsin digestion of air-oxidized, esophageal keratin filaments. Filaments were reconstituted from urea-solubilized esophageal keratins, air-oxidized and digested with various concentrations of trypsin. The samples were then analyzed by SDS-PAGE and immunoblotting. Lanes (1) to (4) of all three panels (a-c) were samples analyzed without reduction, while lanes (1') to (4') were corresponding samples after reduction. (a) Fast Green staining. Lanes (1) and (1') are controls showing intact keratins. I and II mark the 110 kDa and 100 kDa disulfide-crosslinked K4/K13 heterodimers, and the filled and open dots denote K4 and K13 monomers, respectively. Samples in lanes (2) and (2') were treated with 0.001 mg/ml trypsin; lanes (3) and (3') 0.005 mg/ml trypsin; and lanes (4) and (4') 0.01 mg/ml trypsin. Molecular mass standards include carbonic anhydrase (29 kDa), egg ovalbumin (45 kDa), bovine serum albumin (66 kDa), rabbit muscle phosphorylase B (97.4 kDa), beta-galactosidase (116 kDa), and rabbit muscle myosin (205 kDa). (b) AE3 immunoreactivity. (c) AE8 immunoreactivity. Asterisk marks the 90 kDa trypsin-resistant core of the crosslinked heterodimer.

suggests that certain higher-order structure normally stabilized by homodimers is probably totally missing in such filaments.

Since intermolecular disulfide-crosslinking of keratins occurs only in superficial squamous cells (Fig. 9) that are thought to be metabolically inert, it is possible that the cytoplasm of these cells is relatively aerobic and the crosslinking occurs spontaneously between cysteine residues that are spatially favorably oriented. This possibility is supported by our present observation that such a crosslinking pattern can be reproduced in part using *in vitro* reconstituted keratin filaments (Fig. 12).

Functional significance of keratin crosslinking by intermolecular disulfide bonds

We have established in this paper that K4 and K13, the two major differentiation-related keratins in esophageal epithelium, become crosslinked during an advanced stage of differentiation forming both heterodimers (K4/K13) and homodimers (K13-K13; Figs 9-11). In considering the functional significance of these disulfide-stabilized keratin dimers, it is interesting to note that the degrees of disulfide crosslinking of different keratin molecules vary roughly in proportion to the degree by which the epithelium forms a tough and physically durable protective superficial cell layer. Thus simple epithelial keratins contain either very few or no cysteine residues at all, and are usually not crosslinked; esophageal keratins are crosslinked mainly resulting in the formation of dimers which may be compatible with a rather flexible cornified layer; epidermal keratins, on the other hand, formed crosslinked polymers which may account for a tougher stratum corneum structure; finally, hair keratins contain numerous cysteines forming a highly crosslinked and extremely rigid and durable hair fiber. This indicates that the intermolecular crosslinking of keratins is a tissue-specific and terminal differentiation-related event whose function is to achieve various degrees of physical stabilization or hardening of the superficial squamous cells.

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