Isolation and Characterization of Two Polypeptides That Form Intermediate Filaments in Bovine Esophageal Epithelium

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ABSTRACT Cells in the stratified squamous epithelium of bovine esophagus contain abundant tonofilaments measuring 6-10 nm in diameter. Two polypeptides, extracted from esophageal epithelium with 0.05 M Tris, pH 7.4, containing 8 M urea and 25 mM β-mercaptoethanol, comprise 35% of the total extractable protein. These polypeptides have apparent molecular weights of 46,000 and 56,000 daltons and are rich in glutamic acid-glutamine, glycine, and serine. Each polypeptide can be partially purified by DEAE-cellulose chromatography. Mixtures of the purified polypeptides form filaments in vitro that measured 6-10 nm in diameter. Neither polypeptide formed filaments by itself. Filaments formed in vitro give an α-keratin type x-ray diffraction pattern. These data indicate that the tonofilaments in esophageal epithelium are formed primarily from these two polypeptides.

Intermediate filaments are an abundant and characteristic cytoplasmic component of cells in stratified squamous epithelia. They are often referred to as tonofilaments and are morphologically similar in different epithelia: they are 6-10 nm in diameter, they form undulating, unbranched curves in the cytoplasm, their length is often several hundred times their diameter, they often terminate in desmosomes, and they have a tendency to form parallel arrays.

Proteins that form the tonofilaments in cutaneous epithelia have been isolated from calf snout (8, 12, 16, 17), hoof epithelium (18), human epidermis (21), and rat epidermis (2, 23). A large fraction of the extractable protein in such epithelia (as much as 60% in stratum corneum of epidermis) consists of families of polypeptides that can form filaments in vitro. The filaments formed in vitro are morphologically similar to the tonofilaments seen in the intact tissue (12, 16, 18, 21, 23). Each filament-forming polypeptide (FFP) within a given tissue appears to be the product of a separate gene (6); no one is a product or precursor of any of the others (11). Some FFP's found in different cutaneous epithelia, such as in bovine hoof and snout, are identical but other FFP's in these epithelia appear to be tissue-specific (9, 10).

The purpose of this study is to identify the major FFP's in a noncutaneous, stratified squamous epithelium. Several recent immunofluorescence microscopy studies have demonstrated that tonofilaments in noncutaneous epithelia are immunochemically related to the tonofilaments in cutaneous epithelia (5, 22). The morphologic and immunochemical similarities of tonofilaments in different epithelia suggest that the proteins that form tonofilaments in noncutaneous epithelia might be the same as those that form tonofilaments in cutaneous epithelia. This report describes the isolation and characterization of the two major FFP's from the epithelium of calf esophagus. Biochemical comparison of these FFP's with the well characterized FFP's from calf hoof is the subject of a separate report (13).

MATERIALS AND METHODS

Tissue Preparation

Segments of esophagus, extending from the level of the thyroid gland to 3 cm above the stomach (rumen), were obtained from freshly slaughtered calves. The surrounding musculature was trimmed away and the epithelium was separated from underlying connective tissue by heating to 56°C for 30 s in water.

Light Microscopy

Transverse sections of esophagus, 0.3 x 1.0 cm, were fixed overnight in buffered 10% formalin and embedded in paraffin. 5-μm sections were stained with hematoxylin and eosin and photographed with Panatomic-X through a green filter.

Electron Microscopy

1 x 2-mm pieces of esophagus, from which the musculature had been removed, were placed in half-strength Karnovsky's fixative (7) for 2 h at 4°C, washed with...
acetate-veronal buffer, postfixed with 1% buffered osmium tetroxide, washed, stained in block with uranyl acetate, rapidly dehydrated, and embedded in Epon (4). Silver sections of tissue were placed on carbonized, formvar-coated copper grids, stained with uranyl acetate and lead citrate (24), and examined at 80 kV in a Siemens 100 electron microscope.

Negative Staining of Filaments and Tissue Extraction

Suspensions of filaments were spread on carbon-coated mica chips, transferred to carbon-coated holey grids, and stained with 1% uranyl acetate.

Isolation of Individual Polypeptides

SDS polyacrylamide gel electrophoresis was performed on 14 x 14 x 0.15-cm gel slabs consisting of a 12.5% separating gel and a 5% stacking gel as described in the accompanying paper (13).

Single-well, SDS polyacrylamide slab gels, prepared as described above, were loaded with extracts containing 100-300 µg of protein, and individual polypeptides were separated by electrophoresis and recovered from the gels described in the accompanying paper (13).

Amino Acid Analysis

Single polypeptides, isolated by preparative SDS polyacrylamide electrophoresis as described above, were redissolved in 0.01 M sodium borate, pH 7.6, containing 8 M urea, incubated for 30 min at 37°C with an equal volume of Dowex AG-1-X2 (Bio-Rad Laboratories, Richmond, Calif.) to remove the SDS (25), dialyzed against two changes of 1,000 vol of 0.01 M sodium borate, pH 7.6, and lyophilized. 20 µg of each polypeptide were hydrolyzed in 6 N HCl at 110°C, and the amino acids were quantitated on a Durrum-500 analyzer (Durrum Instrument Corp., Sunnyvale, Calif.). Cysteic acid was measured in samples of a 48,000-dalton polypeptide. When such E2 preparations were mixed with fractions containing an equal amount of E2, they were eluted by step gradient of KCl at a gravity flow rate of 0.5 ml/min.

Protein Quantitation

Protein was assayed by the method of Bramhall et al. (1) using bovine serum albumin (Miles, Laboratories, Inc., Elkhart, Ind.) as standard. The relative amount of protein in Coomasie Blue-stained bands on SDS polyacrylamide gels was approximated by scanning the gels with a scanning densitometer (Helena Laboratories, Beaumont, Tex.).

RESULTS

Morphology of Esophageal Epithelium

The entire length of bovine esophagus is lined by a parakeratotic, stratified squamous epithelium that is uninterrupted by mucous glands, sweat ducts, or other adnexal structures (Fig. 1a). The cytoplasm of the epithelial cells contains profuse arrays of 6-10-nm filaments, but there are no keratohyalin granules or keratinosomes (Fig. 1c). The esophageal epithelium is cleanly separated from the underlying connective tissue by immersing the tissue in water at 56°C for 30 s (Fig. 1b).

Extraction of Esophageal Polypeptides

Two polypeptides comprise ~35% of the total extractable protein of esophageal epithelium. Their molecular weights, estimated by relative mobility in SDS polyacrylamide electrophoresis, are 56,000 daltons (E1) and 46,000 daltons (E2) (Fig. 2). The insolubility of these two polypeptides in low-salt buffer allows them to be separated from the majority of other esophageal proteins by a serial extraction procedure. Extraction of the tissue with 0.05 M Tris, pH 7.4, solubilizes most of the esophageal proteins; extraction of the residue with 0.05 M Tris, pH 7.4, containing 8 M urea and 0.025 M β-ME, solubilizes E1 and E2 (Fig. 2). 40-50% of the E1 and E2 can be extracted with urea alone. Total extraction is possible only in the presence of β-ME. E1 and E2 can be only partially extracted with 5 M urea or 0.1 M sodium citrate, pH 2.6, or 1 mM NaOH. They cannot be extracted with 0.6 M KCl.

Filament Formation

Filaments form in the urea extract when the urea is removed by dialysis. Dialysis of a urea extract, containing 2 mg/ml of protein, against 5 mM Tris, pH 7.4, causes the solution to become opalescent. When the opalescent solution is spread on a holey grid, stained with uranyl acetate, and examined in the electron microscope, unbranched filaments are seen (Fig. 3). They are 6-10 nm in diameter and vary in length from 0.1 to 1.0 µm. More long filaments and fewer short filaments are formed when β-ME is added to the dialysis buffer, a phenomenon previously described for in vitro filament formation in urea extracts from hoof epidermis (19). More than 90% of the protein in the opalescent solution is found in the pellet after ultracentrifugation at 200,000 g for 1 h, indicating that the bulk of the protein is in a filamentous form.

To study the interaction of E1 and E2, samples consisting predominantly of E1 or E2 were obtained by DEAE-cellulose column chromatography (Fig. 4). A fraction containing E1, eluted with 20 mM KCl and one containing E2 at 80 mM KCl (Fig. 5). Each fraction was dialyzed against 5 mM Tris containing β-ME, stained with uranyl acetate, and examined for filaments. None were found. When fractions containing E1 were mixed with fractions containing an equal amount of E2, dialyzed against Tris-β-ME, and stained with uranyl acetate, long filaments were observed. The filaments were 6-10 nm in diameter, unbranched, and had a tendency to form side-to-side association. Pellets of these filaments were analyzed by SDS polyacrylamide gel electrophoresis, and E1 and E2 were present in equal amounts. Some E2 fractions contained small amounts of a 48,000-dalton polypeptide. When such E2 preparations were mixed with E1 and the urea was removed by dialysis, the amount of the 48,000-dalton polypeptide found in the pellet was proportional to the amount in the original "E2" preparation.

X-ray Diffraction

Filaments were formed in vitro from a combination of DEAE fractions 32 and 92 (see Fig. 5). X-ray diffraction
analysis of these filaments revealed a meridional arc at 5.09 Å and an equatorial arc at 9.8 Å (Fig. 3).

Amino Acid Composition

The amino acid composition of E₁ and E₂, purified (Fig. 2) by SDS polyacrylamide slab gel electrophoresis, is shown in Table I. These polypeptides are rich in glutamine, glycine, and serine. Their amino acid compositions are similar to those of tonofilament polypeptides isolated from calf hoof epidermis (18). No tryptophane was detected; based on the sensitivity of the assay and the amounts of protein analyzed, there are fewer than two tryptophane molecules per molecule of polypeptide.
DISCUSSION

The criteria used by others to identify filament-forming polypeptides in cutaneous epithelia were that they should be abundant, relatively insoluble, and have the capacity to assemble in vitro into filaments that were 10 nm in diameter and exhibited an \( \alpha \)-keratin type x-ray diffraction pattern (8, 12, 16-18, 23). These operational characteristics were used to identify and isolate the FFP’s from esophageal epithelium. The stratified squamous epithelium of calf esophagus was chosen for study because it was readily prepared free of nonepithelial elements and because the most completely characterized tonofilament proteins are those of bovine hoof epidermis.

The major polypeptides in the urea extract of esophageal epithelium have molecular weights of 46,000 and 56,000 daltons; those in urea extracts of hoof epidermis have molecular weights of 49,000, 51,000, 54,000, 57,000, 61,000, and 65,000 daltons in the same gel system (13). Because the urea extract of esophagus contains a family of polypeptides different from that in hoof, what is the evidence that \( E_1 \) and \( E_2 \) are indeed the esophageal tonofilament polypeptides? (a) The urea extract, as well as a mixture of partially purified \( E_1 \) and partially purified \( E_2 \), form filaments in vitro that are 6-10 nm in diameter. (b) The abundance of \( E_1 \) and \( E_2 \) is commensurate with the abundance of tonofilaments observed by electron microscopy in esophageal epithelial cells. (c) \( E_1 \) and \( E_2 \) can be extracted only with reagents that extract hoof and human epidermal FFP’s, and their amino acid compositions are similar to those of hoof FFP’s (18). A disulfide reducing agent is necessary for complete extraction of \( E_1 \) and \( E_2 \), just as it is required for extraction of hoof (18) and human epidermal (21) FFP’s. (d) Filaments formed by a mixture of \( E_1 \) and \( E_2 \) exhibit an \( \alpha \)-keratin type x-
ray diffraction pattern. This diffraction pattern appears to be a common finding for a variety of 6–10-nm filaments (20) and implies a coiled coil arrangement for the polypeptides in the filament (3).

Although E₁ and E₂ are the major FFP's in esophageal epithelium, they may not be the only ones. Several minor polypeptides with molecular weights of 48,000, 51,000, and 53,000 daltons are also found in the urea extracts. When filaments are formed from urea extracts of esophagus, these minor polypeptides are quantitatively recovered through several cycles of assembly-disassembly-assembly. It remains to be shown whether these polypeptides can form filaments themselves, whether they are required for the formation of filaments by E₁ plus E₂, or whether they become associated with filaments that have already formed.

The relationship of the esophageal FFP's to other polypeptides that form intermediate filaments is unclear, and the occurrence of so many different polypeptides that form intermediate filaments, even within a single tissue, raises many questions. Why are there so many and how did they arise? Do their biochemical differences imply different functions? What is the common biochemical basis for the similar morphology of the filaments they form? Identification and isolation of individual FFP's from different tissues is a necessary step toward answering these questions.

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REFERENCES
1. Bramhall, S., N. Noack, M. Wu, and J. R. Loewenberg. 1969. A simple colorimetric method for determination of protein. Anal. Biochem. 31:146-148.
2. Bird, M. M., R. H. Gray, and I. A. Bernstein. 1977. Tonofilament protein from newborn rat epidermis. J. Biol. Chem. 252:2127-2133.
3. Crick, F. H. C. 1952. Is a-keratin a coiled-coil? Nature (Lond.). 170:882-883.
4. Farquhar, M. G., and G. E. Palade. 1965. Cell junctions in amphibian skin. J. Cell Biol. 26:263-291.
5. Franke, W. W., B. Applehans, E. Schmid, C. Freundstein, M. Osborn, and K. Weber. 1979. Identification and characterization of epithelial cells in mammalian tissues by immunofluorescence microscopy using antibodies to prekeratin. Differentiation. 15:7-25.
6. Fuchs, E., and H. Green. 1979. Multiple keratins of cultured human epidermal cells are translated from different mRNA molecules. Cell. 17:573-582.
7. Kamovsky, M. J. 1963. A formaldehyde-glutaraldehyde fixative of high osmolality for use in electron microscopy. J. Cell Biol. 27:27, Pt. 2.iii:17-17 (Abstr.).
8. Lee, L. D., and H. P. Baden. 1976. Organization of the polypeptide chains in mammalian keratin. Nature (Lond.). 264:377-378.
9. Lee, L. D., J. Kohliho, and H. P. Baden. 1979. Intraspies heterogeneity of epidermal keratins isolated from bovine hoof and stout. Biochem. J. 177:187-196.

TABLE I
Amino Acid Compositions

|    | E₁   | E₂   |
|----|------|------|
| Asp| 7.47 | 8.45 |
| Thr| 3.32 | 5.49 |
| Ser| 8.95 | 8.59 |
| Glx| 13.86| 15.94|
| Pro| 0.84 | 1.08 |
| Gly| 16.01| 15.53|
| Ala| 9.29 | 7.04 |
| Val| 7.51 | 4.11 |
| Met| 1.25 | 1.95 |
| Ile| 4.46 | 4.70 |
| Leu| 8.66 | 10.50|
| Tyr| 2.10 | 2.31 |
| Phe| 3.80 | 3.33 |
| His| 0.08 | 0.33 |
| Lys| 5.91 | 4.53 |
| Arg| 4.63 | 5.39 |
| CysA| 1.83 | 0.74 |

Molecular weight
56,000 46,000

Amino acid analyses were performed on esophageal polypeptides that had been isolated and purified by sequential extraction and preparative gel electrophoresis. A three-point, timed hydrolysis was performed. Maximum values for Val and Ileu are shown. Values extrapolated to zero time are shown for serine and threonine. CysA and Tyr were determined on separate samples. The remaining values are the average of three determinations.
10. McGuire, J., and L. M. Milstone. 1979. Homologies in the keratins of calf hoof and snout epithelium. Clin. Res. 27:446a.

11. McGuire, J., L. M. Milstone, M. Osborne, and L. Inglis. Keratins in cultured human keratinocytes are stable. In Biochemistry of Normal and Abnormal Epidermal Differentiation. I. A. Bernstein and M. Seiji, editors. University of Tokyo Press. Tokyo, Japan. 327-340.

12. Matoltsy, A. G. 1965. Soluble prekeratin. In Biology of the Skin and Hair Growth. A. G. Lyne and B. F. Short, editors. Angus and Robertson, Sydney, Australia. 291-306.

13. Milstone, L. M., and J. McGuire. 1981. Different polypeptides form the intermediate filaments in bovine hoof and esophageal epithelium and in aortic endothelium. J. Cell Biol. 88:312-316.

14. Moore, S. 1963. On the determination of cystine as cystic acid. J. Biol. Chem. 238:235-237.

15. Peake, B. R., Ferenczi, and K. Kovacs. 1974. A new acid hydrolysis method for determining tryptophan in peptides and proteins. Anal. Biochem. 60:45-50.

16. Radlack, K. M. 1952. The proteins of the mammalian epidermis. In Advances in Protein Chemistry. M. L. Anson, K. Bailey, and J. T. Edsall, editors. Academic Press, Inc., New York. 253-290.

17. Skerrow, D. 1974. The structure of prekeratin. Biochem. Biophys. Res. Commun. 59:1311-1316.

18. Steinert, P. M., and W. W. Idler. 1975. The polypeptide composition of bovine epidermal α-keratin. Biochem. J. 151:603-614.

19. Steinert, P. M., W. W. Idler, and S. B. Zimmerman. 1976. Self-assembly of bovine epidermal keratin filaments in vitro. J. Mol. Biol. 108:547-567.

20. Steinert, P. M., S. B. Zimmerman, J. M. Starger, and R. D. Goldman. 1978. Ten-nanometer filaments of hamster BHK cells and epidermal keratin filaments have similar structures. Proc. Natl. Acad. Sci. U. S. A. 75:6098-6101.

21. Sun, T. T., and H. Green. 1978. Keratin filaments of cultured human epidermal cells. J. Biol Chem. 253:2053-2060.

22. Sun, T. T., C. Shih, and H. Green. 1979. Keratin cytoskeletons in epithelial cells of internal organs. Proc. Natl. Acad. Sci. U. S. A. 76:2813-2817.

23. Toczala, T., and I. M. Freedberg. 1972. Epidermal structural proteins. Biochem. Biophys. Acta. 263:382-396.

24. Venetie, H. H., and R. Coggeshall. 1965. A simplified lead citrate stain for use in electron microscopy. J. Cell Biol. 25:407-408.

25. Weber, K., and D. J. Kuster. 1971. Reversible denaturation of enzymes by sodium dodecyl sulfate. J. Biol. Chem. 246:4504-4509.