Vol. 103, No. 1, 1981 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 16, 1981 Pages 347-354

TONOFILAMENT PROTEIN, A KERATIN FROM RAT EPIDERMIS

Linda W. DeLap, Roberta K. Brabec, Robert H. Gray, and I. A. Bernstein

Department of Environmental and Industrial Health
The University of Michigan, Ann Arbor, Michigan 48109

Received September 28,1981

SUMMARY: Tonofilament protein from newborn rat epidermis was found to be antigenically related to mouse keratins. Tonofilament protein, like keratins, could be extracted with 8 M urea in Tris buffer. The yield was enhanced by the presence of dithiothreitol. A higher molecular weight (64,000 dalton) protein antigenically cross-reacting with tonofilament protein but with a slightly different amino acid composition was also extracted.

A leucine-rich protein of molecular weight 58,000 was previously isolated from newborn rat epidermis (1). Antibody to this protein localized over tonofilaments in all cell layers of the rat epidermis but not in the dermis. The amino acid composition of this tonofilament protein was noted to be similar to those of other epidermal fibrous proteins (2, 3, 4), and its molecular weight was close to that reported for a mouse keratin (5) and for a rat epidermal fibrous protein (6). Further work, reported here, has demonstrated the antigenic relatedness of tonofilament protein to mouse keratin and to a higher molecular weight protein of slightly different amino acid composition also obtained from newborn rat epidermis. The time course of incorporation of [^{3}H]-leucine into these two proteins *in vivo* was also studied.

MATERIALS AND METHODS

Mouse keratins K_1 and K_2 and rabbit antiserum against K_2 were kindly provided by Drs. P. M. Steinert and S. H. Yuspa, respectively (National Cancer Institute, Bethesda, MD).

Tonofilament protein was prepared as described by Brysk *et al.* (1), with two modifications. The DEAE-cellulose column was eluted with a linear gradient of zero to 0.4 M NaCl in 8 M urea containing 0.05 M Tris-HCl (pH 8.0 at 25°).

Abbreviations: DTT, dithiothreitol; SDS, sodium dodecyl sulfate; Tris, tris-(hydroxymethyl) aminomethane

0006-291X/81/210347-08$01.00/0

[^{3}H]leucine-labeled material eluting near 0.1 M NaCl was pooled. The gel filtration step was replaced by preparative SDS polyacrylamide gel electrophoresis (7).

Rabbit antiserum to tonofilament protein was obtained after 4 weekly subcutaneous injections of New Zealand rabbits with 300 µg of tonofilament protein in 0.05 M sodium phosphate pH 7.5 containing 0.1% SDS, emulsified with an equal volume of complete Freund's adjuvant. Electron microscopic immunolocalization, carried out as previously described (8), confirmed that the antibody bound to tonofilaments in newborn rat epidermis. Ouchterlony double diffusion was carried out in 1% agarose containing 0.5% Triton X-100, 0.1% SDS, 0.9% NaCl, and 0.1% sodium azide (9).

Material which was antigenically related to tonofilament protein was subsequently obtained by extraction of epidermis with urea plus DTT as follows. Skins from CFN strain rats, 1 to 3 days old, were submerged in water at 56° for 30 sec to allow separation of the epidermis from the dermis. Between 10 and 40 epidermal sections were ground in liquid nitrogen with a mortar and pestle and then stirred vigorously for 30 min in 10 volumes (ml/g) of 8 M urea, 0.05 M Tris-HCl pH 8.5 at 25°, 0.01 M DTT (solution A). The extract was obtained by centrifugation at 20,000 g for 20 min. This was applied to a column (1 x 45 cm) of Whatman DE-52 DEAE cellulose equilibrated with solution A (with 1 mM DTT) and the column was washed with 200 ml of solution A. A linear gradient of from zero to 0.4 M NaCl in solution A (total volume 400 ml) was then applied and 5 ml fractions were collected. Fractions which were eluted at 0.05 to 0.12 M NaCl were combined, concentrated by pressure dialysis, and dialyzed against 10 volumes of 8 M urea, 0.02 M sodium acetate, 1 mM DTT, pH 4.5 (solution B). The dialyzed material was applied to a 1 x 25 cm column of Whatman CM-52 carboxymethyl cellulose equilibrated with solution B. This column was washed with 100 ml of solution B and then eluted with solution A containing 0.4 M NaCl. Eluted material was concentrated by pressure dialysis, dialyzed against 0.1% SDS, 0.01 M sodium phosphate, pH 7.5, and subjected to preparative SDS gel electrophoresis (7). Two proteins were obtained, both of which cross-reacted with tonofilament protein antibody.

To study the time course of incorporation of [^{3}H]leucine into the two cross-reacting proteins, newborn littermate rats were injected intraperitoneally with 50 µCi of [^{3}H]leucine (50 Ci/mmol, New England Nuclear, Inc., Boston, MA). The rats were returned to the mother until sacrifice, in groups of 2 or 3, at 1, 6, 12, and 24 h after injection. Epidermis was obtained and extracted with urea as described above, except that DTT was omitted from solution A. The resulting pellet was then extracted with solution A containing 0.01 M DTT as usual. Each extract was applied to a 1.5 x 7 cm column of DEAE cellulose. Columns were washed with 50 ml of solution A with 1 mM DTT and then eluted with 40 ml of solution A containing 1 mM DTT and 0.25 M NaCl. The eluted material was concentrated by pressure dialysis and dialyzed against 0.01 M sodium phosphate pH 7.5, 0.1% SDS. 200 µg samples [Lowry protein (10)] of these protein preparations were subjected to SDS polyacrylamide gel electrophoresis. Gels were stained with Coomassie blue. The bands corresponding to the low and high molecular weight cross-reacting proteins were cut out and digested in 1 ml 30% H_2O_2 overnight at 50°, then counted in 15 ml ACS (Amersham) in a Packard Tri Carb scintillation counter.

Protein samples (125 µg) were hydrolyzed in 6 N HCl at 110° for 24 h. Amino acid analyses were carried out on a Beckman 120 B analyzer.

Analytical SDS polyacrylamide gel electrophoresis was performed using the Miles "SAGE" kit (Miles Laboratories, Elkhart, IN).

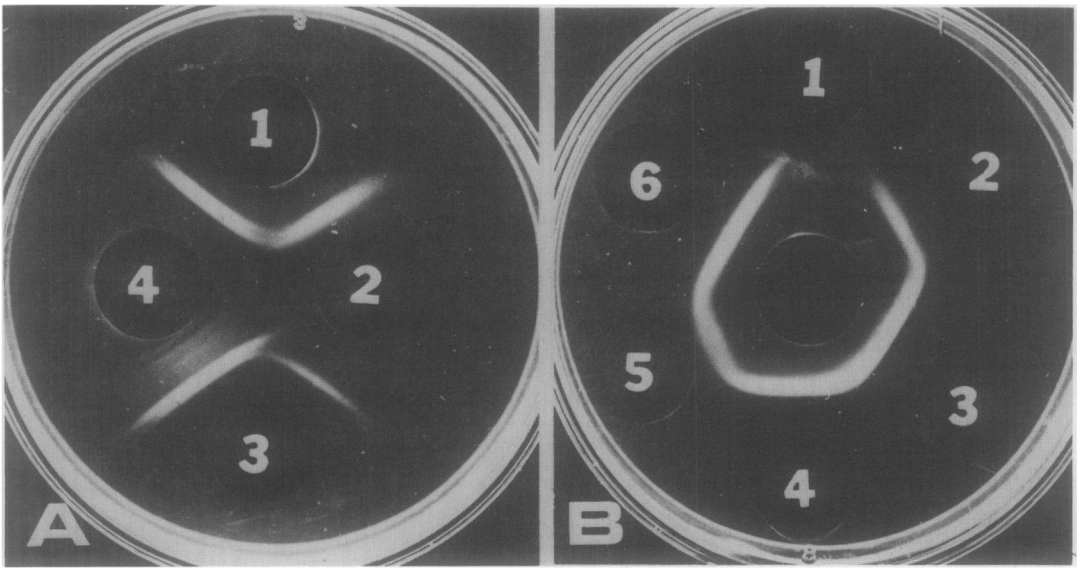

Fig. 1A Double immunodiffusion analysis of rat tonofilament protein and mouse keratin K_2. Well 1, anti-tonofilament protein immunoglobulin; Well 2, tonofilament protein, 0.1 mg/ml; Well 3, anti-mouse keratin K_2 serum; Well 4, mouse keratin K_2, 0.1 mg/ml.

1B Double immunodiffusion analysis of mouse keratin K_1 (Well 1, 0.5 mg/ml, mouse keratin K_2 (Well 2, 0.1 mg/ml), tonofilament protein (Well 3, 0.2 mg/ml), low molecular weight cross-reacting protein (Well 4, 0.2 mg/ml), high molecular weight cross-reacting protein (Well 5, 0.6 mg/ml), urea-DTT extract of newborn rat epidermis (Well 6). Center well, anti-tonofilament protein immunoglobulin.

RESULTS

The antigenic relatedness of tonofilament protein to mouse keratin was shown by double immunodiffusion (Fig. 1A). Antibody to tonofilament protein (well 1) gave a reaction of identity with tonofilament protein and mouse keratin K_2. Antibody to K_2 (well 3) gave a reaction of partial identity, indicating the presence of antigenic sites in K_2 which were not present in tonofilament protein.

Urea-DTT extracts of rat epidermis contained material which reacted with the tonofilament protein antibody (Fig. 1B). Purification by ion exchange chromatography and preparative gel electrophoresis yielded two polypeptides, one of which was evidently tonofilament protein, based on amino acid compo-

Table 1. Amino acid compositions of tonofilament protein and of antigenically cross-reacting proteins (CRPs)

Amino Acid	Tonofilament Protein	Low Molecular Weight CRP*	High Molecular Weight CRP
aspartic acid	8.4	8.6	9.3
threonine	3.7	3.2	3.6
serine	10.8	9.2	10.8
glutamic acid	12.8	14.5	13.5
proline	2.4	2.1	1.9
glycine	24.3	24.6	22.6
alanine	4.9	5.1	3.9
cysteine	n.d.	n.d.	n.d.
valine	4.0	3.0	4.6
methionine	1.2	1.4	1.8
isoleucine	3.0	3.0	3.8
leucine	7.7	8.2	6.8
tyrosine	3.8	3.8	2.6
phenylalanine	2.8	2.9	3.9
histidine	1.3	1.6	0.8
lysine	4.5	4.1	4.7
arginine	4.5	4.8	5.5

*Presumably urea-extracted tonofilament protein
n.d. - not detectable

sition and molecular weight (Table 1, Fig. 2). The other, higher molecular weight cross-reacting protein differed somewhat in amino acid composition. By SDS polyacrylamide gel electrophoresis, the molecular weight of this protein was estimated to be 64,000 daltons. These two proteins were significantly lower in molecular weight than the corresponding mouse keratins K_1 and K_2. Antibody to tonofilament protein appeared to react much more strongly with it than with the higher molecular weight cross-reacting protein (Fig. 1B).

These two proteins could be separated by DEAE ion exchange chromatography of the urea extract, carried out as described in Materials and Methods but with 0.025 M rather than 0.05 M Tris-HCl. Under these conditions a large peak of UV-absorbing, non-cross-reacting material was eluted at the beginning of the NaCl gradient. The high molecular weight cross-reacting protein was eluted by 0.04 M NaCl and tonofilament protein by 0.07 M NaCl (Fig. 2, e and f; Fig. 3).

The time course of incorporation of [^{3}H]leucine into tonofilament protein and into the higher molecular weight cross-reacting protein was determined.

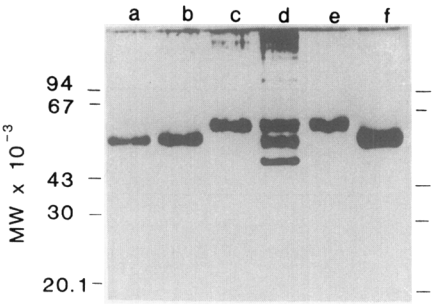

Fig. 2 SDS polyacrylamide gel electrophoresis, tonofilament protein, and antigenically cross-reacting protein. (a) Tonofilament protein, (b) low molecular weight cross-reacting protein, presumably tonofilament protein, (c) high molecular weight cross-reacting protein, (d) urea extract of newborn rat epidermis, (e) peak eluted from DEAE cellulose with 0.04 M NaCl, (f) peak eluted from DEAE cellulose with 0.07 M NaCl.

As described in Materials and Methods, the proteins were purified and the protein content and radioactivity were determined. In this experiment, separate preparations were made from material extracted with urea alone and from material extracted from the resulting pellet with urea plus DTT. The results, presented in Table 2, show that the ratio of specific activities of

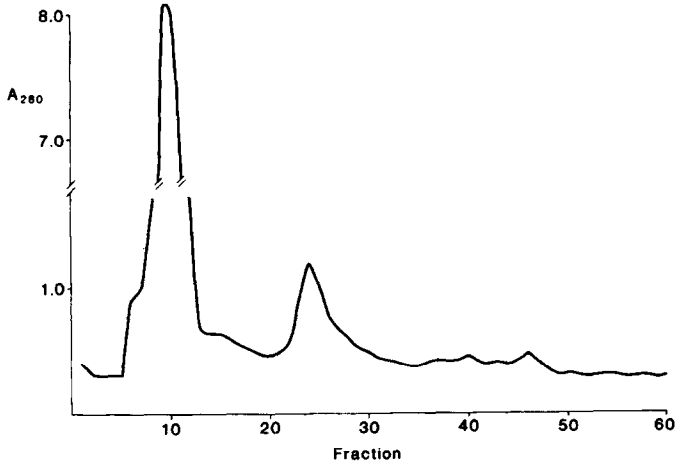

Fig. 3 Elution pattern of urea-DTT extracted material applied to DEAE-cellulose. The column was washed and then eluted with a 300 ml linear gradient from zero to 0.2 M NaCl. 5 ml fractions were collected. Fraction 14 corresponds to 0.04 M NaCl, fraction 24 to 0.07 M NaCl.

Table 2. Time course of incorporation of [^{3}H]leucine into tonofilament protein (TFP) and into higher molecular weight, antigenically cross-reacting protein (CRP), extracted with urea alone or with urea and DTT

Time After Injection	Extraction Medium	Cpm/mg		Ratio of Cpm/mg, TFP/CRP
		TFP	CRP	
1 h	urea	2980	2880	1.03
	urea + DTT	1450	1040	1.39
6 h	urea	4360	4180	1.04
	urea + DTT	1760	1860	0.95
12 h	urea	3520	3760	0.94
	urea + DTT	1700	1440	1.18
24 h	urea	6090	4740	1.28
	urea + DTT	1520	1400	1.09

tonofilament protein and of cross-reacting protein does not change over the course of 24 h. Also, no trend was seen with time in the ratio of specific activities of material purified from the urea extract and of material purified from the residue extracted with urea plus DTT.

DISCUSSION

It was previously observed that antibody to tonofilament protein did not cross-react with bovine epidermal keratin (M. Brysk, unpublished observation). However, antibody raised against tonofilament protein prepared as described did cross-react with mouse keratins. This could be explained by the greater species difference between rat and bovine proteins.

The antigenic relatedness of tonofilament protein to mouse keratin suggested that it could be extracted with 8 M urea and a thiol reducing agent, as are keratins (11, 5, 12). The 58,000 dalton antigenically cross-reacting protein extracted in this manner and purified by ion exchange chromatography and preparative SDS polyacrylamide gel electrophoresis probably represents tonofilament protein. In addition a higher molecular weight cross-reacting protein was obtained, as expected from the results of Huang et al. (6),

Steinert et al. (5), and Milstone (13). It is of interest that antibody to tonofilament protein reacted much more strongly to it than to the higher molecular weight cross-reacting protein. In contrast, antibody to mouse keratin K_1 reacted equally well with mouse keratin K_2 (5).

The results of the time course study do not indicate that a precursor-product relationship exists between the cross-reacting protein and tonofilament protein; there was no change in the ratio of their specific activities with time after [^{3}H]leucine injection. Analysis of the amino acid composition (14) and of the polypeptide fragments produced by partial enzymatic hydrolysis (15) of other keratins has led other investigators to dismiss the possibility that the larger keratins are precursors of the smaller ones. It is of interest that there was also no clear trend in ratio of specific activities of tonofilament protein or of cross-reacting protein extracted with urea to that extracted with urea plus DTT. It was expected that this ratio might decrease with time after injection, as disulfide bonds formed in the newly synthesized keratins (12).

In conclusion, it seems appropriate to regard tonofilament protein as a keratin. Since tonofilaments are a type of intermediate-sized filament, these results together with the previous immunolocalization studies (8) support the thesis that keratins are proteins of epidermal intermediate filaments (16).

ACKNOWLEDGMENTS

Supported by Grants Nos. 5 R01 AM15206 and 5 T32 AM07236 from the U.S. Public Health Service, National Institutes of Health.

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