Trichohyalin, an Intermediate Filament–associated Protein of the Hair Follicle

Joseph A. Rothnagel and George E. Rogers
Department of Biochemistry, University of Adelaide, Adelaide, South Australia 5000, Australia

Abstract. A precursor protein associated with the formation of the citrulline-containing intermediate filaments of the hair follicle has been isolated and characterized. The protein, with a molecular weight of 190,000, was isolated from sheep wool follicles and purified until it yielded a single band on a SDS polyacrylamide gel. The M_r 190,000 protein has a high content of lysine and glutamic acid/glutamine residues and is rich in arginine residues, some of which, it is postulated, undergo a side chain conversion in situ into citrulline residues. Polyclonal antibodies were raised to the purified protein, and these cross-react with similar proteins from extracts of guinea pig and human follicles and rat vibrissae inner root sheaths. Tissue immunochemical methods have localized the M_r 190,000 protein to the trichohyalin granules of the developing inner root sheath of the wool follicle. We propose that the old term trichohyalin be retained to describe this M_r 190,000 protein.

Immunoelectron microscopy has located the M_r 190,000 protein to the trichohyalin granules but not to the newly synthesized filaments. This technique has revealed that trichohyalin becomes associated with the filaments at later stages of development. These results indicate a possible matrix role for trichohyalin.

THE hardened structures of the medulla and inner root sheath (IRS)[1] tissues of the hair and hair follicle have been shown to be composed of insoluble proteins that are readily distinguishable from *a*-keratin (30). The major distinguishing features of these proteins include the presence of citrulline together with a high glutamic acid/glutamine content (26, 28, 34, 39, 40). Protein-bound citrulline is derived by a posttranslational modification of arginine residues mediated by the enzyme peptidyl-arginine deiminase (34). In addition, the citrulline-rich proteins are cross-linked by isopeptide bonds formed between lysine and glutamine residues, by the action of a transglutaminase and not by the disulfide bonds that characterize keratins (16).

Medulla cells develop from the undifferentiated cells just above the dermal papilla and become the central core of cells in the hair fiber. As these cells differentiate, accumulations of electron-dense, amorphous granules appear in their cytoplasm. Some workers distinguish between these medullary granules and those with similar morphology, termed trichohyalin granules, of the IRS. However, histochemical, biochemical, and immunochemical studies have suggested that the granules of the medulla and IRS share common characteristics (29, 30, 33, 34). Hence, in the present discussion, the term trichohyalin will be applied to the granules of both tissues. At later stages of development, the number and size of the granules increase, and in a rapid transformation the granules coalesce, forming a hardened amorphous material (24). In general, the hardened proteins of the medulla lack organization, although filamentous structures have been observed (30).

By comparison, the IRS develops from cells alongside the dermal papilla. During development these cells produce the electron-dense trichohyalin granules, first described by Vörner (45). Later, these cells develop filaments that are of the intermediate type (37) and appear to be closely associated with the trichohyalin granules (24, 27, 30). At the final stage of development the granules disappear and the cells become filled with protein filaments, orientated parallel to the fiber axis (2, 24, 30). With further maturation, the filaments coalesce and harden and the cells fuse, presenting a homogenous appearance (2, 27).

Thus, the process of development is similar in both medulla and IRS tissues, but the differences in the morphology of their final products, generally amorphous versus filamentous, still remains to be explained. Histochemical evidence (29, 33), autoradiographic analysis (33), and ultrastructural studies (30, 31) support the view that trichohyalin granules contain the precursors to the citrulline-containing proteins of fully differentiated medulla and IRS tissues. A partially purified fraction from guinea pig hair follicles had been previously characterized as being derived from trichohyalin granules (34). This arginine-rich fraction, termed trichohyalin precipitate (TR-PPT), was shown to contain suitable substrates for both peptidyl-arginine deiminase and hair follicle transglutaminase (34). Histochemical and immunochemical studies have located hair follicle transglutaminase and suitable substrate proteins in the cells of the medulla and IRS (5).

Notwithstanding these studies, it was not understood why

[1] *Abbreviation used in this paper:* IRS, inner root sheath; TR-PPT, trichohyalin precipitate.

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the citrulline-rich proteins of the medulla differ so markedly in their morphology from those of the IRS. Furthermore, these studies could not reveal whether the arginine-rich protein, trichohyalin, is a precursor to the IRS filaments or whether it is a component of the interfilamentous matrix. This study was aimed at resolving these questions. The present work reports the isolation of trichohyalin from sheep wool follicles in an undegraded form. Antiserum against the sheep follicle protein was used to identify the trichohyalins of guinea pig, rat, and human follicles. Immunoelectron microscopy has unequivocally located the M_r 190,000 protein to the trichohyalin granules of the hair follicle. The possible role of trichohyalin in the medulla and IRS tissues is discussed with respect to this new evidence.

Materials and Methods

Preparation of Follicle Proteins

Intact wool and hair roots were obtained from Border-Leicester × Dorset Horn sheep and shorn dorsal skin of young guinea pigs (300 g) by epilation with fast-setting dental resin (Vertex, Dentimex, Zeist, Holland) and stored in liquid nitrogen before extraction. Human hair follicles were obtained using a wax method similar to that described by Clarke and Rogers (6). Rat vibrissae follicles were plucked and their IRSs were dissected as described before (26).

Follicles were gently dispersed in 7 M guanidine-HCl, 50 mM Tris-HCl, pH 7.6, 1 mM EDTA (10 ml/g tissue) for 10 min on ice. Two other extraction conditions were tested for their ability to minimize proteolysis in tissue samples. Follicles were either incubated on ice for 10 min in 8 M urea, 50 mM Tris-HCl, pH 7.6, 1 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, and 10 mM *p*-tosyl-L-arginine methyl ester, or incubated at 100°C in 1% (wt/vol) SDS, 5% (vol/vol) 2-mercaptoethanol, 50 mM Tris-HCl, pH 7.6, 1 mM EDTA for 10 min.

The extract was centrifuged at 38,000 *g* for 10 min to remove insoluble material, and the supernatant was filtered through a glass fiber disk (Millipore Corp., Bedford, MA) to remove extraneous hair fibers. The solution was then concentrated fivefold (YM10 membrane, Amicon Corp., Danvers, MA) and stored at −20°C. At these temperatures the extracts could be stored indefinitely without detectable degradation. Unless otherwise stated, all procedures were carried out at 4°C.

Purification of Trichohyalin

Wool follicle extracts were chromatographed on a molecular sieving CL-4B column (85 × 1.5 cm, Pharmacia, Uppsala, Sweden). This column was equilibrated with 7 M guanidine-HCl, 50 mM Tris-HCl, pH 7.6, 100 mM NaCl, and 1 mM EDTA. 2 ml of the extract (10 mg/ml) was chromatographed at a flow rate of 24 ml/h, and 8-ml fractions were collected. Fractions containing trichohyalin were dialyzed exhaustively against water at 4°C. Precipitable material was collected by centrifugation, 38,000 *g* for 10 min, and stored as a precipitate at −20°C.

Analytical Procedures

Samples were diluted 1:1 with 2× SDS sample buffer, boiled for 2–5 min, and electrophoresed in 7.5% acrylamide slab gels according to the procedure of Laemmli (20). After electrophoresis the proteins were visualized by the method of Swank and Munkres (43). Samples from guanidine extracts were either diluted fivefold with 8 M urea or dialyzed against water to avoid precipitation of guanidine in the SDS sample buffer.

Acid-urea gels with a running pH of 2.7 were prepared according to Hurley's (18) modification of the Panyim and Chalkley method (23).

Amino acid analysis was kindly performed by Mr. A. S. Inglis, C.S.I.R.O., Division of Protein Chemistry, Melbourne.

Phosphate analysis on guinea pig TR-PPT was determined by a modification of the colorimetric procedure of Sumner (42) as described by Bitte and Kabat (3).

In Vivo Labeling of Follicle Proteins

A 300-g guinea pig was injected intraperitoneally with 3 mCi [^{32}P]phosphate, and the follicles were harvested 2 h later. Follicle proteins were extracted by the urea procedure as described above. Aliquots of the soluble material were electrophoresed on a 7.5% SDS polyacrylamide gel and autoradiographed.

Preparation of Antibodies

Final purification of the M_r 190,000 precursor was achieved by preparative SDS gel electrophoresis of trichohyalin-enriched fractions. These 7.5% (wt/vol) acrylamide gels contained 0.3% (wt/vol) *N,N*-bisacrylylcystamine (Bio-Rad Laboratories, Richmond, CA), which forms a cross-link cleavable by thiols (14). The M_r 190,000 protein was detected by Coomassie Blue staining, and the band, estimated to contain 0.2 mg protein, was excised from the gel. The gel slice was washed in 3 M Tris-HCl, pH 8.0, and solubilized in 0.5 ml 5% (vol/vol) 2-mercaptoethanol in phosphate-buffered saline (PBS), 10 mM Na phosphate, pH 7.3, 0.9% (wt/vol) NaCl, emulsified in 0.5 ml Freund's complete adjuvant, and injected subcutaneously into rabbits. The animals did not suffer any obvious side effects from either the acrylamide or the reducing agent. Booster injections of the M_r 190,000 protein in incomplete adjuvant (0.2 mg/rabbit) were given after 8 d. Preimmune sera and antisera were stored at −20°C in 1-ml aliquots.

Immunoradiography

Immunoradiography was performed according to the procedure of Burnette (4). Samples were electrophoresed on SDS slab gels, transferred electrophoretically to nitrocellulose, and reacted with antiserum diluted 40-fold in 10 mM Tris-HCl, pH 7.4, 0.9% (wt/vol) NaCl and 5% (wt/vol) bovine serum albumin. Specific antigen-antibody complexes were detected by ^{125}I-labeled *Staphylococcus* protein A (Amersham, U.K.) and autoradiography.

Immunoelectron Microscopy

For these studies, the M_r 190,000 antiserum was purified by protein A-Sepharose CL-4B chromatography (17), and the IgG fraction was rechromatographed on an affinity column of the sheep follicle M_r 190,000 protein covalently linked to Sepharose 4B (Pharmacia). The purified antibody was dialyzed overnight against distilled water, concentrated by evaporation to 0.25–0.5 mg/ml, and stored at −20°C. Rat vibrissae IRSs were fixed in 4% (vol/vol) paraformaldehyde, 0.25% (vol/vol) glutaraldehyde in 100 mM Na phosphate, pH 7.3, for 1 h at 4°C. The IRSs were washed with 1 M NH_4Cl for 30 min at 4°C, and then in PBS buffer for 5 min at 4°C. Dehydration of the tissue and subsequent resin infiltration and polymerization were performed according to the manufacturer's instructions for Lowicryl K4M resin (Chemische Werke Lowi, Waldkraiburg, Federal Republic of Germany). Sections were cut at 400 and 20 nm. The thick sections were stained with 1% (wt/vol) toluidine blue and examined under a light microscope to locate specific regions. The thin sections were mounted on parlodion-covered 200 mesh copper grids and treated with Blotto (19) after the mixture, 5% (wt/vol) milk powder in PBS, had been clarified by centrifugation at 38,000 *g* for 10 min. The concentrated and purified antibody was diluted 20-fold in PBS and reacted with the sections overnight at 4°C. The sections were then rinsed with PBS before incubation with protein A-gold (15-nm particles, Janssen Pharmaceutica, Beerse, Belgium), diluted 20-fold in PBS, for 30 min at 25°C. Sections were rinsed free of unbound protein A-gold with water and stained for 5 min with saturated uranyl acetate in 70% (vol/vol) ethanol. Sections were rinsed sequentially with ethanol and water before viewing in a Seimens Elmiskop 102 electron microscope.

Results

Isolation of Intact Trichohyalin

In earlier studies, S-carboxymethyl-modified keratins were separated from the arginine-rich trichohyalin fraction by ion-exchange chromatography. This fraction, designated TR-PPT by Rogers et al. (34), yielded several bands on pH 2.7 and SDS acrylamide gels (Fig. 1), and because of this complexity, trichohyalin was thought to consist of a family of related proteins (32). From the present study it is clear that these procedures produce considerable degradation of trichohyalin. Fig. 2 shows the progressive degradation of sheep trichohyalin after S-carboxymethylation, detected by immunoradiography. This degradation is believed to be caused by the action of proteases released during the disruption of hair follicle cells rather than by the S-carboxymethylation step itself. Therefore, to limit proteolytic activity and preserve trichohyalin intact, all manipulations were carried out on ice and the S-carboxymethylation step was omitted. Instead, hair follicles were

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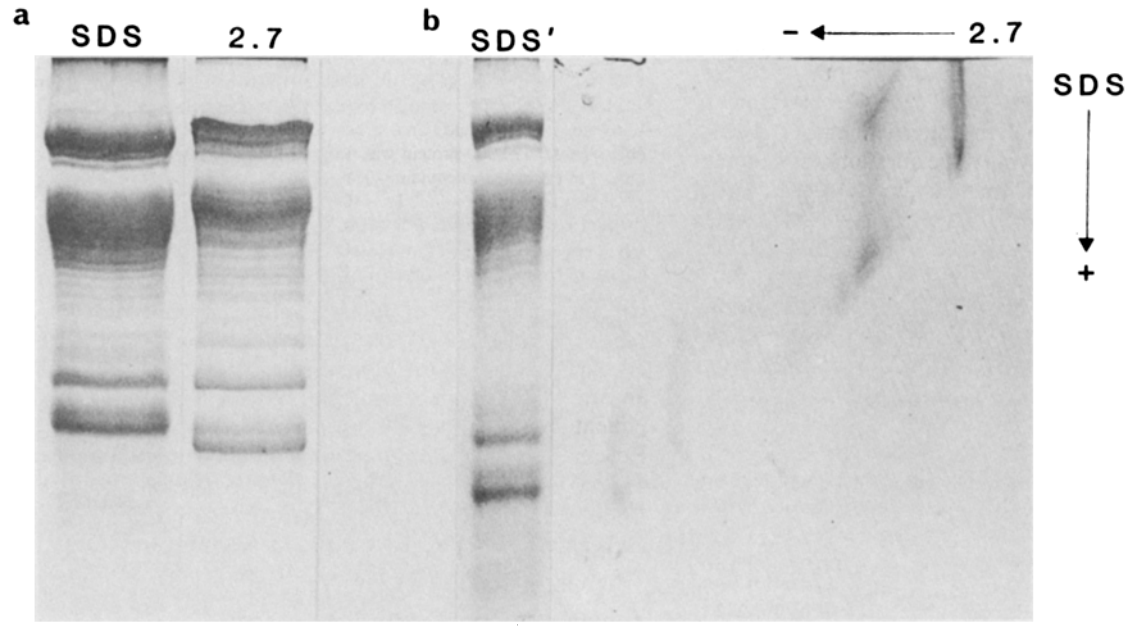

Figure 1. (*a*) Electrophoresis of guinea pig TR-PPT on 10% acrylamide SDS and pH 2.7 gels. The proteins were visualized by Coomassie Blue staining. (*b*) Two-dimensional electrophoresis of guinea pig TR-PPT. First dimension (horizontal) electrophoresis on a 7.5% acrylamide gel at pH 2.7. Second dimension (vertical): electrophoresis on a 7.5% acrylamide gel in SDS. SDS', TR-PPT electrophoresed on a 7.5% acrylamide SDS gel for comparison.

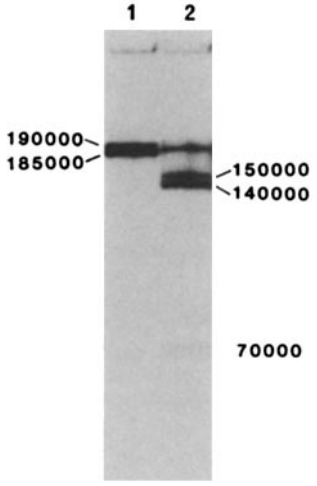

Figure 2. Immunoradiograph of sheep follicle extracts separated on a 7.5% acrylamide SDS gel and reacted with antibody to sheep trichohyalin. Lane *1*, follicle proteins extracted in the urea buffer; lane *2*, as for lane 1 but S-carboxymethylated. Note the two protein bands with molecular weights 190,000 and 185,000 in lane 1.

extracted in buffered 7 M guanidine lacking reducing agents (see Materials and Methods). Under these conditions, the keratin proteins are not solubilized to any great extent, and an enrichment of the M_r 190,000 protein is observed (Fig. 3). The simple gel electrophoretic profile observed for trichohyalin obtained by this procedure contrasts with the complex pattern seen in earlier studies (Fig. 1).

However, even with this procedure, trichohyalin was susceptible to breakdown, and in most preparations a second protein of molecular weight 185,000 was observed (see Fig. 2). Two other extraction conditions were tested for their ability to minimize proteolytic digestion of trichohyalin (see Materials and Methods). Neither the urea nor the SDS/2-mercaptoethanol extraction procedure afforded greater protection to trichohyalin from proteolytic degradation than the guanidine method. In addition, the former procedures extracted keratins more efficiently than did the guanidine buffer, and they were therefore less useful for isolating trichohyalin (Fig. 3).

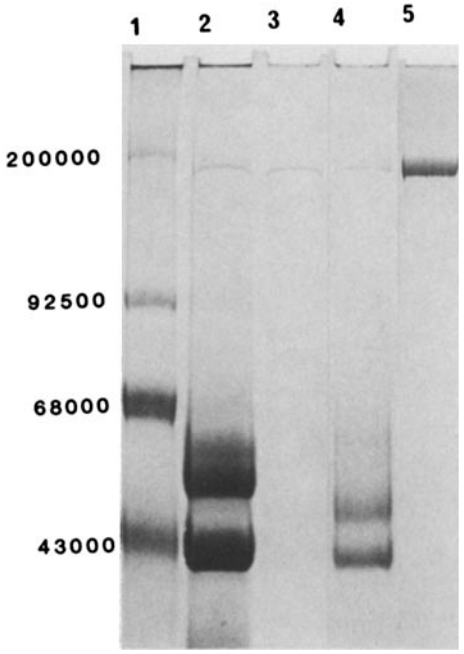

Figure 3. Sheep follicle proteins electrophoresed on 7.5% acrylamide SDS gels and visualized by Coomassie Blue staining. Lane *1*, protein standards (Bethesda Research Laboratories, Gaithersburg, MD) with molecular weights indicated; lane *2*, follicle proteins extracted in the SDS/2-mercaptoethanol buffer; lane *3*, follicle proteins extracted in the guanidine buffer; lane *4*, follicle proteins extracted in the urea buffer; and lane *5*, trichohyalin-enriched fraction (see Fig. 4).

Molecular Sieving Chromatography

Molecular sieving chromatography was used to rapidly separate trichohyalin from the lower molecular weight keratins. Fig. 4 shows the elution profile of wool follicle proteins extracted in guanidine buffer. Column fractions were dialyzed extensively against water, and the precipitates were analyzed

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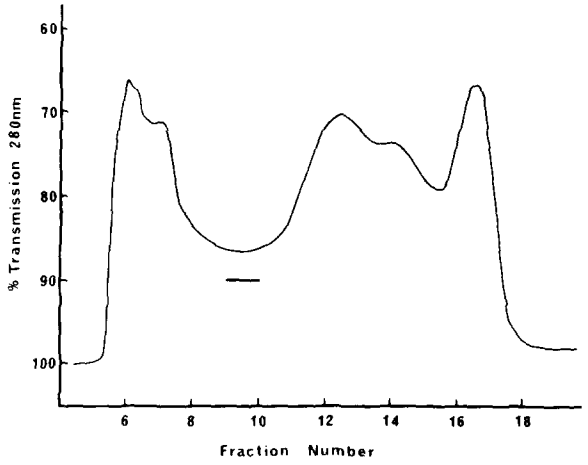

Figure 4. Chromatography of sheep follicle protein extracts on Sepharose CL-4B. The bar indicates the fraction (fraction 9) most enriched for trichohyalin (see Fig. 3).

Table I. Amino Acid Composition of Trichohyalin

Amino acid	Wool follicle trichohyalin
	mol percent
Aspartate/asparagine	6.1
Threonine	3.0
Serine	5.5
Glutamate/glutamine	17.0
Proline	4.0
Citrulline	0.0
Glycine	6.4
Alanine	5.3
Half-cystine	0.7
Valine	4.1
Methionine	1.0
Isoleucine	2.9
Leucine	8.5
Tyrosine	2.1
Phenylalanine	2.7
Lysine	11.6
Histidine	2.7
Arginine	16.5

by SDS gel electrophoresis to locate the fractions containing the M_r 190,000 precursor. With this procedure, trichohyalin could be obtained with >95% purity, as judged by Coomassie Blue-stained gels (Fig. 3).

Amino Acid Composition of Trichohyalin

The fraction most enriched for the precursor (fraction 9, Fig. 4) was analyzed for its amino acid composition (Table I). Sheep trichohyalin contains little or no cysteine but has a relatively high content of glutamic acid/glutamine, lysine, and arginine. Citrulline and orinithine were not observed. In these respects, sheep trichohyalin is similar to the arginine-rich fraction (TR-PPT) from guinea pig hair follicles (34).

Phosphate Determination

Trichohyalin appears not to be phosphorylated. This was determined by the colorimetric assay of Sumner (42) on guinea pig TR-PPT and by SDS gel analysis of guinea pig hair follicle proteins after an intraperitoneal injection of [^{32}P]phosphate. Although radioactivity could be detected in hair follicles after 2 h, neither trichohyalin nor the keratins were measurably phosphorylated (data not shown). This is in direct contrast to the epidermal situation, where both keratins (13) and filaggrin (21) are extensively phosphorylated.

Molecular Weight Determination of Trichohyalin

The molecular weight of trichohyalin was determined by its migration in an SDS gel relative to the migration of proteins with known molecular weights. The molecular weight of sheep trichohyalin was determined to be 190,000 (Fig. 3). By comparison with the wool follicle protein, trichohyalin has a molecular weight of 200,000 in the guinea pig, 210,000 in the rat, and 220,000 in the human (Fig. 5). It is interesting that an M_r 200,000 protein had been observed as a minor component in the earlier study on guinea pig trichohyalin by Rogers et al. (34). It is clear from the present study that the M_r 200,000 band represents the undegraded form of trichohyalin and that the major protein bands observed in the earlier study were breakdown products (compare Figs. 1 and 5).

Localization of Trichohyalin in the Hair Follicle

Immunoelectron microscopy has shown unequivocally that the M_r 190,000 protein is located in the trichohyalin granules of both the medulla (Fig. 6) and IRS (Fig. 7). Note that only the trichohyalin granules were labeled and that the apparently emerging and closely-associated IRS filaments did not react. Likewise, there was no staining of the filaments of the fiber cortex (Fig. 6). Interestingly, anti-trichohyalin staining was observed in those cells that had recently undergone the transition from a granular to a filamentous state (Fig. 8). In later stages of IRS development, where the filaments had coalesed to form a hardened, homogeneous structure, anti-trichohyalin staining again was not evident (Fig. 9). Presumably, the antigenic determinants of the fully mature cells were unavailable to the antibody, perhaps because of steric limitations, protein conformational changes, or protein degradation. The possibility of degradation needs to be considered because of the susceptibility of trichohyalin to proteolytic breakdown. The specificity of the antibody staining reaction was demonstrated by a variety of control procedures. Absorption of the M_r 190,000 antiserum with trichohyalin abolished the staining of the trichohyalin granules (Fig. 10). Application of protein A-gold alone did not result in staining of the granules.

Discussion

These studies have identified trichohyalin as a single, high molecular weight, nonphosphorylated protein located in the trichohyalin granules of medulla and IRS cells. In addition, the protein has a characteristically high content of arginine, glutamic acid/glutamine, and lysine residues. The trichohyalins of sheep, guinea pig, rat, and human follicles were identified and have molecular weights of 190,000, 200,000, 210,000 and 220,000, respectively.

It had been believed that trichohyalin consisted of a family of related arginine-rich proteins with molecular weight heterogeneity (32, 34). Within this context, the initial aspects of the study were aimed to characterize further TR-PPT. The electrophoretic profile of TR-PPT on SDS gels was similar to the oligomeric series observed for the filaggrins (7, 25, 44). Recent studies have shown that the filaggrin oligomers are

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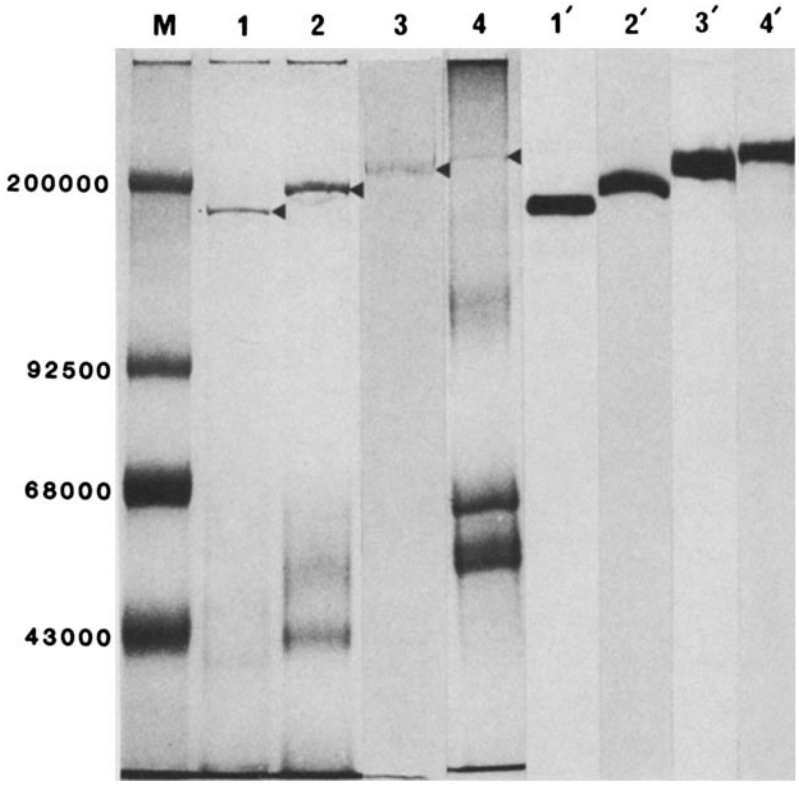

Figure 5. Protein extracts from sheep, guinea pig, rat, and human follicles electrophoresed on a 7.5% acrylamide SDS gel. Proteins were visualized by Coomassie Blue staining. Lane *M*, protein standards; lane *1*, sheep follicle proteins; lane *2*, guinea pig follicle proteins; lane *3*, rat vibrissae IRS proteins; and lane *4*, human follicle proteins. The proteins marked with arrowheads were identified as trichohyalin by immunoradiography of follicle extracts reacted with antibody raised against the sheep M_r 190,000 protein. Lane *1′*, immunoradiograph of sheep follicle proteins; lane *2′*, guinea pig follicle proteins; lane *3′*, rat vibrissae IRS proteins; and lane *4′*, human follicle proteins.

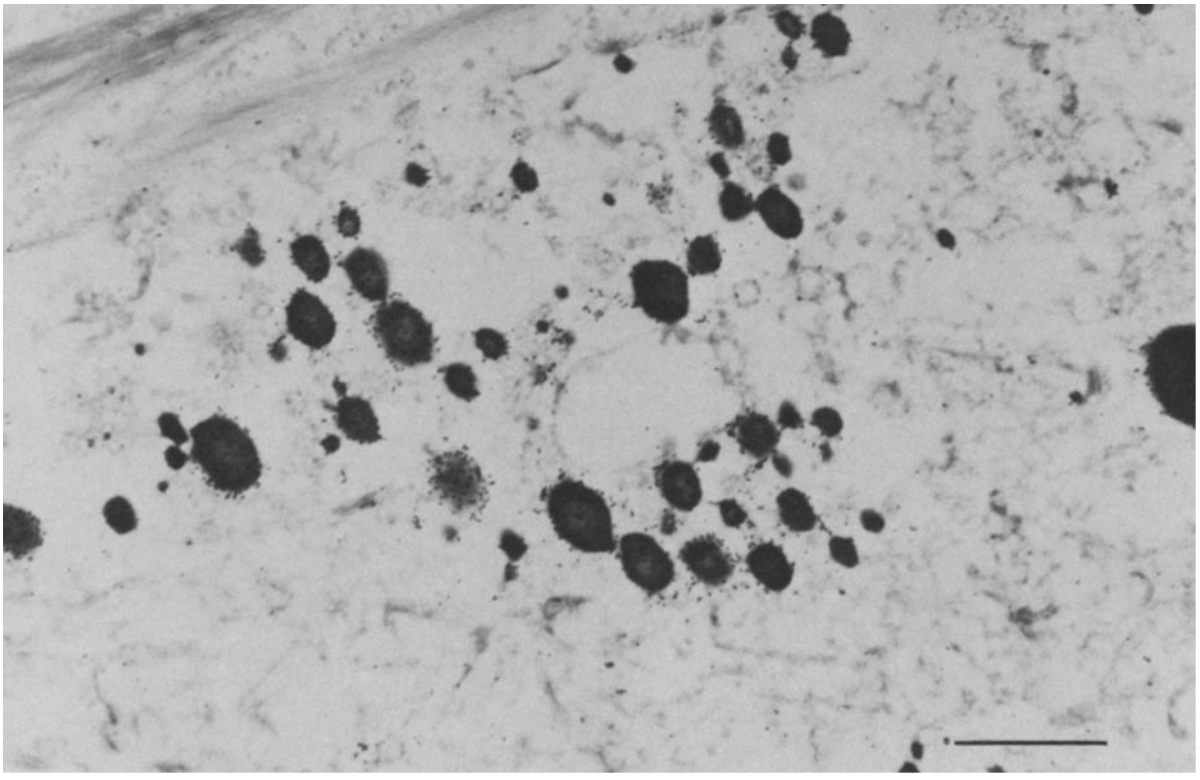

Figure 6. Immunoelectron microscopy of a rat vibrissa follicle. Shown is a section reacted with anti-trichohyalin and detected with protein A-gold. The field of view shows the granules of the medulla and part of the fiber cortex. Note that the keratin intermediate filaments of the cortex have not reacted with the antibody. Bar, 1 μm.

the proteolytic breakdown products of a high molecular weight precursor (25, 35). The evidence of the present study suggests that the observed complexity of TR-PPT also arose from the degradation of a high molecular weight protein by proteolytic activity. Analysis of TR-PPT by two-dimensional gel electrophoresis has demonstrated that the proteins of the arginine-rich fraction have identical charge-mass ratios. This result suggests that trichohyalin may be composed of similar

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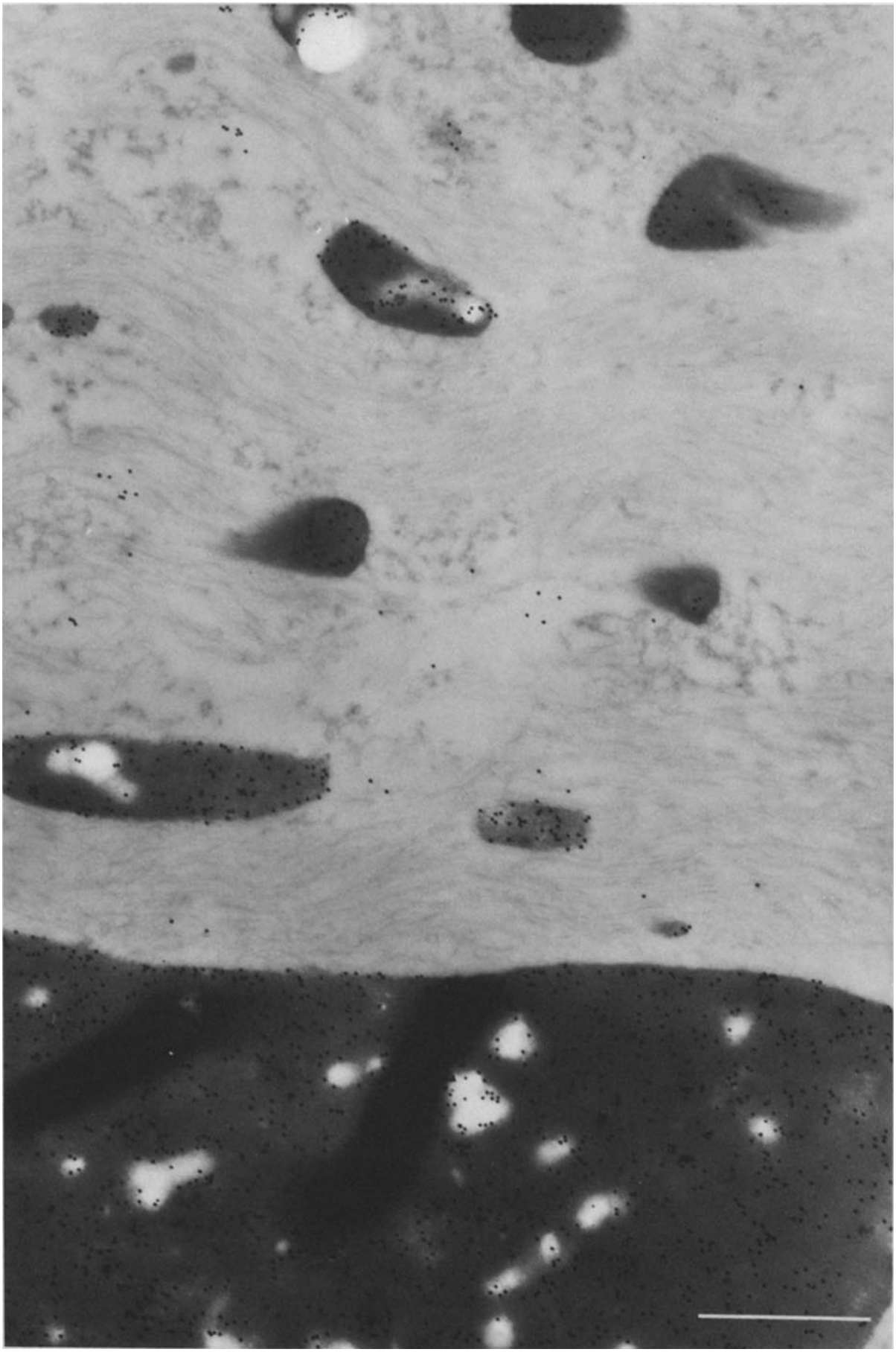

Figure 7. Immunoelectron microscopy of a rat vibrissa follicle reacted with anti-trichohyalin and detected with protein A-gold. The field of view shows the trichohyalin granules and associated filaments of the IRS. Bar, 1 μm.

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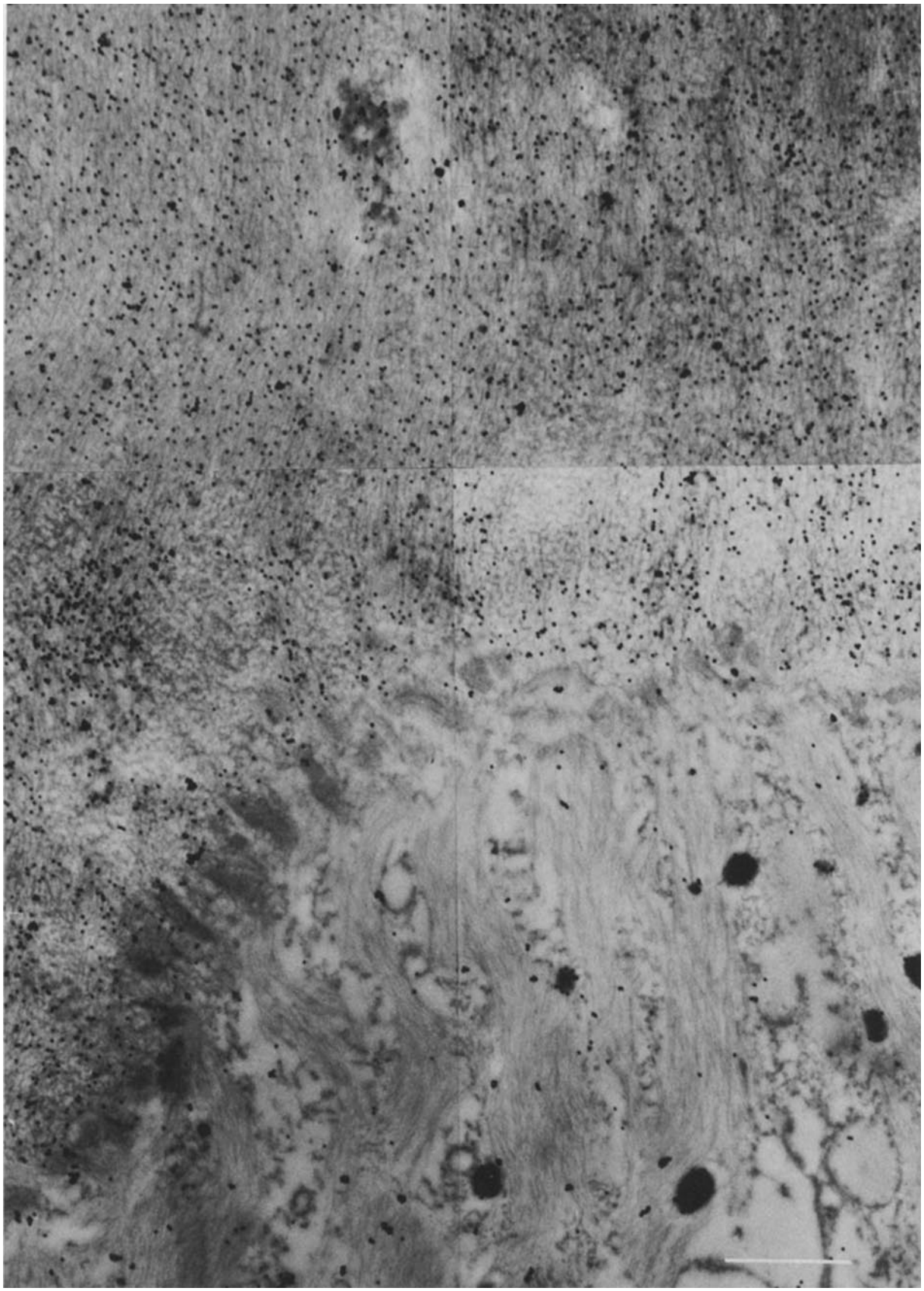

Figure 8. Immunoelectron microscopy of a rat vibrissa follicle reacted with anti-trichohyalin and detected with protein A-gold. This montage shows the division between the granular/filamentous and fully filamentous cells of the IRS. Bar, 1 μm.

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Figure 9. Immunoelectron microscopy of a rat vibrissa follicle reacted with anti-trichohyalin and detected with protein A-gold. The field of view shows a filamentous cell of the IRS, adjacent to a more mature cell where the filaments have begun to coalesce and harden. Bar, 1 μm.

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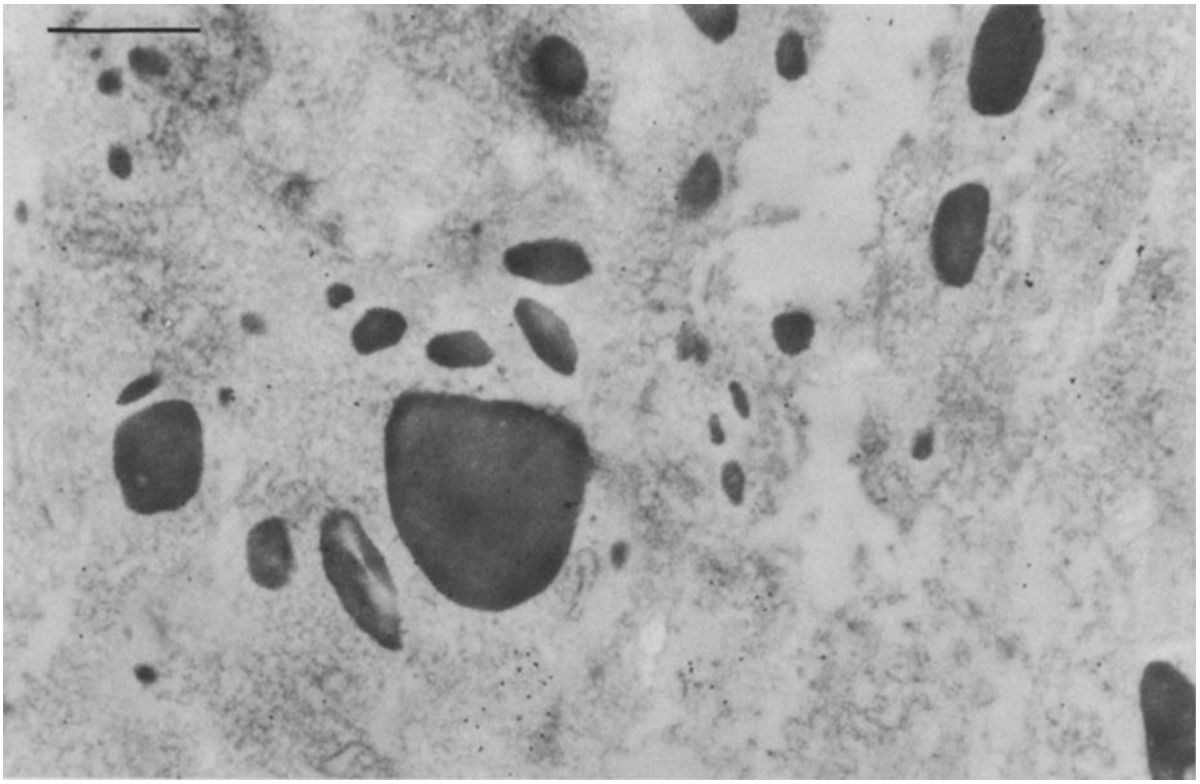

Figure 10. Trichohyalin granules of a rat vibrissa IRS after reaction with absorbed antibody to sheep trichohyalin. The specific antiserum had been reacted wtih pure antigen (M_r 190,000 protein) before incubation with the follicle section. Bar, 1 μm.

repeating domains that are bounded by protease-sensitive regions. Such a tandem repeating structure has been postulated for profilaggrin (22). As is the case for the filaggrins, trichohyalin may undergo specific proteolytic processing during hair growth, giving rise to a smaller functional unit. However, the evidence suggests that trichohyalin is not normally exposed to proteases, since under controlled conditions the protein can be observed as a single high molecular weight species in follicle extracts (Fig. 5). This implies that the supposed proteolytic processing of trichohyalin would have to occur after the proteins are rendered insoluble by glutamyl-lysine cross-links.

It is possible that endogenous proteases may have been released by the disruption of hair follicle cells during the extraction procedures. Proteases have long been recognized as playing a major role in the catabolism of epidermal proteins (10). In the hair follicle it has been postulated that the degradation of the IRS occurs by the action of proteases produced in the tissue or by resident bacteria (12). These proteases warrant further study. They appear to be very hardy proteins and remain active in the presence of strong denaturants such as urea and SDS. Some of the enzymes are not of the arginine-specific serine protease class, as inhibitors such as phenylmethylsulfonyl fluoride and TAME were only partially effective in their protection of trichohyalin.

The application of immunoelectron microscopy has unequivocally confirmed the presence of the M_r 190,000 protein in the trichohyalin granules. On this evidence, the M_r 190,000 protein was termed trichohyalin, but it is possible that these granules are composed of more than one type of protein, as is the case for keratohyalin granules (1). It is observed by immunoelectron microscopy that, although the antibody to the M_r 190,000 protein reacts with the trichohyalin granules of the IRS cells, it does not bind to the newly synthesized filaments adjacent to those granules. Yet, later in the development of these cells, when the components of the granules are dispersed, the antibody is bound throughout the filamentous regions. This evidence suggests a matrix role for trichohyalin.

It is of interest therefore, to compare trichohyalin with filaggrin (9, 38), a matrix protein associated with epidermal keratin filaments and derived from keratohyalin granules (8). It is known that filaggrin is a substrate for peptidyl-arginine deiminase (15) and it is thought that the conversion of basic arginine residues to neutral citrulline is important to the catabolism of filaggrin (15). Although the immunoradiographic data suggest that trichohyalin is not processed in vivo to a smaller protein, the sensitivity of trichohyalin to proteolytic degradation is similar to that of profilaggrin, and like filaggrin, trichohyalin may be composed of repeated domains.

The proposed matrix function of trichohyalin can be reconciled with the observed properties of both the medulla and the IRS tissues. A matrix role for trichohyalin would suggest that the citrulline residues associated with IRS filaments (36) are derived from trichohyalin fragments, cross-linked to the filaments at their nonhelical terminal domains. The nonhelical regions of epidermal keratin filaments have been shown to be important sites of interaction with other proteins such as filaggrin (41). An analogous situation exists in the hair fiber, since the high-sulfur proteins of the fiber matrix are believed to form extensive disulfide cross-links with the relatively cysteine-rich, nonhelical domains of the low-sulfur keratin filaments (11, 37).

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In proposing the function of trichohyalin as a matrix component, it is of paramount importance to consider the implications for the granules of the hair medulla. It has always been difficult to reconcile the similar chemistry of the medulla and IRS (citrulline residues and cross-links) with the differences in morphology. The matrix hypothesis leads directly to the conclusion that the citrulline-rich proteins of the medulla are composed of extensively cross-linked trichohyalin, forming the hardened amorphous material characteristic of these cells.

Although the matrix role for trichohyalin is the favored conclusion from this study, it is clear that further studies at the biochemical level are required. These should include the determination of the primary structure of trichohyalin. The amino acid sequence of trichohyalin, obtained either by direct protein sequencing or from its cDNA, would aid in defining the nature of trichohyalin and determine whether trichohyalin consists of tandemly repeated domains as has been suggested for profilaggrin. In addition, the primary structure data would provide insights into the local environment of the arginine residues that are converted by peptidyl-arginine deiminase. The biological role of citrulline in these tissues is not known. The immunoelectron microscopy study suggests that the contents of the trichohyalin granules are dispersed very rapidly throughout the IRS cells. The two states, granular/filamentous and fully filamentous, apparently occur within adjacent cells. An intermediate state, where the antibody might be expected to bind to arrays of filaments streaming from the granules to form the filamentous regions, was not observed in this study. From the amino acid data (Table 1) and the binding properties to DEAE-cellulose (34), it can be deduced that trichohyalin is an essentially neutral molecule with the basic charges of arginine and lysine balanced by the acidic charges of aspartic and glutamic acid. Therefore, the conversion of arginine residues to neutral citrulline would leave trichohyalin with an overall negative charge. It is possible that electrostatic repulsion between the negatively charged molecules might be involved in the dispersal of the granules from these cells. In addition, the change in the distribution of charge along the molecule may promote the interaction between trichohyalin and the terminal domains of the IRS filaments. Future studies should ascertain whether trichohyalin (both arginine and citrulline-rich forms) can form macrofibrils in vitro when mixed with IRS filaments. A study of this type has defined the role of filaggrin, where it potentiated the aggregation of epidermal, IRS, vimentin, and desmin intermediate filaments into highly ordered linear structures resembling macrofibrils (38).

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