Proc. Natl. Acad. Sci. USA
Vol. 78, No. 12, pp. 7360–7364, December 1981
Biochemistry

Ultrastructural identification of extension aminopropeptides of type I and III collagens in human skin

(immunoelectron microscopy/procollagen fibril growth)

RAUL FLEISCHMAJER*, RUPERT TIMPL†, LEENA TUDERMAN‡§, LAURIE RAISHER*, MANFRED WIESTNER†¶, JEROME S. PERLISH*, AND PETER N. GRAVES*

*Department of Dermatology, Mount Sinai School of Medicine, One Gustave L. Levy Place, New York, New York 10029; †Max-Planck-Institut fur Biochemie, 8033 Martinsried Bei Munchen, Federal Republic of Germany; and ‡Department of Biochemistry, College of Medicine and Dentistry of New Jersey-Rutgers Medical School, Piscataway, New Jersey 08854

Communicated by Hans Popper, August 21, 1981

ABSTRACT **Human skin was labeled with purified antibodies against type I and III collagens and against their extension aminopropeptides by using the ferritin technique. Both aminopropeptides were visualized mainly along thin collagenous fibrils (diameter, 20–40 nm) and rarely in nonfibrillar regions of the skin. The labeling showed a periodicity of 60–65 nm resembling the *D* (67 nm) stagger of collagen molecules. Blocking of antibodies with aminopropeptides and treatment of tissues with procollagen NH_2-terminal protease abolished labeling. Antibodies against type I collagen uniformly labeled ≈80% of the fibrils (diameter, 20–80 nm), while reaction with antibodies against type III collagen was restricted to thin fibrils. It is currently thought that the aminopropeptides of procollagen molecules are cleaved after they are released from the cell and before fibril formation. Our data indicate that aminopropeptides are removed at the fibrillar level and that fibril growth can be regulated by extracellular procollagen processing.**

Type I and III collagens are major components of the dermis and are organized into fibrils varying considerably in diameter. Distinct growth of fibrils is observed during development and may be disturbed in certain skin diseases (1). The mechanism of controlling this process is unknown. It has also not been established whether type I and III collagens are present in the same fibrils. Both collagens can form *D*-staggered fibrils *in vitro* showing identical cross-striations when examined in the electron microscope (2). Because specific antibodies against various collagens and procollagens are available (3, 4), it is feasible to identify distinct types of collagenous proteins at the ultrastructural level *in situ*, as was recently done for type III collagen (5).

Interstitial collagens are synthesized in the form of procollagens that possess additional extension amino- and carboxypropeptides. Each precursor-specific peptide is removed by specific proteases (procollagen NH_2-terminal and COOH-terminal proteases) presumably after release of procollagens from the cell (6). Cell and organ culture studies indicated different kinetics in the processing of type I and III procollagens (7–9). After cleavage, the aminopropeptides persist for some time in the extracellular space, as shown by immunofluorescence staining with antibodies against these peptides (3, 4).

A functional role for extension aminopropeptides in the control of fibril growth was first suggested in studies of dermatosparactic animals, which, due to a defective NH_2-terminal protease, accumulate an intermediate form of type I procollagen (pN-collagen) in the skin and other organs (10, 11). The collagenous fibrils appeared thin and hieroglyphic (12, 13) and could be stained with antibodies against the aminopropeptide by using a ferritin label (13). Small amounts of type I and III pN-collagens could also be extracted from the skin of growing animals (14–18). It was suggested (3, 14, 19) that these aminopropeptides are structural elements of immature or thin fibrils and that their controlled release regulates further growth. In the present study, we provide additional evidence for this possibility by immunoelectron microscopy.

MATERIALS AND METHODS

Skin Specimens. Normal human skin was obtained from three male and one female normal volunteers (35–59 years old). Biopsies were carried out on the arm or forearm under Xylocaine anesthesia and specimens were used immediately. Each specimen was sectioned into an upper papillary and a lower reticular portion and these were studied separately.

Preparation of Antibodies. Antibodies against bovine type I and III collagens and pN-collagens were raised in rabbits and rendered specific for the particular type of collagen or extension aminopropeptide by immunoabsorption (3). Purified antibodies consisted mainly of IgG and were dissolved in phosphate-buffered saline pH 7.2/0.02% sodium azide. As shown previously (4), each of the antibodies showed strong crossreactivity with the corresponding human antigens.

Immunoelectron Microscopy. Labeling and fixation were carried out at 4°C with a modification of a previously described procedure for direct staining (13). Skin specimens were cut into slices (1 × 0.2–0.5 mm) and fixed in 1% glutaraldehyde in 0.16 M cacodylate buffer, pH 7.4, for 20 min. They were then rinsed three times for 10 min each with buffer and three times for 1 hr each with 0.15 M Tris·HCl, pH 7.4. Alternatively, skin specimens were fixed in 1% 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide/0.35% glutaraldehyde (20). The two procedures gave similar results. Fixed samples were incubated with 50–80 μg of purified antibody in 0.1 ml of phosphate-buffered saline with gentle shaking for 24 hr. After removal of antibodies, the specimens were washed in several changes of 0.15 M Tris·HCl, pH 7.4 (16 hr), and incubated for 24 hr in 0.1 ml of ferritin-labeled goat anti-rabbit IgG diluted 1:5 (Nordic Immunology, Tolburg, The Netherlands). The samples were then washed for 24 hr with 0.15 M Tris·HCl, pH 7.4, and three or four times (10 min each) with 0.16 M cacodylate buffer, pH 7.4, and fixed for 2 hr in 3% glutaraldehyde/0.16 M cacodylate, pH 7.4. Sections were fixed in 1% OsO_4 for 90 min, dehydrated with graded alcohol (50–100%), and embedded in Epon. Blocks were sec-

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

§ Present address: Biozentrum der Universitaet Basel, Basel, Switzerland.
¶ Present address: Veterinary School, University of Munich, Munich, Federal Republic of Germany.

tioned into 60- to 80-nm-thick sections in a Porter–Blum ultramicrotome. These sections were stained with 1% aqueous phosphotungstic acid/1% uranyl acetate/Reynold's lead citrate and examined in an electron microscope.

Control Experiments. For negative controls, specific antibodies were replaced by equivalent amounts of rabbit IgG obtained from animals that had not been immunized or by buffer. In addition, equal volumes of antibodies against aminopropeptides type I or III (0.5–0.8 m/ml) and the respective purified aminopropeptides (1 mg/ml in phosphate-buffered saline) were incubated for 1 hr at 37°C. After centrifugation (30 min, 34,000 × g), the supernatants were used in the labeling experiments.

Removal of Extension Aminopropeptide of Type I Procollagen by NH_2-Terminal Protease. The enzyme was purified from chicken embryo tendon extract and showed no other proteolytic activity except cleavage of aminopropeptides from type I procollagen (21). It was also active against human type I pN-collagen but did not cleave human type III pN-collagen (unpublished results). Skin specimens were incubated with ≈0.04 unit of N-protease in 0.1 ml of 0.05 M Tris·HCl, pH 7.4/0.15 M NaCl/5 mM $CaCl_2$ for 3 hr at 30°C. These samples were rinsed with cacodylate buffer, pH 7.4, and then subjected to the fixation and labeling procedure. Controls were incubated for 3 hr at 30°C in buffer.

RESULTS

The indirect ferritin–antibody technique was used to visualize particular structures of collagen and procollagen in human skin. Antibodies specific for type I and type III collagens and for the irrespective extension aminopropeptides produced positive labeling in >90% of all tissue sections examined. Thus, the two-step procedure used allowed sufficient penetration of the tissue slices by the first antibody and the antibody–ferritin conjugate. Most of the ferritin label appeared to be associated with fibrillar structures having the typical cross-striation of collagen fibers (Figs. 1–3). Type I collagen was mainly detected in regions in which fibrils with a diameter of 40–100 nm were abundant (Fig. 1*A*), although some thin fibrils (20–40 nm) were also labeled. In the same regions, antibodies against the type I aminopropeptide mainly labeled thin fibrils (Fig. 1*B*). Labeling was observed along longitudinally sectioned and around cross-sectioned fibrils. Antibodies against the aminopropeptide occasionally showed reactions in regions of the skin devoid of fibrillar structures (Fig. 2*A*).

The localization of antibodies against type III collagen and against its extension aminopropeptide appeared to be restricted to boundary zones of the dermis that lack thick fiber bundles, such as around blood vessels or areas adjacent to the epidermal–dermal junction. Here, fibrils 20–60 nm in diameter (predominantly 20–40 nm) were distinctly stained (Fig. 3). Both antibodies usually did not react in areas with abundant type I collagen fibrils such as shown in Fig. 1. Usually, no labeling was observed in nonfibrillar areas.

The uptake of label was specific as shown by three different controls. Treatment of skin samples with buffer or normal IgG and then with the antibody–ferritin conjugate produced no staining except for a few randomly scattered ferritin particles. The same negative result was obtained by blocking antibodies against aminopropeptides with their specific antigens before their penetration into tissue slices (Fig. 2*B*). This showed that

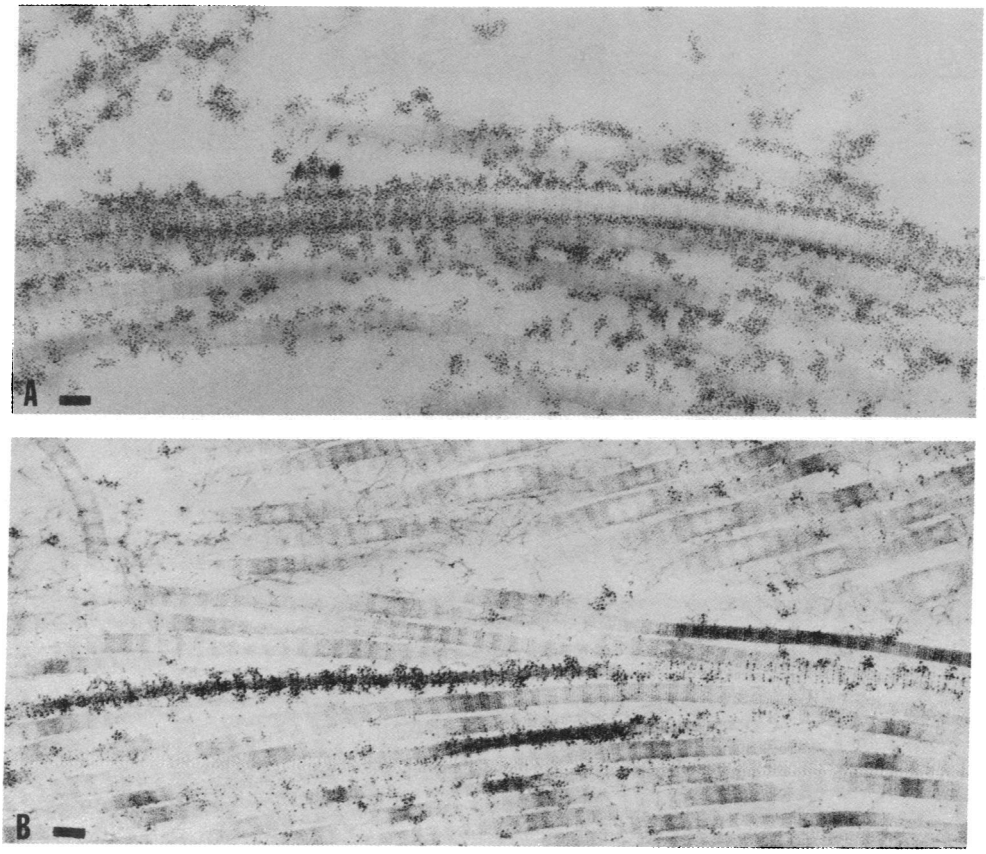

FIG. 1. Comparison of staining patterns obtained with antibodies against type I collagen (*A*) and against the extension aminopropeptide of type I collagen (*B*) in areas of human skin densely packed with cross-striated fibrils. Compare the preferential staining of thin fibrils shown in *B* with the more uniform reaction shown in *A*. Bars = 100 nm. (×38,600.)

binding of antibodies onto fibrils occurred at the antigen-binding site and not at another structure that might have acquired affinity for collagen due to the acidic elution conditions used during purification (3). Finally, treatment of skin samples with procollagen NH_2-terminal protease, an enzyme specific for the aminopropeptide of type I collagen (21), abolished the reaction with antibodies against this aminopropeptide (Fig. 2*C*). No denaturation of collagen fibrils was noted after this enzymatic digestion. Treatment with buffer instead of with enzyme did not alter the usual staining patterns.

Antibodies against extension aminopropeptides showed a periodic distribution at 60–65 nm along fibrils well-exposed in longitudinal sections (Figs. 1–3). This agrees with chemical and immunochemical data (6, 22) suggesting that aminopropeptides should be localized along fibrils at intervals corresponding to the *D* stagger (67 nm) of collagens. Antibodies against type I collagen showed a more uniform labeling pattern, as expected from previous data (23) showing several antigenic determinants along the entire length of the molecule. Frequently, labeling around cross-sectioned fibrils occurred in a concentric fashion (Fig. 3*A Inset*). Other, presumably tangentially cut, cross sections were asymmetrically labeled and occasionally they could not be clearly distinguished from conglomerates of ferritin particles in structurally amorphous regions.

Staining patterns were correlated to fibril diameters in a morphometric analysis (Fig. 4). The evaluation was based on selected distinctly labeled regions in the tissue sections that apparently had been well penetrated by antibodies. Only cross-striated fibrils in longitudinal sections that showed a perpendicular arrangement of a least 10 ferritin particles along a distance of 20–30 nm were considered as positively labeled. The data showed that the extension aminopropeptide of type I collagen was associated exclusively with thin fibrils (diameter 20–40 nm). This was not due to poor accessibility because antibodies against type I collagen reacted with a larger variety of fibrils (diameter 20–80 nm) resembling the size distribution of collagenous fibrils in the skin (Fig. 4*A*). About 15% of the fibrils had a diameter >80 nm, and these were particularly found in densely packed areas. They were not very well labeled except in the periphery, indicating lack of sufficient antibody penetra-

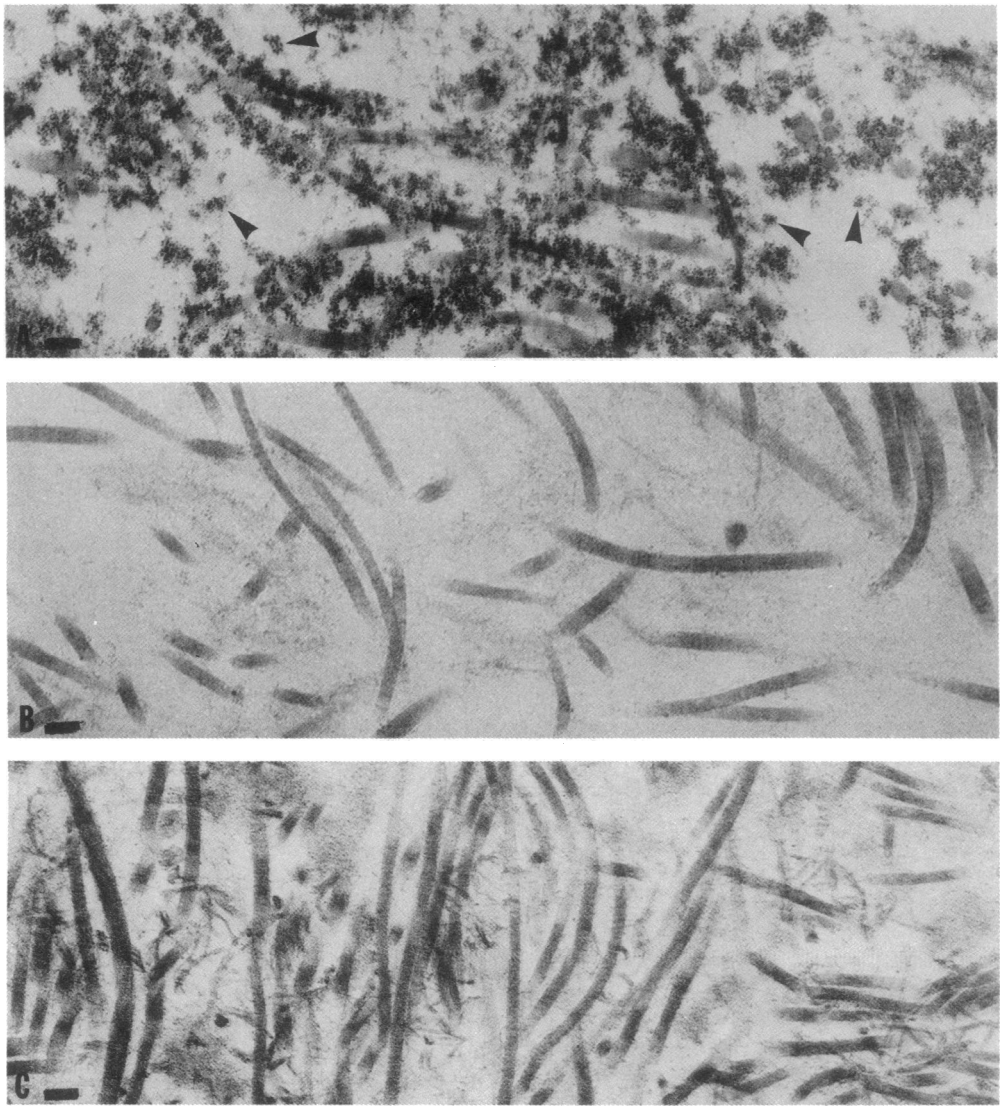

FIG. 2. Staining patterns of skin sections with antibodies against the extension aminopropeptide of type I collagen. (*A*) Many thin collagenous fibrils are heavily stained and there is also some reaction with nonfibrillar regions (►); thick fibrils are not labeled. (*B*) There is no reaction after the antibodies have been absorbed with the aminopropeptide. (*C*) Skin specimens previously treated with procollagen NH_2-terminal protease do not stain. Bars = 100 nm. (×39,700.)

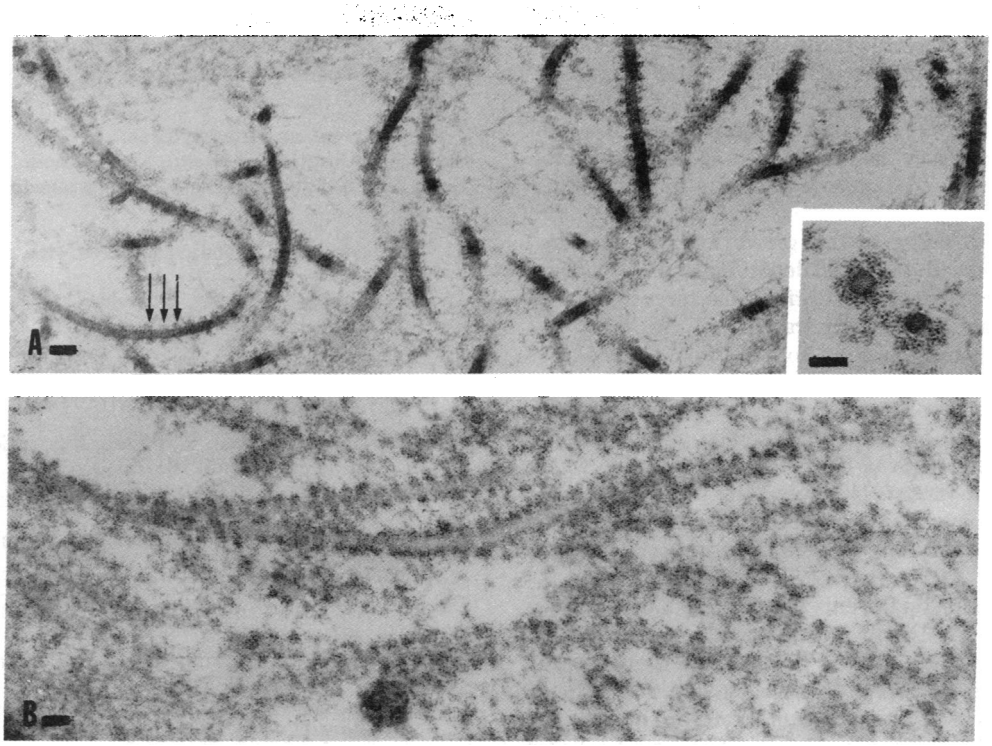

FIG. 3. Staining patterns with antibodies against the extension aminopropeptide of type III collagen in a boundary region of human dermis. (*A*) Distance between arrows is ≈60 nm. (×33,000.) (*Inset*) A few cross-sectioned fibrils stained by the same antibodies. (×46,300.) (*B*) Labeling of type III collagen. (×33,000.) Bars = 100 nm.

tion. The reaction patterns of antibodies against type III collagen (5) and those against its aminopropeptide were similar and included a large proportion of fibrils of 20-to40-nm diameter and a significant fraction of the 40- to 60-nm-diameter fibrils (Fig. 4*B*).

DISCUSSION

Immunoelectron microscopy data showed that extension aminopropeptides are extracellular constituents of normal skin mainly associated with 20- to 40-nm-thick fibrils. These aminopropeptides are apparently not yet released from procollagen molecules, as indicated by the *D*-staggered periodicity of ferritin staining and by their removal from the fibrils after treatment with procollagen NH_2-terminal protease. Thus, certain fibrils in the tissue still possess, at least at their outer surface, procollagen or pN-collagen molecules. The latter possibility is more likely because of the demonstration of small but significant amounts of type I and III pN-collagens in normal skin (14–18).

Chemical (22) and physical (24) studies have shown that extension aminopropeptides are elongated and consist of a short triple helix (≈12 nm long) and a noncollagenous domain with the approximate dimensions 1.5 × 14 nm. An even more compact shape is indicated by rotary shadowing of single pN-collagen molecules (K. Kuhn, personal communication). This structure could easily fit into the hole region (length, 40 nm) of collagen microfibrils. Because a single pN-collagen molecule contains two (type I) or three (type III) of these non-collagenous domains, each at least as thick as a collagen molecule, they could interfere with the further ordered association of microfibrils into larger fibrils for steric reasons. The extension carboxypropeptides are even bulkier (25, 26) and may not fit into the hole region on the outside of collagen fibrils. No ultrastructural data are yet available on the persistence and localization of the carboxypropeptides in the extracellular space.

Our data support previous suggestions (3, 4, 19) that removal of extension aminopropeptides is an extracellular process involved in the control of fibril growth and locate a potential site of NH_2-terminal protease action to the fibrillar level. Different NH_2-terminal proteases are apparently required for the conversion of type I and type III procollagens (21, 27). As shown in organ culture, the aminopropeptides are released from type

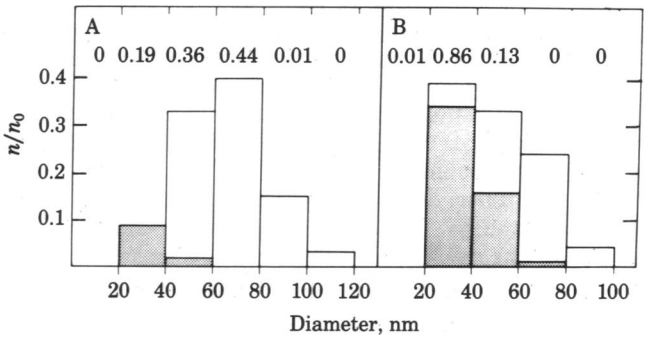

FIG. 4. Diameter of collagen fibrils from areas rich in type I collagen (*A*) and type III collagen (*B*). The fibrils in *A* were stained with antibodies against the extension aminopropeptide of type I collagen and those in *B* were stained with antibodies against the extension aminopropeptide of type III collagen. Each column indicates the fraction (n/n_0) of fibrils evaluated within the size category, and shaded area of the column shows the relative proportion of stained fibrils. In a similar study, the fibrils were stained with antibodies against type I and type III collagen; these results are indicated by the numbers in *A* and *B*, respectively. Note that the distribution of fibrils with antibodies against type I collagen closely resembles the distribution of fibrils in the 20- to 80-nm range. Larger type I collagen fibrils were less efficiently stained, presumably due to lack of antibody penetration. The data are based on examination of 500–750 individual fibers in each experiment. n, Number of fibrils observed within a given diameter range; n_0, Number of fibrils counted.

III procollagen much more slowly than the carboxypropeptides (9). Studies with cultured fibroblasts indicated an inverse order of cleavage for type I procollagen (7, 8). This may be due to a particular high activity of type I procollagen NH_2-terminal protease in cell cultures since the rates of cleavage of carboxypropeptides from type I and III procollagens were similar in other studies (9).

We propose a mechanism of fibril growth for both type I and III collagen starting with the initial deposition of pN-collagens. This could occur by assembly of fibrillar structures from large molecular aggregates produced intracellularly (28) or by deposition of single molecules. A controlled proteolytic release of aminopropeptides is then required to allow further growth in thickness. Specific procollagen NH_2-terminal proteases are the regulatory agents and cessation or inhibition of their activity may terminate the growth of type III collagen fibrils, which even in their larger variants (40–6 nm) still retained the aminopropeptide. A long persistence of type III pN-collagen was also found by *in vivo* labeling (29) and by immunofluorescence analysis of a large variety of tissues (3, 4).

The growth-terminating events are perhaps more complex for type I collagen fibrils. The crucial role of removing the extension aminopropeptide to allow fibril growth is emphasized by the exclusive association of pN-collagen with thin fibrils in normal skin and by the thin and irregular nature of pN-collagen fibrils in dermatosparactic skin (12, 13). Thick type I collagen fibrils (40–80 nm) apparently lack the aminopropeptide, which is not due to a technical artifact preventing appropriate antibody penetration. Other mechanisms may operate, such as interaction with proteoglycans, which could mask an outer layer of pN-collagen in the thick fibrils or prevent further deposition of pN-collagen (or both). Studies on developing tendon have shown a considerable switch in the ratio between chondroitin sulfate proteoglycan and dermatan sulfate proteoglycan when the type I collagen fibrils start to grow substantially in diameter (30). Dermatan sulfate proteoglycan is then eventually found in a *D*-staggered arrangement on the outside of the fibrils (31). Another control could be by fibronectin, which interacts with growing collagen fibrils (32).

The question could also be raised as to whether the type I propeptide has a special affinity for a unique group of thin fibrils and thus be not involved in the regulation of fibril diameter. In this regard, preliminary data from similar labeling experiments carried out in 10 day-old chicken embryo skin showed selective labeling of thin fibrils, while thick collagen fibrils were not labeled (unpublished results).

The mechanism discussed above is compatible with several other observations. It has been found that pN-collagen type I *in vitro* forms much thinner fibrils when compared with collagen type I (19). X-ray diffraction of dermatosparactic fibrils shows, depending on the pN-collagen content, a poorly ordered microfibrillar structure (33). Implantation of dermatosparactic skin in millipore chambers into normal animals allowed the formation of a normal tissue architecture (34). This suggested that thick fibrils have been formed by fusion of thin fibrils after removal of the extension aminopropeptides by procollagen NH_2-terminal protease supplied by the host. A similar fusion may normally operate in the growth of type I collagen fibrils.

The lack of complete correspondence in the staining patterns of types I and III collagen (Fig. 4) indicates that the two protein species are not necessarily located in the same fibrils. This possibility is strengthened by the observation that areas containing many fibrils in parallel arrangement do not react very well for type III collagen. Boundary skin regions in which the fibrils are not in close contact (Figs. 2 and 3), show preferential staining for type III collagen but also some staining for type I collagen.

This study was supported by Grants AM27608 and AM07376 from the National Institutes of Health, North Atlantic Treaty Organization Grant 1960, and the Deutsche Forschungsgemeinschaft.

1. Fleischmajer, R., Damiano, V. & Nedwich, A. (1971) *Science* **71,** 1019–1021.
2. Wiedemann, H., Chung, E., Fujii, T., Miller, E. J. & Kuhn, K. (1975) *Eur. J. Biochem.* **51,** 363–368.
3. Nowack, H., Gay, S., Wick, G., Becker, U. & Timpl, R. (1976) *J. Immunol. Meth.* **12,** 117–124.
4. Timpl, R., Wick, G. & Gay, S. (1977) *J. Immunol. Methods* **18,** 165–182.
5. Fleischmajer, R., Gay, S., Perlish, J. S. & Cesarini, J. P. (1980) *J. Invest. Dermatol.* **75,** 189–191.
6. Fessler, J. H. & Fessler, L. I. (1978) *Annu. Rev. Biochem.* **47,** 129–162.
7. Davidson, J. M., McEneany, L. S. G. & Bornstein, P. (1975) *Biochemistry* **14,** 5188–5194.
8. Fessler, L. I. & Fessler, J. H. (1979) *J. Biol. Chem.* **254,** 233–239.
9. Fessler, L. I., Timpl, R. & Fessler, J. H. (1981) *J. Biol. Chem.* **256,** 2531–2537.
10. Lenaers, A., Ansay, M., Nusgens, B. V. & Lapière, C. M. (1971) *Eur. J. Biochem.* **23,** 533–543.
11. Becker, U., Timpl, R., Helle, O. & Prockop, D. J. (1976) *Biochemistry* **15,** 2853–2862.
12. Fjolstad, M. & Helle, O. (1974) *J. Pathol.* **112,** 183–188.
13. Wick, G., Olsen, B. R. & Timpl, R. (1978) *Lab. Invest.* **39,** 151–156.
14. Veis, A., Anesey, J., Yuan, L. & Levy, S. J. (1973) *Proc. Natl. Acad. Sci. USA* **70,** 1464–1467.
15. Byers, P. H., McKenney, K. H., Lichtenstein, J. R. & Martin, G. R. (1974) *Biochemistry* **13,** 5243–5248.
16. Timpl, R., Glanville, R. W., Nowack, H., Wiedemann, H., Fietzek, P. P. & Kuhn, K. (1975) *Hoppe-Seyler's Z. Physiol. Chem.* **356,** 1783–1792.
17. Lenaers, A. & Lapière, C. M. (1975) *Biochim. Biophys. Acta* **400,** 121–131.
18. Smith, B. D., McKenney, K. H. & Lustberg, T. J. (1977) *Biochemistry* **16,** 2980–2985.
19. Lapiere, C. M. & Nusgens, B. (1974) *Biochim. Biophys. Acta* **342,** 237–246.
20. Willingham, M. C. & Yamada, S. S. (1979) *J. Histochem. Cytochem.* **27,** 947–960.
21. Tuderman, L., Kivirikko, K. & Prockop, D. J. (1978) *Biochemistry* **17,** 2948–2954.
22. Timpl, R. & Glanville, R. W. (1981) *Clin. Orthoped.* **158,** 230–248.
23. Timpl, R. (1976) in *Biochemistry of Collagen*, eds. Ramachandran, G. N. & Reddi, A. H. (Plenum, New York), pp. 319–375.
24. Engel, J., Bruckner, P., Becker, U., Timpl, R. & Rutschmann, B. (1977) *Biochemistry* **16,** 4026–4033.
25. Hoffmann, H.-P., Olsen, B. R., Chen, H. T. & Prockop, D. J. (1976) *Proc. Natl. Acad. Sci. USA* **73,** 4304–4308.
26. Olsen, B. R., Guzman, N. A., Engel, J., Condit, C. & Aase, S. (1977) *Biochemistry* **16,** 3030–3036.
27. Nusgens, B. V., Goebels, Y., Shimkai, H. & Lapière, C. M. (1980) *Biochem. J.* **191,** 699–706.
28. Bruns, R. R., Hulmes, D. J. S., Therrien, S. F. & Gross, J. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 313–317.
29. Limeback, H. F. & Sodek, J. (1979) *Eur. J. Biochem.* **100,** 541–550.
30. Scott, J. E., Orford, C. R. & Hughes, E. W. (1981) *Biochem. J.* **195,** 573–581.
31. Scott, J. E. & Orford, C. R. (1981) *Biochem. J.* **197,** 213–216.
32. Kleinman, H. K., Wilkes, C. M. & Martin, G. R. (1981) *Biochemistry* **20,** 2325–2330.
33. Cassidy, K., Eikenberry, E. F., Olsen, B. & Brodsky, B. (1980) *Lab. Invest.* **43,** 542–546.
34. Shoshan, S., Segal, N., Traub, W., Salem, G., Kuhn, K. & Lapière, C. M. (1974) *FEBS Lett.* **41,** 269–274.