The Carboxylpropeptide of Type I Procollagen in Skin Fibrillogenesis

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Previous studies suggested that the aminopropeptide of type I procollagen may initiate fibril formation. The purpose of this investigation was to study the location of the carboxylpropeptide of type I procollagen during collagen fibrillogenesis. Chick embryonic and posthatching skin specimens were studied by immunofluorescence and immunoelectron microscopy and by immunoblotting with antibodies against the amino and carboxylpropeptide of type I procollagen. The carboxylpropeptide was demonstrated at the surface of collagen fibrils, 20–40 nm in diameter (10-day embryos) and in fibrils, 40–65 nm (21-day embryos). In addition, the carboxylpropeptide was found at the cell surface and free in the ground substance. The aminopropeptide was only seen in fibrils, 20–30 nm in diameter, as previously reported. Ratios of pN-collagen/pC-collagen increased from 16 days embryonic to 3 and 9 days postembryonic skins. This study suggests that both pN-collagen (aminopropeptide plus collagen) and pC-collagen (carboxylpropeptide plus collagen) participate in fibrillogenesis. *J Invest Dermatol 89:212–215, 1987*

The major fibrillar collagens, type I, II, and III, share in common the property to polymerize in the extracellular matrix and form fibrils with a characteristic 60 nm periodicity. The dermis contains about 85–90% of type I collagen. This collagen is synthesized in a precursor form known as procollagen with extension propeptides at the amino and carboxyl ends [1]. Based on in vitro experimentation, it is classically accepted that both the amino propeptides and carboxylpropeptides are cleaved by specific proteases before the collagen molecules assemble in a quarter-staggered arrangement and form fibrils [2]. More recently, it has been shown that the aminopropeptide may participate in initial fibrillogenesis and possibly regulate fibril diameter in skin during embryogenesis as well as during adult life [3–5]. Immunoelectron microscopy revealed the presence of the aminopropeptides at the fibril surface, at regular 60 nm intervals. The carboxylpropeptide was also noted during skin embryogenesis at the surface of fibrils, but in a random distribution [3]. The electron microscopic data were initially interpreted as suggesting that the carboxylpropeptide was present as a free peptide following excision from the procollagen molecule by a specific carboxylpropeptidase [3]; however, additional studies on bone embryogenesis revealed that the carboxylpropeptide may participate in collagen fibrillogenesis [6]. The purpose of the present study was to further characterize the role of the carboxylpropeptide during skin embryogenesis using immunofluorescence, immunoelectron microscopy, and immunoblotting.

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Abbreviations:
PAGE: polyacrylamide gel electrophoresis
pC-collagen: carboxylpropeptide plus collagen
pN-collagen: aminopropeptide plus collagen
SDS: sodium dodecyl sulfate

MATERIALS AND METHODS

Skin was obtained from White Leghorn chick embryos at 10, 11, 16, and 21 days of development and from 3- and 9-day-old posthatching chickens.

Preparation of Antibodies The aminopropeptide and carboxylpropeptide of type I procollagen were prepared from the medium of chick tendons in organ culture as previously described [7,8]. The antisera obtained from rabbits were tested by radioimmunoassays. IgG fractions were purified by Protein A-Sepharose chromatography and showed the same specificity as previously reported [7,8].

Immunofluorescence Microscopy Immunofluorescence microscopy was performed on 21-day embryonic and 2- and 10-day posthatching skins. Frozen sections, about 8 μm thick were treated with antibodies against the aminopropeptides and carboxylpropeptides [9]. Rabbit IgG from nonimmunized animals were used as controls.

Immunoelectron Microscopy Ten- and 21-day chick embryo skins were fixed for 20 min in 1% glutaraldehyde, washed with Tris-HCl buffer, pH 7.3, and successively incubated with specific antibodies for 24 h followed by 24-h incubation with ferritin-tagged rabbit anti-IgG antibodies [3]. Then, specimens were handled as for regular electron microscopy and stained with uranyl acetate and lead citrate. For control experiments, the specimens were treated with equal amounts of rabbit IgG from nonimmunized animals. In other experiments, the specific antibodies were blocked with their corresponding propeptides and after centrifugation, the supernatants were used for labeling [3].

Immunoblotting Collagen was extracted from skin specimens into 0.125 M Tris-HCl buffer, pH 6.8 containing 2% sodium dodecyl sulfate (SDS) in the presence of proteinase inhibitors phenylmethylsulfonyl fluoride (3 mg/L), p-chloromercuribenzoate (3 mg/L), 0.01 M EDTA, and 0.5 mM iodoacetamide using a mortar and pestle followed by heating in a boiling water bath (100°C) for 5 min. Supernatants were obtained by centrifugation at 12,000 *g* for 30 min at room temperature, reduced with β-

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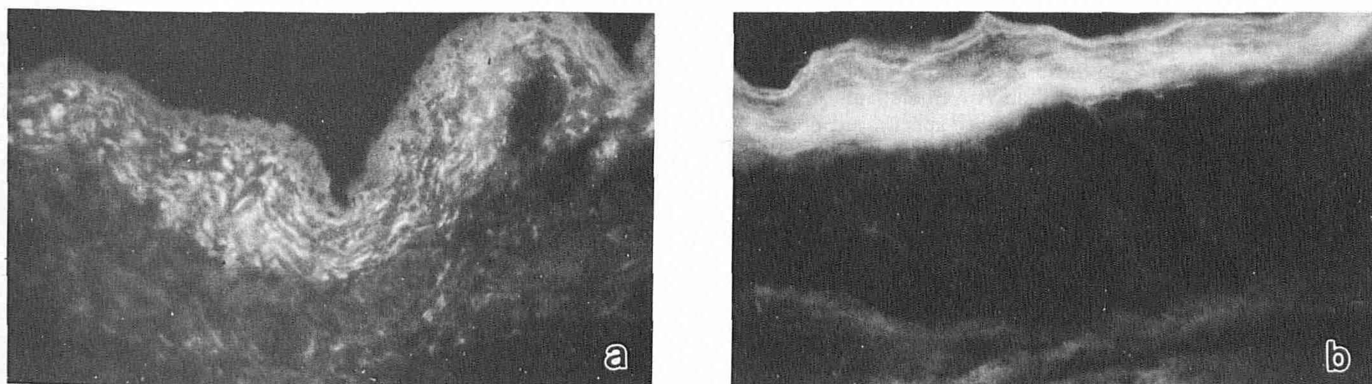
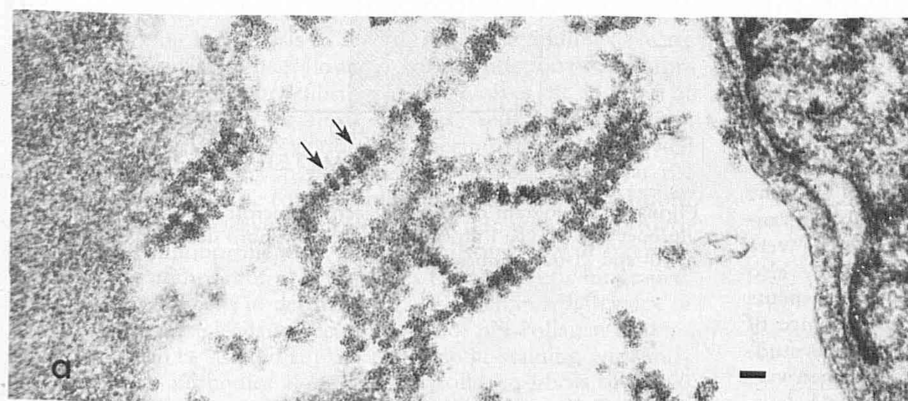
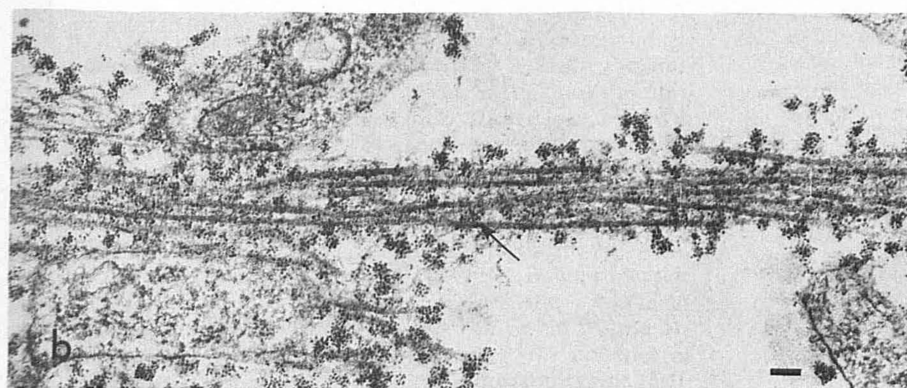


Figure 1. Indirect immunofluorescence microscopy of 21-day embryonic skin (*a*) antibody against the aminopropeptide of type I procollagen; (*b*) antibody against the carboxylpropeptide of type I procollagen.





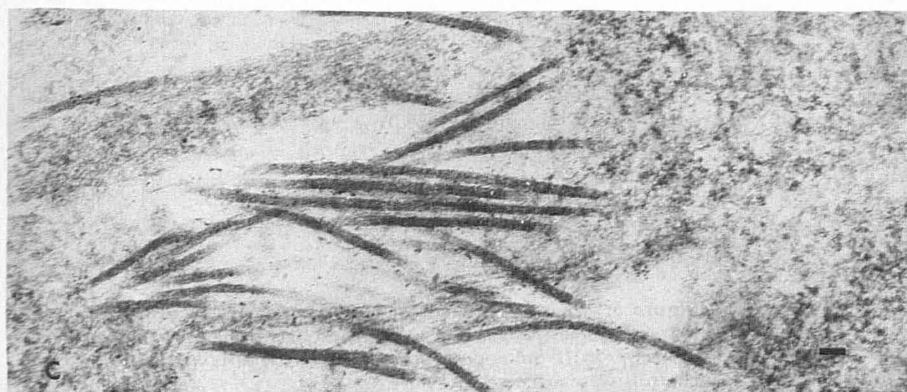


Figure 2. Indirect immunoelectron microscopy of 10-day chick embryo skin with ferritin (*a*) aminopropeptide of type I procollagen antibodies showing labeling of collagen fibrils, about 20 nm in diameter at 60 nm periodicity (*arrows*); (*b*) carboxylpropeptide of type I procollagen showing labeling at the cell surface and collagen fibrils about 25 nm in diameter. There is some labeling free in the ground substance. Note bridging between collagen fibrils (*arrow*); (*c*) no labeling after blocking the antibody with the carboxylpropeptide. Uranyl acetate lead citrate. *Bars* = 100 nm

mercaptoethanol and subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) [10]. Proteins were transferred from the SDS-PAGE gel to a nitrocellulose sheet and subjected to autoradiography using ^{125}I-labeled Protein A (Amersham, Arlington Heights, Illinois) following treatment with specific antibodies or nonimmune rabbit IgG as previously described [11]. Ratios of aminopropeptide/carboxylpropeptide collagen (pN/pC collagen) were determined by scanning the autoradiographs using a Beckman DU8 spectrophotometer. The areas under the peaks corresponding to procollagen, pN, and pC collagen were calculated and used to determine the ratios of pN to pC collagen.

RESULTS

Immunofluorescence microscopy with the aminopropeptide of type I procollagen revealed fluorescence throughout the dermis consisting of distinct short streaks, parallel to the skin surface, and separated by bands of nonfluorescent areas. This pattern of fluorescence suggested distinct isolated areas containing the aminopropeptide. On the other hand, staining with the carboxylpropeptide antibodies gave a more diffuse pattern of fluorescence (Fig 1). This pattern was observed in all specimens studied. Immunoelectron microscopy of 10-day chick embryo skins with the aminopropeptide antibodies revealed labeling of fibrils, 20–30 nm in diameter, at 60 nm periodicity, as previously described [4]. The carboxylpropeptide was apparent at cell surfaces, free in the extracellular matrix and in close proximity to collagen fibrils (Fig 2). Numerous cells revealed ferritin aggregates at or near the cell membrane (Fig 3). Intracellular labeling of cytoplasm and rough endoplasmic reticulum was also noted when the cell membrane was disrupted. Collagen fibrils, about 20–40 nm (10 days) and 40–65 nm (21 days) in diameter (Fig 4) showed labeling with the carboxylpropeptide antibodies at their surface, usually in a random fashion. In addition, the carboxylpropeptide antibodies were noted forming bridges between collagen fibrils and were also found free in the ground substance (Fig 2). Blocking experiments showed no labeling. Immunoblotting revealed the presence of procollagen, pN-collagen and pC-collagen in all specimens studied (Fig 5); however, the ratios of pN-collagen/pC-collagen varied with the age of the tissue studied (Table I). Thus, at 11 days,

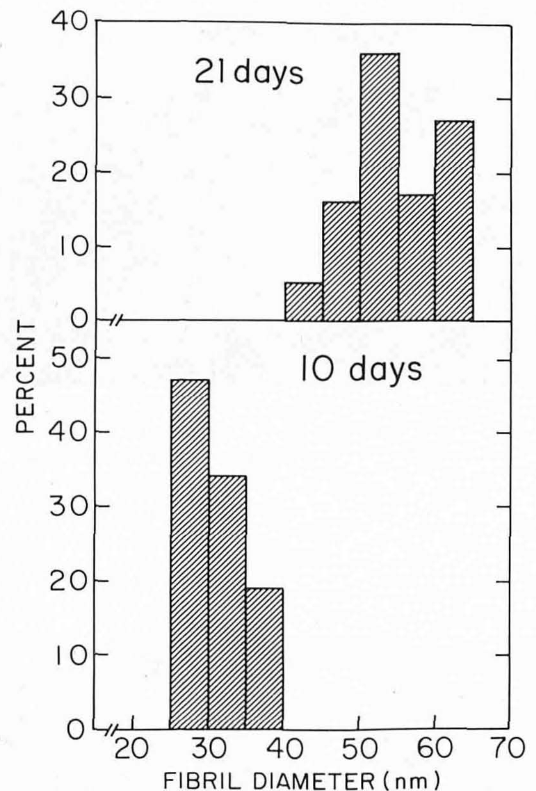


Figure 4. Histogram of immunoelectron microscopy with the carboxylpropeptide of type I procollagen, showing fibril diameter distribution of 10- and 21-days embryonic skin.

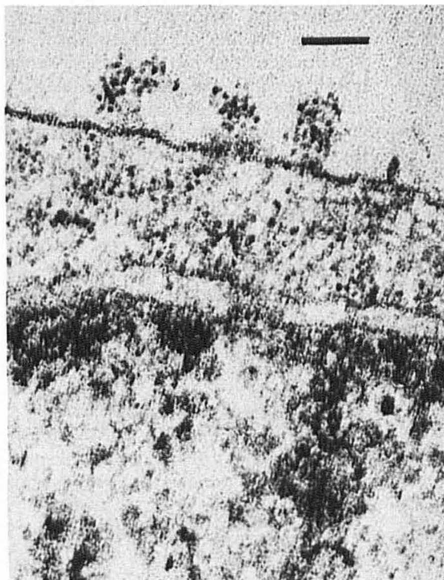


Figure 3. Indirect immunoelectron microscopy with antibody against the carboxylpropeptide. Note labeling at or near cell membrane and cytoplasm. Uranyl acetate–lead nitrate. *Bar* = 100 nm.

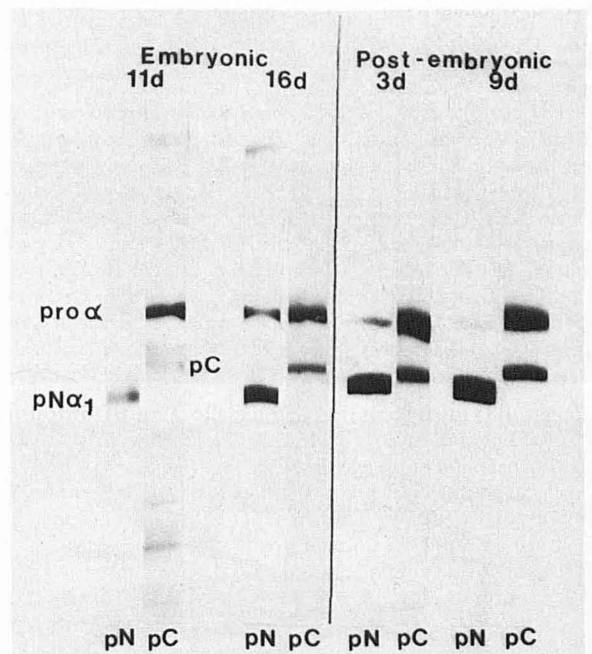


Figure 5. Western immunoblot analysis of embryonic (*11d, 16d*) and postembryonic (*3d, 9d*) chicken skin extracts using antibodies directed against proα1(I) aminopropeptide and proα1(I) carboxylpropeptide, IgG controls were negative.

Table I. Ratio of pN/pC Collagen at Various Stages of Chicken Skin Development

	Age (days)	pN/pC Collagen
Embryonic	11	5.7
	16	2.4
Postembryonic	3	5.6
	9	9.4

pN/pC collagen = Aminopropeptide/carboxylpropeptide collagen.

the ratio was 5.7, which dropped to 2.4 at 16 days; however, the ratio increased at 3 and 9 days posthatching (5.6 and 9.4, respectively).

DISCUSSION

Previous experimental studies suggest that the aminopropeptide of type I and type III procollagen plays a role in fibrillogenesis. Thus, in vivo studies demonstrated the aminopropeptide at the surface of presumably growing fibrils in both embryonic skin and bone [3,5,6]. In addition, in vitro experiments also demonstrated that pN-collagen can be made to polymerize into thin fibrils [12]. Presumably, the aminopropeptide could fit into the gap (space between adjacent collagen molecules) within fibrils.

As a working hypothesis to account for these findings, we are currently considering the following model: initiation of fibril formation followed by lateral fibril growth involves the addition of pN-collagen molecules to fibril surfaces. This is followed by cleavage of the aminopropeptide by a specific protease, followed by the addition of new pN-collagen molecules. Note that the model postulates a role for pN-collagen in limiting lateral fibril growth; failure to cleave the aminopropeptide will limit growth because the aminopropeptide on the fibril surface will interfere with further addition of pN-molecules through steric hindrance. This is what happens in dermatosparaxis wherein a deficiency in the aminoprotease leads to accumulation of pN-collagen in skin. The model can be used to explain the periodic staining with anti-N-propeptide antibodies along type III collagen fibrils of 40–60 nm, in the following way. Lateral growth of type III fibrils involves pN-collagen until the fibrils reach full size, 40–60 nm. At this point, the processing of the aminopropeptide ceases and the lateral growth of the fibril stops. For type I collagen the aminopropeptide would participate in fibrillogenesis until the fibril reaches about 20 nm. Beyond this point, further lateral growth must involve other mechanisms. The current study and a previous one with bone suggests that the carboxylpropeptide may also participate in fibrillogenesis. In bone the propeptide can be revealed (through antibody staining) at the surface of fibrils ranging from 20–100 nm in diameter. These fibrils, mostly above 40 nm, are distinctly larger than those labeled by the aminopropeptide antibody. In the current study, the carboxylpropeptide was identified in fibrils ranging in diameter from 40–65 nm in 10- and 21-day-old embryonic skin. By immunoblotting, the presence of pC-collagen in embryonic (11,16 day) and postembryonic (3,10 day) specimens could be demonstrated.

What do these results mean? Among several alternatives, one possibility is that C-propeptides, products of procollagen processing, are arranged at intervals along the surface of larger collagen fibrils. A second possibility is that pC- or procollagen molecules are localized along the surface of collagen fibrils. The differential staining of small and large type I fibrils with antibodies against the amino- and carboxylpropeptides may be a reflection of differential rates of processing at the amino and carboxyl ends of procollagen molecules at the surface of small and large fibrils or in their proximity. Thus, one may hypothesize that both pN-collagen and pC-collagen molecules participate in the formation of collagen fibrils in vivo. In this regard, it is also noteworthy that both the aminopropeptide and carboxylpropeptide of type I procollagen have been shown to inhibit, by feedback mechanisms, the synthesis of collagen and procollagen mRNA [13–15]. Data from our laboratory and others suggest that collagen propeptides not only regulate intracellular collagen synthesis but also participate in the mechanism of fibril formation, which is an extracellular event.

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