Biosynthesis of Type IV Procollagens†

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ABSTRACT: The biosynthesis of type IV collagen was studied in mouse tumor and parietal yolk sac tissues in vitro. Two major collagenous polypeptides were observed in each system with apparent molecular weights of 185K and 170K. These polypeptides and two other minor constituents were analyzed and found to have ratios of 3- to 4-hydroxyproline, of total hydroxyproline to proline, and of hydroxylysine to lysine characteristic of type IV collagen. The patterns of CNBr peptides obtained from the proteins from the tumor and parietal yolk sac were identical. Several observations indicated that the 185K and 170K peptides are different gene products. Pulse–chase studies showed no conversion of the 185K chain to the 170K chain, and tissue labeled in the presence of α,α'-dipyridyl, an inhibitor of prolyl and lysyl hydroxylases, also produced two chains. Pepsin attacked the two chains at different rates and produced different products. Unlabeled collagen extracted from the tumor matrix contained two smaller chains but had a pattern of CNBr peptides similar to that obtained from the labeled chains. Antibody prepared against the two smaller unlabeled chains precipitated the larger biosynthetic polypeptides. These observations indicate that the 185K and 170K polypeptides are precursors of the smaller forms. Since the ratio of the radiolabeled 185K and 170K chains varied in different preparations, the chains may be present in different molecules.

Basement membranes are extracellular matrices located between parenchymal cells and adjacent connective tissue (Vracko, 1974). Basement membranes are widely distributed in the body and serve as supportive structures for epithelial and endothelial cells, as a scaffolding in repair processes, and as a filter blocking the passage of macromolecules from blood to tissues. Although basement membranes contain collagenous protein, termed type IV collagen, they have a homogeneous and largely nonfibrillar appearance. Basement membranes occur in only small amounts in normal tissues, and their constituents have also proven difficult to characterize due to their insolubility.

The EHS sarcoma, a transplantable mouse tumor, produces an extracellular matrix of basement membrane (Orkin et al., 1977) and represents a useful source of basement membrane constituents. So far, a type IV collagen (Orkin et al., 1977; Timpl et al., 1978, 1979a), a large noncollagenous protein, laminin (Timpl et al., 1979b), and a heparan sulfate proteoglycan (J. R. Hassell, P. Gehron Robey, H.-J. Barrach, J. Wilczek, S. I. Rennard, and G. R. Martin, unpublished experiments) have been isolated from the tumor matrix. The collagenous protein from the tumor has a composition very similar to the composition reported for type IV collagen from authentic basement membranes. Further, antibody to the tumor collagen binds to all authentic basement membranes examined (Timpl et al., 1978; Yaoita et al., 1978). These observations suggested that the tumor matrix and authentic basement membranes contain similar collagens.

However, opposing evidence exists on the molecular structure of type IV collagen. The collagenous protein from the tumor was found to contain two chains of 160000 and 140000 daltons. In contrast, earlier studies had suggested that basement membrane collagen contained a single type of α chain, designated α1(IV) (Kefalides, 1971, 1973), although several investigators have suggested more heterogeneity (Daniels & Chu, 1975; Sato & Spiro, 1976; Orkin et al., 1977; Dixit, 1978; Timpl et al., 1978, 1979c; Tryggvason & Kivirikko, 1978; Kresina & Miller, 1979). Biosynthetic studies suggested that basement membrane collagen was synthesized as a single chain weighing between 135000 and 180000 daltons depending on the system studied (Grant et al., 1972; Clark et al., 1975; Howard et al., 1976; Kefalides et al., 1976; Minor et al., 1976). Conversion of the biosynthetic product to a smaller component, such as is found in the conversion of procollagens to collagen, was observed once in lens capsule (Grant et al., 1972) but not subsequently in parietal yolk sac (Minor et al., 1976) or lens capsule (Minor et al., 1976; Heathcote et al., 1978; Dehm & Kefalides, 1978a). Most models of basement membrane collagen suggest that it is a procollagen-like molecule with substantial noncollageneous segments which are retained in the matrix.

We have found that cells from the EHS sarcoma can be used in organ culture to study the biosynthesis of type IV collagen. The chains synthesized by the tumor cells have been compared to the material produced by pareital yolk sac endoderm. Two similar chains of 185000 and 170000 daltons are synthesized by both tissues, and these appear to be distinct gene products, designated the pro α1(IV) and pro α2(IV) chains. The tumor matrix contains shortened forms of these two chains, indicating that processing to smaller molecules occurs in vivo.

Experimental Procedures

Preparation of Radiolabeled Type IV Procollagens. The EHS tumor (Orkin et al., 1977) was maintained by serial passage intramuscularly into the hind limbs of C57Bl/6J mice. After 3 weeks, tumors were harvested for extraction and purification of unlabeled material (Timpl et al., 1978) or incubated with labeled amino acids for biosynthetic studies. The site in which the EHS tumor is grown affects its biosynthetic activity in vitro. Very little incorporation of labeled amino acids into protein was obtained with tissue from tumors grown in a subcutaneous site. In contrast, tissue from an intramuscular site incorporated substantial amounts of labeled amino acids into protein. Histological studies indicated that there were more cells and less matrix in tumors grown in the intramuscular site (not shown). For the labeling studies, 2 g of the tumor was minced and rinsed several times with 0.15

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M NaCl–0.02 M sodium phosphate, pH 7.4 (PBS),[1] and 100 units of penicillin and 100 μg of streptomycin per 100 mL. Subsequently, the tumor tissue was rinsed with Dulbecco–Vogt medium containing 20% dialyzed fetal calf serum, 75 μg/mL ascorbic acid (Sigma), and 50 μg/mL BAPN (Aldrich Chemical Co.). In most cases, proline- and glutamine-free medium was utilized, and in some cases, the medium also lacked lysine. The tissue suspension was centrifuged at 1600 rpm for 3 min. The pellet was resuspended in 4 volumes of medium, and the mixture was incubated for 30 min at 37 °C in a moist atmosphere of 5% CO_2 and 95% air. In some cases, 0.3 mM α,α′-dipyridyl, an inhibitor of prolyl and lysyl hydroxylases, was added to the media to cause the accumulation of unhydroxylated type IV procollagen. After the preincubation period, either 5 μCi/mL [^{14}C]proline (>250 mCi/mmol, Amersham) or [^{14}C]lysine (>300 mCi/mmol, Amersham) was added. The incubation was continued for 5 h with agitation. All further procedures were carried out at 4 °C. The tissue and medium were centrifuged, and the pellet was washed twice with PBS unless otherwise described. The tissue was then homogenized (1 mL of tissue per 9 mL of solution) in ice-cold 0.5 M acetic acid containing 20 mM EDTA (Sigma) and 8 mM MalNEt (Sigma) and extracted overnight. The suspension was dialyzed against the same solution and clarified by centrifugation at 27000*g* for 30 min. Collagenous protein was precipitated from the supernatant fluid with 1.71 M NaCl. The precipitate was collected by centrifugation, dissolved in acetic acid containing protease inhibitors, and dialyzed overnight. The undissolved material was removed by centrifugation, and the supernatant fluid was dialyzed against 0.1 M acetic acid and lyophilized. The media contained less than 10% of the labeled nondialyzable hydroxyproline produced and was not studied further.

The acetic acid solution of type IV collagen was dialyzed against 0.5 M NaCl and 0.05 M Tris-HCl, pH 7.4, and clarified by centrifugation. The supernatant fluid was then dialyzed sequentially against solutions of the same buffer containing 1.71, 2.56, or 4.5 M NaCl. The precipitates forming at each NaCl concentration and the 4.5 M NaCl supernatant fluid were collected separately and analyzed.

In the preparation of labeled parietal yolk sac procollagen, pregnant mice were killed on the 11th day of gestation and the uteri were removed. The embryos were detached, and the tissue containing the parietal endoderm, the underlying basement membrane, and trophoblast (PEMT) was dissected free from the chorioallantoic placenta as described by others (Clark et al., 1975). PEMT tissue from 12 embryos was used and incubated in a total volume of 10 mL as described above for the tumor tissue. After incubation, the tissue and medium were separated by centrifugation, the tissuse was homogenized, and procollagen was extracted and purified as described above.

In pulse–chase experiments, the tumor tissue was pulsed in media with [^{14}C]proline (5 μCi/mL) for 1 h. Subsequently, half of the sample was removed and the rest was incubated for an additional 24 h in fresh complete medium containing unlabeled proline. At the time the samples were taken, tissue and media were pooled and collagenous protein was extracted and purified as described above.

Polyacrylamide Slab Gel Electrophoresis and Autoradiography. Polyacrylamide slab gel electrophoresis was usually performed with a Tris–glycine buffer system containing sodium dodecyl sulfate as described by Laemmli (1970) using 5 or 10% separating gels and 3% stacking gels. Urea (0.5 M) was included in the solutions to improve the separation of the collagen chains. The gels were stained with Coomassie Brilliant Blue, and labeled bands on the gels were detected by autoradiography. Quantitation of the bands on the developed film was achieved with a Helena densitometer equipped with a peak integrator.

Amino Acid Analysis of Radioactive Material from Polyacrylamide Gels. Sections of the gels containing radioactive polypeptide chains were localized by autoradiography, cut from the gel, and rehydrated. The paper backing was removed, and the gel slice was hydrolyzed in 6 mL of 6 N HCl at 110 °C for 20 h. The hydrolysates were filtered, evaporated, and analyzed on a Beckman analyzer using a procedure previously described (Piez et al., 1963). The effluent from the column was collected separately every 2 min and assayed for radioactivity by using a commercial scintillation solution and a liquid scintillation counter.

Enzymic Digestions. Bacterial collagenase digestion was carried out at 30 °C for 18 h with 50 units of purified collagenase (Advanced Biofactures Corp.) in 0.1 mL of 0.05 M Tris-HCl, pH 7.4, containing 37 mM calcium acetate, 3 mM $CaCl_2$, and 7 mM MalNEt. Incubations with pepsin were performed on samples dissolved in 0.5 M acetic acid with a pepsin/protein ratio of 1:20, using type I collagen as a carrier. The mixture was incubated at 15 °C for 6 or 24 h, after which the samples were neutralized with NaOH and then dialyzed against water and lyophilized.

Immunoprecipitation. Purified antibodies raised against tumor type IV collagen containing the 160K- and 140K-dalton chains were kindly provided by Dr. J.-M. Foidart of the National Institute of Dental Research. Samples containing labeled hydroxylated or unhydroxylated type IV procollagen were dissolved in 0.05 M Tris-HCl, pH 7.4, containing 0.2 M NaCl. The sample (0.05 mL) was incubated for 1 h at 20 °C with 0.02 mL of antitype IV collagen antibody containing 15 μg of protein. Goat antirabbit IgG (100 μg, Miles Laboratories) was added, and the incubation was continued for 1 h. The precipitate was collected after centrifugation through a discontinuous sucrose gradient (0.5 and 1 M sucrose) and examined by electrophoresis.

Other Procedures. Samples to be cleaved with CNBr were dissolved in 5 mL of 70% formic acid containing 1 mM DDT and flushed with nitrogen. A 10-fold excess of CNBr (Kodak) was added, and the samples were incubated at 30 °C for 4 h. The sample was diluted 10 times with water and lyophilized prior to $NaDodSO_4$–polyacrylamide gel electrophoresis.

For reduction and alkylation under nondenaturing conditions, samples were dissolved in 0.5 M acetic acid and dialyzed against 0.4 M NaCl and 0.1 M sodium phosphate, pH 7.6. The samples were reduced with DTT (3.2 mg/mL), and the mixtures were shielded from light and stirred at 4 °C overnight. Alkylation was achieved by adding iodoacetate (16.7 mg/mL) to the mixture. The pH was kept above 7.5 by addition of NaOH, and the samples were stirred for 1 h. This procedure was repeated once, and the samples were desalted by dialysis and then lyophilized.

Results

Comparison of Tumor and Yolk Sac Collagens. Preliminary studies established that [^{14}C]proline was incorporated into protein at a linear rate by incubated tumor as well as parietal yolk sac tissue (PEMT) (not shown). In the case of the tumor tissue, hydroxy[^{14}C]proline accounted for ~7% of

[1] Abbreviations used: BAPN, β-aminopropionitrile fumarate; EDTA, ethylenediaminetetraacetic acid; MalNEt, *N*-ethylmaleimide; DTT, dithiothreitol; FCS, fetal calf serum; CNBr, cyanogen bromide; $NaDodSO_4$, sodium dodecyl sulfate; PBS, phosphate-buffered saline.

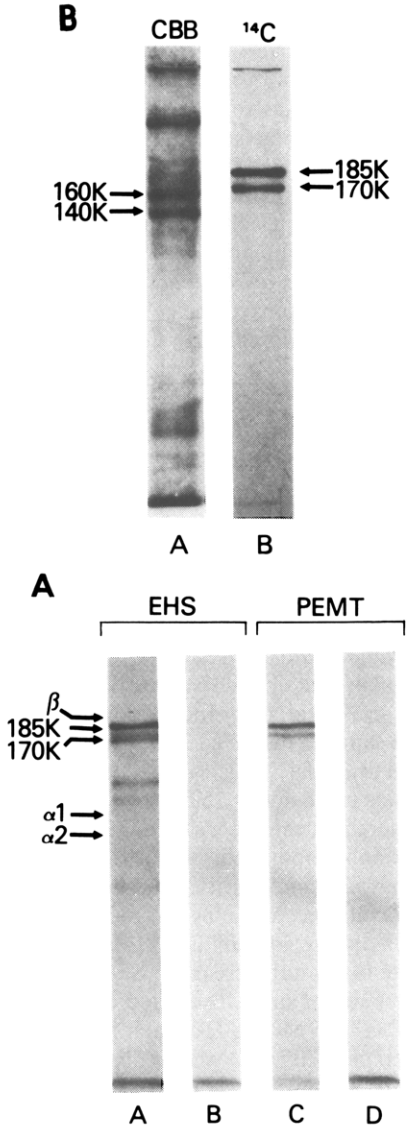

FIGURE 1: (A) $NaDodSO_4$–polyacrylamide gel electrophoresis of [^{14}C]proline-labeled type IV procollagen synthesized by EHS and PEMT tissues. The tissue was incubated for 4 h in Dulbecco–Vogt medium containing 20% dialyzed FCS, 75 μg/mL ascorbate, 50 μg/mL BAPN, and 5 μCi/mL [^{14}C]proline. The protein was extracted with 0.5 M acetic acid in the presence of protease inhibitors and precipitated with 1.71 M NaCl. Samples were run on 5% slab gels in Tris–glycine buffer containing $NaDodSO_4$ and 0.5 M urea, after reduction with dithiothreitol. (A) EHS type IV procollagen; (B) EHS type IV procollagen after digestion with bacterial collagenase; (C) PEMT type IV procollagen; (D) PEMT type IV procollagen after digestion with bacterial collagenase. (B) $NaDodSO_4$–polyacrylamide gel electrophoresis on unlabeled collagen and [^{14}C]proline-labeled EHS type IV procollagen. Tissue sample was incubated for 4 h under the standard incubation conditions in the presence of 5 μCi/mL [^{14}C]-proline. The collagenase protein was extracted with 0.5 M acetic acid in the presence of protease inhibitors and precipitated with 1.71 M NaCl. A sample was run on $NaDodSO_4$–polyacrylamide gel electrophoresis, and the gel was stained with Coomassie Brilliant Blue and dried, followed by autoradiography. (A) Coomassie Brilliant Blue stain; (B) autoradiogram.

the incorporated [^{14}C]proline. On the basis of the known ratio of 4-hydroxyproline to proline in the type IV collagen from the tumor (Timpl et al., 1978), ~12% of the incorporated radiolabeled proline was estimated to be in collagen. In comparison, PEMT incorporated less than 0.1 of the amount of label into protein as did the tumor tissue. The amount of collagenous protein in media and tissue was analyzed, and 90% of the collagenous protein was found in the tissue fraction.

Table I: Relationship of 3-Hydroxy[^{14}C]proline, 4-Hydroxy[^{14}C]proline, and [^{14}C]Proline in Protein Synthesized by the EHS Tumor and PEMT[a]

source	M_r	3-OH-[^{14}C]Pro (cpm)	4-OH-[^{14}C]Pro (cpm)	[^{14}C]Pro (cpm)	3-OH-[^{14}C]Pro/4-OH-[^{14}C]Pro × 100	OH-[^{14}C]Pro/total ^{14}C act. × 100
EHS	185K	2267	24 144	12 254	9.4	68.3
	170K	961	11 728	6 983	8.2	64.5
	125K	742	9 109	6 221	8.2	61.3
	115K	491	5 240	3 159	9.4	64.5
PEMT	185K	1338	16 220	9 121	8.3	65.8
	170K	252	2 680	2 010	9.4	59.3

[a] The tissue was incubated for 5 h in Dulbecco–Vogt medium containing 20% dialyzed FCS, 75 μg/mL ascorbate, and 50 μg/mL BAPN. The protein was extracted with 0.5 M acetic acid in the presence of protease inhibitors and precipitated with 1.71 M NaCl. Automatic amino acid analyses were carried out on hydrolysates from polypeptide chains in polyacrylamide gels.

Table II: Relationship of Hydroxy[^{14}C]lysine in Collagenous Protein Synthesized by the EHS Tumor[a]

M_r	hydroxy-[^{14}C]lysine (cpm)	[^{14}C]lysine (cpm)	hydroxy-[^{14}C]lysine/total ^{14}C act. × 100
185K	586	150	79.6
170K	488	146	77.0
125K	470	160	74.6
115K	442	152	74.4

[a] The tissue was incubated for 5 h in Dulbecco–Vogt medium containing 20% dialyzed FCS, 75 μg/mL ascorbate, and 50 μg/mL BAPN. The protein was extracted with 0.5 M acetic acid in the presence of protease inhibitors and precipitated with 1.71 NaCl. Automatic amino acid analyses were carried out on hydrolysates from polypeptide chains in polyacrylamide gels.

After extraction of the labeled tumor tissue and PEMT with 0.5 M acetic acid, ~60% of the peptide-bound hydroxy-[^{14}C]proline was precipitated by 1.71 M NaCl. Fractions prepared in this manner were examined by electrophoresis. The protein did not migrate into the gel without reduction (not shown). After reduction, two prominent radioactive polypeptides, with molecular weights of 185K and 170K, were observed in the preparations from both the tumor and PEMT (Figure 1A). Two other polypeptides with apparent molecular weights of 125K and 115K were noted in the tumor extract. These lower molecular weight components represented less than 25% of the label in the four polypeptides and in many preparations less than 10%. All four polypeptides were collagenous since they were destroyed by purified bacterial collagenase. In addition, the regions of the gel containing the individual labeled components were hydrolyzed and found to contain substantial amounts of 3- and 4-hydroxy[^{14}C]proline and hydroxyl[^{14}C]lysine. The ratios of hydroxyproline to proline and of hydroxylysine to lysine in the various polypeptides were similar to those found in type IV prepared from the tumor (Timpl et al., 1978), PEMT (Clark et al., 1975), and other tissues producing basement membranes (Kefalides, 1973) (Tables I and II).

The collagenous proteins in the extracts of tumor tissue and PEMT were fractionated by salt precipitation at neutral pH. Previous studies (Orkin et al., 1977) had shown that type IV collagen from the tumor tissue is precipitated by 1.71 M NaCl while types I, II, and V collagens are not. When this fractionation was carried out with the labeled proteins, we observed that only the 185K and 170K polypeptides were precipitated.

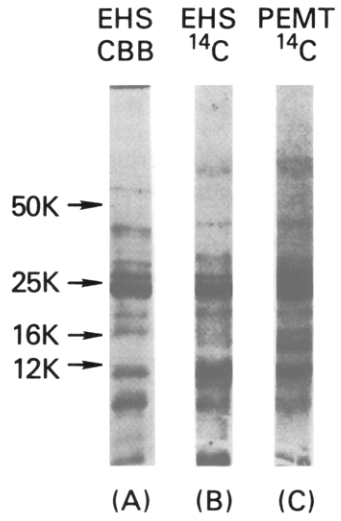

FIGURE 2: $NaDodSO_4$–polyacrylamide gel electrophoresis of CNBr-digested EHS type IV collagen (160K and 140K) and [^{14}C]proline-labeled type IV procollagens (185K and 170K) from EHS and PEMT tissues. Type IV collagen was prepared from the EHS tumor matrix and purified as described in the text. Type IV procollagens were prepared from EHS and PEMT tissues following incubation with [^{14}C]proline as desribed in the text. Following treatment with CNBr, the samples were run on a 10% acrylamide separating gel following reduction of disulfide ions. (A) EHS type IV collagen stained with Coomassie Brilliant Blue (CBB); (B) ^{14}C-labeled EHS type IV procollagen; (C) ^{14}C-labeled PEMT type IV procollagen.

The ratio of the 185K and 170K polypeptides varied in different preparations between 1.7:1 and 4:1 in the initial preparation as well as in the 1.71 M NaCl precipitate. These studies indicate that the minor components, 125K and 115K, are not present in the same protein as the two larger chains. On the basis of their size, as well as their content of 4-hydroxyproline and of hydroxylysine, the two smaller chains could be a constituent of a different collagen, possibly type V (Burgesson et al., 1976; Chung et al., 1976). Indeed these chains may be produced by muscle cells or fibroblasts present along with the tumor cells isolated from the intramuscular site. However, we have not characterized these components further.

The collagen from the tumor and from PEMT tissues, containing only the 185K and 170K polypeptides, was digested with CNBr. Very similar patterns of peptides were obtained from the collagens from each source (Figure 2). These results indicate that the tumor and the yolk sac tissue produce very similar collagen chains. To date we have not been able to resolve these two chains by chromatography on CM-cellulose or by DEAE-cellulose ion-exchange chromatography or by molecular sieve chromatography.

Differences in the susceptibility of the two chains to pepsin were observed. Since previous studies indicated that disulfide bonds present in type IV collagen cause it to be resistant to pepsin (Dehm & Kefalides, 1978a,b; Timpl et al., 1978), samples containing the 185K and 170K chains of type IV collagen were reduced and alkylated under nondenaturing conditions prior to incubation with pepsin. The products obtained were studied by gel electrophoresis (Figure 3). After 6 h of pepsin treatment both the 185K and 170K chains were destroyed. A polypeptide of 175K presumably derived from the 185K peptide was formed. Additionally, polypeptides with molecular weights of 120K, 110K, 75K, 48K, and 20K were observed. After digestion for 24 h, the 175K polypeptide was further degraded, while the proportions of the 110K and 48K peptides were increased. These results indicate that the 185K polypeptide is first cleaved to a 175K polypeptide which can

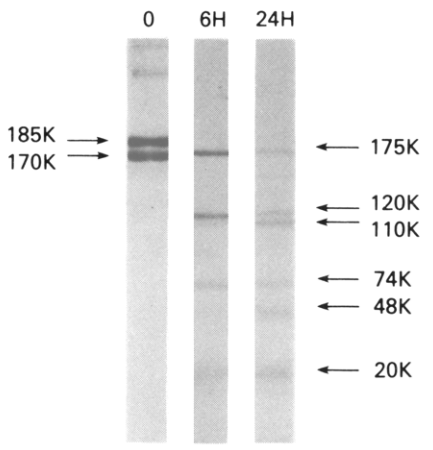

FIGURE 3: Pepsin digestion of reduced and alkylated EHS type IV procollagen. Type IV procollagens containing 185K and 170K chains were reduced with dithiothreitol and alkylated with iodoacetate as described under Experimental Procedures. Pepsinization was carried out at 15 °C by using a pepsin/protein ratio of 1:20. (A) EHS type IV procollagen; (B) after pepsin digestion for 6 h; (C) after pepsin digestion for 24 h.

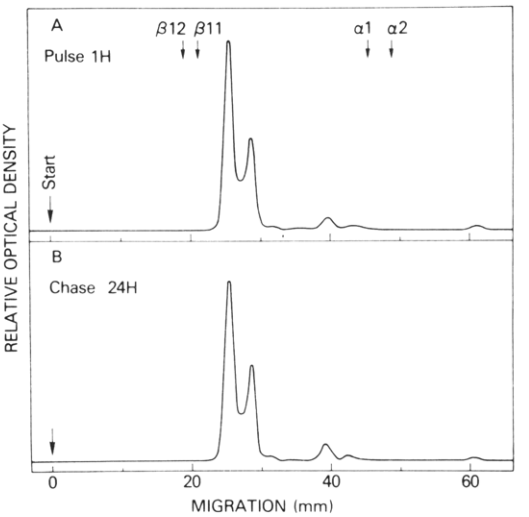

FIGURE 4: Pulse–chase study of EHS type IV procollagen. The tissue was pulsed with 5 μCi/mL [^{14}C]proline for 1 h under normal incubation conditions, after which the medium was changed and the pulse was chased for 24 h. The procollagen was extracted with 0.5 M acetic acid in the presence of protease inhibitors and precipitated with 1.71 M NaCl. Samples were run on $NaDodSO_4$–polyacrylamide gel electrophoresis after reduction, and densiometry was carried out on the autodiagram. (A) After 1-h pulse; (B) after 24-h chase.

then undergo further degradation. Cleavage of the 170K polypeptide is rapid and more extensive.

Further evidence that the 185K and 170K chains are dissimilar was obtained from pulse–chase experiments. These studies were carried out to determine if the 185K polypeptide underwent conversion to the 170K polypeptide with time and whether the proportions of the 125K and 115K polypeptides increased as would be expected for degradation products. However, there was no change in the proportions of the chains present after the chase period (Figure 4), indicating that the 170K chain is not derived from the 185K peptide and that the 125K and 115K polypeptides do not arise as degradation products. These results indicate that all the various peptides are independently synthesized during the labeling period. Tumor tissue was cultured in the presence of α,α'-dipyridyl to block the hydroxylation of prolyl and lysyl residues and thus inhibit the secretion and processing of collagen. Labeled protein was extracted from the tissue, and amino acid analysis

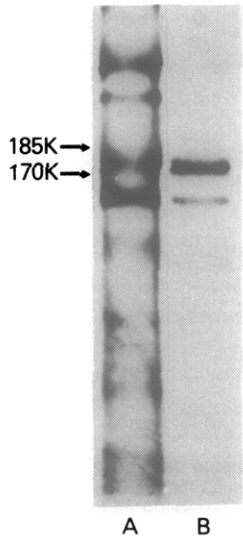

FIGURE 5: Immunoprecipitation of unhydroxylated EHS type IV procollagen. Incubation of tumor tissue was carried out in the presence of 0.3 mM α,α'-dipyridyl to inhibit hydroxylation of collagenous chains. The tissue was then extracted with 0.05 M Tris-HCl, pH 7.4, containing 0.5 M NaCl, 20 mM EDTA, 8 mM MalNEt, 10 μM phenylmethanesulfonyl fluoride, and 1 mM *p*-(hydroxymercuri)benzoate. Extracted protein was incubated with antibodies to EHS type IV collagen (160K and 140K chains) in 0.05 M Tris-HCl and 0.2 M NaCl, pH 7.4, followed by incubation with goat antirabbit IgG. Immunoprecipitates were collected through a sucrose gradient and studied by $NaDodSO_4$–polyacrylamide gel electrophoresis. (A) Starting material; (B) immunoprecipitated unhydroxylated type IV procollagen.

of this protein showed that the formation of hydroxyproline has been completely suppressed. Part of the extract was lyophilized, dissolved in buffer containing reducing agent, and electrophoresed. Another aliquot was neutralized and incubated with antibodies against type IV collagen from the tumor. The precipitate that formed was electrophoresed as above. In the immunoprecipitate two labeled chains were present which coelectrophoresed with the two chains in the original extract. These had apparent molecular weights of 175K and 140K when compared with the migration of α chains and β components of type I collagen (Figure 5). Presumably these two chains correspond to the two chains synthesized in the absence of α,α'-dipyridyl, and the differences in their migration result from the absence of hydroxyproline or hydroxylysine and the carbohydrate attached to hydroxylysine. The demonstration of two underhydroxylated chains is consistent with their being separate gene products.

Comparison of Biosynthetic Chains with Those Extracted from the Tumor. The two major polypeptides (185K and 170K) synthesized by the tumor cells are considerably larger than the two chains (160K and 140K) present in the extract of the tumor tissue. Several studies indicate that they are closely related. For example, the ratios of 3- to 4-hydroxyproline, of hydroxyproline to proline, and of hydroxylysine to lysine of the newly synthesized chains (Tables I and II) were similar to the ratios observed in the chains purified from the tumor extract (Timpl et al., 1978). Furthermore, antibody to the smaller chains precipitates the larger labeled chains. In addition, examination of the material from the incubated tumor by autoradiography and with stain revealed the presence of all four peptides in the same extract (Figure 1B). Treatment of samples containing either 185K and 170K labeled polypeptides or the 160K and 140K polypeptides extracted from the tumor with CNBr produces similar peptide patterns upon $NaDodSO_4$–polyacrylamide gel electrophoresis (Figure 2). The simplest explanation for these data is that the larger peptides are precursors for the smaller peptides and that conversion is inefficient in vitro.

Discussion

Four collagenous chains differing in size were synthesized by the tumor in vitro. All four chains contained high amounts of 3- and 4-hydroxyproline and of hydroxylysine as expected for basement membrane collagen. However, the two predominant polypeptide species, the 185K and 170K chains, were precipitated by 1.71 M NaCl at neutral pH while the two minor components were not. The solubility properties of the larger chains correspond to those observed previously with type IV collagen (Orkin et al., 1977; Timpl et al., 1978), and these studies indicate that the other minor components are not in the same molecule as the larger chains. The cultured parietal yolk sac tissue was also found to produce chains which correspond to the 185K and 170K chains from the tumor. These contained similar levels of 3- and 4-hydroxyproline as the comparable chains from the tumor tissue. Further, examination of the pattern of peptides produced by cleavage of these chains with CNBr indicated similarities. These results confirm the close relationship between the tumor collagen and that from authentic basement membranes suggested by previous immunolocalization studies (Timpl et al., 1978; Yaoita et al., 1978). In contrast to previous reports that parietal yolk sac produces a single type IV chain (Howard et al., 1976; Minor et al., 1976; Heathcote et al., 1978), we find two chains in the extract from the tissue. The two chains migrate as a closely spaced doublet on a 5% $NaDodSO_4$ gel and may not have been readily resolved previously, since the distribution of label in those studies was assessed in slices of the gel rather than by autoradiography. It has recently been reported that amniotic fluid cells (Crouch & Bornstein, 1979) and endothelial cells (Kay et al., 1979) synthesize two type IV procollagen-like chains which resemble in size the 185K and 170K chains described in this study.

A variety of observations indicate that the two chains are distinct gene products rather than related as precursor–product. First, no conversion of the 185K to the 170K chain was observed in pulse–chase experiments. Second, two chains were obtained from tissue labeled with α,α'-dipyridyl, which inhibits the hydroxylation of prolyl and lysyl residues in collagens and delays subsequent processing. The pepsin experiments indicate that the 170K protein is degraded more extensively by the enzyme than the 185K chains. The 185K chain appears to be converted to a slightly smaller component migrating in a position intermediate between the 185K and 170K chains. The more extensive degradation of the 170K chain could occur due to cleavages in the helical region of the molecule containing the chain or due to cleavages in nonhelical sequence if such regions exist. Previous work showed that trypsin yields material that forms larger segment-long-spacing crystallites than pepsin (Timpl et al., 1978). Since such crystallites visualize the helical portions of the collagen molecules, it is likely that bonds in the helical domain of type IV collagen are cleaved by pepsin.

Newly synthesized type IV collagen contains chains larger than α chains which resemble the pro α chains of other collagens (Howard et al., 1976; Minor et al., 1976; Heathcote et al., 1978). In general, previous studies have shown no conversion of these chains to a lower molecular weight protein, as seen, for example, in the conversion of types I–III procollagens to their respective collagens. Such studies suggest that the newly synthesized form of the collagen is the form deposited in the matrix. In contrast, our studies show that

the tumor matrix contains collagenous protein with smaller chains (160K and 140K) than the newly synthesized proteins (185K and 170K). Both sets of chains can be observed in the collagen prepared from the same sample of labeled tumor tissue. The larger chains are not readily demonstrated in the unlabeled tumor extracts. While the smaller chains are not apparent in the labeled protein, there is little question that they represent forms of the same chains based on peptide mapping and immunological studies. Fibroblasts synthesize procollagen type I in culture but do not convert it efficiently to collagen. Presumably, the culture media may lack some factor necessary for the procollagen peptidases. In our studies the larger chains presumably represent the polypeptides of type IV procollagens and the smaller chains the polypeptides of type IV collagens.

References

Burgesson, R. E., Adli, F. A., Kaitila, I. I., & Hollister, D. W. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 2579–2583.

Chung, E., Rhodes, R. K., & Miller, E. J. (1976) *Biochem. Biophys. Res. Commun. 71*, 1167–1174.

Clark, C. C., Tomichek, E. A., Koszalka, T. R., Minor, R. R., & Kefalides, N. A. (1975) *J. Biol. Chem. 250*, 5259–5267.

Crouch, E., & Bornstein, P. (1979) *J. Biol. Chem. 254*, 4197–4204.

Daniels, J. R., & Chu, G. H. (1975) *J. Biol. Chem. 250*, 3531–3537.

Dehm, P., & Kefalides, N. A. (1978a) *J. Biol. Chem. 253*, 6680–6686.

Dehm, P., & Kefalides, N. A. (1978b) *Biol. Chem. Basement Membr., [Proc. Int. Symp.], 1st, 1976*, 235–238.

Dixit, S. N. (1978) *FEBS Lett. 85*, 153–157.

Grant, M. E., Kefalides, N. A., & Prockop, D. J. (1972) *J. Biol. Chem. 247*, 3539–3544.

Heathcote, J. G., Sear, C. H. J., & Grant, M. E. (1978) *Biochem. J. 176*, 283–294.

Howard, B. V., Macarak, E. J., Gunson, D., & Kefalides, N. A. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 2361–2364.

Kay, E., Fareed, G. L., Ruoslahti, E., & Fessler, J. H. (1979) *Annu. Meet. Fed. Am. Soc. Exp. Biol., 63rd*, 817.

Kefalides, N. A. (1971) *Biochem. Biophys. Res. Commun. 45*, 226–234.

Kefalides, N. A. (1973) *Int. Rev. Connect. Tissue Res. 6*, 63–104.

Kefalides, N. A., Cameron, J. D., Tomichek, E. A., & Yanoff, M. (1976) *J. Biol. Chem. 251*, 730–733.

Kresina, T. F., & Miller, E. J. (1979) *Biochemistry 18*, 3089–3097.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.

Minor, R. R., Clark, C. C., Strause, E. L., Koszalka, T. R., Brent, R. L., & Kefalides, N. A. (1976) *J. Biol. Chem. 251*, 1789–1794.

Orkin, R. W., Gehron, P., McGoodwin, E. B., Martin, G. R., Valentine, T., & Swarm, R. (1977) *J. Exp. Med. 145*, 204–220.

Piez, K., Eigner, E. A., & Lewis, M. S. (1963) *Biochemistry 2*, 58–66.

Sato, T., & Spiro, R. G. (1976) *J. Biol. Chem. 251*, 4062–4070.

Timpl, R., Martin, G. R., Bruckner, P., Wick, G., & Wiedemann, H. (1978) *Eur. J. Biochem. 84*, 43–52.

Timpl, R., Bruckner, P., & Fietzek, P. (1979a) *Eur. J. Biochem. 95*, 255–263.

Timpl, R., Rohde, H., Gehron Robey, P., Rennard, S., Foidart, J. M., & Martin, G. R. (1979b) *J. Biol. Chem. 254*, 9933–9937.

Timpl, R., Risteli, J., & Bachinger, H. P. (1979c) *FEBS Lett. 101*, 265–271.

Tryggvason, K., & Kivirikko, K. I. (1978) *Nephron 21*, 230–235.

Vracko, R. (1974) *Am. J. Pathol. 77*, 314–338.

Yaoita, H., Foidart, J.-M. & Katz, S. I. (1978) *J. Invest. Dermatol. 70*, 191–193.