Characterization of a Type IV Procollagen Synthesized by Human Amniotic Fluid Cells in Culture*

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Fetal epithelioid cells, isolated from human amniotic fluid, synthesize and secrete a type IV-like procollagen characterized by a unique pattern of cyanogen bromide (CNBr)-produced peptides. The procollagen is disulfide-bonded and, after migration, migrates on sodium dodecyl sulfate-polyacrylamide gel electrophoresis as a doublet between collagen β components and pro-α1(I) chains. No conversion of the procollagen to collagen or to procollagen intermediates is observed in cell culture. The procollagen was purified by salt fractionation and ion exchange chromatography; its amino acid composition resembles that of collagenous proteins extracted from basement membranes, with a high 3- and 4-hydroxyproline and hydroxylysine content and low levels of alanine and arginine. The major products obtained after limited proteolytic digestion of the protein retain interchain disulfide bonds and, after reduction, migrate on sodium dodecyl sulfate-polyacrylamide gel electrophoresis near intact pro-α1(I) chains. The procollagen is secreted efficiently by amniotic fluid cells despite almost complete inhibition of peptide hydroxylation but, unlike type I procollagen, the secreted underhydroxylated chains lack interchain disulfide bonds. Since these cells also secrete fibronectin and elaborate an extensive extracellular matrix, the system should prove useful in the study of cell-matrix interactions.

Basement membranes are specialized connective tissue structures disposed between epithelial or endothelial cells and underlying tissues. These structures contain at least one collagenous protein and one or more noncollagenous glycoproteins that are synthesized and secreted by the associated epithelial or endothelial cells, or both (1). However, the exact molecular compositions of basement membranes are likely to be tissue-specific and probably vary during development and with aging and disease.

Collagen chains have been isolated from pepsin digests of the renal glomerulus, posterior corneal endothelium, and lens capsule basement membranes, and Kefalides (2) has proposed that basement membranes contain a distinct collagen, [α1(IV)]₃, composed of three identical α chains. Other investigators have reported a more heterogeneous mixture of collagenous components in the glomerular basement membrane (3-5).

Although the size and organization of collagenous components in basement membrane remain controversial, it is generally agreed that basement membrane collagens are distinct from interstitial collagens. Basement membrane, or type IV, collagens are characterized by elevated but variable levels of 3-hydroxyproline, low total amino acid content, and elevated ratios of 4-hydroxyproline to proline and hydroxylysine to lysine. The hydroxylysine residues are almost glycosylated with galactose or glucosylgalactose. Cysteine is generally present, whereas alanine and arginine are significantly reduced relative to their occurrence in types I, II, and III collagen (1).

The in vitro biosynthesis of basement membrane procollagen has been demonstrated in several systems, including the chick lens epithelium (6), rat kidney glomerulus (7, 8), rat parietal yolk sac endoderm (9, 10), rabbit corneal endothelium (11), bovine aortic endothelium (12), and rat lens capsule (13). A type IV-like procollagen synthesized by the basement membrane-producing, murine EHS sarcoma in vivo has also been described (14, 15). However, to date there has been no structural characterization of a type IV procollagen synthesized by human cells in vitro or in vivo. Cell culture offers a distinct advantage in the study of these procollagens since the secondary changes that accompany the extraction and isolation of the protein from a tissue can be minimized.

We have recently described the synthesis and secretion of fibronectin and collagenous proteins by E, AF, and F human amniotic fluid cells in culture (16, 17). In this paper, we report the synthesis of a type IV-like procollagen (termed AF1) by amniotic fluid cells and present a structural characterization of the protein. It should be emphasized that we do not have direct evidence that AF1 is a component of basement membranes. However, the similarity of this procollagen to proteins extracted from basement membranes makes this association likely.

EXPERIMENTAL PROCEDURES

Materials
Powdered DMEM medium, penicillin G, and streptomycin sulfate were purchased from Grand Island Biological; trypsin (1:250) was from ICN Pharmaceutical. Bacterial collagenase (form III) was from Advance Biofabrics. L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin, α-chymotrypsin (three times crystallized), and pepstatin A were from Worthington. Peptatin A was from the Protein Research Foundation (Osaka, Japan). CNBr was from Eastman. DEAE and CM-cellulose were from Whatman. l-[2,3-3H]Proline (92 Ci/mmol) was from Whatman.

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1 The abbreviations used are: DMEM medium, Dulbecco-Vogt modified Eagle’s medium; CNBr, cyanogen bromide; Tris/saline, 0.15 M NaCl, 50 mM Tris-HCl, pH 7.5; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; β-AFN, β-aminopropionitrile fumarate; PhCH₂SO₂F, phenylmethanesulfonfyl fluoride; SLS, segment long spacing.
Type IV Procollagen Synthesized by Cells in Culture

Methods

Cell Isolation and Culture—Cloned and mass cultured amniotic fluid cells were isolated from second trimester amniotic fluid as previously described (16). Cell types were identified on the basis of cellular and clonal morphology as described by Hoehn et al. (18). Cells were propagated and subcultured as described previously (16).

Metabolic Labeling—Labeling and pulse-chase experiments were performed using just-contiguous cultures of amniotic fluid cells (transfer 2 to 3). For these studies, DME medium was supplemented with sodium ascorbate (50 μg/ml), β-APN (80 μg/ml), and penicillin/streptomycin (10 μg/ml) and phenol. After a 2 h incubation in DME medium, and chased in medium containing 20 mM L-proline with 20 to 40 μCi/ml of [3H]proline or 5 μCi/ml of [14C]proline under denaturing conditions was achieved using a composite gel in the presence of inhibitors as described under "Methods." Proteins were resolved by SDS-PAGE on a composite slab gel (see "Methods") after reduction with 50 mM dithiothreitol. Fibronectin (FN) and type IV procollagen were identified (see text). ProAF2, pAF2, and AF2 designate pro-α1, α2, and α3 chains, respectively, of [pro-α1(I)]3 (17). FN labeled proteins in AF cell culture medium and cell layer. Just-confluent cultures of AF cells were labeled for 24 h with [3H]proline-labeled protein when E or AF cells were performed in 8 M urea, 50 mM Tris-HCl, pH 8.5, as described by Monson et al. (21).

Proteolytic Cleavage of AF1—For digestion with pepsin, AF1 in 0.5 N acetic acid was dialyzed against freshly prepared 0.2 mM NH4HCO3 at 4°C. Immediately prior to addition of the protease, 1 mg CAcl was added to a final concentration of 0.5 mM. For digestion with bacterial collagenase, the sample was incubated for 6 h at 37°C with 5 μg/ml of bacterial collagenase. For digestion with trypsin and chymotrypsin, the sample was incubated for 6 h at 15°C with 5 μg/ml of L-1tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin or a-chymotrypsin and the reaction was terminated by the addition of 0.1 M CaCl2. The digests were lyophilized at least twice prior to analysis by SDS-PAGE. Samples were digested with human skin collagenase (2 μg/ml or 1:100 enzyme:substrate) for 24 h at 25°C in Tris/saline containing 10 mM CaCl2.

Amino Acid Analysis—Protein hydrolysates were analyzed on a Beckman model 121 analyzer modified for single column microanalysis. Determinations of radioactive hydroxyproline and proline were made essentially as described by Krus and Bornstein (22).

RESULTS

SDS-PAGE—The proteins secreted by amniotic fluid cells in culture have been examined by SDS-PAGE before and after incubation with bacterial collagenase (16, 17). The major protein in the medium of E and AF cell cultures is a glycoprotein structurally and immunochemically related to plasma cold-insoluble globulin and identified as fibronectin (16). Fibronectin accounted for more than 40% of the total nondialyzable [3H]proline-labeled protein when E or AF cells were cultured.

![Fig. 1. Densitometric scans of fluorescence autoradiograms of [3H]proline-labeled proteins in AF cell culture medium and cell layer. Just-contiguous cultures of AF cells were labeled for 24 h with [3H]proline in the presence of ascorbate and β-APN and processed in the presence of inhibitors as described under "Methods." Proteins were resolved by SDS-PAGE on a composite slab gel (see "Methods") after reduction with 50 mM dithiothreitol. Fibronectin (FN) and collagenous proteins are identified (see text). ProAF2, pAF2, and AF2 designate pro-α1, α2, and α3 chains, respectively, of [pro-α1(I)]3. (17). A, culture medium; B, 0.5 N acetic acid extract of cell layer; C, 0.5 N acetic acid residue of cell layer; D, AF cells labeled for 4 h with [3H]proline during cell attachment and spreading. Cells were detached from the dish with 0.02% EDTA and the substratum-attached material was solubilized in SDS-PAGE sample buffer containing dithiothreitol.](http://www.jbc.org/)
labeled for 24 h in the presence of ascorbate. In contrast, the major protein in the medium of amniotic fluid fibroblasts (AF cells) has been identified as type I procollagen (17).

Four prominent bands which were sensitive to bacterial collagenase were resolved by SDS-PAGE of AF cell medium after labeling for 24 h with [3H]proline in the presence of ascorbate (Fig. 1A). These bands migrated between the positions of fibronectin and α1(I) chains. The closely spaced bands migrating immediately below fibronectin, and more slowly than fibroblast pro-α1(I) chains, appear to be related and have been designated AF1 chains. Although interclonal variation in the relative intensity of the two bands has been observed, the intensity of the upper band was consistently greater than or equal to that of the lower band. Both bands are derived from disulfide-bonded trimers (see below) and, like type I procollagen chains, can be labeled with radioactive mannose, glucosamine, cysteine, and tyrosine. The remaining bands in the culture medium (pro-AF2 and p-AF2) and an additional band in the cell layer (AF2) are derived from a disulfide-bonded procollagen (type I trimer) which contains three identical pro-α chains immunologically and biochemically related to pro-α1(I) (17).

Prominent collagenase-sensitive bands, co-migrating with culture medium AF1, were observed in the 0.5 M acetic acid extract of cell layer homogenates (Fig. 1B). Furthermore, AF1 was enriched, relative to the other collagenous components, in the acetic acid-insoluble fraction of AF cell layers (Fig. 1C) and in the “microexudate” or substrate-attached material remaining on the culture dish when cells were detached with EDTA (Fig. 1D). AF1 procollagen and fibronectin together accounted for the majority of the radiolabeled protein in this material when cells were labeled during attachment and spreading.

AF1 procollagen was observed to be the major collagenous protein in E cell cultures. However, due to the relative difficulty in subculturing E cells and the limited growth potential of these cells in vitro (18), we have confined our biosynthetic and structural studies of AF1 to the protein synthesized by AF cells.

DEAE- and CM-Cellulose Chromatography—When AF culture medium was precipitated with 20% ammonium sulfate and chromatographed on DEAE-cellulose in urea at 4°C, AF1 procollagen was eluted prior to the start of the salt gradient (17). Under these conditions, types I and III procollagens and [pro-α1(I)]3 were eluted within the gradient. AF1 procollagen, obtained in this fashion, was nearly pure as assessed by SDS-PAGE, except for minor low molecular weight contaminants. The procollagen eluted from CM-cellulose near the position of type I procollagen (Fig. 2). Digestion of the native procollagen, obtained by DEAE-chromatography, with pepsin at 4°C for 24 h, generated a disulfide-bonded protein (AFIPep) which migrated on SDS-PAGE near type I procollagen and which, after reduction, migrated as two chains near pro-α1(I). Pepsin-treated AF1 procollagen eluted earlier than intact AF1 procollagen from CM-cellulose (Fig. 2). AF1 procollagen, prepared by sequential ammonium sulfate precipitation and chromatography on DEAE- and CM-cellulose migrated as a closely spaced doublet but was otherwise electrophoretically pure (Fig. 3). This preparation was used for amino acid analysis and peptide mapping.

Molecular Sieve Chromatography—When AF1, radiolabeled with [3H]proline, was chromatographed on 5% agarose, the radioactivity eluted between the void volume and collagen

Fig. 2. CM-cellulose chromatogram of [3H]proline-labeled AF1 procollagen prepared by ammonium sulfate precipitation (20% w/v at 4°C) and DEAE-cellulose chromatography. Cells were labeled and the medium was harvested as described under “Methods." Pepstatin A (1 μg/ml) was added at acid pH and the solution was dialyzed against 0.04 M sodium acetate, 6 M urea, pH 4.8, prior to chromatography at 42°C. The arrow indicates the start of the gradient (0 to 0.1 M NaCl). Conductivities were measured at room temperature. The positions of elution of α1(I), α2(I), α2, and pepsin-treated AF1 procollagen (AF1red) are indicated. The eluent, prior to the start of the gradient, contained predominantly low molecular weight material. Recovery of radioactivity in the major peak was greater than 50%.

Fig. 3. SDS-PAGE of AF1 procollagen in the presence of dithiothreitol. The procollagen was prepared by ammonium sulfate precipitation from culture medium, followed by sequential DEAE- and CM-cellulose chromatography. Proteins were stained with Coomassie blue. Left, AF1 procollagen. A single band migrating above the position of γ components was observed prior to reduction. Right, rat skin collagen. The position of migration of pepsin-treated AF1 procollagen is indicated by an arrow.
γ components. After reduction and alkylation under denaturing conditions, the major radioactive peak, which corresponded to reduced AFl chains, eluted between β components and pro-α(1) chains (data not shown).

**Amino Acid Analysis**—The amino acid compositions of AFl procollagen and pepsin-treated AFl procollagen are shown in Table I. The compositions are characterized by a high 3-hydroxyproline content, high ratios of 4-hydroxyproline to proline and hydroxylysine to lysine, and low levels of total imino acids, alanine, and arginine. The low glycine content and relatively high levels of tyrosine, methionine, cysteine, and hydrophobic amino acids resemble analyses reported for type I procollagen (21). Although AFl is slightly enriched in glycine and imino acids, relative to AFl, the composition of this protein indicates the retention of nontriple helical sequences. The amino acid composition of AFl is similar to that of the EHS murine sarcoma procollagen isolated from a basement membrane-producing tumor (Table I; Ref. 15). Similarities in composition also exist among these proteins and pepsin-resistant collagens isolated from glomerular basement membrane (Fractions C and D in Ref. 3) and components isolated from basement membrane by sonication, reduction and alkylation, and extraction with SDS (4). The composition of AFl resembles that of the type IV collagen chain isolated by pepsin digestion of human glomerular basement membrane (Table I; Ref. 2).

**Cyanogen Bromide Cleavage**—AFl procollagen, labeled with [3H]proline, was cleaved with cyanogen bromide. The resulting peptides were resolved by SDS-PAGE in the presence and absence of reducing agents (Fig. 4). The patterns are distinct from those obtained with human types I, II, III, or A-B collagens or with types I and III procollagens.

**Protease Cleavage**—When AFl procollagen, labeled with either [3H]proline or [3H]mannose, was incubated with pepsin for 24 h at 4°C or for 6 h at 15°C or 30°C, the major product retained disulfide bonds and mannose label and migrated on SDS-PAGE near the position of intact type I procollagen (Fig. 5). After reduction, one or two chains were observed which migrated just below the position of AFl chains. Low molecular weight, collagenase-sensitive fragments were frequently generated in small amounts (Fig. 5), but components with the mobility of interstitial α chains were not observed. The most abundant low molecular weight fragments appeared after reduction and migrated with apparent molecular weights of approximately 60,000 and 45,000. The same result was obtained when AFl procollagen was reduced and alkylated under non-denaturing conditions and then treated with pepsin or when pepsin-treated AFl procollagen was reduced and alkylated prior to a second pepsin treatment. In contrast,

![Fig. 4. Densitometric scans of fluorescence autoradiograms of CNBr peptides of [3H]proline-labeled AFl procollagen, as resolved by SDS-PAGE on a 12.5% separating gel. AFl procollagen was purified by salt fractionation and ion exchange chromatography from AF cell culture medium as described in the text. A, CNBr peptides, unreduced; B, CNBr peptides, reduced with dithiothreitol. The positions of migration of marker types I, II, and III CNBr peptides are indicated.](http://www.jbc.org/)

![Fig. 5. Fluorescence autoradiograms of AFl procollagen, labeled with [3H]proline, as resolved by SDS-PAGE before and after limited digestion with pepsin (100 μg/ml, 6 h, 15°C). The position of migration of α1(I) is indicated. Note the two minor fragments which migrate below the position of α1(I) chains when pepsin-treated AFl procollagen is examined in the presence of dithiothreitol (DTT).](http://www.jbc.org/)
Type IV Procollagen Synthesized by Cells in Culture

Procollagen → Fn → Af

AF  F  AF  F

+ + + +

+ DTT - DTT

α,α'-dipyridyl

FN  proα1(I)  proα2, pNα1  pNα2

FIG. 6. Fluorescence autoradiograms of AF and F cell medium proteins synthesized in the presence and absence of α,α'-dipyridyl, as resolved by SDS-PAGE. Just-confluent control cultures of AF and F cells were labeled for 24 h with [3H]proline in DME medium supplemented with ascorbate and β-APN. Treated cultures were preincubated and labeled in ascorbate-free medium containing 0.1 mM α,α'-dipyridyl. Medium and cell layer proteins were harvested into inhibitors and aliquots were prepared for SDS-PAGE (see "Methods"). The positions of migration of unreduced fibronectin (FN) and procollagen are indicated at the upper left-hand corner of the figure. The positions of migration of reduced AF and F cell procollagen and collagen chains synthesized in control cultures are also indicated. The intense band, third from top, in Lane 5 represents the type I trimer-derived pro-α chain. In the presence of α,α'-dipyridyl, this band migrates as pro-α (Lane 6). DTT, dithiothreitol.

Likewise, when F cells were labeled in the presence of α,α'-dipyridyl, the secreted type I procollagen chains were largely disulfide bonded (Fig. 6, compare Lanes 4 and 8) and migrated more rapidly on SDS-PAGE than the chains synthesized by control cultures (Fig. 6, compare Lanes 7 and 8). A significant proportion of the F cell procollagen was retained in the cell layer in the presence of α,α'-dipyridyl.

When fibroblast type I procollagen was digested with pepsin for 12 to 24 h at 0-4°C without reduction and alkylation, complete conversion to α chains was observed. Disulfide-bonded products with the approximate mobility of pepsin-treated AF1 procollagen were also obtained after digestion of AF1 procollagen with trypsin and chymotrypsin.

AF1 was not a substrate for human skin collagenase under conditions which provided complete digestion of type I collagen to A and B fragments (not shown).

Biosynthetic Studies—When AF cells were labeled with [3H]proline for 24 h, 10 to 20% of the nondialyzable radioactivity released into the culture medium was recovered in AF1 procollagen. However, a significant proportion of the newly synthesized AF1 remained associated with the cell layer (Fig. 1, B and C). The synthesis and secretion time for AF1 procollagen was estimated from short term, continuous labeling studies to be at least 60 min, compared with a time of less than 30 min for fibroblast type I procollagen (not shown).

When AF cells were labeled with [3H]proline for 24 h in the presence of 0.1 mM α,α'-dipyridyl, used to block hydroxylation and glycosylation, both incorporation of radioactivity and secretion of radiolabeled protein were reduced by only 10 to 15%, whereas the per cent hydroxylation of [3H]proline in medium protein was reduced from a normal level of 20 to 25% to less than 0.5%. Nearly normal amounts of AF1 procollagen were secreted into the culture medium in the presence of α,α'-dipyridyl. However, the protein lacked interchain disulfide bonds (Fig. 6, compare Lanes 2 and 6) and migrated more rapidly than AF1 procollagen synthesized by control cultures (Fig. 6, compare Lanes 5 and 6). Although type I trimer-derived pro-α chains showed a similar increase in migration rate on SDS-PAGE when synthesized in the presence of α,α'-dipyridyl, the secreted protein was largely disulfide-bonded (Fig. 6, compare Lanes 1 and 2 with 5 and 6) and some of the procollagen was retained in the cell layer (not shown).

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In pulse-chase studies, the total radioactivity in AF cell medium procollagen remained essentially constant for chase periods of up to 48 h (Fig. 7A). No change in the appearance or mobility of AFl on SDS-PAGE was observed during this period. Processing of AFl in the cell layer was also not observed (not shown). However, during the same chase period, [pro-α1(I)]3, was extensively converted in the culture medium to [α1(I)]3 (Fig. 7B).

**DISCUSSION**

Human fetal AF cells, isolated from second trimester amniotic fluid, synthesize and secrete a type IV-like procollagen (AFl) in cell culture. Like the interstitial procollagens, AFl is a disulfide-bonded glycoprotein that contains both collagenous and noncollagenous domains. The amino acid composition of AFl is similar to that of the type IV procollagen isolated from the murine EHS sarcoma (15) and is characterized by a low total amino acid, alanine, and arginine content, relative to the interstitial procollagens. The protein also contains heteropolysaccharide chains as evidenced by labeling with d-[2-3H]mannose. The cyanogen bromide peptide pattern is distinct from those of types I, II, III, and A-B collagens when examined by SDS-PAGE. It is of interest that AFl is also synthesized in small amounts by fibroblasts in culture.3 The protein may, therefore, be widely distributed in tissues.

When newly synthesized AFl procollagen was examined by SDS-PAGE after reduction, two closely spaced chains migrating between β components and pro-α chains were consistently observed. Since we have not resolved these chains without prior reduction, it remains uncertain whether the two chains are derived from the same or different procollagen molecules. The chains may be genetically distinct, alternatively, they may share the same amino acid sequences with differences in the extent or type of post-translational modifications, or both. The appearance of more than one chain on SDS-gels after reduction has also been observed for the EHS parietal yolk sac (9), glomerular epithelial cell (26), rat lens capsule basement membrane (13), and the EHS sarcoma procollagen (14). It is possible that the triple helical domain of AFl and other type IV procollagens is longer consistent with a reported SLS crystallite length of 3500 Å for EHS sarcoma procollagen (14). It is possible that the triple helical domain of AFl and other type IV procollagens is longer consistent with a reported SLS crystallite length of 3500 Å for EHS sarcoma procollagen (14). On the other hand, SLS crystallites of bovine anterior lens capsule basement membrane collagen, isolated by limited pepsin digestion, had a length of approximately 3000 Å, i.e. the length of interstitial collagen (27). Unfortunately, differences in the extent and type of post-translational modifications, possible differences in the relative size of the collagenous and noncollagenous domains, the anomalous behavior of both collagenous and noncollagenous regions in SDS (28, 29), and the generally poor resolving power of molecular sieves preclude a more precise determination of molecular weight for AFl procollagen.

It is, however, unlikely that the higher apparent molecular weight of AFl, relative to pro-α1(I) chains, results exclusively from differences in post-translational modification since AFl chains synthesized in the presence of α,α'-dipyridyl migrated significantly more slowly on SDS-PAGE than fibroblast pro-α1(I) chains synthesized in the presence of this inhibitor (Fig. 6, compare Lanes 6 and 8). In addition, the AFl chains synthesized in the presence of tunicamycin, an inhibitor of asparaginyl-linked heteropoly saccharide chain synthesis (30), migrated more slowly than pro-α1(I) chains on SDS-PAGE.2 On the basis of these observations, we suggest that AFl chains are either longer or assume a more extended conformation in SDS and CaCl2 than pro-α1(I) chains. Further structural characterization, including independent estimates of the molecular weight of the collagenous and noncollagenous domains, and compositional analysis of the isolated CNBr peptides should provide a more satisfactory molecular weight estimate.

Despite clear differences in primary structure, amino acid composition, and apparent size, several observations suggest that the overall structural organization of type IV procollagen is similar to that of the interstitial procollagens. Circular dichroism and viscometric studies have indicated that type IV procollagens and collagen contain a triple helical domain (15, 31, 32). SLS crystallite studies have confirmed these findings and have indicated that the length of the major triple helical domain is at least as long as that of type I collagen (15, 27, 33). In addition, the amino acid compositions of AFl and the EHS procollagen are similar to those of type I procollagen in their low content of glycine and high levels of methionine, cysteine, tyrosine, and hydrophobic amino acids, relative to the collagenous domains of the interstitial procollagens. Although type IV collagens do not form periodic collagen fibrils in vivo, the isolation of lysine-derived cross-links from basement membranes has suggested that type IV collagen is a substrate for lysyl oxidase (34). Finally, the major collagenase-resistant peptides of AFl procollagen are similar to those of type I procollagen, both in apparent size and in the distribution of interchain disulfide bonds.2

When AFl procollagen was incubated with pepsin, chymotrypsin, or trypsin, under conditions which produce rapid cleavage of type I procollagen to collagen, a fragment was generated which migrated between AFl and γ components on SDS-gels and which contained interchain disulfide bonds and mannose. After reduction, one or two chains were observed which migrated near the position of pro-α1(I) chains. The cleavage resulted in a loss in apparent molecular weight of about 15,000/chain. Since pepsin-treated AFl procollagen is enriched in imino acids, glycine, and hydroxylysine relative to intact AFl (Table I), it seems likely that a noncollagenous extension is lost as a result of these treatments. The major pepsin-resistant fragment of AFl procollagen is not completely collagenous, however, since the glycine and imino acid content remain lower than would be expected for a continuous triple helix. It, therefore, seems likely that pepsin-treated AFl procollagen contains one or more noncollagenous, protease-

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1 E. Crouch and P. Bornstein, unpublished observation.
2 P. Killen and G. E. Striker, personal communication.
3 E. Crouch and P. Bornstein, unpublished observation.
resistant domains which contain mannose and possibly interchain disulfide bonds.

We have occasionally observed the generation of minor fragments which migrate more rapidly than α chains on SDS-gels during pepsin treatment of AF1. Two of these fragments (molecular weights of approximately 45,000 and 60,000) are collagenase-sensitive and appear to be derived from molecules which migrate near the position of pepsin-treated AF1 procollagen prior to reduction. The fragments are comparable in molecular weight to collagenous fragments generated by limited pepsin digestion of EHS procollagen (15) and lens capsula (27). A disulfide-bonded collagenous fragment (55,000 daltons after reduction) has also been isolated from pepsin digests of vascular intima (23) and placenta.4 These results suggest that there may be one or more short noncollagenous sequences, or collagenous regions which are relatively susceptible to pepsin cleavage, within the hol. The existence of pepsin-sensitive regions within the major helical domain is consistent with generation of truncated SLS crystallites when collagens were prepared by prolonged pepsin digestion (15, 27). Similarly, a trypsin-sensitive site within the helical domain has been reported for type III collagen (35).

AF1 procollagen is not processed in the culture medium or cell layer under conditions in which [pro-al(I)], and F cell type I procollagen are extensively converted to p-collagens and collagen. These findings indicate that AF1 procollagen does not undergo processing or, alternatively, that the necessary processing mechanisms are absent or inactive in the AF culture system. Although the conversion of newly synthesized basement membrane collagens to "α chains" and lower molecular weight components has been reported for chick lens and rat glomerulus in organ culture (6, 8), no conversion has been observed for the procollagens synthesized by rat parietal yolk sac (10) or rat lens capsula (13) in vitro or in the EHS sarcoma in vivo (14, 15).

We have observed a prolonged synthesis + secretion time for AF1 procollagen, relative to that of type I procollagen secreted by human fibroblasts. Comparable delays, on the order of 60 min, were reported for the secretion of type IV procollagen by chick lens and rabbit corneal endothelial cells and by rat parietal yolk sac (6, 9, 11). The delay observed in chick cells was accompanied by an apparent delay in the acquisition of disulfide bonds and a pepsin-resistant conformation (36) and has been attributed to the time required for the increased post-translational modification of type IV chains. However, our experiments with αα'-dipyridyl (Fig. 6) and tunicamycin5 suggest that heteropolysaccharide chains and normal hydroxylation and glycosylation of the collage nous domains are not required for the secretion of AF1 procollagen. In agreement with these observation, Maragoudakis et al. (37) have reported that the rat parietal yolk sac procollagen is secreted at a normal rate in the presence of αα'-dipyridyl or GPA 1734, another iron chelator, despite a reduction in the extent of proline hydroxylation to less than 10% of normal. The intracellular delay could, therefore, reflect other differences in the synthesis or transcellular transport of type IV procollagen.

It is of interest that type IV procollagen, secreted in the presence of αα'-dipyridyl, lacks interchain disulfide bonds, whereas type I procollagen secreted by fibroblasts under these conditions is largely disulfide-bonded. This observation suggests that the mechanism of assembly of the two collagen types, or the location of the interchain disulfide bonds, differs, but further studies are required.

AF cells in culture elaborate a complex pericellular and extracellular matrix which has been shown by immunochemical methods to contain fibronectin (16) and collagenous proteins. We have described the results of experiments which indicate that AF1 and fibronectin are major cell-derived components that are deposited on the culture dish during cell attachment and spreading as well as during continuous culture. The AF cell matrix, therefore, should be valuable in studying the possible roles of these components in such diverse processes as adhesion, spreading, motility, matrix synthesis and turnover, and cell differentiation.

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