Fetal Calf Ligament Fibroblasts in Culture Secrete a Low Molecular Weight Collagen with a Unique Resistance to Proteolytic Degradation

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ABSTRACT A highly unusual collagen was secreted by fibroblasts cultured from 150- and 270-d-old fetal calf nuchal ligaments. Purification revealed that this protein (which may be synthesized in a higher molecular weight form) was precipitated at unusually high concentrations of ammonium sulfate and was also eluted from DEAE-cellulose at greater salt concentrations than were types I and III procollagens. On SDS PAGE, the collagenous protein exhibited an M_r of approximately 12,750 that was not altered in the presence of reducing agent. The low molecular weight collagen (FCL-1) was sensitive to bacterial collagenase and had a [^{3}H]glycine content comparable to that found in type I procollagen, although the [^{3}H]Hyp to [^{3}H]Pro ratio was 0.43. FCL-1 was not cleaved by human skin collagenase, mast cell protease, trypsin, Staphylococcal V8 protease, or proteinase K at 37°C. The collagen was susceptible to trypsin, but not to V8 protease, only after heating at 80°C for 30 min. Preliminary structural studies indicate that FCL-1 was resistant to cleavage by CNBr but exhibited limited proteolysis with pepsin.

Both 150- and 270-d-old fibroblasts produced comparable levels of interstitial (types I and III) procollagens, which comprised ~70% of the total protein secreted into the culture medium. However, 270-d-old (term) fibroblasts secreted ~50% more FCL-1, as percent of total culture medium protein, in comparison to the cells from the earlier gestational stage. This collagen may therefore play a role in the development of the nuchal ligament.

Fibroblasts cultured from fetal bovine ligamentum nuchae, a tissue that contains high levels of elastin and is prominent in the necks of grazing mammals, have been used to study the biosynthesis of several major proteins of the connective tissue stroma, including the procollagens, microfibrillar glycoproteins, and elastin (1, 2). Recent experiments by Mecham et al. (2) have indicated that the extracellular matrix stabilizes the phenotypic expression of elastin by fetal calf ligament (FCL)[1] fibroblasts in vitro. In addition, cells from calves in the third trimester of development synthesized greater amounts of both total protein and elastin in comparison to those from earlier gestational stages. Other studies on developing sheep nuchal ligament demonstrated a positive relationship between the augmentation, at term, of tropoelastin synthesis and elastin mRNA levels (3). These workers also showed a concomitant decrease in type I procollagen mRNA.

As new members are added to the collagen gene family, it has become apparent that there is a striking diversity both in the molecular structure and in the functional roles assumed by these proteins (for a review, see reference 4). In addition to the interstitial (types I, II, and III), basement membrane (type IV), and pericellular matrix and/or basement membrane associated (type V) collagens, several collagenous proteins with apparently shorter, or interrupted, triple helical sequences have been recently described. Thus, type VI (intimal) collagen was characterized as a pepsin-resistant fragment from vascular tissues (5–7). Other disulfide-bonded low molecular weight collagens have been isolated from mammalian and avian cartilage after pepsin treatment (8–12). A 59,000-mol-wt collagen was recently purified from chicken embryo chondrocytes without prior pepsin digestion. The synthesis of this protein, which appeared to be distinct from the other collagen

[1] *Abbreviations used in this paper:* FCL, fetal calf ligament; DME, Dulbecco-Vogt-Modified Eagle's Medium; β-APN, β-aminopropionitrile fumarate; DEAE, diethylaminoethyl; CM, carboxymethyl; $PhCH_2SO_2F$ phenylmethane sulfonylfluoride; MalNEt, *N*-ethylmaleimide; BSA, bovine serum albumin; DTT, dithiothreitol; Tris-saline, 0.15 M NaCl, 50 mM Tris-HCl, pH 7.5.

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types, was increased in differentiated cells (13) and in cells cultured within collagen gels (14). Recent experiments in our laboratory have resulted in the identification of a novel class of collagenous components that is similar to endothelial cell (EC) collagen and is secreted by several normal mesenchymal and malignant cell lines in vitro (15; H. Sage, G. Balian, A. Vogel, and P. Bornstein, manuscript submitted for publication).

We now report that cultures of FCL fibroblasts secrete an unusual collagenous protein that we have identified in a low molecular weight form. This collagen is increased in cells from a later gestational age.

MATERIALS AND METHODS

Cell Culture and Metabolic Labeling: Fibroblasts were isolated from *ligamenta nuchae* of 150- and 270-d-old bovine fetuses as described by Mecham et al. (2). The cells were maintained in Dulbecco's modified Eagle's medium (DME)[1] supplemented with penicillin (100 U/ml), streptomycin (50 μg/ml), (Gibco Laboratories, Grand Island, NY) and 10% by volume fetal calf serum (Rehatuin F.S., Armour Pharmaceutical Co., Kankakee, IL). Cells were subcultured in a 1:1 (by volume) solution of 0.02% EDTA and 0.025% trypsin at a split ratio of 1:3.

Nearly confluent FCL cells were metabolically labeled in either primary culture or after one passage. At these stages of subculture, the cells exhibited similar plating efficiencies, growth rates, and morphologic characteristics. Cultures were preincubated in 4 ml (per 75-cm^2 flask) or 8 ml (per 150-mm dish) of DME containing 50 μg/ml sodium ascorbate and 64 μg/ml β-APN (β-aminopropionitrile fumarate) for 30 min–1 h. After removal of preincubation media, the cells were exposed to fresh media containing 25 μCi/ml each of 2,3-[^{3}H]Pro (35 Ci/mmol, New England Nuclear, Boston, MA) and 2-[^{3}H]Gly (23 Ci/mmol, Amersham/Searle Co., Arlington Heights, IL) for 21 h. The culture media were subsequently processed in the presence of proteinase inhibitors as previously described (16).

Analytical Methods: Radiolabeled culture medium proteins were fractionated by ammonium sulfate precipitation at two concentrations (20% and 20–50%, weight to volume ratio), followed by chromatography on DEAE-cellulose (DE-52, Whatman Laboratory Products Inc., Clifton, NJ) at 4°C. This column was equilibrated in a 6 M urea, 50 mM Tris-HCl buffer, pH 8.0, containing 2.5 mM EDTA and 0.2 mM $PhCh_2SO_2F$; elution of bound proteins was performed with a linear gradient, from 0–200 mM NaCl, in 400 ml of equilibration buffer.

Measurement of [^{3}H]Hyp, [^{3}H]Pro, and [^{3}H]Gly was determined by amino acid analysis after hydrolysis of protein in constant boiling HCl *in vacuo* at 110°C for 24 h.

Cleavage of collagens with CNBr was performed as previously described (16), with the addition of a desalting step on a PD-10 column (Pharmacia Fine Chemicals, Piscataway, NJ) in 0.1 M acetic acid, prior to lyophilization of the reaction products. SDS-PAGE was performed on discontinuous methylene (bis)polyacrylamide slab gels (17) containing 0.5 M urea, and the gels were processed for autoradiography according to methodology described by Sage and Bornstein (16).

Proteolysis was conducted in general as previously described (16). Samples of standard, purified procollagens and of FCL-1 collagen were dissolved at room temperature in Tris-saline (~50,000 cpm/10–20 μl). Trypsin (Worthington Biochemical Corp., Freehold, NJ; TPCK, 142 U/mg), proteinase K (EM Biochemicals, Darmstadt, Federal Republic of Germany), and Staphylococcal V8 protease (Miles Laboratories, Inc., Elkhart, IN) were dissolved at 1 mg/ml in Tris-saline immediately before use. Human skin collagenase (600 μg/ml) (a gift from H. Welgus, Washington University), bacterial collagenase (Advance Biofactures, Lynbrook, NY; Form III, 2,700 U/mg), and mast cell protease (40 μg/ml) (a gift from M. Everitt, University of Washington) were used as in previous studies (16). All incubations with collagenases contained 3.3–6.6 mM Ca^{++} and were terminated by the addition of EDTA to 10 mM. Reaction mixtures containing trypsin and mast cell protease were terminated using $PhCH_2SO_2F$ in a fourfold molar excess over the enzyme concentration. Incubations were performed at the temperatures and enzyme concentrations specified in the figure legends, and the reaction products were analyzed by SDS PAGE after addition of an equal volume of electrophoresis buffer containing 0.5 M urea (17) and heating 1 min at 80°C.

For digestion with pepsin (Worthington Biochemical Corp; two times crystallized, 2,828 U/mg), 10 μl of 0.5 N acetic acid and 1 μl pepsin (1 μg, in H_2O) were added to 8 μl of sample in Tris-saline (0.15 M NaCl, 50 mM Tris-HCl, pH 7.5). The reaction was conducted at 4°C for 50 min and was terminated by the addition of pepstatin (Peninsula Laboratories, Inc., Belmont, CA) to a fourfold molar excess over pepsin, followed by 20 μl SDS PAGE buffer.

RESULTS

FCL fibroblasts in culture secrete several proteins that have been identified as components of the extracellular matrix, including fibronectin and types I and III procollagen. We used chromatography of the radiolabeled culture medium proteins on DEAE-cellulose, followed by SDS PAGE of the pooled fractions, to separate and to quantitate several of these components. An initial precipitation step with 20% ammonium sulfate removed >95% of the procollagens and >80% of the fibronectin. Analysis of proteins that were precipitated from the culture medium of 270-d-old cells at a concentration of 20–50% ammonium sulfate revealed a more complex pattern (Fig. 1 and unpublished data). In addition to residual pro- and p-collagens (peaks I, II, and III), fibronectin (peak III), and several other proteins of M_r <50,000, a band corresponding to a small collagenous polypeptide (referred to as FCL-1) was observed in peak IV (Fig. 1). In addition, a second smaller collagenous polypeptide (referred to as FCL-2) was observed in peak IV. A very similar pattern was obtained with 150-d-old cells.

Recoveries of radiolabeled interstitial and FCL-1 collagens from the culture media of both 150- and 270-d-old cells are shown in Table I. Although 270-d-old cells secreted more counts per minute (cpm) in total protein than 150-d-old cells, when compared on a per cell basis, both cell populations secreted proportionally equal amounts of interstitial procollagens (71% of the total protein cpm in the culture medium [Table I]). In contrast, FCL-1 represented 1.7% and 2.7% of the total ^{3}H counts per minute in the culture medium of 150-

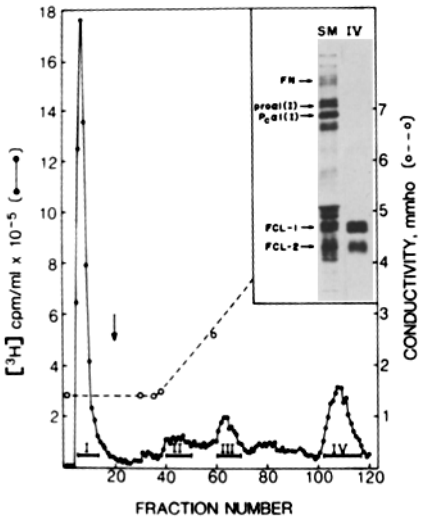

FIGURE 1 Fractionation of FCL culture medium proteins by ion-exchange chromatography and analysis by SDS PAGE. 270-d-old FCL cells were incubated with [^{3}H]Pro and [^{3}H]Gly as described in Materials and Methods. Radiolabeled culture medium proteins that were precipitated in the range of 20–50% ammonium sulfate were chromatographed on DEAE-cellulose. Conductivities were read at 4°C. Arrows indicate inception of gradient, and roman numerals indicate pooled fractions. (*Inset*) The starting material (*SM*) and peak IV were analyzed by SDS PAGE on a 3%/6%/10% composite gel in the presence of 50 mM DTT. Radioactive proteins were visualized by fluorescence autoradiography. Fibronectin (*FN*) and type I pro- and p-collagen chains have been identified. Low molecular weight collagens have been denoted FCL-1 and FCL-2.

and 270-d-old cells, respectively (Table I). Thus the secreted level of this protein was increased by ~50% in the older cells.

Preliminary experiments had indicated that FCL-1 was sensitive to the enzyme bacterial collagenase. Accordingly, we measured radioactive Hyp, Pro, and Gly levels in the peak fractions from DEAE-cellulose that contained the low molecular weight FCL-1 collagen and compared them to the values obtained for type I pro, p-, and collagens secreted by the same cells. As shown in Table II, the type I collagen fraction had a [^{3}H]Hyp to [^{3}H]Pro ratio of 0.92, and a ratio ([^{3}H]Hyp + [^{3}H]Pro) to [^{3}H]Gly of 2.4. In contrast, the [^{3}H]Hyp to [^{3}H]Pro ratio for FCL-1 was 0.43, but the ratio of ([^{3}H]Hyp + [^{3}H]Pro) to [^{3}H]Gly was also 2.4. These values indicate that FCL-1 contains collagenous sequences, although the level of Hyp relative to Pro was somewhat lower than that expected for a procollagen. In addition, the data suggested that FCL-1 has a similar relative content of (Hyp + Pro) and Gly as was found for type I procollagen and collagen. This observation provides further evidence for the collagenous nature of FCL-1.

On the basis of the mobilities of α1(I) and α2(I) CNBr-derived peptides on SDS PAGE, we estimated the M_r of FCL-1 to be approximately 12,750 (Fig. 2). FCL-1 was therefore similar in size to α1(I)-CB3 (Fig. 2, *inset*). When the sample containing FCL-1 was subjected to SDS PAGE on a longer, higher percentage acrylamide gel than that shown in Fig. 1 (*inset*), a second band became apparent and was termed FCL-2 (Fig. 2, *inset*). This protein, which was also shown to be sensitive to bacterial collagenase (see below), migrated on SDS PAGE with an apparent M_r of 3,900 (Fig. 2). FCL-2 appeared to be a minor component of the FCL-1 containing fraction, and preliminary data suggest that it could be related to FCL-1. The level of FCL-2, relative to FCL-1, was difficult to quantitate by fluorescence autoradiography due to its lower molecular weight and potential loss from the gel during the processing procedure.

TABLE I

*Recovery of Interstitial and FCL-1 Collagens from the Culture Media of 150- and 270-d-old FCL Fibroblasts**

	[^{3}H]cpm × 10^{-8}, 0–20% precipitate	[^{3}H]cpm × 10^{-7}, 20–50% precipitate	Interstitial collagens, % of total cpm‡	FCL-1 collagen, % of total cpm§
d				
150	1.33	2.10	70.5	1.72
270	1.61	2.99	70.5	2.65

* FCL fibroblasts (17–150 mm dishes of 150-d-old and 13–150 mm dishes of 270-d-old cells) were incubated 21 h in serum-free DMEM supplemented with sodum ascorbate and β-APN, containing 25 μCi/ml 2,3-[^{3}H]Pro and 2-[^{3}H]Gly. The cpm have been normalized to represent equal numbers of cells from both fetal ages.

‡ Types I and III pro-, p-, and collagens recovered after DEAE-cellulose chromatography of 0–20% ammonium sulfate precipitate (<2% of interstitial collagens was recovered in 20–50% fraction), expressed as percent of total precipitated cpm.

§ Cpm in FCL-1 collagen after DEAE-cellulose chromatography expressed as percent of total precipitated cpm. Scanning densitometry of slab gels was performed to assess the fractional percent of peak IV represented by FCL-1. Recoveries from ion-exchange chromatography were similar, within 9%, for 150- and 270-day-old preparations.

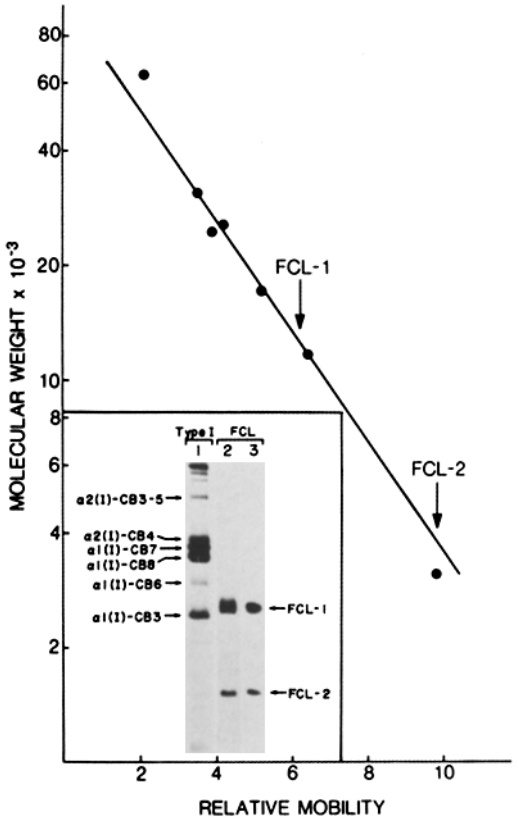

FIGURE 2 Molecular weight determination of FCL collagen based on SDS PAGE analysis. The following CNBr peptides derived from a reference standard rat skin type I collagen were resolved on a 12.5% separating SDS gel, with a 6% stacking gel: α2-CB3-5, α2-CB4, α1-CB7, α1-CB8, α1-CB6, α1-CB3, and α1-CB2. Molecular weights were calculated from the mean residue weights based on the sequence for each peptide (22). The positions of migration of the two low molecular weight collagenous proteins secreted by FCL cells are indicated by arrows (FCL-1 and FCL-2). (*Inset*) SDS PAGE analysis (6%/12.5% gel, in the presence of 50 mM DTT) of CNBr peptides of [^{3}H]proline- and glycine-labeled type I collagen from FCL cells (lane *1*), FCL-1 collagen, before (lane *2*) and after (lane *3*) incubation with CNBr. Several CNBr-derived peptides from α1(I) and α2(I) collagen chains are identified.

TABLE II

*Levels of Hydroxyproline, Proline, and Glycine in Collagens Secreted into the Culture Medium by FCL Fibroblasts**

	4-[^{3}H]Hyp, cpm	[^{3}H]Pro, cpm	[^{3}H]Gly, cpm	Hyp_T/Pro‡	(Hyp_T + Pro)/Gly
Type I Collagen§	463,766	505,690	400,615	0.92	2.42
FCL-1 Collagen‖	21,992	67,168	38,978	0.43	2.46

* FCL fibroblasts (270 d) were incubated 21 h in serum-free DME containing sodium ascorbate (50 μg/ml) and β-APN (64 μg/ml), and 25 μCi/ml each of 2,3-[^{3}H]Pro and 2-[^{3}H]Gly. The cpm have been normalized to represent 1–150-mm dish of 270-d-old cells.

‡ Hyp_T: total 3-Hyp + 4-Hyp. This value is an underestimate of the level of the 3-isomer, as 2,3-[^{3}H]Pro was used as the substrate for 3-prolyl hydroxylase.

§ Isolated by DEAE-cellulose chromatography of a 20% ammonium sulfate precipitate of culture medium protein. This fraction was further purified by chromatography on CM-cellulose under denaturing conditions and contained, by SDS PAGE analysis, proα1(I), proα2(I), α1(I), and several p-collagen intermediates.

‖ Isolated by DEAE-cellulose chromatography of a 20–25% ammonium sulfate precipitate of FCL 270-d-old culture medium protein, as shown in Fig. 2 (*inset*; lane *2*). This preparation contained 10–20% FCL-2.

The mobility of FCL-1 and -2 on SDS PAGE was apparently unaffected by the presence of reducing agent, indicating a lack of interchain disulfide bonds (data not shown). It was also of interest that neither of these proteins was cleaved by CNBr under conditions that produced extensive cleavage of type I collagen (Fig. 2, *inset*, lanes *1–3*). Although reduction of methionine residues was not performed before CNBr treatment, the efficient cleavage of the type I collagen that was synthesized by FCL cells and processed by similar procedures used for FCL-1 and -2 suggests that significant oxidation of methionines did not occur.

The structural properties of FCL-1 were investigated by the use of enzymatic digestion. This collagen demonstrated a remarkable resistance to proteolytic degradation. It was not cleaved by human skin collagenase under conditions that produced the characteristic TC^A and TC^B fragments from interstitial native type III collagen (Fig. 3, compare *A* and *B*). That both FCL-1 and FCL-2 contain collagenous sequence(s), however, was shown by their sensitivity to bacterial collagenase (Fig. 3, *C* and *D*, lanes *BC*). In contrast, at 37°C FCL-1 was not cleaved by Staphylococcal V8 protease, trypsin, or proteinase K (Fig. 3, *C*, lanes *V8* and *T*, and *E*, lane *PK*), under conditions which produced extensive fragmentation of globular, noncollagenous protein (e.g., a protein of M_r 43,000 that occasionally co-purifies with FCL-1) and collagen α-chains (not shown). A limited sensitivity to pepsin was observed after a 1–2 h incubation at 4°C (Fig. 3, *E*, lane *P*); however, this lability most probably reflects a random, limited unfolding of the triple helix rather than the presence of discrete, pepsin-sensitive sites. Control incubation of FCL-1 in acetic acid, followed by heating in Laemmli buffer, produced no change in mobility on SDS PAGE; types I and III procollagen, incubated with pepsin under the same conditions used for FCL-1, were cleaved to α-chain-containing molecules (not shown).

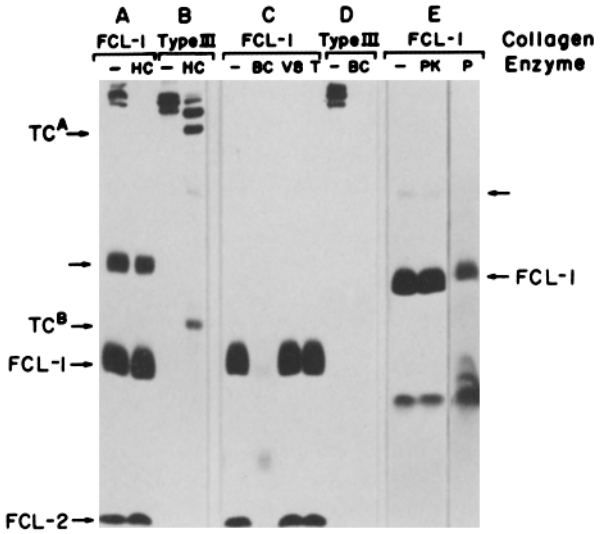

FIGURE 3 Susceptibility of low molecular weight FCL-1 collagen to enzymatic degradation. Samples of FCL collagen were incubated with proteases as indicated below, and the reaction products were analyzed by SDS PAGE under reducing conditions on 5%/10% slab gels. FCL-1 (150-d-old preparation) (*A*) and type III procollagen (*B*), isolated by DEAE-cellulose chromatography from FCL fibroblast medium, were incubated with 1.2 μg human skin collagenase (*HC*) for 14 h at 22°C. FCL-1 (270-d preparation) (C) was incubated with 0.2 μg bacterial collagenase (*BC*) for 15 min at 37°C, 2 μg Staphylococcal V8 protease (*V8*) for 15 min at 37°C, and 2 μg trypsin (*T*) for 15 min at 37°C. Type III procollagen (*D*) was incubated with bacterial collagenase (*BC*) as in C. FCL-1 (270-d preparation) (*E*) was incubated with 1 μg proteinase K (*PK*) for 1 min at 37°C. Digestion with pepsin (*P*) was performed as described in Material and Methods. Note that E was a separate gel from gels *A–D*. Arrow indicates a noncollagenous protein of M_r 43,000 that occasionally co-purifies preparations of FCL-1. TC^A and TC^B represent collagenase cleavage products of type III collagen.

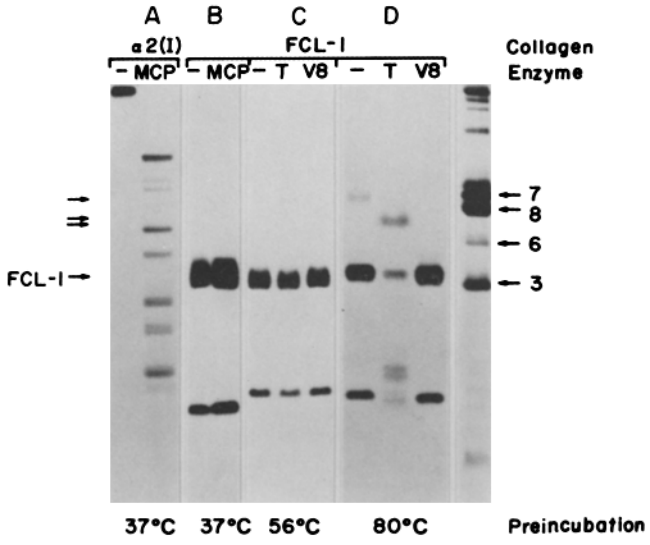

FIGURE 4 Susceptibility of low molecular weight FCL collagen to neutral proteases. Samples of FCL collagen were incubated with enzymes under the conditions described below, and the reaction products were analyzed by SDS PAGE on 6%/12% slab gels in the presence of 50 mM DTT. (*A*) Reference standard rat skin α2(I) chain was incubated with 0.16 μg mast cell protease (*MCP*) for 30 min at 37°C. (*B*) FCL-1 (270-d cell preparation) was incubated as described in *A*. (C) FCL-1 (270-d cell preparation) was heated for 30 min at 56°C, before incubation with 2 μg trypsin (*T*) or 4 μg Staphylococcal V8 protease (*V8*) for 30 min at 37°C. (*D*) FCL-1 (150-d-old preparation) was heated for 30 min at 80°C, before incubation with 2.5 μg trypsin (*T*) or 2.5 μg Staphylococcal V8 protease (*V8*) for 1 h at 37°C. Lane on far right represents type I collagen after cleavage with CNBr; several peptides from the α1(I) chain are identified. Arrow indicates a noncollagenous protein of M_r 43,000, and double arrows identify a stable peptide produced by trypsin cleavage of the M_r 43,000 protein.

Since the resistance of FCL-1 to trypsin, SV8 protease, chymotrypsin (not shown), and proteinase K were thought to be at least partially conformation-dependent, FCL-1 was heated for up to 1 h at both 56°C and at 80°C before incubation with trypsin and SV8. As shown in Fig. 4 *C*, preincubation at 56°C had no effect on the subsequent lability of FCL-1 to these enzymes. However, preincubation at 80°C caused FCL-1 to become trypsin-sensitive, although this collagen was still resistant to cleavage by SV8 (Fig. 4 *D*). Both trypsin and SV8 produced extensive fragmentation of denatured types I, III, and V (pro)collagens after preincubation of these samples at 56°C for 15 min (not shown).

Since mast cell protease has been shown to produce specific cleavages in native type IV collagen, and in denatured α-chains of all collagen types, we incubated α2(I) and FCL-1 with this enzyme at 37°C. As shown in Fig. 4, *A* and *B*, extensive fragmentation of α2(I) was achieved, but FCL-1 remained intact. Identical results were obtained when these

samples were heated 30 min at 56°C, before incubation with mast cell protease at 37°C (not shown).

DISCUSSION

Fetal calf ligamentum nuchae fibroblasts in culture secrete a low molecular weight collagen-like protein that was unusually resistant to proteolytic degradation. This protein, termed FCL-1, contains collagenous sequences by the following criteria: (*a*) sensitivity of the polypeptide chain to bacterial collagenase to produce peptides of M_r <4,000 (Fig. 3*C*); (*b*) a Hyp to Pro ratio of 0.43 (Table II); and (*c*) a (Hyp + Pro) to Gly ratio identical to that of type I procollagen synthesized concomitantly by the FCL cells (Table I). However, FCL-1 was found to be distinct from other previously described collagen types by the following set of criteria: (*a*) selective precipitation in 20–50% ammonium sulfate; (*b*) elution from DEAE-cellulose at approximately 160 mM NaCl; (*c*) an apparent subunit molecular weight of approximately 12,750; (*d*) absence of interchain disulfide bonds; (*e*) resistance to CNBr and human skin collagenase; (*f*) limited susceptibility to pepsin; and (*g*) resistance to several neutral proteases even at high temperatures. Cleavage was in fact only observed with trypsin following preincubation of FCL-1 at 80°C for 30 min.

FCL-1 was produced by cells from both 150- and 270-d-old fetuses, and comprised from 1.7–2.7%, respectively, of the total [^{3}H]Pro- and [^{3}H]Gly-labeled protein secreted into the culture medium (Table I). Interstitial procollagen production (types I and III) comprised 71%, in both cell populations, of the total culture medium protein.

FCL-1 is significantly smaller than the recently described low molecular weight collagen secreted by chondrocytes in vitro (13, 14). This protein, which has an M_r of 59,000 and lacks disulfide bonds, was cleaved by pepsin to a stable product of M_r 45,000. In contrast to FCL-1, it exhibited more extensive prolyl hydroxylation and multiple CNBr cleavage sites, resulting in peptides of M_r <10,000. Incubation with human skin collagenase produced a stable fragment of M_r 32,000 (13). This low molecular weight chondrocyte collagen, and FCL-1, are both distinctly different from the "short-chain" collagens isolated by pepsin digestion of vascular (5) and cartilagenous (8) tissues. These latter proteins are presumably derived from larger, triple helical molecules and are cross-linked by disulfide bonds. Although we have used precautions to avoid proteolysis of FCL-1 during its characterization, we cannot exclude the possibility that it represents the product of physiological processing of a higher molecular weight form.

We currently do not have a good explanation for the unusual protease resistance of FCL-1. In the absence of interchain disulfide bonds or a very high imino acid content (relative to other collagens), one would expect the helical stability to be even lower than that of collagens of higher molecular weight. It is possible that FCL-1 could form protease-resistant complexes with other components in solution, such as the 43,000-mol-wt protein, with which it co-purifies. Additional information, such as a complete amino acid composition, will be useful in explaining the unusual finding.

In contrast to collagens that have interruptions within the triple helix, such as type IV (see reference 4 for a review), recent studies on the M_r 59,000 chondrocyte collagen and on FCL-1 provide a precedent for the existence of collagen gene products, possibly containing nontriple-helical domains, that are substantially smaller than collagen pro α chains associated with types I–V. Recent studies on the complete nucleotide sequences of two collagen genes from the nematode *Caenorhabditis elegans* indicate that these genes are considerably shorter than the chicken or sheep proα2(I) gene and would appear to encode collagenous proteins of M_r 30,000 (18). The newly characterized type VII collagen, which has an extended triple helical domain and an α-chain molecular weight of 170,000 (19), provides additional evidence that the collagen gene family is heterogeneous with respect to the size of its individual components.

The significance of FCL-1 to the cell system from which it was derived is at present not known. FCL fibroblasts in vitro produce copious amounts of extracellular matrix, some components of which are used in the assembly of collagen and elastic fibers (1, 2). Others have found (R. Mecham, manuscript submitted for publication) found that 150-d-old cells, which produced very low levels of tropoelastin, could be induced to synthesize higher levels when plated on a heat-killed ligament or when analyzed as tissue minces. These studies suggest that the extracellular matrix stabilizes the tropoelastin-synthesizing phenotype of FCL cells which is maximally induced at later gestational stages (e.g., 270 d).

We have presented preliminary evidence that the production of FCL-1 is increased in 270-d-old cells compared to 150-d-old cells. It is therefore of interest that the M_r 59,000 collagen recently characterized from chondrocytes was preferentially recovered from the zone of hypertrophy in the developing chick embryo tibiotarsus (20). These authors also found that chondrocytes from earlier stages of differentiation in vivo adopted a differentiated phenotype in culture that was accompanied by increased production of the low molecular weight collagen. As a matrix component (14), this collagen could conceivably influence cell shape, motility, and subsequent biosynthetic events at the cell surface, as has been described for embryonic corneal fibroblasts embedded in collagen gels (21). Studies are in progress to examine the relationship of FCL-1 to the genetic program of the calf ligament fibroblasts at various stages of fetal development.

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