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Fetal membrane collagens: Identification of two new collagen alpha chains

(collagen polymorphism/cyanogen bromide peptide chromatography/electrophoresis)

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ABSTRACT **Human fetal membranes contain two new genetically distinct collagen polypeptide chains which are subunits of one (or two) new molecular species of collagen. These new polypeptide chains, which we have tentatively named αA and αB, have been directly compared with the polypeptide chain subunits of Types I, II, and III human collagen and Type IV collagen from bovine lens capsule. Both αA and αB exhibit characteristic profiles on carboxymethyl-cellulose chromatography and sodium dodecyl sulfate/polyacrylamide gel electrophoresis. The distribution of methionine residues along both new chains is different from known collagen chains as manifest by distinctly different cyanogen bromide peptide profiles on carboxymethyl-cellulose chromatography and/or sodium dodecyl sulfate/polyacrylamide gel electrophoresis. Both αA and αB exhibit contents of imino acids and glycine typical of collagens, and comparison with the observed and reported compositions of collagen chains of Types I–IV collagens reveals notable differences, particularly in the content of alanine, leucine, isoleucine, and the basic amino acids, lysine, hydroxylysine, and arginine.**

The new collagen species containing both αA and αB may be separated in the native (triple-helical) state from other native collagen species by differential salt precipitation. The observations that both chains coprecipitate in the same narrow NaCl range, and that the ratio of αA:αB is constant, suggest the possibility of a single new species of collagen with a subunit structure αA $[\alpha B]_2$.

The molecular heterogeneity of collagen is now well established (1–4), and at present, at least four genetically distinct species of collagens (Types I–IV) have been identified in human (3, 5–10) and other mammalian tissues (11–16). Type I collagen is present in skin, bone, and tendon, and is composed of two identical polypeptide chain subunits, the α1 Type I chains, and a third structurally different polypeptide subunit, the α2 Type I chain (5, 6, 12). In current notation, Type I collagen has the subunit structure $[\alpha 1(I)]_2\alpha 2(I)$. Type II collagen is present in cartilage, and is composed of three identical α chains different in primary structure from α1(I) and α2(I); the subunit structure of Type II collagen is thus $\alpha 1(II)_3$ (7, 14, 17). Type III collagen is found in skin, blood vessels, and fetal membranes and is composed of three identical α chains different than α1(I), α2(I), or α1(II) chains, and has the structure $\alpha 1(III)_3$ (7–10, 15, 18). Type IV collagen is present in basement membranes such as found in glomerulus and lens capsule of the eye, and is composed of three identical α1(IV) chains (3, 11, 16).

In addition to the five genetically distinct α chains which compose the four recognized collagen types, other collagen polypeptides have been isolated from bovine (19), avian (20, 21), and rodent (22) tissues and may represent subunits of additional collagen types.

We wish to report the isolation and characterization of two new collagen polypeptide chains which we operationally designate αA and αB, and which differ from previously reported collagen α chains. These materials were observed in human fetal membranes together with Types I and III collagen, and represent 5–10% of the total collagen. The available evidence suggests that these genetically distinct chains compose a new variety of collagen.

MATERIALS AND METHODS

Preparation of Total Fetal Membrane Collagens. Normal human placentas of varying gestational ages (16–40 weeks) were collected and stored in normal saline at 4° for less than 24 hr. The amniotic and chorionic membranes were excised and manually separated, soaked in distilled water for 48 hr at 4°, washed, and homogenized thoroughly in cold distilled water with a Polytron homogenizer. The homogenates were solubilized by limited pepsin digestion (23), and the solubilized collagens were precipitated from the clarified supernate by addition of NaCl to a concentration of 1 M. The precipitate was dissolved in cold 1 M NaCl, 50 mM Tris·HCl at pH 7.5, titrated to pH 8.6 to inactivate residual pepsin, and reprecipitated by exhaustive dialysis against 0.01 M Na_2HPO_2. The recovered precipitate was redissolved in, and dialyzed against, 0.5 M CH_3COOH and lyophilized.

For collagen extractability studies, fresh membranes were separated, washed briefly with distilled water, homogenized in water, and lyophilized. Two hundred milligrams (dry weight) of amnion or chorion homogenate was suspended and collected sequentially in 200 ml of the following solutions: H_2O; 1 M NaCl, 50 mM Tris·HCl at pH 7.5; 4 M guanidine, 50 mM Tris·HCl at pH 7.5; 4 M guanidine, 50 mM Tris·HCl at pH 7.5, 10 mM dithiothreitol; and 0.5 M CH_3COOH. Extractions were performed at 4° for 48 hr with constant stirring. After each extraction, the insoluble residue was harvested, and washed once in 50 ml of the same extracting solvent; the supernatant and wash were pooled and dialyzed for the hydroxyproline assay (24).

Differential NaCl Precipitation. Routine differential salt precipitation was performed by dissolving total collagens of amnion, chorion, or both (1 mg/ml) in 1 M NaCl, 50 mM Tris·HCl at pH 7.5, and successively dialyzing versus 1.7 M, 2.6 M, and 4.0 M NaCl containing 50 mM Tris·HCl at pH 7.5. The precipitates obtained at these NaCl concentrations were harvested by centrifugation (5.2×10^5 *g*-min), dissolved in and dialyzed against 0.5 M CH_3COOH, and lyophilized. To further define the salting-out behavior of collagens precipitating between 2.6 and 4.0 M NaCl, several 4.0 M NaCl precipitates were redissolved at 0.75 mg/ml concentration in 2.6 M NaCl, 50 mM

Abbreviations: CMeC, carboxymethyl-cellulose; $NaDodSO_4$/PAGE, sodium dodecyl sulfate/polyacrylamide gel electrophoresis; CB-peptides, cyanogen bromide peptides.

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Tris·HCl at pH 7.5, and dialyzed successively against solutions of higher NaCl concentration in 0.1 M NaCl steps to a final NaCl concentration of 4.5 M.

Preparation of Standard Collagens. Human Type I and III collagens and human Type II collagen were prepared from fetal membranes and costal cartilage, respectively, by limited pepsin digestion and precipitation as described above, and the constituent α chains isolated by carboxymethyl-cellulose (CMeC) chromatography. Total amino acid analyses of these materials were comparable to published values (6, 8, 25) with some variations in the degree of hydroxylation of proline and lysine residues. Preparations of purified Type IV collagen isolated by pepsin digestion from bovine lens capsule were the kind gift of Dr. N. Kefalides.

Radioactive human Type I procollagen was isolated as described by Smith *et al.* (26), and pepsin digested as described above; radioactive α1(I) and α2(I) chains were isolated by CMeC chromatography.

Collagenase Digestion. Collagenase digestions were performed essentially as described by Peterkofsky and Diegelmann (27) with Form III Clostridial collagenase (2700 units/ml; Advance Biofactures Corp.).

Analytical Methods. Methods for the CMeC chromatography of whole collagen chains (28), or of cyanogen bromide (CB) peptides (29), prepared as described by Miller and Lunde (25), have been previously reported. Discontinuous sodium dodecyl sulfate/polyacrylamide gel electrophoresis ($NaDodSO_4$/PAGE) was performed as described by Laemmli (30) on slab gels, and modified slightly for whole collagen chains or cyanogen bromide peptides. Details of these procedures are given in the figure legends. Samples for amino acid analysis were hydrolyzed in 6 N HCl at 110° for 24 hr *in vacuo*, and duplicate or triplicate analyses done on a Beckman model 120B analyzer with a previously published method (31).

RESULTS AND DISCUSSION

Sequential extractions of human fetal membranes as described in *Materials and Methods* solubilize a total of less than 2% of the tissue hydroxyproline from either amnion or chorion. In contrast, approximately 80% of the total hydroxyproline is solubilized and recovered after limited pepsin digestion and collagen isolation. Electrophoretic analysis of the pepsin-solubilized total collagens of chorion and amnion is depicted in Fig. 1, channels 11 and 12 respectively, and is compared with the patterns of Types I, II, III, and IV collagens in channels 1 through 4. Both amnion and chorion contain materials whose electrophoretic mobilities are equivalent to those of the α1 and α2 chains of Type I collagen. In addition, both chorion and amnion contain a number of materials of apparently higher molecular weight. Of particular interest are two minor constituents appearing above the A1(I) position. The first of these, labeled αA, has a slightly lower electrophoretic mobility than α1(I), and the second, labeled αB, has a significantly lower mobility. Reduction of these total collagens with 10 mM dithiothreitol prior to electrophoresis results in a marked decrease in very high-molecular-weight materials, and a large increase in stainable collagen in the α1(I) position consistent with the presence of large amounts of high-molecular-weight disulfide bonded Type III collagen. However, reduction does not affect the mobility or apparent amounts of either αA or αB. Similarly, repeat pepsin digestion of total collagens does not alter the electrophoretic profiles under conditions sufficient to quantitatively convert codigested Type I procollagen to α1(I) and α2(I). Yet, digestion with nonspecific protease-free Clostridial collagenase under conditions not affecting the electrophoretic mobility of codigested bovine serum albumin, or repeat pepsin digestion of heat denatured total collagens, yields complete hydrolysis of all observed materials (data not shown).

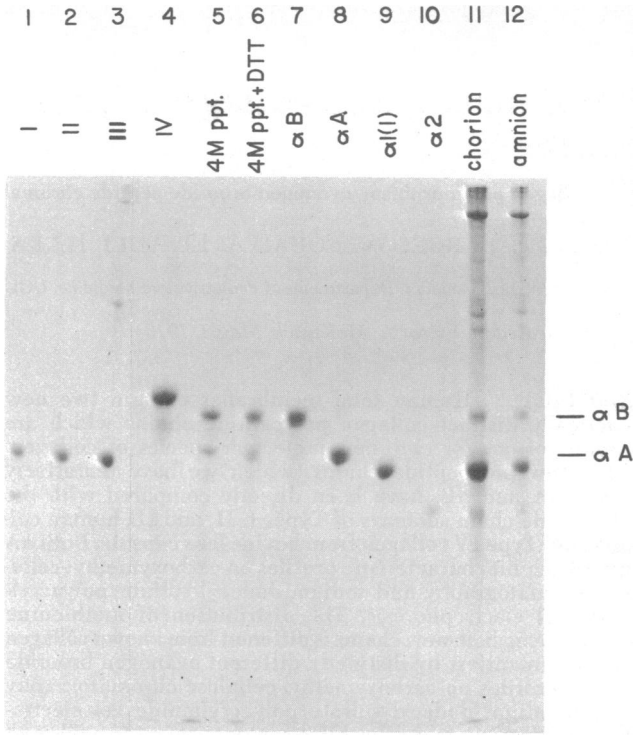

FIG. 1. (0.2%) $NaDodSO_4$/4% polyacrylamide gel electrophoretogram of collagen α chains. Channels 1–4 contain collagen Types I–IV, respectively; (Types III and IV were reduced with dithiothreitol); channels 5 and 6 contain the collagens precipitating (ptt.) between 2.6 and 4.0 M NaCl (see *text*) before and after reduction with dithiothreitol (DTT), respectively; channels 7–10 contain the indicated α chains; and channels 11 and 12 contain the total pepsin-solubilized collagens of chorion and amnion, respectively. The electrophoretic position of αA and αB are indicated on the right. Discontinuous $NaDodSO_4$/PAGE was modified from Laemmli (29) to contain 0.2% $NaDodSO_4$ in both gel and electrode buffers.

Differential salt precipitation has been used to separate native molecules of different collagen types (8, 32). Total collagens were subjected to differential salt precipitation as described in *Materials and Methods*, and flocculent precipitates were observed at 1.7, 2.6, and 4.0 M NaCl. As expected, the 1.7 M NaCl precipitate consists predominantly of Type III collagen, whereas the 2.6 M NaCl precipitate contains predominately Type I collagen as judged by analysis of whole chains and/or cyanogen bromide peptides by $NaDodSO_4$/PAGE and CMeC chromatography. The 4.0 M precipitate contains αA and αB, and typically contains a small and variable amount of Type I collagen, but occasionally contains only αA and αB. A representative electrophoretic profile of this precipitate before and after reduction with dithiothreitol is shown in Fig. 1, channels 5 and 6 respectively, and it is apparent that the vast majority of material is αA and αB, whose mobilities are unaffected by disulfide reducing reagents. Comparison of the electrophoretic mobilities of these materials with standard collagens of Types I–IV, also isolated by limited pepsin digestion, reveals characteristic and reproducible differences. The Type IV collagen used for this comparison electrophoresis migrated as a single band of approximately 140,000 molecular weight. Other Type IV collagen preparations, however, have been isolated with reported variations of molecular weight from 93,000 to 140,000 (3, 11, 33), and electrophoretic mobilities intermediate between the α1(I)

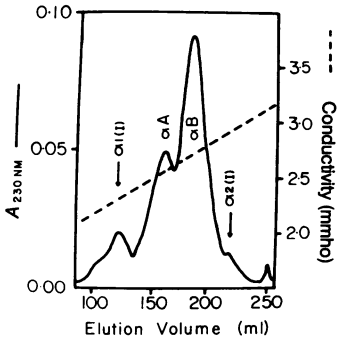

FIG 2. CMeC chromatogram of fetal membrane collagen isolated by differential salt precipitation between 2.7 and 4.0 M NaCl. The sample and equilibration buffer consisted of 0.04 M sodium acetate at pH 4.8, and 6.0 M urea. The column was developed with a superimposed 300 ml linear gradient from 0 to 80 mM NaCl at 42°. Arrows indicate the elution positions of α1(I) and α2(I). Absorbance at 230 nm (——); conductivity in mmho (- - - -).

position and that observed for the present Type IV preparation (32).

The collagens precipitated with 4 M NaCl were denatured and chromatographed on CMeC; a representative elution profile is shown in Fig. 2. Two major unresolved peaks are observed and identified as αA and αB by electrophoresis (Fig. 1, channels 8 and 7 respectively). The minor initial peak is α1(I), and the minor peak eluting with the trailing shoulder of the αB peak is α2(I). Although quantitation of the relative amounts of αA and αB is precluded by peak overlap, the absorbance distribution, αA:αB, appears to be approximately 1:2.

The elution profiles of αA and αB from CMeC are unlike those for pepsin-isolated α1(I) and α2(I), α1(II), and that reported for α1(IV) (11). The αB chain coelutes with α1(III) chains (8).

Amino acid analyses of electrophoretically homogeneous αA and αB are shown in Table 1, and are compared with the observed and reported compositions of human collagen Types I–IV. Both αA and αB exhibit contents of proline, hydroxyproline, glycine, lysine, and hydroxylysine typical of collagen. Each of the chains is substantially different from all other collagen chains in content of alanine, and there are notable differences in isoleucine, leucine, arginine, and lysine plus hydroxylysine. The extent of hydroxylation of proline is comparable to that of Types I, II, and III collagens, but less than that observed for Type IV collagen. Lysine residues demonstrate levels of hydroxylation intermediate between values for Types I, II, and III collagens, and Type IV collagen.

On the basis of the $NaDodSO_4$/PAGE mobilities, CMeC elution positions, and amino acid compositions, αA and αB are either new collagen polypeptide chains, or result from modification of previously described collagen chains sufficient to produce large changes in the above properties. The observed electrophoretic behavior of αA and αB would be consistent with

Table 1. The amino acid compositions of human collagen α chains

Residue	Residues per 1000 residues						
	α1(I)*	α2(I)*	α1(II)†	α1(III)‡	α1(IV)§	αA*	αB*
3-Hydroxyproline¶	1.1	1.2	2.0	N.R.	11	2.5	2.9
4-Hydroxyproline	114	105	99	125	130	109	109
Aspartic acid	46	45	42	42	51	51	50
Threonine	18	18	20	13	23	26	19
Serine	35	30	27	39	37	31	26
Glutamic acid	77	70	89	71	84	84	91
Proline	118	114	121	107	61	97	118
Glycine	330	331	333	350	310	319	322
Alanine	119	105	100	96	33	52	46
Valine	19	35	18	14	29	27	18
Half cystine	N.D.	N.D.	N.R.	2	8	N.D.	N.D.
Methionine	5.3	4.7	9	8	10	11	8
Isoleucine	8.2	16	9	13	30	16	19
Leucine	20	33	26	22	54	35	39
Tyrosine	2.1	3.2	1	3	6	1.8	2.1
Phenylalanine	13	11	13	8	27	14	12
Hydroxylysine	10	12	14	5	44	24	35
Lysine	27	20	22	30	10	18	20
Histidine	4	10	2	6	10	11	7.5
Arginine	49	51	51	46	33	68	50
$\frac{\text{3- + 4-OH-Proline}}{\text{Proline + 3- + 4-OH-proline}}$	0.49	0.48	0.46	0.54	0.70	0.54	0.49
$\frac{\text{OH-Lysine}}{\text{Lysine + OH-lysine}}$	0.27	0.38	0.39	0.14	0.81	0.57	0.64

N.D., not determined. N.R., not reported.

* Human fetal membranes were the source of these samples which were hydrolyzed and sequentially chromatographed under identical conditions. The data are uncorrected for hydrolytic losses of threonine, serine, methionine, and tyrosine.

† Human articular cartilage type II composition calculated from Miller and Lunde (24).

‡ Human dermis, aorta, and uterine leiomyoma type III composition from Chung and Miller (8).

§ Human glomerular basement membrane type IV composition from N. A. Kefalides (11).

¶ The color constant for equimolar amounts of 3-hydroxyproline:4-hydroxyproline:proline were assumed to be 1.00/0.28/0.71 as reported by Piez *et al.* (13). Other color constants were empirically determined.

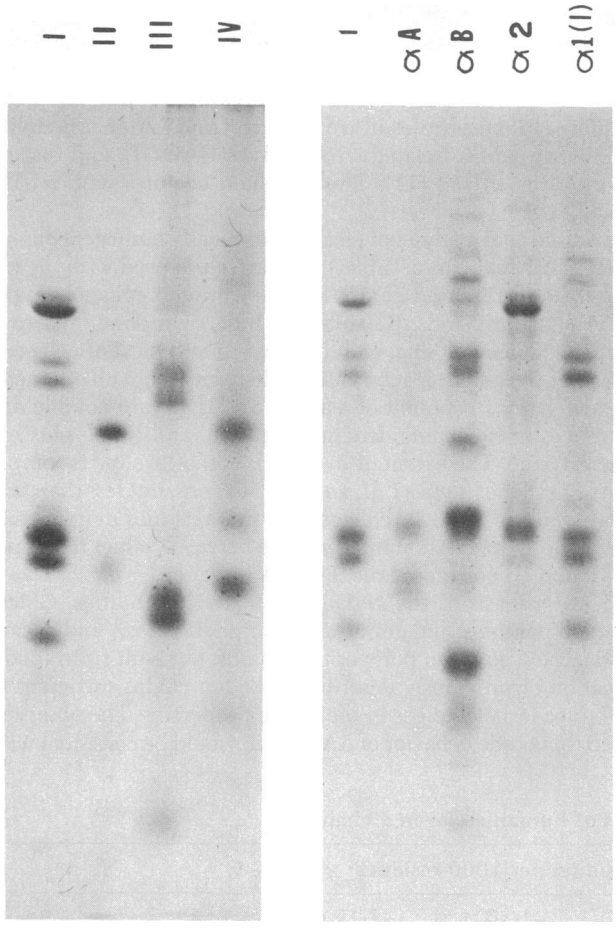

FIG. 3. $NaDodSO_4$ (0.2%)/10% polyacrylamide gel electrophoretograms of CnBr-peptides derived from αA, αB, and the chains of Type I–IV collagens. The content of each channel is indicated.

the possibility of addition of collagen-like peptide material to α1(I), α2(I), α1(II), or α1(III), or a proteolytic excision of a portion of an α1(IV) chain.

To investigate these possibilities, cyanogen bromide peptides were prepared and evaluated by $NaDodSO_4$/PAGE and CMeC chromatography. In Fig. 3 are depicted the CB-peptide electrophoretic profiles of $[\alpha 1(I)]_2\alpha 2(I)$, α1(II), α1(III), α1(IV), αA, αB, α2(I), and α1(I). The size distribution profiles for CB-peptides of each α chain are reproducible and characteristic for that chain. Comparison of the profile generated by αB with those of the other α chains (including αA) reveals no significant similarities. Similar comparisons between αA and α1(I), α2(I), α1(II), α1(III), and αB yields the same conclusion, but the profiles of αA and α1(IV) are similar in pattern although different in mobility. Fig. 4 demonstrates the observed CMeC elution profiles of CB-peptides derived from the α chains of human Types I, II, and III collagen, and of αA and αB. For these studies, radioactive purified α1(I) or α1(I) and α2(I) were codigested and cochromatographed with the α chain of interest and served as internal chromatographic markers. Comparison of the CB-peptide elution patterns demonstrates unique profiles for all the tested collagen chains. Recovery of materials in the major chromatographic peaks and subsequent electrophoresis permitted correlations between these two methods. Cross-comparisons of chromatographic and electrophoretic profiles demonstrate that none of the CB-peptides from either αA and αB exhibit both size and charge properties similar to peptides derived from known α chains. Since αA and αB share no

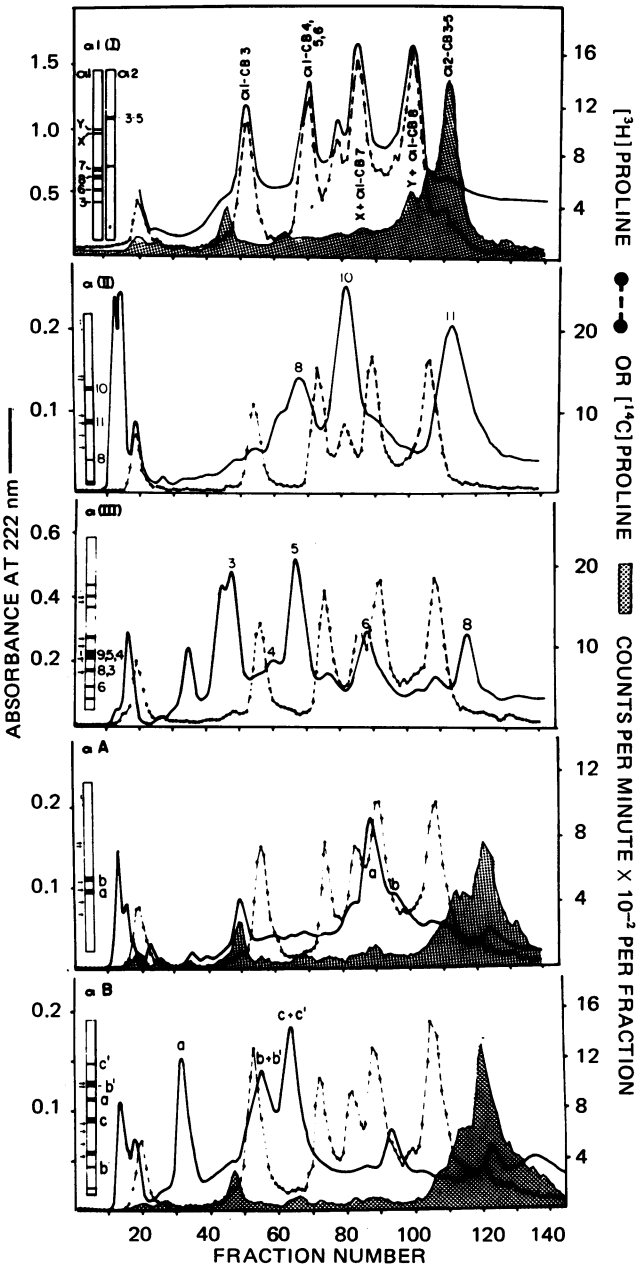

FIG. 4. CMeC chromatograms of CnBr-peptides from collagen chains of Types I, II, III, and αA and αB chains. A 1.6 × 17 cm CMeC column was equilibrated with 0.02 M sodium formate at pH 3.8, and 40 mM NaCl at 42°. Samples (4.7–20 mg) were applied in this buffer, and the chromatograms were developed with a 350 ml linear gradient of 40–155 mM NaCl. The absorbance at 222 nm (——) of CnBr-peptides derived from α1(I), α1(II), α1(III), and αA and αB is shown in the correspondingly marked panel. The profiles of codigested radioactive α1(I) (· - - - ·) and α2(I) (shaded area) are also shown. The chromatographic position and identity of individual CB-peptides is indicated by number for α1(I), α1(II), and α1(III) according to current convention.

Major chromatographic peaks were recovered and analyzed by 0.2% $NaDodSO_4$/10% PAGE. Composites of those electrophoretic results are depicted on the left of each panel; and for comparison, the electrophoretic positions of α1(I) CB-peptides are indicated by arrows.

common CB-peptides with the α chains of Types I, II, and III collagen, they cannot be simple modifications of these α chains, nor can they represent pepsin-resistant precursors of these α chains.

The possibility that one or both new collagen chains might

be a proteolytic degradation product of an α1(IV) chain is suggested by their slightly higher electrophoretic mobilities and by the similarities of the CB-peptide electrophoretic patterns of αA and α1(IV). That this cannot be the case is demonstrated for the αB chain by the very substantial differences in CB-peptide profile (Fig. 3), and for both chains by significant differences in amino acid compositions (Table 1). Although amino acid composition may be altered by proteolytic excision of a portion of a molecule, in order to generate the observed differences between αA or αB and α1(IV), a strikingly nonrandom distribution of 3- and 4-hydroxyproline, proline, alanine, isoleucine, leucine, phenylalanine, and arginine in α1(IV) would be required. But examination of the published amino acid compositions of isolated CB-peptides derived from bovine lens capsule Type IV (3, 16) reveals a uniform distribution of these residues throughout the molecule. Further, the amino acid compositions of Type IV collagens varying in reported molecular weight from 93,000 to 140,000 are similar, but are substantially different from αA or αB (3, 16, 33).

On the basis of the data reported here, αA and αB are distinct from, and cannot be modifications of, known collagen α chains. Moreover, comparison of αA and αB with the available data on other collagenous materials, such as bovine cardiac collagen (19), rat dentine collagen (21), and avian lens, vitreous and scleral collagens (20), reveals differences in amino acid compositions and other properties. We conclude that αA and αB are new collagen α chains. The amino acid compositions and CB-peptide data indicate that these new collagen chains are structurally different proteins, and therefore, are distinct gene products. At least seven identifiable structural genes for collagen α chains must, therefore, exist in the human genome to account for the observed structural diversity of human collagens.

Differential salt precipitation of total fetal membrane collagens yields a purified fraction (the 4.0 M NaCl precipitate) containing αA and αB, and in some preparations, is essentially free of other α chains (Fig. 1, channels 4 and 5). This fraction is resistant to pepsin re-digestion, implying that αA and αB are constituents of native (triple-helical) collagen molecules. These observations pose two possibilities: the two chains are either associated into a single triple-stranded collagen molecular species, or alternatively, compose two different collagen molecular species.

Repeat differential salt precipitation of 4.0 m NaCl precipitates as described in *Materials and Methods* yields only a single precipitate between 3.6 and 3.7 M NaCl. This precipitate contains both αA and αB in a ratio of 0.50 ± 0.02 by NaDodSO$_4$/PAGE densitometry under conditions in which integrated stain intensity is proportional to protein concentration. Similar quantitation of three separately prepared 4.0 M NaCl precipitates not subjected to reprecipitation yields a ratio of 0.53 ± 0.01. Further, CMeC chromatography of 4.0 M NaCl precipitates repeatedly yields an absorbance profile of αA:αB consistent with a 1:2 ratio (Fig. 2). In every instance in more than 40 preparations of fetal membranes analyzed by NaDodSO$_4$/PAGE, the apparent relative amounts of stainable materials in the αA and αB positions were constant, and consistent with a 1:2 ratio.

Present evidence does not exclude the possibility that two separate new collagen species [$(\alpha A)_3$ and $(\alpha B)_3$] are fortuitously present in a constant relative ratio, and have identical solubility behavior in neutral salt solutions. However, the simplest explanation of the available data is the presence of a single new collagen species with a subunit structure of $\alpha A[\alpha B]_2$.

Although αA and αB are genetically distinct from the chains of Type IV collagen, this conclusion does not argue that these new chains could not be components of the morphologically prominent basement membranes of fetal membranes (34).

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