Human type V collagen was purified from placenta and found to contain α1(V), α2(V), and α3(V) chains in varying ratios. Using any of three independent non-denaturing methods (phosphocellulose chromatography, high-performance ion-exchange chromatography on IEX-540 DEAE, and ammonium sulfate precipitation), this preparation could be resolved into two fractions. Analysis of the two fractions by sodium dodecyl sulfate-polyacrylamide gel electrophoresis indicated that one fraction contained α1(V) and α2(V) in a 2:1 ratio and the other contained α1(V), α2(V), and α3(V) in a 1:1:1 ratio. When the crude placental type V collagen was electrophoresed under non-denaturing conditions, two bands were observed, one co-migrating with purified α1(V), α2(V), and the other co-migrating with the fractions containing α1(V), α2(V), and α3(V) chains in a 1:1:1 ratio. Electrophoresis in a second dimension under denaturing conditions confirmed that the fast-migrating band contained (α1(V))2α2(V) and that the slow-migrating band contained the three chains in equimolar ratio. CD spectra of the two fractions and resistance to trypsin-chymotrypsin digestion confirmed that the two fractions contain triple helical collagens. Thermal denaturations were monitored by the changes in CD signal at 221 nm. The two fractions purified by ammonium sulfate precipitation melted at 39.1 and 36.4 °C for the (α1(V))2α2(V) and α1(V)α2(V)α3(V) fractions, respectively. Trypsin cleavage of these two native fractions at temperatures near melting produced completely different fragmentation patterns, indicating different partial unwinding sites of the α1(V) and α2(V) chains in the two preparations and thus different molecular assemblies.

Our data demonstrate the existence of two different molecular assemblies of type V collagen in human placenta consisting of (α1(V))2α2(V) and α1(V)α2(V)α3(V) heterotrimers.

Type V collagen was originally described as a component of choriionic and amniotic membranes (1). Two α chains were described and designated αA and αB and subsequently α2(V) and α1(V), respectively (2). The stoichiometry of these two chains has been established to be (α1(V))2α2(V) (3, 4). In addition, the existence of (α1(V))3 molecules has been demonstrated in Chinese hamster lung cell cultures (5). The stability of (α1(V))3 has been demonstrated by in vitro reconstitution studies while (α2(V))3 homotrimers have been shown to be unstable (4). A third chain, clearly distinguishable by its electrophoretic mobility and by its peptide map, after enzymatic and CNBr cleavages, was found to copurify with α1(V) and α2(V) when purified from human placenta (6). It was called originally αC (6) and later α3(V) (2). Its amino acid composition was similar to the other two type V collagen α chains.

The nature of the molecular assembly that contains the α3(V) chain is still controversial. Rhodes and Miller (7) have shown that the placental type V collagen preparation can be resolved into two fractions by phosphocellulose chromatography. One fraction contains α1(V) and α2(V) in a 2:1 ratio and the other contains α1(V), α2(V), and α3(V) in a 1:1:1 ratio. Since the two fractions showed CD spectra characteristic of native collagen, this was taken as evidence for the existence of a (α1(V) α2(V) α3(V) heterotrimer. On the other hand, Madri et al. (8) have presented data obtained by gel electrophoresis under non-denaturing conditions suggesting the existence of native (α3(V))3 molecules. In both experiments, the separations were done in the presence of 2 M urea.

In this communication, we present two new separation methods by which native human placenta type V collagen can be resolved into two fractions, one containing α1(V) and α2(V) in a 2:1 ratio, and one containing α1(V), α2(V), and α3(V) in a 1:1:1 ratio. One of these methods, using ammonium sulfate precipitation in acetic acid, avoids the use of any potential denaturing agent.

MATERIALS AND METHODS

Type V Collagen from Placenta—Amnion and chorion were dissected away from human placentas and the placentas were suspended in cold distilled water at 4 °C for three days with two changes per day. The placentas were then ground in a meat grinder and suspended in cold distilled water at 4 °C for 2 days with two changes per day. Each change was accomplished by filtering and squeezing the ground placenta through a cheesecloth. The ground placentas were then suspended in 0.5 M sodium acetate at 4 °C for 16 h, filtered, and squeezed through cheesecloth and washed extensively with cold distilled water. The placentas were then weighed and suspended in 0.5 M acetic acid (300 g/liter, wet weight). Pepsin (Sigma) was added (300 mg/liter) and the tissue was digested for 16 h at 4 °C. The insoluble residue was pelleted by centrifugation (27,000 × g for 1 h). Type V collagen was then purified by differential salt precipitation as outlined in Fig. 1.

Type V Collagen from Fetal Membranes—(α1(V))2α2(V) collagen was prepared from amnion and chorion as described by Bentz et al. (4).

Ammonium Sulfate Precipitation—The placental type V collagen preparation containing α1(V), α2(V), and α3(V) was dissolved in 0.5
was stirred for 2 days at 4 °C. The precipitate was collected by centrifugation at 20,000 g for 30 min. Solid ammonium sulfate (10.7 mg/ml of the original solution) was added to the supernatant. The resulting solution was stirred at 4 °C for 2 days and then centrifuged as above. The pellets of the two precipitations and the final supernatant were dialyzed against 0.1 M acetic acid.

Phosphocellulose Chromatography—The procedure described by Rhodes and Miller (7) was followed. A column (1.8 x 9 cm) was packed with sieved and washed PC-11 phosphocellulose (Whatman) and equilibrated at 15 °C with pH 8.2, 0.04 M Tris-HCl buffer containing 0.17 M NaCl and 2 M urea. Up to 80 mg of crude type V collagen (4 mg/ml) in the starting buffer was applied to the column and eluted with a 500-ml linear NaCl gradient (0.17-0.47 M) in the same buffer at a flow rate of 60 ml/h.

Ion-exchange High-performance Liquid Chromatography—Microscale separation of native type V collagen was achieved by chromatography on a IEX-540 DEAE column (4 x 300 mm) (Altex) using a Beckman 334 chromatograph.

The column was equilibrated at 0.8 ml/min with a 0.025 M Tris-HCl buffer, pH 7.5, containing 2 M urea. The collagen was dissolved in 0.1 M acetic acid (200 µg in 0.2 ml), further diluted to 1.0 ml with the starting buffer, and applied to the column. A linear NaCl gradient (0.1-0.4 M) over 60 min at the same flow rate was used for the elution. The effluent was monitored at 214 nm with a Beckman Model 160 detector and collected in 0.8-ml fractions. After the chromatography, the column and the pumps were washed by flushing distilled water for 30 min through the system, followed by methanol/water (1:10) for another 30 min.

Native and Proteolytic Digestions—The conformation of native type V collagen preparations were judged by their resistance to a trypsin-chymotrypsin digestion exactly as described by Bruckner and Prockop (10). To evaluate trypsin sensitivity of the native molecules at 34, 35, and 37 °C, the collagens were dissolved in 0.1 M acetic acid (1 mg/ml). Aliquots (50 µl) were taken for each assay. They were treated with 10 µl of 1 M Tris base and mixed with 100 µl of 0.1 M Tris-HCl buffer at pH 7.4 containing 0.4 M NaCl. The digestion with trypsin was initiated by adding the enzyme dissolved in distilled water (1 mg/ml) at a substrate/enzyme ratio of 10:1. The mixtures were incubated at the specified temperatures for 30 min. The reaction was stopped by the addition of 10 µl of glacial acetic acid followed by 100 µl of electrophoresis sample buffer. The volume was then reduced to 100 µl by partial evaporation in a Speed-Vac centrifuge evaporator (Savant).

RESULTS

Preparation of Type V Collagen—The overall yield of the preparation procedure in two separate experiments has been 250 and 282 mg of crude type V collagen/1000 g of washed wet tissue. For comparison, the 0.7 M NaCl precipitate yielded about 2 g of other collagen types (mostly type I) /1000 g of wet tissue.

Separations of (α1(V)α2(V)α3(V) and of α1(V)α2(V)α3(V) Collagens—Three non-denaturing methods were found to resolve the crude type V collagen preparation into two different and homogenous fractions, one containing α1(V) and α2(V) in a 2:1 ratio and one containing α1(V), α2(V), and α3(V) in a 1:1:1 ratio. The first method is phosphocellulose chromatography as described by Rhodes and Miller (7). Only 50% of the sample applied eluted in the fractions containing (α1(V))α2(V) and α1(V)α2(V)α3(V) collagens. The electrophoretic analysis of the eluted fractions is presented in Fig. 2 (lanes 2 and 3). The chromatographic profile and the melting curves of the separated collagen were similar to those published by Rhodes and Miller (7). We obtained melting temperatures of 32.0 and 34.0 °C for phosphocellulose-purified α1(V)α2(V)α3(V) and α1(V)α2(V)α3(V) collagens, respectively.

Using an IEX-540 DEAE high-pressure liquid chromatography column we were also able to resolve the crude type V collagen into two similar fractions (Fig. 3). The separated fractions are shown in Fig. 2 (lanes 4 and 5). The native conformation of the separated collagens was confirmed by their resistance to proteolytic cleavage using a trypsin-chymotrypsin digestion as described by Bruckner and Prockop (10).

The abbreviation used is: SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.
Fig. 3. High-performance liquid chromatography of crude type V collagens under nondenaturing conditions. The column (IEX-540 DEAE) was equilibrated in 0.025 M Tris-HCl at pH 7.5 containing 2 M urea. 200 μg of the collagen was dissolved in 200 μl of 0.1 M acetic acid and further diluted to 1 ml with the starting buffer and applied on the column. Elution was performed by a NaCl linear gradient (0 to 6 M) for 60 min at a flow rate of 0.8 ml/min. 0.8-ml fractions were collected. Fractions labeled I, II and III contained α1(V)α2(V)α3(V) and (α1(V))2α2(V) (lanes 4 and 5 in Fig. 2). Fraction I contained nonproteinaceous UV absorbing material.

FIG. 4. Thermal transition curves of the collagens purified by differential ammonium sulfate precipitation. The thermal melting transition midpoint (Tm) for each of the fractions is indicated on the figure.

Ammonium sulfate precipitation in 0.5 M acetic acid also resolved (α1(V))2α2(V) and α1(V)α2(V)α3(V) collagens. The (α1(V))2α2(V) collagen was found to precipitate at 135 mg/ml of (NH4)2SO4 while the α1(V)α2(V)α3(V) fraction remained soluble at 144.7 mg/ml of (NH4)2SO4. This method is very simple and has a very high yield. Up to 80% of the initial crude type V collagen was recovered in the pure fractions which contained usually about equal amounts of collagen. Electrophoreses of the purified fractions were presented in Fig. 2 (lanes 6 and 7). Melting curves of the two fractions were obtained in 0.1 M acetic acid (Fig. 4). The fraction containing (α1(V))2α2(V) had a higher melting temperature (39.1 °C) than the one containing α1(V)α2(V)α3(V) (36.4 °C). Under similar conditions, type I collagen melts at 39.1 °C (9). The salt precipitated fractions were also completely resistant to trypsin-chymotrypsin cleavage.

Electrophoresis of Collagens under Nondenaturing Conditions—Electrophoresis of the crude type V collagen preparation revealed two bands (Fig. 5, gel C). All the fractions purified by the three methods outlined above showed only single bands in nondenaturing electrophoreses. The bands from the fractions containing (α1(V))2α2(V) had migrations corresponding to the fast-migrating band of the crude preparation (Fig. 5, gel A) while the fractions containing α1(V)α2(V)α3(V) had migrations corresponding to the slow-moving band (Fig. 5, gel B). Denatured α chains migrated with the dye front. The second dimension electrophoresis under denaturing conditions confirmed the identity of the slow-moving band with α1(V)α2(V)α3(V) and of the fast-
moving band with $(\alpha_1(V))_2\alpha_2(V)$ (Fig. 6). No collagen chain was found in the dye front of the first-dimension gel for any of our native fractions.

**Susceptibility of the Collagens to Trypsin**—The ammonium sulfate-purified native collagens were treated with trypsin as described under “Materials and Methods.” The first objective of this experiment was to demonstrate that the three chains of $\alpha_1(V)\alpha_2(V)\alpha_3(V)$ denature and become susceptible to general proteolysis simultaneously. This is likely if these three chains are part of the same triple helix. The second objective was to compare the tryptic fragmentation pattern of $\alpha_1(V)\alpha_2(V)\alpha_3(V)$ near its melting point with the tryptic fragmentation pattern of $(\alpha_1(V))_2\alpha_2(V)$ described by Sage et al. (14).

Electrophoresis of the digestion products at 34, 35, and 37 °C are shown on Fig. 7. Partial digestion of $\alpha_1(V)\alpha_2(V)-\alpha_3(V)$ is evident at 34 and 35 °C. At 37 °C, the chains are completely digested. The electrophoreses show that after partial cleavage with trypsin, the intact chains remain in a 1:1 ratio. At 34 and 35 °C, cleavage of the $(\alpha_1(V))_2\alpha_2(V)$ molecule yields two large fragments, one migrating just above intact $\alpha_2(V)$ and the other just under $\alpha_2(V)$. At 37 °C, two large fragments with migrations identical to the fragments called T1 and T2 by Sage et al. (14) are obtained.

These findings go along with the different melting temperatures obtained for the two preparations by CD spectroscopy (Fig. 4). The simultaneous cleavage of the three chains of $\alpha_1(V)\alpha_2(V)\alpha_3(V)$ strongly suggest that these chains participate in a single triple helix.

When incubated with trypsin at room temperature, and under otherwise identical conditions, heat-denatured (56 °C, 5 min) collagen molecules are entirely cleaved into small fragments that migrate with the dye front (data not shown). The observed cleavages must then arise from partial unwinding of the triple helices of the collagen molecules in specific regions. Despite the presence of $\alpha_1(V)$ and $\alpha_2(V)$ chains in the two studied fractions, the fragments generated from the $\alpha_1(V)\alpha_2(V)\alpha_3(V)$ molecule are different from those generated from the $(\alpha_1(V))_2\alpha_2(V)$ molecule. This further demonstrates that the two molecular assemblies are completely distinct and that the partial unwinding regions are different in the two molecules.

**FIG. 7. Cleavage of type V collagen by trypsin.** Equal quantities of native type V collagens (50 µg) were incubated at 34, 35, and 37 °C for 30 min. with trypsin at 1:10 enzyme-to-substrate ratio. The reaction was stopped with 10 µl of glacial acetic acid, and 100 µl of electrophoresis sample buffer was added. SDS-PAGE was done on an 8% slab gel. C, collagens incubated with no enzyme.

**DISCUSSION**

Using three different procedures to separate native collagen molecules, we have shown that a crude placental type V collagen preparation can be resolved into two fractions, one containing $\alpha_1(V)$ and $\alpha_2(V)$ in a 2:1 ratio and one containing $\alpha_1(V)$, $\alpha_2(V)$, and $\alpha_3(V)$ in a 1:1:1 ratio. It was shown that the molecules had kept their native conformations as judged by their melting curves and by their resistance to a trypsincinthrombin digestion (10).

Our data show that these two fractions are distinct entities with very little cross-contamination. The melting curves obtained for the phosphocellulose preparation (7) and for the preparation obtained by salt precipitation indicate that the $\alpha_1(V)\alpha_2(V)\alpha_3(V)$ fraction has a lower denaturation temperature than $(\alpha_1(V))_2\alpha_2(V)$. The sharp transitions observed for $\alpha_1(V)\alpha_2(V)\alpha_3(V)$ (Fig. 4) are not compatible with the presence of large amounts of the $(\alpha_1(V))_2\alpha_2(V)$ collagen which melts two degrees higher. The melting temperatures for the phosphocellulose fractions closely correspond to the one observed by Rhodes and Miller (7).

Our finding that the fractions obtained by salt precipitation have higher melting temperatures could be explained by the presence of some residual nondialyzable urea in the phosphocellulose fractions. It is, however, reassuring to find that the melting points of the preparations obtained by salt precipitation are much closer to physiological temperatures. Furthermore, the extension peptides removed during the pepsin digestion (3) could also contribute *in vivo* to the stabilization of type V collagen.

The fragmentation patterns of the two collagen fractions after trypsin digestion at a temperature near melting (Fig. 7) are different despite the presence of $\alpha_1(V)$ and $\alpha_2(V)$ chains in the two preparations. This can only be explained if these two chains are different in the two preparations in their primary structures or in their triple-helical assemblies. Primary structure differences are difficult to rule out without full sequence analysis. Peptide maps established after CNBr or thrombin cleavages have shown no difference between the chains from the two preparations. The differences between the fragmentation patterns are thus best explained by an alteration of the helix stability caused by the introduction of the $\alpha_3(V)$ chain.

Once a 1:1:1 chain ratio was obtained, the $\alpha_1(V)\alpha_2(V)\alpha_3(V)$ fraction could not be further resolved by any non-denaturing separation technique, *i.e.* column chromatography, salt precipitation, and gel electrophoresis. Furthermore, a sharp thermal transition was observed for this fraction (Fig. 4) and the three chains were shown to become trypsin-sensitive simultaneously. The separation of native type V collagen molecules by gel electrophoresis was reported by Madri et al. (8). These authors concluded that $\alpha_3(V)$ chains form homotrimeres $(\alpha_3(V))_3$. Using exactly the same technique but working with a material much more enriched in $\alpha_3(V)$ chain, we have not confirmed their results. Our data clearly indicate that the slowly migrating electrophoretic band of our preparation contains $\alpha_1(V)$, $\alpha_2(V)$, and $\alpha_3(V)$ chains and not $\alpha_3(V)$ as they claimed. When we did this experiment with our preparations containing the three chains in a 1:1:1 ratio, only the slow-moving band was observed. With preparations containing solely $\alpha_1(V)$ and $\alpha_2(V)$, only the fast-moving band was observed.

The renaturation experiments conducted by Bentz et al. (4) have demonstrated that $(\alpha_2(V))_3$ is unstable while $(\alpha_1(V))_3$ is stable. The existence of a sharp denaturation transition in

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our \( \alpha_1(\text{V})\alpha_2(\text{V})\alpha_3(\text{V}) \) preparation shows that all the molecules it contains must have the same conformational stability. This further supports our claim that all the molecules from this preparation have the same \( \alpha \) chain stoichiometry.

Our data thus firmly establish the existence of two major molecular assemblies in placental type V collagen, \( \alpha_1(\text{V})\gamma\alpha_2(\text{V}) \) and \( \alpha_1(\text{V})\alpha_2(\text{V})\alpha_3(\text{V}) \). We have also designed an efficient method for the separation of these two molecular species in their native conformation. The existence of three different primary structures for the three chains of a collagen molecule is an unusual finding. It has been previously reported only for the collagenous tail of Clq (16) and for the LMW collagen fragment from cartilage (17). The specific function of this new collagen structure will have to be elucidated by further investigation.

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