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Identification of a previously unknown human collagen chain, α1(XV), characterized by extensive interruptions in the triple-helical region

(type XV collagen/cDNA clones/multiple RNAs/extracellular matrix/glycosylation)

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ABSTRACT **A previously unknown collagen cDNA clone, PF19, was isolated from a human placenta library. The 2.1-kilobase insert has a complete open reading frame of 709 amino acids that includes 12 amino acids of the NH_2-terminal domain, a principally collagenous region of 577 residues, and 120 residues of the noncollagenous COOH terminus. The collagenous part of the sequence encoded by PF19 is characterized by 13 interruptions ranging in size from 2 to 45 amino acids. Within four interruptions are consensus sequences for attachment of serine-linked glycosaminoglycans and asparagine-linked oligosaccharides suggesting that this collagen may be extensively glycosylated. A synthetic decapeptide representing a sequence at the beginning of the COOH-terminal noncollagenous domain was used to prepare an antibody in rabbits. This antiserum detected a 125-kDa bacterial collagenase-sensitive protein in Western blots of HeLa cell lysate. Consistent with the size of the collagen chain, Northern blot hybridization revealed a major transcript of 5.3 kilobases and two minor ones of 4.7 and 4.4 kilobases that are present in cultured human fibroblasts but absent from umbilical vein endothelial cells. We propose that the previously unidentified polypeptide described in this report be designated the α1 chain of type XV collagen.**

The large family of collagen proteins is currently comprised of 14 formally recognized types (reviews, refs. 1–3). However, it is clear from partial characterization of newly identified chains (4) and cDNA clones (5, 6) that other collagens exist and definition of their primary structure will further enhance our understanding of these important matrix molecules. The collagens can be divided into two groups, the fibrillar and nonfibrillar collagens (2). The fibrillar collagens, composed of types I, II, III, V, and XI, have the same general chain structure consisting of a dominant 100-kDa uninterrupted collagenous region flanked by conserved COOH and variable NH_2 extension peptides (reviews, refs. 7 and 8). The triple-helical molecules associate in a highly ordered, quarter-staggered array to form the characteristic extracellular fiber or fibrillar aggregates. In contrast to the stereotypic nature of these "classical" collagens, the group of nonfibrillar collagens, types IV, VI–X, and XII–XIV, exhibit extensive diversity in size, sequence, nature of the noncollagenous termini, and organization into supramolecular assemblies (2). The one unifying feature is the occurrence of interruptions within one or more of the principally collagenous regions. The individual polypeptides range in size from 60 to 340 kDa and contain noncollagenous domains of a few to several thousand residues that sometimes account for the major part of the protein (2, 9). Some of the nonfibrillar types form sheet-like and fibrous structures, beaded filaments, hexagonal lattices, or amorphous mats; others are found distributed on the surface of banded fibrils (2).

At least 27 genes encode the 14 hetero- and/or homotrimeric collagen types. With the exception of α3(V), all of the constituent chains are currently represented in cDNA clones from which most of the amino acid residues have been derived. Significantly, some of the new collagen types were discovered from cDNA cloning and sequencing. For instance, α1(XII) was able to be classified from analysis of 120 residues encoded by a chicken tendon fibroblast cDNA clone; although the sequence was similar to type IX, the RNAs had a different tissue distribution (10). Recognition of type XIII collagen was a consequence of DNA sequencing of clones originally found in a human tumor HT1080 library using a mouse α2(IV) probe (11). Recently, the existence of a fifth type IV subunit was disclosed by comparison of amino acids deduced from the cloned human DNA sequence with those of other type IV collagen chains (12–14).

Several years ago, we attempted to isolate additional type V cDNA clones to complement our earlier α2(V) studies on sequence and expression. Our efforts, instead, resulted in the identification of α5(IV) clones (13, 14) and two identical clones with 2.1-kilobase (kb) inserts encoding a previously unknown collagen chain not homologous to types I–XIV. Here, we report on the primary structure and characterization of the unique collagen that we have provisionally named α1(XV).‖

METHODS

Isolation of Collagen cDNA Clones. A 550-base-pair (bp) *Eco*RI fragment encoding the end of the collagenous region and beginning of the COOH propeptide of an unidentified chicken fibrillar collagen chain (M.K.G., unpublished results) was used as probe to screen a human placenta λgt11 cDNA library (Clontech). The avian clone had been isolated using our human α2(V) cDNA clone, NH20 (15). The human library was plated on a lawn of *Escherichia coli* strain Y1090 as described (14). Phage DNA was transferred to duplicate nitrocellulose filters and hybridized to 1.5 ng of ^{32}P-labeled DNA per ml (specific activity, 8×10^8 cpm/μg) in a 35%

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‖The sequence reported in this paper has been deposited in the GenBank data base (accession no. L01697).

formamide/6× SSC (1× SSC = 0.15 M NaCl/0.015 M sodium citrate, pH 6.8) buffer at 37°C for 20 hr. The filters were washed in 2× SSC/0.05% $NaDodSO_4$ first at 21°C and then at 40°C. The phage yielding a positive hybridization signal were plaque-purified and the inserts were ligated into the *Eco*RI site of plasmid Bluescript SK vector (Stratagene).

Nucleotide Sequencing and Sequence Analysis. Nucleotide sequences were determined by the dideoxynucleotide sequencing method using Sequenase enzyme (United States Biochemical). The primers were either vector- or insert-specific 17-mer oligonucleotides synthesized on an Applied Biosystems DNA synthesizer. Nucleotide sequences were fully determined for both strands of the cDNA clone PF19. International Biotechnologies software was used to compile the sequence data. Nucleotide homology comparisons were carried out with GenBank and EMBL data bases, and amino acid homology searches were performed using the Protein Identification Resource (National Biomedical Research Foundation) and Swiss-Prot data bases.

Northern Blot Hybridization of Collagen cDNA Clones. Isolation of endothelial cells (HE) from human umbilical veins has been described (16). One cell line of human fibroblasts, HPF, was derived from tissue discarded after surgery to repair penile hypospadias (16). Another fibroblast cell line, GM3348, was derived from a skin biopsy of a normal teenage male (Coriell Institute for Medical Research). Poly(A) RNA was purified as detailed earlier (17).

One-half microgram of RNA per lane was electrophoresed at 30 V for 22 hr in 1% agarose gels containing 2.2 M formaldehyde. RNA was transferred to nitrocellulose filters and hybridized to 2 ng of ^{32}P-labeled DNA per ml (specific activity, 1–2 × 10^9 cpm/μg) in a 50% formamide/4× SSC solution at 42°C for 20–24 hr (16). Final wash of the filters was at 62°C in 0.5× SSC. cDNA inserts were isolated from human clones: α2(I) Δ26 (2.6 kb), α1(III) E6 (2.4 kb), α1(IV) KK4 (1.7 kb), α2(V) NH20 (1.35 kb) (ref. 16 and refs. therein) and the 2.1-kb α1(XV) clone PF19 (Fig. 1).

Synthetic Peptides and Antibody Preparation. Two 10-amino acid peptides were synthesized using Fmoc (fluoren-9-ylmethoxycarbonyl) chemistry on polyamide/kieselgur resin (18). The sequence of the first peptide, NH_2-Ser-Gly-Ser-Thr-Gln-Leu-Leu-Asn-Glu-Pro-COOH, is found in interruption III and the sequence of the second peptide, NH_2-His-Leu-Val-Ile-Glu-Gly-Thr-Phe-Ile-Tyr-COOH, is located in the beginning of the COOH-terminal noncollagenous domain. Both peptide regions were selected on the basis of National Biomedical Research Foundation and Swiss-Prot data base searches for uniqueness to the PF19 collagen sequence—i.e., not represented in other protein sequences and, in particular, other collagen types. Approximately 2 mg of each peptide was conjugated to 10 mg of keyhole limpet hemocyanin using glutaraldehyde (19) and each peptide solution was divided into eight aliquots. For primary immunization, two aliquots of each peptide were emulsified in Freund's complete adjuvant and injected intradermally into two rabbits. The other aliquots were emulsified in Freund's incomplete adjuvant and given as three booster injections at 3-week intervals.

Electrophoretic Transfer Blotting of Cellular Proteins. HeLa cells were grown in 100-mm plates in Dulbecco's modified Eagle's minimal essential medium containing 10% newborn calf serum and 50 μg of ascorbate per ml. At confluency, the cell layer from one plate (≈10^7 cells) was scraped into 0.5 ml of buffer (pH 7.4) consisting of 270 mM Tris·HCl, 270 mM NaCl, 22 mM *N*-ethylmaleimide, 0.1% Triton X-100, and 200 μg of heat-denatured type I collagen per ml (Sigma). Cells were homogenized in a Teflon/glass homogenizer and the sample was centrifuged at 15,000 × *g* for 5 min. Half of the supernatant was boiled for 5 min after adding sample buffer to give a final concentration of 2% $NaDodSO_4$, 10% glycerol, 0.5 M urea, 1% 2-mercaptoethanol, 0.001% bromphenol blue, and 0.125 M Tris·HCl (pH 6.8). The other half of the sample was brought to a final concentration of 10 mM $CaCl_2$ and 0.18 mg of bacterial collagenase per ml (Sigma, type VII). The sample was incubated at 37°C for 4 hr, mixed with sample buffer, and boiled. Samples were electrophoresed in an 8% polyacrylamide gel containing 0.5 M urea and 0.1% $NaDodSO_4$ (20). The fractionated proteins were electroblotted onto nitrocellulose filters (BA85, Schleicher & Schuell) as described (19). The filters were stained with heparin/toluidine, cut into strips, and destained (19). The filter strips were incubated overnight with a 1:3000 dilution of the type XV antiserum prepared against the COOH-terminal noncollagenous domain peptide. Antibody binding was detected using peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad) and 4-chloro-1-naphthol (21).

RESULTS

Isolation of a Human cDNA Clone Encoding a Different Collagen Sequence. Previously, we reported our first results obtained in screening 400,000 clones from a human placenta library at low stringency with a DNA fragment encoding the collagenous and COOH telo/propeptide junction sequence of an unidentified avian fibrillar collagen chain. Four of the six positive recombinants, characterized by DNA sequencing, were found to correspond to two identical α1(I) clones with 2-kb inserts, an α1(IV) clone containing a 2.8-kb insert, and an α5(IV) clone with a 3-kb insert (13, 14). Successive cycles of plaque purification of the other two independently isolated clones revealed a hybridization signal of equal intensity, and molecular weight determination of the respective *Eco*RI inserts showed that both were 2.1 kb. Results of Southern blot hybridization indicated that the clones were identical and this speculation was subsequently confirmed by DNA sequencing. A restriction map of the clone PF19 is illustrated in Fig. 1. PF19 encodes a complete open reading frame of 709 residues primarily composed of a main collagenous region containing multiple interruptions (Fig. 2). The amino acid sequence of the protein was different from those reported for other collagens, and homology searches of nucleotide and protein data bases confirmed that it represented a previously unknown polypeptide not homologous to the type I–XIV collagen chains.

Primary Structure of a Unique Collagen α Chain. At the beginning of the protein sequence encoded by the clone is a 12-amino acid noncollagenous sequence followed by a predominantly collagenous region of 577 residues (Fig. 2). At the COOH terminus are 120 amino acids of the COOH-terminal noncollagenous domain. Within the collagenous region are 13 interruptions that can be divided into three size classes (Table

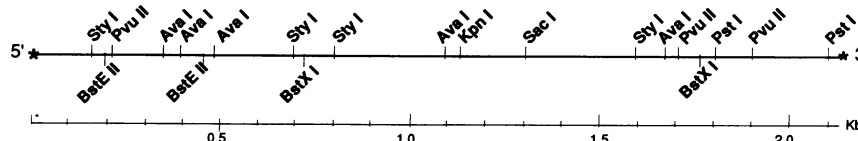

FIG. 1. Restriction map of the human α1(XV) cDNA clone PF19. The *Eco*RI insert of PF19 consists of 2127 coding nucleotides. Both DNA strands were entirely sequenced using vector and insert sequence-specific primers. The restriction sites noted on the map were located by computer analysis and entirely verified by endonuclease restriction digests electrophoresed on agarose or acrylamide gels.

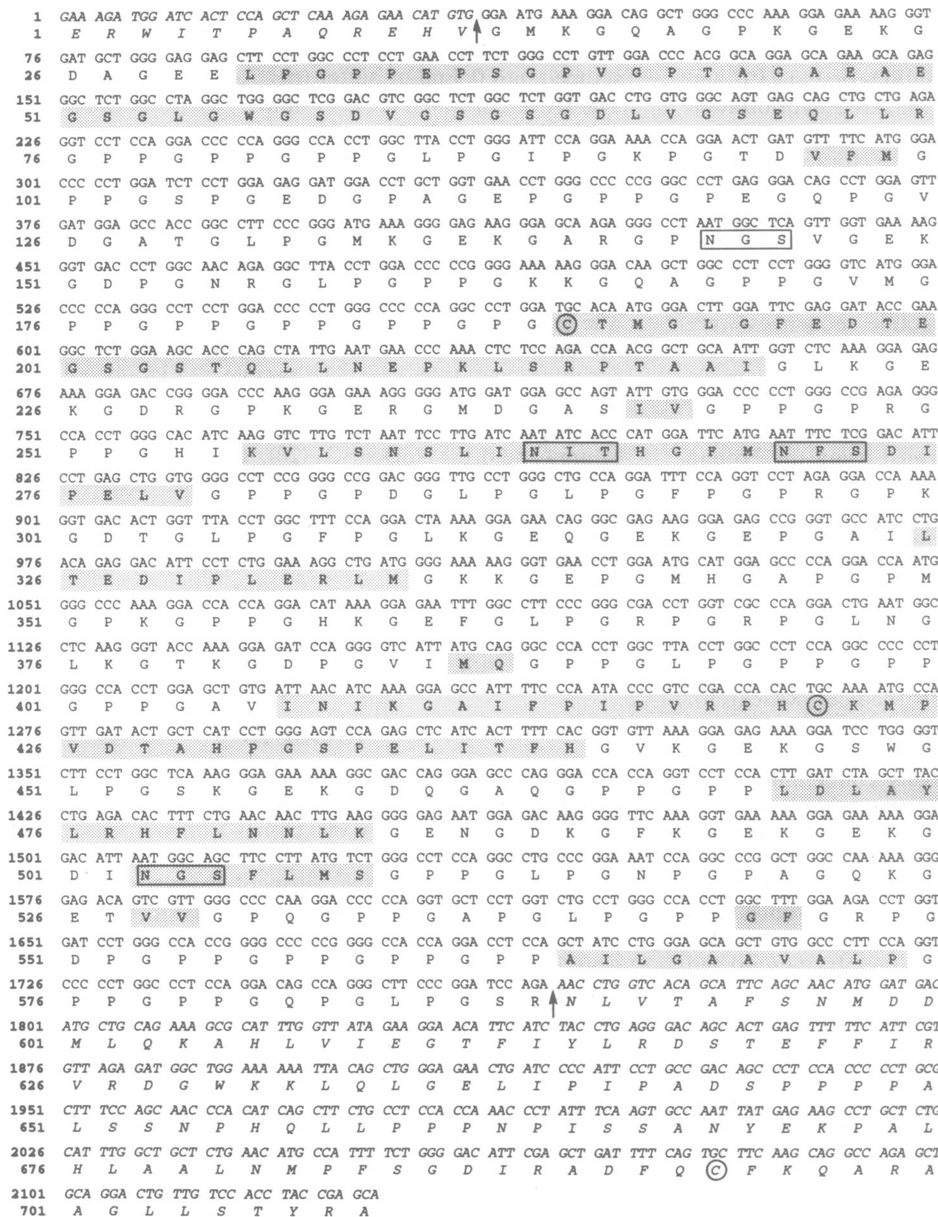

FIG. 2. Nucleotide and encoded amino acid sequence of the PF19 clone. On the top line is the DNA sequence and on the second line is the deduced amino acid sequence shown in single-letter code. Arrows designate the beginning and end of the 577-amino acid discontinuous collagenous region. Interruptions (I–XIII) are highlighted in the shadow areas, cysteines are circled, and potential N-linked glycosylation consensus sequences are boxed.

1). The small interruptions, numbers II, IV, VII, XI, and XII, consist of 2 or 3 residues; the medium size ones, numbers VI, IX, X, and XIII, are 7–14 amino acids in length; and the large interruptions, numbers I, III, V, and VIII, span 24–45 residues. Two cysteines are located in the interruptions; one is the first residue of interruption III and the second is in the center of interruption VIII. (An additional cysteine is found in the noncollagenous COOH terminus.) The collagenous regions flanking most of the interruptions are predominantly short and consist of only 4–7 Gly-Xaa-Yaa triplets. Three other regions include 10, 15, and 17 triplets. The only relatively large collagenous domain, composed of 30 triplets, is positioned between interruptions II and III.

The deduced protein sequence of this collagen was searched for a variety of structural motifs contained within the PROSITE program (22). By this approach six potential carbohydrate attachment sites were identified. Four of these are possible tripeptide acceptor sites for N-linked glycosylation (Asn-Xaa-Ser/Thr, where Xaa or the amino acid following Ser or Thr cannot be Pro; refs. 23 and 24). Two sites, Asn-Ile-Thr and Asn-Phe-Ser, are located in interruption V and another one, Asn-Gly-Ser, is found in interruption X (Fig. 2). A second Asn-Gly-Ser sequence is situated in the collagenous region midway between interruptions II and III. Two putative O-linked glycosaminoglycan attachment sites were also revealed (Table 2). Both sequences, adjacent to each other in interruption I, share the consensus motif (Ser-Gly-Xaa-Gly) employed by this search algorithm. Furthermore, two additional sites containing Ser-Gly dipeptides (Table 2), located in interruptions I and III, fall within the known and predicted consensus sequence in proteoglycan core proteins (25).

Multiple RNA Transcripts Hybridize to the PF19 Collagen Clone. To investigate the nature and size of the mRNA coding for PF19, we first used the clone in Northern blot hybridization to RNA isolated from cultured human fibroblasts. Expecting a low-abundance RNA, if any, we were surprised to find a transcript that was easily identified in a 24-hr autoradiography period. Longer exposures revealed two additional smaller molecular weight bands (a representative picture is included in Fig. 3). Rehybridization of the filter to an α2(V) probe allowed us to assign molecular weight values to the three transcripts hybridizing to the PF19 clone. The major species corresponds to 5.3 kb and the two minor ones are estimated to be 4.7 and 4.4 kb (Fig. 3).

To compare the expression of the RNAs for this collagen with other well-characterized collagen transcripts, we hy-

Table 1. Length and location of type XV collagenous sequences and interruptions

Gly-Xaa-Yaa length*	Interruption†	
	No.	Residues
18	I	45‡
21	II	3
90§	III	31‡¶
21	IV	2
12	V	24§
45	VI	11
51	VII	2
18	VIII	34¶
30	IX	14
18	X	7§
18	XI	2
15	XII	2
18	XIII	10
15	[NC1]¶	

*Amino acids in the Gly-Xaa-Yaa segments between the interruptions. NC1 designates the COOH-terminal noncollagenous domain.
†Identification and size of interruptions I–XIII.
‡Presence of potential O-linked glycosylation site.
§Presence of potential N-linked glycosylation site.
¶Presence of cysteine residue.

bridized PF19 and types I, III, IV, and V clones to Northern blots containing RNAs isolated from two fibroblast cell lines and cultured human umbilical vein endothelial cells. As shown in Fig. 3, PF19 exhibits increased hybridization to the fibroblast RNA that is also enriched for α2(I) transcripts. Neither PF19 nor the α2(I) clone detected any transcripts in endothelial cell RNA preparations at relatively short exposure times that showed type IV and V RNAs (Fig. 3) or at extended autoradiography periods (data not shown) that revealed a minor amount of type III (16).

Identification of a 125-kDa Collagen Chain by Western Blot Analysis. To determine the size of the unique collagen chain, we attempted to prepare immunological probes using two 10-amino acid synthetic peptides. The sequence of one peptide is contained within interruption III and the sequence of the second is located in the beginning of the COOH-terminal noncollagenous domain (*Methods*). Only the NC1 peptide elicited an antigenic response when injected into rabbits and the antiserum was used in Western blots of homogenate from HeLa cells known to express the 5.3-kb RNA. Different dilutions of the antiserum were tested and a dilution of 1:3000 was found to be optimal. As illustrated in Fig. 4, Western blotting of the total HeLa cell homogenate showed a distinct 125-kDa protein band that was eliminated upon treatment of

Table 2. Amino acid sequence and position of potential O-linked glycosylation attachment sites

Potential O-linked glycosylation sites	Sequence position
PPEPSGPVGP * **	34–43
EAEGSGLGWG * **** *	48–57
SDVGSGSGDL ***** *	58–67
DTEGSGSTQL * ***** *	198–207
D/EXEGSGSGXL * ****** *	Consensus

Residues highlighted by asterisks are included in the consensus sequence. The letter X indicates the presence of any amino acid. Position of the sites is according to the numbering of amino acids shown in Fig. 2.

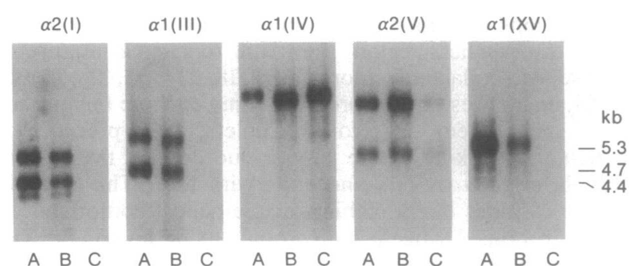

FIG. 3. Northern blot hybridization of collagen clones to fibroblast and endothelial cell RNAs. One-half microgram of poly(A) RNA in each lane was hybridized to human collagen cDNA clones coding for the chains identified above each panel. Lanes A and B, RNAs representing two skin fibroblast cell lines, HPF and GM3348, respectively; lanes C, RNA isolated from human umbilical vein endothelial cells (HE). Autoradiography times were 18 hr for α2(I) and α1(III), 30 hr for α2(V), and 48 hr for α1(IV) and α1(XV).

the sample with bacterial collagenase. The 125-kDa collagenous protein was not detected with serum taken from the rabbit prior to immunization (Fig. 4). [The same pattern was found using homogenate from cultured human skin fibroblasts (data not shown).]

DISCUSSION

In this study, we have presented evidence for the existence of a previously unknown collagen chain discovered by cDNA cloning. The clone PF19 was isolated from a human placenta cDNA library by cross-hybridization to an unrelated 0.5-kb chicken fibrillar collagen clone that also identified types I, III, IV, and XIII human collagen clones found in placenta and endothelial cell libraries. The widespread interspecies homology among these collagen DNAs is likely restricted to nucleotides encoding tandem Gly-Pro-Pro triplets. The 709-amino acid coding sequence deduced from the 2.1-kb PF19 insert bears no similarity to type I–XIV collagens or the collagenous domains found in one of the bullous pemphigoid antigen proteins (5, 6). Nor does it resemble the nonfibrillar collagens in sea urchins or nematodes (26, 27). We propose to name the previously unrecognized polypeptide the α1 chain of type XV collagen.

The 125-kDa protein identified in Western blots using antiserum generated against a synthetic peptide in the type XV sequence suggests that the primary structure shown in Fig. 2 represents more than half of the unique collagen chain. Furthermore, from preliminary inspection of an overlapping 5′ clone, it appears that PF19 encodes the complete triple-helical region. This primarily collagenous domain comprises 577 residues divided into two-thirds Gly-Xaa-Yaa triplets and one-third noncollagenous interruptions. Surprisingly, most of the interruptions separate collagenous segments consisting of only 12–21 amino acids. Unlike several of the other collagen types—i.e., types IX, XII, XIII, and XIV (5, 10, 28–30)—arrangement and size of the interruptions do not support the concept of only a few discrete triple-helical domains. Even if one regards the five 2- or 3-amino acid

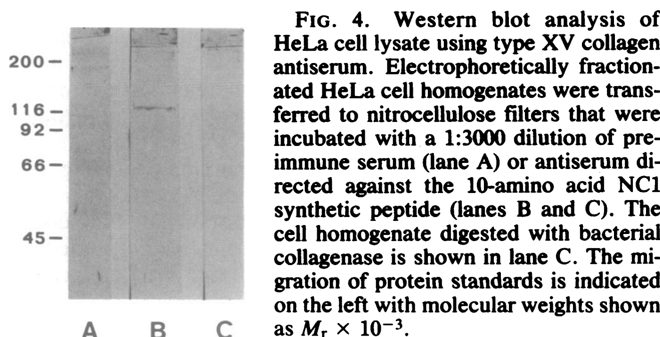

FIG. 4. Western blot analysis of HeLa cell lysate using type XV collagen antiserum. Electrophoretically fractionated HeLa cell homogenates were transferred to nitrocellulose filters that were incubated with a 1:3000 dilution of preimmune serum (lane A) or antiserum directed against the 10-amino acid NC1 synthetic peptide (lanes B and C). The cell homogenate digested with bacterial collagenase is shown in lane C. The migration of protein standards is indicated on the left with molecular weights shown as $M_r \times 10^{-3}$.

interruptions as minor imperfections that are incorporated into a triple-helical region, there still remains a mixture of diverse-size collagenous domains of 18, 114, 35, 45, 71, 30, 18, 55, and 15 residues. Bordering some of these regions are all of the tandem Gly-Pro-Pro triplets; their presence is particularly striking in the 114-residue domain that begins with three such triplets and ends with four. The multiple proline residues likely stabilize triple-helical conformation, especially considering the high probability of those in the Yaa position being hydroxylated.

An unusual combination of eight potential sites for oligosaccharide addition occur in the type XV triple-helical region. All, except one, are found in interruptions. Three possible N-linked acceptor tripeptides are included in interruptions V and X; the fourth spans two Gly-Xaa-Yaa triplets (residues 144–146). Future studies are obviously required to determine whether any or all of these sites are functional. Although there have been reports of similar sequences in a number of collagens, until lately there has been a paucity of experimental data related to this subject. Initial studies showed that the sites in the COOH terminus of type I and the NH_2 terminus of type III were occupied (31, 32). More recent results have demonstrated that N-linked oligosaccharides occupy all of the potential sites present in the collagenous portion of the α1(IV) and α2(IV) 7S domains (33, 34) as well as those in the triple-helical region of all the type VI chains (33).

In contrast, considerable attention was quickly devoted to O-linked glycosylation of type IX collagen upon finding that the α2(IX) subunit contains a glycosaminoglycan chain attached to a serine residue in the noncollagenous NC-3 domain (35). To date, this represents the sole precedent for a combined collagen–proteoglycan molecule but it is plausible that other collagens may be subject to similar posttranslational modifications. Type XV contains four candidate O-linked sites involving serine residues; three are present in the large 45-amino acid interruption no. I and another is located in the 31-residue interruption no. III. The 10-residue segments encompassing the Ser-Gly dipeptide(s) in fact conform to a consensus sequence, Asp/Glu-Xaa-Glu-Gly-Ser-Gly-Ser-Gly-Xaa-Leu (Table 2), which is an expanded version of those known and predicted for a number of proteoglycan core proteins (25). Furthermore, based on hydropathy analysis (36), interruptions I and III are expected to be relatively hydrophilic (data not shown), and potential sites for glycosylation are often disposed in surface-accessible regions having high probabilities for β-turn formation (37).

Hybridization of the PF19 clone to Northern blots of fibroblast poly(A) RNA revealed a major transcript of 5.3 kb and two lower molecular weight ones of 4.7 and 4.4 kb. Both of the smaller RNAs are present in relatively minor amounts. The existence of multiple transcripts is typical of many collagen types. Some of the length differences are due to alternate use of poly(A) attachment sequences and others result from use of alternate promoters and differential splicing (1–3). Although we have yet to ascertain the origin of the three RNAs recognized by the PF19 probe, even the smallest transcript is of sufficient size for translation of the 125-kDa protein.

The α1(XV) RNA is present in moderate levels in human fibroblasts and is absent from human umbilical vein endothelial cells. Accordingly, it is also found in bovine lung fibroblasts and smooth muscle cells, whereas first examination of several bovine endothelial cell lines was negative (data not shown). Though the amount of α1(XV) RNA in cultured cells approximates that of α2(V), comparison of its expression with collagen types I, III, IV, and V shows most similarity to type I. Therefore, we could speculate that along with the majority of collagens, type XV may be distributed in a tissue-specific pattern resembling that of type I collagen.

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