The human α3b is a 'full-sized' laminin chain variant with a more widespread tissue expression than the truncated α3a

Roberto Doliana[a], Isabella Bellina[a], Francesco Bucciotti[a], Maurizio Mongiat[a], Roberto Perris[a], Alfonso Colombatti[b,*]

[a] *Divisione di Oncologia Sperimentale 2, CRO, 33081 Aviano, Italy*
[b] *Dipartimento di Scienze e Tecnologie Biomediche, Università di Udine, 33100 Udine, Italy*

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Abstract **We report the molecular cloning of the human laminin α3b chain variant and its mRNA expression pattern in adult human tissues when compared to the α3a variant. The mRNA encoding for the α3b variant is about 11 kb and the predicted translation product carries the complete set of domains typical for a 'full-sized' laminin α chain. Apart from the similar domain structure of α3b also the sequence of α3 resulted more closely related to the α5 than to the α4 chain. Quantitative analysis of the RNA expression in a broad panel of adult human tissues indicated that the α3b variant is more widely distributed than the α3a shorter variant.**

Key words: α Chain; Cloning; Human laminin; Tissue expression

1. Introduction

Laminins are large cruciform heterotrimeric basement membrane glycoproteins mediating multiple structural and cellular functions [1]. The heterotrimer is composed of genetically distinct but homologous chains denominated α, β and γ [2] which assemble via ionic interactions mediated with high specificity by their coiled-coil domains I and II. Up to date, three β, β1, β2, and β3 [3–7], two γ, γ1 and γ2 [8–11] and six α, α1, α2, α3, α4, α5, and αD [12–26] chains have been described. Common to all the α chains is a unique C-terminus folding into a large globular structure, constituted of five so-called G domains. These are the primary regions of molecular interaction of laminin with other extracellular matrix molecules and with cell surface receptors. The α1, α2, α5, and αD chains have been defined as 'full-sized', as opposed to the α3 and α4 chains which are classified as truncated due to their lack of an extended NH2-terminal region. Each α chain can combine with different β and γ chains to form at least 11 different heterotrimeric laminin isoforms. The α3 chain assembles with β3 and γ2 chains to form laminin 5 [27], with β1 and γ1 chains to originate laminin 6 and with β2 and γ1 to form laminin 7 [28]. Laminin 5, previously denoted epiligrin/kalinin/nicein [29–31], is a major cell ligand in the basement membranes of specialized epithelia where it is integrated via laminins 6 and 7 [28]. The G domain of the α3 chain binds the hemidesmosome via the α6β4 integrin [32] and mutations in each of the laminin 5 chains are associated with junctional forms of epidermolysis bullosa [33]. The α3 chain gene has been reported to give rise to two distinct transcripts [19], now denominated α3a and α3b in man and α3A and α3B in the mouse [34], which display a variable NH_2-terminal region. The shorter transcript, α3a, encoded for a 5,5 kb mRNA, whereas the longer transcript, α3b, is suggested to encode for messages of apparently distinct lengths between the two species.

In this study we report a novel nucleotide sequence of the human α3b that shows that this laminin chain variant has a NH2-terminal domain organization like the full-sized laminin α chains. At the mRNA level the two α3 isoforms display a distinct pattern of expression, with the α3b mRNA having a wider tissue distribution compared to α3a.

2. Materials and methods

2.1. RT-PCR and nucleotide sequencing

Degenerate primers were designed based on the sequences of domains VI and V of the murine laminin α5 chain [25] relying upon the high degree of interchain homology in these domains. Three degenerate primers (sense: *deg-1* 5′ AT(ACT)CA(GA)GG(AGCT)CA(GA)-TA(TC)TG(TC)GA 3′ and antisense: *deg-2* 5′ GC(GA)TG(AGC-T)CC(GA)TG(AG)CA(GA)TT(AG)CA 3′ and *deg-3* 5′ (TC)T-C(AG)CA(AG)(TA)A(AGCT)GG(AG)AA(GA)TT(AG)TA 3′) were used to amplify fragments from total RNA of HUVEC cells or of the human fibrosarcoma cell line HS-913T. The amplification products were cloned into the pGEM-T vector (Promega Corp.) and sequenced by the chain termination method using the Sequenase kit (US Biochemical Corp.). To determine the sequence extending towards the ATG start codon, we applied the RACE method, using the 5′-3′ RACE kit (Boehringer), primer 3 (in RT), primer 4 (first PCR) and primer 5 (nested PCR). To determine the sequence of the mouse α3B laminin chain around the region of the proposed starting codon (34), a degenerate 5′ primer based on the human sequence (sense: 5′ TT(TC)(CGCT)GAGTT(TC)(AT)(GC)(CTG)TGG(CA)GGGG 3′), and a 3′ primer based on the published mouse sequence (antisense: 5′ GTCACAGAGCCATGTGCCCAC 3′) were prepared. The primers were used in RT-PCR to amplify a fragment from total RNA of adult mouse lung. To correct for possible TAQ polymerase errors all sequences were determined from both strands and were repeated on clones obtained from independent PCR products. Database homology searches were performed on the BLAST server at the National Center for Biotechnology Information [35] and sequences were compared by FASTA at the European Bioinformatics Institute server [36].

2.2. Northern blot analysis and RNase protection assay

Ten μg of total RNA isolated from the human kidney 293 cell line was electrophoresed on 0.8% denaturing agarose gels, transferred onto nylon membranes (Hybond N^+, Amersham) and the membranes were incubated according to standard procedures with a [^{32}P]dCTP-labeled probe corresponding to clone 10. A fibronectin-specific probe was used as a reference for mRNA size.

A probe able to discriminate between α3a and α3b was prepared by RT-PCR from mammary gland total RNA (Clontech Laboratories, Inc.) (nucleotides 7–360 GenBank accession No. L34155) and cloned

*Corresponding author. Present address: Divisione di Oncologia Sperimentale 2, CRO, 33081 Aviano, Italy. Fax: +39 (434) 659428. E-mail: acolombatti@ets.it

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into the pGEM-T vector (Promega). [^{32}P]UTP-labeled antisense riboprobes were synthesized from the SP6 promoter with 25 units of SP6 RNA polymerase (Promega) and hybridised overnight with total RNA (10 μg) extracted from a panel of 18 adult tissues (Clontech). The degree of intactness and amount of input RNAs were ascertained by adding a β-actin probe. Post-hybridization procedures were performed using the RPAII kit (Ambion). The RNase protected fragments were run on a sequencing gel, autoradiographed and the specific signals quantified by computer-aided densitometric scanning.

3. Results and discussion

3.1. Molecular cloning of the NH_2-terminal region of the human laminin α3 chain variant

Out of 30 clones obtained with the use of degenerate primers (*deg-1* and *deg-2*) two clones (clone 9 and clone 10) were found to yield distinct restriction patterns which differed from those expected for the known α chains. While the sequence of

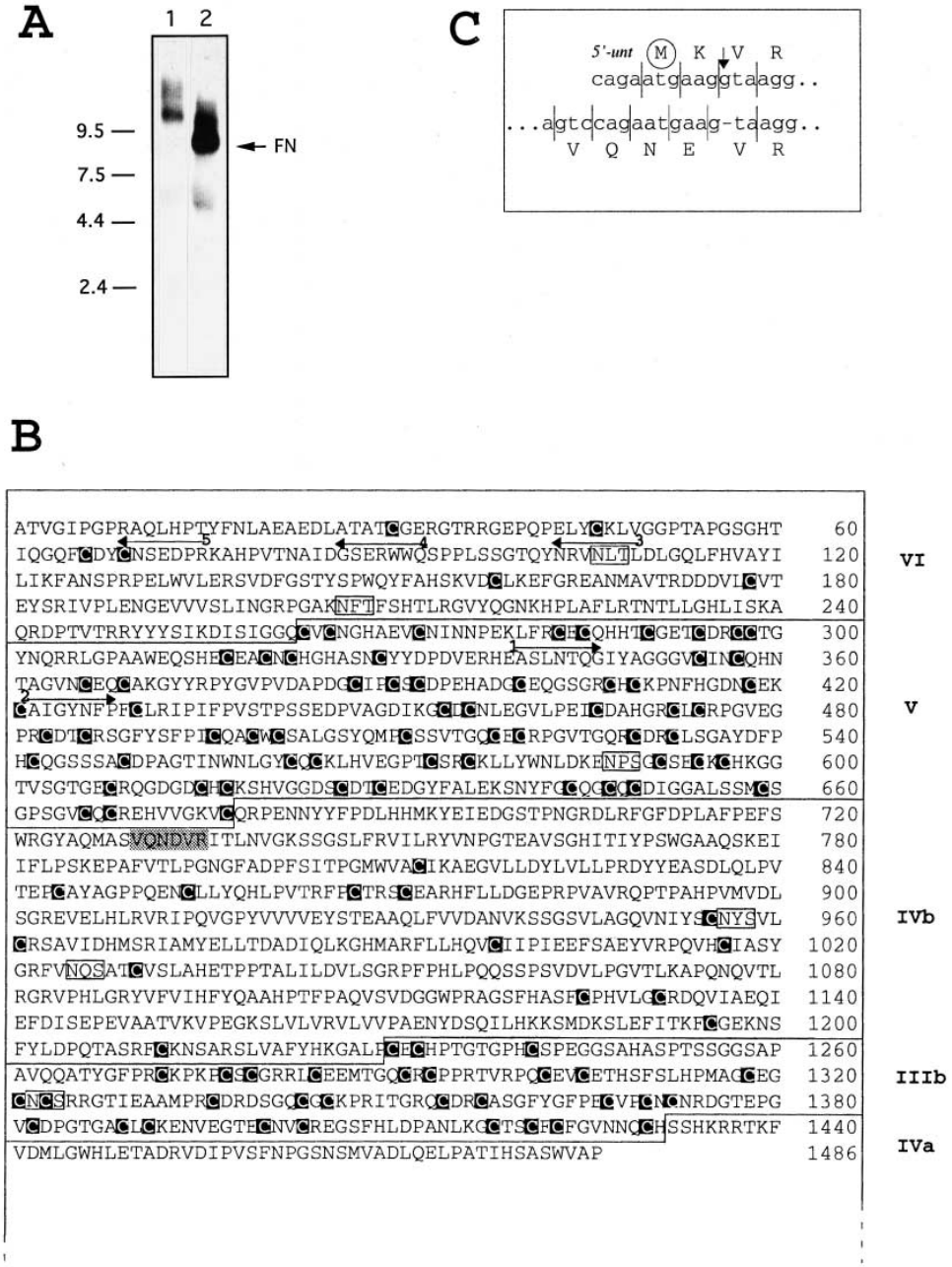

Fig. 1. A: Northern blot analysis of α3b mRNA. Each lane contained 10 μg of total cellular RNA from the embryonal kidney 293 cell line. Lane 1 was hybridized with a α3b-specific probe and lane 2 with a fibronectin- (FN-) specific probe and the blot was exposed for 24 h. Standard size markers are shown on the left. B: Deduced amino acid sequence of the human α3b chain variant NH_2-terminal region. Cysteine residues are highlighted and potential glycosylation sites are boxed. The human sequence corresponding to the partial mouse sequence reported in C (lower row) is boxed in gray. The extension of the conventional laminin domains is indicated on the right [40,41]. The numbered arrows correspond to primers used for PCR and RACE assays. The nucleotide sequence is available from EMBL under accession number AF005258. C: Partial nucleotide and deduced amino acid sequence of the mouse α3B chain variant homologue. Upper row: the sequence reported by Galliano et al. [34]; lower row: the sequence reported in this study. The arrow indicates the base pair that most likely caused the missense translation in [34] resulting in a start codon for methionine (encircled).

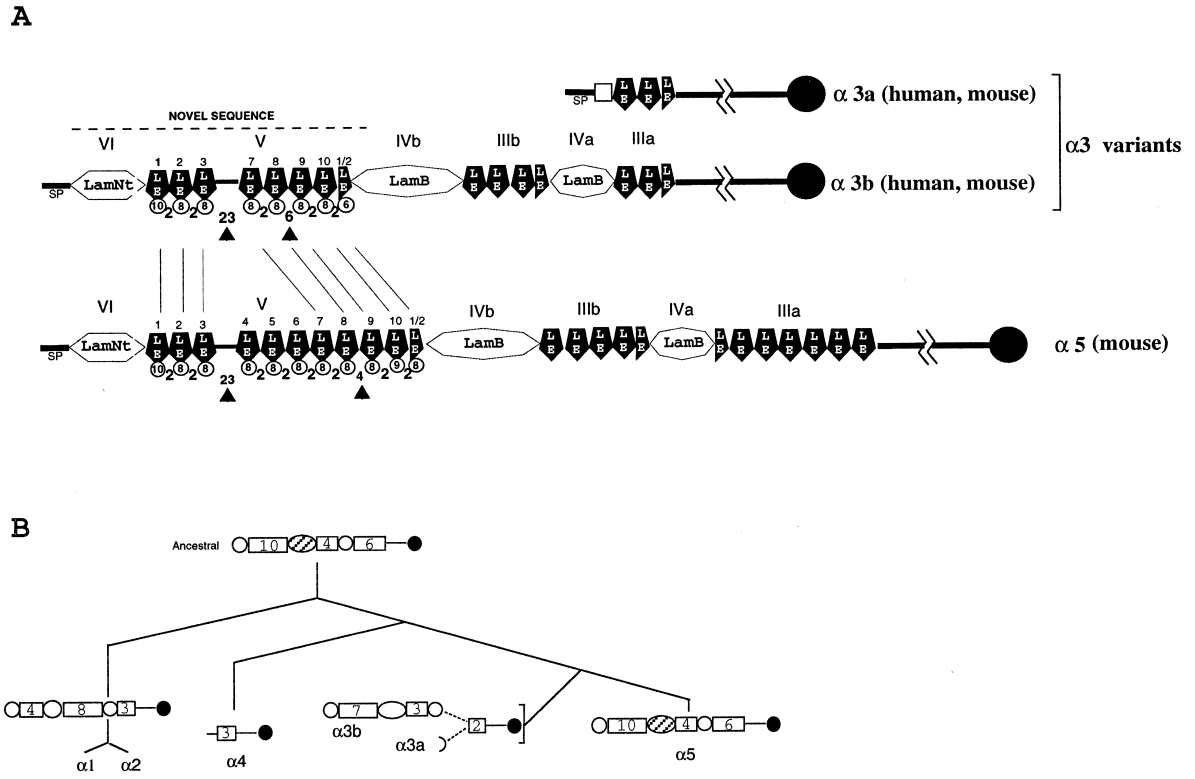

Fig. 2. A: Schematic domain structures of the human α3a and α3b chain variants in relation to the α5 laminin chain. The modules constituting the different domains are designated according to Bork [42]. Plain numbers refer to individual EGF-like modules, whereas encircled numbers correspond to the number of putative cysteines in each module. The number of spacing residues between the EGF modules are in bold. Arrowheads indicate the characteristic longer spacings unique to the α3b and α5 chains. B: Predicted evolutionary scheme modified from Miner et al. [25]. The numbers of intact EGF-like modules of each chain are indicated.

clone 9 was highly homologous to that of the genuine murine α5 chain and likely corresponded to the human α5 chain, the sequence of clone 10 (780 bp) differed slightly from that of clone 9 and was related to but clearly distinct from those of the other known laminin chains. A second overlapping amplification product (clone 11) of 369 bp and extending toward the 3′-end was cloned from the same source using primer 1 (see Fig. 1B) derived from the sequence of clone 10 and one degenerate primer (*deg-3*). Therefore, we hypothesized that these amplification products could represent either part of a new α chain or the presumptive NH_2-terminal region of a predicted variant full-sized α3 form [37]. To test this second hypothesis 10 μg of total RNA of the human fibrosarcoma cell line HS-913T was reversed transcribed using exanucleotides and AMV RT (Promega) and PCR was performed with primer 2 in clone 11 and a primer in the published sequence of the human α3b (nucleotides 301–320 as in GenBank accession No. L34156) using a long extension TAQ polymerase (Boehringer Mannheim). A predominant band of about 3200 bp was reamplified with a nested primer and confirmed its identity. Overlapping digested fragments were then cloned in the pGEM-7zf(-) vector and sequenced. At the 3′-end the novel sequence overlapped by 320 bp the previously published α3b sequence, confirming the gene product identity. With the RACE technique we could further extend the sequencing by about 200 bp towards the 5′-end of the α3b chain transcript. Northern blot analysis of total RNA purified from the 293 cell line and hybridized with a probe specific for the α3b form identified one single band corresponding to a transcript of about 11 kb (Fig. 1A). The novel cDNA (Fig. 1B) spans 4458 bp and corresponds to an open reading frame of 1486 amino acids. The first amino acid of the deduced sequence is an alanine indicating that the fragment does not embody the complete 5′-end of the coding sequence but it extends further upstream compared to murine α3B variant published while this work was in progress [38]. Repeated efforts to utilize the RACE method for identifying these terminal residues have thus far been unsuccessful.

3.2. *Domain structure of the NH_2-terminal region of α3b chain variant and comparison with other laminin α chains*

The laminin α3b chain variant appears to belong to the group of α chains recently defined as 'full-sized' α chains and including α1, α2, α5 and αD. At the amino acid level the highest homology is to the α5 chain, followed by αD, α1 and α2. First, a comparison with domain V of the α5 chain (Fig. 2A) indicates that, in α3b, the EGF-like modules 4, 5, and 6 are skipped and that EGF-like module 10 lacks the unpaired cysteine present in the corresponding module of the α5 chain. To rule out the existence of a transcript with a longer form of the V domain that could suggest alternative splicing of EGF-like modules 4, 5, and 6, a RT-PCR analysis using primers upstream and downstream the three skipped EGF-like modules was performed and in all the α3b-containing tissues and cell lines only a single band of the size corresponding to the α3b transcript originally isolated was amplified (data not shown). Second, another significant similarity between α3b and α5 chains is that the characteristic 2 amino

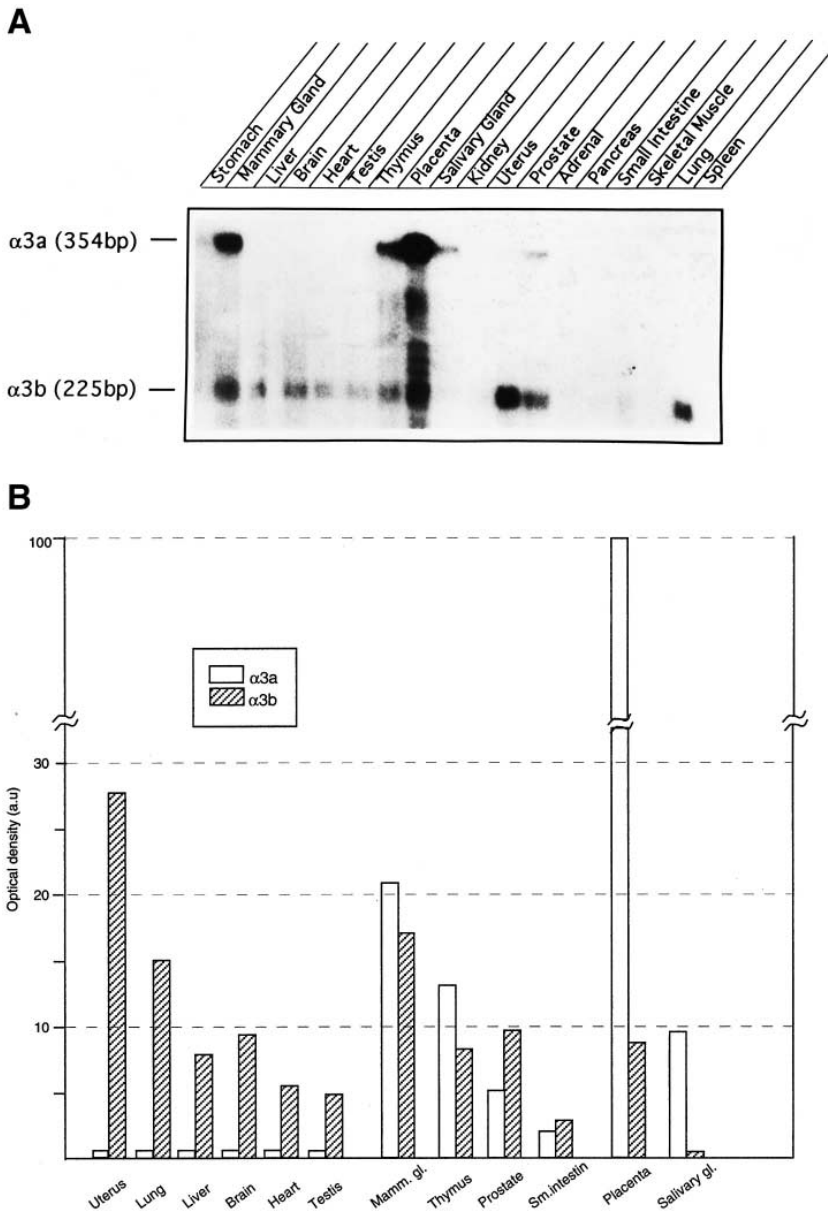

Fig. 3. A: Expression levels of α3a and α3b mRNAs as determined by RNase protection assay. Each lane contains the common probe plus 10 μg of total RNA from the different adult tissues indicated. The products were run on 6% (w/v) sequencing gels and exposed at room temperature. B: Densitometric analysis of autoradiographs as illustrated in A. The optical density scale is in arbitrary units (a.u.) considering the α3a levels in placenta as the maximal optical density (100 a.u.).

acid residues long interspacing of the EGF-like modules in laminins is interrupted in both α3b and α5 chains between EGF-3 and EGF-4 by 23 residues and between EGF-8 and EGF-9 by 6 residues in α3b and by 4 residues in α5. Third, a computer-aided analysis using the FASTA program refined manually in which the common regions of the α3, α4, and α5 chains were compared, indicated that also in the COOH-terminal region the α3 chain was slightly more closely related to the α5 than to the α1-α4 chains. Finally, based on the above analysis, the evolutionary scheme proposed by Miner et al. [25] may be modified as in Fig. 2B in which we propose that the truncated α4 gene evolved separately from a common full-sized progenitor, which later duplicated originating α5 and α3. This evolutionary scheme is a likely possibility in view of the fact that repeated efforts to isolate a α4 full-sized form have failed so far (Iivanainen, A., personal communication).

3.3. Is there an intermediate form between the truncated α3A and the full-sized α3B?

In a recent work, Galliano et al. [34] reported the primary structure of a mouse α3B laminin chain lacking domains VI and V. Repeated unsuccessful attempts were made by us to isolate the corresponding variant from human tissues and cell lines. To determine whether the apparent discrepancy could be due to interspecies differences, the sequence around the predicted initiator ATG of the published mouse α3B chain was amplified from total RNA of adult mouse lung by RT-PCR and a fragment of the expected length (360 bp) was obtained and found to differ by one guanine, just after the

beginning of the coding sequence (Fig. 1C). We next sought to investigate whether any α3 chain of intermediate size exists in the mouse and RNase protection experiments yielded a single protected band of a size corresponding to the long α3B form (data not shown). In support of our data the mouse sequence of Galliano et al. [34] contained an unusual signal peptide, as the calculated score with the von Heijne algorithm [39] was much lower (−4.3) than the averaged score (about 10) for the known signal peptide sequences. This latter finding is consistent with a recent study of Miner et al. (1997) reporting the cloning of a full-sized murine α3B variant with a sequence identical to that determined by us in the region of discrepancy.

3.4. *Differential expression of α3a and α3b chain RNAs in adult human tissues*

The extensive study of the expression of laminin α chains expression during mouse embryogenesis did not investigate the α3a and α3b variants separately [38]. Moreover, while a comparative tissue expression of the two forms by in situ hybridization was reported in selected murine tissues [34], no information is available about the relative ratios of the two α3 chain variants. We have used a riboprobe capable at discriminating between the α3a and α3b transcripts (344 bp and 225 bp for α3a and α3b, respectively) in RNase protection assays on total RNA extracted from a broad panel of adult human tissues (Fig. 3A). This approach allowed for a precise and sensitive quantification of the ratios between the two transcripts in each tissue as well as for a semi-quantitative comparison of the expression levels of each of the RNAs in different tissues. The analysis of the relative amounts (Fig. 3B) of the two variants indicated that there are several patterns of expression: several tissues expressed neither α3a nor α3b; others expressed only the α3b form; other tissues (spleen, stomach, kidney, skeletal muscle, pancreas, and adrenal gland) expressed similar levels of both forms; salivary gland had only α3a. The amounts of α3a and α3b also vary considerably among the positive tissues: placenta was by far the tissue with the highest levels of α3a and the uterus had the highest values of α3b. From this analysis it could be concluded that while the α3a variant is more represented in some epithelial tissues, the α3b variant has a broader tissue expression pattern.

4. Conclusions

The α3a chain variant has been reported to exhibit unique biosynthetic characteristics, as the 200 kDa precursor polypeptide is processed extracellularly at both the NH_2- and COOH-terminal ends, resulting in a mature form of 145 kDa [27]. The exclusive presence of α3b transcripts in some tissues makes it unlikely that this chain is processed in the same way as the α3a. While the reciprocal function of these two α3 chain variants remains to be elucidated, if the α3b chain variant corresponds to an unprocessed full-sized α chain retaining its N-terminal domains VI–IV intact, it may exhibit functional traits analogous to other 'full-sized' α chains such as self-assembly and α1β1/α2β1 integrin recognition. Finally, by sequence comparison between common regions and by domain structural features it appears that the α3 cDNA is more closely related to α5 than to α4.

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