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The human integrin β4B and β4C variants are not expressed in a tissue-specific manner

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Integrins are a large family of heterodimeric cell surface receptors that mediate cell–matrix interactions in important biological processes, such as embryonic development, wound healing, tissue differentiation, and tumor progression [1]. It is common knowledge that integrin α6β4 is unique among the known integrin molecules. It concentrates in hemidesmosomes, the specific cell surface attachment sites for the keratin filament network in stratified and complex epithelia. Moreover, in contrast to all the other α and β subunit cytoplasmic domains, the intracellular portion of the β4 subunit is unusually long. It measures over 1000 amino acids in length and contains in its C-terminal half two pairs of type III fibronectin-like repeats separated by a 142 amino acid connecting segment [2].

In the last decade, a number of studies have reported that the cytoplasmic domain of the integrin β4 subunit mediates both the association with the hemidesmosomal cytoskeleton and the recruitment of signaling adapter proteins participating in signal transduction processes essential for proper cell cycle control. Furthermore, it has been demonstrated that the β4 integrin subunit is highly expressed in some tumor cells, and that this increased expression correlates with metastatic potential of tumor cells [3]. Since the first publication of a partial amino acid sequence of the integrin β4 subunit, a number of different variants have been described [1]. Some of them are known to be generated by proteolytic processing of the mature form of the β4 polypeptide, while others have been ascribed to tissue-specific alternative splicing processes of a pre-mRNA generated by a single copy gene [1]. As to the β4 chain cytoplasmic domain, in addition to the primary most abundant form β4A, a number of variants have been described in humans. Two of them, β4B and β4C, contain in the connecting segment inserts of 53 and 70 amino acids, respectively, which are encoded by distinct exons. Moreover, a β4D variant with a 21 bp deletion, generated by the use of a cryptic splice site within intron 38, and a β4E form with a cytoplasmic domain of only 232 amino acids, generated by retention of intronic sequences that results in a frameshift and introduction of a premature termination codon, have been reported [1]. Like most integrin subunits, the β4 splice variants are always expressed together with the original β4A form. The β4B splice variant, at first isolated from a human keratinocyte cDNA library [2], has also been found by RT-PCR in human placenta and peripheral nerves, as well as in mouse spleen, while the β4C form has been detected exclusively in colon and pancreatic carcinoma cell lines [1]. These findings suggested that the preferential expression of the cytoplasmic variants of the β4 chain could be ascribed to different β4 integrin roles in various physiological and pathological conditions. However, in vitro transfection experiments demonstrated that the β4A and the β4B variants are similar in their ability to associate with other hemidesmosomal components, to co-localize with them in hemidesmosome-like structures and to initiate the formation of hemidesmosomes in β4-deficient keratinocytes [4]. To address still unresolved questions about the biological significance of the β4B and β4C splice variants of integrin β4, we evaluated their quantities in various tissue samples. In a preliminary experiment, the transcription level of the β4 integrin gene in different human normal tissues, cultured primary cell types, and carcinoma cell lines was estimated by dot blot analysis. Using a cDNA probe that recognizes all cytoplasmic splice variants, in normal human keratinocytes and human colon carcinoma cells fairly similar amounts of β4 integrin mRNA were evidenced. By contrast, kidney, placenta, non-small cell lung carcinoma and human umbilical vein endothelial cells exhibited a significantly lower expression level of β4 mRNA (Fig. 1a). Subsequently, using the most sensitive RNase protection assay analysis, we assessed the relative amount of the β4A, β4B and β4C splice variants in the same tissue samples. As shown in Fig. 1b,c, the use of specific riboprobes revealed the presence of the β4A, and to a much lesser extent, of the β4B and β4C splice variants in HUVEC (human umbilical vein endothelial cells), H1264 (non-small cell lung carcinoma cells), DLD1 (human colon carcinoma cells), NHK (normal human keratinocytes), human kidney and placenta. Quantitative densitometry of the hybridization signals confirmed that most of the β4 integrin mRNA encodes the β4A variant and that the β4B and β4C forms represent a minimal amount (less than 2%) in all tissues/cell types analyzed. Similar results were also obtained with colon carcinoma CBS, GEO, and MIP, mammary carcinoma BT474, squamous carcinoma A431, and lung carcinoma CALU1 cell lines (not shown).

To our knowledge, these results indicate for the first time the presence of both β4B and β4C integrin variants at constant ratio in all human tissues analyzed, aside from the transcription level of the β4 integrin mRNA. These data argue against the hypothesized involvement of the cytoplasmic variants of the β4 chain in conferring different functional roles to the α6β4 heterodimer in distinct cell types. The very low tran-

Table 1
Analysis of the RNA splice junction sequence surrounding the exons encoding the β4B and β4C splice variant insertions and evaluation of the matching score to the consensus

	5′ sequence	Score (%)	3′ sequence	Score (%)
Human exon 33	AGgtgaca	73.82	cgttgttcccaagG	80.09
Human exon 35	AGgtagta	69.16	tgtcctgccctagG	81.63
Mean of the *ITGB4*gene splice junctions		83.11 ± 5.1		86.47 ± 3.52

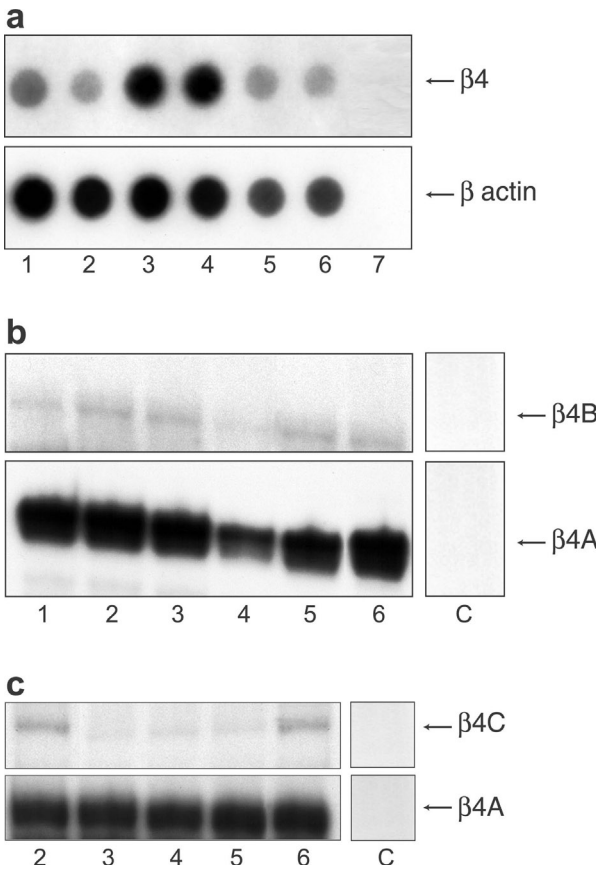

Fig. 1. Evaluation of the human integrin β4 transcription level and quantification of the β4A, β4B and β4C splice variants in different human normal tissues, cultured primary cell types, and carcinoma cell lines. a: Total RNA (1 μg) from different tissues and cultured cells was transferred onto Hybond N nylon membranes (Amersham International, Little Chalfont, UK) using a dot blot apparatus. The membranes were hybridized in QuikHyb hybridization solution (Stratagene, Cedar Creek, TX, USA) with a ^{32}P-labeled β4 integrin cDNA probe encompassing nucleotides 34–665, which recognizes all splice variants. Filters were washed following the manufacturer's instructions and exposed for autoradiography (top). For loading control, hybridization with a ^{32}P-labeled β-actin cDNA probe was carried out (bottom). Quantification of the hybridization signals was performed by densitometric scanning using a GS-750 densitometer (Bio-Rad, Hercules, CA, USA). b,c: Total RNAs (10 μg) from different sources were tested by RNase protection assays. To generate the β4B-specific riboprobe, the following oligonucleotide primers, forward 5′-CGAGACTCTATAATCCTGGC-3′ and reverse 5′-GGTGCACCTGGGATTCAA-3′, were used to amplify from normal human keratinocyte cDNA a 363 bp fragment encompassing the last 52 bp of exon 35, exon 36 and 162 bp of exon 37 of the β4 integrin subunit coding sequence (GenBank Y11107). The PCR fragment, inserted in the dual promoter pCR II Topo plasmid vector (Invitrogen, San Diego, CA, USA), was subjected to retro-transcription using the Sp6 and T7 RNA polymerase (Promega, Madison, WI, USA). The antisense product obtained makes it possible to discriminate between the β4A and the β4B splice variants (311 bp and 363 bp, respectively). The β4C mRNA was evidenced by using the following oligonucleotide primers, forward 5′-GACCTATTGGGCCCATGAAG-3′ and reverse 5′-AGGTCCAGCTCCTCCCCCAG-3′, which amplify from normal human keratinocyte cDNA a specific 361 bp fragment encompassing exons 31, 32 and the first 46 bp of exon 33 of the β4 coding sequence. The RNA probe, generated by transcription of the PCR-cloned fragment, allows discrimination between the β4A and the β4C splice variants (315 bp and 361 bp, respectively). The length of the protected RNA bands was estimated using radiolabeled RNA molecular weight markers (Roche Diagnostic, Monza, Italy). In order to obtain a detectable amount of the minor splice variants in all cell lines/tissues analyzed, loading of the RNase protection assays products was performed utilizing an equivalent amount of radioactivity incorporated. b: Top panel, hybridization signals of β4B variant; bottom panel, hybridization signals of the β4A form. c: Top panel, hybridization signals of β4C variant; bottom panel, hybridization signals of the β4A form. Evaluation of the relative amount of the β4 mRNA isoforms was performed by densitometric analysis. The RNAs utilized were from: HUVEC (lane 1), H1264 (lane 2), DLD1 (lane 3) (kindly provided by Fiorella Guadagni and Carol J. Thiele, respectively), NHK (lane 4), human kidney and placenta (lanes 5, 6) (Clontech Laboratories, Palo Alto, CA, USA). As negative control, human fibroblast total RNA (lane 7) and yeast tRNA (lane C) were used. HUVEC were isolated according to standard protocols, and cultured on fibronectin (Sigma-Aldrich, St. Louis, MO, USA)-coated plates in RPMI 1640 medium supplemented with 10% fetal bovine serum, 50 μg/ml ECGS, 100 μg/ml heparin and 2 mM L-glutamine. H1264 and DLD1 cell lines were cultured in RPMI 1640, supplemented with 10% fetal calf serum. Primary cultures of normal human keratinocytes were established using standard procedures. Total RNA was extracted by lysis of the cell cultures in the presence of guanidine isothiocyanate using standard protocols.

scription level of the β4B and β4C variants suggests that these variants might result from cellular missplicing events, rather than from alternative splicing processes engendering to functional isoforms. Since during pre-mRNA splicing processes exon recognition is strictly dependent on sequence elements residing in the immediate vicinity of the exon–intron borders, analysis of the intronic sequences surrounding the two exons encoding the 53 and the 70 amino acids insertions of the β4B and β4C variants in human β4 integrin gene (GenBank Y11107) was carried out. As summarized in Table 1, the matching score of the splicing sequences to the consensus, calculated according to the formula proposed by Shapiro and Senapathy [5], is considerably lower for both exons compared to the mean value of the entire *ITGB4* gene.

In conclusion, taking into account the almost undetectable level of both β4B and β4C splice variants in all tissues analyzed and the consensus sequence analysis data, we speculate that the β4B and β4C splice variants result from aberrant splicing events occurring at constant ratios. To this end it is worth mentioning that, despite the relatively high number of genetic mutations characterized in the human integrin β4 subunit gene [6], no mutations have so far been identified in the exonic sequences encoding the 53 and 70 amino acid insertions.

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