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An alternative cytoplasmic domain of the integrin β_3 subunit

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ABSTRACT **A cDNA encoding a new form of the shared β subunit (β_3) of the platelet integrin gpIIb/IIIa and the vitronectin receptor was isolated from a placental cDNA library by screening with a β_3 (gpIIIa) DNA probe. This β_3 variant differs from the previously reported β_3 in that the cytoplasmic domain is 8 amino acids shorter and has an alternative, 13-amino acid COOH-terminal peptide. The 3′ untranslated region of the cDNA also differs from the previously reported sequence, while the region coding for the transmembrane domain and extracellular domain is identical to it. Reverse transcription combined with polymerase chain reaction was used to show that human placental tissue and two human cell lines contain the variant mRNA. The sequences of the cDNAs for the previously known β_3 and the variant β_3 described here suggest that the difference between the cytoplasmic domains of these subunits arises as a result of an alternative mRNA splicing. These cytoplasmic domains may provide alternative means for the β_3 integrins to interact with cytoskeletal components.**

Integrins are a group of cell surface, heterodimeric proteins that mediate cell adhesion (1–3). The general structure of integrins is composed of an α and a β subunit, each consisting of a large extracellular domain, a transmembrane domain, and a relatively short cytoplasmic domain (4–7). The integrins form a protein family that can be subdivided into three major groups based on a shared β subunit (1, 2): the β_1 group includes the fibronectin receptor and the very late activation antigens (8); the β_2 group contains the leukocyte receptors LFA-1, Mac-1, and gp150/95 (9), whereas the vitronectin receptor and the gpIIb/IIIa complex make up the β_3 group (7, 10).

The extracellular domain of integrins binds to adhesive proteins such as fibronectin, recognizing in many cases the tripeptide Arg-Gly-Asp as the key structure in the ligand (11). Immunofluorescence studies indicate that integrins mediate an interaction between the extracellular ligands and cytoskeletal elements (12–19). Moreover, the cytoplasmic portion of the avian integrin complex has been reported to interact with talin in direct binding assays (20). These results suggest that integrins connect the extracellular matrix to the cytoskeleton and that the cytoplasmic domain mediates the cytoskeletal interaction. An indication of the importance of the cytoplasmic domain functions is the complete conservation of the β_1 subunits of human and chicken integrins (6, 21).

In this report we describe a cDNA clone that encodes a β_3 variant with a new cytoplasmic domain sequence[§] and provide evidence suggesting that this variant arises through alternative mRNA splicing.

MATERIALS AND METHODS

Isolation of cDNA Clones and DNA Sequencing. cDNA clones were isolated from λgt11 cDNA libraries made from myeloma cell RNA by use of a cDNA cloning kit (Amersham) and from placental RNA (22). A 21-mer oligonucleotide, 5′ CACTGAGAGCAGGACCACCAG 3′, from the sequence of β_3 (23) or inserts from cDNA clones were used for the screening. cDNA inserts were subcloned into the phage vector M13mp19 and sequenced by the dideoxy chain-termination method (24) either manually with deoxyadenosine 5′-[α-[^{35}S]thio]triphosphate as the label or by using an automated DNA sequencer and fluorescent primers (Applied Biosystems model 370A) according to the manufacturer's instructions.

Cell Culture and RNA Isolation. MG63 osteogenic sarcoma cells, originally obtained from the American Type Culture Collection, were grown to near confluency in Dulbecco's medium supplemented with 10% (vol/vol) fetal calf serum. RNA from these cells and from human placental tissue was isolated by using the guanidine isothiocyanate method (25). The HEL erythroleukemia cell RNA was a gift of C. van Beveren (La Jolla Cancer Research Foundation).

Reverse Transcription (RT)/Polymerase Chain Reaction (PCR). RT/PCR was essentially done as described (26). Total RNA (0.4 μg) was reverse transcribed by using 200 units of Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories), 0.4 μg of p(dT)$_{12-18}$, and 2 μg of nuclease-free bovine serum albumin. The total volume was 20 μl. One-tenth of the resulting cDNA was amplified by using the DNA amplification reagent kit and a DNA thermal cycler (Perkin–Elmer/Cetus). One unit of *Thermus aquaticus* (*Taq*) polymerase and each primer at 1 μM were used; the final volume was 50 μl. The following primers were used (the numbering of nucleotides is from ref. 23): 1, extracellular domain 1851–1875; 2, extracellular domain 1879–1903; 3, extracellular domain 2064–2088; 4, cytoplasmic domain β_3 2273–2297; 5, 3′ untranslated region β_3 2559–2583; 6, 3′ untranslated region β_3 3104–3128; 7, 3′ untranslated region β_3 3473–3497; 8, cytoplasmic domain plus 3′ untranslated region alternative sequence 2301′–2331′ (the ′ symbol refers to the variant β sequence); 9, 3′ untranslated region alternative sequence 2408′–2432′. Of the PCR mixture, 15 μl were electrophoretically separated in 2% agarose gels or 3% NuSieve GTG/1% SeaKem GTG agarose gels (FMC), and DNA was visualized with ethidium bromide. *Hae* III fragments of φX174 replicative form DNA (500 ng) were used as molecular size markers (Bethesda Research Laboratories). RNA digestion was performed with 50 μg of ribonuclease A (Sigma) and 14 μg of total RNA in a total volume of 30 μl. Digestion was for 20 hr at 37°C.

RESULTS

β_3 Subunit cDNA Clones. Screening of 3 × 10^5 plaques from an M 21 myeloma cDNA library with a 21-mer oligonucleo-

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Abbreviations: RT, reverse transcription; PCR, polymerase chain reaction.

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§The sequence reported in this paper has been deposited in the GenBank data base (accession no. M25108).

tide probe from the published β_3 cDNA sequence revealed one positive clone. The 1.3-kilobase (kb) cDNA insert from this clone was used to screen 7×10^5 plaques from a placental λgt11 cDNA library, which resulted in the isolation of three positive clones. Partial sequences of two of the clones revealed the same sequence as in the published β_3 sequence (23, 27). The third clone (no. 10) was different. This 1.8-kb clone consisted of 1.0- and 0.8-kb *Eco*RI fragments, and its 5′ end is in the extracellular domain (base number 1254, see ref. 23 for the numbering). The published β_3 sequence and that of clone 10 were found to be identical through the 5′ fragment and part of the 3′ fragment but diverged within the 3′ fragment in the region that encodes the cytoplasmic domain of the β_3 polypeptide. The DNA sequence of the 3′ fragment and the amino acid sequence derived from it are shown in Fig. 1. The variant sequence encodes a cytoplasmic domain in which the COOH-terminal 21 amino acids of the previously known β_3 sequence have been replaced with an alternative 13-amino acid sequence. The 3′ untranslated region, containing polyadenylylation signals and a poly(A) tail, is also different from the part of the β_3 3′ untranslated sequence that has been determined.

Expression of the Variant β_3 mRNA. Our initial attempts to use RNA blotting to study the expression of the mRNA for the variant β_3 were unsuccessful, perhaps because of the low abundance of the mRNA. We therefore turned to the RT/PCR method, which is extremely sensitive and allows as few as 10 molecules of mRNA to be detected (28). Three different oligonucleotide primers derived from the published β_3 sequence were selected as primers at the 5′ end, and two oligonucleotides derived from each of the published and variant β_3 sequences served as 3′ end primers (Fig. 2). Amplified fragments of the sizes predicted on the basis of the primary sequence were obtained for both forms of β_3 with each primer combination tested. Placental RNA, as well as RNA from HEL erythroleukemia cells and MG63 osteogenic sarcoma cells, each gave such fragments (Fig. 3).

Since the amplification method is so sensitive, it was necessary to exclude the possibility that contaminating genomic DNA was the source of the fragments obtained. RNase

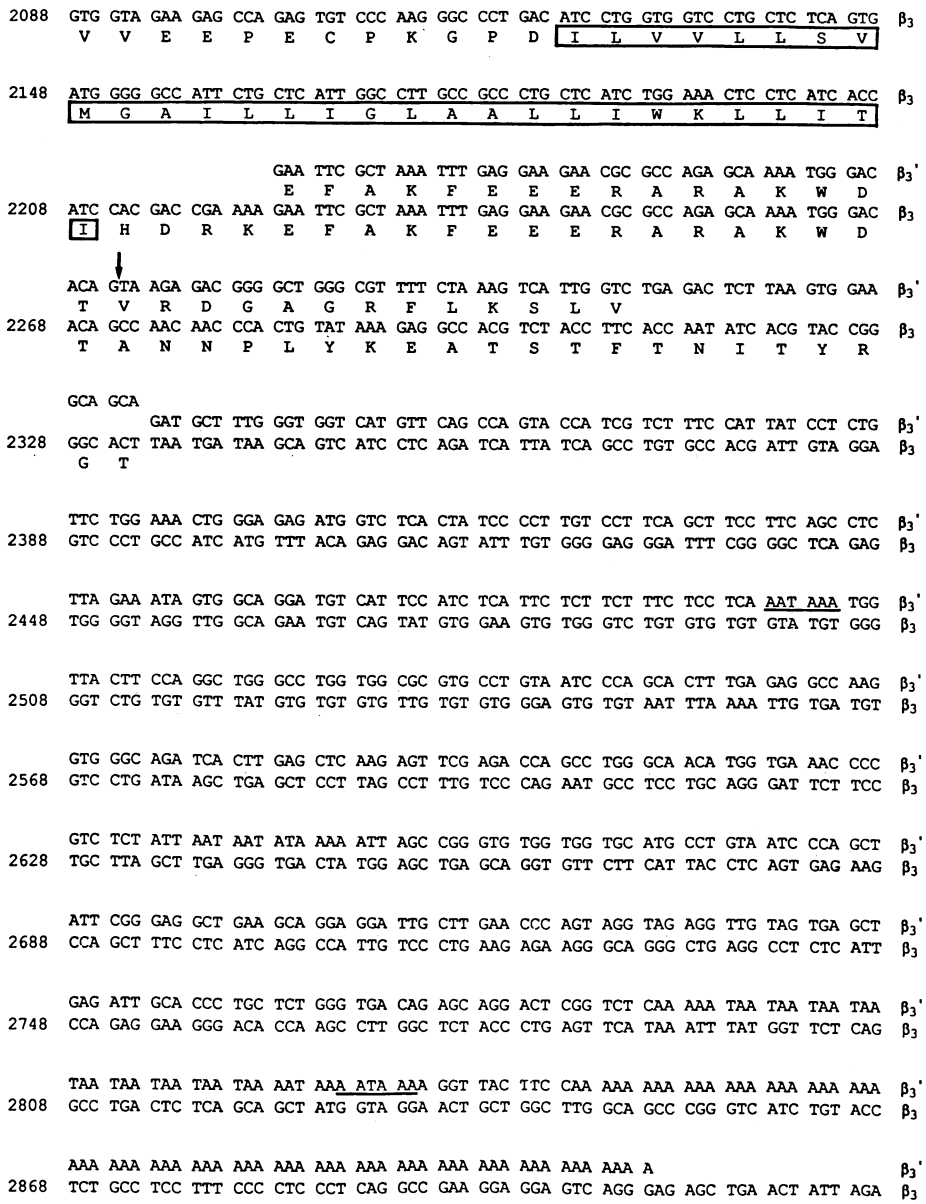

FIG. 1. The cDNA sequence and deduced amino acid sequence of the cytoplasmic domain of variant β_3 subunit. The sequence of the *Eco*RI fragment containing the alternative cytoplasmic domain is shown and referred to as β_3'. Part of the published β_3 sequence is shown for comparison (7, 23). Amino acids are indicated in the single-letter code. The putative transmembrane domain in the β_3 sequence is boxed. The site where the two sequences become different is indicated by an arrow. Polyadenylylation signals are underlined.

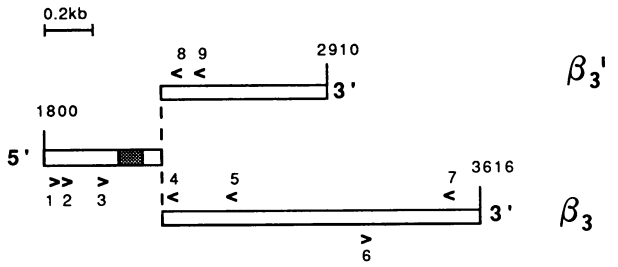

FIG. 2. Oligonucleotide primers used for RT/PCR analysis. The location of each primer used for RT/PCR and the direction of priming are indicated by the symbols > and <. The number of β_3 is from ref. 23 and the numbering of β_3' is from Fig. 1. Primers 1, 2, and 3 are located in the extracellular domain shared by β_3' and β_3; primers 4, 5, 6, and 7 are specific for β_3; and primers 8 and 9 are specific for β_3'. The stippled box indicates the putative transmembrane domain of β_3' and β_3.

treatment, performed prior to the RT/PCR, or omission of the RT reaction were used to distinguish RNA-derived and DNA-derived PCR products. Each of the primer combinations from the β_3 and β_3' sequences gave a fragment that was RNA-derived by these criteria. The results for one primer combination are shown in Fig. 4 (lanes e–h, k, and l).

Further PCR controls showed that, although there was enough DNA present in the RNA preparations to give amplified fragments, no DNA-derived fragments could be obtained with the primers used to detect the β_3' RNA. A fragment, the appearance of which was partially resistant to both RNase treatment of the RNA and omission of the reverse transcription step, was obtained from MG-63 cells and placental RNA when adjacent primers from the 3′ untranslated β_3 region were used (Fig. 4, compare lane a with lanes b and i and compare lane c with lanes d and j). This fragment was also obtained with isolated genomic DNA (data not shown). In contrast, when the 3′ primer came from the β_3' cytoplasmic domain sequence and the 5′ primer came from the shared extracellular domain sequence, no DNA-derived fragments were obtained from RNA (Fig. 4, lanes e–h, k, and l) or genomic DNA (data not shown).

DISCUSSION

We report here evidence for the presence of an alternative cytoplasmic domain in the β_3 integrin subunit. Several features in the structure of the cDNA encoding the newly identified sequence suggest that it corresponds to an alternatively spliced β_3 mRNA. The cDNA covers about one-half of the β_3 coding sequence and, with the exception of the portion coding for the 13 COOH-terminal amino acids, the sequence is identical to the published β_3 sequence (7, 23, 27). Moreover, no DNA-derived fragments were obtained in the PCR when the primers flanked the presumed alternative splice site, suggesting that the β_3 gene contains an intron or introns between the cytoplasmic and extracellular domains. Taken together these results establish alternative mRNA splicing as the likely basis for the existence of the two β_3 cDNA variants.

Our results show that the alternatively spliced β_3 sequence is expressed at the mRNA level. RT/PCR analysis using primers chosen so that one was specific for the shared extracellular domain sequence and the other was specific for the unique cytoplasmic domain or 3′ untranslated sequences of the two β_3 variants yielded the expected fragments when mRNA from human placenta or cultured cells was used as the template. Moreover, controls excluded the possibility that the genomic DNA, rather than RNA, served as the template for the fragments detected. These results, therefore, establish expression of the newly described β_3 sequence at the mRNA

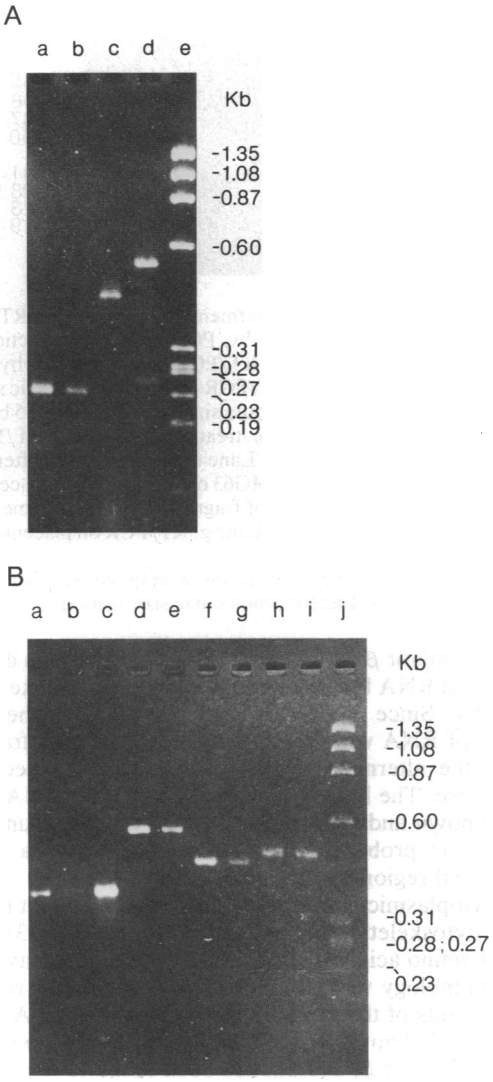

FIG. 3. Detection of mRNA transcripts coding for β_3 subunits by the RT/PCR method. RNA from human tissue and cells was used as a template for RT, and the resulting cDNA was amplified by PCR. The pairs of primers (see Fig. 2) were chosen so that one primer corresponded to a sequence in the extracellular domain, while the other came from the cytoplasmic domain or the 3′ untranslated region of either the known β_3 (*A*) or the variant β_3 (*B*) cDNA sequence. (*A*) Fragments obtained with β_3 primers. Lane a, MG63 osteogenic sarcoma cell RNA; primers 3 and 4; predicted size of fragment = 234 base pairs (bp). Lane b, placental RNA; primers 3 and 4. Lane c, MG63 cell RNA; primers 2 and 4; predicted size of fragment = 419 bp. Lane d, HEL erythroleukemia cell RNA; primers 3 and 5; predicted size of fragment = 520 bp. Lane e, molecular size markers. (*B*) Fragments obtained with primers from the variant β_3 sequence. Lane a, MG63 cell RNA; primers 3 and 9; predicted size of fragment = 369 bp. Lane b, placental RNA; primers 3 and 9. Lane c, HEL cell RNA; primers 3 and 9. Lane d, MG63 cell RNA; primers 2 and 9; predicted size of fragment = 554 bp. Lane e, placental RNA; primers 2 and 9. Lane f, MG63 cell RNA; primers 2 and 8; predicted size of fragment = 452 bp. Lane g, placental RNA; primers 2 and 8. Lane h, MG63 cell RNA; primers 1 and 8; predicted size of fragment = 480 bp. Lane i, placental RNA; primers 1 and 8. Lane j, molecular size markers.

level. We have not yet been able to study its expression at the protein level because our attempts to generate antibodies specific for the cytoplasmic tail of the variant β_3 have not been successful.

The alternatively spliced β_3 appears to be a minor form of β_3. The size of the mRNA coding the alternative β_3 subunit should be about 3.0 kb, as deduced from the primary se-

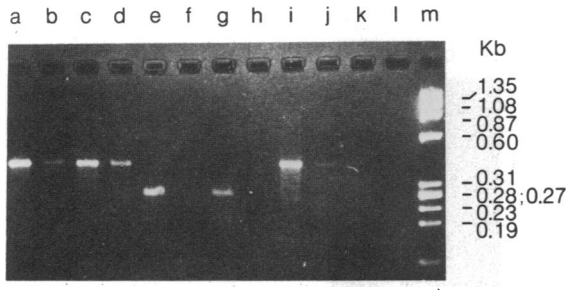

FIG. 4. Effect of RNase treatment or omission of RT on the production of DNA fragments by PCR. RT/PCR reactions and control reactions in which the RT/PCR was preceded by RNase treatment are shown. Lane a, RT/PCR on MG63 osteogenic sarcoma cell RNA; primers 6 and 7; predicted size of fragment = 395 bp. Lane b, same as lane a after RNase treatment. Lane c, RT/PCR on placental RNA; primers 6 and 7. Lane d, same as lane c after RNase treatment. Lane e, RT/PCR on MG63 osteogenic sarcoma cell RNA; primers 3 and 8; predicted size of fragment = 268 bp. Lane f, same as lane e after RNase treatment. Lane g, RT/PCR on placental RNA; primers 3 and 8. Lane h, same as lane g after RNase treatment. Lanes i, j, k, and l, same as lanes a, c, e, and g, respectively, but with the RT reaction omitted. Lane m, molecular size markers.

quence. A minor β_3 transcript of this size has been detected by others in RNA blot analysis of HEL erythroleukemia cell RNA (29). Since the signal was weak even when large amounts of RNA were used for the analysis, this transcript may be the alternatively spliced minor RNA species described here. The large size difference of the mRNA coding for the known and alternative forms of the β_3 subunit (6 kb vs. 3 kb) is probably due to the presence of a long 3′ untranslated region in the 6-kb mRNA (23).

The cytoplasmic domains of integrins are thought to interact with cytoskeletal components (19, 30, 31). The 13 COOH-terminal amino acids of the alternative sequence have virtually no homology with those in the corresponding region of the β subunits of the fibronectin receptor (β_1), LFA (β_2), or the previously known β_3, all of which are homologous to one another (32). The β_1 subunit has been found to be phosphorylated at a tyrosine residue (33) that is surrounded by a sequence resembling tyrosine phosphorylation sites of growth factor receptors (21). Interestingly, although the previously published β_3 sequence contains the tyrosine residue, the alternative β_3 cytoplasmic domain does not. Tyrosine phosphorylation of the cytoplasmic domain has been suggested to alter the ability of a chicken integrin to bind to the extracellular matrix as well as to the cytoskeletal protein talin (3, 31). Moreover, truncation of the cytoplasmic domain of a cell-to-cell adhesion molecule, E-cadherin, abolishes the ability of the cadherin to promote cell aggregation, presumably by interference with the anchorage of the cadherin to the cytoskeleton (34). Therefore, because of its short, tyrosine-deficient cytoplasmic domain, the alternatively spliced β_3 subunit may differ from the other β subunits in its interactions. That the alternative splicing is functionally important is suggested by the presence of an equivalent splicing variation in the β_1 integrin subunit (G. Tarone and F. Altruda, personal communication). These alternative forms may further add to the complexity and diversity of the already versatile integrin family.

Note Added in Proof. Genomic gpIIIa sequence shows that the β_3′ cytoplasmic domain sequence is contiguous in the genomic sequence, while the β_3 sequence is generated through the use of a splice site between nucleotides 2270 and 2271 of the cDNA sequence in Fig. 1 (F. Lanza, L. Fitzgerald, N. Kieffer, and D. Phillips, personal communication).

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