Point Mutations Impairing Cell Surface Expression of the Common β Subunit (CD18) in a Patient with Leukocyte Adhesion Molecule (Leu-CAM) Deficiency

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Abstract

The leukocyte adhesion molecules CD11a/CD18, CD11b/CD18, and CD11c/CD18 (Leu-CAM) are members of the integrin receptor family and mediate crucial adhesion-dependent functions in leukocytes. The molecular basis for their deficient cell surface expression was sought in a patient suffering from severe and recurrent bacterial infections. Previous studies revealed that impaired cell surface expression of Leu-CAM is secondary to heterogeneous structural defects in the common β subunit (CD18). Cloning and sequencing of complementary DNA encoding for CD18 in this patient revealed two mutant alleles, each representing a point mutation in the coding region of CD18 and resulting in an amino acid substitution. Each mutant allele results in impaired CD18 expression on the cell surface membrane of transfected COS M6 cells. One substitution involves an arginine residue (Arg^{593} → cysteine) that is conserved in the highly homologous fourth cysteine-rich repeats of other mammalian integrin subfamilies. The other substitution involves a lysine residue (Lys^{196} → threonine) located within another highly conserved region in integrins. These data identify crucial residues and regions necessary for normal cell surface expression of CD18 and possibly other integrin β subunits and define a molecular basis for impaired cell surface expression of CD18 in this patient. (*J. Clin. Invest.* 1990. 85:977–981.) leukocyte adhesion molecules • integrins • inflammatory response • molecular cloning • genetics

Introduction

Leukocyte adhesion molecule (Leu-CAM)[1] deficiency is a rare inherited disorder of leukocytes characterized clinically by recurrent and often fatal bacterial infections occurring in infants and children (1). Leukocytes from affected individuals have either a complete (type I) or partial (type II) deficiency in surface membrane expression of a family of three glycoprotein heterodimers that mediate leukocyte adhesion-dependent functions such as phagocytosis, chemotaxis, aggregation, adhesion to vascular endothelium, binding to iC3b-coated particles, lymphoid cell proliferation, and cytotoxicity (1–7). The three Leu-CAM, CD11a/CD18 (LFA-1), CD11b/CD18 (Mo1), and CD11c/CD18 (p150.95) each consists of a distinct α subunit of 177, 155, and 130–150 kD, respectively, noncovalently associated with an identical β subunit (CD18) of 94 kD (8, 9). Biosynthesis studies in patients with Leu-CAM deficiency revealed that the α subunits are intrinsically normal and that defective cell surface expression of Leu-CAM is secondary to heterogeneous defects in the common CD18 subunit (10–12).

Molecular cloning studies revealed that Leu-CAM are members of a large family of adhesion receptors called integrins (13). At least three subfamilies of integrins were described, each with a unique common β subunit associated with several α subunits (13–17). The β1 subfamily includes the fibronectin receptor and the very late activation antigens. The β2 integrin subfamily consists of the three Leu-CAM and the β3 subfamily includes the vitronectin receptor and platelet IIb/IIIa. The β1 and β3 integrins have wide cell distribution, recognize the tripeptide arg-gly-asp, and are involved in cell-substrate adhesion, matrix assembly, wound healing, and morphogenesis (1, 13–15). Integrins share several common features such as the heterodimeric structure, homologous α and β subunits, requirement for divalent cations for αβ-complex stabilization and function, and interaction with cytoskeleton and with several ligands, but the structure-function relationship of several conserved domains within their α and β subunits is unclear.

In this report, we identified two mutant alleles in CD18 derived from a patient with partial (type II) Leu-CAM deficiency. Each mutant allele resulted in a single amino acid substitution (mutation A, lys^{196} → thr; mutation B, arg^{593} → cys) in the extracellular region of CD18 and in impaired cell surface expression of CD18 in COS M6 cells. Mutation B occurred in a characteristic and highly conserved cysteine-rich region and mutation A occurred within a second highly conserved 247 amino-acid region, suggesting critical roles for these residues and regions in the cell surface expression of CD18 and possibly that of other β integrins.

Methods

Patient. The patient studied was the first one diagnosed with Leu-CAM deficiency (3, 18). Leukocytes from this patient synthesized normal amounts of a CD18 precursor (10). Only 10–20% of this precursor

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1. *Abbreviations used in this paper:* ABP, actin-binding protein; Leu-CAM, leukocyte adhesion molecules; PCR, polymerase chain reaction.

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underwent carbohydrate processing and cell surface expression (10). Leukocytes from this patient contained normal amounts of a normal-sized CD18 mRNA by Northern blot analysis (10).

Construction and screening of patient-derived cDNA library. Poly(A+) mRNA derived from an established patient-derived EBV-cell line (10) was used to construct a cDNA library inserted into the πH3M plasmid vector and amplified in MC1061/p3 *Escherichia coli* according to published methods (19). 800,000 bacterial colonies were plated and screened using a 2.7-kb CD18-specific cDNA probe kindly provided by Dr. Alex Law (Oxford University, UK) (16). The probe was ^{32}P-labeled using the hexanucleotide labeling method (20). Hybridization was carried out overnight at 42°C in 50% formamide, 5× SSPE (1× SSPE is 0.15 M sodium chloride, 15 mM sodium phosphate, 1 mM EDTA), 5× Denhardt's solution, and 100 μg/ml salmon sperm DNA. Filters were washed twice for 30 min in 2× SSC (1× SSC is 0.15 M sodium chloride, 15 mM sodium citrate) containing 0.1% SDS at room temperature, once in 1× SSC/0.1% SDS at 65°C and once in 0.2× SSC/0.1% SDS at 68°C. Filters were then dried and exposed to Kodak-XOR films. Plasmid DNA was prepared from positive clones and used for nucleotide sequencing. The latter was carried out using the dideoxynucleotide termination method of Sanger et al. (21) and plasmid DNA or cDNA fragments cloned in M13. Both strands of the isolated inserts were sequenced. M13-, plasmid πH3M-, and CD18 cDNA-specific primers were synthesized on an oligosynthesizer (Applied Biosystems, Inc., Foster City, CA).

Site-directed mutagenesis. A Hind III-Eco RI 1.2-kb CD18 cDNA clone (isolated from an HL60 cDNA library) that contained the signal peptide and extended to the unique internal Eco RI site in CD18 (M. A. Arnaout, unpublished observations) was ligated either to wild-type or mutant B CD18 cDNA clones extending from the internal Eco RI site to the 3′ untranslated region of the gene. The full length wild-type or mutant B clones were each subcloned in the unique Hind III-Dra I site of the πH3M expression vector. Mutant A was created using the gapped duplex method for site-directed mutagenesis (22) modified as follows: Blue Script plasmid (containing a 5′ Hind III-Eco RI fragment of wild-type CD18) was linearized with Xba1 (then treated with calf intestinal phosphatase) or digested with Hind III-Eco RI (to remove the CD18 insert). These two plasmids were mixed with an excess of the synthetic and 5′-end phosphorylated 19-mer, O-35lt (ACAAGGAGACAGAGTGCCA) which contains the desired nucleotide mutation (underlined). The mixture was denatured by boiling and renatured by step-wise cooling. The gapped heteroduplex DNA (containing single-stranded region to which the mutant 19-mer is hybridized) was filled and used to transform *E. coli* strain BMH 71-18 mut L. Plasmids containing the mutation were identified by differential hybridization with ^{32}P-labeled mutant 19-mers and the DNA used to retransform *E. coli* JM109. Several positive colonies were identified after rehybridization, and the DNA from one colony was sequenced to verify the mutation and then subcloned into πH3M containing the wild-type CD18.

Transfection studies. A full length human CD11b (Mo1α) cDNA clone was constructed from three isolated cDNA clones (23) and inserted into the πH3M vector (D. M. Fathallah, and M. A. Arnaout, unpublished observations). COS M6 monkey kidney cells (24) were grown in Iscove's modified Dulbecco's medium supplemented with 10% Nu-serum. 30–40% confluent cells in 100-mm plates (Costar Data Packaging Corp., Cambridge, MA) were washed in serum-free medium and transfected by the DEAE-dextran technique as detailed elsewhere (19). 2 μg of supercoiled CD11b cDNA were cotransfected with an equal amount of each of the three CD18 cDNA constructs. Preliminary experiments revealed that cotransfection of CD18 cDNA with CD11b cDNA resulted in formation of $\alpha\beta$ heterodimer and significantly enhanced cell surface expression of the wild-type CD18 subunit in COS cells when compared with transfection with CD18 cDNA alone (N. Dana and M. A. Arnaout, unpublished observations). Cells transfected with CD8 cDNA (kindly provided by Dr. Brian Seed, Massachusetts General Hospital) were used as controls. Transfected cells were grown for 48 h, rinsed, detached (by trypsin-EDTA treatment), and plated into 100-mm petri dishes or into six-well plates (Costar Data Packaging Corp.). After 24 h, confluent monolayers in replicate six-well plates were processed for immunofluorescence analyses, metabolic studies, or radioimmunoassays.

Quantitation of CD18 expression in transfected COS cells. Transfected COS cells in six-well microtiter plates were washed once, then incubated for 30 min at room temperature with saturating amounts of anti-CD18 mAb (in a total volume of 1 ml). The anti-CD18 mAbs TS18 and 60.3 (25, 6), which recognize different epitopes on CD18 (26), were kindly provided by Dr. Steven Burakoff (Dana-Farber Cancer Institute, Boston, MA), and Dr. John Harlan (Seattle, WA), respectively. Cells were washed twice, then incubated with saturating amounts of ^{125}I- or fluorescein-labeled antimouse Ig (30 min, room temperature). Cells were again washed and either examined using an inverted fluorescent microscope (Zeiss, Oberkochen, FRG) or solubilized with 0.1% SDS-0.5 N NaOH in water and extracts counted in a gamma counter. Specific binding (for each replicate, in counts per minute) of mAb was calculated by subtracting background binding (binding to COS cells expressing CD8) from binding observed with COS cells expressing CD18.

Biosynthesis of wild-type or mutant CD18 in transfected COS cells. Confluent monolayers (in 100-mm petri dishes) of transfected COS cells were metabolically labeled with [^{35}S]methionine (0.5 mCi/well) for 16 h, as previously described (10), washed, then solubilized with 1% NP-40 in the presence of 2 mM PMSF. After 15 min on ice, cell lysates were spun at 12,000 *g* for 10 min at 4°C and the supernatant used for immunoprecipitations. The latter were done exactly as detailed elsewhere (10).

Quantitation of CD18 on EBV cells. Cell surface expression of CD18 in EBV cells derived from the patient, his parents, and a normal individual was done exactly as described for COS cells except that the radioimmunoassay was done using 1×10^6 cells/replica in Eppendorf tubes. *P* values were calculated using the paired *t* test.

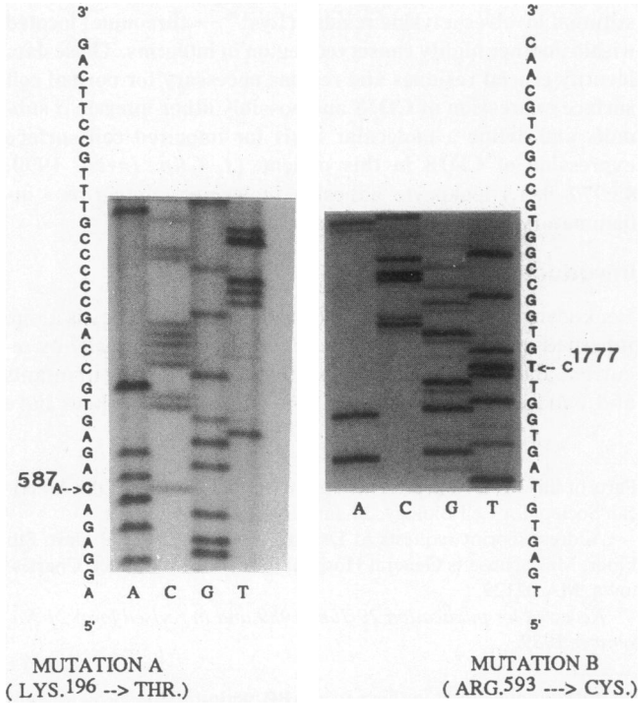

Figure 1. Nucleotide sequence of mutant CD18 cDNAs. An autoradiograph of a sequencing gel showing the two-point mutations in patient-derived CD18 cDNA. There is an A to C transversion at bp 587 (mutation A) and a C to T transition at bp 1,777 (mutation B) (bp 1 = A of the first translation initiation site) (17). Neither mutation A nor B resulted in loss or generation of a restriction enzyme site.

Synthesis and amplification of cDNA fragments spanning mutations A and B. First-strand synthesis of two separate regions of CD18 cDNA from the patient's mother, one spanning mutation A and the other spanning mutation B, was performed using, respectively, the antisense oligonucleotides, O-91 (derived from nt. 676–692) and O-134 (derived from nt. 1981–1998) of CD18 (17), total mRNA derived from a maternal EBV cell line, and the cDNA synthesis system as recommended by the manufacturer (Amersham Corp., Arlington Heights, IL). The first-strand product in each case was then used as a template to amplify two regions, one defined by oligonucleotides O-91 and O-93 (5′-GCCAGGCAGCAGCGTTC-3′, nt. 401–417) and the other defined by O-134 and O-127 (5′-CCAACCAGCGACGT-3′, nt. 1629–1643) using the polymerase chain reaction (PCR) (27). The primers were annealed at 42°C for 2 min, extended at 72°C for 3 min (with 2 U of Taq polymerase), and denatured at 94°C for 1.5 min, for 40 cycles with a final cycle of 7 min for extension in an automatic thermal cycler (Perkin-Elmer Cetus, Emeryville, CA). The PCR products were separated by electrophoresis on a 1.2% agarose gel, the amplified DNA visualized by ethidium bromide staining, electroeluted, ethanol precipitated, and reused as a template for a second round of PCR amplification. Identification of mutations A or B in the PCR products was performed by hybridization of equivalent amounts of cDNA slot blotted on nitrocellulose (Schleicher & Schuell, Inc., Keene, NH), hybridization with the respective 5′-end labeled mutant 19-mers (O-35lt for mutation A; O-138: GTGGTTGTGGCCGGTGCAA, for mutation B) under permissive temperatures followed by a 10-min wash in 6× SSC at the selective temperature (Td) for each oligonucleotide. The respective regions of CD18 cDNA (amplified by PCR using the same sets of oligonucleotides and wild-type or mutant CD18 cDNA) were included, either alone or as a 1:1 mixture, as internal controls for selective hybridizations.

Results and Discussion

Screening of 8×10^5 colonies from a patient-derived cDNA library with a ^{32}P-labeled 2.7-kb CD18 cDNA probe led to isolation of 28 reactive colonies which stratified into four groups by restriction mapping. The insert size ranged from 0.4 to 2.0 kb. Nucleotide sequencing of one or more inserts within each group led to the identification of three point mutations (Fig. 1). The first mutation (mutant A) involved an adenine to cytosine nucleotide transversion at bp 587, resulting in replacement of a lysine^{196} (codon AAA) by a threonine (codon ACA). The second mutation (mutant B) was a cytosine to thymine nucleotide transition at bp 1,777 which replaces the normal arginine at codon 593 with a cysteine. Mutant B was

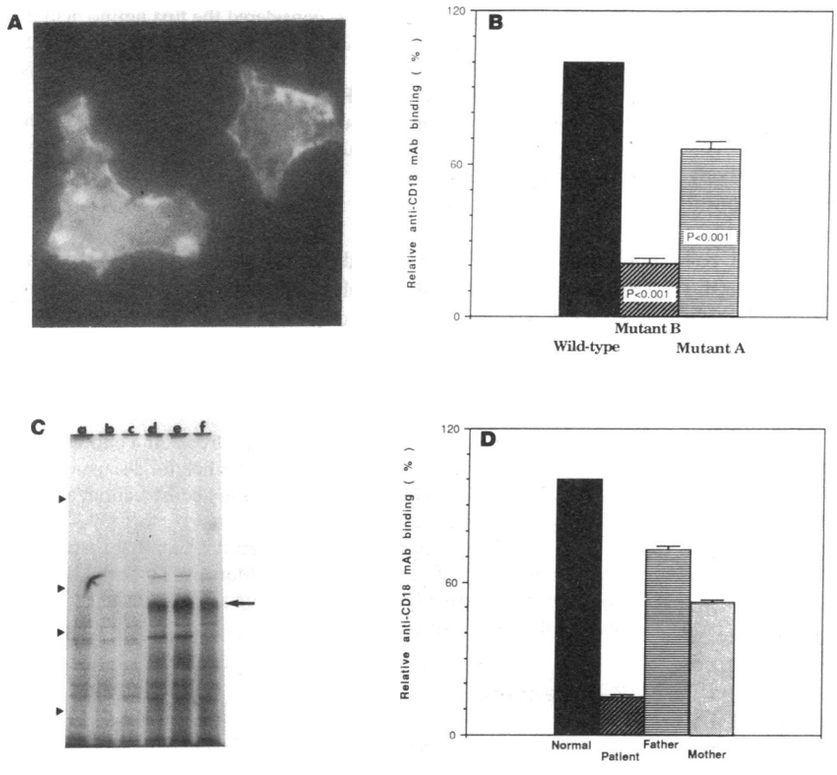

Figure 2. Effects of lysine^{196} to threonine (mutant A) and arginine^{593} to cysteine (mutant B) substitutions on cell surface expression and biosynthesis of CD18 in transfected COS cells. (*A*) Cell surface membrane expression of wild-type CD18 cDNA in COS cells. Expression was measured by staining COS cells cotransfected with CD11b and wild-type CD18 cDNA using an anti–CD18 mAb (TS18) and a fluoresceinated goat antimouse Ig (Meloy Laboratories Inc., Springfield, VA). A similar pattern was observed when cells were stained with a CD11b-specific mAb (2) (not shown). Original magnification = 40. (*B*) Comparison of COS cell surface membrane expression of mutated CD18 cDNA to that of the wild-type. Cell surface expression of wild-type, mutant A, and mutant B CD18 was measured in a radioimmunoassay using the anti–CD18 mAb (TS18) and an affinity-purified ^{125}I-antimouse Ig (New England Nuclear, Boston, MA). Histograms and bars represent, respectively, the mean±SD of three independent experiments, each carried out in triplicate. Expression of wild-type CD18 cDNA in each experiment was assigned a value of 100. Quantitation of mutant B expression on COS cells using 60.3, a second anti–CD18 mAb (6), gave similar results (21±3% of control). (*C*) Biosynthesis of CD18 precursors in transfected COS cells. Autoradiograph of a 7.5% polyacrylamide gel after electrophoresis of immunoprecipitates from metabolically labeled COS cells transfected with wild-type (lanes *a*, *d*), mutant B (lanes *b*, *e*) or mutant A CD18 cDNA (lanes *c*, *f*) using rabbit Igs as a control (lanes *a–c*) and a rabbit anti–CD18 antibody (lanes *d–f*) (10). Arrowheads indicate the position of the molecular weight markers myosin (200 kD), phosphorylase A (92.5 kD), bovine albumin (68 kD), and carbonic anhydrase (40 kD). Normal-sized CD18 protein precursors (*arrow*) were synthesized by COS cells transfected with each of the three CD18 cDNAs. The amounts synthesized were equivalent when compared with radiolabeled actin-binding protein (ABP) immunoprecipitated using a polyclonal antihuman ABP (kindly provided by Dr. John Hartwig, Massachusetts General Hospital) from the same cell lysates. The ratios of each CD18 precursor to that of ABP in each lysate (quantified by densitometric scanning of an autoradiograph) were: 5.2, 6.0, and 5.0 for wild-type CD18, mutant B-CD18, and mutant A-CD18, respectively. (*D*) Quantitation of CD18 expression on the cell surface of EBV-transformed B cells derived from a normal individual, the patient, and his parents. The anti–CD18 mAb TS18 was used as the primary antibody as in Fig. 2 *B*. Histograms and bars represent, respectively, the mean±SD of three independent experiments each carried out in triplicate. Expression of wild-type CD18 in EBV-transformed B cells was assigned a value of 100 in each experiment.

found in one group of isolated clones but not in another (not shown), indicating that the patient was a heterozygote for mutant B. A third nucleotide substitution (guanine[24] → thymine) resulted in a silent mutation (leucine → leucine at codon 8) (not shown). The presence of the point mutations in the coding region of CD18 is consistent with our previous data, which showed presence of normal-sized CD18 precursors intracellularly in leukocytes from this patient (10).

In order to determine the relevance of the observed mutations to the abnormal expression of CD18 in patient's leukocytes, wild-type and mutagenized CD18 cDNA encoding the full-length amino acid sequence of CD18 were constructed, inserted into πH3M expression vector, and transiently expressed in COS M6 monkey kidney cells (Fig. 2). Cotransfection of COS cells with the wild-type CD18 and CD11b cDNA resulted in expression of CD18 on the cell surface (Fig. 2 *A*). COS cells cotransfected with CD11b and mutagenized CD18 cDNA (encoding for either mutant A or mutant B) expressed, respectively, 66 and 20% of the wild-type levels of CD18 on the cell surface (Fig. 2 *B*). These results were confirmed by cytofluorometric analysis of transfected cells as well as by use of a second anti-CD18 mAb, 60.3 (legend, Fig. 2 *B*), suggesting that the differences in surface fluorescence are due to reduced surface expression rather than to decreased binding of mAbs to mutagenized CD18. Impaired surface expression with either mutation was also not due to differences in the amount or size of CD18 precursor synthesized (Fig. 2 *C*). When cell surface expression of CD18 on EBV-transformed B cell lines derived from the patient, his heterozygous parents (4) and a normal individual was determined by a radioimmunoassay, CD18 cell surface expression was 15, 70, and 53% of normal, respectively (Fig. 2 *D*). These data suggested that the patient is a compound heterozygote with CD18 alleles carrying either the threonine or cysteine substitutions inherited, respectively, from the patient's father and mother. Conclusive evidence supporting these findings was independently derived by oligonucleotide hybridization of the PCR-amplified respective regions of CD18 cDNA in the patient's mother. Mother-derived CD18 cDNA selectively hybridized with O-138 (containing mutant B) but not with O-35lt (containing mutant A) (data not shown). The present data also offer an explanation for the partial nature of Leu-CAM deficiency in this patient.

The three β subunits (β1, β2, and β3) of mammalian integrins and the β subunit of Drosophila position-specific antigen are structurally related (14–17, 28, 29). One striking feature of the extracellular portion of these β subunits is a cysteine-rich region consisting of four tandem repeats of an eight cysteine-motif that has no known function. The Arg[593] → Cys mutation in β2 integrin (mutation B) occurs within the fourth cysteine-motif and involves a conserved arginine residue (Fig. 3). This is the first direct demonstration of a critical role for this region in surface membrane expression of the common β subunit of β2 integrins and possibly those of β1 and β3 integrins as well. The β subunits also have a 247-amino acid region with several highly conserved segments (62–87.5% identity among the β subunits) (29, Fig. 3). In the β3 subunit, this region contains an arg-gly-asp binding site (30, 31), suggesting a role for this domain in ligand binding. The Lys[196] → Thr mutation (mutation A) occurs within this conserved region (Fig. 3), suggesting that it plays an additional role in normal surface membrane expression of the β subunits of integrins. In agreement is the finding that a 30-amino acid deletion in the COOH-terminal portion of this region (involving residues 332–361) also impairs cell surface expression of β2 integrins (32).

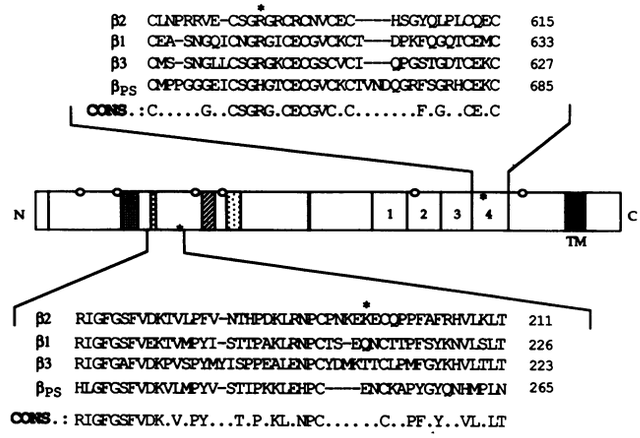

Figure 3. Schematic diagram showing the structure of CD18 subunit, location of the two amino acid substitutions (*), and the alignment of the regions immediately surrounding these mutations with other integrin β subunits. The four cysteine-rich repeats are shown (1–4) and the location of the 247 highly conserved segment is outlined by bold lines. The four marked regions within this segment are 62–87.5% identical among β integrins. The position of the potential *N*-glycosylation sites are represented by circles. TM, N, C refer to the transmembrane region, the NH_2-terminal end, and COOH-terminal end, respectively. The signal peptide at the NH_2-terminal end is boxed. The initiator methionine is considered the first amino acid in each case. The amino acid positions in each β integrin are indicated by numbers in the right margin. The deduced amino acid sequences were aligned by the GAP and PRETTY computer programs using the University of Wisconsin sequence analysis software package. The consensus (Cons.) sequence required exact matching of three of the four compared sequences.

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