Cristina López Rodríguez[○□], Arsenio Nueda[○△], Barbara Grospierre[▲], Francisco Sánchez-Madrid[●], Alain Fischer[▲], Timothy A. Springer[■] and Angel L. Corbí[○]

Unidad de Biología Molecular[○] and Sección de Inmunología, Hospital de La Princesa[●], Madrid, INSERM U132, Hospital Necker Enfants Malades[▲], Paris and the Center for Blood Research, Harvard Medical School[■], Boston

Characterization of two new CD18 alleles causing severe leukocyte adhesion deficiency*

Leukocyte adhesion deficiency (LAD) is an autosomal recessive disease caused by heterogeneous mutations within the gene encoding the common β subunit (CD18) of the three leukocyte integrins LFA-1 (CD11a/CD18), Mac-1 (CD11b/CD18), and p150,95 (CD11c/CD18). Based on the level of expression of CD18 on patient leukocytes, two phenotypes of LAD have been defined (severe and moderate) which correlate with the severity of the disease. We have investigated the molecular basis of the disease in two unrelated severe patients (HS and ZJO). Both patients share a complete absence of CD18 protein precursor and cell surface expression, but they differ in the level of CD18 mRNA, which is normal in HS and undetectable by Northern blot in ZJO. Determination of the primary structure of the patient HS CD18 mRNA revealed a 10 bp deletion between nucleotides 190–200 (CD18 exon 3), which eliminates residues 41–43 and causes a frameshift into a premature termination codon 17 base pairs downstream from the deleted region. The 10-base pair frameshift deletion maps to a region of the CD18 gene where aberrant mRNA processing has been detected in HS and two other unrelated LAD patients. In the ZJO patient, amplification of lymphoblast CD18 mRNA demonstrated the presence of a non-sense mutation in the third nucleotide of the triplet encoding ^{534}Cys (TGC → TGA), within exon 12. Both genetic abnormalities were also detected at the genomic level, and affect the restriction pattern of their corresponding genes, thus enabling the detection of the mutant alleles among healthy heterozygous alleles in family studies. The identification of two new LAD CD18 alleles, either carrying a non-sense mutation (ZJO) or a partial gene deletion (HS), further illustrates the heterogeneity of the genetic alterations in LAD.

1 Introduction

Cellular adhesion plays an essential role in the onset and regulation of immune and inflammatory processes. Most leukocyte adhesive functions are absolutely dependent on the expression and function of the leukocyte-specific heterodimers LFA-1 (CD11a/CD18), Mac-1 (CD11b/CD18), and p150,95 (CD11c/CD18), which comprise the β2 integrin subfamily [1]. Their essential role in leukocyte binding to and migration through the endothelial layer in inflamed tissues is illustrated by the existence of an immunodeficiency disorder termed Leukocyte adhesion deficiency (LAD). LAD is an autosomal recessive disease caused by the deficient expression of the three β2 integrins on the cell surface of leukocytes [1, 2]. LAD patients exhibit delayed umbilical cord separation, impaired pus formation, and recurrent bacterial and fungal infections of soft tissues, as a consequence of the lack of neutrophil migration into sites of inflammation [1–4]. Two clinical phenotypes of LAD (severe and moderate or partial), which normally correlate with the severity of the disease, have been defined according to the level of β2 integrin expression on patient leukocytes [3]. LAD is originated by heterogeneous mutations within the common β2 (CD18) subunit gene [5, 6], which is located on chromosome 21q22 [7]. The diversity of the alterations has led to the definition of several types of LAD (I–V), whose classification is based on the size and levels of the CD18 subunit precursor, the CD18 messenger RNA, and the resulting phenotype [5]. Analysis of severe and moderate LAD CD18 alleles has identified aberrant splicing events [8–11], mis-sense mutations [9–15], and a one bp deletion [14] within the structural region of the CD18 gene.

The molecular analysis of LAD patients has provided insight into the structure-function relationships in β subunit integrins and may identify specific regions within the β2 integrin gene which are more prone to naturally ocurring genetic alterations. In the present report we describe the identification of two new types of genetic abnormalities leading to severe LAD, namely a 10 bp frameshift deletion (patient HS) and a non-sense mutation (patient ZJO). Interestingly, both LAD alleles alter the restriction pattern of the CD18 gene, facilitating their detection among healthy carriers. Our results expand the repertoire of genetic abnormalities causing LAD and further indicate that the heterogeneity of the molecular basis of LAD is comparable to that of other genetic diseases such as thalassemia and hemophilia.

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Correspondence: Angel L. Corbí, Unidad de Biología Molecular (Planta 9). Hospital de la Princesa. Diego de León 62. Madrid 28006. Spain (Fax: 34 13 09 24 96)

Abbreviation: **LAD:** Leukocyte adhesion deficiency

2 Materials and methods

2.1 Patients, LAD cell lines and cell culture

The analyzed LAD patients (HIS and ZJO) have been previously described to exhibit the severe phenotype of LAD [5, 6, 16], with a complete absence of CD18 expression. HS patient leukocytes and lymphoblasts express normal-size CD18 mRNA but lack any detectable CD18 precursor [6, 16]. Lymphoblasts from the ZJO patient have been previously shown to lack detectable levels of CD18 mRNA and protein precursor, without any gross deletion of the CD18 gene [5].

Jurkat leukemic T cells, U937 and KG1 myeloid cells, and the EBV-transformed B cell lines from LAD patients HS [6, 16], ZJO [5], EM [15], SML [5, 14], and KOS [5, 11] were grown in RPMI medium supplemented with 10% FCS, 2 mM glutamine and 50 μg/ml gentamycin, and maintained in a humidified atmosphere with 5% CO_2.

2.2 Polymerase chain reaction (PCR)

2.2.1 Amplification of mRNA (RT-PCR)

Total cellular RNA was prepared by extraction with guanidine thiocyanate and density gradient centrifugation in cesium chloride [17]. cDNA first-strand synthesis was performed on 1 μg of total or polyA$^+$ RNA in 40 μl of a solution containing 0.5 pM random hexamers, 0.5 mM of each deoxynucleotide, 10 mM DTT, 50 mM Tris-HCl pH 8.2, 50 mM KCl, 5 mM $MgCl_2$, and RNase inhibitor and AMV reverse transcriptase at 1 U/μl. The mixture was incubated at 42 °C for 90 min. PCR amplification of the CD18 mRNA was carried out on 10 μl of the cDNA synthesis reaction in 50 μl of a solution containing 0.2 mM of each deoxynucleotide, 2.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl pH 8.3, 1 μM of each oligonucleotide primer and 2.5 units of Taq DNA polymerase. Reaction was performed by 35 cycles of denaturation (94 °C, 1 min) and annealing/extension (62 °C, 3 min), followed by a 10 min extension step at 72 °C. Oligonucleotides used for CD18 mRNA amplification (CD18#1–4) were derived from the CD18 sequence [18, 19] and have been previously reported [15]. PCR products were blunted with the Klenow fragment of *E. coli* DNA polymerase I and ligated into SmaI-digested pUCBM21 (Boehringer, Mannheim) or, alternatively, ligated into pCR-II (Invitrogen). To eliminate the possibility of PCR artifacts, two independent amplifications were performed in each case at least five clones from each amplification were sequenced and analyzed by restriction mapping and hybridization. Control amplifications were carried out in the absence of reverse transcriptase.

2.2.2 Amplification of human genomic DNA

Genomic DNA was isolated by standard procedures [17]. Specific regions within the CD18 gene were amplified under similar conditions as for cDNA amplifications, using 100–500 ng of genomic DNA and 1 μM of each oligonucleotide primer. The sequence of the primers is: CD18#9 (sense): 5′-gcccagagcacccactcacc-3′, CD18#10 (antisense): 5′-caaaagctgggcaggtgggga-3′, CD18#11 (sense): 5′-GCTGCTCTCCCTCGGGTGCG-3′, CD18#18 (sense): 5′-CAACTCCATCATCTGCTCAGGG-3′, CD18#19 (antisense): 5′-GGTCCTCTCGCACTGGCA-CG-3′, CD18#20 (sense): 5′-gttgcagtgagctgagatcacgcc-3′ and CD18#21 (anti-sense): 5′-TCACCGTGGACATAG-CGGGGCCTC-3′, with uppercase and lowercase letters indicating nucleotides derived from exon and intron sequences, respectively [20]. Oligonucleotides CD18#9 and #10 are derived from genomic sequences located 19 bp upstream and 20 bp downstream from exon 3 and were used to amplify exon 3. CD18#11 corresponds to the last 20 nucleotides from exon 2 and was used, in combination with CD18#10 to amplify intron 2 plus exon 3. Oligonucleotides CD18#18 and #19 were utilized for exon 12 amplification and are based on sequences from exons 12 and 13, respectively. The CD18 proximal promoter region was obtained by using oligonucleotides CD18#20 and #21, which are derived from the CD18 promoter sequence between − 904 and − 881 (CD18#20) and between + 112 and + 89 (CD18#21) [21]. PCR products were ligated into pCR-1000 or pCR-II plasmids (Invitrogen) and sequenced by the dideoxy termination method [22], using plasmid- and CD18-specific primers.

2.3 Northern blot

Total RNA from KG1 and the B lymphoblastoid cell lines (20 μg) was separated on formaldehyde-containing agarose gels and transferred onto nitrocellulose filters using standard procedures [17]. After blotting, the nitrocellulose membrane was baked for 90 min in a vacuum oven at 70 °C and prehybridized for 2 h at 42 °C in 6 × SSC, 50% formamide, 5 × Dernhardt's solution, 0.5% SDS and 200 μg/ml of denatured salmon sperm DNA. Hybridization was performed in the same solution containing 10^6 cpm/ml of a 1 kbp EcoRI fragment from the CD18 cDNA [18, 19], which had been previously labeled at an specific activity of 3 × 10^8 cpm/μg. After an overnight incubation at 42 °C, the filter was serially washed in 2 × SSC, 0.5% SDS at room temperature; 1 × SSC, 0.5% SDS at room temperature; 0.3 × SSC, 0.5% SDS at 37 °C; and 0.3 × SSC, 0.5% SDS at 65 °C, and exposed to × Ray film overnight with intensifying screens.

2.4 Transfections and promoter activity assays

The CD18 gene regulatory region contained between − 904 and + 112 was amplified from Jurkat and ZJO genomic DNA using oligonucleotides CD18#20 and CD18#21 and cloned into HindIII/XhoI-cut pXP2 plasmid upstream from the firefly luciferase cDNA [23]. Of the resulting plasmids, 80 μg (pCD18-Luc and pCD18-Luc(ZJO)) was transfected into U937 cells by electroporation [24], and the promoter activity determined 14 h after transfection by measuring the level of luciferase activity produced by each plasmid. To determine the phorbol-ester inducibility of the activity of each promoter fragment, cells were split immediately after electroporation and PMA was added to half of the transfected cells. Transfection efficiencies were normalized by including 20 μg of the CMV-βgal plasmid in each electroporation mixture.

3 Results

3.1 Analysis of LAD patients CD18 mRNA by Northern blot

Previous characterization of HS and ZJO-derived lymphoblasts revealed that HS exhibited normal size CD18 mRNA [6, 16], whereas no detectable CD18 mRNA could be observed in ZJO [5]. Northern blot was performed to confirm the steady-state level of the CD18 mRNA in both cell lines and compare them to the previously characterized LAD cell lines EM, SML and KOS. EM lymphoblasts exhibit a mis-sense mutation in the CD18 gene [15], while the molecular basis for KOS and SML LAD patients has been reported as a point mutation affecting splicing and causing an aberrantly longer CD18 mRNA (ZJO) [11], and a one-bp deletion (SML) [14]. In addition, ZJO and SML express extremely low levels of CD18 mRNA [5]. Northern blot analysis revealed that the HS CD18 mRNA steady-state level is similar to that of EM and considerably higher than those exhibited by the SML and KOS B lymphoblastoid cell-lines (Fig. 1). Conversely, no CD18 mRNA could be detected in ZJO, even in conditions that allow its detection in SML and KOS (Fig. 1). These results suggested that the defect in the HS CD18 mRNA was probably affecting the coding portion of the mRNA, while the deficiency in ZJO could result from an impaired transcription or decreased stability/processing of the CD18 mRNA.

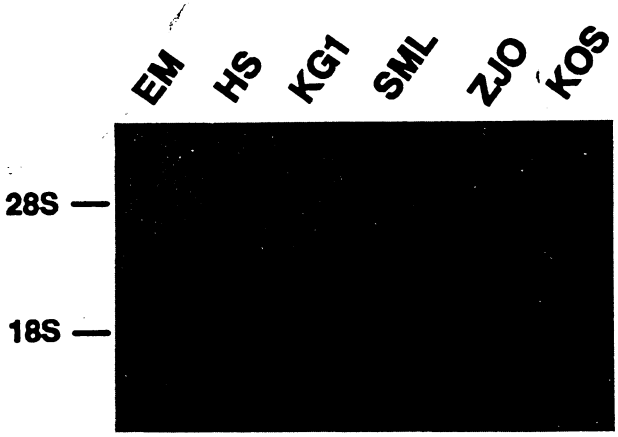

Figure 1. RNA steady-state level for CD18 in the HS- and ZJO-derived cell lines. Northern blot analysis of CD18 mRNA from the B lymphoblastoid cell lines HS, ZJO, EM [14], KOS [5] and SML [5, 12]. The position of the 28S and 18S ribosomal RNA is indicated. The myeloid cell line KG1 was included as a control of normal size CD18 mRNA.

3.2 Molecular basis for LAD in patient HS

To identify the molecular basis for the deficiency, HS polyA$^+$ RNA was reverse-transcribed and amplified with two sets of sense/anti-sense oligonucleotide primers which span the entire CD18 coding region [15]. After two independent amplifications, RT-PCR products were cloned and their nucleotide sequence revealed four differences with the CD18 sequence previously reported [18, 19]. Three of the changes were single nucleotide silent substitutions at Gly^{273} (GGG for GGA), Val^{367} (GTA for GTC), and Val^{441} (GTC for GTT), which probably represent polymorphisms since they have been detected in an unrelated LAD patient [15], and two of them have also been observed during the sequencing of the CD18 gene [20] and Dr. S. K. A. Law, personal communication). In addition, a

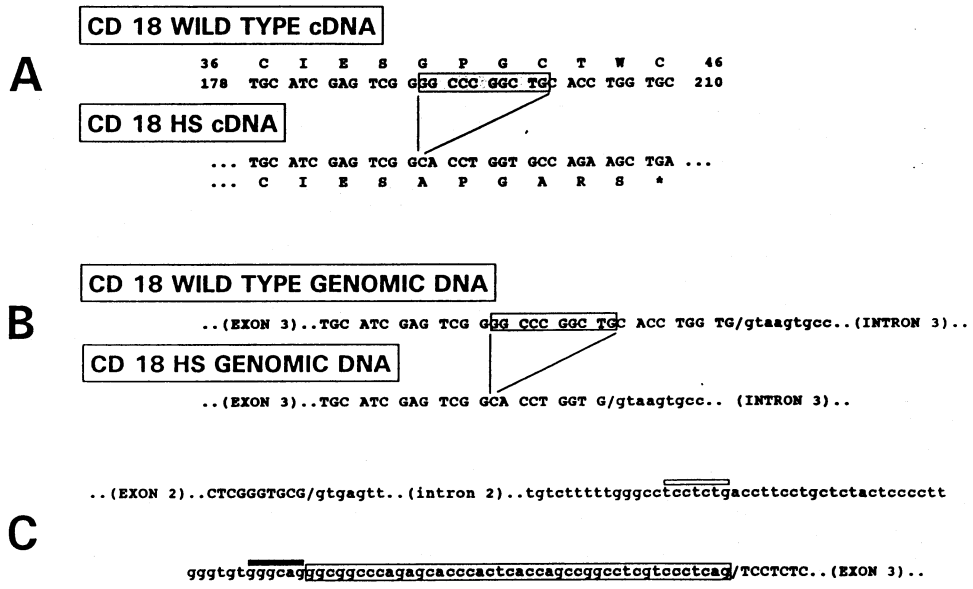

Figure 2. Identification and location of a 10 bp frameshift deletion within the HS CD18 gene. (A) Comparison of the nucleotide sequences of HS and wild type CD18 mRNA around the deletion site. The deleted nucleotides are boxed, and numbers correspond to the CD18 wild type cDNA [18]. (B) Partial sequence of CD18 exon 3 from the HS patient and wild-type genomic DNA. Deleted nucleotides are boxed and exon sequences are denoted by uppercase letters. (C) Sequence of a RT-PCR product containing a 43-nucleotide insertion between exon 2- and exon 3-encoded sequences. Exon sequences are shown in capital letters. Inserted nucleotides are boxed and empty and filled boxes overline the potential alternative branch point sequence and splicing acceptor site, respectively.

ten-nucleotide deletion was detected between the first nucleotide encoding Gly^{40} (GGG) and the third nucleotide for Cys^{43} (TGC) (Fig. 2 A). The deleted sequence (GGCC-CGGCTG) includes nucleotides 191 to 200 from the previously reported CD18 sequence [18, 19] (Fig. 2 A), and is located within the exon 3-encoded region of the CD18 mRNA [20].

To determine whether the deletion was present at the genomic level, the CD18 exon 3 was amplified by PCR on DNA isolated from the HS cell line, using two oligonucleotide primers based on the sequence from the 3′ end of intron 2 (CD18#9) and the 5′ end of intron 3 respectively (CD18#10). Cloning and sequencing of the amplified products revealed that the HS CD18 gene lacks 10 bp within its third exon (Fig. 2 B), confirming the RT-PCR results and demonstrating that the deletion is present in the patient genome. Since the 10-bp deletion causes a frameshift, and due to the presence of an in-frame termination codon 17 bp 3′ from the deletion, the translation of the patient CD18 mRNA predicts an abnormally short β2 protein precursor of 45 amino acids and whose last six residues are translated from a different reading frame than those of the normal molecule (Fig. 2 A). The prediction of a truncation in the N-terminal region of the CD18 precursor is in perfect agreement with the previously described absence of β2 precursor in the HS cell line and correlates with the severe phenotype exhibited by HS [6, 16].

3.3 Aberrant splicing around the third exon in the HS CD18 gene

The 10-bp deletion was detected in all of 20 different RT-PCR clones derived from two independent amplifications; however, 3 clones (1 out of 3 and 2 out of 15 from the two independent amplifications) exhibited an additional 43-bp insertion between nucleotides 130 and 131, precisely at the exon 2-exon 3 junction and located 60 bp upstream from the frameshift deletion (Fig. 2 C). Sequencing revealed that the inserted 43 bp correspond to the 3′ end of intron 2 [20], indicating that these clones were derived from aberrantly spliced CD18 mRNA. To determine the basis for this aberrant splicing event, intron 2 in the HS CD18 gene was sequenced after amplification of genomic DNA with oligonucleotides CD18#10 and CD18#11. The sequence 5′-GGGCAG-3′, which conforms perfectly to the consensus for splicing acceptor sites, was found immediately preceding the last 43 nucleotides in intron 2, and a perfect branch-point consensus sequence (5′-CTCTGAC-3′) [25] was located 33 nucleotides upstream from the aberrant splicing acceptor site, demonstrating that the aberrant splicing has occurred by a normal mechanism (Fig. 2 C). Moreover, the whole HS intron 2 sequence was identical to the intron 2 sequence derived from a CD18 genomic clone isolated from a human genomic DNA library (provided by Drs. A. H. Wright aND S. K. A. Law) [20], suggesting that the aberrant splicing detected in patient HS is not the consequence of additional genetic alterations. Since only 15% of the CD18 mRNA molecules appear to contain the 43 bp insertion, its contribution to the patient phenotype should be negligible. However, the finding of a similar phenomenon in other unrelated LAD patients ([15], and S. K. A. Law, personal communication) suggests that it may be caused by the presence of alterations in nearby regions of the CD18 gene.

3.4 Molecular basis for LAD in patient ZJO

In agreement with previous studies [5], Northern blot analysis revealed the absence of detectable CD18 mRNA in the ZJO-derived B lymphoblastoid cell line (Fig. 1). Since no gross deletion of the CD18 gene has been previously detected in ZJO lymphoblasts DNA [5], we hypothesized that the molecular defect should affect either the transcription or the stability or processing of the patient CD18 mRNA. To test the first hypothesis, the proximal region of the CD18 gene promoter (covering nucleotides between −904 and +82), which has been previously shown to direct the tissue-specific and regulated expression of reporter gene constructs [21], was isolated by PCR, sequenced, and cloned upstream of the luciferase gene in the pXP2 plasmid. Seventy per cent of the amplified molecules exhibited the wild-type sequence, while the remaining molecules showed a T at position −403 within the promoter (Fig. 3 A). Transient expression in U937 cells, which constitutively express CD18, showed that the ZJO CD18 gene proximal regulatory region containing T^{-403} (pCD18-Luc(ZJO)) exhibited similar basal and inducible promoter activity as the CD18 promoter isolated from Jurkat (pCD18-Luc), which constitutively expresses high levels of CD18 (Fig. 3 B). Therefore, the activity of the CD18 gene proximal promoter region is not altered in the ZJO patient,

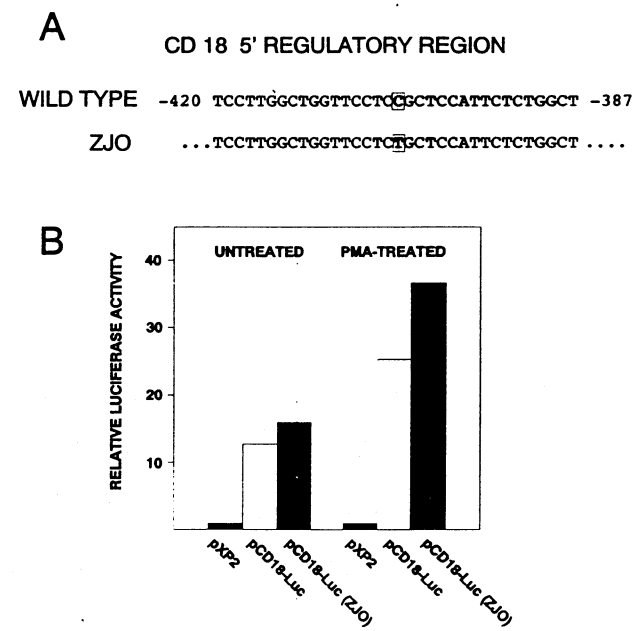

Figure 3. Structural and functional analysis of the ZJO CD18 gene promoter. (A) Presence of a nucleotide substitution ($^{-403}$T-C) in 30% of the PCR clones derived from the amplification of the ZJO CD18 gene promoter. The position of the nucleotide substitution is indicated by boxes. Numbering corresponds to the published sequence for the CD18 gene promoter region [21]. (B) Basal and PMA-inducible activity of the ZJO CD18 gene promoter. pCD18-Luc and pCD18-Luc(ZJO). which contain the CD18 gene proximal promoter region (− 904 to + 112) derived from Jurkat and ZJO, were electroporated into U937 cells. After transfection, half of the transfected cells were treated with PMA and the luciferase activity determined 14 h later. CD18 promoter activity is expressed relative to the luciferase activity produced by the promoterless plasmid pXP2, after correction for transfection efficiency.

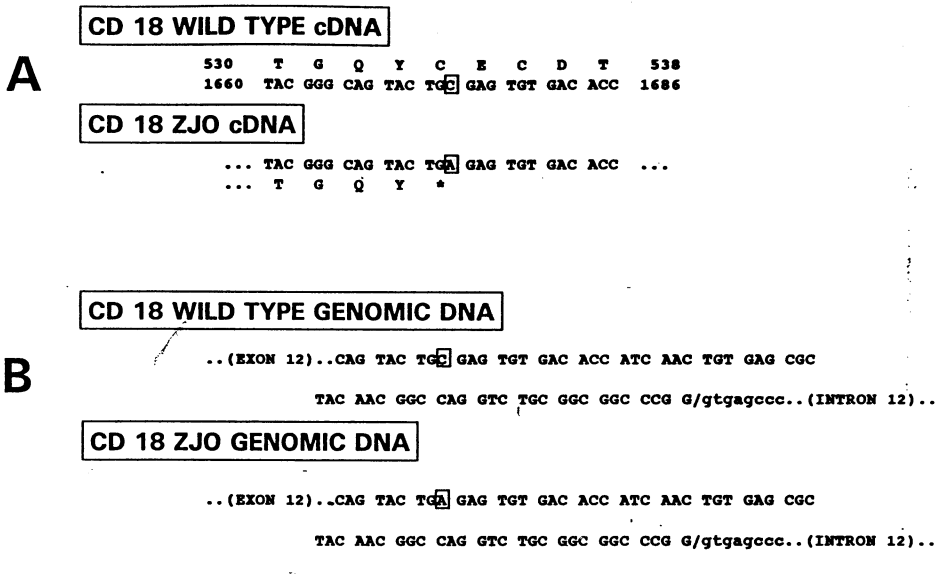

Figure 4. Identification and location of a non-sense mutation in the ZJO CD18 gene. (A) Comparison of the sequence around ^{534}Cys in ZJO and the wild type CD18 mRNA. The position of the non-sense mutation is boxed and the numbers correspond to the published CD18 mRNA sequence [18]. (B) Partial sequence of the CD18 exon 12 derived from the ZJO patient and wild type CD18 genomic DNA. The point mutation is boxed and intronic sequences are shown as lowercase letters.

suggesting that the lack of detectable CD18 mRNA is not due to an altered transcription of the CD18 gene.

The sensitivity of PCR for mRNA detection exceeds that of Northern blot by several orders of magnitude [26]. In an attempt to determine whether CD18 mRNA molecules could be detected in ZJO lymphoblasts, RT-PCR was performed on 1 μg of total ZJO RNA using two primer pairs which cover the whole CD18 mRNA coding region [15]. Two independent amplifications were performed with each set of primers and, in every case, the size of the amplified fragments corresponded to the expected size for CD18-derived products. The RT-PCR products were isolated and cloned, and the sequence of four clones from each amplification revealed the presence of a point mutation at nucleotide 1674, which converts a C into an A (Fig. 4 A). ^{1674}C is the third nucleotide of the triplet encoding ^{534}Cys (TGC) and, therefore, the point mutation causes the appearance of a stop codon (TGA) within the CD18 open reading frame (Fig. 4 A). The affected residue is encoded by exon 12 in the CD18 gene [20], and amplification of CD18 exon 12 from ZJO genomic DNA with oligonucleotides CD18#18 and Cd18#19 further demonstrated that the non-sense mutation is encoded in the patient's genome (Fig. 4 B). Altogether, the above results illustrate the presence of a non-sense mutation within the coding region of the ZJO CD18 mRNA which reflects a genomic DNA mutation. Although the mechanism is unclear, the presence of non-sense mutations along the open reading frame of several genes is known to cause greatly diminished or undetectable steady-state levels of the corresponding mRNA [27–34]. In this sense, the detected non-sense mutation at ^{1674}C could be responsible for the extremely low levels of CD18 mRNA in the ZJO patient (see Sect. 4).

3.5 Detection of the mutant alleles

The identification of the molecular defects of hereditary disorders is of great value for the diagnosis and detection of heterozygous individuals among patient's relatives and the population. In the patients here analyzed, the 10 bp deletion in HS causes the disappearance of an ApaI restriction site, while the non-sense mutation creates a DdeI site within the ZJO CD18 gene. Both altered sites provided useful markers to determine whether the HS and ZJO are homozygous for their respective genetic alterations, as well as for the detection of the mutant alleles. In the case of HS, amplification of CD18 mRNA with oligonucleotides CD18 #1 and CD18 #2 [15] and subsequent digestion with ApaI and EcoRI demonstrated the absence of this specific restriction site in the amplified product (Fig. 5 A). This fact, together with the presence of the deletion in all the sequenced PCR products and the consanguinity in the patient's family [4], strongly suggests that the HS LAD patient is homozygous for the 10-bp frameshift deletion. Amplification of the ZJO CD18 mRNA with oligonucleotides CD18 #7 and CD18 #8 [15] and subsequent digestion of the amplified products with DdeI also showed the usefulness of the newly created restriction site for detection of the mutant allele (Fig. 5 B). Moreover, the homozygosity of the ZJO patient for the non-sense mutation is suggested by (i) the presence of the mutation in all the amplified products, (ii) the normal copy number of the CD18 gene [5], and (iii) the unique restriction pattern observed after DdeI treatment (Fig. 5 B).

4 Discussion

In the present report we have studied the molecular basis for LAD in two unrelated patients (HS and ZJO), and found two new genetic alterations in the CD18 gene resulting in the severe phenotype. The first one is a 10-bp deletion in the third exon which causes a frameshift into a premature stop codon and predicts the synthesis of a CD18 protein truncated at residue 40. The second alteration is a non-sense point mutation at residue 534 which appears to affect the steady-state level of the CD18 mRNA. The presence of both abnormalities alter the restriction pattern of the CD18 gene, thus allowing the detection of the mutant alleles among healthy relatives and its use for genetic counseling.

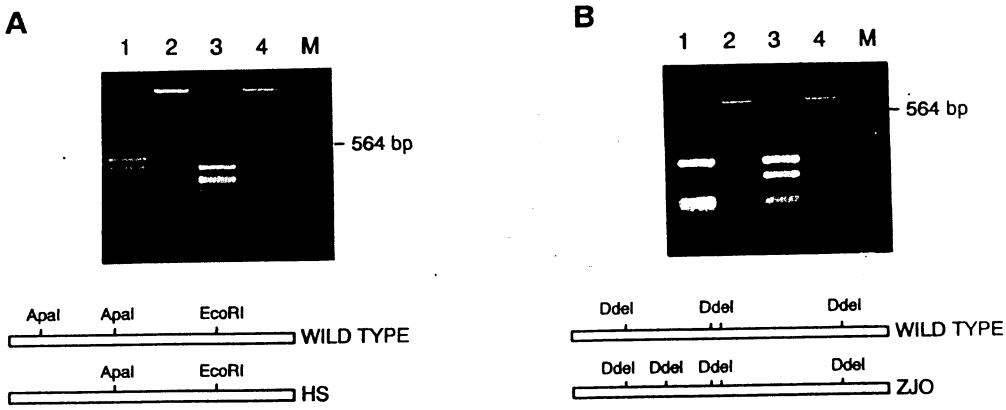

Figure 5. Detection of mutations with restriction enzymes. (A) CD18 mRNA from the HS (lanes 1, 2) and KG1 (lanes 3, 4) cell lines was amplified between nucleotides 51 and 1286 [15], and amplified products were digested with ApaI/EcoRI (lanes 1, 3) or left undigested (lanes 2, 4). The middle-size band in lane 3 contains co-migrating fragments of 366 and 350 bp. (B) Appearance of an additional DdeI site due to the non-sense mutation detected in patients ZJO. CD18 mRNA from ZJO lymphoblasts (lanes 1, 2) and Jurkat (lanes 3, 4) was amplified between nucleotides 1486 and 2166 [18] and digested with DdeI (lanes 1, 3) or left untreated (lanes 2, 4). Lower bands correspond to co-migrating fragments of 110, 105, 96 and 82 bp (lane 1), and 105 and 96 bp (lane 3). HindIII-digested lambda DNA was used as molecular weight marker, and the 564-bp band is indicated (lane M). The relevant restriction sites are indicated on a schematic representation of the amplified fragments drawn to scale.

Patient HS lymphoblasts show a complete lack of β2 integrin cell surface expression and CD18 protein precursor, while exhibiting normal size and levels of the CD18 mRNA [6, 16]. The presence of the frameshift deletion completely explains the patient phenotype, since the truncated β2 protein precursor only retains the 17 N-terminal residues from the mature wild-type molecule and consequently, no functional leukocyte integrin heterodimer can be assembled. Patient HS cannot be ascribed to any of the known types of LAD [5] and constitutes the first case of LAD caused by a genetic defect which affects more than a single nucleotide within the CD18 gene. Given the heterogeneity of the genetic alterations leading to LAD, it is conceivable that additional gene deletions will be identified in other patients. In fact, partial deletions within the gpIIb (13 bp, and 4.5 kbp) and β3 (11 bp) integrin genes have been already identified as the molecular basis for three independent cases of Glansmann's thrombasthenia [35, 36], and a large number of severe cases of hemophilia A and B, and α^0 and β^0 thalassemias, whose molecular defects are also heterogeneous, are due to complete or partial frameshift gene deletions [37, 38].

The systematic study of the amplified HS CD18 mRNa allowed us to detect that 15% of all analyzed clones contained 43 bp from intron 2 which had not been spliced out. Interestingly, analysis of an unrelated LAD patient, whose molecular defect lies on exon 3 in close proximity to the deleted nucleotides in patient HS, has revealed the same "alternative" splicing event (A. H. Wright and S. K. A. Law, unpublished results), and aberrant intron 2 splicing has also been described in a third unrelated patient [15].

Taken together, these results suggest that the aberrant splicing event in intron 2 is not directly involved in the patient HS deficiency and that genetic alterations within this region of the CD18 gene alter the normal RNA processing mechanism, as already proposed for other genes [30].

On the other hand, ZJO represents the only reported LAD patient whose CD18 mRNA level is undetectable by Northern blot ([5] and Fig. 1). Promoter activity analysis indicated that the ZJO CD18 gene promoter region directs the basal and TPA-induced transcription of a reporter gene, demonstrating that this specific regulatory region is not responsible for the absence of CD18 mRNA in the patient lymphoblasts. However, sequencing of the CD18 mRNA coding region revealed the presence of a non-sense mutation at residue 534, which may alter the steady-state level of the CD18 mRNA. Undetectable or decreased steady-state mRNA abundance is a common phenomenon for genes harboring non-sense mutations: premature termination of translation often results in reduced mRNA levels for murine immunoglobulin μ [27], human β-globin [28], triose-phosphte isomerase [29], and dihydrofolate reductase mRNA [30]. These findings have been further substantiated by the identification of the molecular basis for a number of genetic diseases, demonstrating that non-sense mutations in the messenger RNA for β-globins [31], LDL receptor [32], vitamin D receptor [33], and insulin receptor [34] cause considerably reduced levels of the corresponding mRNA. The presence of premature termination codons may alter RNA processing [30], lead to RNA degradation [39, 40] or impair the transport across the nuclear envelope [31], which, in turn, would lead to decreased mRNA abundance. However, the exact mechanism remains unclear and the effect of non-sense mutations on mRNA levels is probably dependent on the analyzed cell type [30, 31]. Given these antecedents and the fact that the ZJO CD18 gene promoter is fully functional, it is very likely that the non-sense mutation in ZJO is directly responsible for the almost undetectable CD18 mRNA levels. However, it is still possible that additional mutations in other regulatory regions or in the 3′ untranslated region of the CD18 gene also contribute to the diminished CD18 mRNA levels.

Detailed biochemical and genetic studies have clearly established that heterogeneous alterations within the CD18

gene constitute the molecular basis for LAD. Five distinct classes of LAD have been defined based on the phenotype of the patient and the size and levels of CD18 mRNA and protein precursor [5]. The genetic causes for LAD in a number of patients has been reported [8–15] and, except for a one-bp deletion, all of them have been identified as point mutations affecting either coding sequences or intronic nucleotides involved in the splicing process. The identification of two additional CD18 alleles from LAD patients further extend the range of molecular defects leading to LAD and suggest that the heterogeneity of its molecular basis is comparable to that of other genetic deficiencies such as hemophilia and thalassemia.

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