Identification of Molecular Defects in a Subject With Type I CD36 Deficiency

By Hirokuzu Kashiwagi, Yoshiaki Tomiyama, Satoru Kosugi, Masamichi Shiraga, Robert H. Lipsky, Yoshi Kanayama, Yoshiyuki Kurata, and Yuji Matsuawa

We performed a molecular analysis of a subject whose platelets and monocytes did not express any cell surface CD36 (designated as a type I CD36 deficiency). Amplification of the 5' half of platelet and monocyte CD36 cDNA (corresponding to nucleotide [nt] 191-1009 of the published CD36 cDNA sequence [Oquendo et al., Cell, 58:95, 1989]) showed that two different-sized CD36 cDNAs existed. One cDNA was of predicted normal size, whereas the other was about 150 bp smaller than that predicted for normal CD36 cDNA. Amplification of the 3' region of CD36 cDNA (nt 962-1714) in this subject showed only normal-sized CD36 cDNA. Cloning and nt sequence analysis of the cDNAs showed that the smaller sized CD36 cDNA had 161-bp deletion (from nt 331 to 491), and a dinucleotide deletion starting at nt position 539. The same dinucleotide deletion was also detected in the normal sized CD36 cDNA. Both deletions caused a frameshift leading to the appearance of a translation stop codon. RNA blot analysis and quantitative assay using the reverse transcription-polymerase chain reaction (RT-PCR) showed that the CD36 transcripts in both platelets and monocytes were greatly reduced. Comparison of the determined cDNA sequences with the genomic DNA sequence for the human CD36 gene showed that the dinucleotide deletion was located in exon 5, and that the 161-bp deletion corresponded to a loss of exon 4. PCR-based analysis using genomic DNA showed that this subject was homozygous for the dinucleotide deletion in exon 5. Except for the dinucleotide deletion, we could not find any abnormalities around exons 3, 4, and 5 including the splice junctions. These results suggested that the deletions in CD36 mRNA were likely to be responsible for instability of the transcripts, and the dinucleotide deletion in exon 5 might affect the splicing of exon 4.

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MATERIALS AND METHODS

Case. A subject (female) having anti-CD36 antibodies (anti-Nak*) was found by screening with platelet-specific antibodies in Osaka Red Cross Blood Center (Osaka, Japan). She is apparently healthy and has no history of easy bleeding. Because she has two children and no history of blood transfusion, the antibodies appeared to be produced during pregnancy. We showed that neither her platelets nor monocytes expressed cell surface CD36 (type I CD36 deficiency).14

Materials. Platelets were obtained from this case, from 5 subjects with type II CD36 deficiency, and from 17 CD36-positive subjects as previously described.15,16 Mononuclear cells from this subject and from 2 CD36-positive subjects were obtained using a Ficoll-Hypaque gradient.17 HEL cells (obtained from Japan Cancer Research Resources Bank [JCRB], Tokyo, Japan) were used as a CD36-negative control.

Amplification of CD36 cDNA and CD36 gene. Extraction of total cellular RNA, and RT-PCR were performed as previously described.15,16 In brief, first-strand cDNA was synthesized from total cellular RNA using M-MLV reverse transcriptase kit (Bethesda Research Laboratories, Gaitherburg, MD) and 100 pmol of antisense primer. A total of 200 pmol of sense primer, 100 pmol of antisense primer, and 5 U of Taq polymerase (Promega Corp, Madison, WI)

CD36 (ALSO KNOWN as glycoprotein IV [GPIV] or GPIIIb) is an 88-kD GP expressed on platelets, monocytes, erythroblasts, capillary endothelial cells, and mammary epithelial cells.1-5 CD36 has been proposed as a receptor for collagen and thrombospondin on platelets,6 and has been shown to play a role in the binding of Plasmodium falciparum-infected erythrocytes to endothelial and melanoma cells.8 Recently, it has also been shown that CD36 is one of the receptors for oxidized low-density lipoproteins and fatty acids.9,10

Platelet CD36 deficiency is present in about 3% of the Japanese population11 and in 0.3% of the US population.12 We recently showed that platelet CD36 deficiency can be divided into two subgroups according to expression of CD36 on their monocytes.13,14 The majority of platelet CD36-deficient individuals also express CD36 on the surface of their monocytes (referred to as type II CD36 deficiency). However, in a few subjects with platelet CD36 deficiency, their monocytes do not express CD36 (referred to as type I deficiency). These individuals are apparently healthy and suffer no obvious hemostatic problems as a result of the absence of CD36. However, we detected anti-CD36 antibodies only in sera from subjects with type I CD36 deficiency, suggesting that these individuals are at high risk to produce anti-CD36 antibodies after a blood transfusion or during pregnancy.14

The molecular basis of CD36 deficiency remains obscure. We previously reported that a 426C → T polymorphism (proline-90 by serine) predominates in the type II deficiency.15 A molecular analysis of the type I CD36 deficiency has not been reported. We now report the first molecular analysis of a subject with type I CD36 deficiency. In this subject, we detected deletions in coding region of CD36 mRNA leading to frameshift and appearance of stop codon. RNA blot analysis and quantitative assay using the reverse transcription-polymerase chain reaction (RT-PCR) showed that greatly reduced amount of CD36 transcripts in platelets and monocytes of this subject. DNA analysis showed that this subject was homozygous for the dinucleotide deletion in exon 5.

From the Second Department of Internal Medicine, Osaka University Medical School; the Department of Blood Transfusion, Osaka University Hospital, Osaka, Japan; and the Cell Biology Department, The Jerome H. Holland Laboratory, American Red Cross, Rockville, MD.

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Address reprint requests to Hirokazu Kashiwagi, MD, The Second Department of Internal Medicine, Osaka University Medical School, 2-2 Yamadaoka, Suita 565, Japan.

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First-round amplification was performed for 30 cycles in a heating and cooling system (TSR-100: IWAKI Glass Corp, Tokyo, Japan). A total of 1 μL of the amplified product was reamplified for 30 cycles with 2.5 pmol of primers in a total volume of 100 μL. The numbers of primer sequences and positions are shown in Table 1 and Fig 1A. PCR conditions were described elsewhere. We amplified the regions of nucleotide (nt) 191-1009 and nt 962-1714. The 3' end after nt 1714 was not examined in this study.

Amplification of genomic DNA across splice junctions for exons 3, 4, and 5 of the CD36 gene was performed using primers developed from intron and exons sequences shown in Table 1 and Fig 1B. About 1 μg of DNA was amplified with 2.5 U of Taq polymerase and 100 pmol of primers in a total volume of 100 μL for 30 cycles. Other sites of the CD36 gene in this subject were not examined in this study.

Quantitative analysis of CD36 cDNA using RT-PCR. To quantify CD36 mRNA in platelets and mononuclear cells, we amplified CD36 cDNA and β-actin cDNA (internal standard) simultaneously using RT-PCR. First-strand cDNA was synthesized from 200 ng of total cellular RNA as described above. In these studies, random hexamer oligonucleotides (Takara CO, Kyoto, Japan) were used instead of the antisense primers. Subsequently, amplification was performed using 2.5 pmol/L of primers, NAKS and NAK6, for CD36, and 1.5 pmol/L of primers, C-1 and C-2, for β-actin. Amplification was performed for 25 cycles, with cycle conditions of 94°C for 1

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**Table 1. Nucleotide Sequences and Locations of Oligonucleotide Primers Used in this Study**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAk1</td>
<td>5'-GTGCCCTAACACTTTACC-3'</td>
<td>nt 176 to 195</td>
</tr>
<tr>
<td>NAk1'</td>
<td>5'-TTACCTCCTGGAAGT-3'</td>
<td>nt 181 to 206</td>
</tr>
<tr>
<td>NAk2</td>
<td>5'-ATACAGCATAGTGAATG-3'</td>
<td>nt 1045 to 1025</td>
</tr>
<tr>
<td>NAk2'</td>
<td>5'-GGAACTGCAATACCTGGC-3'</td>
<td>nt 1009 to 982</td>
</tr>
<tr>
<td>NAk3</td>
<td>5'-ACTGTCGAGCAGATATG-3'</td>
<td>nt 922 to 953</td>
</tr>
<tr>
<td>NAk3'</td>
<td>5'-AGAACTGCAATACCTGGC-3'</td>
<td>nt 962 to 977</td>
</tr>
<tr>
<td>NAk4</td>
<td>5'-GCCTGCTTAGCGATCTG-3'</td>
<td>nt 1745 to 1726</td>
</tr>
<tr>
<td>NAk4'</td>
<td>5'-GCCTAAATGTAATCTC-3'</td>
<td>nt 1714 to 1698</td>
</tr>
<tr>
<td>NAk5</td>
<td>5'-CTTCTTTAGGCAATGACT-3'</td>
<td>nt 8 to 32</td>
</tr>
<tr>
<td>NAk6</td>
<td>5'-AGCCAGGACAGCACCTATG-3'</td>
<td>nt 270 to 250</td>
</tr>
<tr>
<td>IC(-)</td>
<td>5'-GATACAAATTAGCAGTTAC-3'</td>
<td>nt 1986 to 1993 within intron C</td>
</tr>
<tr>
<td>IC(+1)</td>
<td>5'-GATACAAATTAGCAGTTAC-3'</td>
<td>nt 12038 to 12067 within intron D</td>
</tr>
<tr>
<td>ID(-)</td>
<td>5'-TAAGTCAATTGTCACACT-3'</td>
<td>nt 12382 to 12404 within intron D</td>
</tr>
<tr>
<td>ID(+1)</td>
<td>5'-AGATCTAAATTGTCACACT-3'</td>
<td>nt 17798 to 17805 within intron D</td>
</tr>
<tr>
<td>NAK7</td>
<td>5'-GCGAGTTGAGACGTGGAAG-3'</td>
<td>nt 17977 to 17986 within exon 5 (nt 632 to 612 of CD36 cDNA)</td>
</tr>
<tr>
<td>NCO(-)</td>
<td>5'-CCATTGGGCTGCAGGAAAG-3'</td>
<td>nt 17911 to 17886 within exon 5 (nt 567 to 542 of CD36 cDNA)</td>
</tr>
<tr>
<td>NCO(+)</td>
<td>5'-TTCGATGAAGGGCATGATG-3'</td>
<td>nt 12012 to 12034 within intron C</td>
</tr>
<tr>
<td>C-1</td>
<td>5'-GAGCTGACACACCAGACCA-3'</td>
<td>nt 172 to 122 of β-actin cDNA</td>
</tr>
<tr>
<td>C-2</td>
<td>5'-CTCTGCGACACACCAGACCA-3'</td>
<td>nt 642 to 619 of β-actin cDNA</td>
</tr>
</tbody>
</table>

Nucleotide positions from NAk1 to NAk6 are from the published CD36 cDNA sequence. Positions from IC(-) to NCO(+) are from the published CD36 gene sequence. Nucleotide positions of C-1 and C-2 are from the published β-actin cDNA sequence. Nt underlined are mismatched.
minute, 50°C for 1 minute, and 72°C for 2 minutes. Then, 4 μL of amplified sample was added to a PCR buffer containing the same primers to a final volume of 400 μL, and 100 μL was aliquotted into 4 separate tubes. Then, the second-round amplification was performed under the same conditions as the first-round amplification. Reaction tubes were removed at different cycle numbers (15, 18, 21, and 24 cycles), and an equal amount of aliquot was fractionated on 1.5% agarose gels and was stained with ethidium bromide.

**Sequencing of PCR products.** The amplified CD36 cDNA fragments were cloned into the M13 vectors or pBluescript (Stratagene, La Jolla, CA). Sequencing reaction was performed with the dideoxy termination method18 using the sequenase kit version 2 (US Biochemical Corp, Cleveland, OH). At least six clones for each fragment were sequenced to avoid errors resulting from Taq polymerase.

**RNA blot and DNA blot analysis.** Twenty micrograms of total cellular RNA extracted from mononuclear cells or cell lines were size-fractionated by electrophoresis through a 1.0% formaldehyde agarose gel and transferred to nitrocellulose membranes. A cDNA clone encoding CD36 (kindly provided by Dr Brian Seed, Massachusetts General Hospital, Boston, MA) was labeled with [32P]dCTP by the random primer method. After hybridization, the membrane was washed and autoradiographed. To control for RNA loading, the membrane was rehybridized with β-actin cDNA (Nippon Gene, Toyama, Japan).

Extraction of DNA from mononuclear cells was performed according to the methods previously described. Ten micrograms from each DNA sample was digested with five restriction enzymes, HindIII, Pst I, BamHI, EcoRI, and Bgl II, and electrophoresed through a 1.0% agarose gel and transferred to a nitrocellulose membrane. The membrane was treated the same as RNA blot analysis.

**RESULTS**

**Amplification of CD36 cDNA from platelets.** To detect CD36 mRNA in a small amount of platelet RNA pools, we performed second-round amplification using nested primers. Amplification of the 5' half of platelet CD36 cDNA (corresponding to nt 191-1009) showed that two different sized CD36 cDNAs existed; one was apparently normal-sized and the other was about 150 bp smaller (Fig 2). In contrast, we could detect a 2.9-kb band that is normally present. We only detected a trace of approximately 5.5-kb hybridized band in RNA pools from mononuclear cells, which may be an immature transcript of CD36 or a cross-reacted gene (Fig 5). Next, we performed RT-PCR assay. First-round amplification failed to detect CD36-specific bands from mononuclear cell RNA of this subject. In contrast, we could detect CD36 cDNA from mononuclear cells of CD36-positive subjects, which was consistent with RNA blot analysis. Second-round amplification enabled us to detect CD36-specific bands from mononuclear cell RNA of this subject, which was essentially the same as those of platelet CD36 cDNA (ie, the two different sized bands were detected by amplification of 5' half region, and normal-sized continuous...
band was detected by amplification of 3' half; data not shown). We confirmed the existence of both deletions in mononuclear cell CD36 cDNA by sequencing (data not shown). These results suggested that the subject's monocytes contained the same CD36 mRNA as platelets, and the amount of CD36 mRNA in monocytes was greatly reduced.

Quantitative analysis of CD36 mRNA in platelets. Because RNA blot analysis indicated that CD36 transcripts in monocytes of this subject were greatly reduced, we performed coamplification of CD36 cDNA and β-actin cDNA as internal standard using RT-PCR to quantify platelet CD36 mRNA. The intensity of CD36- (nt 8-270; 263 bp) and β-actin- (540 bp) specific bands increased as cycle numbers increased, indicating that the amplification was within the exponential phase. Under these conditions, the ratio of the intensity of CD36 band:β-actin band decreased markedly in this subject compared with that in a CD36-positive subject (Fig 6). Essentially the same result was obtained using monocyte RNA of this subject. These results indicated that CD36 transcripts in platelets of this subject were also reduced similar to those in monocytes.

Analysis of the CD36 gene. A comparison of the determined cDNA sequence with the human CD36 gene sequence for exons 3, 4, and 5 indicated that the dinucleotide deletion (539AC) was within exon 5, and the 161-bp deletion (nt 331-491) corresponded to the loss of exon 4 (see Fig 1B).
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DNA sequence analysis of amplified genomic DNA from the subject showed that the dinucleotide deletion occurred within exon 5 (data not shown). Because all amplified clones (six independent clones were examined) contained the same 2-bp deletion, the subject appeared to be homozygous for the dinucleotide deletion. Because we knew that the CD36 is a single-copy gene located on human chromosome pair 7,24,25 we wanted to confirm that both copies of the subject’s CD36 gene had the dinucleotide deletion. We amplified a region across exon 5 using a mismatched primer that created a new Ssp I site only when the dinucleotide was deleted (Fig 7A). Amplified products from the subject’s CD36 gene were completely digested with Ssp I, indicating that she was homozygous for the dinucleotide deletion (Fig 7B).

To clarify the mechanism that would lead to a loss of exon 4 during CD36 pre-mRNA processing, we first performed DNA blots on the subject’s DNA using five different restriction enzymes. No gross abnormality was detected in the subject’s CD36 gene (data not shown). Next, we sequenced regions around exon 3.4, and 5 including the splice-site junctions. We did not detect any differences between the DNA sequence determined for this subject and a CD36-positive subject in the 5’ splice site of intron C, in the entire sequence of exon 4, or in the 5’ and 3’ splice sites of intron D. The only sequence difference observed occurred at nt 12140 (this nt number was from the determined CD36 genomic sequence by Tang et al23) in the 3’ splice site of intron C (c or t) in the CD36-positive control (INTRON C . . . ttgcatagCAAGTTGTC . . . EXON 4; the position of the sequence divergence is underlined). The subject’s nt at position 12140 was t ( . . . ttgcatagCAAGTTGTC . . . ). Therefore, the normal control DNA sample was heterozygous for a C/T polymorphism at position 12140 of the 3’ splice junction. To examine whether the ‘‘t’’ allele of this polymorphism was specific to this subject, we performed mismatched PCR using primers, NCO(+ ) and NCO(− ). Nucleotide sequences of the primers are shown in Table 1. NCO(+) contains an Nco I site (CCATGG) as an internal control. PCR conditions were the same as described above except that the annealing temperature was raised to 53°C. The amplified products were digested with Nco I. If position 12140 was a “c”, a new Nco I site would be created. Nco I digestion of amplified DNA obtained from another 4, unrelated CD36-positive subjects showed that 2 of 4 CD36-positive subjects were homozygous for the “t” allele, the same as the CD36-deficient subject, whereas the remaining 2 CD36-positive subjects were heterozygous (data not shown). These results clearly indicated that the C/T polymorphism could not solely account for the loss of exon 4 during CD36 pre-mRNA processing.

DISCUSSION

In this report, we performed a molecular analysis of a subject with type I CD36 deficiency, whose platelets and monocytes did not express CD36 on their surface. We detected two different-sized CD36 cDNAs in the subject’s platelets and monocytes and determined that both a 161-bp (nt 331-491) and a dinucleotide (nt 539-540) deletion occurred in the smaller CD36 cDNA. The same dinucleotide deletion (nt 539-540) was present in the larger CD36 cDNA. For personal use only.on September 24, 2017. For personal use only.
Both deletions would be predicted to a frameshift and the appearance of a stop codon. RNA blot analysis and quantitative analysis using RT-PCR showed that platelets and monocytes of this subject contained very few CD36 transcripts compared with that for CD36-positive subjects. CD36 gene structure indicated that the 161-bp deletion in the smaller CD36 cDNA corresponded to a loss of exon 4. We couldn’t find any abnormalities around exon 3, 4, and 5 including the splice junctions except for the dinucleotide deletion in exon 5. We also determined that this subject was homozygous for the deletion.

Concerning the dinucleotide deletion, we observed similar features that have been reported in other examples of gene deletions involving a few nucleotides. (1) A short direct repeat was found around the deletion (. . . TGAGGACACAGCAG . . . ; the nucleotides in lower case letters were deleted). Direct repeats have been found in the immediate vicinity of almost all short gene deletions causing human diseases.26-28 Direct repeats are common feature in a number of recombination-, replication-, or repair-based models of deletion mutagenesis. (2) A consensus sequence (TGA/GA/ GG/TA/C) has been determined for “hot spots” of short deletions occurring in the human genome.28 In this case, TGAGGA sequence is found in the vicinity of the deletion.
The mechanism of exon 4 skipping in the processing of the subject's CD36 mRNA is unclear. DNA blot analysis failed to show any abnormality in the CD36 gene of this subject. Thus, it is unlikely that there is a major rearrangement of genomic structure in the vicinity of the CD36 locus. During the course of performing genomic analysis, we found a single-base polymorphism in the polypyrimidine tract upstream of exon 4. However, this polymorphism was not confined to this individual. Most reported examples of exon loss are because of mutations that change either the conserved sequences at the 5' or 3' splicing-junction sites. However, recent in vitro studies have shown that intronic sequence can also influence splice-site selection. In human diseases, intragenic deletions, as well as missense mutations and nonsense mutations, have been shown to induce exon skipping. An alteration in cis-acting elements binding splicing factors or changes in pre-mRNA secondary structure have been proposed as the basis for these observations. We favor a hypothesis that the dinucleotide deletion in exon 5 might induce aberrant splicing of exon 4 by altering CD36 pre-mRNA secondary structure, although we are not aware of other reports where intragenic mutations affect the splicing of flanking exons. The precise mechanism needs to be clarified in future studies.

Our results also showed that the subject's platelets and monocytes contained very few CD36 transcripts compared with that for CD36-positive subjects. The mechanism of decrease of CD36 transcripts is unclear. However, it has been reported in some genes that prematurely terminated translation caused by nonsense mutations or frameshfts leads to instability. Thus, it is likely that the deletions in CD36 transcripts of this subject is responsible for its instability.

The frequency of the dinucleotide deletion in CD36 gene of subjects with CD36 deficiency is now under investigation. We could not detect the deletion in 5 subjects with type II CD36 deficiency (H. Kashiwagi, unpublished observation), suggesting that the gene frequency of the deletion may be low in CD36-deficient subjects, at least in type II-deficiency subjects. However, the facts that the subject reported here was homozygous for the deletion and that the deletion leads to both platelet and monocyte CD36 deficiency suggest that the deletion may be spread widely in type I CD36 deficiency. Further studies are required to clarify this hypothesis.

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Identification of molecular defects in a subject with type I CD36 deficiency

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