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Research Article

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Molecular Basis of CD36 Deficiency

Evidence That a ^{478}C→T Substitution (Proline90→Serine) in *CD36* cDNA Accounts for CD36 Deficiency

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Abstract

CD36 deficiency is divided into two subgroups: neither platelets nor monocytes express CD36 (type I deficiency), and monocytes express CD36 in spite of the lack of platelet CD36 (type II deficiency). We have already demonstrated that a ^{478}C→T substitution (proline90→serine) in platelet *CD36* cDNA predominates in type II deficiency (Kashiwagi, H., S. Honda, Y. Tomiyama, H. Mizutani, H. Take, Y. Honda, S. Kosugi, Y. Kanayama, Y. Kurata, and Y. Matsuzawa. 1993. *Thromb. Haemostasis.* 69:481–484). In this study, we revealed that monocyte *CD36* cDNA from two type II deficient subjects was heterozygous for C478 and T478 form, while platelet *CD36* cDNA of these subjects consisted of only T478 form. In a type I deficient subject, both platelet and monocyte *CD36* cDNA showed only T478 form. Expression assay using C478 or T478 form of *CD36* cDNA transfected cells revealed that there was an 81-kD precursor form of CD36, and that the maturation of the 81-kD precursor form to the 88-kD mature form of CD36 was markedly impaired by the substitution. The mutated precursor form of CD36 was subsequently degraded in the cytoplasm. These results indicate that the ^{478}C→T substitution directly leads to CD36 deficiency via defects in posttranslational modification, and that this substitution is the major defects underlying CD36 deficiency. (*J. Clin. Invest.* 1995. 95:1040–1046.)

Key words: platelet • monocyte • transient expression • precursor • posttranslational modification

Introduction

CD36 (also known as platelet glycoprotein IV, or IIIb) is a membrane glycoprotein and has been identified in a wide variety of cell types including platelets, monocytes, erythroblasts, capillary endothelial cells, and mammary epithelial cells (1–5). CD36 has been proposed as one of collagen and thrombospondin receptors on platelets (6, 7), and it also plays a role in the binding of *Plasmodium falciparum*-infected erythrocytes to endothelial and melanoma cells (8). Recently, it has been shown that CD36 is one of receptors for oxidized low density lipoproteins and fatty acids, suggesting that CD36 may play a part in atherogenesis and lipid metabolism (9, 10).

Platelet CD36 deficiency is present in ~ 3% of the Japanese population (11, 12), and 0.3% of the U.S. population (13). We and others recently demonstrated that platelet CD36 deficiency can be divided into two subgroups according to expression of CD36 on their monocytes (14, 15). In the majority of platelet CD36 deficiency, their monocytes express CD36 on the surface (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 CD36 deficiency). These individuals are apparently healthy and suffer no obvious hemostatic problems despite the absence of CD36. However, we detected anti-CD36 antibodies only in sera from subjects with type I deficiency, suggesting that these individuals are at high risk to produce anti-CD36 antibodies following a blood transfusion or during pregnancy (14).

We have already demonstrated that a substitution of T for ^{478}C (proline90→serine) in platelet *CD36* cDNA predominates in subjects with type II deficiency (16). In this study, we investigated whether the ^{478}C→T substitution directly leads to CD36 deficiency. Expression assay revealed that there was an 81-kD precursor form of CD36, and that the maturation of the 81-kD precursor form to the 88-kD mature form of CD36 was impaired by the substitution, leading to marked reduction in surface expression of CD36.

Methods

Materials. Platelets and mononuclear cells were obtained from 5 subjects with type II CD36 deficiency, two subjects with type I CD36 deficiency, and 17 CD36 positive subjects as previously described (16–18).

Reverse transcriptase–polymerase chain reaction (RT-PCR),[1] sequencing, and Sau96I digestion. Amplification of *CD36* cDNA, subclonig, and sequencing were performed as previously described (16, 17). Since the ^{478}C→T substitution leads to loss of a Sau96I site at nt. 476, an amplified 198 bp *CD36* cDNA fragment (nt. 386 to 583) was digested with Sau96I as previously described (16). The digested fragments were electrophoresed in a 6% polyacrylamide gel, and visualized by staining with ethidium bromide.

Construction of expression vectors containing C478 or T478 form of CD36 cDNA. Strategy for construction of expression vectors containing

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1. *Abbreviations used in this paper:* *CD36*^{C478}, C478 form of *CD36* cDNA; *CD36*^{T478}, T478 form of *CD36* cDNA; CD36(WT), pCDM8 containing *CD36*^{C478}; CD36(P90S), pCDM8 containing *CD36*^{T478}; PVDF, polyvinylidene difluoride; RT-PCR, reverse transcriptase-polymerase chain reaction; sulfo-NHS-biotin, sulfo-*N*-hydroxysuccinimidobiotin.

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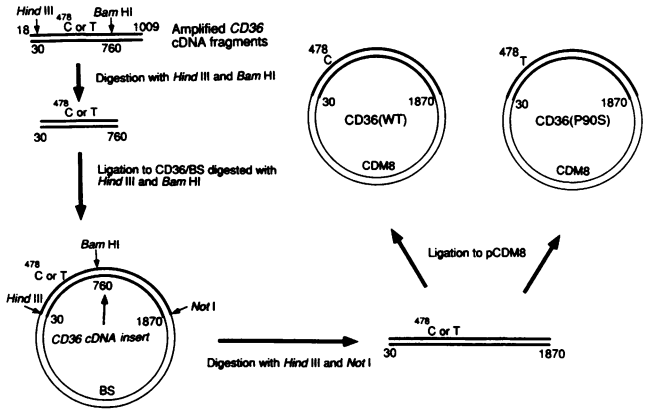

Figure 1. Strategy for construction of CD36 expression vectors. Amplified 992-bp fragments of monocyte *CD36* cDNA from a subject with type II deficiency (case 1) were digested with HindIII and BamHI, and 731-bp fragments were directionally inserted into a full-length *CD36* cDNA treated with the same enzymes. Then *CD36* cDNA fragments (nt.30–1870) which contain the entire coding region were cut out with HindIII and NotI, and were directionally inserted to a mammary expression vector, pCDM8. A plasmid of which nt.478 was C or T, was designated as CD36(WT) or CD36(P90S), respectively.

C478 or T478 form of *CD36* cDNA is shown in Fig. 1. First, a 1038 bp region (nt.8 to 1045) of monocyte *CD36* cDNA from a subject with type II deficiency (case 1) was amplified, as previously described (16). The monocyte *CD36* cDNA of this subject consisted of both C478 and T478 form (see Results). Then, second round amplification was performed using 1 μl of the first round PCR products as a template with nested primers, NAK18-HIND (^{18}CATTTTAAAGAAAGCTTTCC; nucleotide underlined was mismatched) and NAK2′ ($\overline{^{1009}}$AGAACTGCAATACCTGGC). NAK18-HIND has a new HindIII cutting site at nt.30. The amplified 992bp fragment (nt.18–1009) was digested with HindIII and BamHI, and a 731-bp fragment (nt.30–760) was extracted using Gene Clean II kit (Bio 101, La Jolla, CA). Full-length of *CD36* cDNA (19) (1870 bp; kindly provided by Dr. Brian Seed, Masattusetts General Hospital, Boston, MA) was cloned into pBruescript II (Stratagene, La Jolla, CA), which was designated as CD36/BS. The 731-bp fragment was directionally shuttled into CD36/BS digested with HindIII and BamHI. A clone whose nt.478 was C or T, was selected by colony-PCR followed by Sau96I digestion. The sequences of inserted fragments were confirmed with dideoxy termination method (20). Then, an 1841-bp fragment (nt. 30–1870) which contained the entire coding region (nt.211–1626) was obtained with HindIII and NotI digestion of the clone, and was inserted directionally into a mammalian expression vector, pCDM8 (provided by Dr. B. Seed), digested with the same enzymes. A plasmid, of which nt.478 was C, was designated as CD36(WT), and another plasmid, of which nt.478 was T, was designated as CD36(P90S).

The constructed plasmid, CD36(WT) or CD36(P90S), was transfected into 293T cells (kindly provided by Dr. David Baltimore, The Rockefeller University, New York) by the calcium phosphate method as previously described (21). The cells were cultured in Dulbecco's modified Eagle's medium (DME) with 10% heat inactivated FCS. We used pCDM8 containing no inserts for mock transfection.

RNA blot analysis. RNA blot analysis was performed as previously described (22). In brief, 20 μg of total cellular RNA extracted from the transfected cells was size-fractionated by electrophoresis through 1.0% formaldehyde agarose gel and transferred to nitrocellulose membrane. After hybridized with *CD36* cDNA labeled by the random primer method (23), the membrane was washed and autoradiographed.

Flow cytometry. Flow cytometric analysis using platelets or monocytes was performed as previously described (14).

Flow cytometric analysis using transfected cells was performed 2 d after transfection. The transfected cells were detached from dish using 0.125% trypsin and 0.01% EDTA, and were washed with PBS and resuspended in PBS containing 0.1% NaN_3. The cells were incubated with anti-CD36 monoclonal antibody, OKM5 (Ortho Diagnostic Systems, Raritan, NJ), or rabbit polyclonal anti-CD36 antibodies (kindly provided by Dr. Naomasa Yamamoto, the Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan) at 4°C for 30 min, then washed three times with PBS containing 0.1% NaN_3. The cells were subsequently incubated with FITC-conjugated goat F(ab′)$_2$ anti-mouse IgG antibody (Beckton Dickinson Co, Sunnyvale, CA) or FITC-conjugated goat anti-rabbit IgG antibody (Cappel, Durham, NC) at 4°C for 30 min, and washed three times before analysis using a FACScan® flow cytometer (Beckton Dickinson Co).

Surface labeling of platelets or the transfected cells. Platelet surface proteins were biotinylated as described by Smith et al (24). Briefly, platelets obtained by differential centrifugation were suspended in PBS at a final concentration of 2×10^8/ml, and then incubated with 5.5 mg/ml of sulfo-*N*-hydroxysuccinimidobiotin (sulfo-NHS-biotin; Pierce Chemical Co., Rockford, IL) for 30 min on ice. 5 ml of Tris/glycine buffer was added and the suspension incubated for another 10 min on ice. The labeled platelets were washed twice with Tris/glycine buffer.

Surface of the transfected cells was biotinylated 2 d after transfection as previously described (25, 26). Briefly, after three washes with PBS, the cells were suspended in labeling buffer (100 mM Hepes, 150 mM NaCl, pH 8.0) at a concentration of 1×10^7/ml, and sulfo-NHS-biotin was added to the cell suspension. The final concentration of sulfo-NHS-biotin was 0.1 mg/ml. After 30 min incubation at room temperature, the cells were washed once with chilled RPMI1640 and twice with RPMI1640 supplemented with 1% FCS.

Immunoprecipitation. The labeled platelets or transfected cells were lysed with Triton X-100 buffer (10 mM Tris pH 7.5, 0.15 M NaCl, 1 mM phenylmetylsulfonyl fluoride, 50 mM iodoacetamide, 0.1% NaN_3, 1 mM EDTA, 1% Triton X-100) containing soybean trypsin inhibitor (10 μg/ml), leupeptin (1 μg/ml), and aprotinin (1 trypsin inhibitor U/ml). Insoluble material was removed by centrifugation at 10,000 *g* for 15 min at 4°C. Precleared lysates were precipitated with rabbit polyclonal anti-CD36 antibodies. Immunoprecipitates were separated by 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions and electrophoretically transferred to a polyvinylidene difluoride (PVDF) membrane (Immobilon; Millipore Corp., Bedford, MA). After blocking with 0.5% gelatin, 0.05% Tween 20, and 0.1% NaN_3 in PBS, the membrane was incubated with 0.1% horseradish peroxidase conjugated with streptoavidin (Amersham, Arlington Heights, IL) for 1 h. The PVDF membrane was washed three times with PBS containing 0.05% Tween 20, and proteins were revealed with the ECL Western blot detection system (Amersham), and exposed to Kodak X-AR film for 1 to 30 s.

Metabolic label with [^{35}S]methionine and pulse chase. Metabolic labeling of transfected cells was performed one day after transfection as previously described (27). The transfected cells were incubated with methionine-free DME (GIBCO/BRL, Gaitherburg, MD) containing 5 mmol/liter glutamate, 1 mmol/liter sodium pyruvate, 10% dialyzed FCS, and 0.1 mCi/ml of [^{35}S]methionine (Trans ^{35}S label, ICN Biomedicals, Irvine, CA) for 4 h, and lysed with Triton X-100 buffer containing protease inhibitors. Precleared cell lysates were immunoprecipitated with polyclonal anti-CD36 antibodies, and proteins were separated by 7.5% SDS-PAGE and autoradiographed.

For pulse chase labeling, the cells were incubated with 0.4 mCi/ml of [^{35}S]methionine for 30 min and then the medium was changed to DME/10% FCS with 15 mg/liter of nonradioactive methionine. Cells were collected after 0, 1, 2, and 6 h chase and lysed with Triton X-100 buffer. Immunoprecipitation was performed as described above.

Results

Analysis of monocyte CD36 cDNA. We have already demonstrated that platelets from 16 out of 17 CD36 positive subjects

Table I. Summary of Analysis of CD36 cDNA

Phenotype	Case	Platelet *CD36* cDNA	Monocyte *CD36* cDNA
Type I	M.Y.	T478	T478
	K.Y.	deletions in the coding region, and decrease of transcripts*	
Type II	Case 1	T478	C478/T478
	Case 2	T478	C478/T478
	Case 3	T478	nd
	Case 4	T478	nd
	Case 5	No abnormality‡	No abnormality‡
CD36 positive	16 cases	C478	C478
	1 case	C478/T478	C478/T478

* The results of genetic analysis of this subject were described elsewhere (22).
‡ There was no abnormality in the coding region by sequencing. *nd*, not determined.

had C478 form of *CD36* cDNA ($CD36^{C478}$) and that of the remaining one subject had both $CD36^{C478}$ and T478 form of *CD36* cDNA ($CD36^{T478}$). In contrast, platelets from four out of five subjects with type II CD36 deficiency had only $CD36^{T478}$ (16). In this study, we have examined the $^{478}C \rightarrow T$ substitution in monocyte *CD36* cDNA. Results are summarized in Table I. In contrast to platelet *CD36* cDNA, RT-PCR followed by Sau96I digestion showed that in two out of two type II deficient subjects monocytes had both $CD36^{C478}$ and $CD36^{T478}$ (cases 1 and 2). Furthermore, in one type I deficient subject (M.Y.) both platelets and monocytes had only $CD36^{T478}$ (Fig. 2). The substitution was confirmed by sequencing (data not shown). Monocyte

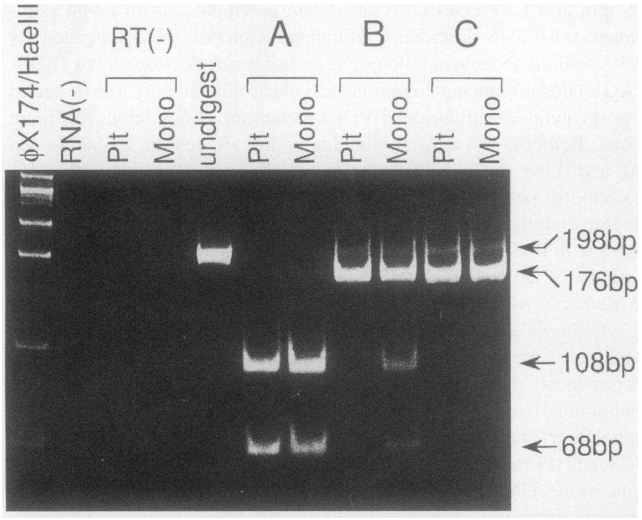

Figure 2. Detection of the $^{478}C \rightarrow T$ substitution in *CD36* cDNA by RT-PCR followed by Sau96I digestion. The 198-bp fragments of *CD36* cDNA (nt. 386 to 583) were amplified and digested with Sau96I. This fragments contain a new Sau96I site at nt.408 generated by mismatched-PCR for internal standard. The absence of 198-bp bands in all samples showed the complete digestion with Sau96I. The $^{478}C \rightarrow T$ substitution leads to loss of a Sau96I site at nt.476. The C478 form of *CD36* cDNA are digested to 108-, 68-, and 22- (invisible) bp fragments, and the T478 form of *CD36* cDNA are digested to 176-, and 22 (invisible)-bp fragments. *RNA(−)*, RT-PCR reaction without RNA samples, *RT(−)*: RT-PCR reaction with no reverse transcriptase; *A*, a CD36 positive subject; *B*, a type II deficient subject (case 1); *C*, a type I deficient subject (M. Y.).

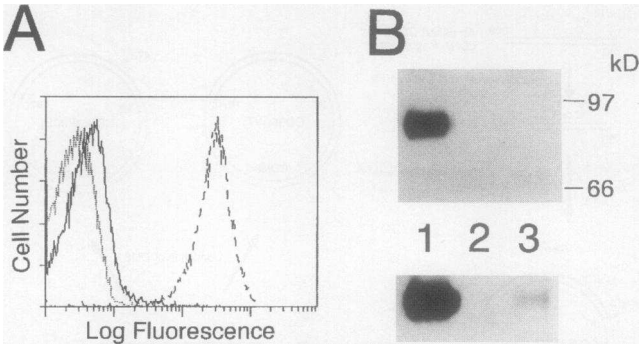

Figure 3. Detection of a trace amount of CD36 on platelets having $CD36^{T478}$. (A) Flow cytometric analysis of platelets from a CD36 positive subject having $CD36^{C478}$ stained with OKM5 (-----), and a type II deficient subject (case 1) stained with OKM5 (——) or IgG_1 control antibody (· · · · ·), followed by incubation with FITC-conjugated goat $F(ab')_2$ anti-mouse IgG. (*B*) Immunoprecipitation of surface labeled platelets using polyclonal anti-CD36 antibodies. Platelets from a CD36 positive platelets (lane *1*) or case 1 (lanes *2* and *3*) was surface labeled with biotin, and lysed. Immunoprecipitation was then performed using rabbit polyclonal anti-CD36 antibodies (lanes *1* and *3*) or normal rabbit serum (lane *2*). Precipitates were separated by 7.5% SDS-PAGE under reducing conditions, and electrophoretically transferred to a PVDF membrane. Proteins were detected by chemiluminescence using a 2-s exposure (*upper panel*) or a 30-s exposure (*lower panel*).

CD36 cDNA from CD36 positive subjects showed the same pattern as that observed in their platelet *CD36* cDNA, i.e., monocytes from 16 out of 17 subjects had $CD36^{C478}$, and those of the remaining one subject had both $CD36^{C478}$ and $CD36^{T478}$. In one type II deficient subject (case 5), we could not detect any abnormalities including the $^{478}C \rightarrow T$ substitution in the entire coding region of platelet and monocyte *CD36* cDNA by sequencing. In another type I deficient subject (K.Y.), we detected deletions in coding region, leading to appearance of premature termination codon, as previously reported (22). These results indicated that monocytes as well as platelets having only $CD36^{T478}$ could not express CD36, and this substitution was frequently detected in subjects with CD36 deficiency. These results also indicated that there was some discrepancy between platelet *CD36* cDNA and monocyte *CD36* cDNA in most type II deficient subjects.

Platelets having only $CD36^{T478}$ express a trace amount of CD36. In a process of analyzing CD36 expression by flow cytometry, we have been aware that platelets from most type II deficient subjects showed a modest reactivity with anti-CD36 monoclonal antibody, OKM5, compared with IgG_1 control. Fig. 3 *A* shows representative data obtained from case 1. Platelets from case 1 showed marked reduction in the expression of CD36 (mean fluorescence intensity: 4.4) as compared with CD36 positive platelets (mean: 510.0). However, a modest reactivity with OKM5 was observed as compared with IgG_1 control (mean: 2.7). To investigate the relationship between this modest reactivity with OKM5 and the molecular basis of CD36 deficiency, we reanalyzed the data of flow cytometry obtained from subjects shown in Table I. Platelets having $CD36^{T478}$ from four type II and one type I deficient subjects showed this modest reactivity with OKM5. However, platelets from one type II deficient subject (case 5) due to an unidentified abnormality other than the $^{478}C \rightarrow T$ substitution and platelets

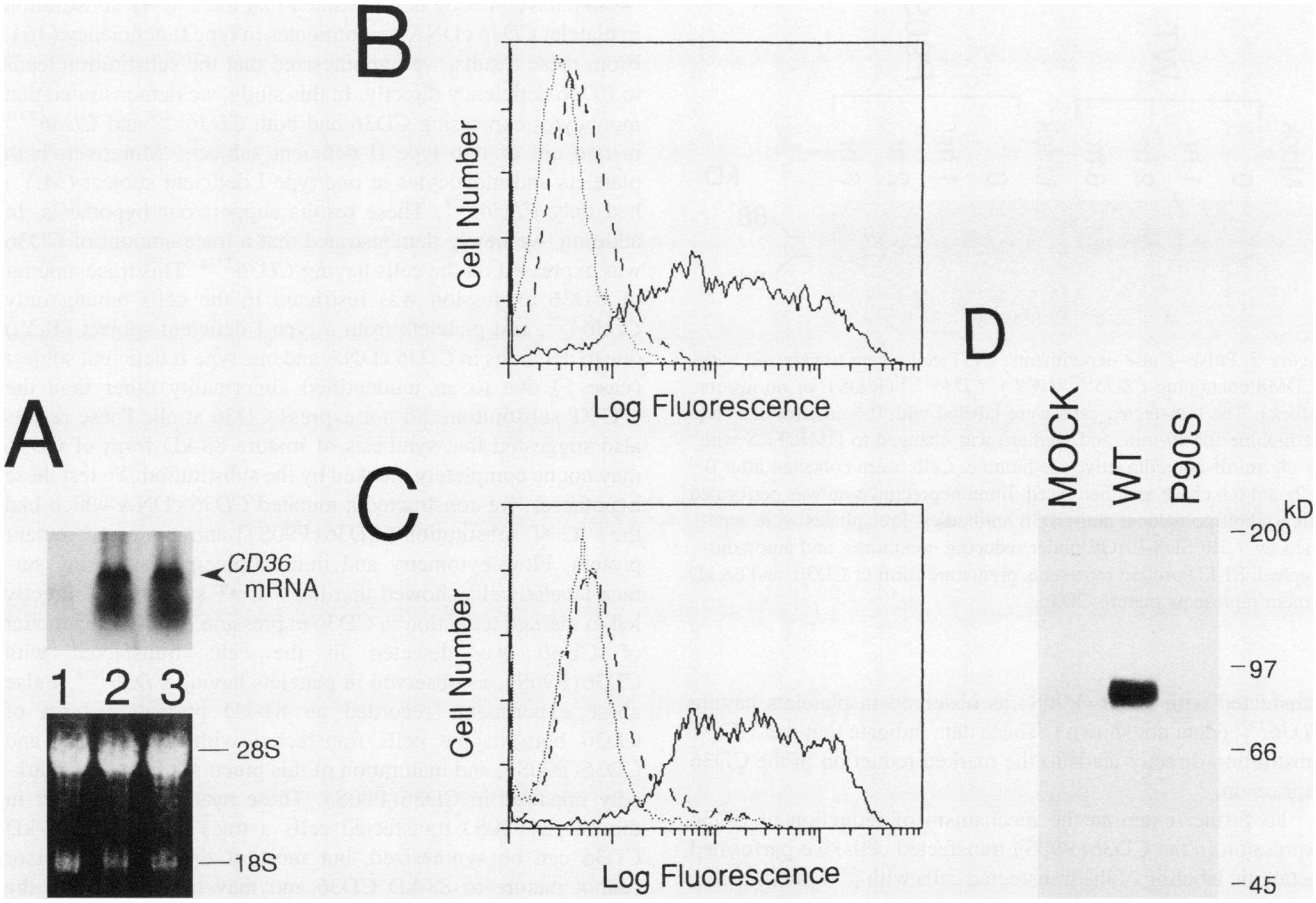

Figure 4. Surface expression of CD36 on the transfected cells. 293T cells were transfected with pCDM8 containing $CD36^{C478}$ (WT), $CD36^{T478}$ (P90S), or no inserts (MOCK). (*A*) RNA blot analysis hybridized with *CD36* cDNA (*upper panel*). Total cellular RNA were obtained from 293T cells transfected with MOCK (lane *1*), CD36(WT) (lane *2*), or CD36(P90S) (lane *3*). Equal amounts of RNA were present in each lane as documented by ethidium bromide staining (*lower panel*). (*B*) (*C*) Flow cytometric analysis of the transfected cells stained with OKM5 (*B*) or polyclonal anti-CD36 antibodies (*C*), followed by incubation with FITC-conjugated goat $F(ab')_2$ anti-mouse IgG or FITC-conjugated goat anti-rabbit IgG, respectively. WT (——); P90S (-----); MOCK (· · · · ·). (*D*) Immunoprecipitation of surface-labeled cells using polyclonal anti-CD36 antibodies. The transfected cells were surface labeled with biotin, and lysed. Immunoprecipitation was then performed using rabbit polyclonal anti-CD36 antibodies. Precipitates were separated by 7.5% SDS-PAGE under reducing conditions, and electrophoretically transferred to a PVDF membrane. Proteins were detected by chemiluminescence using a 1-s exposure.

from a subject with type I deficiency due to deletions in *CD36* cDNA (K.Y.) did not react with OKM5 at all (data not shown). To further examine the nature of the modest reactivity with OKM5, we performed immunoprecipitation using surface labeled platelets. In this experiment, we used polyclonal anti-CD36 antibodies. In an appropriate exposure time, no bands were detected in platelets having $CD36^{T478}$ (Fig. 3 *B, upper panel*). However, when the membrane was overexposed, a trace amount of 88-kD band was detected (Fig. 3 *B,* lower). Essentially the same result was obtained using OKM5 for immunoprecipitation (data not shown). These results suggested that the synthesis of 88-kD CD36 may not be completely blocked by the $^{478}C \rightarrow T$ substitution.

Expression study using vectors containing $CD36^{C478}$ or $CD36^{T478}$. To confirm that the $^{478}C \rightarrow T$ substitution directly leads to marked reduction in the CD36 expression, we constructed expression vectors which contained $CD36^{C478}$ [CD36(WT)], or $CD36^{T478}$ [CD36(P90S)], and expressed CD36 in 293T cells transiently. We confirmed the same efficiency of transfection of CD36(WT) and CD36(P90S) into 293T cells by RNA blot analysis (Fig. 4 *A*).

Flow cytometric analysis using OKM5 showed markedly reduced expression of CD36 antigen on the CD36(P90S) transfected cells (mean; 6.5) compared with the CD36(WT) transfected cells (mean; 416.6). However, fluorescence intensity of the CD36(P90S) transfected cells was slightly increased as compared with that of the cells transfected with vectors containing no inserts (mean; 4.0) (Fig. 4 *B*) or that of the CD36(P90S) transfected cells sensitized with control IgG_1 (mean; 4.0) instead of OKM5, which were consistent with the results obtained from platelets having $CD36^{T478}$. Essentially the same results were obtained using polyclonal anti-CD36 antibodies for flow cytometric analysis instead of OKM5 (Fig. 4 *C*). Immunoprecipitation of surface labeled cells using rabbit polyclonal anti-CD36 antibodies also demonstrated that CD36 was little expressed on the cell surface transfected with CD36(P90S) (Fig. 4 *D*). When the membrane was overexposed, a trace amount of 88 kD band was detectable on the cells

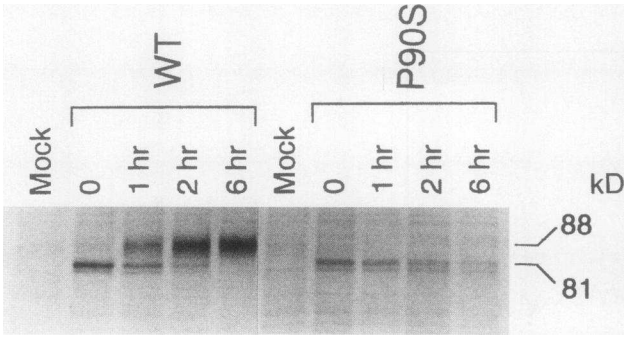

Figure 5. Pulse–chase experiments. 293T cells were transfected with pCDM8 containing *CD36*^{C478} (*WT*), *CD36*^{T478} (P90S), or no inserts (*Mock*). The transfected cells were labeled with 0.4 mCi/ml of [^{35}S]-methionine for 30 min, and medium was changed to DME/FCS with 15 mg/ml of nonradioactive methionine. Cells were collected after 0, 1, 2, and 6 h chase and then lysed. Immunoprecipitation was performed using rabbit polyclonal anti-CD36 antibodies. Precipitates were separated by 7.5% SDS-PAGE under reducing conditions, and autoradiographed. 81-kD protein represents premature form of CD36, and 88-kD protein represents mature CD36.

transfected with CD36(P90S) as observed in platelets having *CD36*^{T478} (data not shown). These data indicate that the ^{478}C→T substitution directly leads to the marked reduction in the CD36 expression.

To further examine the mechanism of reduction of CD36 expression in the CD36(P90S) transfected cells, we performed metabolic labeling of the transfected cells with [^{35}S]methionine. Immunoprecipitation using rabbit polyclonal anti-CD36 antibodies showed that 88- and 81-kD bands were detected in the CD36(WT) transfected cells, whereas, only the 81-kD band was detected in the CD36(P90S) transfected cells (data not shown), indicating that 88-kD CD36 was scarcely synthesized or very rapidly degraded in the CD36(P90S) transfected cells. Pulse chase experiments revealed that CD36 was synthesized as an 81-kD precursor form which was detected immediately after the pulse, and almost all 81-kD protein shifted to the mature 88-kD form of CD36 during the 6-h chase period in the CD36(WT) transfected cells, probably due to glycosylation of the 81-kD protein. In contrast, in the cells transfected with CD36(P90S), the 81-kD form of CD36 did not shift to the mature 88-kD form and was degraded slowly during chasing (Fig. 5). Essentially the same results were obtained using OKM5 for immunoprecipitation instead of rabbit polyclonal anti-CD36 antibodies (data not shown). These results indicate that the ^{478}C→T substitution leads to maturation defect in post-translational modification of premature CD36, and most of premature form of CD36 may be degraded in the cytoplasm.

Discussion

In this study, we demonstrated that (*a*) the ^{478}C→T substitution (proline90→serine) directly leads to the marked reduction in CD36 expression via maturation defect in posttranslational modification of CD36; (*b*) there is an 81-kD precursor form of CD36, and the normal 81-kD precursor form changes to the 88-kD mature form within 6 h; and (*c*) there is some discrepancy between platelet *CD36* cDNA and monocyte *CD36* cDNA in most type II deficient subjects.

We have already demonstrated that the ^{478}C→T substitution in platelet *CD36* cDNA predominates in type II deficiency (16). From these results, we hypothesized that the substitution leads to CD36 deficiency directly. In this study, we demonstrated that monocytes expressing CD36 had both *CD36*^{C478} and *CD36*^{T478} in two out of two type II deficient subjects. Moreover, both platelets and monocytes in one type I deficient subject (M.Y.) had only *CD36*^{T478}. These results support our hypothesis. In addition, we newly demonstrated that a trace amount of CD36 was expressed on the cells having *CD36*^{T478}. This trace amount of CD36 expression was restricted in the cells having only *CD36*^{T478}, and platelets from a type I deficient subject (K.Y.) due to deletions in *CD36* cDNA and one type II deficient subject (case 5) due to an unidentified abnormality other than the ^{478}C→T substitution did not express CD36 at all. These results also suggested that synthesis of mature 88-kD form of CD36 may not be completely blocked by the substitution. To test these hypotheses, we constructed a mutated *CD36* cDNA which had the ^{478}C→T substitution [CD36(P90S)] and expressed mutant protein. Flow cytometry and immunoprecipitation using surface-labeled cells showed that the ^{478}C→T substitution directly led to marked reduction in CD36 expression, but a trace amount of CD36 was detected in the cells transfected with CD36(P90S), as observed in platelets having *CD36*^{T478}. Pulse chase experiments recorded an 81-kD premature form of CD36 both in the cells transfected with CD36(WT) and CD36(P90S), and maturation of this precursor form was markedly impaired in CD36(P90S). These results indicate that in the CD36(P90S) transfected cells a trace amount of 88-kD CD36 can be synthesized, but most of the CD36 precursor cannot mature to 88-kD CD36 and may be degraded in the cytoplasm. The mature 88-kD form of CD36 was hardly detectable in the CD36(P90S) transfected cells by pulse chase experiments, probably due to less sensitivity of this experiment compared with flow cytometry or surface labeling with biotin.

CD36 is a highly glycosylated protein containing 26% of carbohydrate (2), and the *CD36* cDNA sequence shows ten potential *N*-glycosylation sites (19). It has been known that amino acids neighboring consensus sequence (Asn-X-Ser/Thr) of *N*-glycosylation may have significant effect on the rate and extent of glycosylation, because only about one third of the potential Asn-X-Ser/Thr sites in proteins are actually glycosylated (28). Proline90 is located between the first putative *N*-glycosylation site (Asn-79) and the second *N*-glycosylation site (Asn-102) (19). A conformational change derived from the replacement of proline90 by serine may impair accessibility of these neighboring glycosylation sites to the oligosaccharyltransferase. It may be also possible that the substitution affects glycosylation sites other than Asn-79 or Asn-102, if the substitution leads to large conformational change of the CD36 precursor. Recently, it has been shown that CD36 is a member of a gene family whose members are a rat lysosomal protein, LIMPII (29), a drosophila epithelial membrane protein, emp (30), and a human plasma membrane protein, CLA-1 (31). The fact that proline90 is preserved in all these proteins supports our hypothesis that this residue may be essential for normal processing of CD36 (Fig. 6).

In this study, we first described the premature form of CD36 which had a molecular mass of 81 kD. CD36 has been studied mainly using platelet materials, and we have been aware of no other reports of pulse chase assay for analysis of CD36 biosynthesis. It has been shown that the CD36 homologue pro-

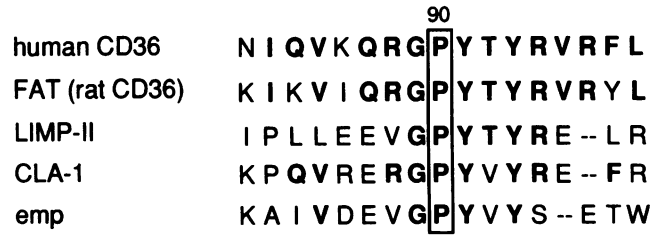

Figure 6. Comparison of amino acid sequences of a *CD36* gene family around proline90 predicted from cDNA sequences. Proline90 is preserved in all members of the *CD36* gene family. Bold letters indicate identical residues. The data of human CD36, FAT, LIMP-II, CLA-I, and emp are from Oquendo et al. (19), Abumrad et al. (10), Vega et al. (29), Calvo et al. (31), and Hart et al. (30), respectively.

tein, LIMP-II, was synthesized as precursor of 72 kD and matured to 74-kD form after 2 h of chase (32). Our present results showed that the 81-kD precursor form of CD36 was synthesized within 30 min, and matured to 88-kD CD36 within 6 h.

Our results also showed that there was a discrepancy between platelet and monocyte *CD36* cDNA in most type II deficient subjects, i.e., monocytes had both $CD36^{C478}$ and $CD36^{T478}$, while platelets had only $CD36^{T478}$. It has been demonstrated that the CD36 is a single copy gene located on chromosome pair 7, and the nt.478 of *CD36* cDNA is corresponding to nt.12293 in exon 4 of the *CD36* gene (33, 34). We have already observed that the *CD36* gene of these type II deficient subjects were heterozygous for C/T at nt.12293 (H. Kashiwagi et al., unpublished observation). These results indicate that there is an abnormal *CD36* allele of which transcripts are hardly detectable in platelets in spite of detection of its transcripts in monocytes. We consider that many of type II deficient subjects are compound heterozygous with this abnormal *CD36* allele which may have platelet specific transcription defect(s) and the allele mutated at proline90. Interestingly, the amount of platelet *CD36* transcripts in one type II deficient subject (case 5) having no abnormalities in the coding region was markedly reduced in spite of detection of normal amount of *CD36* transcripts in monocytes (H. Kashiwagi et al., unpublished observations), suggesting that this subject may be homozygous for the *CD36* allele with platelet specific transcription defect(s). Further studies will be required to determine the basic defects in the abnormal *CD36* allele, including the clarification of normal regulation of *CD36* gene expression.

Subjects with CD36 deficiency are apparently healthy and suffered no hemostatic problems despite the absence of CD36. It is, however, demonstrated that CD36 deficiency is one of causes of refractoriness of HLA-matched platelet transfusion in Japan, especially in type I deficient subjects (11). Moreover, recent findings that CD36 is one of receptors for oxidized LDL and fatty acids suggest that CD36 may play a part in atherogenesis and lipid metabolism (9, 10). It will be of great interest to investigate the uptake of oxidized LDL in CD36 deficient monocytes (and macrophages). The experiments are now under way in our laboratory.

In summary, we demonstrated that the $^{478}C \rightarrow T$ substitution (proline90→serine) in *CD36* cDNA directly leads to CD36 deficiency via defects in posttranslational modification, and this substitution is the major defect underlying CD36 deficiency.

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