Table 1. Characteristics of the 175 patients with systemic lupus erythematosus.

Complication	*Number of patients*	*Female/ Male*	*Median age*	*Age range*
Thrombotic complications	67	61/6	42.9	26-71
Arterial thrombosis	32	27/5	43.3	29-67
Venous thrombosis	35	34/1	42.6	26-71
Fetal loss	14	14/0	31.4	24-40
Thrombocytopenia	14	13/1	41.7	26-70
Without above complications	80	76/4	39.0	10-75

distribution with the Stat Flex program (Ver. 4.2, Artech Inc.) before statistical analysis was performed. We chose the mean + 3SD of each aPL concentration in the 80 controls as the cut-off point. The cut-off values for aCL, anti-β2-GPI, anti-PT, and anti-PS/PT were 398.0, 392.1, 500.8, and 403.2 mAU, respectively. We regarded a result as positive when the absorbance exceeded each cut-off value. In plasma of SLE patients, aCL, anti-β2-GPI, anti-PT, anti-PS/PT, and LA activity was detected in 65 37.1%, 55 31.4%, 95 54.3%, 76 43.4%, and 89 50.9% of 175 patients, r espectively. The prevalence of anti-PS/PT was most strongly correlated with the prevalence of LA activity [odds' ratio OR = 11.4, 95% confidence interval CI = 4.64-27.9]. In our anti-PS/PT ELISA system, purified human prothrombin was added to the phosphatidylserine-coated well. We measured the anti-PS/PT levels using both phosphatidylserine-coated prothrombin+ wells and phosphatidylserine-coated prothrombin– wells. The absorbance of each sample in the prothrombin+ wells and prothrombin– wells was compared for the evaluation of prothrombin dependency of antibodies. In this experiment, all of the samples in the prothrombin+ wells showed clearly higher absorbance levels compared to the prothrombin– wells. These results suggest that the anti-PS/PT detected by our ELISA system was not directed against phosphatidylserine but recognized the complex of phosphatidylserine and prothrombin.

Multivariate logistic analysis confirmed that both anti-PS/PT and anti-β2-GPI were significant, independent risk factors for arterial thrombosis [anti-PS/PT, OR=9.36, 95%CI=2.67–32.8; anti-β2-GPI, OR=4.23, 95%CI=1.02–17.5] and venous thrombosis (anti-PS/PT, OR=7.33, 95%CI=2.51–21.4; anti-β2-GPI, OR=4.10, 95%CI=1.19–14.2) in patients with SLE. It is important to note that the odds ratio for the presence of anti-PS/PT was much higher than that for the presence of anti-β2-GPI. The prevalence of anti-PT was increased in SLE patients with arterial thrombosis and venous thrombosis. However, multivariate logistic regression analysis indicated that the presence of anti-PT was not a risk factor for these thrombotic complications (OR=2.15, 95%CI=0.60–7.70 for arterial thrombosis; OR=1.66, 95%CI=0.58-4.75 for venous thrombosis) because anti-PT was detected in a large number of SLE patients without thrombotic complications 32/95 cases, 40.0%. The prevalence of aCL was significantly higher in SLE patients with arterial thrombosis and venous thrombosis, suggesting that the presence of aCL is an important risk factor for these thrombotic complications. Although a significant correlation was observed between the concentrations of aCL and anti-β2-GPI, 21 32.3% of the 65 aCL-positive patients had no anti- β2-GPI. Because the vast majority of aCL-positive patients who had no anti-β2-GPI 16/21 cases, 76.2% did not have thrombotic complications, the presence of aCL was not reliable as a risk factor for thrombotic complications. Multivariate logistic analysis also indicated that the only significant risk factor for thrombocytopenia was the presence of aCL OR=2.36, 95%CI=0.56–9.91. It has been suggested that the antibodies detected by the standard aCL-ELISA differ from those detected by an anti-β2-GPI-specific ELISA. The anti-β2-GPI ELISA detects antibodies that are specific for a conformationally changed epitope of β2-GPI whereas the standard aCL-ELISA may also detect antibodies that react with some components of human plasma and/or bovine serum proteins bound to the cardiolipin-coated wells.[17] The prevalence of anti-β2-GPI was increased in SLE patients with thrombotic complications but not significantly increased in the SLE patients with thrombocytopenia.

Much effort has been made to clarify the mechanisms underlying arterial and/or venous thrombosis and thrombocytopenia in SLE patients with aPL, including activation of platelets, promotion of the blood coagulation system, and impairment of the endothelial system. However, the precise mechanisms responsible for thrombotic and thrombocytopenic events in these patients remain unclear. Two possible mechanisms by which aPL could cause thrombotic events have been proposed. The first possibility is that aPL bind to phospholipid/plasma protein complexes on the membranes of activated platelets and influence platelet activation and aggregation. The second possibility is that aPL inhibit phospholipid-dependent reactivity of the activated protein C pathway, such as activation of protein C by thrombin/thrombomodulin and/or degradation of factor Va by activated protein C/protein S.

In previous studies,[16,18] we found that SLE patients with both anti-CL/β2-GPI and anti-PS/PT had a quite high prevalence of cerebral infarction, as compared with those with either anti-CL/β2-GPI or anti-PS/PT. Furthermore, when we studied the *in vitro* effects of anti-CL/β2-GPI and/or anti-PS/PT on the enhancement of platelet activation induced by stimulation with a low concentration of adenosine diphosphate ADP, we found that the purified IgG containing both anti-CL/β2-GPI and anti-PS/PT significantly enhanced ADP-induced platelet activation. However, the purified IgG containing either anti-CL/β2-GPI or anti-PS/PT alone did not enhance ADP-induced platelet activation. These results indicate that anti-CL/β2-GPI and anti-PS/PT may co-operate to promote platelet activation, and may be involved in the pathogenesis of cerebral

Table 2. Multivariate logistic analysis.

	LA activity *Sensitivity* *Specificity* *OR 95%CI* *p*	*Arterial thrombosis* *Sensitivity* *Specificity* *OR 95%CI* *p*	*Venous thrombosis* *Sensitivity* *Specificity* *OR 95%CI* *p*	*Fetal loss* *Sensitivity* *Specificity* *OR 95%CI* *p*	*Thrombocytopenia* *Sensitivity* *Specificity* *OR 95%CI* *p*
aCL	60.7% 87.2% 5.60 2.04-15.4 < 0.001	75.0% 71.3% 2.36 0.59-9.91 0.240	42.9% 64.3% 0.64 0.18-2.20 0.475	21.4% 61.5% 1.24 0.19-8.04 0.819	57.1% 64.6% 5.76 1.35-24.5 < 0.05
Anti-β2GPI	49.4% 87.2% 1.53 0.52-4.54 0.442	71.9% 77.6% 4.23 1.02-17.5 < 0.05	48.6% 72.9% 4.10 1.19-14.2 < 0.05	7.1% 66.5% 0.55 0.05-6.29 0.630	21.4% 67.7% 0.46 0.08-2.52 0.368
Anti-PT	67.4% 59.3% 0.88 0.36-2.13 0.773	78.1% 51.0% 2.15 0.60-7.70 0.240	74.3% 50.7% 1.66 0.58-4.75 0.346	14.3% 42.2% 0.25 0.05-1.25 0.092	71.4% 47.2% 3.66 0.90-14.8 0.069
Anti-PS/PT	71.9% 86.1% 11.4 4.64-27.9 < 0.001	84.4% 65.7% 9.36 2.67-32.8 < 0.001	68.6% 62.9% 7.33 2.51-21.4 < 0.001	21.4% 54.7% 1.49 0.26-8.56 0.653	50.0% 57.1% 1.84 0.45-7.46 0.393

aCL, anti-cardiolipin antibodies; anti-β2GPI, anti-β2glycoprotein I antibodies; anti-PT, anti-prothrombin antibodies; anti-PS/PT, anti-phosphatidylserine/prothrombin antibodies; LA, lupus anticoagulant; OR, odds' ratio; CI, confidence interval. LA activity was detected by both dilute Russell viper venom time and STACLOT LA tests. Statistical analysis was performed using univariate and multivariate logistic analysis. An OR was considered statistically significant when the lower limit of the 95% CI was > 1.0. A value of p<0.05 was considered to be statistically significant to indicate a risk factor.

infarction in patients with SLE. On the other hand, when we examined the *in vitro* effects of anti-CL/β2-GPI and/or anti-PS/PT on the anticoagulant activity of activated protein C, we found that purified IgG containing anti-CL/β2-GPI or anti-PS/PT significantly hampered the anticoagulant activity of activated protein C.[15] Our results indicate that anti-CL/β2-GPI and anti-PS/PT independently cause activated protein C resistance, which may contribute as an independent risk factor for venous thrombotic events such as pulmonary embolism, deep vein thrombosis, and thrombophlebitis. These findings raise the possibility that the high frequency of thrombotic complications in SLE patients with anti-CL/β2-GPI and/or anti-PS/PT might be due to enhancement of platelet activation and inhibition of anticoagulant systems.

As mentioned above, several clinical studies have established that the presence of LA activity is the strongest risk factor for thrombotic events in patients with SLE.[10,11] In this study, we confirmed that the prevalence of anti-PS/PT was most strongly correlated with the presence of LA activity. It is thus reasonable to speculate that the presence of anti-PS/PT is the most significant risk factor for both arterial thrombosis and venous thrombosis. However, our finding that anti-PS/PT and anti-β2-GPI were significant independent risk factors for thrombotic events in patients with SLE suggests that not only anti-PS/PT-ELISA but also anti-β2-GPI -ELISA should be performed for the diagnosis of APS.

Although β2-GPI and PT are the major protein targets involved in the binding of aPL to phospholipids, other phospholipid-binding proteins such as protein C, protein S, annexin V, and high- and low-molecular-weight kininogens have also been described as target proteins for aPL. ELISA for aPL against other phospholipid-binding proteins are still under development and will need standardization and extensive evaluation.

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ORIGINAL ARTICLE

Critical role of ADP interaction with $P2Y_{12}$ receptor in the maintenance of $\alpha_{IIb}\beta_3$ activation: association with Rap1B activation

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Summary. *Objective:* Platelet integrin $\alpha_{IIb}\beta_3$ plays a crucial role in platelet aggregation, and the affinity of $\alpha_{IIb}\beta_3$ for fibrinogen is dynamically regulated. Employing modified ligand-binding assays, we analyzed the mechanism by which $\alpha_{IIb}\beta_3$ maintains its high-affinity state. *Methods and results:* Washed platelets adjusted to 50×10^3 μL^{-1} were stimulated with 0.2 U mL^{-1} thrombin or 5 μM U46619 under static conditions. After the completion of $\alpha_{IIb}\beta_3$ activation and granule secretion, different kinds of antagonists were added to the activated platelets. The activated $\alpha_{IIb}\beta_3$ was then detected by fluorescein isothiocyanate (FITC)-labeled PAC1. The addition of 1 μM AR-C69931MX (a $P2Y_{12}$ antagonist) or 1 mM A3P5P (a $P2Y_1$ antagonist) disrupted the sustained $\alpha_{IIb}\beta_3$ activation by ~92% and ~38%, respectively, without inhibiting CD62P or CD63 expression. Dilution of the platelet preparation to 500 μL^{-1} also disrupted the sustained $\alpha_{IIb}\beta_3$ activation, and the disruption by such dilution was abrogated by the addition of exogenous adenosine 5′-diphosphate (ADP) in a dose-dependent fashion. The amounts of ADP released from activated platelets determined by high-performance liquid chromatography were compatible with the amounts of exogenous ADP required for the restoration. We next examined the effects of antagonists on protein kinase C (PKC) and Rap1B activation induced by 0.2 U mL^{-1} thrombin. Thrombin induced long-lasting PKC and Rap1B activation. AR-C69931MX markedly inhibited Rap1B activation without inhibiting PKC activation. *Conclusions:* Our data indicate that the continuous interaction between released ADP and $P2Y_{12}$ is critical for the maintenance of $\alpha_{IIb}\beta_3$ activation.

Keywords: $\alpha_{IIb}\beta_3$ activation, adenosine 5′-diphosphate, $P2Y_{12}$, protein kinase C, Rap 1B.

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Introduction

Platelet $\alpha_{IIb}\beta_3$ (GPIIb-IIIa), a non-covalently associated heterodimer, is a prototypic integrin that functions as a physiologic receptor for fibrinogen and von Willebrand factor (VWF). $\alpha_{IIb}\beta_3$ plays a crucial role in platelet aggregation, a key event of hemostatic plug formation and pathologic thrombus formation [1–3]. Inherited abnormalities in the expression or the function of $\alpha_{IIb}\beta_3$ preclude platelet aggregation, resulting in the bleeding disorder Glanzmann thrombasthenia (GT) [4,5]. Conversely, clinical studies have shown the beneficial effects of $\alpha_{IIb}\beta_3$ antagonists in patients undergoing coronary angioplasty or suffering from unstable angina [6,7]. During thrombogenesis, the affinity of $\alpha_{IIb}\beta_3$ for macromolecular ligands is dynamically regulated [1–3]. In resting platelets, $\alpha_{IIb}\beta_3$ is in a low-affinity state and does not bind soluble macromolecular ligands. However, after exposure to the subendothelial matrix, several mediators such as adenosine 5′-diphosphate (ADP) and thromboxane A_2, or shear stress, platelets become activated and activation signals (inside-out signaling) that induce a high-affinity state of $\alpha_{IIb}\beta_3$ for soluble ligands ($\alpha_{IIb}\beta_3$ activation) are generated. So far, much attention has been directed to the nature of inside-out signaling, and major advances have recently been made regarding the structural basis of $\alpha_{IIb}\beta_3$ activation, resulting in the proposal of 'switchblade' and 'deadbolt' models [8,9].

Previous studies revealed that the activation of $\alpha_{IIb}\beta_3$ is a reversible process [10,11]. When platelets are stimulated with weak agonists such as ADP in the absence of fibrinogen, $\alpha_{IIb}\beta_3$ gradually loses its binding capacity. In contrast,

thrombin induces long-lasting $\alpha_{IIb}\beta_3$ activation even in the absence of fibrinogen. Although it has been suggested that the maintenance of $\alpha_{IIb}\beta_3$ activation is mediated by a protein kinase C (PKC)-dependent pathway, the mechanism by which $\alpha_{IIb}\beta_3$ is kept in a high-affinity state still remains elusive [11].

ADP is actively secreted from platelet dense granules upon platelet activation and is passively released from damaged erythrocytes and endothelial cells [12,13]. Platelets possess at least two major G protein-coupled ADP receptors: $P2Y_1$ is a G_q-coupled receptor responsible for mediating platelet shape change and reversible platelet aggregation through intracellular calcium mobilization [14], whereas $P2Y_{12}$ is a G_i-coupled receptor responsible for mediating the inhibition of adenylyl cyclase and sustained platelet aggregation [15,16]. We previously identified a patient with $P2Y_{12}$ deficiency, OSP-1, caused by a point mutation within the translation initiation codon (ATG to AGG) [17]. Based on our findings in the functional analysis of OSP-1 platelets, we thought that $P2Y_{12}$ might play a role in the maintenance of $\alpha_{IIb}\beta_3$ activation.

In this study, employing modified ligand-binding assays we analyzed the mechanism of sustained $\alpha_{IIb}\beta_3$ activation and demonstrated the critical role of the continuous interaction of released ADP with $P2Y_{12}$ receptor in the maintenance of $\alpha_{IIb}\beta_3$ activation.

Materials and methods

Reagents

ADP, protease-activated receptor 1-activating peptide (PAR1 TRAP, SFLLRNPNDKYEPF), PAR 4-activating peptide (PAR4 TRAP, AYPGKF), thrombin, thromboxane A_2 analog U46619 (9, 11-dideoxy-11α, 9α-epoxymethanoprostaglandin $F_{2\alpha}$), apyrase, prostaglandin E_1 (PGE_1), and prostaglandin I_2 (PGI_2) were purchased from Sigma Aldrich (St Louis, MO, USA). Fluorescein isothiocyanate (FITC)-labeled PAC1, a ligand-mimetic $\alpha_{IIb}\beta_3$-specific monoclonal antibody (mAb) that binds specifically to activated $\alpha_{IIb}\beta_3$, was purchased from BD Biosciences (Mountain View, CA, USA) [18]. PE-labeled anti-CD62P (P-selectin), PE-anti-CD63, and PE-IgG were purchased from Beckman Coulter (Fullerton, CA, USA). AR-C69931MX was a generous gift from Astra-Zeneca (Loughborough, UK). Adenosine 3′-phosphate 5′-phosphate (A3P5P) and yohimbine were purchased from Sigma-Aldrich. The specificities and actions of these antagonists have been described [12]. A specific thromboxane A_2 receptor antagonist, SQ-29548, and a PKC inhibitor, Ro31-8220, were purchased from Cayman Chemical (Ann Arbor, MI, USA) and Merck KGaA (Darmstadt, Germany), respectively. MCI-9042, an antagonist for the serotonin ($5\text{-}HT_2$) receptor, was a gift from Mitsubishi Pharma Corporation (Tokyo, Japan) [19]. PT25-2 mAb, which is specific for and activates $\alpha_{IIb}\beta_3$, was a kind gift from Drs Makoto Handa and Yasuo Ikeda (Keio University, Tokyo, Japan) [20]. FK633, an $\alpha_{IIb}\beta_3$-specific antagonist, was kindly provided from Astellas Pharma Inc. (Osaka, Japan) [21].

Platelet preparation

Washed human platelets were prepared as previously described [22]. In brief, fresh whole blood anticoagulated with 0.15 volume of acid–citrate–dextrose solution [National Institute of Health (NIH) formula A] was obtained from healthy volunteers who had not taken any medication for at least 1 week and centrifuged at $250 \times g$ for 10 min to obtain platelet-rich plasma (PRP). After incubation with 20 ng mL^{-1} PGE_1 for 15 min, the PRP was centrifuged at $750 \times g$ for 10 min, washed three times with 0.05 M isotonic citrate buffer containing 20 ng mL^{-1} PGE_1, resuspended in Walsh buffer (137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 3.3 mM NaH_2PO_4, 3.8 mM HEPES, 0.1% glucose, 0.1% bovine serum albumin, pH 7.4) without PGE_1, and allowed to rest for 30 min before use.

Flow cytometry

Flow cytometric analysis using monoclonal antibodies (mAbs) was performed as previous described with some modifications [21]. Washed platelets adjusted to 50×10^3 μL^{-1} were stimulated with 0.2 U mL^{-1} thrombin or 5 μM U46619 under static conditions for 15 min. After the stimulation, each of the antagonists or Walsh buffer (for the control) was added to the suspensions for an additional 5 min. The platelet suspensions were then incubated with FITC-PAC1 and PE-anti-CD62P (or PE-anti-CD63) for 30 min and analyzed using a flow cytometer (FACScan; Becton Dickinson, Mountain View, CA, USA).

In another set of experiments, after a 15-min stimulation with thrombin, stimulated platelets were diluted with buffer containing 0.2 U mL^{-1} thrombin or 5 μM U46619 for an additional 5 min. The platelet suspensions were then incubated with FITC-PAC1 for 30 min.

Measurement of released ADP in platelet suspensions by high-performance liquid chromatography (HPLC)

Platelet suspensions (200×10^3 platelets μL^{-1}) were stimulated with thrombin or U46619 for 15 min under static conditions and centrifuged at $1000 \times g$ for 10 min. The supernatant was collected into ultra-free centrifugal filter units (Millipore, Bedford, MA, USA) and centrifuged at $10\,000 \times g$ for 1 min. Then samples were analyzed by HPLC. The chromatographic separation of ADP was performed using the SMART system (Amersham Pharmacia Biotech, Uppsala, Sweden) using an ion-exchange column (mono Q 1.6/5) at room temperature. Aliquots (50 μL) of samples or standard mixtures were injected into the column, and ADP was separated using a gradient in which the concentration of elution buffer B (20 mM Tris–HCl, 1 M NaCl, pH 8.0) was increased from 0% to 50% over a period of 20 min and detected at 254 nm. The

flow rate was 0.1 mL min^{-1} and retention time for ADP was 11.8 min.

Rap 1B activation assay

The detection of activated Rap 1B was performed using a pull-down assay kit according to the manufacturer's instructions (EZ-Detect™ Rap1 Activation Kit; Pierce, Rockford, IL, USA). In brief, platelets that had been prestimulated with 0.2 U mL^{-1} thrombin for 15 min were incubated with 1 μM AR-C69931MX, 1 mM A3P5P, 1 μM PGI_2, or Walsh buffer for 5 min, and then the platelets were lyzed with 0.5% Triton X-100 lysis buffer. The guanosine triphosphate (GTP)-form of Rap 1B was pulled down by incubation with glutathione S-transferase (GST)-RalGDS-Rap 1-binding domain (RBD) and glutathione beads for 1 h at 4 °C. After washing with lysis buffer, proteins were eluted from the precipitates with sodium dodecyl sulfate (SDS)-sample buffer with 2-mercaptoethanol at 100 °C for 5 min, and resolved by electrophoresis on a 12% SDS–polyacrylamide gel electrophoresis. After transfer to polyvinylidene fluoride membranes, Rap 1B was detected with rabbit anti-Rap 1B polyclonal antibody. The total Rap 1B in each lysate was detected in samples assayed in parallel. The optical density of the bands was measured using NIH IMAGE software (Bethesda, MD, USA).

PKC activation assay

PKC activation in thrombin-stimulated platelets was detected by immunoblotting using antiphosphoserine PKC substrate antibody (Cell Signaling Technology, Inc., MA, USA). Conventional PKC isozymes phosphorylate substrates contain serine or threonine, with arginine or lysine at the −3,−2 and +2 positions, and hydrophobic amino acids at position +2. This antibody recognizes conventional PKC substrates only when phosphorylated at the serine residues.

Results

Agonist-induced $\alpha_{IIb}\beta_3$ activation on P2Y$_{12}$-deficient platelets

We previously identified a patient with P2Y$_{12}$-deficiency (OSP-1) as result of a homozygous mutation within the translation initiation codon (ATG to AGG) [17]. We stimulated OSP-1 platelets with different kinds of agonists in the presence of FITC-PAC1 for 30 min and then analyzed PAC1 binding using flow cytometry. As shown in Fig. 1, the amount of PAC1 binding to OSP-1 platelets stimulated with 100 μM PAR1-TRAP, 200 μM PAR4-TRAP or 5 μM U46619 was only 12.5% ± 1.1%, 4.6% ± 0.1% and 4.3% ± 2.3% of the control value (mean ± SD, $n = 4$), respectively. In contrast, phorbol 12-myristate 13-acetate (PMA)-induced PAC1 binding was only slightly impaired (92.9% ± 1.9% of control, mean ± SD, $n = 4$). PAR1-TRAP and U46619 are able to induce transient aggregation of OSP-1 platelets, indicating that $\alpha_{IIb}\beta_3$ could be transiently activated with these agonists [17].

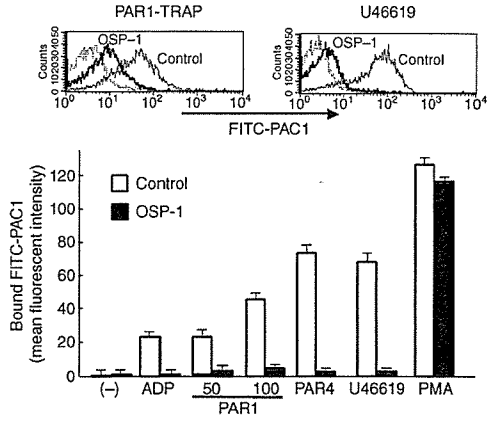

Fig. 1. Agonist-induced $\alpha_{IIb}\beta_3$ activation on platelets from a P2Y$_{12}$-deficient patient, OSP-1. Washed platelets obtained from a patient with P2Y$_{12}$-deficiency (OSP-1) or control subjects (control) were stimulated with 20 μM adenosine 5′-diphosphate (ADP), 50 or 100 μM PAR1-TRAP, 200 μM PAR4-TRAP, 5 μM U46619, or 0.2 μM phorbol 12-myristate 13-acetate (PMA) in the presence of fluorescein isothiocyanate (FITC)-labeled PAC1, and bound PAC1 was analyzed using flow cytometry after 30 min of incubation. (upper panel) Representative histograms of PAC1 binding with 100 μM PAR1-TRAP or 5 μM U46619 stimulation. PAC1 binding to control platelets in the presence of an $\alpha_{IIb}\beta_3$ antagonist (FK633) is shown by a dotted line. (lower panel) Specific PAC1 binding to OSP-1 (closed columns) or control (open columns) platelets calculated using the following formula is shown ($n = 4$): (MFI in the absence of FK633) − (MFI in the presence of FK633).

These findings suggest that the $\alpha_{IIb}\beta_3$ activation may be too short and unstable to be detected by the PAC1-binding assay. From these findings, we assume that released ADP and P2Y$_{12}$-mediated signaling may play a critical role in the maintenance of $\alpha_{IIb}\beta_3$ activation.

Effects of antagonists on the sustained $\alpha_{IIb}\beta_3$ activation induced by thrombin

First, we examined PAC-1 binding at different time points between thrombin stimulation and FITC-PAC1 addition and confirmed that there was no difference in PAC1 binding between time 0 min (thrombin and PAC1 were added at the same time) and time 15 min (PAC1 was added 15 min after thrombin stimulation) (data not shown) [11]. To determine the role of P2Y$_{12}$-mediated signals in the maintenance of $\alpha_{IIb}\beta_3$ activation, an experimental protocol using platelets preactivated with thrombin was then employed (Fig. 2A). After the completion of $\alpha_{IIb}\beta_3$ activation and the induction of α-granules and lysosome secretion by thrombin, each of antagonists was added to the activated platelets. Under these conditions, the stimulated platelets showed long-lasting $\alpha_{IIb}\beta_3$ activation even in the absence of ligand binding. However, the addition of 1 U mL^{-1} apyrase, 1 μM AR-C69931MX (a P2Y$_{12}$ antagonist) or 1 mM A3P5P (a P2Y$_1$ antagonist) disrupted the sustained $\alpha_{IIb}\beta_3$ activation by 87.1% ± 1.4% (mean ± SD, $n = 3$), 91.7% ± 5.3% (mean ± SD, $n = 6$) and 38.2% ± 10.5% (mean ± SD, $n = 4$), respectively, without inhibiting CD62P or CD63 expression (Fig. 2B,C). In

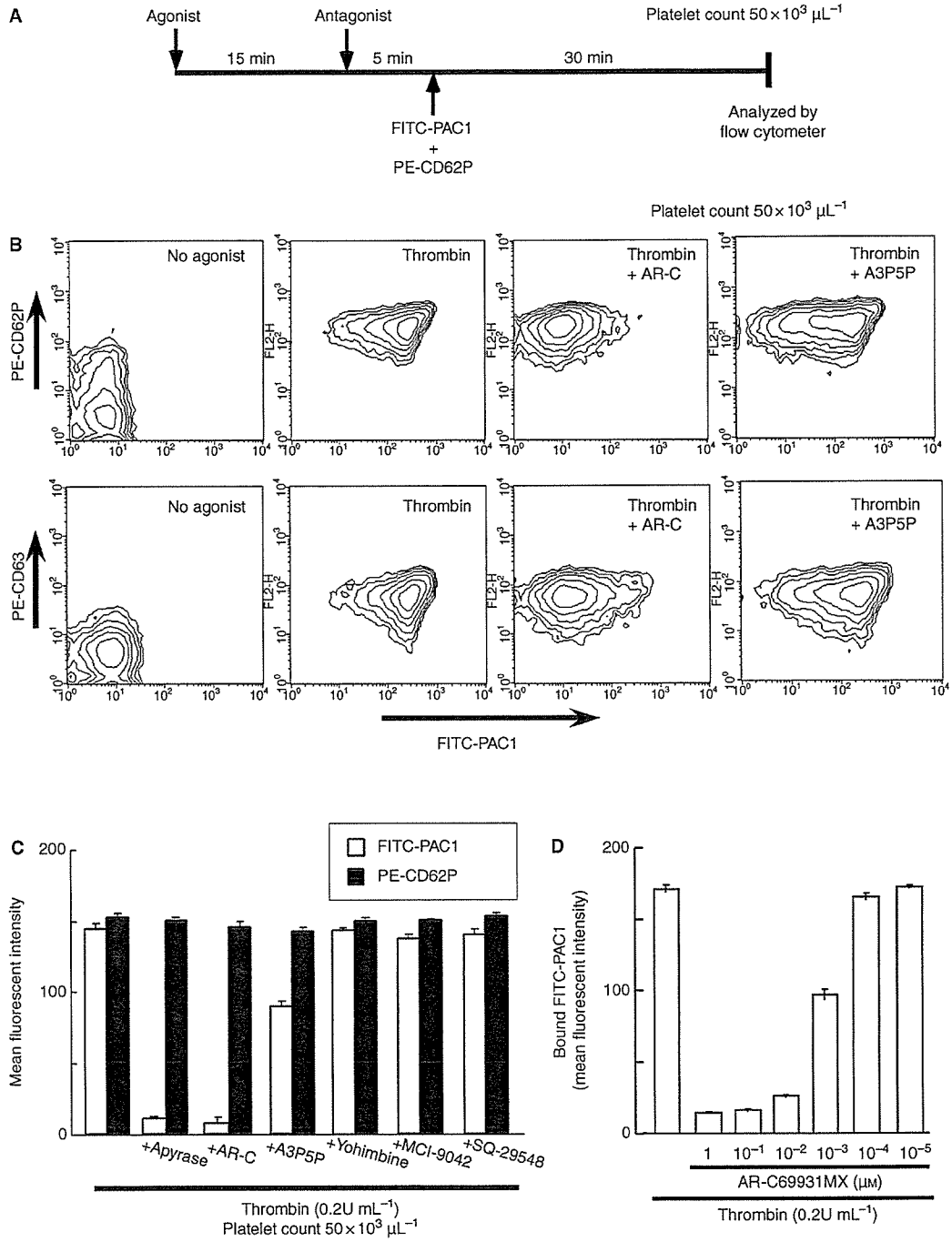

Fig. 2. Effects of receptor antagonists on the sustained $\alpha_{IIb}\beta_3$ activation induced by thrombin. (A) Scheme of the experimental procedure to analyze the effects of antagonists on the sustained $\alpha_{IIb}\beta_3$ activation. (B) One micromolar AR-C69931MX (AR-C) or 1 mM A3P5P was added to thrombin (0.2 U mL^{-1})-stimulated platelets. Representative results obtained using flow cytometry for PAC1 binding and CD62P or CD63 expression are shown. (C) Representative results for specific PAC1 binding in the absence or presence of 1 U mL^{-1} apyrase, 1 mM AR-C, 1 μM A3P5P, 10 μM yohimbine, 1 μM MCI-9042, or 100 nM SQ-29548 (mean ± SD of triplicates) are shown. Similar results were obtained in at least three independent experiments. (D) Dose-dependent inhibition of AR-C on the sustained $\alpha_{IIb}\beta_3$ activation is shown.

addition, AR-C69931MX dose-dependently inhibited the sustained $\alpha_{IIb}\beta_3$ activation with IC_{50} of approximately 1.6 nM (Fig. 2D). This value is very similar to IC_{50} (3.5 nM) for the inhibition of ADP-induced $\alpha_{IIb}\beta_3$ activation [23]. We also examined the effect of 10 μM yohimbine (an adrenergic receptor antagonist), 1 μM MIC-9042 (a 5-HT_2 receptor antagonist) or 100 nM SQ-29548 (a thromboxane A_2 receptor antagonist) on the maintenance of $\alpha_{IIb}\beta_3$ activation. Each of these antagonists showed only negligible effects on PAC1 binding (Fig. 2C).

These results suggest that released endogenous ADP and P2Y$_{12}$ may be required for the maintenance of $\alpha_{IIb}\beta_3$ activation induced by thrombin.

Effects of reduction of the platelet concentration on the sustained $\alpha_{IIb}\beta_3$ activation

Next, we examined if reduction of the released ADP concentration might disrupt the sustained $\alpha_{IIb}\beta_3$ activation. As shown in Fig. 3A, after stimulation with 0.2 U mL^{-1} thrombin for 15 min, platelets (50×10^3 μL^{-1}) were then diluted in Walsh buffer containing 0.2 U mL^{-1} thrombin to produce different platelet concentrations. The reduction of the platelet concentration after thrombin stimulation attenuated PAC1 binding to activated platelets, and PAC1 binding was only 16.9% ± 7.8% of the control value at a concentration of 500 platelets μL^{-1} (83.1% ± 7.8% reduction, mean ± SD, $n = 9$). On the other hand, the addition of PT25-2 mAb to thrombin-activated platelets markedly increased PAC1 binding even at 500 platelets μL^{-1}, indicating that the attenuation of PAC-1 binding is not because of an artifact of the dilution.

Taken together with the data shown in Fig. 2, these results suggest that released component(s), probably ADP, from thrombin-stimulated platelets are critical for the maintenance of $\alpha_{IIb}\beta_3$ activation.

Effects of exogenously added ADP on the sustained $\alpha_{IIb}\beta_3$ activation, and measurement of released ADP by HPLC

To further investigate the role of ADP in the maintenance of $\alpha_{IIb}\beta_3$ activation, we examined the effect of exogenously added ADP on PAC1 binding to platelets at a concentration of 500 platelets μL^{-1}. A series of concentrations of ADP was added to the dilution buffer containing 0.2 U mL^{-1} thrombin. As shown in Fig. 4, the amount of PAC1 binding to platelets was positively correlated with the exogenously added ADP concentration. Even 0.125 μM ADP significantly increased the PAC1 binding, and only approximately 1 μM ADP was sufficient to restore PAC1 binding at 500 platelets μL^{-1} to the levels obtained at 50×10^3 platelets μL^{-1}. The restoration of PAC1 binding by exogenous ADP was almost completely blocked by AR-C69931MX and was partially blocked by A3P5P.

Employing HPLC, we next measured the actual amounts of ADP released from thrombin-stimulated platelets. As shown in Fig. 5, the released ADP concentration was approximately

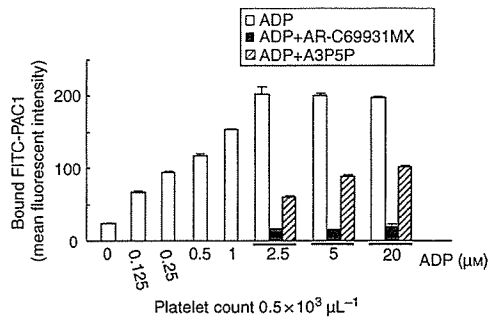

Fig. 4. Effects of exogenously added adenosine 5′-diphosphate (ADP) on the induction of the sustained $\alpha_{IIb}\beta_3$ activation at a platelet concentration of 500 platelets μL^{-1}. After dilution of thrombin (0.2 U mL^{-1})-stimulated platelets to 500 platelets μL^{-1}, the indicated doses of ADP in the absence (open columns) or presence of either 1 μM AR-C69931MX (AR-C, closed columns) or 1 mM A3P5P (shaded columns) were added to the platelet suspension. Representative results for specific PAC1 binding (mean ± SD of triplicates) are shown. Similar results were obtained in three independent experiments.

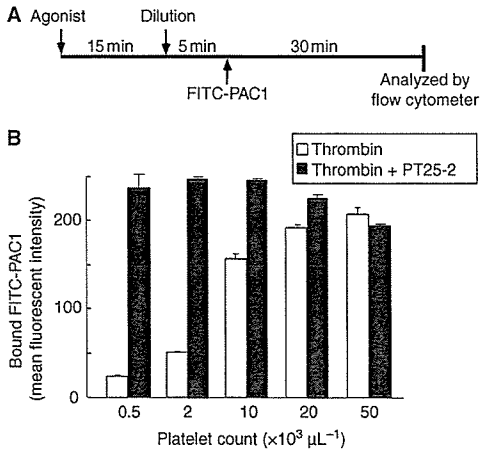

Fig. 3. Effects of platelet dilution on the sustained $\alpha_{IIb}\beta_3$ activation induced by thrombin. (A) Scheme of the experimental procedure to analyze the effects of platelet dilution on the sustained $\alpha_{IIb}\beta_3$ activation. (B) Representative results for specific PAC1 binding to thrombin (0.2 U mL^{-1})-stimulated (open columns) or [thrombin + PT25-2 monoclonal antibody (mAb)]-stimulated platelets (closed columns) at the indicated platelet concentrations (mean ± SD of triplicates) are shown. Similar results were obtained in at least three independent experiments.

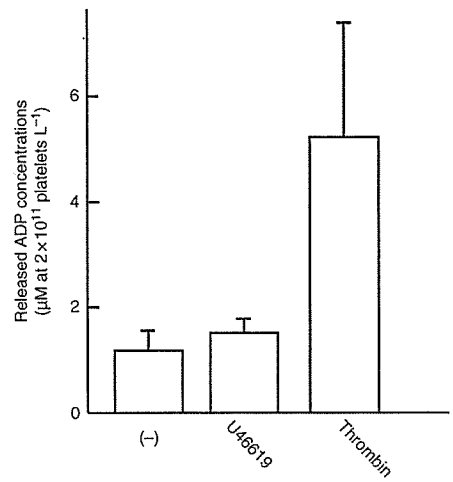

Fig. 5. Measurement of secreted adenosine 5′-diphosphate (ADP) using high-performance liquid chromatography (HPLC). Washed platelets (200×10^3 μL^{-1}) were stimulated with either 5 μM U46619 or 0.2 U mL^{-1} thrombin. After 15 min, platelets were centrifuged at $1000 \times g$ for 10 min, and the concentration of ADP in each supernatant was measured using HPLC as described in Materials and methods ($n = 11$).

5.21 ± 2.17 μM (mean ± SD, $n = 11$) when platelets were stimulated with 0.2 U mL^{-1} thrombin at 2×10^{11} platelets per liter (=200×10^3 μL^{-1}). Extrapolation of the data shown in Fig. 5 indicates that an ADP concentration at 50×10^3 μL^{-1} would be approximately 1.30 μM. These values are compatible with the doses of exogenous ADP that restored the PAC1 binding.

Role of $P2Y_{12}$ in sustained $\alpha_{IIb}\beta_3$ activation induced by U46619

We also examined the effect of the released ADP on the sustained $\alpha_{IIb}\beta_3$ activation induced by U46619. The expression levels of CD62P and CD63 as well as the levels of $\alpha_{IIb}\beta_3$ activation on U46619-stimulated platelets were much lower than those on thrombin-stimulated platelets, and not all the platelets expressed CD62P or activated $\alpha_{IIb}\beta_3$. The amount of PAC1 binding was correlated with the CD62P and CD63 expression levels on U46619-stimulated platelets. Again, AR-C69931MX and A3P5P disrupted the U46619-induced sustained $\alpha_{IIb}\beta_3$ activation by 98.7% ± 1.7% and 48.6% ± 10.2%, respectively (mean ± SD, $n = 3$) (Fig. 6). The disruption of PAC1 binding was also induced by reduction of the platelet concentration, and supplementation with approximately 0.25 μM ADP was sufficient to restore PAC1 binding at 500 platelets μL^{-1} to the levels obtained at 50×10^3 platelets μL^{-1} (Fig. 6). The released ADP concentration induced by U46619 stimulation was only 1.51 ± 0.06 μM at 2×10^{11} platelets per liter (mean ± SD, $n = 11$, approximately 0.38 μM at 50×10^3 platelets μL^{-1}) (Fig. 5). These values are also comparable to the doses of exogenous ADP that restored the PAC1 binding level to that induced by U46619. Moreover, higher levels of exogenous ADP further increased the amount of PAC1 binding. These results demonstrate the critical role of released ADP in the sustained $\alpha_{IIb}\beta_3$ activation on U46619-stimulated as well as thrombin-stimulated platelets.

Role of $P2Y_{12}$ in the increase of PKC and Rap1B activities in thrombin-stimulated platelets

In order to further clarify the mechanism by which thrombin induces sustained $\alpha_{IIb}\beta_3$ activation, we examined the effects of AR-C69931MX and A3P5P on the PKC activation induced by thrombin. PKC activation was detected by immunoblot analysis using the antibody specific for phosphorylated serine residues in PKC substrates. As in the experiment shown in Fig. 2A, platelets were stimulated with 0.2 U mL^{-1} thrombin for 15 min, different kinds of antagonists were added for an additional 5 min, and then PKC activation was examined. The phosphorylation of a 47-kDa protein (pleckstrin) was long-lasting, and the addition of AR-C69931MX or A3P5P did not inhibit the phosphorylation of pleckstrin. In contrast, Ro31-8220 markedly inhibited the phosphorylation of pleckstrin (Fig. 7A).

We next examined the activation of Rap1B (GTP-Rap1B) induced by thrombin. Thrombin induced Rap1B activation at 1 min after thrombin stimulation, and the activation was long lasting, even being observed at 30 min (Fig. 7B). In contrast to the PKC activation, AR-C69931MX as well as PGI_2 markedly inhibited the Rap1B activation. However, even in the presence of AR-C69931MX, the addition of epinephrine restored Rap1B activation (Fig. 7C) as well as $\alpha_{IIb}\beta_3$ activation (data not shown).

Discussion

In the present study, we examined the mechanism by which the high-affinity state of $\alpha_{IIb}\beta_3$ is maintained on platelets stimulated with thrombin or U46619 even in the absence of ligand binding. The long-lasting activation of $\alpha_{IIb}\beta_3$ induced by thrombin or U46619 was inhibited by 1 μM AR-C69931MX by 92% and 99%, and by 1 mM A3P5P by 38% and 49%, respectively. Only negligible inhibitory effects were observed with the tested antagonists for adrenergic receptor, 5-HT receptor or thromboxane A_2 receptor. The $\alpha_{IIb}\beta_3$ activation was also inhibited by the dilution of the platelet preparation. The disruption of $\alpha_{IIb}\beta_3$ activation by the dilution was abrogated by the addition of small amounts of exogenous ADP. The concentrations of ADP required for the restoration of $\alpha_{IIb}\beta_3$ activation at 500 platelets μL^{-1} were similar to those of endogenous ADP released from activated platelets [24]. These findings demonstrate that G_q- and $G_{12/13}$-mediated signaling pathways are not sufficient for the sustained $\alpha_{IIb}\beta_3$ activation, and the interaction of secreted ADP with its receptors, especially $P2Y_{12}$, is necessary for the sustained $\alpha_{IIb}\beta_3$ activation induced by thrombin or U46619.

van Willigen and Akkerman have suggested that the sustained $\alpha_{IIb}\beta_3$ activation is tightly controlled by PKC and a cyclic AMP-sensitive process [11]. However, AR-C69931MX disrupted the $\alpha_{IIb}\beta_3$ activation without inhibiting PKC activation when it was added 15 min after stimulation. Thus, $P2Y_{12}$-mediated signaling seems to be a downstream event from PKC activation. PKC activation induces ADP release from dense granules, and then the released ADP additionally induces $P2Y_{12}$-mediated signaling which is essential for the sustained $\alpha_{IIb}\beta_3$ activation. $\alpha_{IIb}\beta_3$ activation was not disturbed by inhibiting thrombin by treatment with hirudin, suggesting that a transient interaction between thrombin and thrombin receptors (PAR-1 and PAR-4) is sufficient for the sustained activation. In sharp contrast, the sustained $\alpha_{IIb}\beta_3$ activation could be disrupted by inhibiting $P2Y_{12}$-mediated signaling even in the presence of thrombin. Desensitization of $P2Y_{12}$ has recently been demonstrated [25]. However, it is likely that the degree of desensitization is not enough to reduce $\alpha_{IIb}\beta_3$ activation under our experimental conditions, as the amounts of activated $\alpha_{IIb}\beta_3$ did not alter even after 15 min of thrombin stimulation. Recently, unique regulation and relocalization of $P2Y_{12}$ after activation have been demonstrated. Although a substantial amount of $P2Y_{12}$ was rapidly and transiently internalized, most of the $P2Y_{12}$ receptors remained at the plasma membrane even after ADP stimulation [26]. Taken together, these findings indicate that the $P2Y_{12}$ remains

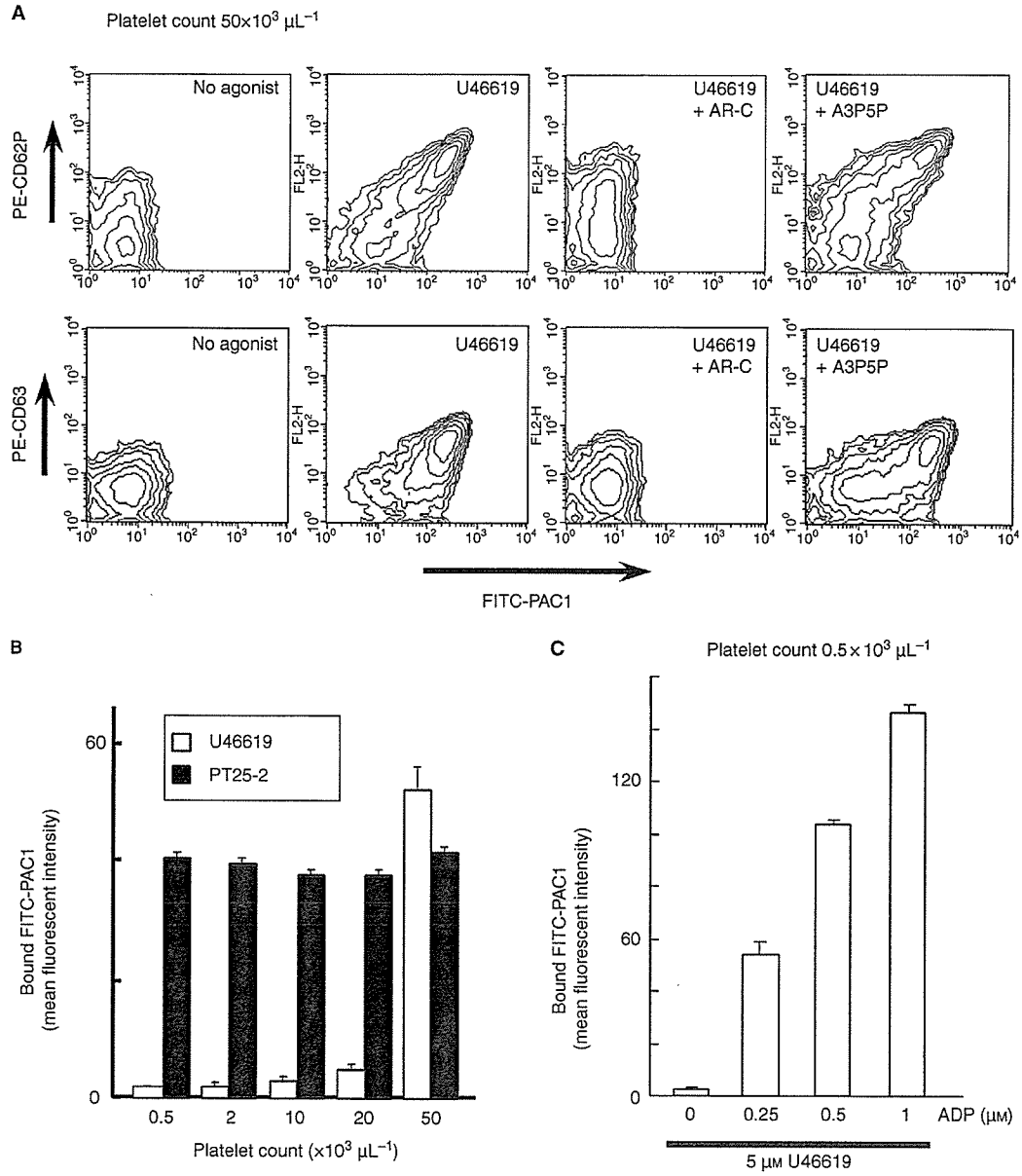

Fig. 6. Sustained $\alpha_{IIb}\beta_3$ activation on platelets stimulated with U46619. (A) Experiments were performed as in Fig. 2 using 5 μM U46619 instead of thrombin. One micromolar AR-C69931MX (AR-C) or 1 mM A3P5P was added to U46619 (5 μM)-stimulated platelets. Representative results obtained using flow cytometry for PAC1 binding and CD62P or CD63 expression are shown. (B) Effects of platelet dilution on the sustained $\alpha_{IIb}\beta_3$ activation induced by U46619. Representative results for specific PAC1 binding to U46619 (5 μM)-stimulated (open columns) or PT25-2-treated platelets (closed columns) at the indicated platelet concentrations (mean ± SD of triplicates) are shown. Similar results were obtained in three independent experiments. (C) Effects of exogenously added ADP on the induction of the sustained $\alpha_{IIb}\beta_3$ activation on U46619-stimulated platelets at a concentration of 500 platelets μL^{-1}. The indicated doses of ADP were added to the U46619-stimulated platelet suspension.

functional after thrombin stimulation and continuous interaction between P2Y$_{12}$ and the released ADP is critical for the sustained $\alpha_{IIb}\beta_3$ activation.

To further clarify the mechanism of the sustained $\alpha_{IIb}\beta_3$ activation by thrombin, we examined the activation of small GTPase Rap 1B during the sustained $\alpha_{IIb}\beta_3$ activation, as Rap 1B has recently been demonstrated to be a regulator of $\alpha_{IIb}\beta_3$ activation in platelets [27]. In response to calcium and DAG, CalDAG-GEFI activates Rap 1B by promoting the release of GDP and the loading of GTP. Both Rap 1B-null mice and CalDAG-GEFI-null mice show impaired $\alpha_{IIb}\beta_3$ activation in response to different kinds of agonists [28,29]. Thrombin induced sustained Rap 1B activation under our experimental conditions. However, the addition of AR-C69931MX as well as PGI$_2$ after a 15-min stimulation disrupted the sustained Rap 1B activation and $\alpha_{IIb}\beta_3$ activation. Because G_z-mediated signaling induced by epinephrine could activate $\alpha_{IIb}\beta_3$ in P2Y$_{12}$-deficient mice [30], we examined the effect of

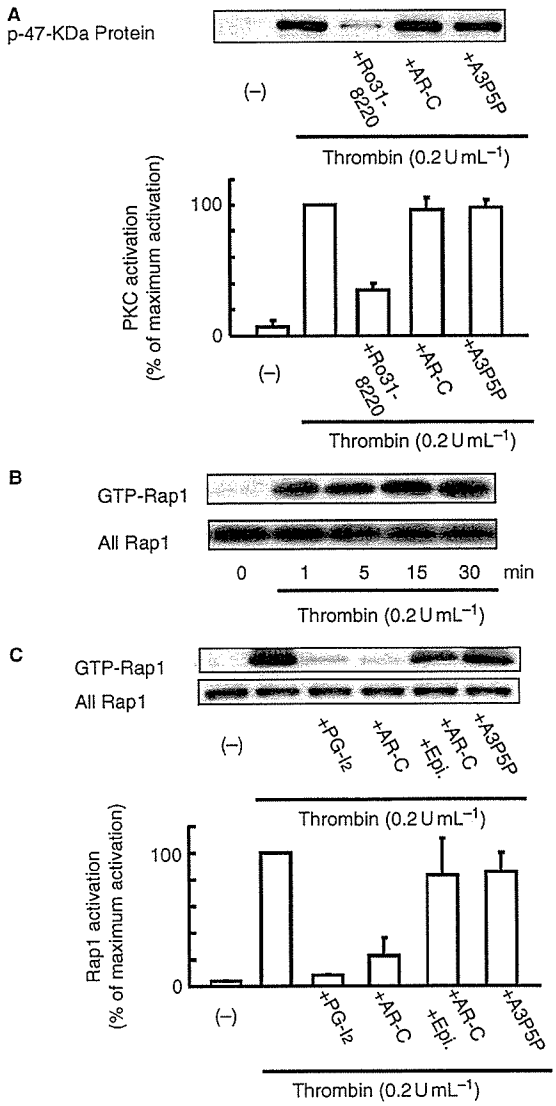

Fig. 7. Effects of $P2Y_{12}$ antagonist on protein kinase C (PKC) and Rap1B activation. (A) (upper panel) Washed platelets (200×10^3 μL^{-1}) were stimulated with 0.2 U mL^{-1} thrombin, and 15 min later 5 μM Ro31-8220, 1 μM AR-C69931MX (AR-C), or 1 mM A3P5P was added to the activated platelets. After 5 min, the platelets were lyzed, and PKC activation was detected by immunoblotting using antiphosphoserine PKC substrate antibody. The phosphorylation of a 47-kDa protein (pleckstrin) is shown. (lower panel) Optical density of the 47-kDa band was measured using NIH IMAGE software, and relative % compared with PKC activation without antagonist (maximum activation) is shown (mean ± SD, $n = 3$). (B) Washed platelets (200×10^3 μL^{-1}) were stimulated with 0.2 U mL^{-1} thrombin, and Rap IB activation was detected by the pull-down assay using GST-RalGDS-RBD followed by incubation with rabbit anti-Rap 1B polyclonal antibody at indicated time points. Total Rap 1B in each lysate was detected in parallel. (C) (upper panel) Washed platelets (200×10^3 μL^{-1}) were stimulated with 0.2 U mL^{-1} thrombin, and 15 min later 1 μM PGI_2, 1 μM AR-C69931MX (AR-C), 1 μM AR-C + 10 μM epinephrine, or 1 mM A3P5P was added to the activated platelets. After 5 min, the platelets were lyzed, and then Rap IB activation was examined. (lower panel) Optical density of activated Rap IB was measured using NIH IMAGE software, and relative % compared with Rap 1B activation without antagonist (maximum activation) is shown (mean ± SD, $n = 3$).

epinephrine under our experimental conditions. Interestingly, the addition of epinephrine induced Rap IB activation even in the presence of AR-C69931MX. Thus, our data demonstrate that there is a close relationship between the sustained $\alpha_{IIb}\beta_3$ activation and Rap 1B activation. Our data are consistent with previous reports that G_i-mediated signaling is necessary for Rap 1B activation [31,32] and also demonstrate for the first time that the continuous stimulation of G_i-mediated signaling is needed for the sustained Rap 1B activation. The patient with $P2Y_{12}$ deficiency (OSP-1) showed the markedly impaired PAC-1 binding in response to different kinds of agonists except for PMA, which is similar to CalDAG-GEFI-null mice [28]. It is possible that PMA may induce CalDAG-GEFI (and Rap1B)-independent signaling pathways to induce and sustain $\alpha_{IIb}\beta_3$ activation [28].

Our present findings also indicate important cautions regarding $\alpha_{IIb}\beta_3$ ligand binding assays. Platelet concentrations and the timing of ligand incubation could influence the ligand-binding capacity of $\alpha_{IIb}\beta_3$. This is especially true of U46619 stimulation, because the amount of ADP released from U46619-stimulated platelets was much lower than that released from thrombin-stimulated platelets. Indeed, the ligand-binding capacity of U46619-stimulated platelets was dramatically influenced by the platelet concentration. In addition, the presence of leukocytes in PRP or whole blood may also modify the results, as leukocyte ecto-nucleotidase (CD39) influences the metabolism of released ADP [33]. Thus, several factors influencing the concentration of released ADP should be taken into account during $\alpha_{IIb}\beta_3$ ligand-binding assays.

A number of studies, including ours, have demonstrated the important role of $P2Y_{12}$ in thrombus stability [17,30,34]. Moreover, recent *in vivo* observations demonstrated that during platelet thrombus formation, circulating platelets were tethered to the luminal surface of growing thrombi by VWF–GPIb interaction. However, more than 95% of tethered platelets were subsequently translocated and/or detached [35,36]. In this study, we obtained the novel finding that the $\alpha_{IIb}\beta_3$ activation could not be sustained at a low concentration of platelets (500 platelets μL^{-1}) without exogenous ADP. Activated $\alpha_{IIb}\beta_3$ on the detached platelets should become inactivated, because the released ADP is immediately diluted by the blood flow. At the luminal surface, activated $\alpha_{IIb}\beta_3$ on the tethered platelets would be maintained only when the platelets are continuously exposed to ADP released from adjacent activated platelets. At the inside of growing thrombi, it appears that platelets are constantly exposed to such high concentrations of released ADP that $\alpha_{IIb}\beta_3$ can be maintained in its high-affinity state in concert with the effects of thrombin and TXA_2. It is possible that ADP concentrations surrounding platelets may largely influence on determining whether platelets participate in thrombus formation or not. Thus, $P2Y_{12}$ may serve as a sensor for thrombogenic status surrounding individual platelets.

In summary, our data demonstrate that the continuous interaction between released ADP and $P2Y_{12}$ is critical for

sustained $\alpha_{IIb}\beta_3$ activation in platelets activated via G_q and $G_{12/13}$-coupled receptors.

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ORIGINAL ARTICLE

Role of DNA methylation for expression of novel stem cell marker CDCP1 in hematopoietic cells

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CDCP1, a novel stem cell marker, is expressed in hematopoietic cell line K562 but not in Jurkat. When *CDCP1* promoter was transfected exogenously, Jurkat showed comparable promoter activity with K562, suggesting that the factor to enhance transcription was present but interfered to function in Jurkat. The reporter assay and si-RNA-mediated knockdown experiment revealed that zfp67, a zinc-finger protein, enhanced *CDCP1* transcription. Amount of zfp67 in Jurkat was comparable with K562, but chromatin immunoprecipitation showed that zfp67 bound to *CDCP1* promoter in K562 but not in Jurkat. There are CpG sequences around the promoter of *CDCP1*, which were heavily methylated in Jurkat but not in K562. Addition of demethylating reagent to Jurkat induced *CDCP1* expression, and increased the zfp67 binding to *CDCP1* promoter. Among normal hematopoietic cells such as $CD34^+CD38^-$ cells, lymphocytes and granulocytes, inverse correlation between proportion of methylated CpG sequences and *CDCP1* expression level was found. Demethylation of CpG sequences in lymphocytes, in which CpG sequences were heavily methylated, induced *CDCP1* expression and its expression level further increased through zfp67 overexpression. The methylation of DNA appeared to regulate the cell-type-specific expression of *CDCP1* through the control of interaction between chromatin DNA and transcription factors.
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Introduction

CDCP1 is a transmembrane protein containing three extracellular CUB domains and an intracellular tyrosine kinase domain.[1–5] This molecule was originally identified as an epithelial tumor antigen through comparison of lung cancer cell lines and normal lung tissues.[1] Very recently, it was demonstrated that the transplantation of human CDCP1-positive bone marrow cells into non-obese diabetic/severe-combined immunodeficient mice gives rise to chimeric hematopoiesis, indicating the CDCP1 to be a novel stem cell marker.[3,4] In hematopoietic system, *CDCP1* is expressed in CD34-positive stem cells but not in differentiated cells. To date, K562 is the only CDCP1-positive hematopoietic cell line, which is derived from the blast crisis of chronic myeloid leukemia (CML).[1,2]

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Recently, the correlation between stem cell marker expression in tumor cells and their malignant grade has been reported in brain tumor.[6] However, the regulatory mechanism for the expression of stem cell markers remained to be elucidated. Here, by comparing two hematopoietic cell lines, the role of DNA methylation for cell-type-specific *CDCP1* expression was examined.

Materials and methods

Cells

Human cell lines used in this study (K562 and Jurkat) were obtained from the American Type Culture Collection (Rockville, MD, USA), and cultured in RPMI1640 (Sigma, St Louis, MO, USA) supplemented with 10% fetal calf serum (FCS, Nippon Bio-supp Center, Tokyo, Japan). K562 are derived from CML in blast crisis and Jurkat are from T-cell leukemia. To determine the effect of methylation on *CDCP1* expression, 2 μM of demethylating reagent 5-aza deoxycytidine (5-aza dC) (Sigma) was added. Peripheral blood cells from healthy volunteers and umbilical cord blood from normal full-term deliveries were collected after informed consent. The present study was performed under the authors' institutional guidelines and was approved by the institutional review boards (No. 535). $CD34^+CD38^-$ cells were purified from umbilical cord blood using FACSAria (BD, Franklin Lakes, NJ, USA). Granulocytes and lymphocytes were purified with Polymorphprep (Axis-Shield, Oslo, Norway) and lymphocyte separation medium (Cappel, Aurora, OH, USA), respectively. Monocytes were purified using the magnetic-activated cell sorter (Miltenyi Biotech, Bergish Gladbach, Germany) according to the manufacturer's guidelines.

Semiquantitative reverse transcription (RT)-PCR analysis

Total RNAs were extracted with RNeasy kit (Qiagen, Valencia, CA, USA) with DNase I treatment. Two micrograms of total RNA were subjected to reverse transcription by Superscript III (Invitrogen, Carlsbad, CA, USA), and the single-strand cDNAs were obtained. One microliter, 0.1 μl or 0.01 μl of the reverse-transcribed product was added to 25 μl of polymerase chain reaction (PCR) mixture containing 1.25 U of *Taq* DNA polymerase (Roche Diagnostics GmbH, Mannheim, Germany) and 25 pmol of each of the primers. The sequence of primers was as follows: 5′-GGGCGCGCATTCATGATCATCCAGG-3′ corresponding to the sequence of exon 7 and 5′-CCTCT GGCTGCAGGAAGGAGCCGCTGGA-3′ corresponding to the sequence of exon 9 of *CDCP1* gene. The sequence of primers for *β-actin* was 5′-GCCGAGCGGGAAATCGTGCG-3′ and 5′-ACG ATGGAGGGGCCGGACTC-3′. PCR conditions were 30 s at

95°C, 30 s at 55°C and 1 min at 72°C for 35 or 40 cycles in *CDCP1* and for 25 cycles in *β-actin*.

Quantification of mRNA levels by real-time RT-PCR

RNA was extracted using an RNeasy kit (Qiagen) with DNase I treatment. The mRNA levels for *CDCP1* and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) genes were verified using TaqMan gene expression assays from Applied Biosystems (ABI, Foster City, CA, USA) as recommended by the manufacturer. The amount of *CDCP1* mRNA was normalized to that of *GAPDH* mRNA.

Transient transfection and luciferase assay

The PCR-amplified fragment containing promoter and a part of exon1 (nucleotide (nt) −150 to +140, +1 means a transcription initiation site) was cloned into pSP-Luc vector containing luciferase gene as reported previously.[7] The reporter plasmid containing the deleted *CDCP1* promoter (nt −130 to +140, nt −110 to +140 and nt −60 to +140), and the reporter plasmid mutated at the binding site for zfp67, a zinc-finger type of transcription factor,[8] were also constructed by PCR. The sequence of *CDCP1* fragment was verified by ABI 3100 sequencer. After the DNA isolation, 5 μg of the reporter plasmid was transiently transfected into K562 and Jurkat, together with 1 μg of the β-galactosidase control vector by using electroporation (Bio-Rad Laboratories, Richmond, CA, USA). Forty-eight hours after the transfection, a luciferase activity was measured and was normalized to β-galactosidase activity as described previously.[7] The normalized value of each reporter plasmid was divided by that of reporter plasmid starting from nt −60, and the resultant value was shown as relative luciferase activity.

Small interfering RNA transfection and immunoblot

The predesigned small interfering RNA (siRNA) against zfp67 was purchased from Ambion Inc. (Austin, TX, USA). The siRNA (150 pmol) was transfected into 2×10^6 of K562 by using Amaxa cell line nucleofector kit (Amaxa Inc., Gaithersburg, MD, USA). After 72 h, cells were harvested, and lysed in a buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% Triton X-100 and 1 mM phenylmethylsulfonyl fluoride. The resulting lysates were separated on 10% sodium dodecyl sulfate (SDS)-polyacrylamide gels, transferred to Immobilon (Millipore, Bedford, MA, USA) and reacted with anti-zfp67 (Abcam, Cambridge, UK) or anti-actin (Sigma). After washing, the blots were incubated with an appropriate peroxidase-labeled secondary antibody, and then reacted with Renaissance reagents (NEN, Boston, MA, USA) before exposure.

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) assay was carried out with ChIP assay kit (Upstate, Lake Placid, NY, USA) according to the manufacturer's instruction. Briefly, K562 and Jurkat were fixed with 1% formaldehyde for 10 min, washed twice with ice-cold phosphate buffer solution (PBS) and sonicated 15 times for 10 s each at power setting '3' with the ultrasonicator (Tomy Seiko, Tokyo, Japan). Soluble chromatin solution was cleared by centrifugation and adjusted to 0.1% SDS, 1% Triton X-100 and 140 mM NaCl. Immunoprecipitation reactions were performed overnight with salmon-sperm DNA/protein A agarose and anti-zfp67, anti-acetyl histone H3 (Upstate) or normal rabbit IgG (for negative control, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). The immunoprecipitates were washed, and reverse-crosslinked at 65°C for 6 h. DNA purified from immunoprecipitates was subjected to PCR analysis. In some experiments, the real-time PCR using Sybr-green system (ABI) was carried out. The sequence of primers for amplifying *CDCP1* promoter (−248 to +214) was as follows: 5′-CAGACTTGG GAAGGAAGACTAAGC-3′ and 5′-AGCAGAACCCCCTAGCAG TGCGATA-3′. DNA directly purified from soluble chromatin solution was used as a positive control (input).

Bisulfite modification and DNA sequencing analysis

One microgram of genomic DNA was modified by sodium bisulfite using the CpGenome DNA modification kit (Chemicon, Temecula, CA, USA). PCR was carried out with the modified DNA as a template. The PCR reaction mixture contained 1 μl of DNA in 25 μl of total volume, which also included 2.5 μl of 10 × PCR buffer II, 5 mM $MgCl_2$, 250 μM deoxynucleoside triphosphate, 1 μM of each primer and 1.25 U *Taq* gold. The PCR buffer II, $MgCl_2$ and *Taq* gold are parts of the AmpliTaq Gold with Gene Amp kit from ABI. The sequence of the primers was as follows: 5′-TAGATTTGGGAAGGAAGATTAAGT-3′ and 5′-AACAACAAAACCCCTAACAATA-3′. The PCR was carried out at 95°C for 10 min, followed by 45 cycles at 95°C for 1 min, 50°C for 1 min and 72°C for 1 min. In this condition, we confirmed that both methylated and unmethylated fragments were amplified at a comparable level. The amplified PCR fragment was purified with QIAquick gel extraction kit (Qiagen), and was subcloned into pGEM-T Easy vector (Promega, Madison, WI, USA). The sequence of the amplified fragment was analyzed using an ABI 3100 sequencer (Applied Biosystems). Ten clones were analyzed for each condition.

In vitro *methylation*

The luciferase construct that contained promoter region of *CDCP1* starting from nt −150 was incubated with *SssI* methylase (New England Biolabs, Beverly, MA, USA) in the presence (methylated) or absence (mock) of *S*-adenosylmethionine, as recommended by the manufacturer. After DNA isolation, 1 μg of the methylated or mock luciferase construct was transiently transfected into K562, together with 0.2 μg of the β-galactosidase control vector using Transfast transfection reagent (Promega). Forty-eight hours after transfection, luciferase activity was measured and normalized to the β-galactosidase activity.

Overexpression of zfp67 *in lymphocytes*

The coding region of *zfp67* was amplified by PCR, and cloned into pIRES2-AcGFP1 vector (Clontech Laboratories, Mountain View, CA, USA). The sequence was verified with an ABI 3100 sequencer. Overexpression of zfp67 in lymphocytes was induced with Human T-cell nucleofector kit (Amaxa) according to the protocol of manufacturer's for unstimulated T cells. Thirty-two hours after transfection, cells were harvested, and RNA was extracted. A part of harvested cells was used for immunoblot to confirm the overexpression of zfp67.

Statistical analysis

The values are shown as the mean ± s.e. of at least three experiments. In bisulfite sequencing, 10 clones were analyzed for each cell type, and the proportion of methylated CpG sequences is shown as the mean ± s.e. of these 10 clones. Statistical comparisons were carried out using Student's *t*-tests.

Results

Semiquantitative RT-PCR demonstrated that K562 expressed *CDCP1* but Jurkat did not (Figure 1a). The real-time quantitative RT-PCR revealed the amount of *CDCP1* mRNA in K562 to be approximately 10 000 times higher than that of Jurkat (Figure 1b), indicating that K562 was a CDCP1-positive, whereas Jurkat was a CDCP1-negative cell line. Then, these two cell lines were used to investigate the regulatory mechanism of *CDCP1* expression.

First, the *cis*-acting motif(s) mediating *CDCP1* transcription was searched using reporter assay. K562 and Jurkat were transfected with the reporter plasmid containing various length of *CDCP1* promoter (Figure 2). In K562, the remarkable luciferase activity was detected when the *CDCP1* promoter started from nt −150, whereas the luciferase activity decreased when the *CDCP1* promoter was deleted to nt −130, −110 and −60 (Figure 2). The luciferase activity was also decreased in Jurkat by deleting the region between nt −150 and −130 (Figure 2). These findings indicated that K562 and Jurkat possessed the *trans*-acting factor(s) to express *CDCP1*, which activated the exogenously transfected *CDCP1* promoter via the motif locating between nt −150 and −130.

Putative binding motifs for transcription factor were searched in the CDCP1 promoter region between nt −150 and −130 using Genomatix software (http://www.genomatix.de/index.html). One binding motif for zinc-finger transcription factor zfp67 (also called c-Krox or Th-POK)[8,9] was found between −139 and −133 (Figure 3a; consensus sequence for zfp67 is GGGAGGG). The mutation at zfp67 binding motif (GGGAGGG to GTAAGGG; the mutated nucleotides were underlined) decreased the luciferase activity in K562 and Jurkat, suggesting that zfp67 transactivated the exogenously transfected *CDCP1* promoter (Figure 3b). To examine whether zfp67 mediated *CDCP1* transcription *in vivo*, zfp67 was knocked down in K562 by siRNA (Figure 3c). The zfp67-knocked down K562 showed the lower expression of *CDCP1* than the original K562 (Figure 3d), indicating that zfp67 transactivate *CDCP1* promoter in K562.

The *in vivo* binding of zfp67 to *CDCP1* promoter was examined with ChIP assay using antibody specific for zfp67. The *CDCP1* promoter was enriched for zfp67 in K562 but not in Jurkat (Figure 4a). Immunoblot analysis revealed the amount of zfp67 in Jurkat to be comparable with K562 (Figure 4b),

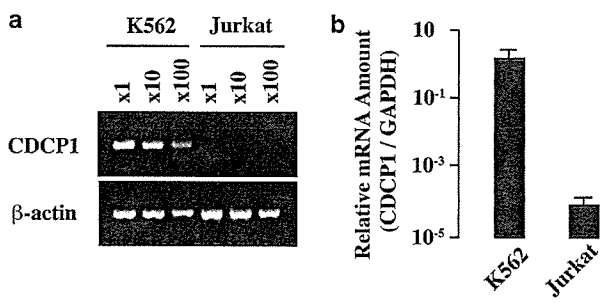

Figure 1 Expression of *CDCP1* gene in K562 and Jurkat. (**a**) Semiquantitative RT-PCR. Two micrograms of total RNA was reverse-transcribed. One microliter, 0.1 μl or 0.01 μl of the RT product (shown as 1×, 10× and 100×, respectively) was amplified for 35 cycles in *CDCP1* and for 25 cycles in *β-actin*. (**b**) Quantification of *CDCP1* mRNA using real-time RT-PCR. The amount of *CDCP1* mRNA was quantified and normalized for the amount of *GAPDH* mRNA. The values represent the mean±s.e. of three experiments. In some cases, the s.e. was too small to be shown by bars.

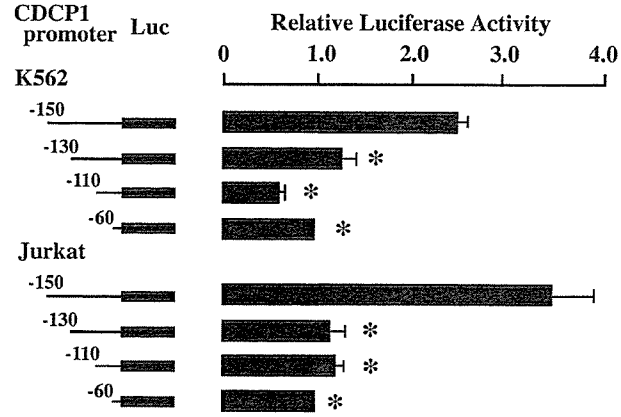

Figure 2 Luciferase assay to search for *cis*-acting element mediating *CDCP1* transcription. Reporter plasmids containing sequentially deleted *CDCP1* promoter were transiently transfected in K562 or Jurkat, together with expression plasmid containing *β-galactosidase* gene. The luciferase activity was normalized to the β-galactosidase activity. The values represent the mean±s.e. of three experiments. $^*P<0.05$ by *t*-test when compared to the value obtained with the reporter plasmid starting from nt −150.

indicating that zfp67 was present in Jurkat but was interfered to bind with the *CDCP1* promoter.

There are many CpG sequences around the transcription initiation site of *CDCP1* gene. As the methylation at CpG sequences is known to inhibit binding of transcription factor by packing chromatin structure,[10] the effect of methylation on *CDCP1* expression was examined by demethylating reagent 5-aza dC. The addition of 5-aza dC for 4 days increased the expression of *CDCP1* mRNA approximately 100 times in Jurkat (Figure 5a). Then, the methylation status of CpG sequences was examined by bisulfite sequencing in K562, Jurkat and Jurkat treated with 5-aza dC. K562 showed a low frequency of methylation, whereas Jurkat showed a high frequency (Figure 5b). The addition of 5-aza dC decreased the methylation frequency in Jurkat (94.1–20.9%). These findings suggest that zfp67 might be interfered to bind with *CDCP1* promoter through packing of chromatin structure as a result of methylation at CpG sequences. To confirm this, the histone acetylation status, which reflects the chromatin decondensation,[10] was examined. ChIP assay revealed *CDCP1* promoter to be enriched for the acetylated histone H3 in K562 but not in Jurkat (Figure 6). The addition of 5-aza dC induced the enrichment of *CDCP1* promoter for acetylated histone H3 in Jurkat, indicating that 5-aza dC caused the chromatin decondensation. The binding of zfp67 to *CDCP1* promoter was also induced in Jurkat by the addition of 5-aza dC (Figure 6).

To confirm the inhibitory effect of CpG methylation on *CDCP1* expression, *in vitro* methylation of *CDCP1* promoter was performed. The luciferase construct containing *CDCP1* promoter was methylated *in vitro* using *Sss*I methylase. The methylated or mock-methylated promoter construct was transiently transfected into K562, and luciferase activity was measured. The methylated promoter construct, but not the mock-methylated one, was resistant to digestion with methylation-sensitive restriction enzyme *Hpa*II (Figure 7a), which confirmed that the CpG sequences of methylated construct were completely methylated *in vitro*. When transfected into K562, luciferase activities for the mock-methylated *CDCP1* promoter were higher than those for methylated one (Figure 7b).

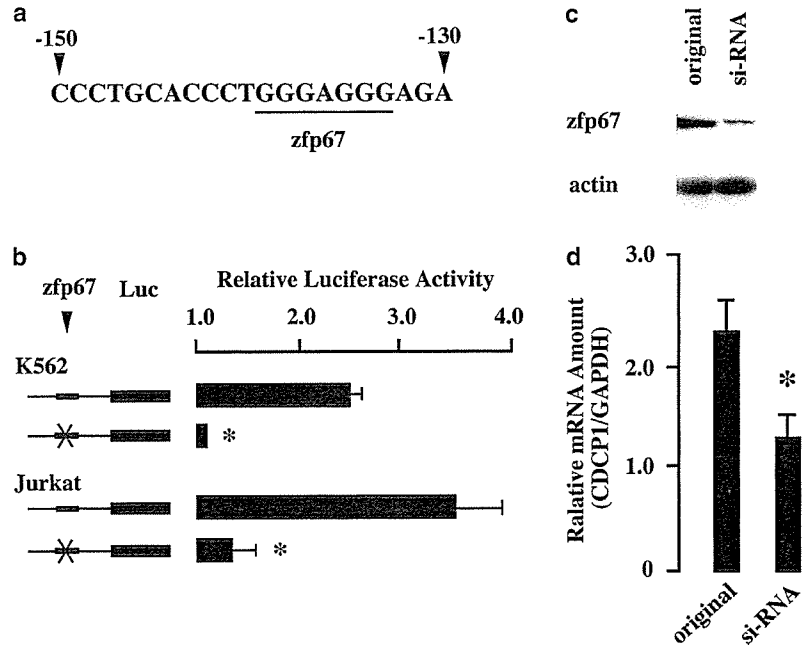

Figure 3 Effect of zfp67 on transcription of *CDCP1*. (**a**) DNA sequence between nt −150 and −130. The putative binding motif for zfp67 was underlined. (**b**) Luciferase assay with the reporter plasmid mutated at the zfp67 binding motif. The values represent the mean±s.e. of three experiments. **P*<0.05 by *t*-test when compared to the value with the reporter plasmid that contained unmutated zfp67 motif. (**c**) Immunoblot analysis. The siRNA against zfp67 was transfected into K562, and the amount of zfp67 was examined with immunoblot. (**d**) Expression of *CDCP1* in zfp67-knocked down K562. By real-time PCR, the amount of *CDCP1* mRNA was quantified and normalized for the amount of *GAPDH* mRNA. The values represent the mean±s.e. of three experiments. **P*<0.05 by *t*-test when compared to the value of original K562.

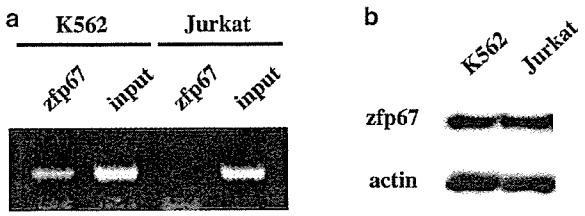

Figure 4 Binding of zfp67 to *CDCP1* promoter. (**a**) ChIP assay for analyzing zfp67 binding to *CDCP1* promoter. The immunoprecipitation with anti-zfp67 enriched *CDCP1* promoter in K562 but not in Jurkat. DNA directly purified from sonicated chromatin was used for positive control (input). (**b**) Immunoblot for zfp67. The amount of zfp67 was compared between K562 and Jurkat.

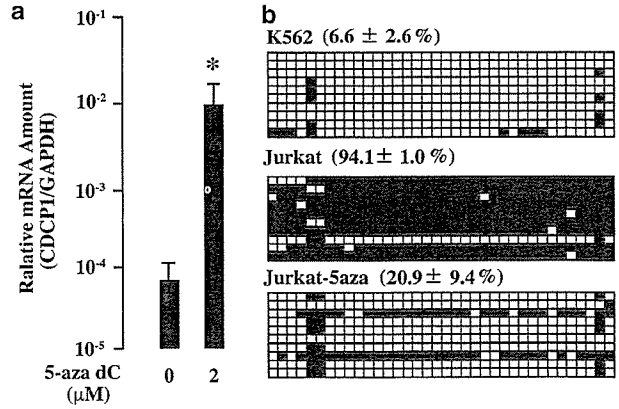

Figure 5 Effect of CpG methylation on the expression of *CDCP1* gene. (**a**) Effect of demethylating reagent 5-aza dC on *CDCP1* expression. Quantitative real-time RT-PCR was carried out with mRNA from Jurkat treated by 5-aza dC for 4 days. The amount of *CDCP1* mRNA was normalized for the amount of *GAPDH* mRNA. The values represent the mean±s.e. of three experiments. **P*<0.05 by *t*-test when compared to the value without treatment of 5-aza dC. (**b**) Methylation status of CpG sequences. Ten clones for each cell line were analyzed by bisulfite sequencing. Open and closed squares denote unmethylated and methylated cytosines, respectively. Numbers in parentheses denote frequency of methylation (mean±s.e. of 10 clones).

The relationship between methylation status of CpG sequences and *CDCP1* expression level was examined in normal hematopoietic cells. The real-time quantitative RT-PCR revealed that the amount of CDCP1 mRNA in $CD34^+CD38^-$ cells was approximately 100 times higher than that of granulocytes, lymphocytes and monocytes (Figure 8a). Proportion of methylated CpG was low in $CD34^+CD38^-$ cells, whereas that in granulocytes, lymphocytes, and monocytes was high (Figure 8b). The addition of 5-aza dC to lymphocytes induced the *CDCP1* expression, which was further increased through the overexpression of zfp67 (Figure 9a and b).

Discussion

CDCP1 was expressed in K562 but not in Jurkat, showing a cell-type-specific expression in hematopoietic cell lines. K562, derived from the blast crisis of CML, can differentiate into erythrocytes or megakaryocytes by stimulation with hemin[11] or phorbol ester.[12] Jurkat express T-cell lineage markers, and thus appears to be more differentiated cell line than K562. Therefore, present results were consistent with the report by Buhring *et al*.[4]

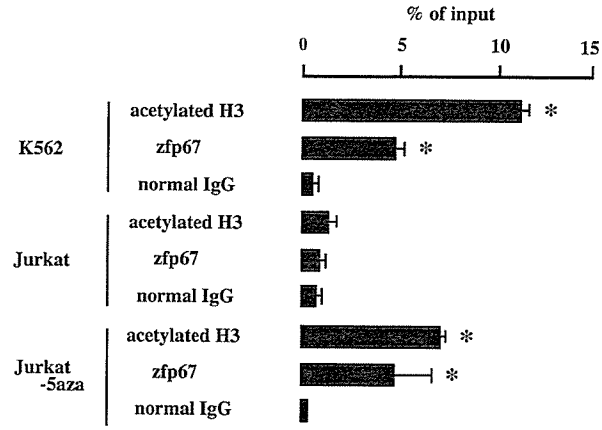

Figure 6 ChIP assay for analyzing acetylation and zfp67 binding on *CDCP1* promoter. The enriched *CDCP1* promoter by immunoprecipitation with anti-acetyl histone H3 and anti-zfp67 was analyzed with real-time PCR. DNA directly purified from sonicated chromatin was used as a positive control, and DNA enriched with normal rabbit IgG was used as a negative control. The values represent the mean ± s.e. of three experiments. In some experiments, the s.e. was too small to be shown. $*P<0.05$ by *t*-test when compared to the value of normal rabbit IgG.

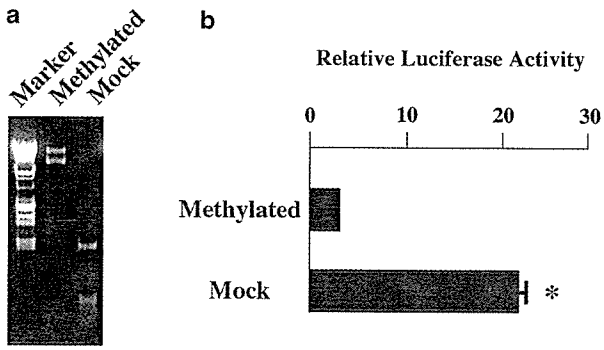

Figure 7 Effect of methylation on *CDCP1* promoter activity. (**a**) Digestion patterns with methylation-sensitive restriction enzyme *Hpa*II of methylated and mock-methylated reporter plasmids. The luciferase reporter plasmid containing *CDCP1* promoter was methylated or mock-methylated *in vitro*. Subsequently, each construct was digested with *Hpa*II and electrophoresed. The molecular weight marker (λDNA digested with *Eco*RI and *Hin*dIII) is also shown. (**b**) Relative luciferase activities of the methylated and mock-methylated *CDCP1* constructs. K562 was transiently transfected with the methylated or mock-methylated reporter construct, together with the expression plasmid containing *β-galactosidase* gene. The luciferase activity is normalized for the β-galactosidase activity. The values represent the mean ± s.e. of three experiments. In some cases, the s.e. is too small to be shown by bars. $*P<0.05$ by the Student's *t*-test versus the value for the methylated construct.

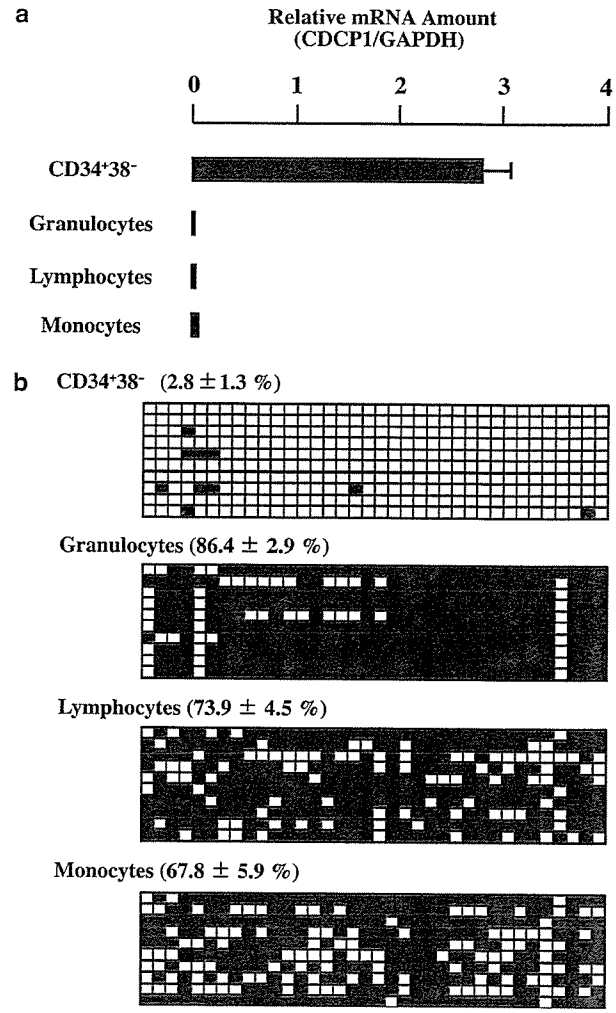

Figure 8 Correlation of methylation status of CpG sequences to *CDCP1* expression level in normal hematopoietic cells. (**a**) Quantification of *CDCP1* mRNA using real-time RT-PCR. The amount of *CDCP1* mRNA was quantified and normalized for the amount of *GAPDH* mRNA. The values represent the mean ± s.e. of at least three experiments. In some cases, the s.e. was too small to be shown by bars. (**b**) Methylation status of CpG sequences. Ten clones for each population were analyzed by bisulfite sequencing. Open and closed squares denote unmethylated and methylated cytosines, respectively. Numbers in parentheses denote frequency of methylation (mean ± s.e. of 10 clones).

that *CDCP1* is expressed only in the undifferentiated state of hematopoietic cells.

The exogenously transfected *CDCP1* promoter was activated in Jurkat and K562, but *CDCP1* was expressed in K562 but not in Jurkat. This might be owing to failure of *trans*-activating factor to activate *CDCP1* transcription in Jurkat. By reporter assay and siRNA experiment, the *trans*-activating factor to express *CDCP1* was demonstrated to be zfp67. As expected, zfp67 existed in Jurkat. ChIP assay revealed that zfp67 bound to *CDCP1* promoter in K562 but not in Jurkat. It is well known that the packing of chromatin structure inhibits the binding of transcription factors, and the packing of chromatin structure is enhanced by methylation of CpG sequences and deacetylation of histone.[10] The proportion of methylated CpG around *CDCP1* promoter was high in Jurkat but low in K562. In addition, the histone around *CDCP1* promoter was highly acetylated in K562 but not in Jurkat. These findings indicate that the chromatin was packed in Jurkat, which inhibits the binding of factor to enhance *CDCP1* transcription. In fact, demethylation of CpG sequences induced the binding of zfp67 and increased the expression level of *CDCP1* in Jurkat.

Role of methylation on *CDCP1* expression was examined in normal hematopoietic cells. As reported by Buhring *et al.*,[4]

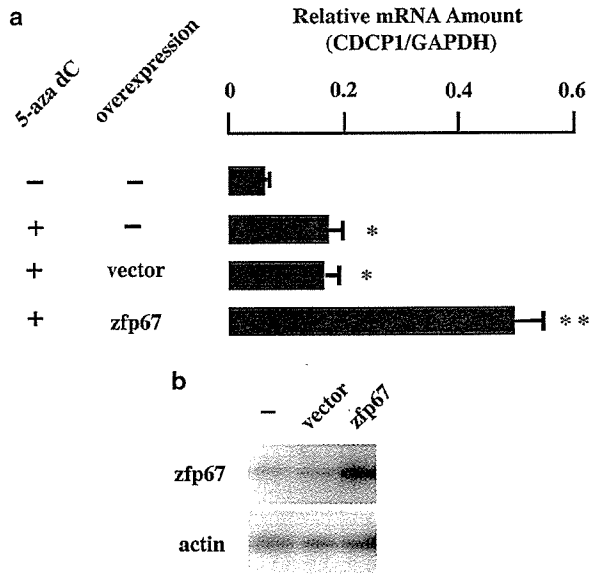

Figure 9 Effect of demethylation and *zfp67* overexpression in lymphocytes. (a) Effect of demethylating reagent 5-aza dC and overexpression of zfp67 on *CDCP1* expression. Quantitative real-time RT-PCR was carried out with mRNA from lymphocytes treated by 5-aza dC (2 μM) for 1 day, or from lymphocytes overexpressing *zfp67* after the treatment of 5-aza dC. The amount of *CDCP1* mRNA was normalized for the amount of *GAPDH* mRNA. The values represent the mean$\pm$s.e. of at least three experiments. *$P<0.05$ by *t*-test when compared to the value without treatment of 5-aza dC. **$P<0.05$ by *t*-test when compared to the value of lymphocytes overexpressing vector alone. (**b**) Immunoblot for zfp67. The amount of zfp67 was examined in lymphocytes not treated with 5-aza dC (−), treated with 5-aza dC followed by overexpression of vector alone (vector) or treated with 5-aza dC followed by overexpression of *zfp67* (zfp67).

$CD34^+CD38^-$ cells, possible human hematopoietic stem cells, abundantly expressed *CDCP1*. In contrast, mature hematopoietic cells such as granulocytes, lymphocytes and monocytes hardly expressed *CDCP1*. This was also consistent with the previous report.[4] The proportion of methylated CpG around *CDCP1* promoter was high in mature hematopoietic cells but low in stem cells, showing the inverse correlation between *CDCP1* expression level and proportion of methylated CpG sequences. Demethylation of CpG sequences in lymphocytes by 5-aza dC treatment induced *CDCP1* expression, and its level increased through zfp67 overexpression. These findings indicated the similar regulatory mechanism of *CDCP1* expression in hematopoietic cells to that in K562 and Jurkat.

Buhring *et al.*[4] demonstrated that *CDCP1* is expressed on cells phenotypically identical to mesenchymal stem/progenitor cells and neural progenitor cells. Thus, CDCP1 is not only a novel marker for immature hematopoietic progenitor cell subsets but also for cells with phenotypes reminiscent of stem/progenitor cells with non-hematopoietic lineages. Besides CDCP1, other stem cell markers of both hematopoietic and non-hematopoietic lineage cells, such as Nanog and Oct-4, possess CpG sequences around the transcription initiation site.[13] Although the relationship between transcription factors and the CpG methylation status in Nanog and Oct-4 gene has not been demonstrated as yet, Deb-Rinker *et al.*[13] recently reported that their CpG sequences were sequentially methylated as differentiation proceeds in human embryonal carcinoma cell line, resulting in the decrease of expression. As demonstrated in the present study, the epigenetic regulation of stem cell marker expression might be a common mechanism for gene silencing in the process of differentiation of hematopoietic cells.

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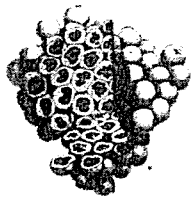

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HOX Decoy Peptide Enhances the Ex Vivo Expansion of Human Umbilical Cord Blood CD34 + Hematopoietic Stem Cells/Hematopoietic Progenitor Cells

Hirokazu Tanaka, Itaru Matsumura, Kiminari Itoh, Asako Hatsuyama, Masayuki Shikamura, Yusuke Satoh, Toshio Heike, Tatsutoshi Nakahata and Yuzuru Kanakura

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TRANSLATIONAL AND CLINICAL RESEARCH

HOX Decoy Peptide Enhances the Ex Vivo Expansion of Human Umbilical Cord Blood CD34$^+$ Hematopoietic Stem Cells/Hematopoietic Progenitor Cells

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Key Words. Ex vivo expansion • Hematopoietic stem/progenitor cells • HOX • Peptide mimetics

ABSTRACT

HOX transcription factors play important roles in the self-renewal of hematopoietic cells. HOX proteins interact with the non-HOX homeobox protein PBX1 to regulate, both positively and negatively, the expression of target genes. In this study, we synthesized a decoy peptide containing the YPWM motif from HOX proteins (decoy HOX [decHOX]), which was predicted to act as a HOX mimetic, and analyzed its effects on self-renewal of human cord blood CD34$^+$ cells. We were able to deliver decHOX into approximately 70% of CD34$^+$ cells. By examining the expression of HOX target genes c-*myc* and *p21*$^{waf1/cip1}$, we confirmed that decHOX enhanced HOX functions. After 7 days of culture in serum-free medium containing a cytokine cocktail, cultures treated with decHOX had approximately twofold-increased numbers of CD34$^+$ cells and primitive multipotent progenitor cells compared with control cells. Furthermore, decHOX-treated cells reconstituted hematopoiesis in nonobese diabetic/severe combined immunodeficiency mice more rapidly and more effectively (more than twofold greater efficiency, as determined by a limiting dilution method) than control cells. decHOX-treated cells were also able to repopulate secondary recipients. Together, these results indicate that in combination with growth factors and/or other approaches, decHOX might be a useful new tool for the ex vivo expansion of hematopoietic stem/progenitor cells.
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INTRODUCTION

Human umbilical cord blood (CB) is a useful source of hematopoietic stem cells (HSCs) for transplantation. In fact, during the last few years, an increasing number of patients have received CB transplants [1]. However, clinical applications of CB are inevitably limited by the fact that the number of HSCs in each CB sample is insufficient for many adult patients. Also, compared with transplantation of HSCs from the bone marrow or HSCs mobilized into peripheral blood, the recovery of hematopoiesis is rather delayed in patients receiving CB transplants, partly because of the insufficient number of transplanted HSCs and progenitor cells and the persistent quiescence of CB HSCs that sometimes accompanies lethal complications [1]. Therefore, it is of particular interest to expand CB HSCs ex vivo and to develop strategies for hastening hematopoietic recovery after CB transplantation in vivo [2]. Regarding strategies for ex vivo expansion, the most important problem is to preserve the functions and properties of HSCs, that is, self-renewal and multipotency, during culturing. At present, the use of cytokines is the most promising and practical strategy for this purpose. To establish the culture conditions most suitable for expansion of HSCs, a number of investigators have used various cytokine combinations [2, 3]. When their effects were compared by long-term reconstitution assays in transplanted mice, the combination of stem cell factor (SCF), FLT3 ligand (FL), thrombopoietin (TPO), and IL-6/soluble IL-6 receptor (sIL-6R) was found to expand HSCs most efficiently, with a 4.2-fold increase in severe combined immunodeficient (SCID)-repopulating cells (SRC) [4]. Several patients have received the transplantation with cytokine-expanded CB HSCs, and these cells were transplanted without serious toxicities [5, 6]. However, although increased numbers of infused CB HSCs were shown to correlate with good outcomes, cytokine-expanded CB HSCs did not shorten the nadir period after transplantation, indicating the

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