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Thrombospondin-bound Integrin-associated Protein (CD47) Physically and Functionally Modifies Integrin $\alpha_{IIb}\beta_3$ by Its Extracellular Domain*

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Integrin-associated protein (IAP/CD47) is a receptor for the C-terminal cell binding domain of thrombospondin (TS). A peptide from the C-terminal cell binding domain, KRFYVVMWKK (4N1K) binds to IAP and stimulates the integrin-dependent cell functions, including platelet aggregation. We investigated the mechanism by which TS-bound IAP modulates the affinity of platelet integrin, $\alpha_{IIb}\beta_3$. Platelet aggregation induced by 4N1K was not completely inhibited by energy depletion with sodium azide and 2-deoxy-D-glucose, although ADP or collagen-induced platelet response was completely inhibited. The binding of ligand-mimetic antibody PAC1 to $\alpha_{IIb}\beta_3$ was also induced in the energy-depleted platelets. In the transfected Namalwa cells, 4N1K induced activation of the $\alpha_{IIb}\beta_3$ with mutated β_3 (Ser-752 to Pro), which is a non-responsive form to inside-out signaling, as well as wild type $\alpha_{IIb}\beta_3$. The truncated form of IAP with only the extracellular immunoglobulin-like (Ig) domain was sufficient for the activation of $\alpha_{IIb}\beta_3$ in Chinese hamster ovary cells, although the IAP-mediated intracellular signaling was abolished, which was monitored by the absence of down-regulation of mitogen-activated protein kinase phosphorylation. Furthermore, the soluble recombinant Ig domain of IAP induced PAC1 binding to $\alpha_{IIb}\beta_3$ on Chinese hamster ovary cells when added with 4N1K. Physical association between the soluble recombinant Ig domain of IAP and purified $\alpha_{IIb}\beta_3$ was detected in the presence of 4N1K. These data indicate that the extracellular Ig domain of IAP, when bound to TS, interacts with $\alpha_{IIb}\beta_3$ and can change $\alpha_{IIb}\beta_3$ in a high affinity state without the requirement of intracellular signaling. This extracellular event would be a novel mechanism of affinity modulation of integrin.

Integrins are heterodimeric transmembrane receptor complexes involved in numerous physiological processes such as angiogenesis, immune response, and hemostasis (1, 2). They function in cell adhesion and signaling by interacting with an extracellular matrix or cellular counter receptors. The adhesive function is subject to rapid regulation referred to as inside-out signaling. A prototypical example of integrin modulation is the transition of platelet $\alpha_{IIb}\beta_3$ (GPIIb-IIIa complex) from a low affinity/avidity state to a state in which it can effectively bind soluble ligands such as fibrinogen (3–5). Recent evidence suggests that physiologically relevant signals are transduced to the integrin through its cytoplasmic domains by intracellular associated factors, such as β_3-endonexin, calcium integrin-binding protein, or cytoskeletal protein, talin (6–8). Studies of gene-targeted murine platelets or cultured megakaryocytes have defined the requirements for intracellular signaling molecules including Syk, phosphatidylinositol 3-kinase, vasodilator-stimulated phosphoprotein, calpain, and Rap1b (9–11). In addition, cell surface membrane proteins including urokinase plasminogen activator receptor, CD98, platelet endothelial cell adhesion molecule-1, and integrin-associated protein (IAP/CD47)[1] are also likely involved in the affinity modulation (12). Thus, the control of platelet $\alpha_{IIb}\beta_3$ seems to be complex. Recent crystallographic determination of the structure of integrin has shown that a bent integrin conformation has low affinity, and an extended structure is linked to high affinity access of macromolecular ligands to its contact site in the integrin head (13, 14). Such a dynamic conformational rearrangement in the integrin extracellular domains can be induced by ligand mimetic peptides, Mn^{2+}, and also by inside-out signaling. However, the molecular basis for regulation of its extracellular conformation is not entirely clear.

IAP is a 50-kDa membrane glycoprotein that has 5 transmembrane-spanning regions and 1 immunoglobulin (Ig)-like extracellular domain (15, 16). It was originally reported to be physically associated with certain integrins including $\alpha_2\beta_1$, $\alpha_V\beta_3$, and $\alpha_{IIb}\beta_3$ (17–19). Blockade of IAP with monoclonal antibody inhibits some aspects of integrin function, and ligation of IAP with activating antibodies induces the modulation of integrin-dependent cell adhesion. A gene-targeting study showed that IAP plays a key role in host defense by participation in migration and activation of leukocytes in response to bacterial infection (20). Subsequently, it was reported that IAP is a receptor for the C-terminal cell binding domain of thrombospondin (TS) (21). TS, its C-terminal cell binding domain, and a peptide from the C-terminal cell binding domain, KRFYVVMWKK (4N1K), all stimulate the integrin-dependent adhesion, spreading, and motility of the cells including endothelial cells, leukocytes, and smooth muscle cells (18, 22). On

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[1] The abbreviations used are: IAP, integrin-associated protein; srIAP, soluble recombinant form of IAP; TS, thrombospondin; FITC, fluorescein isothiocyanate; MAP, mitogen-activated protein; CHO, Chinese hamster ovary; IL-2, interleukin 2; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid.

the other hand, IAP is a ligand for the transmembrane signal-regulatory protein (23). In this case, IAP likely plays a role in macrophage function. The role on hematopoiesis (24) or adhesion of sickle red blood cell (25) was also reported.

In platelets, the functional role of IAP was hardly detected using only a monoclonal antibody against IAP (26). However, the peptide 4N1K induces platelet aggregation (27) and spreading on immobilized fibrinogen or collagen (19). The ability of the peptide depends on its interaction with IAP because platelet response was decreased by treatment with an antifunctional IAP antibody or in platelets from IAP-deficient mice (17). There are reports showing that the binding of 4N1K to IAP initiates intracellular signaling that would result in affinity modulation of $\alpha_{IIb}\beta_3$ (19). 4N1K induces tyrosine phosphorylation of several proteins such as Syk and focal adhesion kinase. The effect of 4N1K is inhibited by pertussis toxin, indicating the participation of a heterotrimeric G_i protein (19). This sort of stimulation of integrin function via G proteins is similar to that caused by agonists through seven transmembrane spanning receptors, such as ADP, epinephrine, and thrombin. Recently cloned ADP receptor (P2Y12) (28) also involves activation of G_i-containing heterotrimeric GTPases. However, these receptors are not necessarily present close to $\alpha_{IIb}\beta_3$. Therefore, the functional significance of the association of IAP with the integrin is not clear.

In this report we investigate the mechanism by which IAP modulates the affinity of $\alpha_{IIb}\beta_3$. We provide evidence that only the extracellular Ig domain of IAP interacts with $\alpha_{IIb}\beta_3$ and can change $\alpha_{IIb}\beta_3$ to a high affinity state without requirement of intracellular signaling when it binds to TS. These phenomena might correspond with the dynamic structural change of integrin. The extracellular event reported here would be a novel mechanism of affinity modulation of integrin.

EXPERIMENTAL PROCEDURES

Materials—Hybridoma cells producing anti-IAP monoclonal antibody B6H121 were purchased from American Type Culture Collection (ATCC; HB-9771, Manassas, VA). The antibody was purified from mouse ascites using HiTrap protein G affinity column (Amersham Biosciences) according to the manufacturer's instructions. T10, a monoclonal antibody that recognizes $\alpha_{IIb}\beta_3$ and inhibits fibrinogen binding, and Tab, an antibody that recognizes α_{IIb}, were kindly provided by Dr. R. P. McEver (Oklahoma Medical Research Foundation, Oklahoma City, OK) (29). Anti-LIBS6, an activating anti-β_3 antibody, was kindly provided by Dr. M. H. Ginsberg (Scripps Clinic, La Jolla, CA) (30). FITC-conjugated PAC1, a ligand-mimetic monoclonal antibody (31), was purchased from BD Biosciences). M2 anti-FLAG antibody was from Eastman Kodak Co.. Anti-phospho-p44/p42 MAP kinase (Thr-202/Tyr-204) antibody and anti-p44/p42 MAP kinase antibody were from New England Biolabs (Beverly, MA). Control antibodies were from Organon Technica (West Chester, PA). FITC-conjugated goat F(ab′)2 anti-mouse immunoglobulins and horseradish peroxidase-conjugated anti-mouse IgG were from TAGO (Burlingame, CA). Human $\alpha_{IIb}\beta_3$ was purified from platelet concentrates as previously described (32). Anti-$\alpha_{IIb}\beta_3$ antibody was raised by immunizing rabbits with purified human $\alpha_{IIb}\beta_3$ according to a standard protocol. IgG was purified by protein A CL-4B-Sepharose (Amersham Biosciences) according to the manufacturer's instructions. Specificity of the antibody was confirmed by Western blot using human platelets. ADP was purchased from Biopool (Ventura, CA). Human fibrinogen was purified as previously described (26). A peptide from the C-terminal cell binding domain of TS, 4N1K (KRFYVVMWKK), and a control scrambled peptide (KVFRWKYVMK) were synthesized by BioSynthesis (Lewisville, TX) and purified by high performance liquid chromatography. CHO-K1 cells and Namalwa cells were grown in Dulbecco's modified Eagle's medium/F-12 and RPMI-1620 (Invitrogen), respectively, and supplemented with 10% fetal bovine serum under 5% CO_2 at 37 °C. Expression vectors for α_{IIb} and β_3 in pBK-EF and CHO cells stably expressing $\alpha_{IIb}\beta_3$ were described previously (26).

Platelet Aggregation—Blood samples were collected from consenting healthy volunteer donors in a 1:10 volume of 3.8% trisodium citrate (w/v). Platelets were isolated from platelet-rich plasma by centrifugation at 800 × *g* for 10 min in the presence of 0.1 μg/ml prostaglandin E1 and 1 unit/ml apyrase. The pellet was resuspended and washed twice in 85 mM sodium citrate, 111 mM dextrose, 71 mM citric acid, pH 7.0, containing prostaglandin E1 and apyrase and then resuspended at a concentration of 3×10^8 platelets/ml in a modified Tyrode-HEPES buffer (138 mM NaCl, 0.36 mM NaH_2PO_4, 2.9 mM KCl, 12 mM $NaHCO_3$, 10 mM HEPES, 5 mM glucose, 1 mM $MgCl_2$, and 1 mM $CaCl_2$, pH 7.4). Platelet aggregation was measured by the addition of peptides (4N1K or control peptide) at 37 °C in an aggregometer (Chrono-log, Havertown, PA), with continuous stirring at 1200 rpm. To inhibit the fibrinogen binding or 4N1K binding, 100 μg/ml T10 or B6H12 was added 10 min before aggregation measurements. B6H12 induced direct aggregation of the platelets from some subjects depending on the polymorphism of Fc receptor, FcγRII. Therefore, the effect of B6H12 was tested using the platelets from "non-responders." To block the intracellular metabolism, platelets were incubated with 0.4% sodium azide and 4 mg/ml 2-deoxy-D-glucose at 37 °C for 1 h. Human fibrinogen was added at a final concentration of 200 μg/ml before the addition of several agonists (100 μM peptides, 5 μg/ml collagen, or 5 μM ADP).

Flow Cytometric Analysis—The affinity state of $\alpha_{IIb}\beta_3$ was determined by flow cytometric analysis using ligand-mimetic monoclonal antibody PAC1. PAC1 is an IgM monoclonal antibody that binds only to the activated form of $\alpha_{IIb}\beta_3$ in the same manner as the physiological ligand (31). Platelets were suspended at a concentration of 2×10^7/ml in a modified Tyrode-HEPES buffer and stimulated with peptides or agonists in the presence of 20 μg/ml FITC-conjugated PAC1 for 20 min at room temperature without stirring. Fifty μl of each aliquot was then diluted with 500 μl of the buffer, and the mixture was directly analyzed by a flow cytometer, Epics XL (Coulter, Fullerton, CA). Single platelets were gated and analyzed. In the case of cultured Namalwa and CHO cells, cells were harvested and resuspended at 1×10^6 cells/ml in a modified Tyrode-HEPES buffer. Cells were incubated with 100 μM peptides and/or antibodies (50 μg/ml LIBS6 for stimulation and 100 μg/ml T10 or B6H12 for inhibition) in the presence of 20 μg/ml FITC-PAC1 for 30 min at room temperature. After washing, cells were stained with 1 μg/ml propidium iodide, and propidium iodide-negative cells were gated to exclude permeabilized dead cells.

To estimate the expression of $\alpha_{IIb}\beta_3$ or IAP, cells were incubated with the first antibody, T10 or B6H12, (10 μg/ml) in Hanks' balanced salt solution with Ca^{2+} containing 1% fetal bovine serum and 0.1% NaN_3. After washing, they were incubated again with FITC-conjugated goat F(ab′)2 anti-mouse immunoglobulins. Each incubation was performed on ice for 1 h. Cells were finally washed and analyzed in the buffer containing propidium iodide. Because the expression levels of wild type and mutant forms of IAP in CHO cells were different, PAC1 binding was standardized by the mean fluorescence intensity of B6H12 binding to each cell type.

Mutational Analysis—Mutant cDNAs of $\alpha_{IIb}\beta_3$ and IAP were constructed by PCR according to the strategy previously described (33). To mutate Ser-752 of β_3, PCR was performed using cDNA of wild type β_3 as a template. The sense primer contained the *Bam*HI site at base 1500 within the β_3 cDNA, and the antisense primer contained the Ser-752 to Pro mutation. After gel purification, the PCR product was used for the second PCR. The second PCR was performed with the same sense primer and another antisense primer that overlapped with the first antisense primer and contained the stop codon of β_3 cDNA and the *Hin*dIII site for cloning. The PCR fragment was digested by *Bam*HI and *Hin*dIII, isolated by gel electrophoresis, and then used to replace the fragment of the wild type β_3 cDNA extending from the *Bam*HI site in the cDNA to the *Hin*dIII site in the multicloning site of the expression vector.

IAP cDNA in the expression vector was previously described (26). Wild type IAP in this study was identical to the so-called form 2, which is the most abundant isoform (15). Two truncated mutants were constructed. In the first construct (IAP Δ291) a stop codon was introduced just after the 5th transmembrane domain, resulting in deletion of the C-terminal cytoplasmic tail after Lys-291. In the second (IAP Δ163) a stop codon was created after the first transmembrane domain, which resulted in deletion after Lys-163. In both cases PCR was performed with the sense primer containing the translation initiation codon and the antisense primer containing the indicated stop codon. In another mutant form (IAP-Tac) the extracellular domain of IAP was fused with the transmembrane and cytoplasmic domains of the IL-2 receptor α chain (Tac antigen/CD25). Primer sets were prepared in which half of the sequence was identical to the end of the extracellular domain of IAP, before Asn-142, and the other half was identical to the beginning of the transmembrane domain of the IL-2 receptor. Two PCR were

performed separately to amplify the extracellular domain of IAP and the transmembrane and cytoplasmic domains of the IL-2 receptor. The PCR products were mixed, and the final PCR was performed using the outside primers.

All constructs were then cloned into the expression vector, pBK-EF, and verified by nucleotide sequencing of the region encoding the PCR products using an automated DNA sequencer (ABI 310, Applied Biosystems, Foster City, CA).

Cells Expressing Recombinant $\alpha_{IIb}\beta_3$ and IAP—Namalwa and CHO cells were transfected using DMRIE-C and LipofectAMINE reagents (Invitrogen), respectively, according to the manufacturer's instructions. Permanent transfectants were selected with G418 as described elsewhere. To establish the cells that stably express $\alpha_{IIb}\beta_3$ and/or IAP, stable cells were sorted by reactivity to the antibody T10 and/or B6H12 using a cell sorter (Epics Elite, Coulter, Fullerton, CA). Cells were cultured again for 5–7, days and the positive clones were obtained.

Immunoprecipitation and Immunoblotting Analysis—To determine the association of $\alpha_{IIb}\beta_3$ and IAP, platelets or transfected cells were lysed in an ice-cold lysis buffer (10 mM Tris, pH 7.4, 100 mM NaCl, 1 mM $CaCl_2$, 0.1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, and 100 μg/ml leupeptin). The lysates were chilled on ice for 1 h followed by centrifugation at 15,000 $\times$ *g* for 10 min. The supernatants were precleared with protein G-agarose (Immunopure Immobilized Protein G, Pierce), and the resultant supernatants were incubated for 1 h with protein G-agarose that was preincubated with antibodies T10, B6H12, or control antibody. Protein G-agarose was washed with the lysis buffer three times. The samples were then separated into two aliquots. One was solubilized with SDS sample buffer under reduced conditions and applied to 7% polyacrylamide electrophoresis gel (PAGE) for the detection of $\alpha_{IIb}\beta_3$. The other was incubated with SDS sample buffer under non-reduced conditions at 60 °C for 30 min and applied to 10% PAGE for IAP. The resolved proteins were then electrophoretically transferred to polyvinylidene difluoride membranes. The membranes were blocked with 10% skim milk in TBS buffer (10 mM Tris, 150 mM NaCl, pH 7.4) and then incubated with anti-$\alpha_{IIb}\beta_3$ rabbit polyclonal serum or 1 μg/ml B6H12 for 2 h. After extensive washing with TBS containing 0.1% Tween 20, antibody binding was detected using peroxidase-conjugated anti-rabbit or anti-mouse IgG and visualized with ECL chemiluminescence reaction reagents (Amersham Biosciences).

MAP Kinase Phosphorylation—To determine the intracellular signaling events, MAP kinase activation was analyzed in the CHO cells expressing IAPs (34). Cells were incubated with 4N1K or control peptide for 10 min and lysed by the addition of an equal volume of 2× lysis buffer (35) (20 mM Tris, pH 7.4, 40 mM KH_2PO_4, 10 mM sodium orthovanadate, 40 mM molybdic acid, 80 mM sodium pyrophosphate, 0.2 mM trifluoroperazine, 2 mM EGTA, 20 mM benzamidine, 2 mM phenylmethylsulfonyl fluoride, 200 μg/ml of leupeptin, and 2% Triton X-100). After removal of the insoluble fraction by centrifugation, cell lysates were subjected to immunoblotting with anti-phospho-MAPK antibody. Immunoblotting was performed as described above, except that blocking was with 2% bovine serum albumin. To reprobe with anti-MAPK antibody to detect the total MAPK levels, membranes were incubated in stripping buffer (62.5 mM Tris, pH 6.7, 100 mM 2-mercaptoethanol, 2% SDS) at 70 °C for 1 h, washed, and then used for the second immunoblotting.

Recombinant Soluble Form of IAP and Pull-down Assay—An expression vector that sequentially introduces a FLAG epitope, His tag, and a stop codon at 3′-end of cDNA in pBK-EF was kindly provided by Dr. K. Fukudome (Saga Medical College, Saga City, Japan) (36). To make recombinant soluble IAP (rsIAP), the extracellular domain of IAP until Asn-142 was PCR-amplified, digested, and ligated to this vector. Human 293 cells were transfected and selected with G418. High producing clones were screened in culture dishes by agarose diffusion immunoassay for secreted protein as previously described (37). Immunoassay was performed with B6H12 and the anti-FLAG antibody, M2. The cells producing the highest amount of rsIAP were grown until semi-confluent, and the culture medium was changed to serum-free medium for 293 cells, SFMII (Invitrogen). After 5 days of culture, the medium was collected, and the rsIAP was purified by a His-Trap chelating column (Amersham Biosciences) according to the manufacturer's instructions. The purification procedure was repeated twice, and the protein concentration was determined by BCA protein assay (Pierce). The effects of rsIAP on the affinity state of $\alpha_{IIb}\beta_3$ in CHO cells were analyzed by flow cytometer.

To analyze the association of $\alpha_{IIb}\beta_3$ and rsIAP *in vitro*, pull down assay was performed. Purified human $\alpha_{IIb}\beta_3$ (32) and rsIAP (10 μg each) were mixed in a buffer containing 50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 1% CHAPS, and then 100 μg peptide was added in a total volume of 100 μl for 3 h. Protein G-agarose, which was preincubated with 5 μg of antibodies Tab or M2, was added and further incubated for 1 h. The protein G-agarose was subsequently washed three times with the above buffer. Bound proteins were released in the SDS sample buffer and then determined by immunoblotting.

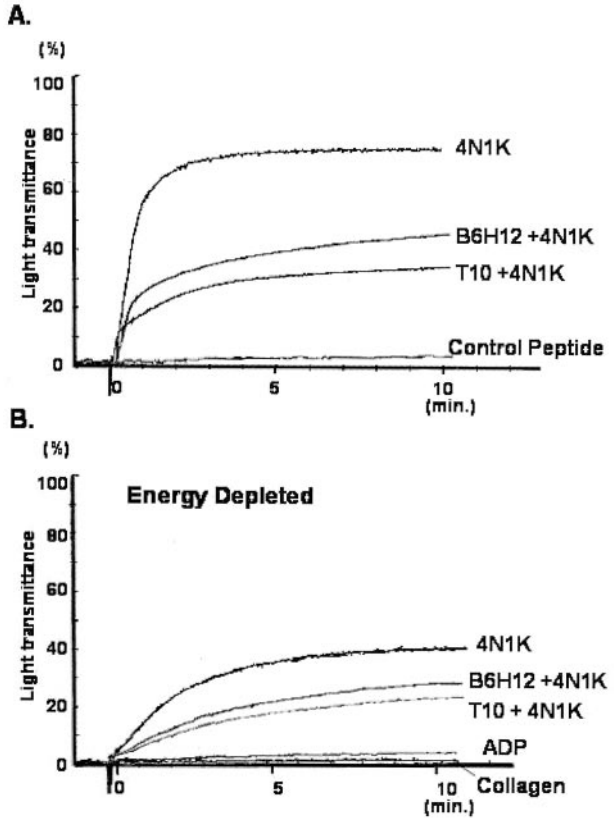

FIG. 1. **Platelet aggregation induced by 4N1K peptide was partially mediated by IAP, $\alpha_{IIb}\beta_3$, and intracellular metabolism.** *A*, washed human platelets were prepared in a modified Tyrode-HEPES buffer (3×10^8/ml), and aggregation was measured by the addition of 100 μM peptides (4N1K or control peptide). To inhibit the fibrinogen binding or 4N1K binding, 100 μg/ml T10 or B6H12 was added 10 min before the addition of 4N1K. Platelet aggregation was determined by measuring the transmission of light through a 200-μl sample with constant stirring (1200 rpm) in an aggregometer at 37 °C. The transmission of the modified Tyrode-HEPES buffer was set as 100%. The base line (0%) was set using a platelet suspension before the addition of agonists. The extent of aggregation was recorded for 10 min after the addition of the agonist. *B*, to block the intracellular metabolism, platelets were incubated with 0.4% sodium azide and 4 mg/ml 2-deoxy-D-glucose at 37 °C for 1 h, and then aggregation was measured. Human fibrinogen was added at a final concentration of 200 μg/ml before the addition of several agonists (100 μM 4N1K, 5 μg/ml collagen, or 5 μM ADP). Inhibition with antibodies (T10 and B6H12) was also measured. These results were confirmed in four separate experiments using platelets from different donors.

RESULTS

4N1K Induces the Activation of $\alpha_{IIb}\beta_3$ on Human Platelets—4N1K peptide, which is from the C-terminal cell binding domain of TS, induced aggregation of washed human platelets dose-dependently, with a maximal response at 100 μM. The mutated control peptide did not. Pretreatment with an inhibitory anti-$\alpha_{IIb}\beta_3$ antibody, T10, partially blocked the aggregation by ~50%. A functional antibody for IAP, B6H12, also partially inhibited 4N1K-induced aggregation (Fig. 1*A*). We next tested platelets in which intracellular response was prevented (energy-depleted). The mechanism of inside-out signaling is not entirely clear, but it is known that the affinity modulation of platelet $\alpha_{IIb}\beta_3$ requires metabolic energy and can be abolished by pretreatment with sodium azide and 2-deoxy-

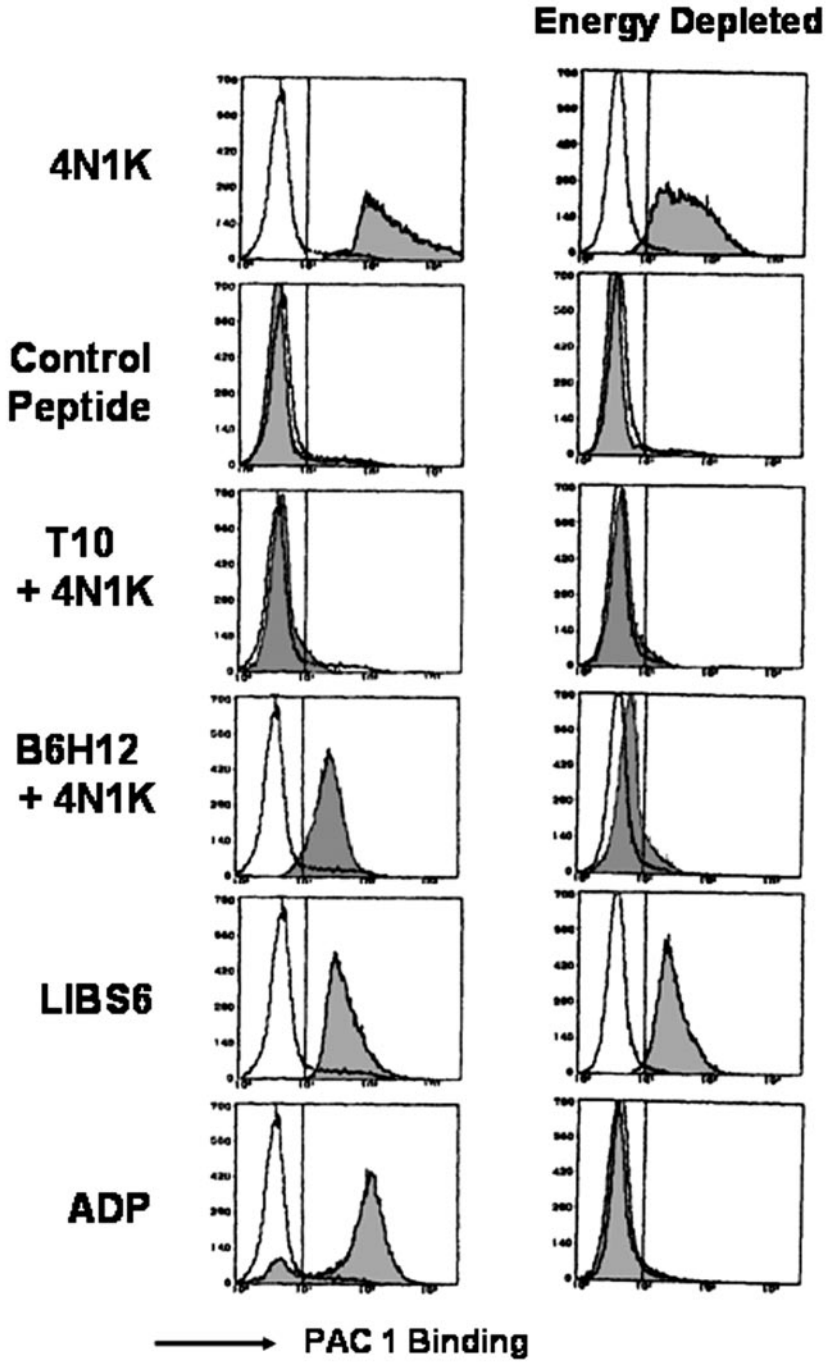

FIG. 2. **Activation of platelet $\alpha_{IIb}\beta_3$ induced by 4N1K peptide.** The affinity state of $\alpha_{IIb}\beta_3$ was determined by flow cytometric analysis using ligand-mimetic monoclonal antibody, PAC1. Washed platelets (*left*) or energy-depleted platelets (*right*) were stimulated with 100 μM peptides (4N1K or control peptide) or agonists (50 μg/ml LIBS6 or 5 μM ADP) in the presence of 20 μg/ml FITC-conjugated PAC1 for 20 min at room temperature. To analyze the involvement of IAP, 100 μg/ml B6H12 was added 10 min before the addition of 4N1K. Each aliquot was then diluted, and the PAC1 binding was analyzed by flow cytometer. Histograms were obtained from the gated single platelets. Open histograms indicate fluorescence obtained with FITC-conjugated control antibody, and filled histograms represent PAC1 binding. These results were confirmed in three separate experiments using platelets from different donors.

D-glucose (6). In the treated platelets, aggregation induced by collagen or ADP was completely abolished. However, 4N1K induced aggregation in the presence of fibrinogen, although the aggregation was decreased compared with non-treated platelets. This aggregation was also partially inhibited by antibody T10 or B6H12 (Fig. 1*B*). This result suggested that 4N1K induced platelet aggregation in part through agglutination, which was not dependent on $\alpha_{IIb}\beta_3$ and IAP, as reported recently (38), but also indicated the presence of aggregation mediated by $\alpha_{IIb}\beta_3$ and IAP. The latter part of the response partially depended on intracellular metabolism.

The activation state of $\alpha_{IIb}\beta_3$ was estimated by the binding of ligand mimetic antibody, PAC1 (Fig. 2). 4N1K induced PAC1 binding to platelets, whereas control peptide did not. The binding was completely inhibited by T10 and also partially inhibited by B6H12. In the energy-depleted platelets, 4N1K still induced PAC1 binding, although the binding induced by ADP was completely blocked. B6H12 largely inhibited the binding in this case. The effects were similar to that of activating antibody, LIBS6. These results suggested that there are pathways in which 4N1K induces the activation of $\alpha_{IIb}\beta_3$ via IAP, and some pathways do not require the intracellular signaling. In the following study, we used the expression system in the heterologous cultured cells since 4N1K did not induce the agglutination of these cells.

4N1K Activates $\alpha_{IIb}\beta_3$ Independently with Intracellular Signaling Events—To determine the mechanism underlying the activation of $\alpha_{IIb}\beta_3$ induced by 4N1K, an expression study was performed in a human B-lymphocytic cell line, Namalwa cells. These cells expressed a high amount of intrinsic IAP (Fig. 3*A*). Wild type cDNA of $\alpha_{IIb}\beta_3$ was transfected into the cells, and stable clones were selected. In the transfected cells, the asso-

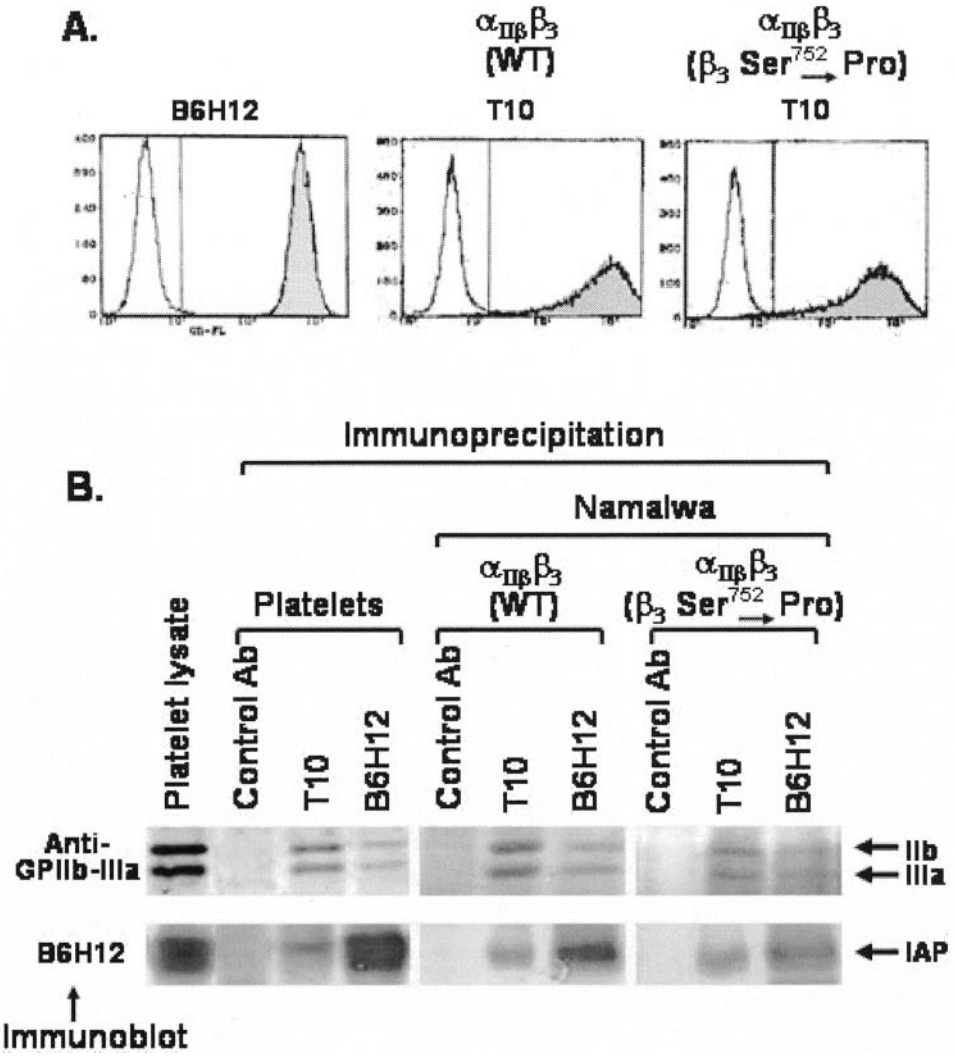

FIG. 3. **Characterization of the transfected Namalwa cells expressing wild type and mutant $\alpha_{IIb}\beta_3$.** Namalwa cells were transfected with cDNAs of wild type α_{IIb} and β_3 or mutant β_3, which contained single amino acid substitution (Ser-752 to Pro) in the cytoplasmic domain, and the permanent transfectants were established. *A*, expression of IAP and $\alpha_{IIb}\beta_3$ was analyzed by flow cytometer. The *left panel* indicates the surface expression of IAP in the parental Namalwa cells analyzed by B6H12. The *middle* and *right* panels indicate expression of $\alpha_{IIb}\beta_3$ in the transfectants with wild type (*WT*) and mutant $\alpha_{IIb}\beta_3$ analyzed by T10. *Open histograms* represent fluorescence obtained with control antibody, and *filled histograms* represent binding of the indicated antibody. *B*, association between IAP and $\alpha_{IIb}\beta_3$ was analyzed by immunoprecipitation. Cells were lysed and immunoprecipitated with antibodies T10, B6H12, or control antibody (*Ab*). After SDS-PAGE, $\alpha_{IIb}\beta_3$ was detected by immunoblotting with anti-$\alpha_{IIb}\beta_3$ rabbit polyclonal antibody, and IAP was detected with B6H12. A control experiment using human platelets is also shown.

ciation of $\alpha_{IIb}\beta_3$ and IAP was detected because $\alpha_{IIb}\beta_3$ and IAP was co-immunoprecipitated by T10 and B6H12, as observed in platelets (Fig. 3*B*). The activation of $\alpha_{IIb}\beta_3$ was analyzed with these cells. 4N1K induced PAC1 binding although control peptide did not. PAC1 binding was almost completely blocked by T10 and B6H12, indicating that activation of $\alpha_{IIb}\beta_3$ was thoroughly mediated by IAP in these cells (Fig. 4). Treatment with sodium azide and 2-deoxy-D-glucose did not abolish the binding of PAC1 (not shown). We prepared mutant cDNA of β_3 (Ser-752 to Pro) and its transfectants. Platelets containing $\alpha_{IIb}\beta_3$ with this mutation fail to bind fibrinogen or aggregate upon agonist stimulation probably because intracellular signaling cannot be transduced to the cytoplasmic domains (39). The mutant β_3 formed a complex with α_{IIb} and associated with IAP in the transfected cells, as did wild type β_3, which was demonstrated by immunoprecipitation assay (Fig. 3*B*). 4N1K induced PAC1 binding to the mutant $\alpha_{IIb}\beta_3$ as well as to the wild type. The binding was also likely mediated by IAP because of B6H12 inhibition (Fig. 4). The same results were observed in the three independent stable transfectants. The results further support the theory that the activation of $\alpha_{IIb}\beta_3$ can be induced by 4N1K independently with intracellular signaling events.

Activation of $\alpha_{IIb}\beta_3$ by 4N1K Does Not Require the Transmembrane and Cytoplasmic Domains of IAP—We next examined the effects of deletion mutants of IAP on the activation of $\alpha_{IIb}\beta_3$. For this purpose, we used CHO cells since in our previous study (26) intrinsic IAP of CHO cells did not affect the function of human $\alpha_{IIb}\beta_3$, although CHO cells may express hamster IAP. In addition, 4N1K did not induce the activation of $\alpha_{IIb}\beta_3$ in CHO cells, indicating that 4N1K peptide derived from human TS fails to bind hamster IAP or fails to affect the $\alpha_{IIb}\beta_3$ even if the peptide is bound to it. Wild type IAP and two truncated mutants were prepared. However, a mutant with a single transmembrane domain (IAP Δ163) was not effectively expressed on the cell surface. Therefore, another mutant form was created in which the extracellular domain of IAP was fused with the transmembrane and cytoplasmic domains of an irrelevant transmembrane protein, IL-2 receptor (Tac antigen). Three constructs (Fig. 5*A*: wild type IAP, IAP Δ291, and IAP-Tac) were co-transfected into CHO cells with the cDNA of $\alpha_{IIb}\beta_3$. The established clones expressed comparable amounts of $\alpha_{IIb}\beta_3$ (Fig. 5*B*). All IAP constructs were recognized by anti-

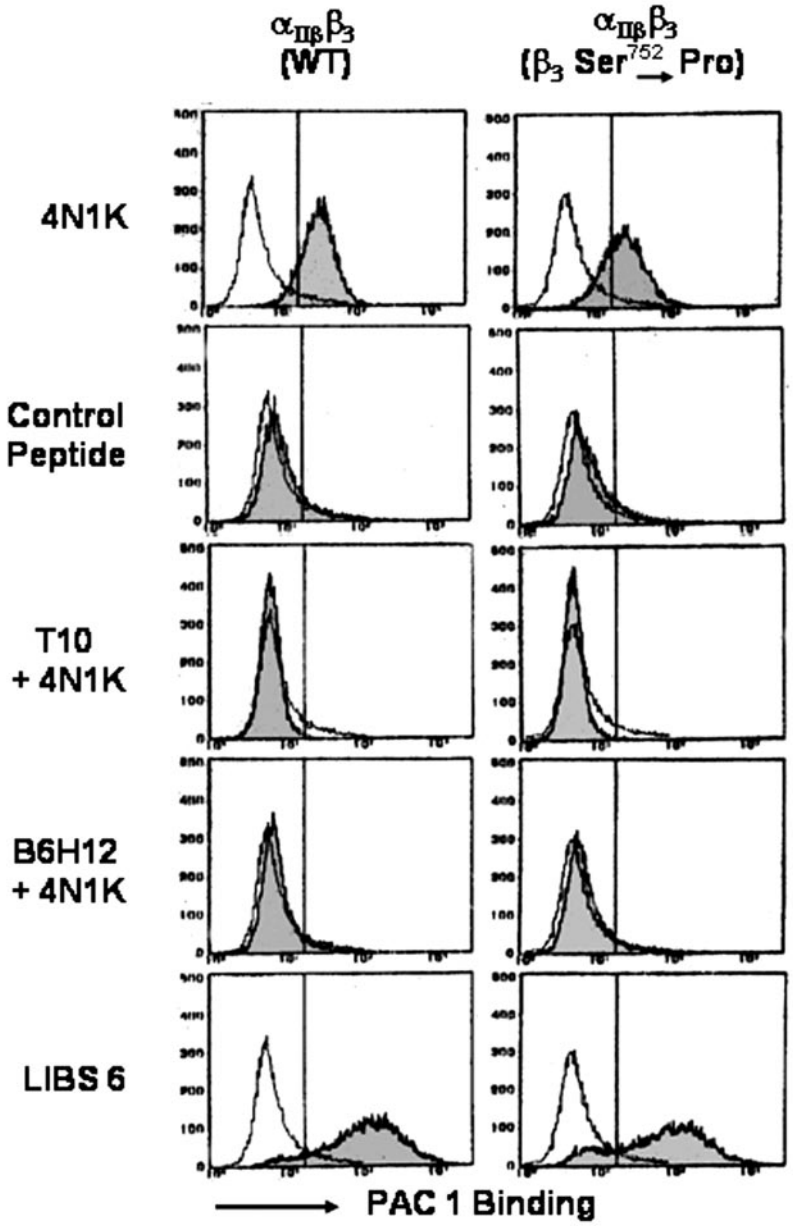

FIG. 4. **Activation of wild type and mutant $\alpha_{IIb}\beta_3$ by 4N1K peptide in transfected Namalwa cells.** The affinity state of $\alpha_{IIb}\beta_3$ in Namalwa cells was determined by the binding of PAC1 antibody. Cells expressing wild type $\alpha_{IIb}\beta_3$ (*left*) or the mutant form (Ser-752 to Pro) (*right*) were incubated with 100 μM peptides (4N1K or control peptide) or activating antibody (50 μg/ml LIBS6) in the presence of 20 μg/ml FITC-conjugated PAC1 for 20 min at room temperature. For the inhibition study, 100 μg/ml B6H12 or T10 was added 10 min before the addition of 4N1K. *Open histograms* indicate fluorescence obtained with FITC-conjugated control antibody, and *filled histograms* represent PAC1 binding. The same results were observed in three independent stable transfectants.

body B6H12 and detected by immunoblotting at an expected molecular size (Fig. 5*C*). The association of $\alpha_{IIb}\beta_3$ with the wild type or mutant form IAP (IAP Δ291) was detected by immunoprecipitation. However, the association with IAP-Tac was hardly detected (Fig. 5*D*).

4N1K induced PAC1 binding to all the cells expressing wild type IAP, IAP Δ291, and IAP-Tac, whereas the binding was not observed with control peptide (Fig. 6*A*). T10 and B6H12 inhibited the binding, as observed in Namalwa cells (not shown). Because the expression level of the three IAP forms varied (Fig. 5*B*), PAC1 binding was standardized by each surface expression level. Namely, mean fluorescence intensity of PAC1 binding in the presence of 4N1K was divided by mean fluorescence intensity of B6H12 binding and calculated as an activation index. The activation index was ~0.8 with IAP Δ291 and 0.5 with IAP-Tac, whereas the index with wild type was 1.0. We tested at least three independent clones expressing each IAP construct and obtained the same results.

To confirm that the construct with only the extracellular domain of IAP did not cause the intracellular signaling, we analyzed the phosphorylation of MAP kinase. It was reported that the binding of 4N1K to IAP caused G_i-mediated strong inhibition of MAP kinases, a unique phenomenon among IAP-mediated signal pathways (34). 4N1K caused down-regulation of MAP kinase phosphorylation (mainly p44) in the CHO cells expressing the wild type and the mutant IAP (IAP Δ291) (Fig. 6*B*), although total MAP kinase levels were not changed (not shown). In contrast, 4N1K did not influence the phosphorylation in CHO cells expressing IAP-Tac, indicating that intracellular signaling was not induced by the mutant form with only the extracellular domain of IAP. We concluded that the alteration of $\alpha_{IIb}\beta_3$ to the active form induced by 4N1K was possible by only the extracellular domain of IAP.

4N1K-bound Extracellular Domain of IAP Can Associate with $\alpha_{IIb}\beta_3$ and Activate It—We further analyzed the effects of rsIAP. Purified rsIAP was recognized by B6H12 and detected as a single band at ~30 kDa in immunoblotting (Fig. 7*A*). Using CHO cells that expressed a high amount of $\alpha_{IIb}\beta_3$ alone, the affinity state of $\alpha_{IIb}\beta_3$ was analyzed. To our surprise, the addition of srIAP together with 4N1K caused PAC1 binding to $\alpha_{IIb}\beta_3$ on the surface of CHO cells (Fig. 7*B*). With the control peptide or with only srIAP or 4N1K, PAC1 binding was not

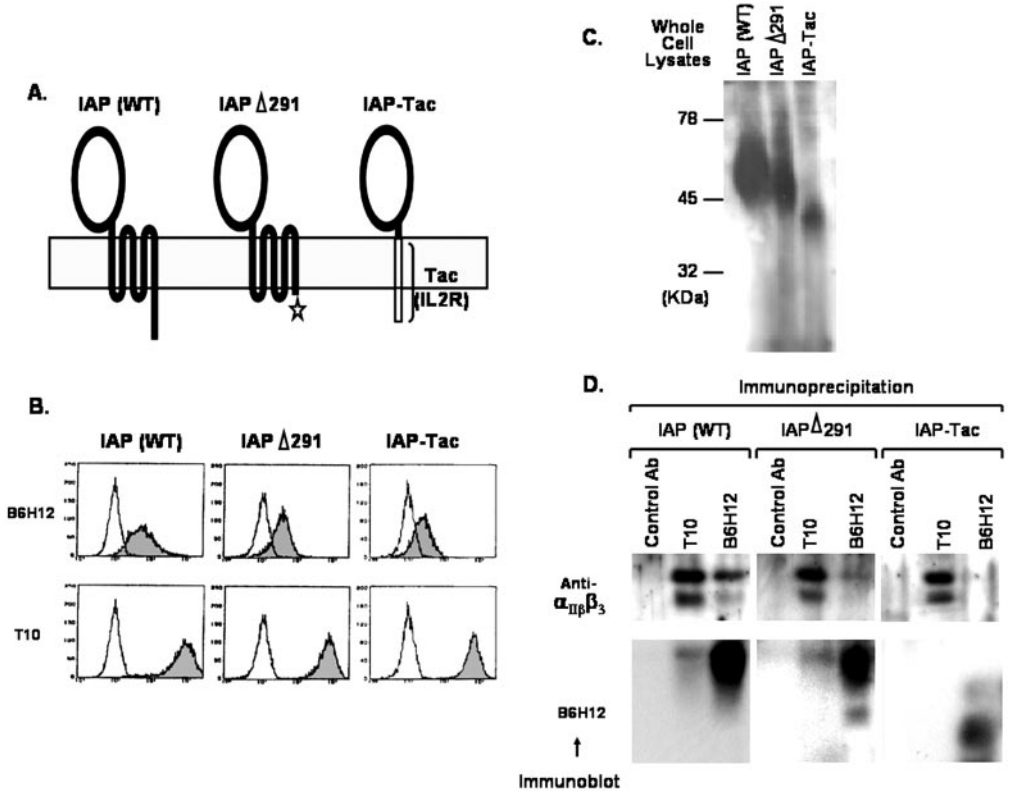

FIG. 5. **Characterization of the CHO cells expressing wild type and mutant forms of IAP with $\alpha_{IIb}\beta_3$.** CHO cells were transfected with cDNAs of wild type or mutant forms of IAP together with cDNA of $\alpha_{IIb}\beta_3$, and the permanent transfectants were established. *A*, schematics of the three forms of IAP analyzed in this study. Wild type (*WT*) IAP has a single extracellular Ig domain, five transmembrane-spanning regions, and a short cytoplasmic tail. In the truncated mutant (IAP Δ291), a stop codon was introduced just after the 5th transmembrane domain, resulting in deletion of the C-terminal cytoplasmic tail. In the third mutant form (IAP-Tac), the extracellular domain of IAP was fused with the transmembrane and cytoplasmic domains of IL-2 receptor α chain (Tac antigen/CD25). *B*, expression of IAP and $\alpha_{IIb}\beta_3$ was analyzed by flow cytometer. The *upper panel* indicates the surface expression of IAP analyzed by B6H12, which recognizes the extracellular Ig domain. The *lower panel* indicates expression of $\alpha_{IIb}\beta_3$ analyzed by T10. *Open histograms* represent fluorescence obtained with control antibody, and *filled histograms* represent binding of the indicated antibody. *C*, using the cell lysate of each transfectant, the molecular size of the three IAP forms was determined by immunoblotting with B6H12. *D*, association between IAP and $\alpha_{IIb}\beta_3$ was analyzed by immunoprecipitation. Cells were lysed and immunoprecipitated with antibodies T10, B6H12, or control antibody. After SDS-PAGE, $\alpha_{IIb}\beta_3$ or IAP was detected by immunoblotting using anti-$\alpha_{IIb}\beta_3$ rabbit polyclonal antibody or B6H12.

induced. Although the physical association of rsIAP and $\alpha_{IIb}\beta_3$ was hardly detected by immunoprecipitation using the CHO cells (not shown), the data suggested that the rsIAP functionally interacted with $\alpha_{IIb}\beta_3$ and caused its activation in the presence of 4N1K.

To detect the association of srIAP with $\alpha_{IIb}\beta_3$, we finally tried a pull-down assay using purified proteins in the relatively mild detergent, 1% CHAPS (Fig. 8). After the two proteins were mixed, $\alpha_{IIb}\beta_3$ was precipitated by anti-α_{IIb} antibody, Tab, and srIAP was precipitated by M2, which recognized the FLAG epitope introduced at the C-terminal end of srIAP. When 4N1K was present, a significant amount of srIAP was coprecipitated with $\alpha_{IIb}\beta_3$ by Tab, whereas without the peptide or with the control peptide, only a faint band of srIAP was detected. Conversely, $\alpha_{IIb}\beta_3$ was detected in the precipitates by M2 only when 4N1K was added. These results indicated that the binding of 4N1K to the extracellular Ig domain of IAP facilitated the physical association with $\alpha_{IIb}\beta_3$.

DISCUSSION

In this study, we investigated the mechanism by which IAP modulates the affinity of platelet integrin, $\alpha_{IIb}\beta_3$. The obtained evidence was as follows. (*a*) Platelet aggregation induced by 4N1K was not completely inhibited by energy depletion. The binding of PAC1 to $\alpha_{IIb}\beta_3$ was also observed in the energy-depleted platelets. (*b*) In the transfected Namalwa cells, 4N1K induced activation of $\alpha_{IIb}\beta_3$ with mutated β_3 (Ser-752 to Pro) as well as wild type $\alpha_{IIb}\beta_3$. (*c*) In CHO cells, the truncated form of IAP with only the extracellular Ig domain was sufficient for activation of $\alpha_{IIb}\beta_3$, although IAP-mediated intracellular signaling was abolished. (*d*) srIAP induced PAC1 binding to the $\alpha_{IIb}\beta_3$ on CHO cells when added with 4N1K. Physical association between srIAP and purified $\alpha_{IIb}\beta_3$ was detected in the presence of 4N1K. These results led us to conclude that the Ig domain of IAP when bound to TS associates with the extracellular domain of $\alpha_{IIb}\beta_3$ and changes it to the active form without the requirement of intracellular signaling.

Tulasne *et al.* (38) recently reported that 4N1K induces platelet agglutination in addition to aggregation, and the agglutination is independent of $\alpha_{IIb}\beta_3$. Our data corresponded in part with this since anti-$\alpha_{IIb}\beta_3$ inhibitory antibody only partially inhibited platelet aggregation by 4N1K. They further concluded that platelet aggregation or agglutination was not mediated by IAP because the ability of 4N1K to stimulate aggregation was not altered in the platelets from IAP-deficient mice. However, this is a still controversial. In previous studies, Chung *et al.* conclude that the effect of 4N1K or TS on platelet aggregation was absolutely dependent on IAP because the platelets from IAP-deficient mice did not form aggregates in response to 4N1K or TS on the collagen surface (17) and also showed little spreading of the B6H12-treated platelets on immobilized fibrinogen (19). The disparity in findings may have resulted from different measurement conditions and is proba-

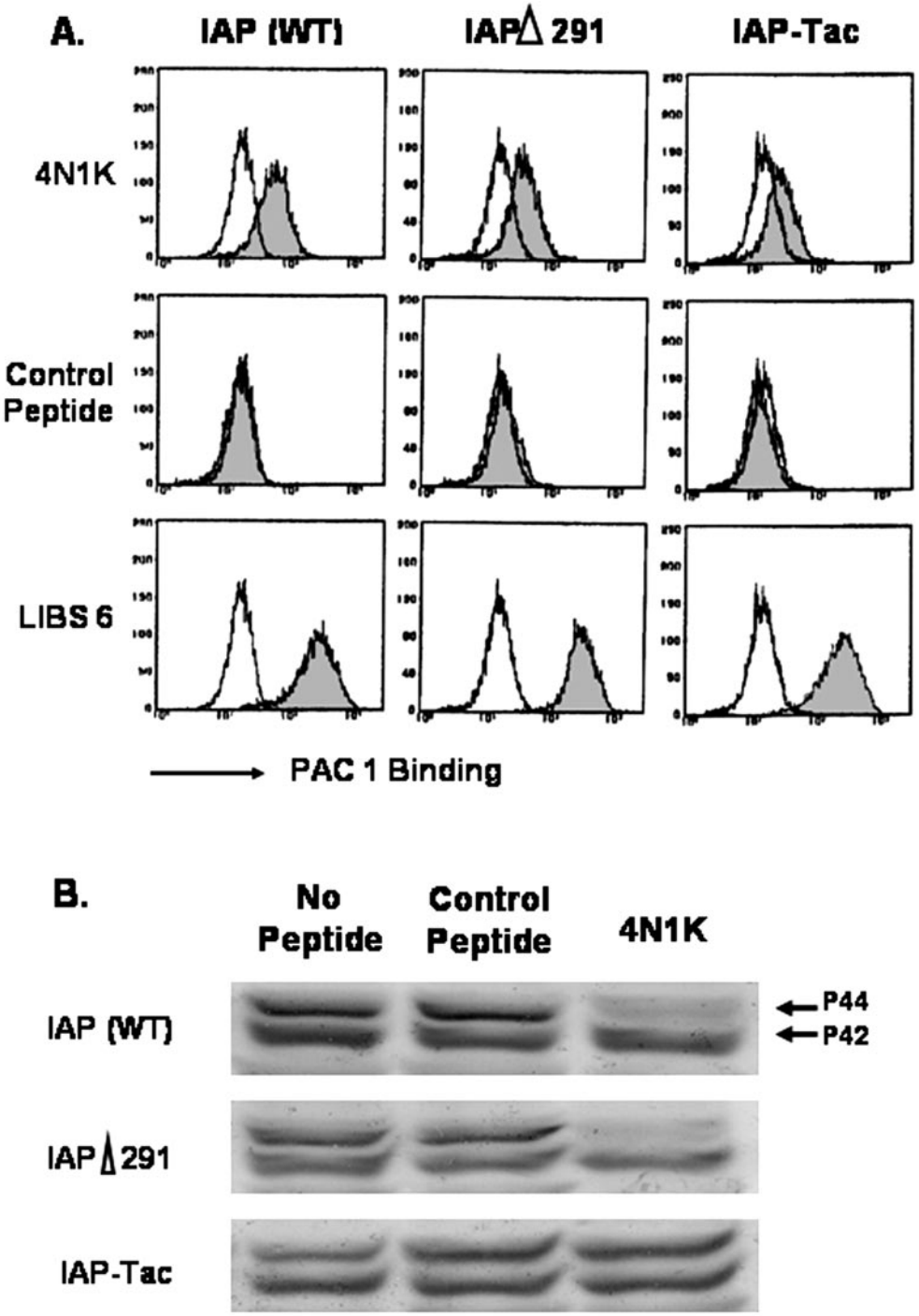

FIG. 6. **Activation of $\alpha_{IIb}\beta_3$ in CHO cells expressing wild type and mutant forms of IAP by 4N1K without intracellular signaling.** *A*, the affinity state of $\alpha_{IIb}\beta_3$ in CHO cells was determined by PAC1 binding. Cells expressing wild type (*WT*) or mutant forms of IAP (IAP Δ291 and IAP-Tac) were incubated with 100 μM peptides (4N1K or control peptide) or activating antibody (50 μg/ml LIBS6) in the presence of 20 μg/ml FITC-conjugated PAC1. *Open histograms* indicate fluorescence obtained with FITC-conjugated control antibody, and *filled histograms* represent PAC1 binding. *B*, to determine the intracellular signaling events, MAP kinase activation was analyzed by immunoblotting in these CHO cells. After incubation with 4N1K or control peptide for 10 min, cells were lysed and subjected to immunoblotting with anti-phospho-MAPK antibody. These results were confirmed with three independent clones expressing each IAP.

bly due to difficulty in separating the platelet agglutination and aggregation.

Our results supported the presence of IAP-mediated platelet aggregation since B6H12 partially inhibited it. The data suggested that there are at least three components by which platelets form aggregates in response to 4N1K. The first part is aggregation, which requires inside-out signaling. This was shown by the decrease in aggregation or intensity of PAC1 binding in the energy-depleted platelets compared with non-treated platelets. The second is agglutination, which was shown by the residual response of the energy-depleted platelets in the presence of antibodies to $\alpha_{IIb}\beta_3$ and IAP. The third is aggregation, which was represented by the deceased part by the antibodies in the energy-depleted platelets. The last aggregation does not require intracellular signaling but is mediated by $\alpha_{IIb}\beta_3$ and IAP.

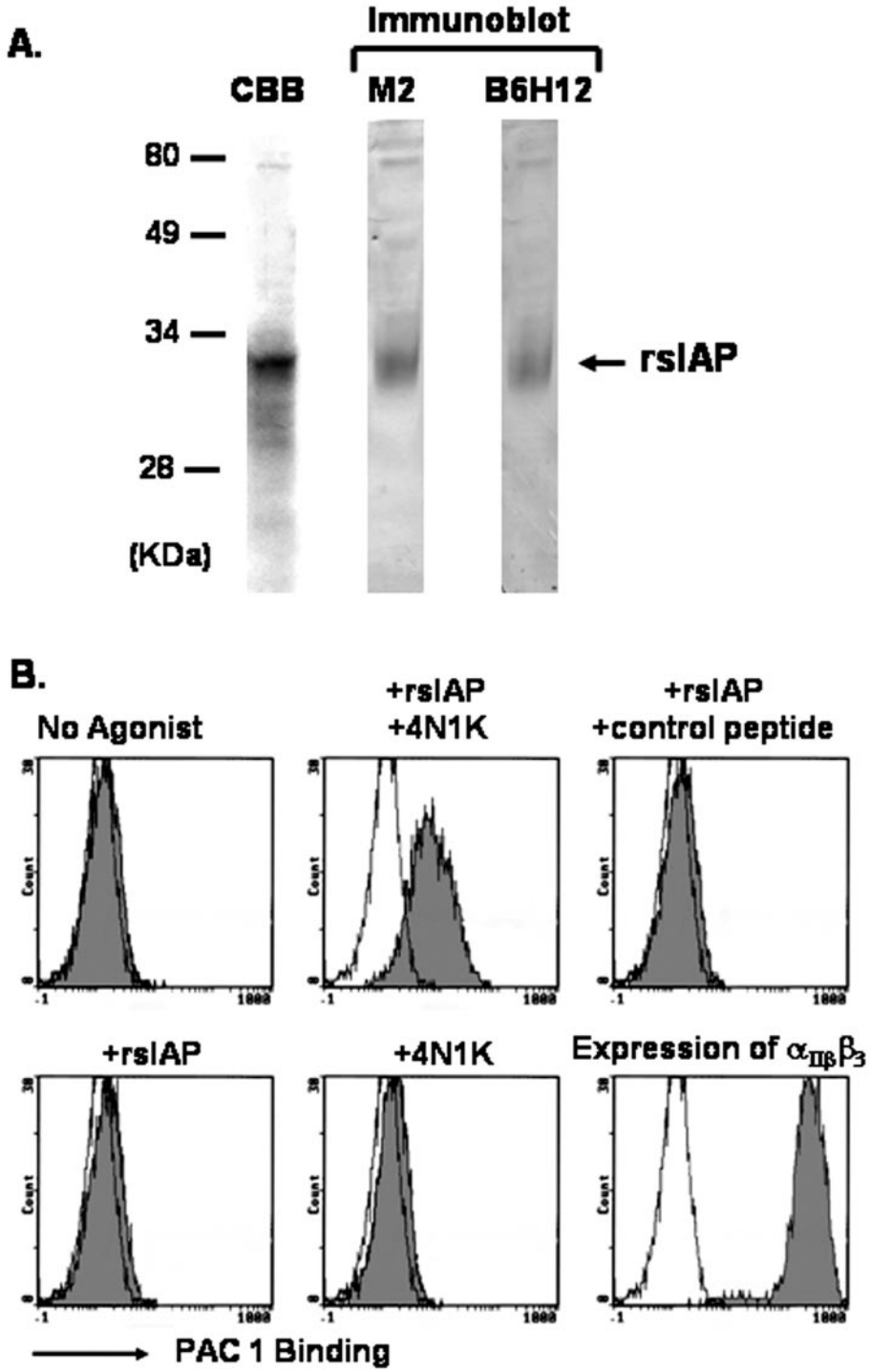

FIG. 7. **Recombinant soluble IAP caused PAC1 binding to the $\alpha_{IIb}\beta_3$ on CHO cells in the presence of 4N1K.** *A*, the extracellular domain of IAP was expressed and purified as rsIAP. The purified protein was analyzed by SDS-PAGE followed by Coomassie Brilliant Blue staining (*CBB*) and by immunoblotting using B6H12 and anti-FLAG antibody, M2. *B*, the effects of rsIAP on the affinity state of $\alpha_{IIb}\beta_3$ were analyzed in CHO cells. CHO cells expressing $\alpha_{IIb}\beta_3$ were incubated with 50 μg/ml rsIAP plus 100 μM 4N1K in the presence of 20 μg/ml FITC-conjugated PAC1, and the PAC1 binding was analyzed by flow cytometer. As controls, cells were also incubated with rsIAP alone, 4N1K alone, or rsIAP plus control peptide. *Open histograms* indicate fluorescence obtained with FITC-conjugated control antibody, and *filled histograms* represent PAC1 binding. Expression of $\alpha_{IIb}\beta_3$ on the analyzed CHO cells was determined by T10. These results were confirmed in three separate experiments.

In previous studies using cultured cells, the effects of 4N1K were clearly demonstrated probably because cells other than platelets did not show agglutination. 4N1K can modulate the function of $\alpha_V\beta_3$ on endothelial or melanoma cells and $\alpha_2\beta_1$ on smooth muscle cells (15, 34). IAP-expressing cells spread much more rapidly on vitronectin in the presence of 4N1K (18). The attachment and migration of smooth muscle cells on matrices through $\alpha_2\beta_1$ was dramatically stimulated by 4N1K, and this increased migration was blocked by antibodies recognizing either $\alpha_2\beta_1$ or IAP (22). In smooth muscle cells from IAP-deficient mice, 4N1K did not stimulate migration (34). All these experiments represent clear proof that the 4N1K peptide acts through IAP to augment integrin functions. In this study, 4N1K induced PAC1 binding to $\alpha_{IIb}\beta_3$ in Namalwa and CHO cells, and the effects were dependent on IAP since PAC1 binding was completely inhibited by B6H12 in these cells.

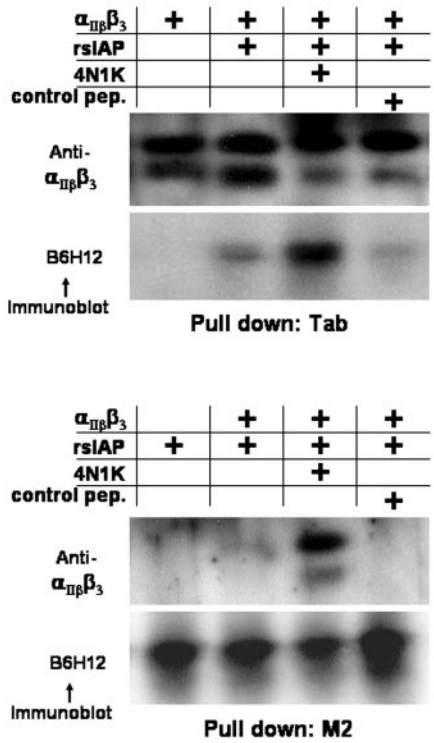

FIG. 8. **Association of purified $\alpha_{IIb}\beta_3$ and rsIAP determined by pull down assay.** Purified $\alpha_{IIb}\beta_3$ and rsIAP (10 μg each) were mixed in a presence of 100 μg of peptide (4N1K or control peptide) as indicated *above* the *panels*. $\alpha_{IIb}\beta_3$ or rsIAP was pulled down by anti-α_{IIb} antibody Tab- or anti-FLAG antibody M2-bound protein G-agarose, respectively. Bound proteins were released in SDS sample buffer and then determined by immunoblotting using anti-$\alpha_{IIb}\beta_3$ rabbit polyclonal antibody or B6H12. These results were confirmed in three separate experiments.

It has been reported that the activation of G_i-containing heterotrimeric GTPases is involved in intracellular signaling induced by IAP (15, 16). 4N1K caused an immediate and dramatic fall in cAMP levels within the cells, including platelets. The inhibition of phosphorylation of MAP kinase was also likely mediated by G_i protein (34). Another study showed that the Fc receptor γ-chain was associated with signaling in 4N1K-induced platelet aggregation (38). Our data also suggested the presence of these signaling pathways in addition to the direct activating mechanism of $\alpha_{IIb}\beta_3$. Indeed, platelet aggregation induced by 4N1K was partially inhibited by energy depletion. By this treatment, G_i-coupled or Fcγ chain-associated signaling was completely abolished; this was monitored by the absence of ADP or collagen-induced response since it is known that the major platelet ADP receptor, P2Y12, is coupled with G_i (28) and collagen receptor GPVI is coupled with the Fcγ chain (40). The binding of PAC1 to the energy-depleted platelets was detected but decreased compared with that of non-treated platelets. 4N1K induced the activation of the mutant $\alpha_{IIb}\beta_3$ (Ser-752 to Pro) but to a lower extent than the activation of wild type $\alpha_{IIb}\beta_3$ in Namalwa cells. Activation of $\alpha_{IIb}\beta_3$ was induced with the truncated form of only the extracellular Ig domain of IAP (IAP-Tac) in CHO cells, but when PAC1 binding was standardized by its surface expression, the PAC1 intensity was approximately half compared with that with wild type IAP. One possible explanation for this is a lower efficiency of the association with $\alpha_{IIb}\beta_3$ since the association between IAP-Tac and $\alpha_{IIb}\beta_3$ was hardly detected by immunoprecipitation. However, it might account for the absence of intracellular signaling.

The structural requirement for physical and functional association was examined only for IAP association with $\alpha_V\beta_3$. Lindberg *et al.* (41) demonstrate that the Ig domain of IAP was required for functional cooperation with $\alpha_V\beta_3$. Cells expressing only Ig domain attached to the plasma membrane with a glycan phosphoinositol anchor or a CD7 single transmembrane segment facilitated the ligand binding function of $\alpha_V\beta_3$. They also showed that these constructs were hardly immunoprecipitated with $\alpha_V\beta_3$ or only a small minority of total $\alpha_V\beta_3$ was coprecipitated. Our data support the importance of Ig domain also in the association with $\alpha_{IIb}\beta_3$. Using a similar construct of IAP-Tac and the recombinant soluble Ig domain of IAP, we further demonstrated direct evidence that upon binding to TS, the Ig domain can physically associate with the extracellular domain of $\alpha_{IIb}\beta_3$ with a higher affinity and then change the $\alpha_{IIb}\beta_3$ to the active form. It was suggested that the physical association requires a higher affinity interaction between IAP and integrins than functional association since the association between IAP-Tac and $\alpha_{IIb}\beta_3$ was not detected by immunoprecipitation, which was in agreement with the above report. The multiple membrane-spanning domain of IAP may stabilize the physical association with integrins (42).

Rebres *et al.* (43) report the presence of a long range disulfide bond between the extracellular and membrane-spanning domains of IAP (Cys-33 and Cys-263). They demonstrated that IAP with the mutation of the cysteines showed impaired signal transduction and reduction of binding to another IAP ligand, signal-regulatory protein. But loss of the disulfide bond did not affect the association with $\alpha_V\beta_3$. Therefore, this disulfide bond is likely to be important for signaling events rather than interaction with integrins. The construct IAP-Tac or rsIAP used in our study theoretically did not contain this disulfide bond. However, consistent with their report, these recombinant forms were sufficient for interaction with $\alpha_{IIb}\beta_3$.

Cells can regulate the integrin-mediated response by changing their affinity for ligands. Rapid changes in affinity have been widely documented among a number of integrins including not only β_3 but also β_1, β_2, and β_7 classes (2). The regulation of integrin function or inside-out signaling is believed to be mediated by the cytoplasmic domains. The interaction between integrin α and β subunit cytoplasmic tails or the linkage with cytoplasmic proteins can regulate the affinity of the extracellular domain by an allosteric mechanism (8). This may involve transmission of long range dynamic conformational rearrangements, namely from a bent conformation to a high affinity extended structure (13). Our data suggested that one of the signals that can induce such conformational change may be transduced at the extracellular domains of $\alpha_{IIb}\beta_3$ by the Ig domain of IAP, which may not require the long range conformational rearrangement from its cytoplasmic tail. Ligand mimetic peptides or Mn^{2+} can directly induce a conformational change in integrin extracellular domains (13). Several activating monoclonal antibodies, including LIBS6, may also change the extracellular conformation. The mechanism of $\alpha_{IIb}\beta_3$ activation by TS-bound IAP Ig domain might resemble that by such antibodies. The activating antibodies usually recognize the β subunit of the integrin. Some mutations within the cysteine-rich region of the β subunit can confer the naturally active form of $\alpha_{IIb}\beta_3$ integrin (44). Thus, it should be determined which subunit of $\alpha_{IIb}\beta_3$ integrin would be a binding site for TS-bound IAP. In addition, further study is needed to determine whether the phenomenon observed here could be widely demonstrated among other integrins.

Nonetheless, the extracellular event demonstrated in this study would facilitate the dynamic change in the conformation of the integrin extracellular domain and represents a novel mechanism of affinity modulation of integrins.

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REFERENCES

1. Hynes, R. O. (2002) *Nat. Med.* **8,** 918–921
2. Liddington, R. C., and Ginsberg, M. H. (2002) *J. Cell Biol.* **158,** 833–839
3. Phillips, D. R., Prasad, K. S., Manganello, J., Bao, M., Nannizzi, and Alaimo, L. (2001) *Curr. Opin. Cell Biol.* **13,** 546–554
4. Shattil, S. J., Kashiwagi, H., and Pampori, N. (1998) *Blood* **91,** 2645–2657
5. Woodside, D. G., Liu, S., and Ginsberg, M. H. (2001) *Thromb. Haemostasis* **86,** 316–323
6. Kashiwagi, H., Schwartz, M. A., Eigenthaler, M., Davis, K. A., Ginsberg, M. H., and Shattil, S. J. (1997) *J. Cell Biol.* **137,** 1433–1443
7. Naik, U. P., Patel, P. M., and Parise, L. V. (1997) *J. Biol. Chem.* **272,** 4651–4654
8. Vinogradova, O., Velyvis, A., Velyviene, A., Hu, B., Haas, T., Plow, E., and Qin, J. (2002) *Cell* **110,** 587–597
9. Hauser, W., Knobeloch, K. P., Eigenthaler, M., Gambaryan, S., Krenn, V., Geiger, J., Glazova, M., Rohde, E., Horak, I., Walter, U., and Zimmer, M. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 8120–8125
10. Azam, M., Andrabi, S. S., Sahr, K. E., Kamath, L., Kuliopulos, A., and Chishti, A. H. (2001) *Mol. Cell. Biol.* **21,** 2213–2220
11. Bertoni, A., Tadokoro, S., Eto, K., Pampori, N., Parise, L. V., White, G. C., and Shattil, S. J. (2002) *J. Biol. Chem.* **277,** 25715–25721
12. Fenczik, C. A., Sethi, T., Ramos, J. W., Hughes, P. E., and Ginsberg, M. H. (1997) *Nature* **390,** 81–85
13. Takagi, J., Petre, B. M., Walz, T., and Springer, T. A. (2002) *Cell* **110,** 599–611
14. Arnaout, M. A., Goodman, S. L., and Xiong, J. P. (2002) *Curr. Opin. Cell Biol.* **14,** 641–651
15. Brown, E. J., and Frazier, W. A. (2001) *Trends Cell Biol.* **11,** 130–135
16. Brown, E. J. (2002) *Curr. Opin. Cell Biol.* **14,** 603–607
17. Chung, J., Wang, X. Q., Lindberg, F. P., and Frazier, W. A. (1999) *Blood* **94,** 642–648
18. Gao, A. G., Lindberg, F. P., Dimitry, J. M., Brown, E. J., and Frazier, W. A. (1996) *J. Cell Biol.* **135,** 533–544
19. Chung, J., Gao, A. G., and Frazier, W. A. (1997) *J. Biol. Chem.* **272,** 14740–14746
20. Lindberg, F. P., Bullard, D. C., Caver, T. E., Gresham, H. D., Beaudet, A. L., and Brown, E. J. (1996) *Science* **274,** 795–798
21. Gao, A. G., Lindberg, F. P., Finn, M. B., Blystone, S. D., Brown, E. J., and Frazier, W. A. (1996) *J. Biol. Chem.* **271,** 21–24
22. Wang, X. Q., and Frazier, W. A. (1998) *Mol. Biol. Cell* **9,** 865–874
23. Seiffert, M., Brossart, P., Cant, C., Cella, M., Colonna, M., Brugger, W., Kanz, L., Ullrich, A., and Buhring, H. J. (2001) *Blood* **97,** 2741–2749
24. Furusawa, T., Yanai, N., Hara, T., Miyajima, A., and Obinata, M. (1998) *J. Biochem.* **123,** 101–106
25. Brittain, J. E., Mlinar, K. J., Anderson, C. S., Orringer, E. P., and Parise, L. V. (2001) *Blood* **97,** 2159–2164
26. Fujimoto, T., Fujimura, K., Noda, M., Takafuta, T., Shimomura, T., and Kuramoto, A. (1995) *Blood* **86,** 2174–2182
27. Dorahy, D. J., Thorne, R. F., Fecondo, J. V., and Burns, G. F. (1997) *J. Biol. Chem.* **272,** 1323–1330
28. Hollopeter, G., Jantzen, H. M., Vincent, D., Li, G., England, L., Ramakrishnan, V., Yang, R. B., Nurden, P., Nurden, A., Julius, D., and Conley, P. B. (2001) *Nature* **409,** 202–207
29. McEver, R. P., Bennett, E. M., and Martin, M. N. (1983) *J. Biol. Chem.* **258,** 5269–5275
30. Huang, M. M., Lipfert, L., Cunningham, M., Brugge, J. S., Ginsberg, M. H., and Shattil, S. J. (1993) *J. Cell Biol.* **122,** 473–483
31. Shattil, S. J., Cunningham, M., and Hoxie, J. A. (1987) *Blood* **70,** 307–315
32. Fujimoto, T., Fujimura, K., and Kuramoto, A. (1991) *Thromb. Haemostasis* **66,** 598–603
33. Fujimoto, T., Stroud, E., Whatley, R. E., Prescott, S. M., Muszbek, L., Laposata, M., and McEver, R. P. (1993) *J. Biol. Chem.* **268,** 11394–11400
34. Wang, X. Q., Lindberg, F. P., and Frazier, W. A. (1999) *J. Cell Biol.* **147,** 389–400
35. Fujimoto, T., and McEver, R. P. (1993) *Blood* **82,** 1758–1766
36. Fukudome, K., Kurosawa, S., Stearns, Kurosawa, D. J., He, X., Rezaie, A. R., and Esmon, C. T. (1996) *J. Biol. Chem.* **271,** 17491–17498
37. Ushiyama, S., Laue, T. M., Moore, K. L., Erickson, H. P., and McEver, R. P. (1993) *J. Biol. Chem.* **268,** 15229–15237
38. Tulasne, D., Judd, B. A., Johansen, M., Asazuma, N., Best, D., Brown, E. J., Kahn, M., Koretzky, G. A., and Watson, S. P. (2001) *Blood* **98,** 3346–3352
39. Chen, Y. P., Djaffar, I., Pidard, D., Steiner, B., Cieutat, A. M., Caen, J. P., and Rosa, J. P. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 10169–10173
40. Watson, S. P. (1999) *Thromb. Haemostasis* **82,** 365–376
41. Lindberg, F. P., Gresham, H. D., Reinhold, M. I., and Brown, E. J. (1996) *J. Cell Biol.* **134,** 1313–1322
42. Green, J. M., Zhelesnyak, A., Chung, J., Lindberg, F. P., Sarfati, M., Frazier, W. A., and Brown, E. J. (1999) *J. Cell Biol.* **146,** 673–682
43. Rebres, R. A., Vaz, L. E., Green, J. M., and Brown, E. J. (2001) *J. Biol. Chem.* **276,** 34607–34616
44. Kashiwagi, H., Tomiyama, Y., Tadokoro, S., Honda, S., Shiraga, M., Mizutani, H., Handa, M., Kurata, Y., Matsuzawa, Y., and Shattil, S. J. (1999) *Blood* **93,** 2559–2568