**50-kD Integrin-Associated Protein Does Not Detectably Influence Several Functions of Glycoprotein IIb-IIIa Complex in Human Platelets**

By Tetsuro Fujimoto, Kingo Fujimura, Masaaki Noda, Toshiro Takafuta, Takeshi Shimomura, and Atsushi Kuramoto

A 50-kD integrin-associated protein (IAP) has been reported to be associated with β₃ integrins and to modulate their function, especially vitronectin receptor in human erythroleukemia (HEL) cells and leukocyte response integrin in neutrophils. We studied the involvement of IAP in the function of platelet β₃ integrin, glycoprotein (GP) IIb-IIIa complex. IAP was a widely distributed protein and was also expressed in the cells that do not have β₃ integrin. Platelets from a patient with thrombocytopenia, which lack GPIIb and IIa, expressed IAP as well as normal platelets. Neither platelet aggregation nor intracellular Ca²⁺ elevation after stimulation was influenced by the anti-IAP antibody, B6H12, which was reported to be inhibitory for other β₃ integrins. The expression level of GPIIb-IIIa complex was not influenced by coexpression of human IAP in the transfected Chinese hamster ovary (CHO) cells. IAP did not facilitate the binding of soluble fibrinogen to the CHO cells expressing GPIIb-IIIa complex. Furthermore, cell adhesion onto the immobilized fibrinogen via GPIIb-IIIa complex was not inhibited by B6H12 in HEL cells and was not altered by coexpression of human IAP in CHO cells. We concluded that expression of IAP is regulated independently with that of GPIIb-IIIa complex and that IAP does not influence the function of GPIIb-IIIa complex.

© 1995 by The American Society of Hematology.

**MATERIALS AND METHODS**

**Materials.** Hybridoma cells producing anti-IAP monoclonal antibody, B6H12, were purchased from American Type Culture Collection (ATCC; HB-9771, Rockville, MD). The antibody was purified from mouse ascites using HiTrap protein G affinity column (Pharmacia, Uppsala, Sweden) according to the manufacturer’s instructions. P2 (anti–GPIIb-IIIa) and S2 (anti–GPIIb) were purchased from Immunotech (Marseille, France). LM609 (anti-vitronectin receptor) was kindly provided by Dr D.A. Cheresh (Scrpps Research Institute, La Jolla, CA). Control IgG (mouse myeloma IgG) was from Organon Technica (West Chester, PA). Fluorescein isothiocyanate (FITC)-conjugated goat F(ab')₂, antimouse IgGs were from TAGO (Burlingame, CA). FITC-conjugated streptavidin was from CalTag (South San Francisco, CA). Aequorin was from Wako Pure Chemical (Osaka, Japan). Propidium iodide (PI), ADP, epinephrine, phorbol myristate acetate (PMA), poly-L-lysine, and Cy3-
tin was from Pierce (Rockford, IL). Prostaglandin IAP and GPIIb-IIIa complex 2175 from Green Cross was selected with lipofectin and permanent transfected were previously described. Vitronectin and lipofectin were from GIBCO BRL (Gaithersburg, MD).

Flow cytometric analysis. Platelets from healthy volunteers and a patient with thrombasthenia were obtained after informed consent was obtained according to the declaration of Helsinki. Flow cytometric analysis was performed as described previously.20 Platelets were fixed with 1% paraformaldehyde in HEPES buffer (137 mM NaCl, 2.7 mM KCl, 3.3 mM NaH2PO4, 1 mM MgCl2, 4 mM L-HEPES, and 5.6 mM glucose, pH 7.4), washed, and suspended at 2 x 10^6/mL in the buffer described above. Each aliquot (50 µL) was incubated with the appropriate first antibody (10 µg/mL). After washing, platelets were incubated again with FITC-conjugated goat F(ab') subclass IgG (40 µg/mL) and the sequence was verified. Platelet aggregation study was performed as described previously.22 Platelet aggregation was recorded using a Platelet Ionized Calcium Aggregometer (PICA; Chrono-Log, Havertown, PA).

Concentration of the intracellular calcium ion was measured using a flow cytometer (Cytroharon Absolute; Ortho Diagnostic System, Raritan, NJ). Human neutrophils were isolated from heparinized blood using MonoPoly Resolving Media (Flow Laboratories, North Ryde, Australia) according to the manufacturer's instructions and the bottom layer was also collected as an erythrocyte fraction. For cases of neutrophils, erythrocytes, and cultured cells (CHO, HL-60, and 293 cells), cells were stained in the same manner except that they were not fixed and were incubated in Hank's Balanced Salt Solution with Ca2+ (HBSS) containing 1% bovine serum (FBS) and 0.1% NaN3 instead of HEPES buffer. After the last wash, some cells were also stained with 1 µg/mL PI to exclude permeabilized dead cells.

Expression vectors and transfection to CHO cells. The entire coding lesion of the IAP cDNA was amplified from human neutrophil cDNA by means of a polymerase chain reaction (PCR) method using a DNA thermal cycler (Perkin Elmer Cetus, Norwalk, CT). Primers were designed according to the published sequence23 (Bio-Synthesis, Lewisville, TX). PCR products were cloned into pGEM-T vector (Promega, Madison, WI) and the sequence was verified using an automated DNA sequence (Model 373A; Applied Biosystems Inc, Foster City, CA). The cDNA was ligated to a plasmid expression vector, pBKF-EF, which contains a powerful promoter from human elongation factor-1α (EF-1α) gene24 and neomycin-resistance gene. EF-1α promoter was also cloned using the PCR method and replaced with cytomegalovirus (CMV) promoter in a plasmid pBKF-CMV (Stratagene, La Jolla, CA). cDNAs of platelet GPIIb and IIIa were cloned from the HEL cell cDNA library using a colony hybridization method, according to the published sequences.25 CHO cells were grown in a mixture of D-MEM/F-12 (GIBCO BRL) supplemented with 10% FBS, 200 U/mL penicillin, and 200 µg/mL streptomycin under 5% CO2 at 37°C. Transfection was performed using lipofectin and permanent transfected cells were selected with G418, as described previously.25 To establish the CHO cells that stably express both GPIIb-IIIa complex and IAP, stable cells expressing GPIIb-IIIa were firstly established and the cDNA of IAP was transfected into these cells. Ten days after culture in G418-containing medium, cells were selected by reactivity to the antibody B6H12 using an immunomagnetic cell selection system (Dynal, Oslo, Norway). Cells were harvested and incubated with B6H12, followed by Dynabeads with covalently bound antihuman IgG. Positive cells were then collected using a magnetic particle concentrator and cultured again for 3 to 4 days. These procedures were repeated three times.

Measurement of fibrinogen binding using a flow cytometer. Fibrinogen binding to platelets and CHO cell transfecteds in solution was measured using a flow cytometer.26 Cells were washed and suspended in HEPES buffer containing 1 mM MgCl2 and 1 mM CaCl2. We added 10 µmol/L PMA for stimulated cells or 1 µmol/L PGE1 for nonstimulated cells and various concentration of biotinylated fibrinogen. The mixture was then incubated for 15 minutes at room temperature without stirring. The final incubation volume was 50 µL and the cell concentration was 4 x 10^9/mL. Fibrinogen was biotinylated with NHS-LC-biotin, as described previously.27 Five microliters of streptavidin-FITC conjugate was then added and the mixture was incubated for an additional 15 minutes. Finally, 500 µL of HEPES buffer containing 1 µg/mL PI was added and the mixture was directly analyzed using a flow cytometer. PI-negative cells and single particles especially for platelets were gated. A control aliquot for each reaction was prepared under the same condition except that 5 mMol/L EDTA was added instead of CaCl2. The difference of mean fluorescence intensity between in the presence of Ca2+ and EDTA was estimated as a specific binding.

Cell adhesion assay. Adhesion assays of HEL cells and transfected CHO cells were performed according to a previously published method28 with a slight modification. Ninety-six-well enzyme immunoassay/ radioimmunoassay (EIA/RIA) plates (Costar, Cambridge, MA) were coated with 100 µL of HBSS containing 20 µg/mL fibrinogen, 5 µg/mL vitronectin, 100 µg/mL BSA, or 100 µg/mL poly-L-lysine overnight. Plates were washed three times with HBSS and blocked with 1 mg/mL BSA. A total of 1 x 10^6 cells were added per well in the presence of 40 µg/mL of the appropriate antibody or 10 mmol/L EDTA. The plates were centrifuged at 100g for 5 minutes and then incubated at 37°C for 1 hour. Plates were washed four times with HBSS containing 1 mg/mL BSA to remove the unbound cells and the bound cells were fixed with 1% paraformaldehyde. Cells were stained with 0.5% Crystal Violet for 15 minutes, rinsed, and solubilized with 1% sodium dodecyl sulfate (SDS). The absorbance at 540 nm was measured on a microtiter plate reader (SLT-Lab Instruments, Salzburg, Austria).

RESULTS

Expression of IAP in a variety of cells and thrombasthenic platelets. We prepared an anti-IAP monoclonal antibody, B6H12, from the hybridoma cell line that was purchased from ATCC. The antibody recognized one relatively broad band at approximately 50 kDa from platelets and neutrophil membrane proteins when analyzed by immunoblot analysis (data not shown), confirming the reactivity of the antibody. Using this antibody, we first analyzed the expression of IAP in a variety of cells using a flow cytometer (Fig 1). IAP was expressed on platelets, neutrophils, and erythrocytes that have no known β subunits, corresponding with the results of previous reports.29 Several analyzed cell lines, including HL-60 and HEL, all expressed IAP. Furthermore, 293 cells, which belong to a fibroblastic cell line and do not have a β subunit, also expressed IAP. These data suggest that IAP is a very widely distributed protein and that the expression of
IAP does not require the presence of $\beta_3$ integrins. Only CHO cells were negative, probably because the hamster IAP was not recognized by the antihuman IAP, B6H12. CHO cells were then used in the following expression study.

The expression of IAP was also analyzed in platelets from a patient with thrombasthenia, which is an inherited bleeding disorder. Thrombasthenic platelets do not aggregate because the patient's platelets lack the GPIIb-IIIa complex. As shown in Fig 2, GPIIb-IIIa complex was reduced in the patient's platelets. However, the expression of IAP was observed as well in normal platelets. These data indicate that IAP can be expressed independently even though the expression of GPIIb-IIIa complex was impaired.

Effects of B6H12 on platelet function. We next determined the effects of anti-IAP antibody on several platelet functions. The antibody B6H12 was reported to have an epitope on the extracellular Ig-like loop of IAP and to inhibit the function of vitronectin receptor in HEL cells, the oxidative burst mediated by leukocyte response integrin in neutrophils, or the integrin-mediated $\text{Ca}^{2+}$ influx in endothelial cells, thus acting as an inhibitory antibody for IAP. Platelet aggregation was measured in the presence of the antibody. However, as shown in Fig 3, platelet aggregation induced by ADP, collagen, or epinephrine was not influenced by B6H12 (up to 100 $\mu$g/mL) at all, although the anti-GPIIb-IIIa complex antibody P2 completely inhibited the aggregation. Intracellular calcium elevation after thrombin or collagen stimulation was also measured using the Aequorin method, but was not influenced by the antibody B6H12 (Fig 4). Clot retraction using whole blood was not also inhibited by B6H12 (data not shown). These data indicate that B6H12, an inhibitory antibody for IAP, does not inhibit platelet function, whereas previous reports showed that the antibody does inhibit integrin function in HEL cells, neutrophils, and endothelial cells.

Expression of IAP on CHO cells. To determine whether the expression of IAP and GPIIb-IIIa complex could affect each other, expression studies in CHO cells were performed. We first prepared CHO cells stably expressing GPIIb-IIIa complex and transfected IAP cDNA into these cells. CHO cells stably expressing both IAP and GPIIb-IIIa complex were established by selection for the reactivity of B6H12, as described in the Materials and Methods. Although IAP was expressed additionally, the expression level of GPIIb-IIIa complex was not changed either in transient or stable cells (Fig 5). In the other experiments, we prepared the CHO cells stably expressing IAP first and then transfected cDNA of GPIIb-IIIa complex. The expression level of GPIIb-IIIa complex was compared with that in the parental CHO cells transfected with GPIIb-IIIa cDNA. However, the expression level of GPIIb-IIIa was not affected by the presence of IAP (data not shown). These data indicate that the expression of IAP and GPIIb-IIIa complex was regulated independently.

Fibrinogen binding to the transfected CHO cells. For these two types of CHO cell transfectants (expressing GPIIb-IIIa complex alone or expressing both IAP and GPIIb-IIIa complex), fibrinogen binding in solution was measured using...
a flow cytometer. GPIIb-IIIa complex was observed in the immunoprecipitate by B6H12 from the latter CHO cell lysates (data not shown). Platelets were used as a positive control and the experiments were performed at a very low concentration to avoid cell aggregation. We tried to stimulate cells with PMA because it is reported that protein kinase C activity closely correlates with agonist-induced activation of GPIIb-IIIa complex in platelets and at least several kinases participating in the intracellular signalling should also be activated in CHO cells. Using this method, when we added the increasing amount of fibrinogen, a small fluorescence shift was observed even in the parental CHO cells, depending on the concentration of fibrinogen, which was probably due to the nonspecific binding. Therefore, a control experiment was prepared in the buffer containing EDTA instead of calcium for each reaction and the difference of the mean fluorescence intensity was estimated as specific fibrinogen binding. In the control platelets, a fluorescence shift due to the fibrinogen binding was observed when the cells were stimulated. In contrast, in the GPIIb-IIIa-CHO cells, fibrinogen binding was not observed at all despite coexpression with IAP and cell stimulation (Fig 6A). The changes of relative mean fluorescence intensity in the various concentrations of fibrinogen are shown in Fig 6B. We concluded that IAP does not facilitate soluble fibrinogen binding to the CHO cells expressing GPIIb-IIIa complex.

Cell adhesion on fibrinogen-coated surface. We finally examined the effects of IAP and anti-IAP antibody on cell

![Graphs showing effects of B6H12 on platelet aggregation reaction.](image)

Fig 3. Effects of B6H12 on platelet aggregation reaction. PRP was incubated with 40 \( \mu \)g/mL of B6H12, P2 (anti-GPIIb-IIIa complex), or control mouse IgG for 5 minutes and then 5 \( \mu \)mol/L ADP (left), 2 \( \mu \)g/mL collagen (middle), or 1 \( \mu \)g/mL epinephrine (right) was added.
adhesion to fibrinogen-coated surface via the GPIIb-IIIa complex. GPIIb-IIIa complex in the transfected recombinant form or on relevant megakaryocytic cells has the ability to promote cell adhesion to a fibrinogen-coated surface, although GPIIb-IIIa complex can bind soluble fibrinogen only on activated platelets. Furthermore, all of the reported effects of anti-IAP antibody were observed in a cell adhesion assay of the immobilized adhesive protein. We examined the adhesion of HEL cells onto a fibrinogen- or vitronectin-coated surface (Fig 7). HEL cells express GPIIb-IIIa complex, $\alpha_\beta$, and IAP. The inhibitory antibody, B6H12, also blocked the adhesion to vitronectin surface in our hands, confirming previous reports. However, adhesion to fibrinogen surface was not influenced at all. Adhesion of the transfected CHO cells onto fibrinogen surface was also examined (Fig 8). These two kinds of CHO cells (expressing GPIIb-IIIa complex alone or both GPIIb-IIIa complex and IAP) expressed comparable levels of GPIIb-IIIa complex. Adhesion to the immobilized fibrinogen was not significantly different between GPIIb-IIIa-CHO and GPIIb-IIIa plus IAP-CHO cells. The antibody B6H12 did not show inhibitory effects for both cells. As a control experiment, the effect of B6H12 on the adhesion of these CHO cells onto vitronectin surface via hamster $\alpha_\beta$ was tested. B6H12 showed an inhibitory effect when the cells expressed human IAP.

**DISCUSSION**

Our first conclusion in this study is that the expression of IAP is regulated independently with that of GPIIb-IIIa complex. IAP was fully expressed in the cells that do not have $\beta$ integrins and in thrombasthenic platelets that lack the GPIIb-IIIa complex. GPIIb-IIIa complex did not affect the expression level of each other in the transfected CHO cells. In many cases, the complexed form of the membrane surface proteins requires the presence of all subunits for their complete surface expression, and each subunit cannot localize stably on the cell surface independently, eg, in the case of GPIIb-IIIa or GPIb-V-IX complex in platelets. However, our data show that association of IAP and GPIIb-IIIa complex is not critical for their surface expression. Brown et al first showed that GPIIb-IIIa complex can be coimmunoprecipitated with IAP by anti-IAP antibody, but the stoichiometry of the association is not yet known. IAP and GPIIb-IIIa complex are expressed on the cell surface through an independent pathway and, after that, some might associate on the surface.

The second conclusion is that IAP does not influence the function of GPIIb-IIIa complex. Platelet aggregation or intracellular Ca$^{2+}$ elevation was not inhibited by B6H12. The binding of soluble fibrinogen to GPIIb-IIIa complex, as observed in platelets, was not reproduced by coexpression of IAP in CHO cells. Cell adhesion onto the immobilized fibrinogen was not inhibited by B6H12 in HEL cells and was not altered by coexpression of IAP in CHO cells. CHO cells probably have a hamster IAP that can be recognized by polyclonal anti-IAP antibody as previously reported, but it is not certain whether hamster IAP can associate with human...
**Fig 5.** Effect of IAP on the expression of GPIIb-IIIa complex in CHO cells. Stable cells expressing GPIIb-IIIa were first established (upper panel) and the cDNA of IAP was transfected. Transient (middle panel) and stable cells (lower panel) established using the immunomagnetic cell selection method were analyzed for reactivity with BSH12 (left) and P2 (right) using a flow cytometer.

**Fig 6.** Soluble fibrinogen binding to platelets and CHO cell transfectants was measured using a flow cytometer. (A) Typical histogram data with 400 μg/mL biotinylated fibrinogen are shown. Platelets (left), CHO cells expressing GPIIb-IIIa complex (right), or CHO cells expressing both IAP and GPIIb-IIIa complex (middle) were analyzed after stimulation with 10 μmol/L PMA (upper panel) or without stimulation (lower panel; Non-Stim.). The expression pattern of IAP and GPIIb-IIIa complex in these CHO cells are shown in Fig 5. Control bindings were measured in the presence of 5 mmol/L EDTA. (B) The difference of mean fluorescence intensity between in the presence of Ca²⁺ and EDTA was estimated as a specific binding, and the dose-dependency of the added fibrinogen is shown.
GPIIb-IIIa complex or in functional form even if associated. Therefore, we performed the experiments using coexpression of human IAP. In platelets and possibly in HEL cells, IAP and GPIIb-IIIa complex are both present under the natural conditions. However, we could not obtain evidence that IAP is involved in the function of GPIIb-IIIa complex under any experimental conditions. The binding of soluble fibrinogen requires a significant change in the affinity of the GPIIb-IIIa complex. The lower affinity of the receptor is sufficient for cell attachment onto immobilized fibrinogen because adhesion of HEL cells or CHO cells with GPIIb-IIIa complex of reduced affinity can occur. However, IAP did not affect the GPIIb-IIIa complex of either affinity status. In contrast, the adhesion of HEL cells or the CHO cells expressing human IAP onto vitronectin surface was inhibited, confirming the previous report of Lindberg et al. They showed that the binding of vitronectin-coated beads to HEL cells via $\alpha_\beta_3$ receptor was blocked by B6H12. They also showed that, in CHO cells, once cells were transfected with human IAP, which probably can associate with hamster $\alpha_\beta_3$, B6H12 then inhibited the binding to vitronectin via hamster $\alpha_\beta_3$, whereas B6H12 did not inhibit the binding of parental CHO cells to vitronectin. In our experiments, B6H12 did not inhibit the CHO cell binding to fibrinogen via GPIIb-IIIa complex, even though human IAP was transfected to CHO cells (Fig 8). Their group also indicated that IAP does not associate with all integrins, did not affect the binding of HEL cells to fibronectin via $\alpha_\beta_1$, and did not affect $\beta_1$, or $\beta_2$-dependent function of leukocytes. All these data suggest that the functional involvement of IAP is receptor type-specific and is restricted even among $\beta_i$ integrins. The functional significance of the association of IAP and GPIIb-IIIa complex in platelets is not certain at present. However, the discrepancy of the effects of IAP on the function of $\alpha_\beta_1$ and GPIIb-IIIa complex would be of interest when the activation mechanisms of these $\beta_i$ integrins are considered. Schwartz et al. showed the inhibitory effects of B6H12 on a slow Ca$^{2+}$ influx into endothelial cells on adhesion to fibronectin or vitronectin surface, suggesting that IAP is closely associated with calcium channel activity. We previously reported that GPIIb-IIIa complex is involved in Ca$^{2+}$ influx in platelets. It would be an attractive hypothesis that IAP itself may be a channel molecule because it has a multi-membrane-spanning structure. However, B6H12 did not show any effects on platelet intracellular Ca$^{2+}$ elevation.

The mechanisms by which cytoplasmic signals cause the affinity modulation of the platelet GPIIb-IIIa complex are still not clear. A point mutation (Ser752→Pro) in the cytoplasmic domain of the $\beta_3$ subunit results in a defect in the activation of this integrin. Overexpression of the cytoplasmic domain of the $\beta_3$ as a chimeric protein competitively reduced the integrin affinity, suggesting that the cytoplasmic domain of $\beta_3$ may be a target for intracellular signaling to activate the integrin function. On the other hand, the conformational changes induced by RGD-peptides are reversible in intact platelets. Deletion of the cytoplasmic domain of the $\alpha_{\text{IIB}}$ subunit renders the GPIIb-IIIa complex constitutively active, suggesting that the cytoplasmic domain of the $\alpha_{\text{IIB}}$ may interact with negative regulatory elements. All of these results indicate that intracellular signals for the affinity modulation of GPIIb-IIIa complex are transduced by factors associated with its cytoplasmic domains. Such signals are probably cell-type specific, be-
cause recombinant GPIIb-IIIa complex in the transfected heterologous cells cannot be converted to the active state. We have begun exploring the involvement of already known molecules in the functional modulation of GPIIb-IIIa complex and consider IAP to be a good candidates, although it is not known whether IAP is associated with the integrin at its extracellular or cytoplasmic domain. However, we could not obtain supportive evidence in this study. We performed similar experiments with another molecule called cell adhesion regulator (CAR), which was reported to be a myristoylated and tyrosine-phosphorylated cytoplasmic protein able to increase cell adhesion to a collagen type I-coated surface. However, we could not observe any effects of the CAR molecule on the function of GPIIb-IIIa complex. The affinity modulation of GPIIb-IIIa complex might be regulated by as yet unknown but probably megakaryocyte-platelet lineage-specific novel molecules.

REFERENCES

16. O'Toole TE, Mandelman D, Forsyth J, Shattil SJ, Ploew EF, Ginsberg MH: Modulation of the affinity of integrin $\alpha_{IIb}\beta_3$ (GPIIb-IIIa) by the cytoplasmic domain of $\alpha_{IIb}$. Science 254:845, 1991
17. Filardo EJ, Cherecis DA: A $\beta$ turn in the cytoplasmic tail of the integrin $\alpha_{IIb}$ subunit influences conformation and ligand binding of $\alpha_{IIb}\beta_3$. J Biol Chem 269:4641, 1994
32. Lopez JA, Weisman S, Sanan DA, Sih T, Chambers M, Li CQ: Glycoprotein (GP) $Ib\beta$ is the critical subunit linking GP Ibo


50-kD integrin-associated protein does not detectably influence several functions of glycoprotein IIb-IIIa complex in human platelets
T Fujimoto, K Fujimura, M Noda, T Takafuta, T Shimomura and A Kuramoto