

# Analysis of the Roles of 14-3-3 in the Platelet Glycoprotein Ib-IX-mediated Activation of Integrin $\alpha_{IIb}\beta_3$ Using a Reconstituted Mammalian Cell Expression Model

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**Abstract.** We have reconstituted the platelet glycoprotein (GP) Ib-IX-mediated activation of the integrin  $\alpha_{IIb}\beta_3$  in a recombinant DNA expression model, and show that 14-3-3 is important in GPIb-IX signaling. CHO cells expressing  $\alpha_{IIb}\beta_3$  adhere poorly to vWF. Cells expressing GPIb-IX adhere to vWF in the presence of botrocetin but spread poorly. Cells coexpressing integrin  $\alpha_{IIb}\beta_3$  and GPIb-IX adhere and spread on vWF, which is inhibited by RGDS peptides and antibodies against  $\alpha_{IIb}\beta_3$ . vWF binding to GPIb-IX also activates soluble fibrinogen binding to  $\alpha_{IIb}\beta_3$  indicating that GPIb-IX mediates a cellular signal leading to  $\alpha_{IIb}\beta_3$  activation. Deletion of the 14-3-3-binding site in GPIb $\alpha$  inhibited GPIb-IX-mediated fibrinogen binding to  $\alpha_{IIb}\beta_3$  and cell spreading on vWF. Thus, 14-3-3 bind-

ing to GPIb-IX is important in GPIb-IX signaling. Expression of a dominant negative 14-3-3 mutant inhibited cell spreading on vWF, suggesting an important role for 14-3-3. Deleting both the 14-3-3 and filamin-binding sites of GPIb $\alpha$  induced an endogenous integrin-dependent cell spreading on vWF without requiring  $\alpha_{IIb}\beta_3$ , but inhibited vWF-induced fibrinogen binding to  $\alpha_{IIb}\beta_3$ . Thus, while different activation mechanisms may be responsible for vWF interaction with different integrins, GPIb-IX-mediated activation of  $\alpha_{IIb}\beta_3$  requires 14-3-3 interaction with GPIb $\alpha$ .

**Key words:** platelet • glycoprotein Ib-IX • integrin • 14-3-3 • von Willebrand factor

**P**LATELET adhesion to the subendothelial matrix plays a critical role in thrombosis and hemostasis. Initial platelet adhesion is mediated by the interaction between a platelet receptor for von Willebrand factor (vWF), the glycoprotein Ib-IX complex (GPIb-IX), and matrix-bound vWF (Sakariassen et al., 1979, 1986; Savage et al., 1996, 1998). This interaction is particularly important under high shear flow conditions. Subsequently, platelets firmly adhere and spread on vWF, and aggregate to form a primary thrombus (Weiss et al., 1986; Savage et al., 1996, 1998). Platelet spreading is dependent upon activation of integrin  $\alpha_{IIb}\beta_3$  interaction with the RGD sequence in vWF, and platelet aggregation is dependent upon activation of integrin  $\alpha_{IIb}\beta_3$  binding to soluble fibrinogen (for reviews see Phillips et al., 1991; Ruggeri and Ware, 1993; Du and Ginsberg, 1997). Thus far, the GPIb-IX-mediated

signaling pathway leading to integrin activation has been unclear.

GPIb-IX consists of three subunits: GPIb $\alpha$ , GPIb $\beta$ , and GPIX. GPIb-IX is loosely associated with glycoprotein V. The NH<sub>2</sub>-terminal domain of GPIb $\alpha$  contains binding sites for vWF and thrombin (for reviews see Lopez, 1994; Ware, 1998). In vivo, binding of vWF to GPIb-IX does not occur unless vWF first interacts with exposed subendothelial matrix components such as collagen VI (Mazzucato et al., 1999). Collagen probably induces a conformational change in vWF exposing the binding site for GPIb $\alpha$  (Mazzucato et al., 1999). In vitro, the effect of collagen on vWF is mimicked by desialation of vWF or by vWF modulators such as ristocetin or botrocetin which similarly induce vWF binding to the same binding pocket in the NH<sub>2</sub>-terminal region of GPIb $\alpha$  (Berndt et al., 1988; Vicente et al., 1988; Andrews et al., 1989). The cytoplasmic domain of GPIb $\alpha$  contains a binding site for filamin (also called actin-binding protein or ABP-280), which links GPIb-IX to cross-linked actin filamental structures underlining the plasma membrane (the membrane skeleton; Fox, 1985a,b). We found that an intracellular signaling molecule, 14-3-3 $\zeta$ , is also associated with GPIb-IX (Du et al., 1994), and a binding site

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for 14-3-3 $\zeta$  is located in a 15-amino acid residue region (residues 595–610) at the COOH terminus of GPIb $\alpha$  (Du et al., 1996), distinct from the binding site for filamin (residues 536–568; Andrews and Fox, 1992). Binding of 14-3-3 to GPIb $\alpha$  is regulated by phosphorylation of GPIb $\alpha$  at serine<sup>609</sup> (Bodnar et al., 1999). 14-3-3 binding also involves an additional 14-3-3-binding site in GPIb $\beta$  regulated by protein kinase A (PKA; Andrews et al., 1998; Calverley et al., 1998). The 14-3-3 family of highly conserved intracellular proteins interacts with serine phosphorylated intracellular proteins (Furukawa et al., 1993; Fanti et al., 1994; Freed et al., 1994; Fu et al., 1994; Pallas et al., 1994; Acs et al., 1995; Bonnefoy et al., 1995; Conklin et al., 1995; Liu et al., 1996; Meller et al., 1996), and regulates their functions (Fanti et al., 1994; Ford et al., 1994; Li et al., 1995; Zha et al., 1996; Tzivion et al., 1998). A recognition motif, RSXpSXP, has been identified in several 14-3-3 ligands (Muslin et al., 1996; Yaffe et al., 1997). Interaction with RSXpSXP-motif-containing proteins requires helix G of 14-3-3 $\zeta$  (Gu and Du, 1998). In contrast, GPIb $\alpha$  binding requires the helix I region of 14-3-3 $\zeta$  distinct from the site required for binding of RSXpSXP-containing ligands (Gu and Du, 1998).

In this study, we have established a CHO cell expression model for studying GPIb-IX-mediated integrin activation. We show that deletion of the 14-3-3-binding site in the COOH terminus of GPIb $\alpha$  inhibits GPIb-IX-induced integrin activation. Further, we show that expression of a dominant negative mutant of 14-3-3 containing the GPIb-IX-binding site also inhibits vWF induced integrin activation. Thus, 14-3-3 plays important roles in vWF-induced GPIb-IX signaling leading to  $\alpha_{IIb}\beta_3$  activation. In addition, deleting both the filamin and 14-3-3-binding sites in GPIb $\alpha$  enhanced cell spreading on vWF. This did not require  $\alpha_{IIb}\beta_3$ , but did require an endogenous integrin. The same deletion mutant, however, failed to mediate vWF-induced fibrinogen binding, suggesting that  $\alpha_{IIb}\beta_3$  activation was inhibited. Thus, different mechanisms may be responsible for vWF interaction with different integrins after GPIb-IX-mediated initial adhesion.

## Materials and Methods

### Reagents

An anti-peptide antibody, anti-Ib $\alpha$ C, recognizing the COOH-terminal domain of GPIb $\alpha$  has been described previously (Du et al., 1996). The monoclonal antibodies WM23 and AK2 against GPIb $\alpha$ , and purification of vWF and botrocetin were as described previously (Berndt et al., 1985; Andrews et al., 1989). The monoclonal antibody against GPIb $\alpha$ , P3221, was kindly provided by Dr. Zaverio Ruggeri (The Scripps Research Institute, La Jolla, CA). mAbs D57 and 15 against integrin  $\alpha_{IIb}\beta_3$  were kindly provided by Dr. Mark Ginsberg (The Scripps Research Institute, La Jolla, CA). Monoclonal antibody, 4F10, against integrin  $\alpha_{IIb}\beta_3$  complex was kindly provided by Dr. Virgil Woods (University of California at San Diego, CA). Monoclonal antibody against GPIb $\alpha$ , SZ2, monoclonal antibody against human  $\beta_3$ , SZ21, and monoclonal antibody against vWF, SZ29, were generous gifts from Dr. Changgeng Ruan (Suzhou Medical College, Suzhou, China; Ruan et al., 1987a,b); cDNA clones encoding  $\alpha_{IIb}$  and  $\beta_3$  in CDM8 vector were kindly provided by Dr. Mark Ginsberg. In some experiments, botrocetin was also purchased from Centerchem. Ristocetin was purchased from Sigma Chemical Co.

DNA encoding wild-type and mutant 14-3-3 $\zeta$  was described previously (Du et al., 1996; Gu and Du, 1998). The wild-type and mutant 14-3-3 $\zeta$  were subcloned into pEGFP-C2 vector (Clontech) between EcoRI and

XbaI sites. The constructs encode a wild-type or a mutant 14-3-3 $\zeta$  fused to the COOH terminus of green fluorescent protein (GFP)<sup>1</sup>.

### Cell Lines Expressing Recombinant Proteins

Transfections of cDNA into CHO cells were performed according to the previously described methods using Lipofectamine (BRL; Du et al., 1996). Selection markers (CDneo and CDhygro; Invitrogen) were cotransfected with desired DNA at a 1:10 ratio. Stably transfected cell lines were selected in selection media containing 0.5 mg/ml G418 and/or 0.2 mg/ml hygromycin, and further selected by mass cell sorting using antibodies recognizing GPIb $\alpha$  (P3221) and/or integrin  $\alpha_{IIb}\beta_3$  (D57). The following cell lines were established: cells expressing GPIb-IX complex (1b9) (Du et al., 1996); cells expressing integrin  $\alpha_{IIb}\beta_3$  (2b3a); cells coexpressing GPIb-IX and  $\alpha_{IIb}\beta_3$  (123); and cells coexpressing integrin  $\alpha_{IIb}\beta_3$  and GPIb-IX mutants with truncated GPIb $\alpha$  cytoplasmic domains at residues 591 ( $\Delta$ 591/2b3a) and 559 ( $\Delta$ 559/2b3a) (Du et al., 1996). Cells expressing comparable levels of integrins or/and GPIb-IX were further selected by cell sorting and monitored by flow cytometry.

### Cell Adhesion Assay

Microtiter wells were coated with 10  $\mu$ g/ml vWF or fibrinogen in PBS at 4°C overnight. Cells in Tyrode's buffer in the presence of 5  $\mu$ g/ml botrocetin were incubated in ligand-coated microtiter wells for 30 min at 37°C in a CO<sub>2</sub> incubator. As adhesion of the GPIb-IX and integrin-transfected CHO cells to vWF does not require botrocetin, botrocetin was omitted in some experiments. After three washes, cell spreading was examined under an inverted microscope (20 $\times$  objective lens). In quantitative assays, 50  $\mu$ l of 0.3% *p*-nitrophenyl phosphate in 1% Triton X-100, 50 mM sodium acetate, pH 5.0, was added to microtiter wells and incubated at 37°C for 1 h. The reaction was stopped by adding 50  $\mu$ l of 1 M NaOH. Results were determined by reading OD at 405 nm wave length. A standard curve of acid phosphatase reaction was established by adding the acid phosphatase substrate to various known numbers of the same cells in parallel wells. Acid phosphatase assay of the standards confirmed that the OD value was proportional to cell number. The rate of cell adhesion was estimated from the ratio of the numbers of adherent cells to that of total cells.

### Fluorescence Microscopy

Cells were allowed to adhere and spread on vWF- or fibrinogen-coated glass chamber slides (Nunc). After three washes, cells were fixed by adding 4% paraformaldehyde in PBS. In experiments that required cell permeabilization, cells were permeabilized by adding 0.1 M Tris, 0.01 M EGTA, 0.15 M NaCl, 5 mM MgCl<sub>2</sub>, pH 7.4, containing 0.1% Triton X-100, 0.5 mM leupeptin, 1 mM PMSF, and 0.1 mM E64. The cells were then incubated with 20  $\mu$ g/ml of various antibodies at 22°C for 1 h. After three washes, cells were further incubated with fluorescein- or rhodamine-labeled secondary antibodies at 22°C for 30 min. To stain the actin filaments, rhodamine-labeled phalloidin (Sigma Chemical Co.) was also added. After additional washes, cells were photographed under a fluorescence microscope. In some experiments, the data were collected by a cooled CCD camera and surface area quantitated using Image-Pro Plus (Media Cybernetics).

### Flow Cytometry Analysis of vWF Binding and vWF-induced Fibrinogen Binding

Fluorescein-labeling of fibrinogen was prepared as described previously (Chen et al., 1994). Cells expressing recombinant proteins were harvested and suspended in modified Tyrode's buffer (Du et al., 1991). Cells ( $\sim 1 \times 10^7$ /ml) were incubated for 30 min with 15  $\mu$ g/ml fluorescein-labeled fibrinogen in the presence of 20  $\mu$ g/ml vWF and 1 mg/ml ristocetin. As a negative control, cells were also incubated with fluorescein-labeled fibrinogen in the presence of 1 mg/ml ristocetin but in the absence of vWF. RGDS peptide (1 mM) was added in parallel assays for estimation of specific fibrinogen binding to the integrin. We showed previously that 1 mM RGDS completely abolished fibrinogen binding to integrin  $\alpha_{IIb}\beta_3$  while 1 mM RGD had no effect (Du et al., 1991). Fibrinogen binding was analyzed by flow cytometry.

1. *Abbreviations used in this paper:* GFP, green fluorescent protein; GP, glycoprotein; PGE<sub>1</sub>, prostaglandin E<sub>1</sub>; PKA, protein kinase A; vWF, von Willebrand factor.

For vWF binding, the cells in Tyrode's buffer were incubated for 30 min at 22°C with vWF in the presence 1 mg/ml ristocetin. After washing, the cells were further incubated for 30 min with a monoclonal antibody against vWF, SZ29, and then analyzed by flow cytometry.

### Immunoprecipitation

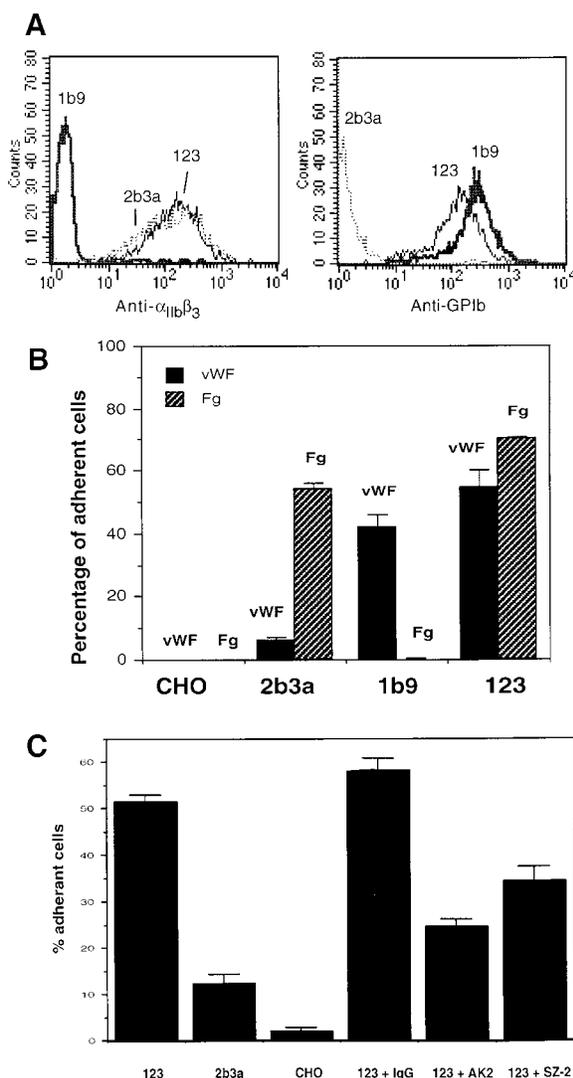
CHO cells coexpressing integrin  $\alpha_{IIb}\beta_3$  with wild-type GPIb-IX (123 cells) or GPIb-IX mutant  $\Delta 591$  were solubilized as previously described (Gu and Du, 1998). Cell lysates were incubated with 10  $\mu$ g of WM23 against GPIb $\alpha$  or mouse IgG (Sigma Chemical Co.) at 4°C for 1 h and further incubated for 1 h after addition of protein G-conjugated Sepharose beads (Sigma Chemical Co.). After three washes, the bead-bound proteins were analyzed by SDS-PAGE and Western blotting with a rabbit anti-GPIb $\alpha$  antibody or a rabbit antibody against 14-3-3 $\zeta$  (Du et al., 1996). Reaction of the antibodies was visualized using an enhanced chemiluminescence kit (Amersham-Pharmacia).

### Results

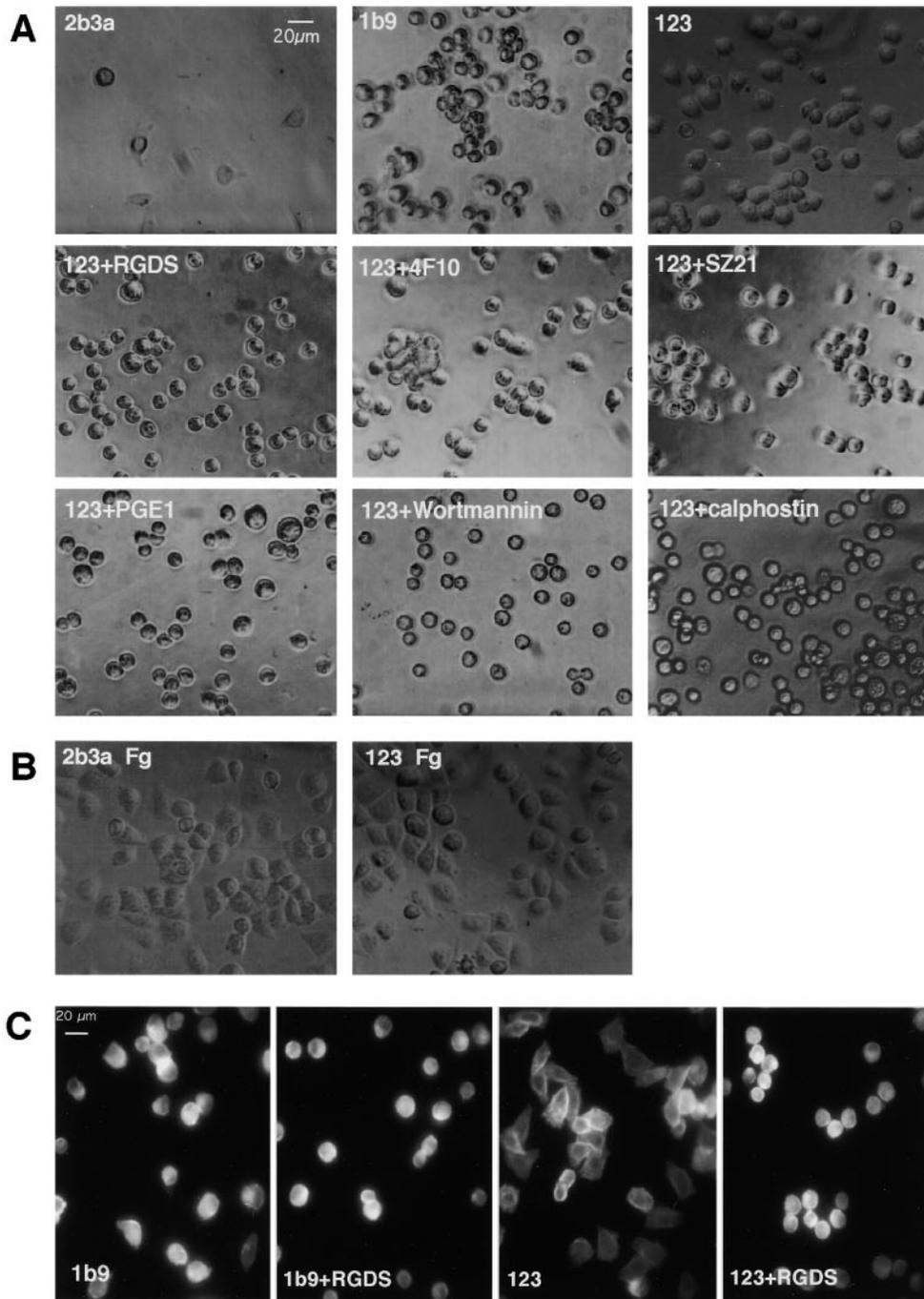
#### Roles of GPIb-IX and $\alpha_{IIb}\beta_3$ in Mediating Cell Adhesion to vWF

To analyze the roles of GPIb-IX and integrin  $\alpha_{IIb}\beta_3$  in vWF-mediated platelet adhesion and activation, stable CHO cell lines were established that express one of the two platelet receptors for vWF: GPIb-IX (1b9 cells) or integrin  $\alpha_{IIb}\beta_3$  (2b3a cells). A stable cell line was also established that expressed both GPIb-IX and integrin  $\alpha_{IIb}\beta_3$  at levels comparable to 1b9 and 2b3a cells, respectively (123 cells; Fig. 1 A). These cells were incubated in vWF-coated microtiter wells for 30 min in the presence of botrocetin, which binds to vWF and mimics the effects of subendothelial matrix to induce vWF binding to GPIb-IX (Andrews et al., 1989). As a positive control, these cells were also incubated in fibrinogen-coated microtiter wells. Adherent cells were quantitated with an acid-phosphatase assay. As shown in Fig. 1, <10% of the 2b3a cells (expressing only  $\alpha_{IIb}\beta_3$ ) adhered to the vWF-coated surface compared with ~55% adhesion to fibrinogen, suggesting only a background level of vWF-integrin interaction. This result is consistent with previous work showing a low affinity state of  $\alpha_{IIb}\beta_3$  expressed in CHO cells (O'Toole et al., 1990), and is also consistent with results obtained in platelets showing that integrin  $\alpha_{IIb}\beta_3$  interacts poorly with vWF without prior activation (Savage et al., 1992). The possibility of defective integrin function and expression in 2b3a cells can be excluded, as both the 2b3a cells and 123 cells but not 1b9 cells adhered to immobilized fibrinogen which is known to interact with integrin  $\alpha_{IIb}\beta_3$  without prior activation (Coller, 1980; Savage et al., 1995; Fig. 1 B). Thus, vWF is a poor ligand for unactivated integrin  $\alpha_{IIb}\beta_3$ .

In contrast to cells expressing  $\alpha_{IIb}\beta_3$  (2b3a cells), those expressing GPIb-IX (1b9 cells) or those expressing both GPIb-IX and integrin  $\alpha_{IIb}\beta_3$  (123 cells), adhered to vWF-coated wells in the presence of botrocetin (Fig. 1 B). As platelet adhesion to immobilized vWF occurs in the absence of vWF modulators (Savage et al., 1992), we further examined the adhesion of 123 cells to immobilized vWF without botrocetin treatment. Fig. 1 C shows that 123 cell adhesion to vWF does not require botrocetin, indicating that adhesion of CHO cells expressing GPIb-IX and  $\alpha_{IIb}\beta_3$  to vWF is similar to platelet adhesion. Furthermore, adhesion of 123 cells to vWF was inhibited by monoclonal antibodies against vWF-binding site of GPIb $\alpha$  (Fig. 1 C).



**Figure 1.** Adhesion of cells expressing recombinant receptors for vWF. (A) Cells expressing integrin  $\alpha_{IIb}\beta_3$  (2b3a), cells expressing GPIb-IX (1b9) and cells expressing both GPIb-IX and  $\alpha_{IIb}\beta_3$  (123) are described in Materials and Methods. These cells were detached and incubated with biotin-labeled monoclonal antibody WM23 (against GPIb $\alpha$ ) at 22°C for 30 min, and then incubated with phycoerythrin-labeled streptavidin and FITC-labeled monoclonal antibody D57 (against integrin  $\alpha_{IIb}\beta_3$ ) at 22°C for 30 min. The cells were then analyzed by flow cytometry. (B) The cells described in A were incubated in vWF-coated microtiter wells for 30 min at 37°C in the presence of 5  $\mu$ g/ml botrocetin. Untransfected CHO cells were also used as a negative control. To compare the function of  $\alpha_{IIb}\beta_3$  expressed in 123 and 2b3a cells, cells are also incubated with fibrinogen (Fg)-coated wells. After washing, the adherent cells were quantitated by an acid phosphatase assay as described in Materials and Methods. Shown in the figure are the results from triplicate samples (mean  $\pm$  SD). (C) 123 cells, 2b3a and CHO cells were also allowed to adhere to (10  $\mu$ g/ml) vWF-coated microtiter wells in the absence of botrocetin. 123 cells were also preincubated with control IgG, or the monoclonal antibodies, AK2 and SZ2 against the vWF-binding site of GPIb $\alpha$ , and then allowed to adhere to vWF-coated wells in the absence of botrocetin. Note that AK2 and SZ2 inhibited cell adhesion to vWF.



**Figure 2.** GPIb-IX activates integrin-vWF interaction and integrin-dependent cell spreading on vWF. (A) Three CHO cell lines, 1b9 (expressing GPIb-IX), 2b3a (expressing  $\alpha_{IIb}\beta_3$ ), and 123 (expressing GPIb-IX and integrin  $\alpha_{IIb}\beta_3$ ) were incubated in the microtiter plates coated with 10  $\mu\text{g/ml}$  vWF for 30 min at 37°C in the presence of 5  $\mu\text{g/ml}$  botrocetin. 123 cells were also incubated in the vWF-coated wells in the presence of 2 mM of the integrin antagonist peptide, RGDS (123+RGDS), a monoclonal antibody specific for integrin  $\alpha_{IIb}\beta_3$  complex (123+4F10) and a monoclonal antibody against human  $\beta_3$  (123+SZ21) at 100  $\mu\text{g/ml}$ , 500 ng/ml prostaglandin E1 (PGE1), 25 nM calphostin C, and 0.1  $\mu\text{M}$  Wortmannin. (B) To compare  $\alpha_{IIb}\beta_3$  function, 2b3a cells and 123 cells were incubated in microtiter wells coated with 10  $\mu\text{g/ml}$  fibrinogen. After washing, the adherent cells were photographed under a phase contrast microscope (20 $\times$  objective lens). (C) Cells expressing GPIb-IX (1b9) and coexpressing both  $\alpha_{IIb}\beta_3$  and GPIb-IX (123) were allowed to adhere to botrocetin-treated vWF-coated glass slides in the absence or presence of RGDS peptide. After fixation and permeabilization, cells were stained with rhodamine-labeled phalloidin, and photographed under a fluorescence microscope (40 $\times$  objective lens).

These results suggest that, as in platelets, GPIb-IX is required for cell adhesion to vWF in this CHO cell expression model.

### ***GPIb-IX Induces Integrin-vWF Interaction and Integrin-dependent Cell Spreading on vWF***

Under a microscope, most adherent 1b9 cells (expressing GPIb-IX only) on vWF showed a rounded morphology similar to nonadherent cells (Fig. 2 A). In contrast, 123 cells (coexpressing GPIb-IX and  $\alpha_{IIb}\beta_3$ ) spread on the vWF-coated surface (Fig. 2 A). Spreading of 123 cells was

abolished by RGDS peptide (Fig. 2 A), indicating that spreading was mediated by integrins. Spreading of 123 cells was also inhibited by the monoclonal antibody 4F10, against human  $\alpha_{IIb}\beta_3$  complex, and by anti-human  $\beta_3$  antibody SZ21 (Fig. 2 A). These data indicated that spreading was mainly mediated by integrin  $\alpha_{IIb}\beta_3$  and that endogenous integrins were unlikely to play a major role. It is unlikely that coexpression of GPIb-IX with  $\alpha_{IIb}\beta_3$  in the 123 cell line resulted in constitutively active integrin  $\alpha_{IIb}\beta_3$ , as 123 cells did not bind to soluble fibrinogen without prior activation (data not shown, see Fig. 3). Thus, vWF binding to GPIb-IX induces integrin-vWF interaction and inte-

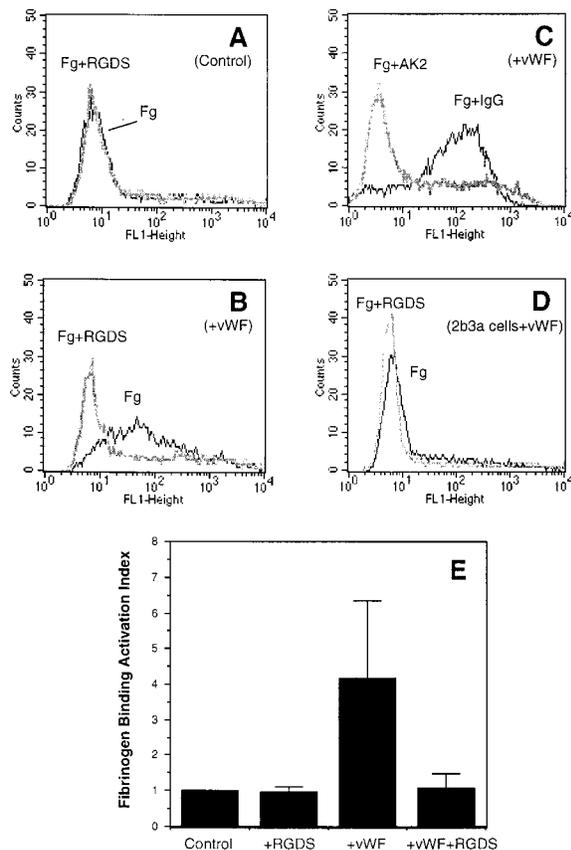
grin-mediated cell spreading. To examine whether GPIb-IX-mediated signaling pathway in CHO cells mimics that in platelets, we examined the effects of platelet activation inhibitors. We found that the PGE1, which elevates intracellular cAMP, wortmannin, and calphostin C, which inhibit PI-3 kinase and PKC, respectively, also inhibited GPIb-IX and integrin-dependent CHO cell spreading on vWF. Thus, GPIb-IX expressed in CHO cells induced integrin interaction with vWF in a manner similar to that in platelets.

To further exclude the possibility that integrin function in 123 cells may differ from that in the 2b3a cell line, we also examined integrin-mediated cell spreading on immobilized fibrinogen. Both 2b3a cells and 123 cells fully spread on immobilized fibrinogen (Fig. 2 B), suggesting that  $\alpha_{IIb}\beta_3$  expressed in both cell lines functioned in a similar manner. As shown above, only a small percentage of 2b3a cells adhere to vWF. Some of these adherent cells, however, also spread on vWF, suggesting that the background level GPIb-IX-independent interaction of  $\alpha_{IIb}\beta_3$  with vWF in a small percentage of 2b3a cells can also mediate cell spreading.

To examine the morphological changes in more detail, the adherent cells were stained with fluorescently labeled phalloidin, and examined by fluorescence microscopy under high magnification. Only 5% of 1b9 cells spread on vWF (Fig. 2 C). Most 1b9 cells did not spread or only poorly spread on vWF. However, 58% of these poorly spread cells showed limited filopodium- or lamellipodium-like structures extending to the vWF-coated surface (Fig. 2 C) which was inhibited by RGDS peptide. This indicates a low level interaction between vWF and an endogenous integrin, which is consistent with the results obtained by Cunningham et al. (1996). In contrast to 1b9 cells, ~70% of 123 cells (expressing both GPIb-IX and integrin  $\alpha_{IIb}\beta_3$ ) fully spread, which was inhibited by RGDS peptide (Fig. 2 C). These results show that GPIb-IX induces integrin  $\alpha_{IIb}\beta_3$  interaction with vWF which is responsible for 123 cell spreading on vWF.

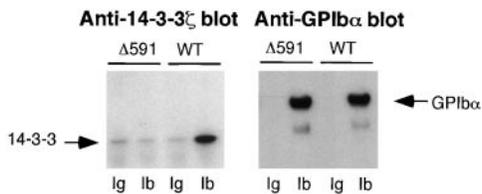
### vWF-induced Fibrinogen Binding to Integrin $\alpha_{IIb}\beta_3$ in CHO Cells

Two possible mechanisms could explain GPIb-IX-induced integrin-vWF interaction in the CHO cell expression model: (a) GPIb-IX may induce a cellular signal that increases the affinity of integrin for vWF (activation); or (b) the GPIb-IX binding to vWF may allow access of integrin to vWF, e.g., by changing the conformation of vWF. To differentiate between these two possibilities, we examined whether vWF activated integrin binding to another ligand of  $\alpha_{IIb}\beta_3$ , soluble fibrinogen, in 123 cells. It is known that integrin  $\alpha_{IIb}\beta_3$  binds soluble fibrinogen only after the integrin is activated (for reviews, see Du and Ginsberg, 1997; Phillips et al., 1991). FITC-labeled fibrinogen was incubated with 123 cells in the presence of ristocetin which is known to induce soluble vWF binding to GPIb-IX and vWF-dependent platelet aggregation but not to induce fibrinogen-dependent platelet aggregation in the absence of vWF (for review, see Ware, 1998). As expected, there was no specific fibrinogen binding to 123 cells exposed only to ristocetin, indicating that ristocetin alone does not induce



**Figure 3.** GPIb-IX-induced soluble fibrinogen binding to integrin  $\alpha_{IIb}\beta_3$ . (A) Cells expressing both GPIb-IX and integrin  $\alpha_{IIb}\beta_3$  (123 cells) were incubated with FITC-labeled fibrinogen (15  $\mu$ g/ml) and 1 mg/ml ristocetin in the absence (Fg) or presence of RGDS peptide (Fg+RGDS). (B) 123 cells were incubated with FITC-labeled fibrinogen, ristocetin, and 20  $\mu$ g/ml purified human vWF in the absence of RGDS (Fg) or in the presence of RGDS (Fg+RGDS). (C) Control mouse IgG or a monoclonal antibody against GPIb $\alpha$ , AK2, were added to 123 cells. The cells were then incubated with FITC-labeled fibrinogen, ristocetin and vWF. Note that the increased binding of fibrinogen in the presence of vWF was inhibited by RGDS and by AK2. (D) 2b3a cells expressing  $\alpha_{IIb}\beta_3$  only were incubated with FITC-labeled fibrinogen, vWF and ristocetin. Cells in A–D were analyzed for fibrinogen binding by flow cytometry. (E) 123 cells were incubated with FITC-labeled fibrinogen in the presence of ristocetin only (Control), ristocetin and RGDS (+RGDS), ristocetin and vWF (+vWF), or ristocetin, vWF and RGDS (+vWF+RGDS) at 22°C for 30 min and examined for fibrinogen binding as described in A. Activation of fibrinogen binding was quantitated and expressed as an activation index which is the ratio of the fluorescence intensity (Geo mean) of sample cells over the fluorescence intensity (Geo mean) of the control 123 cells (not stimulated with vWF). Shown in E are the results of 4 experiments (mean  $\pm$  SD). Student's *t* test revealed that the difference between control and vWF-stimulated (+vWF) fibrinogen binding is highly significant ( $P < 0.001$ ).

specific fibrinogen binding to integrin  $\alpha_{IIb}\beta_3$  (Fig. 3 A). When both vWF and ristocetin were present, however, there was significant binding of fibrinogen. vWF-induced fibrinogen binding was inhibited by RGDS peptide (Fig. 3, B and E), and was also inhibited by an anti-GPIb $\alpha$  mono-



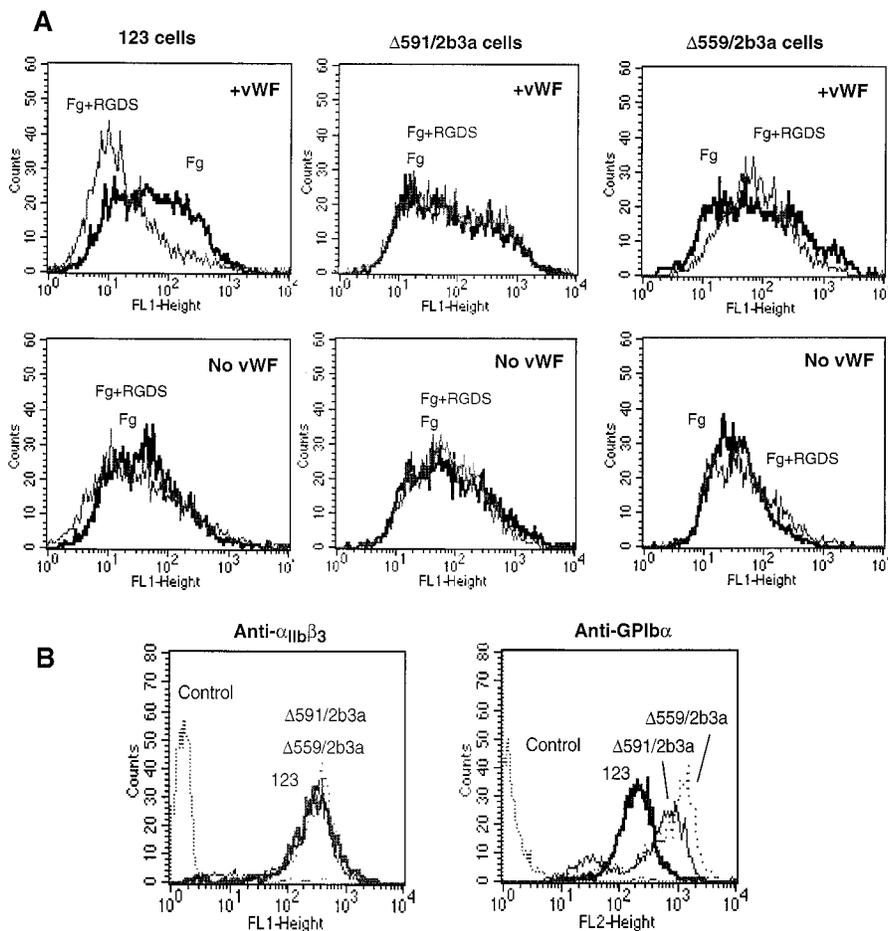
**Figure 4.** Coimmunoprecipitation of endogenous CHO cell 14-3-3 with GPIb-IX. Cells were stably transfected with integrin  $\alpha_{IIb}\beta_3$  together with wild-type GPIb-IX (123) or GPIb-IX mutants with GPIb $\alpha$  cytoplasmic domain truncated at residue 591 to delete 14-3-3-binding site ( $\Delta 591/2b3a$ ). The 123 cells and  $\Delta 591/2b3a$  cells were solubilized and immunoprecipitated with the monoclonal antibody, WM23, against GPIb $\alpha$  (Ib) or control mouse IgG (Ig). Immunoprecipitates were analyzed by SDS-PAGE and immunoblotted with a rabbit antibody against 14-3-3 $\zeta$  or a rabbit anti-GPIb $\alpha$  antibody. Note that 14-3-3 $\zeta$  was coimmunoprecipitated with wild-type GPIb-IX but not  $\Delta 591$ .

clonal antibody, AK2, known to inhibit ristocetin-induced vWF binding to GPIb-IX (Fig. 3 C). Furthermore, vWF did not induce specific fibrinogen binding to 2b3a cells (Fig. 3 D), suggesting that vWF-induced fibrinogen binding to integrin  $\alpha_{IIb}\beta_3$  requires vWF interaction with GPIb-IX. Thus, vWF interaction with GPIb-IX not only stimulates vWF- $\alpha_{IIb}\beta_3$  interaction, but also induces the integrin to bind soluble fibrinogen. These data indicate that risto-

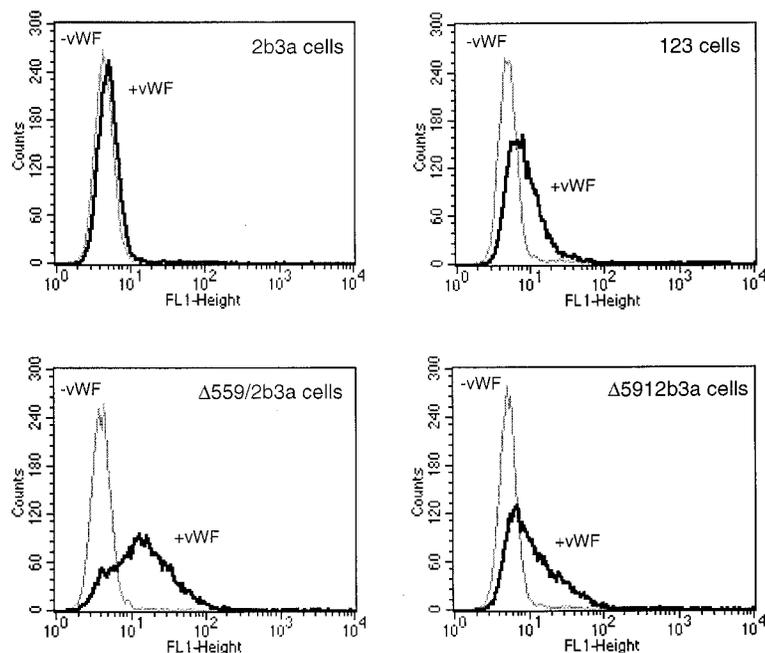
cetin-dependent vWF binding to GPIb-IX induces a cellular signal that activates the ligand-binding function of  $\alpha_{IIb}\beta_3$ .

### Effects of GPIb $\alpha$ Cytoplasmic Domain Deletion Mutagenesis on 14-3-3-binding Function of GPIb-IX

We showed previously (Du et al., 1996) that the intracellular signaling molecule 14-3-3 $\zeta$  binds to a site in the COOH-terminal 15 residues (residues 595–610) of the cytoplasmic domain of GPIb $\alpha$ . To investigate the role of 14-3-3 in GPIb-IX-mediated activation of  $\alpha_{IIb}\beta_3$  in the CHO cell model, we established a CHO cell line ( $\Delta 591/2b3a$  cells) that coexpresses  $\alpha_{IIb}\beta_3$  and a mutant GPIb-IX,  $\Delta 591$ , that lacks the 14-3-3-binding site (18 residues) at the COOH terminus of GPIb $\alpha$ , but retains the functional filamin-binding domain in GPIb $\alpha$  (Cunningham et al., 1996; Du et al., 1996). As shown in Fig. 4, wild-type GPIb-IX expressed in CHO cells (123 cells) coimmunoprecipitates with an endogenous CHO cell 14-3-3 protein reactive with anti-14-3-3 $\zeta$  antibodies (Fig. 4). The mutant GPIb-IX ( $\Delta 591$ ), however, failed to coimmunoprecipitate endogenous CHO cell 14-3-3 (Fig. 4). As a control, we also immunoblotted the same immunoprecipitates with an anti-GPIb $\alpha$  antibody, and observed that similar amounts of GPIb $\alpha$  were immunoprecipitated from both the  $\Delta 591/2b3a$  cells and 123 cells (expressing wild-type GPIb-IX; Fig. 4). Thus, the  $\Delta 591$  mutant GPIb-IX is defective in binding to endogenous 14-3-3.



**Figure 5.** Deletion of the 14-3-3-binding site in the cytoplasmic domain of GPIb $\alpha$  inhibited GPIb-IX-induced integrin activation. Cells were transfected with integrin  $\alpha_{IIb}\beta_3$  together with wild-type GPIb-IX (123), a GPIb-IX mutant lacking the 14-3-3-binding site ( $\Delta 591/2b3a$ ), or a GPIb-IX mutant with deleted filamin and 14-3-3-binding sites ( $\Delta 559/2b3a$ ). (A) The 123 cells,  $\Delta 591/2b3a$  and  $\Delta 559/2b3a$  cells were incubated with FITC-labeled fibrinogen (20  $\mu\text{g/ml}$ ) at 22°C for 30 min in the presence of ristocetin only (1 mg/ml; No vWF) or in the presence of 20  $\mu\text{g/ml}$  vWF and ristocetin (+vWF). To estimate nonspecific binding, these cells were also incubated with FITC-labeled fibrinogen in the presence of 1 mM RGDS peptide (+RGDS) known to specifically inhibit fibrinogen binding to  $\alpha_{IIb}\beta_3$ . The cells were then analyzed by flow cytometry. (B) 123 cells or  $\Delta 591/2b3a$  cells were incubated with biotin-labeled WM23 (against GPIb $\alpha$ ) and FITC-labeled D57 (against  $\alpha_{IIb}\beta_3$ ). As controls, CHO cells expressing GPIb-IX only or  $\alpha_{IIb}\beta_3$  only were also incubated with these antibodies at 22°C for 30 min. After washing and further incubation with phycoerythrin-labeled streptavidin, the cells were diluted in Tyrode's buffer and analyzed by flow cytometry.



**Figure 6.** Effects of truncation mutations on ristocetin-induced vWF binding to GPIb-IX. The 2b3a cells (expressing  $\alpha_{IIb}\beta_3$  only), 123 cells,  $\Delta 591/2b3a$  cells and  $\Delta 559/2b3a$  cells were incubated with (+vWF) or without (No vWF) vWF in the presence of 1 mg/ml ristocetin. The cells were then stained with a monoclonal antibody, SZ29, specific for human vWF, and analyzed by flow cytometry to estimate vWF binding.

### ***GPIb-IX-mediated Activation of the Integrin $\alpha_{IIb}\beta_3$ Requires the 14-3-3-binding Site in GPIb $\alpha$***

To determine whether deletion of the 14-3-3-binding site in GPIb $\alpha$  affects GPIb-IX-mediated activation of the integrin  $\alpha_{IIb}\beta_3$ , we examined whether ristocetin-induced vWF binding to the mutant GPIb-IX stimulates the binding of FITC-labeled fibrinogen to  $\Delta 591/2b3a$  cells. Fig. 5 A shows that vWF induces soluble fibrinogen binding to 123 cells which is inhibited by RGDS peptide. In contrast, vWF-induced fibrinogen binding to  $\Delta 591/2b3a$  cells is absent. It is unlikely that the defect in fibrinogen binding to  $\Delta 591/2b3a$  cells results from naturally occurring mutations developed in the CHO cells during selection as the  $\Delta 591/2b3a$  cells are established by mass sorting of cells reactive with both antibodies against  $\alpha_{IIb}\beta_3$  and GPIb-IX and not by single cell cloning. It is also unlikely that the inhibition of integrin activation results from defective binding of vWF as vWF binding to  $\Delta 591/2b3a$  cells is not negatively affected (Fig. 6). As  $\Delta 591/2b3a$  cells adhered and spread on fibrinogen (Fig. 7), the possibility of a defective integrin function can be further excluded. Thus, our data indicate that the 14-3-3-binding site of GPIb $\alpha$  plays an important role in GPIb-IX-mediated integrin activation.

We also examined vWF-induced fibrinogen binding to a CHO cell line ( $\Delta 559/2b3a$ ), expressing integrin  $\alpha_{IIb}\beta_3$  and a truncation mutant GPIb-IX lacking both the 14-3-3-binding domain and filamin-binding domain of GPIb $\alpha$ . No specific fibrinogen binding was detected in this cell line suggesting that inhibition of  $\alpha_{IIb}\beta_3$  activation by deleting the 14-3-3-binding site of GPIb $\alpha$  was not reversed by further deletion of the filamin-binding site of GPIb $\alpha$  (Fig. 5).

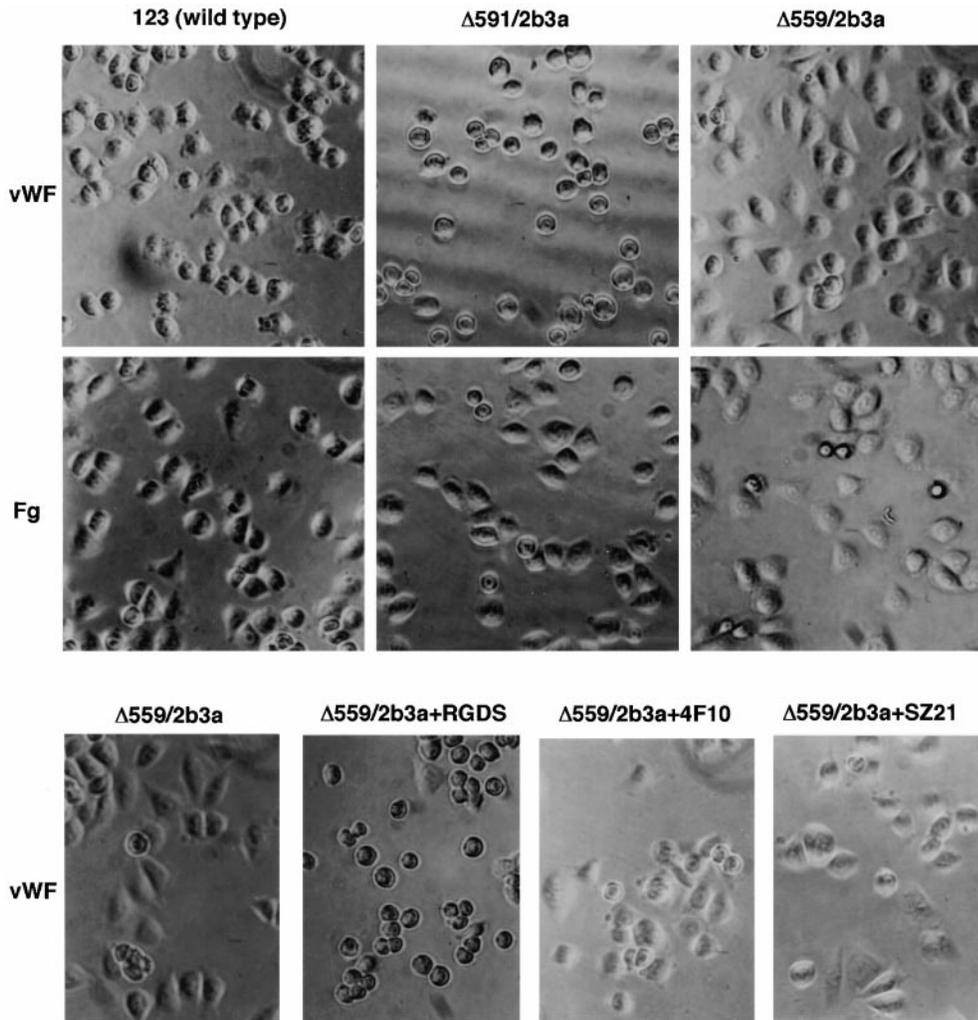
### ***The 14-3-3-binding Site of GPIb-IX Is Involved in GPIb-IX-induced Integrin-vWF Interaction and Cell Spreading on vWF***

To investigate whether 14-3-3 binding plays a role in

GPIb-IX-induced integrin-vWF interaction and integrin-dependent cell spreading on vWF, the 123 cells and  $\Delta 591/2b3a$  cells were allowed to adhere to vWF-coated microtiter wells. As examined under the microscope, ~70% of the 123 cells were spread on both vWF- and fibrinogen-coated microtiter wells. In contrast, only a small percentage (~30%) of  $\Delta 591/2b3a$  cells appeared spreading on vWF, indicating that GPIb-IX-induced integrin-vWF interaction was inhibited (Fig. 7). To quantitate the cell spreading objectively, cells adherent to vWF were permeabilized and stained with rhodamine-labeled phalloidin. Fluorescently stained cells in randomly selected fields were quantitated for cell surface area using Image-Pro Plus software (Media Cybernetics). As shown in Fig. 8, the average surface area of  $\Delta 591/2b3a$  cells were about half of that of 123 cells, indicating that the spreading of the mutant cell line was significantly reduced but not totally abolished. Since  $\Delta 591/2b3a$  cells adhered and spread on fibrinogen in a manner similar to 123 cells, the ligand-binding function of  $\alpha_{IIb}\beta_3$  and the integrin-mediated spreading process was not impaired in the  $\Delta 591/2b3a$  cell line. Thus, inhibition of GPIb-IX- and integrin-dependent spreading on vWF in this cell line is unlikely to be caused by a defect in ligand-binding function of  $\alpha_{IIb}\beta_3$  or in the integrin's post-ligand occupancy events. These data suggest that 14-3-3 $\zeta$  binding to the COOH-terminal region of GPIb $\alpha$  plays an important role in GPIb-IX-mediated activation of integrin  $\alpha_{IIb}\beta_3$ . Spreading of a small percentage of mutant cells reflects a background level of  $\alpha_{IIb}\beta_3$ -vWF interaction or the interaction of vWF with the endogenous CHO cell integrin (see Fig. 2 C).

### ***Effects of Disruption of GPIb-IX Interaction with Filamin on vWF Interaction with Integrins***

It has been shown previously (Cunningham et al., 1996; Du et al., 1996) that truncation of GPIb-IX at residue 559



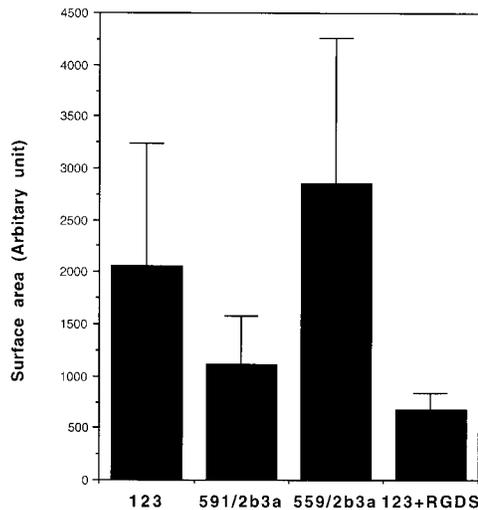
**Figure 7.** Effects of truncation mutants of GPIb-IX on the GPIb-IX- and integrin-dependent cell spreading on vWF. Cell lines were established that coexpressed integrin  $\alpha_{IIb}\beta_3$  together, either with wild-type GPIb-IX (123), or a GPIb-IX mutant with the GPIb-IX cytoplasmic domain truncated at residue 591 to delete the 14-3-3-binding site ( $\Delta 591/2b3a$ ), or at residue 559 to delete both 14-3-3- and filamin-binding sites ( $\Delta 559/2b3a$ ). The cells were incubated in microtiter wells coated with 10  $\mu\text{g/ml}$  vWF or 10  $\mu\text{g/ml}$  fibrinogen. Adherent cells were photographed under phase contrast microscope (20 $\times$  objective lens).  $\Delta 559/2b3a$  cells were also allowed to adhere to vWF in the presence of RGDS peptide (1 mM) or an antibody against  $\alpha_{IIb}\beta_3$  complex, 4F10 (100  $\mu\text{g/ml}$ ), or an antibody against  $\beta_3$ , SZ21.

( $\Delta 559$ ) of GPIb-IX abolishes filamin and 14-3-3 binding to GPIb-IX, and induces GPIb-IX-dependent cell spreading in the absence of integrin  $\alpha_{IIb}\beta_3$  (Cunningham et al., 1996). To investigate functional effects of this truncation mutation on GPIb-IX-dependent vWF interaction with different integrins, we coexpressed  $\Delta 559$  with integrin  $\alpha_{IIb}\beta_3$  ( $\Delta 559/2b3a$ ). Not only did the  $\Delta 559/2b3a$  cells exhibit no defect in spreading, but they actually showed enhanced spreading on vWF compared with 123 cells (Figs. 7 and 8). The spreading of  $\Delta 559/2b3a$  cells was significantly inhibited by RGDS peptide but poorly inhibited by anti- $\alpha_{IIb}\beta_3$  antibody 4F10 and anti- $\beta_3$  antibody SZ21 (Fig. 7), suggesting that an endogenous integrin plays a significant role. This result is consistent with previous studies showing that CHO cells expressing the same mutant of GPIb-IX spread on vWF in the absence of integrin  $\alpha_{IIb}\beta_3$  (Cunningham et al., 1996). However, when incubated with soluble fibrinogen in the presence of vWF and ristocetin, no specific binding of fibrinogen to  $\Delta 559/2b3a$  cells was detected (see Fig. 5). These results indicate that deletion of both 14-3-3 and filamin-binding sites of GPIb-IX inhibited GPIb-IX-mediated activation of fibrinogen binding to  $\alpha_{IIb}\beta_3$ , but enhanced the interaction of vWF with an endogenous integrin (which only plays a very limited role in wild-type GPIb-

IX-mediated cell spreading (see Fig. 2 C). Thus, it appears that two different mechanisms may be involved in the vWF interaction with integrins: a GPIb-IX-mediated 14-3-3-dependent mechanism that induces an activation signal leading to the activation of integrin  $\alpha_{IIb}\beta_3$ , and an alternative mechanism that allows the interaction of vWF with an unidentified integrin. The latter mechanism becomes significant only when the association of GPIb-IX with the membrane skeleton structure is disrupted.

#### ***Inhibition of GPIb-IX- and Integrin-dependent Cell Spreading by a 14-3-3 Fragment Containing the GPIb-IX-binding Site***

We have recently shown that GPIb-IX binds to a site in the helix I region of 14-3-3 $\zeta$ , distinct from the sites required for 14-3-3 $\zeta$  binding to RSXP motif containing ligands such as c-Raf (Gu and Du, 1998). To verify that 14-3-3 $\zeta$  plays a role in GPIb-IX signaling, we constructed cDNAs encoding fusion proteins of green fluorescent protein (GFP) with wild-type 14-3-3 $\zeta$  (GFP-1433) as well as a small fragment of 14-3-3 $\zeta$  containing the GPIb-IX-binding site (1433T12, residues 188–231; Gu and Du, 1998). Transient expression of the wild-type and the mutant 14-3-3



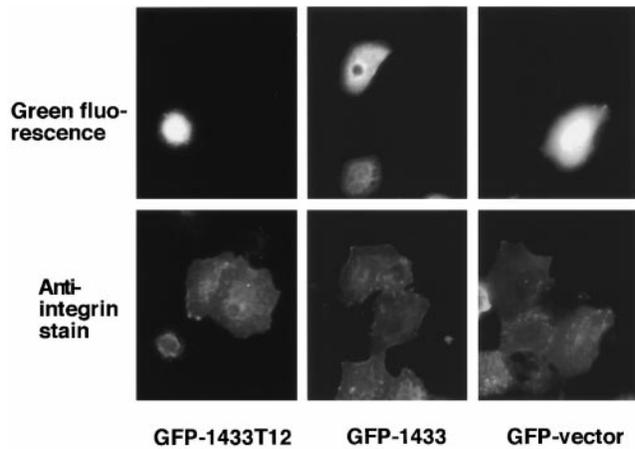
**Figure 8.** Quantitation of the effects of truncation mutations on cell spreading. The 123,  $\Delta$ 591/2b3a, and  $\Delta$ 559/2b3a cells were incubated for 1 h at 37°C in vWF-coated chamber slides, washed, fixed, and permeabilized. The cells were then stained with rhodamine-labeled phalloidin, and photographed under a Nikon fluorescence microscope using a cooled CCD camera. To estimate the surface area of round cells, RGD-treated 123 cells were allowed to adhere to vWF and stained with rhodamine-labeled phalloidin as above. Three randomly selected fields (for each cell line) were then analyzed for surface area of adherent cells using Image-Pro Plus software. Results were expressed as mean  $\pm$  SD (for 123 cells,  $n = 200$ ; for  $\Delta$ 591/2b3a cells,  $n = 180$ ; for  $\Delta$ 559/2b3a cells,  $n = 192$ ; for RGD-treated 123 cells,  $n = 60$ ). The difference between 123 cell and  $\Delta$ 591/2b3a cells is highly significant ( $P < 0.00001$ ).

was indicated by the emission of green fluorescence (Fig. 9). After transfection of pEGFP vector alone,  $\sim$ 70% of 123 cells adhere and spread on vWF. Cells expressing GFP-1433 fusion protein showed an increase in the percentage of spreading (85%), suggesting that overexpression of 14-3-3 enhanced cell spreading on vWF-coated surface (Fig. 9). In contrast, 90% of the cells expressing GFP-1433T12 fusion protein are rounded (Fig. 9), and the rest (10% cells) only partially spread on vWF (not shown). These results suggest that the small fragment of 14-3-3 $\zeta$  inhibited the function of endogenous 14-3-3 in a dominant negative fashion.

## Discussion

In this study, we show that GPIb-IX binding to vWF induces signals that activate the ligand-binding function of integrin  $\alpha_{IIb}\beta_3$  and integrin-dependent cell spreading using a reconstituted CHO cell expression model. We show that vWF-induced GPIb-IX signaling is inhibited by deletion of the 14-3-3-binding sites in the cytoplasmic domain of GPIb $\alpha$  (Figs. 5 and 7). Thus, our study indicates that interaction between GPIb-IX and 14-3-3 plays an important role in GPIb-IX-mediated signaling leading to activation of integrin  $\alpha_{IIb}\beta_3$ .

Understanding the intracellular signaling mechanism induced by ligand binding to the platelet vWF receptor,



**Quantitation of cell spreading on vWF (n=3)**

Cells	GFP-1433T12	GFP-1433	GFP vector
Round	90.7 $\pm$ 1.1%	14.7 $\pm$ 5.1%	30.0 $\pm$ 4.0%
Spread	9.3 $\pm$ 1.1%	85.3 $\pm$ 5.1%	70.0 $\pm$ 4.0%

**Figure 9.** A small fragment of 14-3-3 $\zeta$  containing the GPIb $\alpha$ -binding site inhibited GPIb-IX-induced cell spreading. Truncation mutation of 14-3-3 $\zeta$  and mapping of the GPIb $\alpha$ -binding site of 14-3-3 $\zeta$  were reported previously (Du et al., 1996; Gu and Du, 1998). A truncation mutant of 14-3-3 $\zeta$  (T12) contains the binding site for GPIb $\alpha$  (residues 181–231; Gu and Du, 1998). Vectors encoding GFP, GFP-14-3-3 $\zeta$  fusion protein (1433), and GFP-T12 fusion protein were transiently transfected into 123 cells (stably expressing integrin  $\alpha_{IIb}\beta_3$  and GPIb-IX) using lipofectamine. After 48 h, cells were detached and then allowed to adhere and spread on botrocetin-treated vWF-coated glass slides. Cells were then fixed and stained with an antibody against integrin  $\alpha_{IIb}\beta_3$  (D57) followed by rhodamine-labeled goat anti-mouse IgG. Cells expressing GFP-T12, GFP-14-3-3 $\zeta$ , or GFP are shown by emission of green fluorescence. Shown in the pictures are typical results from three experiments. To quantitate cell spreading, green fluorescent cells were counted under the fluorescence microscope, percentages of round and spread cells from three experiments are shown in the table (mean  $\pm$  SD).

GPIb-IX, as well as platelet signaling in general, has been hampered by the lack of specific means to interfere with platelet signaling intracellularly at a molecular level. Studies on the GPIb-IX-induced platelet activation by biochemical approaches have shown that ligand binding to GPIb-IX induces a series of intracellular biochemical changes such as generation of thromboxane A<sub>2</sub> (Kroll et al., 1991), production of phosphatidic acid (Kroll et al., 1991), activation of PI-3 kinase (Jackson et al., 1994), increase in the cytoplasmic calcium level (Kroll et al., 1991; Ikeda et al., 1993), and activation of protein kinases such as protein kinase C (Kroll et al., 1991, 1993; Chow et al., 1992) and tyrosine kinases (Razdan et al., 1994; Asazuma et al., 1997). The consequence of these intracellular signaling events is the activation of integrin  $\alpha_{IIb}\beta_3$  (De Marco et al., 1985a,b; Gralnick et al., 1985; Kroll et al., 1991; Savage et al., 1992, 1996). However, specific molecular pathways leading to these signaling events are unclear. In many cell types, ad-

vances in understanding the molecular mechanisms of intracellular signaling are often achieved with the use of recombinant DNA transfection techniques to express specific intracellular signaling molecules or to specifically alter the function of such a molecule. As platelets do not have nuclei and cannot be maintained in culture, it is difficult to use recombinant DNA approach directly. Thus, we have developed a model system in a CHO cell line expressing both the human integrin  $\alpha_{IIb}\beta_3$  and GPIb-IX. In our CHO cell expression model, GPIb-IX mediates signaling leading to the activation of integrin  $\alpha_{IIb}\beta_3$  in a manner similar to that observed in platelets: (a) vWF binding to GPIb-IX in our CHO cell model not only induces integrin-vWF interaction but also induces soluble fibrinogen binding to the integrin  $\alpha_{IIb}\beta_3$ , suggesting that GPIb-IX is unlikely to be simply presenting  $\alpha_{IIb}\beta_3$  to vWF or inducing changes in vWF, but is inducing a cellular signal that activates the ligand-binding function of the integrin (Fig. 3). This is consistent with previous findings in platelets showing vWF binding to GPIb-IX initiates signaling leading to integrin  $\alpha_{IIb}\beta_3$  activation (De Marco et al., 1985a,b; Gralnick et al., 1985; Kroll et al., 1991; Savage et al., 1992, 1996). (b) We show that GPIb-IX-induced integrin-dependent cell spreading on vWF was inhibited by prostaglandin  $E_1$  ( $PGE_1$ ), and the protein kinase C inhibitor calphostin C (Fig. 2). These inhibitors also inhibit vWF-induced integrin activation in platelets (Coller, 1981; De Marco et al., 1985a; Savage et al., 1992; Kroll et al., 1993; Kroll et al., 1991, 1993). Reconstitution of the platelet GPIb-IX-mediated activation of  $\alpha_{IIb}\beta_3$  in CHO cells is thus significant to further understanding the GPIb-IX-mediated signaling using specific molecular biological approaches.

In our CHO cell expression model, the vWF modulator, ristocetin, was used to induce binding of soluble vWF to GPIb-IX expressed in CHO cells. It is known that platelets do not bind to soluble vWF under physiological conditions. At the site of vascular injury, vWF binds to exposed subendothelial matrix proteins such as collagen. Collagen binding causes the exposure of the GPIb-IX-binding site in vWF probably by inducing a conformational change (Mazzucato et al., 1999). Shear stress may play a role in the conformational change of vWF induced by the subendothelial matrix (Siediecki et al., 1996). In vitro, the effect of subendothelial matrix proteins on vWF can be mimicked by desialation of vWF (De Marco et al., 1985a), natural occurring mutations in vWF (De Marco et al., 1985b; Gralnick et al., 1985) or binding of artificial vWF modulators such as botrocetin (Andrews et al., 1989) and ristocetin (Berndt et al., 1985; Kroll et al., 1991). Although there is a report that ristocetin may flocculate fibrinogen (Scott et al., 1991) and thus may increase nonspecific binding of fibrinogen, ristocetin-induced platelet aggregation in platelet-rich plasma is dependent on vWF binding to GPIb-IX, indicating that ristocetin cannot directly induce fibrinogen binding to integrin  $\alpha_{IIb}\beta_3$ . Binding of vWF to platelets induced by ristocetin and other in vitro methods is similar to vWF binding induced by subendothelial matrix under flow conditions. In both cases vWF binds to essentially the same ligand-binding pocket on GPIb-IX in the  $NH_2$ -terminal region of GPIb $\alpha$ , and can be inhibited by the same mono-

clonal antibodies (e.g., AK2) directed against the  $NH_2$ -terminal region of GPIb-IX (Berndt et al., 1988; Vicente et al., 1988, 1990; Fredrickson et al., 1998). Furthermore, vWF binding to GPIb-IX induced by vWF modulators, desialation or mutations initiate similar platelet responses to that observed when platelets adhere to matrix-bound vWF. These responses include activation of PKC, elevation of intracellular calcium, release of thromboxane  $A_2$ , release of granule contents, and activation of the integrin  $\alpha_{IIb}\beta_3$  (Kroll et al., 1991; Savage et al., 1992, 1996, 1998). For these reasons, ristocetin as a modulator of vWF binding to GPIb-IX has been commonly used in clinical and research laboratories. Since all available evidence indicates that GPIb-IX signaling is initiated by vWF binding to the  $NH_2$  terminus of GPIb $\alpha$  (Ware, 1998), and the experiments are controlled such that the effects of ristocetin alone can be excluded (Fig. 3), differences in the methods of induction of vWF binding (desialation, mutation, modulators, or shear stress) are unlikely to be a significant factor causing GPIb-IX signaling to diverge into dramatically different pathways. Thus, data obtained using vWF modulators such as ristocetin are relevant to understanding GPIb-IX signaling during platelet adhesion to the subendothelial matrix in vivo.

We have shown previously that 14-3-3, an intracellular signaling molecule, bound to the cytoplasmic domain of GPIb-IX, and that its binding was dependent upon the COOH-terminal region of GPIb $\alpha$  (Du et al., 1996) and the helix I region of 14-3-3 (Gu and Du, 1998). In this study, we have investigated the role of 14-3-3 in GPIb-IX signaling in the CHO cell expression model and show that deletion of the 14-3-3-binding site in the COOH terminus of GPIb $\alpha$  inhibits GPIb-IX-mediated  $\alpha_{IIb}\beta_3$  activation. As deletion of the COOH-terminal domain of GPIb $\alpha$  did not negatively affect vWF binding to GPIb-IX (Fig. 6), it is unlikely that the inhibition in integrin activation resulted from a loss of vWF binding function of the mutant GPIb-IX. Since this GPIb-IX mutant still interacts with filamin at a nearby site (Cunningham et al., 1996), it is also unlikely that a gross disturbance of the tertiary structure of the GPIb $\alpha$  cytoplasmic domain or loss of the interaction with the filamin-membrane skeleton caused the inhibition in signaling. Consistent with the importance of 14-3-3 in vWF-induced signaling, the small dominant negative fragment of 14-3-3 that contains the GPIb $\alpha$ -binding site (Gu and Du, 1998) also inhibited GPIb-IX-mediated integrin activation (Fig. 7). Thus, we conclude that 14-3-3 binding to GPIb-IX plays an important role in wild-type GPIb-IX-mediated signaling leading to integrin activation. This is the first identified early link between GPIb-IX and the integrin activation pathway.

Filamin binding to the central region of the GPIb $\alpha$  cytoplasmic domain links GPIb-IX to the membrane skeleton structure (cross-linked short actin filaments) underlining the membrane (Fox et al., 1988). The association of GPIb-IX with this structure is important for platelets to maintain a discoid shape (Lopez et al., 1998). A membrane skeleton-like structure can also be seen in the CHO cells expressing GPIb-IX complex (Du, X., unpublished data). It has been shown previously that truncation of GPIb $\alpha$  at residue 559 abolished association of GPIb-IX with the filamin-membrane skeleton. Cells expressing this truncated

mutant GPIb-IX spread on vWF without coexpression of  $\alpha_{IIb}\beta_3$  (Cunningham et al., 1996), a process that is inhibited by RGDS peptides (Du, X., unpublished data). Consistent with this, we found that  $\Delta 559/2b3a$  cells expressing this mutant GPIb-IX and integrin  $\alpha_{IIb}\beta_3$  showed an enhanced spreading on vWF which was poorly inhibited by anti- $\alpha_{IIb}\beta_3$  antibodies that blocked ligand-binding sites but was significantly inhibited by RGDS peptide (Fig. 7), suggesting that an RGDS-dependent endogenous integrin is responsible. In contrast to the truncation mutant, cells expressing wild-type GPIb-IX spread poorly on vWF in the absence of  $\alpha_{IIb}\beta_3$  (Fig. 2). This suggests that the function of this endogenous CHO cell integrin to mediate cell spreading on vWF is restrained by the membrane skeleton association with GPIb-IX and enhanced by disruption of this association. When coexpressed with integrin  $\alpha_{IIb}\beta_3$ , however, wild-type GPIb-IX is able to induce cell spreading on vWF (Fig. 2) and soluble fibrinogen binding to integrin  $\alpha_{IIb}\beta_3$ . Thus, GPIb-IX-mediated activation of  $\alpha_{IIb}\beta_3$  is not restrained by the association of GPIb-IX with the membrane skeleton. This suggests that the functions of  $\alpha_{IIb}\beta_3$  and the endogenous integrin are regulated by GPIb-IX via different mechanisms. Indeed, we showed that GPIb-IX-mediated activation of integrin  $\alpha_{IIb}\beta_3$  involves the binding of 14-3-3 to the COOH terminus of GPIb $\alpha$ . In contrast, the  $\Delta 559/2b3a$  cells expressing the mutant GPIb-IX lacking both filamin and 14-3-3-binding sites were defective in vWF-induced fibrinogen binding (Fig. 5). It remains unclear what types of endogenous CHO cell integrin are responsible for cell spreading on vWF in the absence of  $\alpha_{IIb}\beta_3$ , and what mechanisms are involved in the upregulation of their function when GPIb-IX is dissociated from the membrane skeleton. CHO cells express an endogenous vitronectin receptor ( $\alpha_v$  complexed with  $\beta_1$  or possibly  $\beta_5$ ) and  $\alpha_5\beta_1$ , both of which are inhibited by RGDS peptides (Chen et al., 1995; Felding-Habermann and Cheresch, 1993; Zhang et al., 1995). As these CHO cell integrins are known to interact with immobilized ligands without prior activation, one possibility is that binding of vWF to GPIb-IX may change the conformation of vWF or bring vWF to the vicinity of the endogenous integrin and thus allow their interaction via a localized signaling mechanism. Disruption of GPIb-IX association with the membrane skeleton may allow free lateral movement of GPIb-IX to the vicinity of these integrins without the restraint by the membrane skeleton structure, thus enhancing cell spreading on vWF. In contrast, the function of  $\alpha_{IIb}\beta_3$  to interact with vWF and soluble fibrinogen is known to require prior activation via intracellular signaling (Phillips et al., 1991; Savage et al., 1992). Thus, vWF-induced activation of  $\alpha_{IIb}\beta_3$  is not restrained by the membrane skeleton structure, but requires a 14-3-3-dependent signaling mechanism.

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