Disabled-2 is a novel αIIb-integrin-binding protein that negatively regulates platelet-fibrinogen interactions and platelet aggregation

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Summary

Platelet aggregation plays a pivotal role in the haemostatic process and is involved in the pathological counterpart of arterial thrombosis. We have shown that the adapter protein disabled-2 (DAB2) is expressed abundantly in platelets. In this study, DAB2 was found to distribute in the platelet α-granules and was released from the granular compartment upon platelet activation. The secreted DAB2 binds to the extracellular region of αIIbβ3 integrin on the platelet surface through the phosphotyrosine-binding domain. The DAB2-platelet interactions result in the inhibition of agonist-induced platelet aggregation with the exception of thrombin, a DAB2 protease that renders DAB2 inactive. Biochemical and mutational analysis revealed that the DAB2 cell-adhesion Arg-Gly-Asp (RGD) motif (amino acid residues 64-66) and the αIIb-integrin–fibrinogen-binding region (amino acid residues 171-464) are important for the DAB2-platelet interactions. Such interactions compete for the binding of αIIb integrin with fibrinogen and provide a mechanism for DAB2 to inhibit platelet aggregation. Accordingly, the synthetic RGD-motif-containing DAB2 peptide PDARGDKM also elicited anti-platelet aggregation activity. These findings demonstrate for the first time that DAB2 is an αIIb-integrin-binding protein that plays a novel role in the control of platelet-fibrinogen interactions and platelet aggregation.

Key words: Disabled-2, Fibrinogen, αIIbβ3 Integrin, Platelet aggregation, RGD motif

Introduction

Human platelets are anucleated cells derived from megakaryocytes and represent the second most numerous blood cells in the peripherial blood (George, 2000). Upon vessel wall damage, platelets adhere to the exposed endothelial cell surface and collagen fibers through the platelet glycoprotein (GP) Ib-IX-V complex on the platelet surface (Savage et al., 1996). Intracellular signaling following platelet adhesion results in platelet activation and degranulation of α-granules and δ-granules. The release of granular contents further facilitates the local recruitment of platelets and induces platelet aggregation that is mediated by the interaction between fibrinogen and the activated αIIbβ3 integrin. Finally, fibrin formation results in stabilization of the haemostatic plug and prevention of blood loss.

Deregulation of platelet function is involved in many pathophysiological processes. Disruption of the fibrinogen–αIIbβ3-integrin interactions due to quantitative and/or qualitative defects in αIIbβ3 integrin results in Glanzmann's thrombasthenia with impaired haemostasis and increased risk of bleeding (Quinn et al., 2003). Conversely, excessive and inappropriate platelet activation results in abnormal thrombus formation that leads to the development of stroke and myocardial infarction. Platelets are also involved in the promotion of tumor metastasis with an increase in angiogenesis and osteoclastic activity (Gupta and Massague, 2004; Trikha and Nakada, 2002; Boucharaba et al., 2004). These studies clearly indicate that platelet function is important for the maintenance of normal human physiological haemostasis and is crucial in pathological development of human diseases.

Regardless of the activation process, the soluble agonists for platelet aggregation usually stimulate a common effector pathway involving αIIbβ3 integrin inside-out signaling (Calderwood, 2004). The interaction between platelet and fibrinogen further triggers outside-in signaling and αIIbβ3 integrin activation. Because of its importance in platelet aggregation, the binding of αIIbβ3 integrin to fibrinogen was extensively studied. αIIbβ3 Integrin was shown to recognize a stretch of amino acids HHLGGAKQAGDV at the C-terminus of the fibrinogen γ-chain through the αIIb integrin subunit (Kloczewiak et al., 1982). αIIbβ3 integrin also binds to the cell-adhesion Arg-Gly-Asp (RGD) motifs that are present in the fibrinogen α-chain: the sequence RGDS at residues 572-

575, and the sequence RGDF at residues 95-98 (Plow et al., 1985). Amino acid residues 109-171 and 204-231 of the β3 integrin subunit also contain the fibrinogen-binding site (D'Souza et al., 1988; Charo et al., 1991). These studies suggest that fibrinogen binding to αIIbβ3 integrin is a complex and probably multistep process.

Disabled-2 (DAB2) is an adapter protein that is implicated in negative regulation of αIIbβ3-integrin-mediated fibrinogen adhesion and cell signaling (Huang et al., 2004). Like other adapter proteins, DAB2 elicits its function through interaction with other cellular factors. This is mainly mediated through the N-terminal phosphotyrosine binding (PTB) domain, the aspartic-acid–proline–phenylalanine (DPF) motif and the C-terminal proline-rich region (Calderwood et al., 2003; Huang et al., 2004; Wang et al., 2002; Hocevar et al., 2001; Hocevar et al., 2003). Accordingly, DAB2 acts to control a variety of cellular processes including growth factor and hormone signaling, endocytosis and cell-adhesive function (Xu et al., 1998; Inoue et al., 2002; Morris et al., 2002; Kowanetz et al., 2003; Zhou et al., 2003). In the hematopoietic system, DAB2 is abundantly expressed in human platelets (Huang et al., 2004) and is upregulated through platelet-derived growth factor (PDGF) autocrine signaling (Tseng et al., 2005) during megakaryocytic differentiation of human leukemic cell lines and $CD34^+$ hematopoietic pluripotent stem cells (Tseng et al., 2001; Tseng et al., 2003; Huang et al., 2004). DAB2 negatively regulates αIIbβ3-integrin-mediated fibrinogen adhesion and cell signaling in a S24-phosphorylation-dependent manner that promotes DAB2 membrane translocation and the subsequent interaction with the β3 integrin cytoplasmic tail (Huang et al., 2004). The latter study provides evidence that the interaction between DAB2 and platelet αIIbβ3 integrin is of significance in platelet function. Whether or not DAB2 has a role in platelet activation and aggregation remains to be elucidated.

In this study, we report for the first time that DAB2 is associated with the α-granules of human platelets. During platelet activation, DAB2 is released and bound to the extracellular region of αIIbβ3 integrin on the platelet surface. Such binding involves the DAB2 RGD motif and the αIIb-integrin–fibrinogen binding region. Consequently, DAB2 competes for platelet-fibrinogen binding and elicits anti-platelet aggregation activity. These findings contribute to our understanding of DAB2 in platelet function and perhaps shed new light on platelet physiology.

Results

Subcellular localization of DAB2 in human platelets

DAB2 is a major platelet protein in human peripheral blood (Huang et al., 2004). To examine the subcellular localization of platelet DAB2 more closely, we analyzed platelet homogenates by using linear sucrose-density-gradient centrifugation to separate different platelet organelles from the bulk of cytosolic and plasma membrane proteins. Western blot analysis of the gradient fractions revealed two distinct pools of DAB2 (Fig. 1A). DAB2 was partly present in the low-density fractions (fractions one to four), which contained most of the cytoplasmic and plasma membrane proteins, and in fractions of higher densities (~50% sucrose, fractions 11 to 14), in which intracellular granules are expected. This distribution was paralleled by P-selectin and PF-4, the marker proteins of α-granules. The presence of a minor amount of the α-granule markers in the low-density fractions can be explained by a partial rupture of the granules during homogenization (Mairhofer et al., 2002).

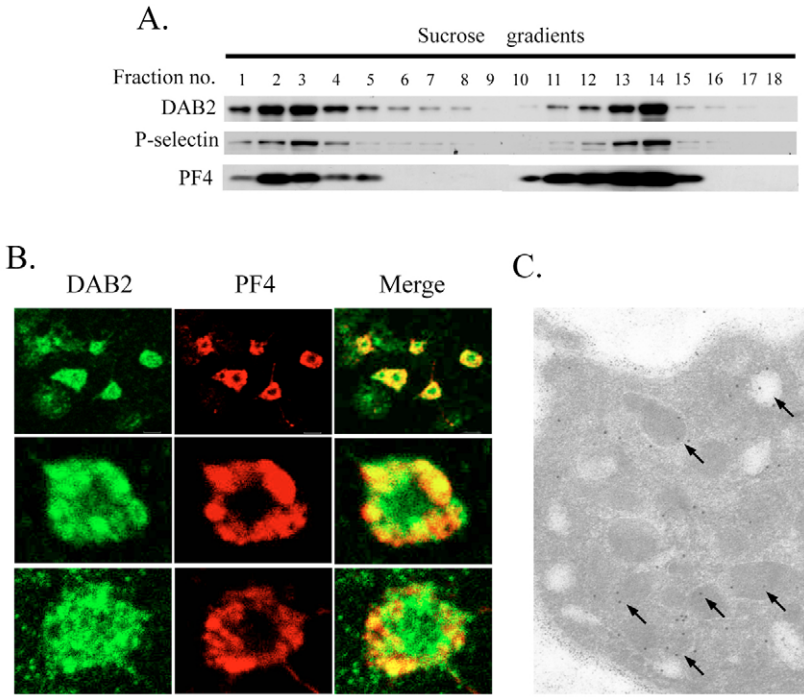

Fig. 1. Subcellular localization of DAB2 in human platelets. (A) Association of DAB2 with platelet α-granules. Platelet homogenates (2×10^9) were separated by sucrose-density-gradient (30-60%) centrifugation. Eighteen fractions (700 μl each) were collected from the top and aliquots were subjected to western blot analysis using the anti-p96 (DAB2), anti-P-selectin and anti-PF4 antibodies. (B) Colocalization of DAB2 with PF4. Immunofluorescence staining of DAB2 (green) and PF4 (red) was performed with platelets cytospun on a glass slide and was observed by confocal microscopy. The image of individual platelets was obtained by using confocal microscopy analytical software to enlarge and focus the indicated platelet. (C) Immunoelectron microscopy of resting platelets revealed the presence of DAB2 in α-granules. Washed platelets were immunostained with anti-DAB2 antibody. After labeling with 10-nm colloidal-gold-conjuagated protein G, the subcellular distribution of DAB2 was observed by electron microscopy. Arrows indicate positive staining of the immunogold particle. Original magnification 15,000×.

To further delineate that DAB2 is present in α-granule, human platelets were subjected to co-immunofluorescence staining with anti-DAB2 and anti-PF4 antibodies. DAB2 displayed a cluster and granular staining pattern that partly overlapped with the PF4 staining signal in a confocal microscopy analysis (Fig. 1B), suggesting that DAB2 is colocalized with the α-granule protein PF4. To obtain a better resolution for the subcellular distribution of DAB2, human platelets were subjected to immunoelectron microscopy analysis with anti-DAB2 antibody. Our data revealed that most of the DAB2 immuostaining signals were present in the α-granules and surface-connected canalicular system with trace amount in the cytoplasm (Fig. 1C). These observations were consistent with the results of sucrose-gradient fractionation and confocal microscopy analysis. DAB2 is thus considered as a platelet protein mainly distributed in the α-granule.

Platelet DAB2 is released during agonist-induced platelet aggregation in a PKC-dependent manner

Platelets are activated by various stimulants in vitro that results in fusion of the α-granules with the plasma membrane and surface-connected canalicular system. Subsequently, the α-granular contents are released to trigger serial biochemical reactions that induce platelet adhesion and aggregation. We studied the fate of DAB2 in this process. Washed platelet suspensions were treated with different concentrations of thrombin, TPA, collagen and the thromboxane A2 receptor agonist U46619 in the presence of EDTA. In this experimental condition, platelet activation and α-granule secretion occurred without platelet aggregation. The platelet pellet and the suspension medium into which protein is secreted were separated for western blot analysis to determine whether DAB2 is released during platelet activation. The presence of PF4 protein in the suspension medium was employed as a positive control for the release of α-granules. Like PF4, DAB2 was released into the suspension medium in a dose-dependent manner after agonist-induced platelet activation (Fig. 2A). The lack of an abundant cytosolic platelet protein PKCα in the suspension medium and the bulk of DAB2 protein in the platelet pellet indicates that the presence of DAB2 in the suspension medium was not due to extensive lysis of platelets during the assay. When the assays were performed in the absence of EDTA, platelet aggregation occurred. Accordingly, the amount of DAB2 in the suspension medium was reduced perhaps because of trapped or increased binding of DAB2 on the platelet surface (data not shown). These data indicate that release of DAB2 is associated with the secretion of platelet granules.

PKC is involved in the process of platelet granule secretion. The pan-PKC inhibitor Ro-31-8220, which inhibits platelet dense and α-granule secretion (London, 2003), was used to determine whether DAB2 release is PKC-dependent. At a concentration of 20 μM, Ro-31-8220 inhibited the release of both DAB2 and PF4 into the suspension medium during agonists-induced platelet activation (Fig. 2B). Based on the observations that DAB2 is mainly distributed in the α-granule, these results indicate that the release of platelet DAB2 is likely to associate with α-granule secretion in a PKC-dependent manner.

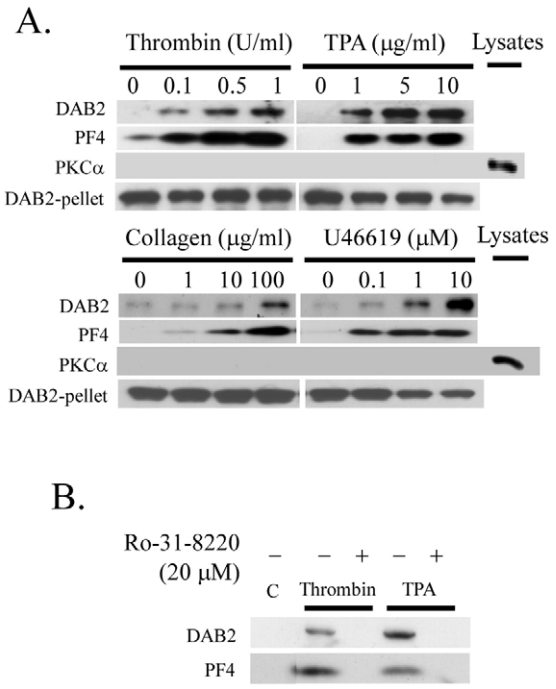

Fig. 2. DAB2 is released during agonist-induced platelet aggregation in a PKC-dependent manner. (A) Release of DAB2 during agonist-induced platelet aggregation. Washed platelets (1.4×10^8) were stimulated with the indicated concentration of agonists. The platelet pellet and the suspension medium into which protein is secreted were separated for western blot analysis with anti-p96, anti-PF4 and anti-PKCα antibodies. The lysates of platelet pellets were also used as a control to demonstrate the presence of PKCα in the platelet pellet but not the suspension medium. (B) PKC-dependent DAB2 release during platelet aggregation. Washed platelets with or without pretreatment of the pan-PKC inhibitor Ro-31-8220 (20 μM) were stimulated by thrombin (0.1 U/ml) or TPA (1 μg/ml). The suspension medium was collected for western blot analysis with anti-96 (DAB2) and anti-PF4 antibodies. The suspension medium from resting platelets was used as a control (C) for the experiment.

DAB2 binds to platelets and megakaryocytic differentiating K562 cells

To delineate whether or not the agonist-induced release of DAB2 binds to the platelet surface, resting and agonists-treated platelets were immunolabeled with anti-DAB2 antibody followed by FITC-conjugated secondary antibody and were subjected to flow cytometry. The non-specific trapping of secondary antibody into platelet aggregates was also determined for calculation of specific DAB2 binding. In the resting phase, DAB2 was barely detectable on the platelet surface (Fig. 3A); the percent of platelets showing DAB2 binding was 1.67±0.76% (n=6). For the activated platelets, the percent of platelets showing detectable DAB2 binding was agonist-dependent, with 12.21±0.94% for thrombin (0.1 U/ml; n=4), 67.54±2.52% for TPA (1 μg/ml; n=6), 29.68±2.95% for collagen (10 μg/ml; n=4), 46.34±5.84% for U46619 (1 μM; n=5), and 59.04±5.84% for TRAP (10 μM; n=5), respectively. Binding did not change even in the presence of EDTA (data not shown). These data reveal that platelet DAB2 was released and interacted with the activated platelet surface. In addition, DAB2 binding to thrombin-activated platelets is significantly different to the platelets activated by other agonists (P<0.001).

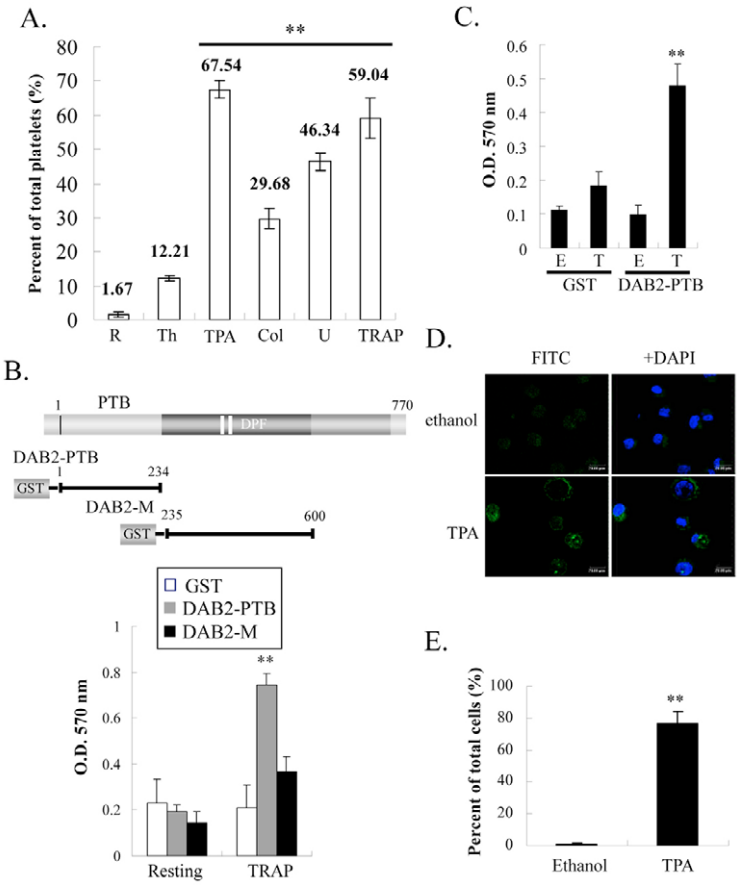

Fig. 3. DAB2 binds to platelets and megakaryocytic differentiating K562 cells. (A) DAB2 binds to the surface of activated platelets. Washed platelets (1.5×10^8) were either untreated (R, resting platelets) or were stimulated with thrombin (Th, 0.1 U/ml), TPA (1 μg/ml), collagen (Col, 10 μg/ml), U46619 (U, 1 μM) or TRAP (10 μM) for 10 minutes. Platelets were analyzed by flow cytometry after incubation with anti-DAB2 (1:100 dilution) and FITC-conjugated goat anti-mouse secondary antibody. The percent of platelets with specific DAB2 binding is presented. (B) The PTB domain of DAB2 is crucial for the interaction with platelets. Resting or TRAP-stimulated (10 μM) platelets (1.5×10^8/well) were added for 3 hours to 24-well plates pre-coated with DAB2-PTB, DAB-M or control GST proteins (100 μg/ml). Platelet adhesion was then quantified by Crystal Violet assay. (C) TPA-treated (T, 10 ng/ml) or vehicle control ethanol-treated (E, 0.01%) K562 cells (5×10^5 cells/well) were added to plates pre-coated with DAB2-PTB or control GST protein (100 μg/ml). Cell adhesion was quantified by Crystal Violet assay. (D,E) TPA- and ethanol-treated K562 cells (5×10^5) were incubated with DAB2-PTB for 3 hours. Binding of DAB2 was analyzed by incubating K562 cells with FITC-conjugated anti-GST antibody, counterstained with 4′,6-diamidino-2-phenylindole (DAPI) and observed by confocal microscopy (D). The percent of K562 cells with DAB2-PTB binding was determined by flow cytometry (E). The data represent the mean ± s.d. of three to six experiments. **P<0.001 when compared with thrombin-treated platelets (A), TRAP-stimulated platelet adhesion to GST (B) and ethanol-treated K562 cell adhesion (C), or binding to DAB2-PTB (E).

Owing to the relatively high percentage of DAB2 binding in TRAP-treated platelets, TRAP was used as a representative platelet agonist for molecular characterization of the interactions between DAB2 and platelets.

Because we did not succeed in generating full-length and C-terminal DAB2 recombinant proteins, GST recombinant proteins encoding the N-terminus (DAB2-PTB, aa. 1-234) and middle region (DAB2-M, aa 235-600) of DAB2 were used in the following experiments to further demonstrate the interaction between platelets and extracellular DAB2. Resting and TRAP-activated platelets were added onto non-tissue culture plates that had been pre-coated with DAB2-PTB, DAB2-M, or the GST control protein (100 μg/ml) in an in vitro adhesion assay (Fig. 3B). For the resting platelets, there was no increase in platelet adhesion to DAB2-PTB and DAB2-M compared with the GST control protein. However, TRAP-stimulated platelets increased their adhesion to DAB2-PTB compared with DAB2-M and GST (n=5; P<0.001). These data indicate that recombinant DAB2-PTB acts like secreted platelet DAB2 in its interaction with the platelet surface.

To explore whether or not the extracellular form of DAB2 also binds to megakaryocytic cells, K562 cells were added to plates pre-coated with DAB2-PTB or control GST protein. The cell adhesion was then quantified by Crystal-Violet-staining assay. Only non-specific background adhesion to DAB2-PTB and GST was observed for the solvent-vehicle control, ethanol-treated non-differentiating K562 cells (Fig. 3C). Following TPA treatment to induce megakaryocytic differentiation, the adhesive activity of K562 cells towards DAB2-PTB was enhanced by more than fourfold (n=4; P<0.001), indicating that an interaction occurred between K562 cells and extracellular DAB2. To further delineate such an interaction, soluble DAB2-PTB was mixed with K562 cells and the binding of DAB2-PTB on the cell surface was detected by immunofluorescence staining using FITC-conjugated anti-GST antibody. As shown in Fig. 3D, a fluorescence signal was detected on the cell surface when DAB2-PTB was mixed with the TPA-treated, megakaryocytic differentiating K562 cells. No binding of DAB2-PTB was found for the control cells. Flow cytometric analysis was further used to quantify DAB2-PTB binding (Fig. 3E). More than 76.66±7.47% of the megakaryocytic differentiating cells displayed DAB2-PTB binding, compared with 0.80±0.68% for control cells (n=3; P<0.001). No binding of GST with K562 cells was observed (data not shown), thereby ruling out the possibility that the interaction of DAB2-PTB with TPA-treated K562 cells was due to the GST region of the recombinant protein. These data support the notion that DAB2 binds to both the platelet and megakaryocytic cell surface.

DAB2 is an αIIbβ3-integrin-binding protein

αIIbβ3 and αvβ3 are the two main platelet integrins. To define whether or not these integrins are involved in DAB2-to-platelet binding, the functional-blocking monoclonal antibodies (mAbs) 7E3 (αVβ3- and αIIbβ3-specific), 10E5 (αIIbβ3-specific) or LM609 (αVβ3-specific), and the RGDS peptide that competes with the interaction between the RGD sequence and cellular integrins were added to the assay

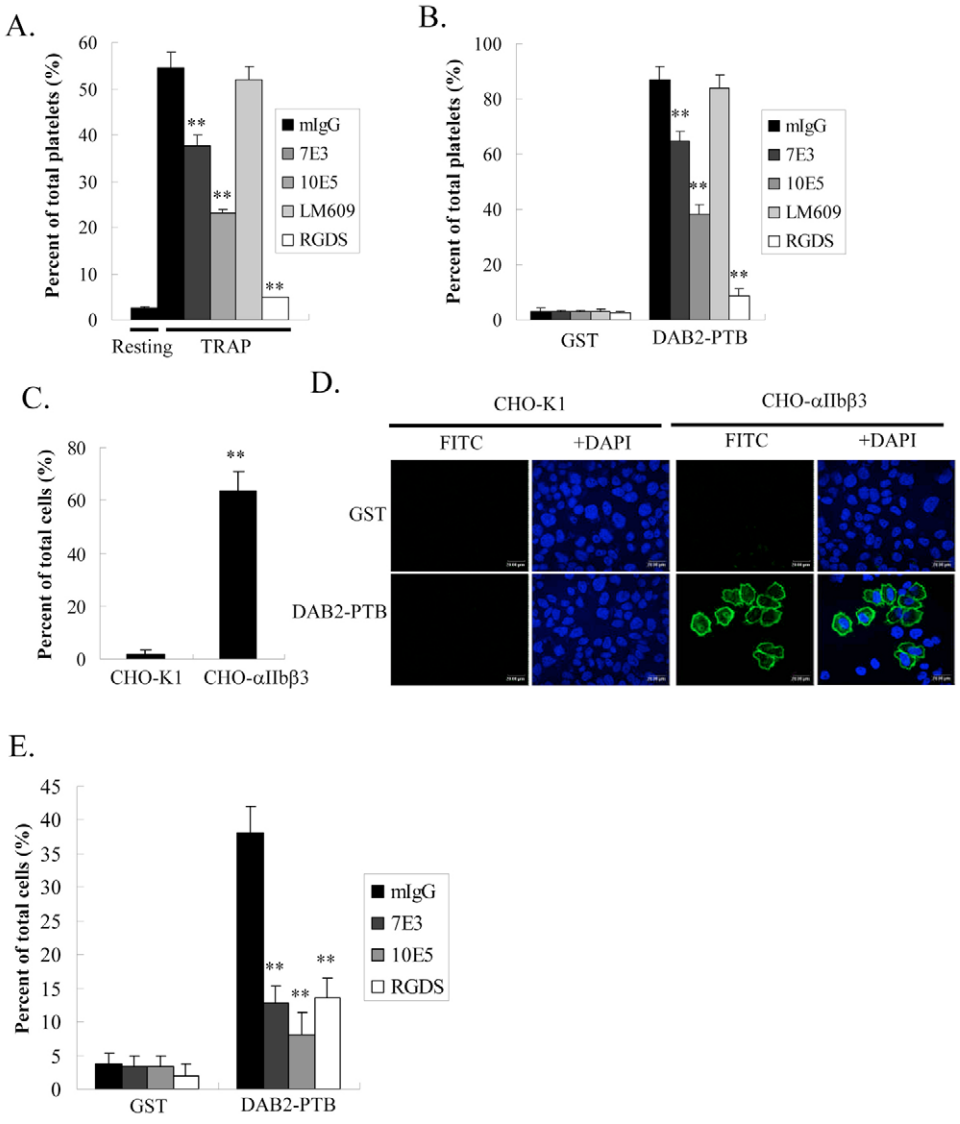

Fig. 4. αIIbβ3 integrin mediates DAB2 binding to platelets and the surface of CHO-αIIbβ3-integrin cells. (A) Effect of the functionally blocking mAbs and the RGDS peptide on the interaction of DAB2 with platelets. Resting or TRAP-stimulated (10 μM) platelets (1.5×10^8) were incubated with the indicated mAbs (20 μg/ml) or RGDS peptide (100 μM) for 30 minutes. The percent of platelets with DAB2 binding was determined by flow cytomery after incubating the platelets with anti-DAB2 (H110) antibody (1:200 dilution) and FITC-conjugated donkey anti-rabbit secondary antibody. (B) Effects of functional blocking mAb and RGDS peptide on the binding of recombinant DAB2-PTB to platelet surface. TRAP-stimulated platelets (1.5×10^8) were incubated with the mAb or RGDS peptide at room temperature for 30 minutes. DAB2-PTB or control GST protein (100 μg/ml) was added into the assay mixture and incubated for 3 hours. DAB2-PTB binding was detected as described in A except that an anti-GST antibody was used. (C) Expression of αIIbβ3 integrin on CHO-K1 and CHO-αIIbβ3-integrin cells. The percent of cells with αIIbβ3 integrin expression was determined by flow cytometry using 10E5 (20-30 μg/ml) and FITC-conjugated goat anti-mouse secondary antibody. (D) Binding of DAB2 to the surface of CHO-αIIbβ3-integrin cells. DAB2-PTB or control GST protein (100 μg/ml) were added to the cultured CHO-K1 and CHO-αIIbβ3-integrin cells for 2 hours. After incubation with FITC-conjugated anti-GST antibody (green) and counterstaining with DAPI (blue), confocal microscopy analysis was performed to visualize DAB2 binding. (E) Effects of function blocking mAbs and RGDS peptide on DAB2 interaction with CHO-αIIbβ3-integrin. The CHO-αIIbβ3-integrin cell suspension was incubated with the indicated mAbs or the RGDS peptide for 30 minutes. Then, DAB2-PTB or control GST protein (100 μg/ml) were added into the cell suspension and incubated for 3 hours. DAB2 binding was determined by flow cytometry using FITC-conjugated anti-GST antibody. The data represent the mean ± s.d. of three to six experiments. $^{**}P<0.001$ compared with TRAP-stimulated platelets treated with mIgG (A and B), CHO-K1 cells (C) or CHO-αIIbβ3-integrin cells pre-treated with mIgG (E).

mixture during TRAP-induced platelet activation. The percent of platelets with binding of platelet-secreted DAB2 was determined by flow cytometry using the anti-DAB2 (H110) antibody. As shown in Fig. 4A, the mouse control IgG antibody (mIgG) and LM609 did not affect DAB2 binding. By contrast, DAB2 binding to platelets was blocked by 7E3, 10E5 and the RGDS peptide (n=3; P<0.001). Increasing concentration of 7E3 further demonstrated a dose-dependent inhibition of DAB2 binding (data not shown). When experiments were performed in the presence of recombinant DAB2-PTB, pre-treatment with 7E3, 10E5 and the RGDS peptide also blocked the interaction between TRAP-activated platelets and DAB2-PTB in an in vitro adhesion assay (n=4; P<0.001). By contrast, LM609 had no effect on interactions of platelets and DAB2-PTB (Fig. 4B). These data suggest that αIIbβ3 integrin but not αvβ3 integrin is the main platelet integrin that mediates DAB2 binding to the activated platelets.

To further demonstrate the interaction between DAB2 and αIIbβ3 integrin, the CHO-αIIbβ3-integrin cell line that stably expresses αIIbβ3 integrin and its parental cell line CHO-K1 (not expressing αIIbβ3-integrin) were used for the binding assay with DAB2-PTB. αIIbβ3 integrin was expressed in 63.45±7.20% of CHO-αIIbβ3-integrin and 1.68±1.79% of CHO-K1 (n=6; P<0.001) as determined by flow cytometry using mAb 10E5 that specifically recognizes the αIIbβ3-integrin complex (Fig. 4C). Binding assays with DAB2-PTB or control GST protein revealed that DAB2 bound the cell surface of CHO-αIIbβ3-integrin cells but not CHO-K1 cells (Fig. 4D). Similar findings were observed for the K562 cells that transiently expressed αIIbβ3 integrin (data not shown).

The binding of DAB2-PTB can be blocked by 7E3, 10E5 and the RGDS peptide (n=5; P<0.001), suggesting the specific interaction between DAB2-PTB and αIIbβ3 integrin (Fig. 4E). These data indicate that αIIbβ3 integrin mediates DAB2 binding to the cellular surface of platelets and αIIbβ3 integrin-expressing cells.

DAB2 interacts with the αIIb integrin extracellular domain through the RGD motif

To gain insight into the interaction between DAB2 and αIIbβ3 integrin at the molecular level, we mapped the binding of DAB2 to αIIbβ3 integrin. The recombinant proteins encoding different regions of the αIIb integrin (Fig. 5A, constructs a-d) and β3 (Fig. 5A, construct e) extracellular domains were generated. Cell extracts of DAB2-overexpressing K562 cells were first pulled down with GST-recombinant proteins a and e (Fig. 5B). DAB2 was found to interact with recombinant protein a but not e, suggesting that the extracellular domain of αIIb integrin interacts with DAB2. Further mapping of the DAB2-binding region in αIIb integrin using the GST recombinant proteins b, c and d demonstrated that DAB2 bound to αIIb integrin at the region spanning aa 171-464 (construct d), which contains major fibrinogen-binding and γ-chain-peptide-binding sequences (aa 294-314).

We noticed a RGD motif essential for mediating the integrin and extracellular matrix interaction located at the N-terminus of DAB2 (Fig. 5C). The evolutionary conservation of the RGD motif of DAB2 and its flanking sequences in mammalian species, such as human, rat and mouse, suggests that RGD is important for DAB2 function. Hence, in the following experiments, we determined whether or not RGD plays a role in the interaction of DAB2 with αIIb integrin. A peptide (DAB2-RGD) containing the DAB2-RGD sequence PDARGDKM was synthesized and added to the GST-pull-down assay with GST–αIIb-integrin-171-464 (construct d). The interaction of αIIb integrin and DAB2 was abrogated in the presence of DAB2-RGD peptide (Fig. 5D). In addition, a peptide containing the mutated RGD sequence PDARGEKM (D→E) (DAB2-RGE) lost its effect on the interference of αIIb-integrin–DAB2 interactions. To further demonstrate that DAB2 RGD is important for platelet-DAB2 interactions, resting or TRAP-stimulated platelets were added to plates pre-coated with DAB2-PTB or DAB2-D66E (carrying the RGD→RGE mutation) for the in vitro adhesion assay (Fig. 5E). The activated platelets adhered to the pre-coated DAB2-PTB plates but not to plates coated with DAB2-D66E, demonstrating the involvement of the RGD motif in DAB2-platelet interactions. These data indicate that DAB2 RGD is involved in the interaction between DAB2 and αIIb integrin, and is important for the binding of DAB2 to platelets and megakaryocytic differentiating cells.

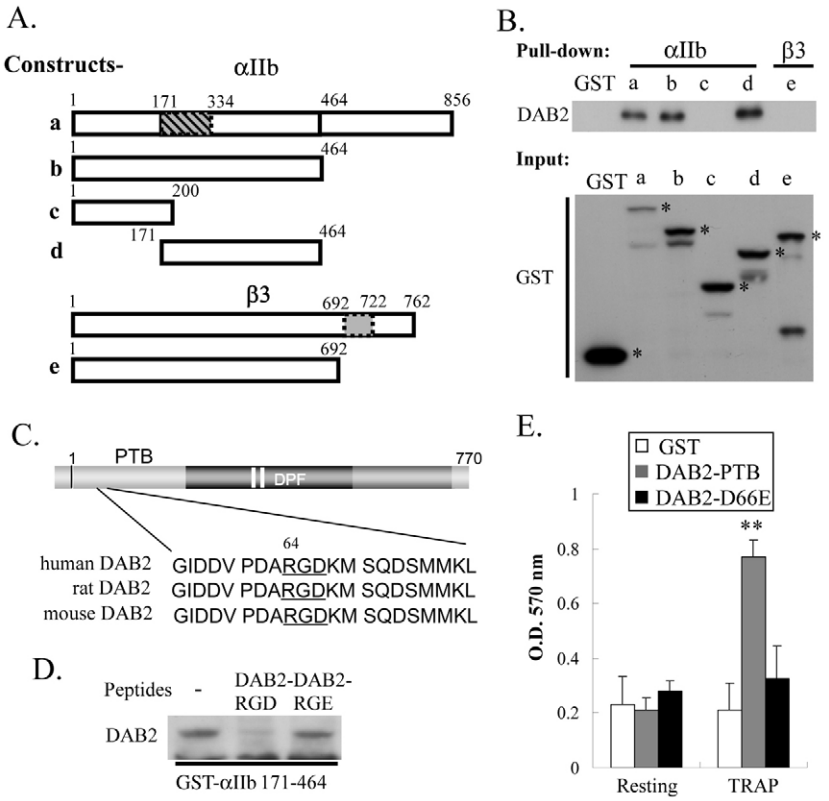

Fig. 5. Mapping the interaction of αIIbβ3 integrin and DAB2. (A) Schematic representation of the recombinant GST-αIIb integrin and β3 integrin mutant proteins. The shaded box of αIIb integrin (aa 171-334) represents the fibrinogen-binding site. The shaded region (aa 692-722) of β3 integrin represents the transmembrane domain. (B) Amino acids 171-464 of αIIb integrin interact with DAB2. Cell lysates (1 mg) of DAB2-transfected K562 cells were collected for GST-pull-down assays using the indicated GST-αIIb integrin (constructs a-d) and GST-β3 (construct e) proteins or control GST proteins (25 μg). The pull-down lysates (upper panel) and the aliquots of the input GST-proteins (lower panel) were subjected to western blot analysis with anti-DAB2 and anti-GST antibodies. The expected band for each purified protein is denoted by asterisk. (C) Schematic representation of the RGD motif and its flanking sequences for human, rat, and mouse DAB2. (D) The DAB2-RGD peptide interferes with DAB2–αIIb-integrin interaction. Cell lysates (1 mg) of DAB2-transfected K562 cells were subjected to GST-pull-down analyses with GST-αIIb-integrin aa 171-464 (construct d) in the absence or presence of DAB2-RGD and DAB2-RGE peptide (100 μg/ml). The pull-down lysates were subjected to western blot analysis using anti-DAB2 antibody. (E) The resting or TRAP-stimulated (10 μM) platelets (1.5×10^8) were added into the plates pre-coated with GST, DAB2-PTB or DAB2-D66E (100 μg/ml) for 3 hours at 37°C. Platelet adhesion was then quantified by the Crystal Violet assay. The data represent the mean ± s.d. (n=5). **P<0.001 compared with the TRAP-stimulated platelet adhesion to GST control protein.

DAB2 modulates in vitro platelet aggregation through the DAB2 RGD motif

To elucidate the function of DAB2 release and its interaction with αIIb integrin during platelet activation, we performed an in vitro platelet aggregation assay to analyse whether or not DAB2 affects agonist-induced platelet aggregation. Recombinant DAB2-PTB proteins were added to the assay mixture. We found that DAB2-PTB delayed and inhibited TPA-, collagen-, U46619- and TRAP-stimulated platelet aggregation (Fig. 6A). By

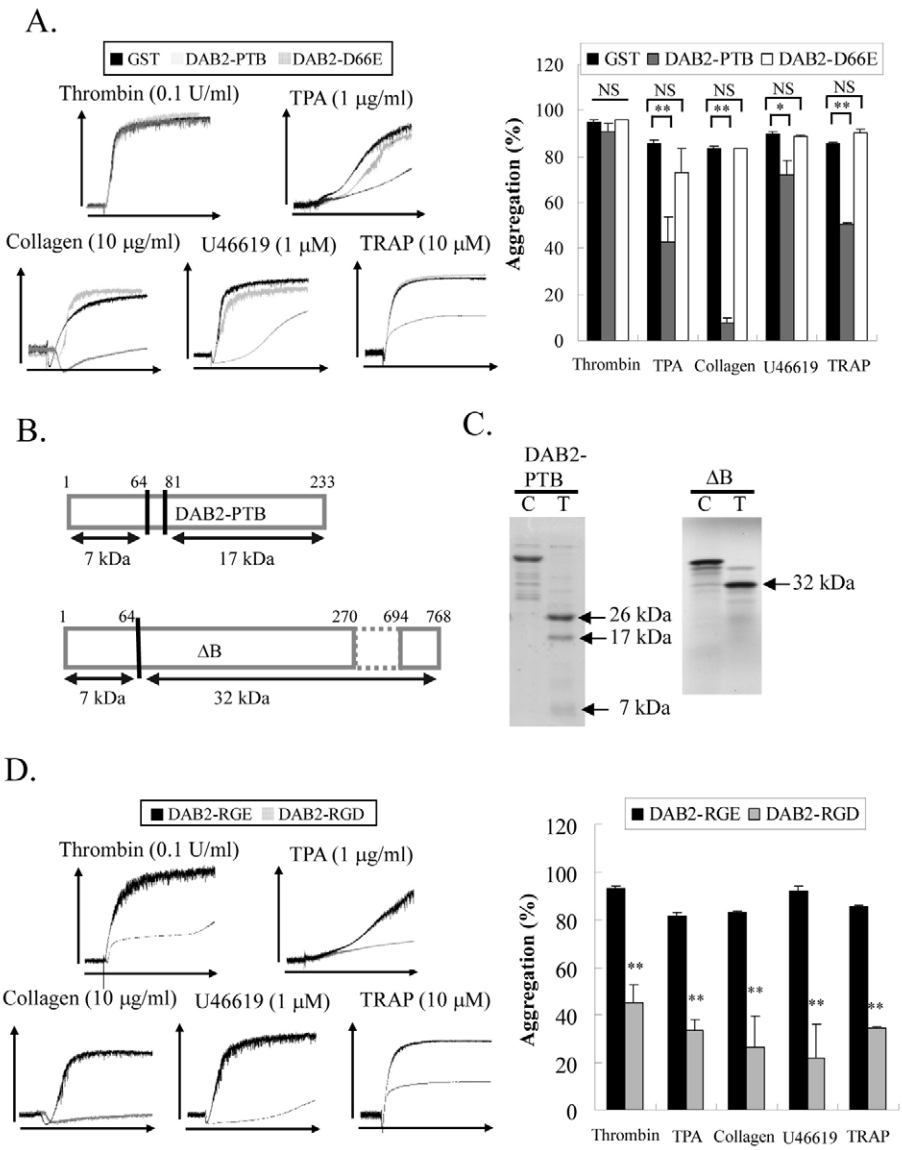

Fig. 6. The RGD motif is involved in DAB2-mediated inhibition of agonist-induced platelet aggregation. (A) DAB2 selectively inhibits agonist-induced platelet aggregation. Platelet aggregation was induced by the indicated concentration of agonists in the presence of GST, DAB2-PTB or DAB2-D66E (100 μg/ml). The platelet aggregation curves (left panel) and the percent of aggregation (right panel) were obtained and quantified by using a platelet aggregometer. (B) Schematic representation of the putative thrombin-cleavage sites in human DAB2-PTB and rat ΔB. The putative thrombin-cleavage sites and the size of the cleavage product for human DAB2-PTB and rat ΔB were shown. (C) Thrombin is a DAB2 protease. The recombinant human DAB2-PTB and in vitro transcription- and translation-derived [S^{35}]-methionine-labeled rat ΔB were incubated in the absence (C) or presence (T) of thrombin (10 U/ml). Aliquots of the reaction mixtures were fractionated on SDS-PAGE and were visualized by Commassie Blue staining (DAB2-PTB) or autoradiography (ΔB). (D) The DAB2-RGD peptide inhibits agonist-induced platelet aggregation. Platelet aggregation was induced by agonists at indicated concentration in the presence of DAB2-RGD or DAB2-RGE peptides (100 μg/ml). The platelet aggregation curves (left panel) and the percent of aggregation (right panel) was obtained and quantified by using a platelet aggregometer. The data represent the mean ± s.d. of three to four experiments. *P<0.05 and **P<0.001, compared with platelet aggregation induced by the same agonist in the presence of control GST protein (A) or DAB2-RGE (D). NS, not significant.

contrast, DAB2 was relatively ineffective on thrombin-mediated platelet aggregation. To explain this distinct feature of DAB2, we analyzed human DAB2 protein sequences and found three putative thrombin cleavage sites in the DAB2-PTB recombinant protein; two within the DAB2-PTB domain (at aa 64 and 81) and one at the site of fusion between GST and DAB2 (Fig. 6B). In an in vitro cleavage study, thrombin (10 U/ml) cleaved DAB2-PTB and, as predicted, generated cleavage protein products of approximately 7 kDa, 17 kDa and 26 kDa (Fig. 6C). Under conditions of platelet aggregation, thrombin (0.1 U/ml) partially cleaved DAB2-PTB within 10 minutes, with complete cleavage after extended incubation time (16 hours, data not shown). Similarly, a rat p82-DAB2 mutant ΔB with a deletion of the majority of the C-terminus corresponding to the rat DAB2 PTB (Tseng et al., 1999) also contained a putative thrombin cleavage site (aa 64) overlapping the RGD motif and was cleaved by thrombin in vitro to generate a 32-kDa cleavage product (Fig. 6C). Hence, thrombin cleavage of DAB2 might result in the loss of DAB2 inhibitory activity on platelet aggregation. These data indicate that the RGD motif of DAB2 is potent in the regulation of platelet aggregation. In addition, thrombin cleavage of DAB2 might result in the decrease of DAB2 binding to the platelet surface (Fig. 2A) and the loss of DAB2 inhibitory activity on thrombin-induced platelet aggregation.

To define the role of the DAB2 RGD motif in the regulation of platelet aggregation, DAB2-D66E recombinant protein encoding DAB2 RGD mutant was added to the platelet aggregation assay mixture. Unlike DAB2-PTB, DAB2-D66E did not inhibit agonist-induced platelet aggregation (Fig. 6A). To further demonstrate the involvement of the DAB2 RGD motif in the regulation of platelet aggregation, the DAB2-RGD peptide (100 μg/ml) was added to the assay mixture. We found that DAB2-RGD peptide inhibited platelet aggregation induced by the soluble agonists (Fig. 6D). The inhibition of thrombin-induced platelet aggregation by the DAB2-RGD peptide might be due to the high concentration of peptide that can outcompete the proteolytic effect of thrombin on DAB2. The inhibitory effect on platelet aggregation was not observed when DAB2-RGE peptide was used in the assay. The DAB2 RGD motif is

thus important for the anti-platelet-aggregation-activity of DAB2.

DAB2 inhibits the interaction between platelet and/or megakaryocytic cells and fibrinogen

Platelet aggregation involves the interaction between platelets and fibrinogen. Our data indicate that DAB2 and fibrinogen share a common binding region in αIIb integrin of platelets. Whether or not the soluble and extracellular form of DAB2 competes and disrupts the platelet-fibrinogen interaction was examined in cell adhesion assays using immobilized fibrinogen (Fig. 7A). Upon activation by TRAP, platelet adhesion to fibrinogen was increased approximately threefold. The increase in platelet adhesion was partially inhibited when DAB2-PTB was present in the assay mixture (Fig. 7A). This effect was not observed in the presence of GST or DAB2-D66E. Consistent with these observations, the DAB2-RGD wild-type but not the DAB2-RGE mutant peptide also competed for fibrinogen adhesion of platelets (Fig. 7B). We have also analyzed fibrinogen interaction in K562 cells in cell adhesion assays. Cultured K562 cells had little fibrinogen adhesive activity. Upon TPA-induced megakaryocytic differentiation, adhesion and/or binding of K562 cells to fibrinogen was increased significantly (Fig. 7C). The fibrinogen interaction of TPA-treated cells was diminished in a dose-dependent manner when the DAB2-RGD peptide was present in the assay medium (Fig. 7C,D). By contrast, DAB2-RGE peptide had no effect on the interaction of cells with fibrinogen. These results indicate that DAB2 can competitively interfere with the platelet- and/or megakaryocyte-fibrinogen interaction through the RGD motif.

Discussion

DAB2 is a multifunctional protein involved in many biological processes and signaling pathways. Most of the functions attributed to DAB2 are related to its nature as a cytoplasmic adapter protein; it modulates epithelial-to-mesenchymal transition (Prunier and Howe, 2005), cytoskeleton organization, gene transcriptional activity and cell signaling of various receptor protein tyrosine kinases. DAB2 is also a component of the trafficking vehicle and regulates receptor-mediated endocytosis (Morris and Cooper, 2001); it drives clathrin-coat assembly by synchronizing cargo selection and displays the properties of a cargo-specific adapter protein (Mishra et al., 2002). In this study, several new aspects of DAB2 function in platelets are reported (Fig. 8). In addition to being an intracellular adapter protein, platelet DAB2 is distributed abundantly in the platelet α-granules and is released in a PKC-dependent manner during platelet activation. Using megakaryocytic differentiating K562 cells, we have previously reported that DAB2 interacts with the cytoplasmic tail of β3 integrin and negatively regulates αIIbβ3 integrin activation as well as its downstream signaling (Huang et al., 2004). Here, we further show that DAB2 secreted by platelets can bind to the platelet and megakaryocytic cell surface through αIIbβ3 integrin, and interfere with platelet-fibrinogen interactions, thereby demonstrating for the first time that DAB2 is a negative regulator in platelet aggregation. Whether or not DAB2 secretion during platelet activation plays some physiological role is subjected to further investigation. Accordingly, a major focus for our future work is to develop means to measure the DAB2 concentration in plasma and to determine whether or not DAB2 constitutes a variable factor that affects the magnitude of platelet aggregation and thrombus formation in vivo.

Platelet α-granules represent a unique storage compartment that stores both endogenously synthesized proteins from

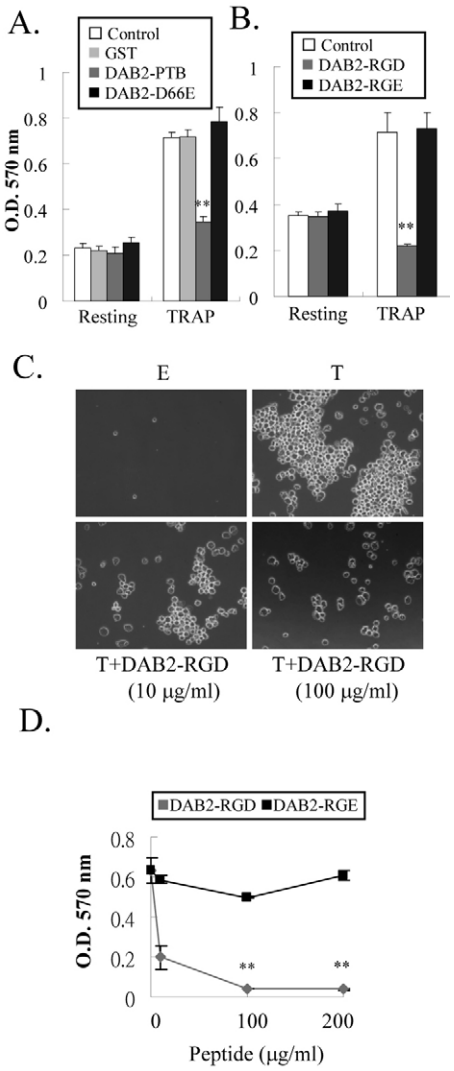

Fig. 7. DAB2 inhibits the interaction between platelet and/or megakaryocytic cells and fibrinogen. (A,B) The RGD motif is involved in DAB2-mediated inhibition of TRAP-stimulated platelet adhesion to fibrinogen. Resting and TRAP-stimulated (10 μM) platelets were added to plates pre-coated with fibrinogen (10 μg) in the absence (control) or presence of soluble GST, DAB2-PTB and DAB2-D66E (100 μg/ml) (A), or DAB2-RGD and DAB2-RGE peptides (100 μg/ml) (B) for 3 hours at 37°C. Platelet adhesion with fibrinogen was quantified in Crystal Violet assays. (C,D) Dose-dependent inhibition of K562 cell adhesion to fibrinogen by DAB2-RGD peptide. Ethanol-treated (E) or TPA-treated (T, 10 ng/ml) K562 cells were added to plates pre-coated with fibrinogen (10 μg) in the presence of the indicated concentration of DAB2-RGD or DAB2-RGE peptides. K562-cell adhesion was observed by inverted microscopy (C, 100×). Cell adhesion of TPA-treated cells was also quantified in Crystal Violet assays (D). Data represent the mean ± s.d. (n=4). **P<0.001 compared with platelets stimulated with TRAP only (A and B) or TPA-treated cells in the absence of DAB2 peptide (D).

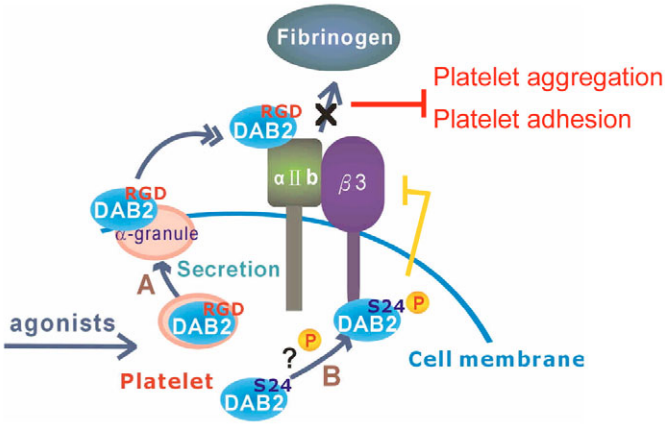

Fig. 8. Model for the effects of platelet DAB2 on platelet aggregation. DAB2 is secreted from platelets upon agonist-induced platelet aggregation. Secreted DAB2 binds to the extracellular region of αIIb integrin and interferes with the αIIbβ3-integrin–fibrinogen interaction that results in the inhibition of platelet aggregation. Whether or not Ser24 phosphorylation of platelet DAB2 plays a role during platelet activation and aggregation still needs to be established.

megakaryocytes, the platelet precursor cells, as well as proteins exclusively recruited from plasma by receptor-mediated endocytosis and correct intracellular targeting. Our studies revealed that DAB2 expression is induced and closely associated with megakaryocytic maturation of K562 and $CD34^+$ hematopoietic pluripotent progenitor cells (Tseng et al., 2003; Huang et al., 2004), suggesting that platelet DAB2 probably derived from protein biosynthesis in mature megakaryocytes. During platelet formation, the α-granule represents a post-multivesicular body in the endocytic pathway with αIIbβ3-integrin-mediated endocytosis as an important route for the delivery and uptake of functional proteins such as fibrinogen (Heijnen et al., 1998). Upon receptor internalization, DAB2 is widely recognized as an adaptor protein that binds to the NPxY motif of various receptors, including αIIbβ3 integrin, and is present in the trafficking vehicles (Calderwood et al., 2003; Maurer and Cooper, 2005). Accordingly, cytosolic DAB2 is likely to be accompanied by other functional proteins and is probably delivered into α-granule cells through vesicular trafficking during the biogenesis and maturation of α-granules. This notion might also explain the observations that DAB2 is located in both cytoplasm and the α-granule compartment of platelet.

Platelet aggregation can usually be interpreted by measuring aggregation amplitude, which represents the difference between baseline (where the agonist was added) and the point at which maximum aggregation was achieved. In addition, curve type, smoothness and aggregation stability also provide insight to the extent of platelet aggregation (Cornell and Randolph, 2002). We noted that the platelet aggregation curve is usually more intense with a jagged aggregation curve for thrombin-, collagen- and U46619-stimulated platelets, whereas the platelet aggregation is either delayed or less intense for TPA- and TRAP-stimulated platelets. Interestingly, relatively high DAB2 binding to the platelet surface was observed for TPA- and TRAP-stimulated platelets. In addition, the aggregation curve is usually smooth when recombinant DAB2 was added into the assay mixture during platelet aggregation. Based on the association between the amount of DAB2 binding to platelets and the shape of platelet aggregation curve, we postulate that DAB2, in addition to other cellular factors such as fibrinogen, is probably involved in regulating the magnitude of platelet aggregates. The fact that DAB2 competes with fibrinogen binding to αIIb integrin further provides evidence for this notion and it is worth further analysis.

The molecular basis for DAB2 binding to the platelet surface and acting as a negative regulator of platelet aggregation was also investigated in this study. We demonstrated that the binding of DAB2, either released endogenously or added exogenously, to the platelet surface was inhibited by the RGDS peptide and the mAbs that functionally block αIIbβ3 integrin but not αvβ3 integrin. According to the well-known style of binding of these mAbs and the peptide (Coller et al., 1986; Cheresh and Spiro, 1987), we conclude that DAB2 interacts specifically with the extracellular region of αIIbβ3 integrin. It has been shown by mutational analysis that the DAB2 RGD motif that is conserved in several mammalian species is crucial for DAB2 binding the αIIb integrin aa sequence at position 171-464, which overlaps with the fibrinogen γ-chain binding site (Gulino et al., 1992; Taylor and Gartner, 1992). DAB2 is thus considered as an endogenous αIIbβ3 integrin fibrinogen-receptor antagonist and is added to the list of endogenous substances and proteins that interfere with platelet aggregation, such as nitric oxide (Emerson et al., 1999), N-acetyl-L-cysteine (Anfossi et al., 2001), high-molecular weight kininogen (Chavakis et al., 2002) and semaphorin 3A (Kashiwagi et al., 2005).

Although both DAB2 and fibrinogen binds to αIIb integrin, they follow a different binding model and have distinct effects on platelet aggregation. First, DAB2–αIIbβ3-integrin interaction is Ca^{2+} independent; binding was still significant, even in the presence of EDTA. This is different to fibrinogen, because divalent cations are required for fibrinogen binding to αIIbβ3 integrin (Loftus et al., 1994). Second, αIIbβ3 integrin resides in cell membranes at a thermodynamic equilibrium of resting, active and clustered states (Li et al., 2004). Platelets adhere to immobilized fibrinogen without activation of αIIbβ3 integrin, whereas the binding to soluble fibrinogen depends on αIIbβ3-integrin activation. The experimental evidence presented here, indicates that the binding of immobilized and soluble DAB2 to platelets occurs only when αIIbβ3 integrin is activated by soluble agonists. For resting platelets, no interaction was observed between DAB2 and αIIbβ3 integrin. Although the binding of DAB2 to CHO cells stably expressing αIIbβ3 integrin (which presumably is in the resting state) seems to contradict our hypothesis, we can not rule out that a portion of the αIIbβ3 integrin in these cells is actived and can therefore interact with DAB2. Hence, we conclude that DAB2 is a αIIb-integrin-binding protein whose binding mode depends on the activation of αIIbβ3 integrin and is different from fibrinogen. In summary, we describe for the first time that DAB2 is a αIIb-integrin-binding protein that is secreted during platelet activation and acts as a negative regulator for platelet-to-fibrinogen binding and platelet aggregation. These findings contribute to our understanding of DAB2 in platelet function and the mechanisms involved in platelet aggregation.

Materials and Methods

Materials

The CHO-αIIbβ3-integrin cells were a kind gift from Yoshikazu Takada (The Scripps Research Institute, CA). Monoclonal antibodies (mAbs) 7E3 and 10E5 were a kind gift from Berry S. Coller (Rockfeller University, NY). Anti-p96 antibody was purchased from Transduction Laboratory (San Jose, CA). Anti-P-selectin antibody was purchased from R&D Systems (Minneapolis, MN). Anti-platelet factor 4 (PF4), anti-DAB2 (H-110) and fluorescein isothiocyanate (FITC)-conjugated anti-GST and goat anti-mouse antibodies were from Santa Cruz Biotechnologies (Santa Cruz, CA). The mAb LM609 was from Chemicon (Temecula, CA). The synthetic peptides of DAB2-RGD and DAB2-RGE were from MDBio, Inc. (Taipei, Taiwan). Collagen (type I) was purified from bovine Achilles tendon and homogenized in 25 mM acetic acid. The pan protein kinase C (PKC) inhibitor Ro-31-8220 was purchased from CalBiochem (San Diego, CA). The fibrinogen, TPA, U46619, thrombin, and the RGDS and TRAP (SFLLRN) peptides were purchased from Sigma (St Louis, MO). The LR White and 10-nm colloidal-gold-conjugated protein IgG were purchased from Electron Microscopy Sciences (Fort Washington, PA).

Plasmids

The αIIb integrin expression plasmid was a kind gift of Mark H. Ginsberg (The Scripps Research Institute, CA). The β3 integrin expression plasmid was a kind gift of Erkki Ruoslahti (The Burnham Institute, CA). The design and construction methods of pGEX4T-DAB2-PTB, DAB2-M, DAB2-D66E, αIIb integrin, and β3 integrin mutants are available upon request.

Sucrose-gradient assay for subcellular fractionation of platelet

Peripheral blood was drawn from healthy, drug-free volunteers. Platelets were isolated and fractionated as described previously (Huang et al., 2004; Mairhofer et al., 2002). The washed platelets (2×10^9) were homogenized in a Thermo French pressure cell (150 psi) and the platelet lysate was centrifuged (2000 ***g*** for 10 minutes at 4°C) to obtain pellets of unhomogenized platelet. The supernatant was laid on top of a linear sucrose-density gradient (30-60% sucrose) and centrifuged at 200,000 ***g*** for 2 hours. Eighteen fractions (700 μl each) were collected from the top of the gradient and subjected to western blot analysis.

Immunofluorescence staining and immunoelectron microscopy analysis

For immunofluorescence staining, washed platelets were cytospun on a glass coverslip, fixed with 3.7% formaldehyde solution at 37°C for 15 minutes, and permeabilized with 0.1% Triton X-100 at 4°C for 10 minutes. After several washes with 1× phosphate-buffered saline (PBS), fixed cells were blocked with 5% bovine serum albumin (BSA) and incubated with anti-p96 and anti-PF4 antibodies (1:50) at 4°C overnight. Platelets were washed three times with 1× PBS and incubated with FITC-conjugated goat anti-mouse (for p96) and Rodamine-conjugated donkey anti-goat (for PF4) secondary antibodies (1:200) at room temperature for 1 hour. Finally, the cells were mounted and observed using confocal microscopy. As a negative control, platelets were incubated with the secondary antibody only upon which no signal was detected.

For immunoelectron microscopy, washed platelets were fixed for 1 hour at 4°C in 0.1% glutaraldehyde, washed, embedded with LR White medium resin on grids and sectioned. The sections were preincubated with 1× PBS, containing 5% BSA for blocking of non-specific binding sites, followed by incubation with the anti-p96 antibody at 4°C overnight. Grids were rinsed three times, incubated with 10-nm colloidal-gold-conjugated protein G (1:40) in PBS with 1% BSA. Sections were then counterstained with 3% uranyl acetate and lead citrate and examined with a JEM-1230 transmission electron microscope (JEOL Ltd., Tokyo, Japan). As a negative control, platelets were incubated with the colloidal-gold-conjugated protein G without primary antibody upon which no signal was detected.

DAB2-binding assay and functional competition with integrin-blocking antibodies

For analysis of DAB2 binding to secreted platelets, platelets were incubated with the anti-DAB2 (p96 or H-110) antibody (1:100) without fixation and permeabilization, followed by incubation with the FITC-conjugated secondary antibody. To analyze binding of recombinant DAB2, platelets or culture cells were incubated with the indicated recombinant DAB2 protein at room temperature for 3 hours. Cells were washed three times with 1× PBS and incubated with FITC-conjugated anti-GST antibody (1:200) at room temperature for 2 hours. After several washes with 1× PBS, DAB2 binding was analyzed by flow cytometry or confocal microscopy.

To assess the effects of the functionally blocking mAbs and the RGDS peptide on DAB2 binding, platelets or CHO cells were preincubated with 7E3 (αVβ3-integrin- and αIIbβ3-integrin-specific), 10E5 (αIIbβ3-integrin-specific), LM609 (αVβ3integrin-specific) (20 μg/ml) or RGDS peptide (100 μM) for 30 minutes at room temperature. DAB2-binding assays were then performed as described above.

Platelet and K562 cell adhesion assay

The assay for cell adhesion of K562 cells to fibrinogen was performed as described previously (Huang et al., 2004). To assay for platelet adhesion, 3×10^8 platelets per well in 500 μl 0.5% BSA-PBS were added to a 24-well non-tissue culture plate pre-coated with fibrinogen (10 μg) or recombinant DAB2 (100 μg) and blocked with 5% BSA. After a 3-hour incubation at 37°C in the presence or absence of platelet agonist, plates were washed with 2 ml 0.5% BSA-PBS buffer for three times. The adhesive platelets were then fixed with 1% glutaraldehyde and stained with 0.5% Crystal Violet. Finally, the dye was eluted with Sorenson's buffer (Tseng et al., 1998) and the OD was measured at 570 nm. For quantification, adhesion to untreated plates was measured and substracted from the adhesion to fibrinogen and/or recombinant proteins.

GST pull-down assay

The assay was performed as previously described (Huang et al., 2004). An excessive amount of glutathione (20 mM) was added to elute the GST-fusion protein and any associated protein from the beads, and subjected to western blot analysis.

Platelet aggregation assay

Washed platelets were adjusted to a concentration of 3×10^8 platelets/ml with Tyrode's buffer and maintained at 37°C for 1 minute while stirring. Platelet aggregation was initiated by addition of agonists, and light transmission was monitored using a platelet aggregometer (Payton, Stouffville, ON, Canada) connected to the PowerLab data acquisition and recording system (ADInstrument, Castle Hill, NSW, Australia).

In vitro transcription and translation, and thrombin-cleavage analysis

In vitro transcription and translation was performed using the TNT-coupled reticulocyte-lysate system as described by the manufacturer (Promega). One aliquot (20 μl) of [^{35}S]-methionine-labeled translational products was mixed with thrombin protease (10 U) at room temperature for 16 hours and was fractionated on a SDS-PAGE for autoradiography. For thrombin cleavage of DAB2-PTB (~25 μg), the reaction was performed in 1× thrombin-cleavage buffer (20 mM Tris-HCl pH 8.4, 150 mM NaCl, and 2.5 mM $CaCl_2$) at room temperature for 16 hours. The cleavage products were resolved on a SDS-PAGE and were detected by Coomassie Blue staining.

Statistical analysis

Student's *t*-test was used for statistical analysis. $P<0.05$ was considered statistically significant.

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References

Anfossi, G., Russo, I., Massucco, P., Mattiello, L., Cavalot, F. and Trovati, M. (2001). N-acetyl-L-cysteine exerts direct anti-aggregating effect on human platelets. *Eur. J. Clin. Invest.* **31**, 452-461.

Boucharaba, A., Serre, C.-M., Gres, S., Saulnier-Blache, J. S., Bordet, J.-C., Guglielmi, J., Clezardin, P. and Peyruchaud, O. (2004). Platelet-derived lysophosphatidic acid supports the progression of osteolytic bone metastases in breast cancer. *J. Clin. Invest.* **114**, 1714-1725.

Calderwood, D. A. (2004). Integrin activation. *J. Cell Sci.* **117**, 657-666.

Calderwood, D. A., Fujioka, Y., de Pereda, J. M., Garcia-Alvarez, B., Nakamoto, T., Margolis, B., McGlade, C. J., Liddington, R. C. and Ginsberg, M. H. (2003). Integrin β cytoplasmic domain interactions with phosphotyrosine-binding domains: a structural prototype for diversity in integrin signaling. *Proc. Natl. Acad. Sci. USA* **100**, 2272-2277.

Charo, I. F., Nannizzi, L., Phillips, D. R., Hsu, M. A. and Scarborough, R. M. (1991). Inhibition of fibrinogen binding to GPIIb-IIIa by a GPIIIa peptide. *J. Biol. Chem.* **266**, 1415-1421.

Chavakis, T., Boeckel, N., Santoso, S., Voss, R., Isordia-Salas, I., Pixley, R. A., Morgenstern, E., Colman, R. W. and Preissner, K. T. (2002). Inhibition of platelet adhesion and aggregation by a defined region (Gly-486–Lys-502) of high molecular weight kininogen. *J. Biol. Chem.* **277**, 23157-23164.

Cheresh, D. A. and Spiro, R. C. (1987). Biosynthetic and functional properties of an Arg-Gly-Asp-directed receptor involved in human melanoma cell attachment to vitronectin, fibrinogen and von Willebrand factor. *J. Biol. Chem.* **262**, 17703-17711.

Coller, B. S., Peerschke, E. I., Seligsohn, U., Scudder, L. E., Nurden, A. T. and Rosa, J. P. (1986). Studies on the binding of an alloimmune and two murine monoclonal antibodies to the platelet glycoprotein IIb-IIIa complex receptor. *J. Lab. Clin. Med.* **107**, 384-392.

Cornell, R. F. and Randolph, T. R. (2002). The minimum concentration of fibrinogen needed for platelet aggregation using ADP. *Clin. Lab. Sci.* **15**, 30-36.

D'Souza, S. E., Ginsberg, M. H., Lam, S. C. T. and Plow, E. F. (1988). Chemical cross-linking of arginyl-glycyl-aspartic acid peptides to an adhesion receptor on platelets. *J. Biol. Chem.* **263**, 3943-3951.

Emerson, M., Momi, S., Paul, W., Alberti, P. F., Page, C. and Gresele, P. (1999). Endogenous nitric oxide acts as a natural antithrombotic agent in vivo by inhibiting platelet aggregation in the pulmonary vasculature. *Thromb. Haemost.* **81**, 961-966.
George, J. N. (2000). Platelets. *Lancet* **355**, 1531-1539.
Gulino, D., Boudignon, C., Zhang, L. Y., Concord, E., Rabiet, M. J. and Marguerie, G. (1992). Ca(2+)-binding properties of the platelet glycoprotein IIb ligand-interacting domain. *J. Biol. Chem.* **267**, 1001-1007.
Gupta, G. P. and Massague, J. (2004). Platelets and metastasis revisited: a novel fatty link. *J. Clin. Invest.* **114**, 1691-1693.
Heijnen, H. F., Debili, N., Vainchencker, W., Breton-Gorius, J., Geuze, H. J. and Sixma, J. J. (1998). Multivesicular bodies are an intermediate stage in the formation of platelet alpha-granules. *Blood* **91**, 2313-2325.
Hocevar, B. A., Smine, A., Xu, X. X. and Howe, P. H. (2001). The adaptor molecule Disabled-2 links the transforming growth factor β receptors to the Smad pathway. *EMBO J.* **20**, 2789-2801.
Hocevar, B. A., Mou, F., Rennolds, J. L., Morris, S. M., Cooper, J. A. and Howe, P. H. (2003). Regulation of the Wnt signaling pathway by disabled-2 (Dab2). *EMBO J.* **22**, 3084-3094.
Huang, C. L., Cheng, J. C., Liao, C. H., Stern, A., Hsieh, J. T., Wang, C. H., Hsu, H. L. and Tseng, C. P. (2004). Disabled-2 is a negative regulator of integrin αIIbβ3-mediated fibrinogen adhesion and cell signaling. *J. Biol. Chem.* **279**, 42279-42289.
Inoue, A., Sato, O., Homma, K. and Ikebe, M. (2002). DOC-2/DAB2 is the binding partner of myosin VI. *Biochem. Biophys. Res. Commun.* **292**, 300-307.
Kashiwagi, H., Shiraga, M., Kato, H., Kamae, T., Yamamoto, N., Tadokoro, S., Kurata, Y., Tomiyama, Y. and Kanakura, Y. (2005). Negative regulation of platelet function by a secreted cell repulsive protein, semaphorin 3A. *Blood* **106**, 913-921.
Kloczewiak, M., Timmons, S. and Hawiger, J. (1982). Localization of a site interacting with human platelet receptor on carboxy-terminal segment of human fibrinogen gamma chain. *Biochem. Biophys. Res. Commun.* **107**, 181-187.
Kowanetz, K., Terzic, J. and Dikic, I. (2003). Dab2 links CIN85 with clathrin-mediated receptor internalization. *FEBS Lett.* **554**, 81-87.
Li, R., Bennett, J. S. and DeGrado, W. F. (2004). Structural basis for integrin αIIbβ3 clustering. *Biochem. Soc. Trans.* **32**, 412-415.
Loftus, J. C., Smith, J. W. and Ginsberg, M. H. (1994). Integrin-mediated cell adhesion: the extracellular face. *J. Biol. Chem.* **269**, 25235-25238.
London, F. S. (2003). The protein kinase C inhibitor Ro318220 potentiates thrombin-stimulated platelet-supported prothrombinase activity. *Blood* **102**, 2472-2481.
Mairhofer, M., Steiner, M., Mosgoeller, W., Prohaska, R. and Salzer, U. (2002). Stomatin is a major lipid-raft component of platelet α-granules. *Blood* **100**, 897-904.
Maurer, M. E. and Cooper, J. A. (2005). Endocytosis of megalin by visceral endoderm cells requires the Dab2 adaptor protein. *J. Cell Sci.* **118**, 5345-5355.
Mishra, S. K., Keyel, P. A., Hawryluk, M. J., Agostinelli, N. R., Watkins, S. C. and Traub, L. M. (2002). Disabled-2 exhibits the properties of a cargo-selective endocytic clathrin adaptor. *EMBO J.* **21**, 4915-4926.
Morris, S. M. and Cooper, J. A. (2001). Disabled-2 colocalizes with the LDLR in clathrin-coated pits and interacts with AP-2. *Traffic* **2**, 111-123.
Morris, S. M., Arden, S. D., Roberts, R. C., Kendrick-Jones, J., Cooper, J. A., Luzio, J. P. and Buss, F. (2002). Myosin VI binds to and localizes with Dab2, potentially linking receptor-mediated endocytosis and the actin cytoskeleton. *Traffic* **3**, 331-341.
Plow, E. F., Pierschbacher, M. D., Ruoslahti, E., Marguerie, G. A. and Ginsberg, M. H. (1985). The effect of Arg-Gly-Asp-containing peptides on fibrinogen and von Willebrand factor binding to platelets. *Proc. Natl. Acad. Sci. USA* **82**, 8057-8061.
Prunier, C. and Howe, P. H. (2005). Disabled-2 (Dab2) is required for transforming growth factor betw-induced epithelial to mesenchymal transition (EMT). *J. Biol. Chem.* **280**, 17540-17548.
Quinn, M. J., Byzova, T. V., Qin, J., Topol, E. J. and Plow, E. F. (2003). Integrin αIIbβ3 and its antagonism. *Arterioscler. Thromb. Vasc. Biol.* **23**, 945-952.
Savage, B., Saldivar, E. and Ruggeri, Z. M. (1996). Initiation of platelet adhesion by arrest onto fibrinogen or translocation on vonWillebrand factor. *Cell* **84**, 289-297.
Taylor, D. B. and Gartner, T. K. (1992). A peptide corresponding to GPIIb alpha 300-312, a presumptive fibrinogen gamma-chain binding site on the platelet integrin GPIIb/IIIa, inhibits the adhesion of platelets to at least four adhesive ligands. *J. Biol. Chem.* **267**, 11729-11733.
Trikha, M. and Nakada, M. T. (2002). Platelets and cancer: implications for antiangiogenic therapy. *Semin. Thromb. Hemost.* **28**, 39-44.
Tseng, C. P., Ely, B. D., Li, Y., Pong, R. C. and Hsieh, J. T. (1998). Regulation of rat DOC-2 gene during castration-induced rat ventral prostate degeneration and its growth inhibitory function in human prostatic carcinoma cells. *Endocrinology* **139**, 3542-3553.
Tseng, C. P., Ely, B. D., Pong, R. C., Wang, Z., Zhou, J. and Hsieh, J. T. (1999). The role of DOC-2/DAB2 protein phosphorylation in the inhibition of AP-1 activity. An underlying mechanism of its tumor-suppressive function in prostate cancer. *J. Biol. Chem.* **274**, 31981-31986.
Tseng, C. P., Huang, C. H., Tseng, C. C., Lin, M. H., Hsieh, J. T. and Tseng, C. H. (2001). Induction of disabled-2 gene during megakaryocyte differentiation of k562 cells. *Biochem. Biophys. Res. Commun.* **285**, 129-135.
Tseng, C. P., Huang, C. L., Huang, C. H., Stern, A., Cheng, J. C., Tseng, C. H. and Chiu, D. T. Y. (2003). Disabled-2 small interfering RNA modulates cellular adhesive function and MAPK activity during megakaryocytic differentiation of K562 cells. *FEBS Lett.* **541**, 21-27.
Tseng, C. P., Chang, P., Huang, C. L., Cheng, J. C. and Chang, S. S. (2005). Autocrine signaling of platelet-derived growth factor regulates disabled-2 expression during megakaryocytic differentiation of K562 cells. *FEBS Lett.* **579**, 4395-4401.
Wang, Z., Tseng, C. P., Pong, R. C., Chen, H., McConnell, J. D., Navone, N. and Hsieh, J. T. (2002). The mechanism of growth-inhibitory effect of DOC-2/DAB2 in prostate cancer. Characterization of a novel GTPase-activating protein associated with N-terminal domain of DOC-2/DAB2. *J. Biol. Chem.* **277**, 12622-12631.
Xu, X. X., Yi, T., Tang, B. and Lambeth, J. D. (1998). Disabled-2 (Dab2) is an SH3 domain-binding partner of Grb2. *Oncogene* **16**, 1561-1569.
Zhou, J., Scholes, J. and Hsieh, J. T. (2003). Characterization of a novel negative regulator (DOC-2/DAB2) of c-Src in normal prostatic epithelium and cancer. *J. Biol. Chem.* **278**, 6939-6941.