Disabled-2 (DAB2) is an adapter protein that is up-regulated during megakaryocytic differentiation of hematopoietic cells and is abundantly expressed in platelets. In this study, the role of DAB2 in integrin αIIbβ3-mediated matrix protein fibrinogen adhesion and cell signaling was investigated. In K562 cells differentiating to the megakaryocytic lineage, down-regulation of DAB2 by DAB2 small interfering RNA augmented integrin αIIbβ3 activation and resulted in an increase in cell adhesion to fibrinogen. Ectopic expression of DAB2 reversed the DAB2 small interfering RNA effect or, by itself, decreased fibrinogen adhesion of K562 cells. Mutational analysis revealed that a DAB2 Ser24 phosphorylation mutant (S24A) abrogated the inhibitory function of DAB2. The spatial and temporal association/interaction of DAB2 and platelet integrin αIIbβ3 (CD61) in both megakaryocytic cells and platelets led us to examine the effect of Ser24 phosphorylation on the interaction between DAB2 and integrin β3. Through cellular localization and co-immunoprecipitation analysis, we demonstrate for the first time that Ser24 phosphorylation promotes membrane translocation of DAB2 and its subsequent interaction with integrin β3 thereby defining a mechanism for DAB2 in regulating integrin αIIbβ3 activation and inside-out signaling. Consistent with the effect on fibrinogen adhesion, Ser24 phosphorylation of DAB2 was also involved in the negative regulation of αIIbβ3-induced T cell factor transcriptional activity. In contrast, the S24A mutant acted like wild-type DAB2 and inhibited both β-catenin- and plakoglobin-mediated T cell factor transactivation. Hence, DAB2 elicits distinct regulatory mechanisms in αIIbβ3 and β-catenin/plakoglobin signaling in a Ser24 phosphorylation-dependent and -independent manner, respectively. These findings indicate Ser24 phosphorylation as a molecular basis for DAB2 acting as a negative regulator in αIIbβ3 inside-out signaling and contribute to our understanding of DAB2 in megakaryocytic differentiation and platelet function.

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Disabled-2 (DAB2) is an adapter protein that has been implicated in growth factor signaling (1, 2), endocytosis (3–5), cell adhesion function (6, 7), and hematopoietic cell differentiation (8). Like other adapter proteins, DAB2 elicits its function through interaction with other cellular proteins. DAB2 interacts with Grb2, myosin VI, SMAD2/3, DIP1/2, Dvl-3, the integrin β subunit, and c-Src through the N-terminal phosphotyrosine-binding (PTB) domain and the C-terminal proline-rich region (1, 4, 5, 9–13). These interactions have been shown to modulate cytoskeleton organization, transcriptional activity, and cell signaling of various receptor protein-tyrosine kinases. DAB2 thus plays in a pivotal role in the control of cellular homeostasis.

In cells, protein phosphorylation of DAB2 modulates its functional activity during growth factor signaling, megakaryocytic differentiation, macrophage spreading, and cell cycle progression. To date, protein kinase C (PKC) and Cdc2 are the only two known DAB2 kinases (14, 15). The major PKC phosphorylation site has been mapped to Ser24. This phosphorylation site is essential for the inhibitory function of DAB2 in 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced AP-1 gene transcription (14). In addition, the cell cycle-dependent phosphorylation of DAB2 by Cdc2 promotes the association of DAB2 with Pin1, a peptidylprolyl isomerase that regulates the rate of DAB2 dephosphorylation. These findings indicate that DAB2 phosphorylation is important as a regulator of cell proliferation and differentiation.

An appropriate level of DAB2 expression is necessary for maintaining normal cellular and physiological function. In tumors, abnormal expression of DAB2 has been reported in ovarian, breast, prostate, colon, and metastatic pancreatic cancers (16–19). Homozygotic disruption of DAB2 by knockout experiments demonstrates that DAB2 is essential for embryonic development and kidney transport (20, 21). The up-regulation of DAB2 during megakaryocytic differentiation of several hematopoietic leukemic cell lines, including K562, HEL, and MEG-01, has been observed in recent studies in our laboratory; these results indicate a potential function of DAB2 in hematopoietic cell differentiation (8). DAB2 has also been shown to play a role in modulating cell adhesion and the kinetics of MAPK activation using vector-based RNA interference methodology (7). Among the adhesion-related molecules, integrins are a family

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†† The abbreviations used are: PTB, phosphotyrosine-binding; PKC, protein kinase C; TPA, 12-O-tetradecanoylphorbol-13-acetate; MAPK, mitogen-activated protein kinase; TCF, T cell factor; GST, glutathione S-transferase; HCV, hepatitis C virus; siDAB2, DAB2 small interfering RNA; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid.
of major receptors involved in cell adhesion to extracellular matrix proteins and cell-cell adhesion (22–24). In mammalian cells, all integrins are heterodimers composed of 18 a and 8 b subunits noncovalently linked (23). It is clear that integrins bind to other cell-surface receptors to activate a large variety of signaling pathways that modulate many cellular biological functions, including proliferation, survival/apoptosis, shape, polarity, motility, gene expression, and differentiation (22, 23).

In megakaryocytes and platelets, the major integrin αIbβ3 is expressed at high density in an inactive state (25). Upon platelet activation, αIbβ3 is activated from within the cells (inside-out signaling) so that platelets can bind their major ligand in plasma, fibrinogen, leading to platelet aggregation and thrombosis (26, 27). A model for talin-induced inside-out signaling activation and αIbβ3 conformational changes has been proposed (28). This is mediated through binding of the talin FERM domain or the PTP-like subdomain to the β3 cytoplasmic tail NPXY motif (29, 30). Consequently, the outside-in signaling due to the binding of extracellular ligand further activates a variety of αIbβ3-associated signaling molecules, including focal adhesion kinase, integrin-linked kinase, and Grb2, and transmits signals that modulate gene transcription and cytoskeleton organization (23, 31). One such protein, the integrin-linked kinase, is known to associate with the integrin β3 subunit in an activation-dependent manner, and it has been speculated to be part of the process controlling affinity and avidity changes in integrin αIbβ3 (32). In addition, integrin-linked kinase overexpression leads to the activation of the T cell factor (TCF)/β-catenin signaling pathway, which in turn regulates integrin-mediated cell signaling and gene transcription (33).

In this study, the function of DAB2 in integrin αIbβ3-mediated fibronogen adhesion and cell signaling was investigated. We report that DAB2 negatively regulates integrin αIbβ3 activation, leading to the inhibition of αIbβ3-mediated fibronogen adhesion and TCF transcriptional activity. We further demonstrate that Ser24 phosphorylation is crucial for membrane translocation of DAB2 and its interaction with the integrin β3 cytoplasmic tail, thereby defining Ser24 phosphorylation as a key step in eliciting DAB2 inhibitory function in integrin αIbβ3 activation and inside-out signaling. These findings contribute to our understanding of DAB2 in megakaryocytic differentiation and platelet activation.

### EXPERIMENTAL PROCEDURES

**Materials**—The anti-p66 and anti-PAC-1 antibodies were purchased from BD Biosciences. The anti-T7 tag antibody was purchased from Novagen (Madison, WI). The anti-integrin β3 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-human CD61 antibody was purchased from Serotec (Oxford, United Kingdom). The Dual-Luciferase assay system reagents and pCI-neo vector were purchased from Promega (Madison, WI). Fibrinogen, TPA, and ingenol mebutate (T85384) were from Calbiochem. The PKC inhibitors GF 109203X, Go6983, and GF1209040 (S2631) were purchased from Sigma.

**Plasmids**—To construct pCI-siDAB2, an ~400-bp BglII-DraIII fragment containing the mouse U6 promoter (~315 to +1), dabin2 targeting sequence (nucleotides 2112–2133), and the -TTTTR termination signal of RNA polymerase III was isolated from Dab2-2112 and cloned into the SpeHI site of pCI-neo as a replacement for the cytomegalovirus promoter and any associated protein from the beads. The eluted proteins were then used for Western blot analysis with antibodies to DAB2 and Integrin-mediated Cell Adhesion

The PCR product of full-length β-catenin cDNA was cloned into the StuI site of pIRE5-hyg2 to generate pIRE5-hyg2-β-catenin. For the TCF-luciferase reporter construct pTf4RE-luc, the chemically synthesized oligonucleotide with four copies of TCF4-binding sites was cloned into pIRE5neo2 between the Asel and EcorI sites to generate pIRE5neo2-Tf4RE. A DNA fragment bearing the β-globin basic promoter fused to luciferase, previously isolated from pGL3-Basic, was placed into the BamHI site of pIRE5neo2-Tf4RE so that pTf4RE-luc. For small interfering RNAs of firefly luciferase and hepatitis C virus (HCV) 5′-noncoding region, the complementary oligonucleotides F1S (5′-CTCAATGGGAGAAGATGCTTCGTAATAGG-3′) and F1AS (5′-GGATCCTGGAGGCTCAGTGCTGCATTCAATAGG-3′) and HCV-5′-CACCGGCCTTCCGAGGCTTCAGCTTG-3′(5′-GGATCAACCCGCTATGCTGCGCCAGTGGACGGTTCGA-3′) were annealed, respectively. The ligation between the annealed oligonucleotides and pTOPO-U6 at the EcoRV and Bsil cloning sites generated F1 and HCVsi, respectively.

The plakoglobin expression plasmid was a kind gift of Dr. Pamela Cowin (New York University School of Medicine). The integrin αIb expression plasmid was a kind gift of Dr. Mark H. Ginsberg (Scripps Research Institute). The integrin β3 expression plasmid was a kind gift of Dr. Erkki Ruoslahti (Barnham Institute). pTOPO-U6, Dab2-2112, pCI-neo-dHAB2, pCI-neo-His6, pGST-AB, ps2, AB, apoB, and pS2-S2A4 (S2A4) have been described previously (2, 7, 14, 34).

**Plasmid Insertion of DAB2 into Cell Adhesion (siDAB2) Stable Cell Lines**—The K562 subline K562-9 was transfected with 6 μg of pCI-siDAB2 or pCI-neo with LipofectAMINE 2000 reagent. 48 h after transfection, cells were diluted with RPMI 1640 medium containing 600 μg/ml G418 for single cell cloning in 96-well plates. Following 4 weeks of selection and expansion, the stable lines were characterized by Western blot analysis and maintained in selective medium containing 300 μg/ml G418 for biological and functional analysis.

**Cell Aggregation and Cell Adhesion Assay**—The assay for cell aggregation and cell adhesion to plates was performed as described previously (7). For assay of cell adhesion to fibrinogen, 5 × 10^5 cells/well in 500 μl of medium plus 500 μl of 0.5% bovine serum albumin and T5 medium (10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM CaCl2, and 2 mM MgCl2) were added to a 24-well non-tissue culture plate precoated with fibrinogen (10 μg). After incubation for 3 h at 37 °C, the medium was removed and washed three times with 2 ml of warmed 0.5% bovine serum albumin and T5 medium. The cell number was determined by crystal violet staining. The adhered cells were fixed with 1% glutaraldehyde and stained with 0.5% crystal violet for 15 min. Finally, the dye was eluted with 25% acetic acid.

**Flow Cytometry Analysis**—For analysis of PAC-1 binding and CD61 expression, K562 cells were incubated with the fluorescein isothiocyanate-conjugated anti-PAC-1 antibody (20 μl) and with the mouse anti-human CD61 monoclonal antibody (1 μl) followed by the fluorescein isothiocyanate-conjugated anti-mouse antibody and analyzed by flow cytometry. Flow cytometry analysis was then performed using the FACSscan system with CellQuest software (BD Biosciences).

**Immunoprecipitation Analysis**—K562 cells were lysed in lysis buffer (10 mM Tris-HCl (pH 7.4), 100 mM NaCl, 0.5 mM CaCl2, 0.5 mM MgCl2, 2% CHAPS, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 200 μM sodium orthovanadate, 10 mM sodium fluoride, and 1 mM EGTA) and subjected to immunoprecipitation and Western blot analysis as described previously (7).

**GST Pull-down Analysis**—The recombinant GST fusion proteins were expressed and affinity-purified from bacteria as described by the manufacturer (Amersham Biosciences). The GST fusion protein (~25 μg) was then incubated overnight with 1 μg of GST-Sepharose beads in lysis buffer (20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM CaCl2, and 2 mM MgCl2) and washed three times with wash buffer (20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM CaCl2, and 2 mM MgCl2) before elution with glutathione-Sepharose beads. After three washes with lysis buffer, excess glutathione (20 μM) was added to elute the GST fusion protein and any associated protein from the beads. The eluted proteins were then resolved by SDS-PAGE followed by Western blot analysis.

**Luciferase Activity Assay**—Following transient transfection of the integrin αIbβ3 and expression constructs, cells were plated at 5 × 10^4 cells/well in 24-well culture plates, and cells were cultured for 24 h. The transfected cells were lysed with passive lysis buffer, and an equal amount of cell extract was mixed with luciferase assay reagent II. The firefly luciferase activity was measured with a Lumat LB 9507 tube luminometer.

**Immunofluorescence Staining**—Cells were directly incubated with antibody (for CD61) or fixed with 3.7% formaldehyde solution at 37 °C for 30 min and permeabilized with 0.1% Triton X-100 (for DAB2). After several washes with 1× phosphate-buffered saline, the fixed cells were blocked with 10% fetal bovine serum and incubated with the indicated antibody.
antibody at room temperature for 1 h. The cells were washed three
times with 1× phosphate-buffered saline and incubated with fluores-
cin isothiocyanate-conjugated secondary antibody (1:500) at room
temperature for 1 h. The nucleus was visualized by staining with 4',6-
diamidino-2-phenylindole. Finally, the cells were cytospun on a glass
slide and mounted, ready for observation by confocal microscopy.

Isolation of CD34+ Cells from Cord Blood and ex Vivo Megakaryo-
cytic Differentiation—Mononuclear cells were isolated from umbilical
cord blood using Ficoll-Paque Plus (1.077 g/ml). After centrifugation at
1600 rpm for 30 min, the mononuclear cells were transferred to a 50-ml
tube, followed by several washes with 1× phosphate-buffered saline.
The cell suspension was mixed with Fc receptor-blocking reagents and
Microbeads conjugated to the mouse anti-human CD34 monoclonal
antibody and subjected to an autoMACS separator (Miltenyi Biotech). The
enriched CD34+ cells were induced to megakaryocytic differentiation
by culture in Iscove's modified Dulbecco’s medium supplemented with
200 μg/ml transferrin, 10 ng/ml thrombopoietin, 10 μg/ml insulin,
0.8 μg/ml lecithin, 0.78 μg/ml cholesterol, 0.28 μg/ml linoleic acid,
1% bovine serum albumin, and 28 mg/ml CaCl2. Megakaryocytic differenti-
ation was confirmed by the presence of a CD61 surface marker using
flow cytometry analysis.

Platelet Isolation—Peripheral blood was drawn from healthy drug-
free volunteers. Approximately 60 ml of blood was mixed with the
anticoagulant solution (acid/citrate/dextrose) at a ratio of 9:1 and cen-
trifuged at 2000 rpm for 10 min to obtain platelet-rich plasma. The
platelets were gently resuspended in control prostaglandin E1 buffer
supplemented with 50 units/ml heparin and centrifuged at 2800 rpm for
8 min. After several washes with Tyrode’s buffer, the platelet lysates
were isolated for Western blot analysis.

Cellular Fractionation—K562 cells were resuspended in extraction
buffer (20 mM Tris-HCl (pH 7.4), 2 mM EDTA (pH 7.4), 10 mM EGTA (pH
7.4), 0.25 M sucrose, 50 mM β-mercaptoethanol, 0.4 mM leupeptin, and
0.3 mM phenylmethylsulfonyl fluoride) and homogenized with a Dounce
homogenizer. The cell lysates were centrifuged at 100,000 × g for 1 h to
obtain the soluble cytosolic fraction. The resulting pellets were washed
twice and dissolved in extraction buffer supplemented with 0.5% Triton
X-100 for 30 min. Following centrifugation at 49,000 × g for 30 min, the
supernatant was collected as the particulate fraction.

Statistical Analysis—Student’s t test was used for statistical analy-
sis. p < 0.05 was considered statistically significant.

RESULTS

Effects of siDAB2 on K562 Cell Adhesion to Culture Plates and Fibroinogen—To elucidate the role of DAB2 in blood cell
differentiation and adhesion. K562-9 cells, a clonal subline of
K562 retaining the properties of TPA-induced DAB2 and inte-
grin β3 expression as well as the characteristics of megakaryo-
cytic morphological changes, were stably transfected with the
human-specific siDAB2 expression plasmid. The siDAB2 stable
line K562-si19 had significantly reduced basal and TPA-in-
duced DAB2 protein levels in comparison with the vector con-
trol cells (K562-V7) and the parental cells (K562-9) (Fig. 1A).
Both K562-si19 and K562-V7 cells displayed identical cell
growth rates and did not adhere to the culture plates under
general culture conditions (Fig. 1, B and C). Upon TPA-medi-
ated megakaryocytic differentiation, K562-si19 cells did not
undergo cell-cell aggregations, as observed in K562-V7 and

FIG. 1. Characterization of K562 cells stably expressing siDAB2. A, down-regulation of DAB2 in K562-si19 cells. The vector control
(K562-V7) and siDAB2 (K562-si19) stable clones were seeded at a density of 3 × 10^5 cells/ml and treated with 10 ng/ml TPA (T) or a vehicle control
(ethanol (E)) for 48 h. The total cell lysates were collected and analyzed by Western blotting using anti-p96 (DAB2) antibody. The expression of
β-actin was used as a control for equal protein loading. B, growth curves of siDAB2 and vector control K562 stable lines. The K562-V7 (V7) and
K562-si19 (si19) cells were plated at a density of 3 × 10^4 cells/10-cm culture dish. The total cell numbers were counted at 12, 24, 48, 80, and 100 h
after seeding. The data represent the mean ± S.E. of six assays in a representative experiment. C, decrease in K562-si19 cell-cell aggregation and
increase in K562-si19 cell adhesion to cell culture plates upon TPA-mediated megakaryocytic differentiation. The K562-V7 and K562-si19 cells
were plated at a density of 3 × 10^5 cells/ml, followed by ethanol or TPA treatment for 48 h. The cell adhesive properties were observed by
phase-contrast microscopy, and representative fields are shown (magnification ×400). D, increase in K562-si19 cell adhesion to fibrinogen during
TPA-induced megakaryocytic differentiation. The indicated K562 stable lines (K562-V7 and K562-si19) were cultured and treated with ethanol or
TPA for 48 h. The cells were counted, and a total of 5 × 10^4 cells were seeded on the 24-well plates precoated with 10 μg of fibrinogen. After 3 h
of incubation, the number of cells adhering to fibrinogen was determined by crystal violet staining and quantified using a value of A_{570} nm. The data
represent the mean ± S.E. of three assays in a representative experiment. Similar results were obtained in three independent experiments. *p <
0.05 compared with K562-V7-T cells.
K562-9 cells. In contrast, K562-si19 cells adhered to the cell culture plates (Fig. 1C). These results are consistent with our previous findings (7) and indicate that DAB2 plays a role in cell adhesion during megakaryocytic differentiation of K562 cells.

Fibrinogen is a major matrix protein involved in the function of megakaryocytes and platelets in peripheral blood. Hence, the effects of siDAB2 on fibrinogen adhesion were determined using both K562-si19 and K562-V7 cells. Under usual growing conditions, these two stable lines have little fibrinogen adhesive activity and do not adhere well to plates precoated with fibrinogen. In the presence of TPA, K562-V7 fibrinogen adhesion increased by >30-fold. For K562-si19 cells, the adhesive activity for fibrinogen was increased by >111-fold compared with K562-V7-E control cells and by 2.5-fold compared with K562-V7-T cells (Fig. 1D).

The effect of siDAB2 on fibrinogen adhesion was further demonstrated in transient transfection experiments. The transfection efficiency was ~58% according to a control transfection with an enhanced green fluorescent protein expression plasmid (data not shown). Similar to the results with K562-si19 cells, transfection of the siDAB2 plasmid Dab2-2112 significantly increased fibrinogen adhesion of K562 cells in the presence of TPA (Fig. 2A). However, small interfering RNAs targeting firefly luciferase (Ff1) and the HCV 5′-noncoding region (HCVsi) did not alter cell adhesion to fibrinogen. Most significantly, the effect of Dab2-2112 on cell adhesion could be reversed by coexpression of rat DAB2 p82, which was not the target of and was not down-regulated by Dab2-2112 (Fig. 2B). These data indicate that DAB2 is a negative regulator that modulates fibrinogen adhesion of K562 cells.

Fig. 2. Specificity of siDAB2 in the increase in cell adherence to fibrinogen. A, effect of various small interfering RNA expression plasmids on fibrinogen adhesion of K562 cells. The plasmids for vector control (pTOPO-U6 (pU6)), Dab2-2112 (2112), and small interfering RNAs for firefly luciferase (Ff1) and the HCV 5′-noncoding region (HCVsi) were transiently transfected into K562 cells by LipofectAMINE 2000. 24 h after transfection, the cells were plated at a density of 6 × 10^5 cells/10-cm culture dish and treated with 10 ng/ml TPA (T) or a vehicle control (ethanol (E)) for 48 h. The fibrinogen adhesion assay was performed, and the results were quantified as described under "Experimental Procedures." **, p < 0.01 compared with pU6-T. B, rat DAB2 reverses the effect of Dab2-2112 on fibrinogen adhesion. K562 cells were transfected with the indicated plasmids, followed by treatment with ethanol or TPA for 48 h. The levels of DAB2 expression were determined by Western blot analysis (upper panel). Fibrinogen adhesion was determined and quantified as described under "Experimental Procedures." The data represent the mean ± S.E. of three assays in a representative experiment. Similar results were obtained in two independent experiments. *, p < 0.05 compared with Dab2-2112-T.
cytic differentiation of K562 cells. To further reveal the temporal association of DAB2 and integrin \(\alpha_{IIb}\beta_3\), selective pharmaceutical agents implicated in the PKC signaling pathways, including GF 109203X (conventional and novel PKC inhibitor), Go6976 (novel PKC inhibitor), rottlerin (PKC \(\alpha\) inhibitor), and the phorbol ester ingenol 3,20-dibenzoate (selective PKC activator), were used to treat K562 cells, and their effects on DAB2 and integrin \(\alpha_{IIb}\beta_3\) expression were examined. The PKC inhibitors suppressed TPA-induced DAB2 and integrin \(\alpha_{IIb}\beta_3\) in a dose-dependent manner, whereas the selective PKC \(\alpha\) activator ingenol 3,20-dibenzoate, which induces K562 cell megakaryocytic differentiation (37), increased both DAB2 and integrin \(\beta_3\) expression with a maximal induction at a concentration of 0.1 \(\mu\)M (Fig. 4B). Similarly, the MEK1 (mitogen-activated protein kinase/extracellular signal-regulated kinase kinase-1) inhibitor U0126 (8) and the platelet-derived growth factor receptor inhibitor STI571 also inhibited TPA-induced DAB2 and integrin \(\beta_3\) expression concomitantly (data not shown). These results indicate that DAB2 and integrin \(\beta_3\) are coexpressed and co-regulated during megakaryocytic differentiation.

The temporal association of DAB2 and integrin \(\beta_3\) was also analyzed using an ex vivo megakaryocytic differentiation model. CD34\(^+\) hematopoietic pluripotent progenitor cells were isolated from umbilical cord blood and induced by thrombopoietin to undergo megakaryocytic differentiation. In the CD34\(^+\) progenitor cells, DAB2 and integrin \(\beta_3\) (CD61) were barely detected by Western blotting and flow cytometry analysis, respectively (Fig. 4C). Following 10 days of thrombopoietin treatment of CD34\(^+\) cells, both DAB2 and integrin \(\beta_3\) were induced, with \(\sim 87\%\) of the cells expressing the CD61 megakaryocytic differentiation surface marker (Fig. 4C). In accord with these findings, platelets (but not neutrophils) isolated from the blood of healthy volunteers expressed highly abundant amounts of DAB2 and integrin \(\beta_3\) (Fig. 4D). Given that the proteins that participate in the same pathway or that are part of the same protein complex are often coexpressed and co-regulated (38), these results indicate that DAB2 and integrin \(\beta_3\) are likely to have a functional link that may account for the effects of siDAB2 on the argumentation of integrin \(\alpha_{IIb}\beta_3\) activation and fibrinogen adhesion.

DAB2 Interacts with Integrin \(\beta_3\) in a Time- and Ser\(^{24}\) Phosphorylation-dependent Manner—Multiple proteins have been shown to interact with the integrin \(\beta_3\) subunit and to

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2 P. Chang, J.-C. Cheng, S.-S. Chang, and C.-P. Tseng, manuscript in preparation.
regulate integrin \(\alpha_{\text{IIb}}\beta_3\) activity. The temporal and spatial association of DAB2 and integrin \(\beta_3\) has prompted us to examine whether or not there is a direct interaction between them. Immunoprecipitation of the TPA-treated K562 cells using anti-DAB2 antibody H-110, but not the control antibody, revealed that integrin \(\beta_3\) associated with DAB2 in vivo (Fig. 5A, left panels). The association occurred in a time-dependent manner (Fig. 5A, right panels). Strong association of DAB2 and integrin \(\beta_3\) occurred in the platelets as well (Fig. 5B). Ectopic expression of T7-tagged DAB2 in K562 cells also revealed a time-dependent association of DAB2 and integrin \(\beta_3\) (Fig. 5C). These results indicate that endogenous and exogenous DAB2 proteins bind integrin \(\beta_3\) in both megakaryocytic cells and platelets. Further analysis with recombinant proteins revealed that the GST-\(\Delta\B\) fusion protein, containing the N terminus of DAB2, bound integrin \(\beta_3\) present in TPA-treated K562 cell lysates (Fig. 5D), whereas the GST-\(\Delta\B\) fusion protein, containing the integrin \(\beta_3\) cytoplasmic tail, bound DAB2 in TPA-treated K562 cell lysates (Fig. 5E). Hence, the PTB domain of DAB2 and the cytoplasmic tail of integrin \(\beta_3\) are involved in the association of DAB2 with integrin \(\beta_3\).

To determine whether or not TPA/PKC-mediated Ser\(^{24}\) phosphorylation affects the binding of DAB2 to integrin \(\beta_3\), either T7-tagged wild-type DAB2 (p82) or the S24A mutant was transfected into K562 cells, followed by TPA treatment for 24 h. Co-immunoprecipitation analysis with the anti-T7 tag antibody revealed that S24A bound less to integrin \(\beta_3\) compared with the p82 control (Fig. 5F). These results demonstrate that Ser\(^{24}\) phosphorylation is important in the binding of DAB2 to integrin \(\beta_3\).

Ser\(^{24}\) Phosphorylation Promotes DAB2 Membrane Translocation and Is a Key Step in DAB2 Regulation of Integrin \(\alpha_{\text{IIb}}\beta_3\)-mediated Fibrinogen Adhesion—The decrease in binding of S24A to integrin \(\beta_3\) prompted us to determine the effect of Ser\(^{24}\) phosphorylation on the cellular localization of DAB2. Either p82 or S24A was transfected into K562 cells for immunofluorescence staining with the anti-T7 tag antibody. In the absence of TPA, both p82 and S24A displayed cytoplasmic staining (Fig.
DAB2 and Integrin-mediated Cell Adhesion

To determine whether or not Ser\textsuperscript{24} phosphorylation of DAB2 plays a role in regulating integrin α\textsubscript{IIb}β\textsubscript{3}-mediated fibrinogen adhesion, various DAB2 constructs were cotransfected with Dab2-2112 into K562 cells to assess their effect on reversing siDAB2-mediated increase in fibrinogen adhesion (Fig. 7, A and B). In the presence of TPA, the two DAB2 deletion mutants, ΔB and ΔN, behaved like p82 and reduced fibrinogen adhesion to the level of vector control cells (Fig. 7C). In contrast, coexpression of S24A did not alter the siDAB2-mediated increase in fibrinogen adhesion, indicating the involvement of DAB2 Ser\textsuperscript{24} phosphorylation in fibrinogen adhesion. To confirm this, K562 cells were transfected with p82 and S24A, respectively. In the presence of TPA, p82 (but not S24A) reduced fibrinogen adhesion (Fig. 7D). These results reveal that Ser\textsuperscript{24} phosphorylation of DAB2 is important to elicit its negative regulatory role in α\textsubscript{IIb}β\textsubscript{3}-mediated fibrinogen adhesion.

Ser\textsuperscript{24} Phosphorylation of DAB2 Is Required for Its Inhibitory Function in α\textsubscript{IIb}β\textsubscript{3}-mediated (but Not β-Catenin- and Plakoglobin-mediated) TCF Transcriptional Activity—The importance of DAB2 Ser\textsuperscript{24} phosphorylation in integrin α\textsubscript{IIb}β\textsubscript{3} signaling was further delineated for the activation of the TCF/β-catenin pathway, which has been implicated in cell adhesion signaling. We first characterized the expression of a luciferase reporter construct, pTcf4RE-luc, which contains four copies of the TCF4-responsive element at the 5'-end of the β-globin basic promoter, during megakaryocytic differentiation of the siDAB2 stable line. An increase in luciferase activity was observed in both K562-si19 and K562-V7 cells in the presence of TPA (Fig. 8A).
The luciferase activity was significantly higher in K562-si19 cells than in K562-V7 control cells. To determine whether or not the α<sub>IIb</sub>β<sub>3</sub> signaling relates to the activation of TCF transcriptional activity, we transiently coexpressed α<sub>IIb</sub> and β<sub>3</sub> with pTcf4RE-luc in K562 cells. Although integrin α<sub>IIb</sub> or β<sub>3</sub> alone did not affect the level of luciferase expression (data not shown), coexpression of integrins α<sub>IIb</sub> and β<sub>3</sub> resulted in a ~2–3-fold increase in luciferase activity, which was further enhanced in the presence of TPA (Fig. 8B). In addition, expression of p82 resulted in inhibition of the α<sub>IIb</sub>β<sub>3</sub>-mediated increase in pTcf4RE-luc luciferase activity. These results indicate that TCF activation is part of the integrin α<sub>IIb</sub>β<sub>3</sub> downstream signals and that DAB2 acts as a negative regulator in α<sub>IIb</sub>β<sub>3</sub>-mediated TCF signaling.

The role of DAB2 Ser<sup>24</sup> phosphorylation in integrin α<sub>IIb</sub>β<sub>3</sub>-mediated TCF transactivation was also characterized by cotransfection experiments with various DAB2 mutants. The results show that ΔB and ΔN behaved like wild-type DAB2 p82 and inhibited integrin α<sub>IIb</sub>β<sub>3</sub>-mediated increases in pTcf4RE-luc luciferase activity (Fig. 8B). The inhibitory effect is specific because expression of the membrane protein phospholipid scramblase-His<sub>9</sub> did not alter the luciferase activity. In contrast, S24A did not abrogate integrin α<sub>IIb</sub>β<sub>3</sub>-mediated pTcf4RE-luc expression. These data indicate that Ser<sup>24</sup> phosphorylation is necessary for DAB2 to elicit its inhibitory function in α<sub>IIb</sub>β<sub>3</sub> signaling.

In transient transfection experiments, we also found that DAB2 repressed both β<sub>3</sub>-catenin- and plakoglobin-mediated increases in pTcf4RE-luc luciferase activity (Fig. 8, C and D). To further demonstrate whether or not Ser<sup>24</sup> phosphorylation of DAB2 also plays a role in its inhibitory function in β<sub>3</sub>-catenin- and plakoglobin-mediated TCF transactivation, mutant forms of DAB2 were cotransfected with pTcf4RE-luc in the presence of either β<sub>3</sub>-catenin or plakoglobin. Analysis of the luciferase activity revealed that all DAB2 mutants, including S24A, ΔB, and ΔN, reduced β<sub>3</sub>-catenin- and plakoglobin-induced luciferase activity (Fig. 8, C and D). These data indicate that Ser<sup>24</sup> phosphorylation is not crucial for DAB2 to inhibit β<sub>3</sub>-catenin- and plakoglobin-mediated TCF transcriptional activity. Therefore, DAB2 regulates α<sub>IIb</sub>β<sub>3</sub> and β<sub>3</sub>-catenin/plakoglobin signaling through different mechanisms in a Ser<sup>24</sup> phosphorylation-dependent and -independent manner, respectively.

**DISCUSSION**

DAB2 is up-regulated during megakaryocytic differentiation of human leukemic cell lines (7, 8) and CD34<sup>+</sup> hematopoietic pluripotent stem cells. Accordingly, DAB2 is highly expressed in human platelets. It has been shown that the platelet integrin α<sub>IIb</sub>β<sub>3</sub> is a differentiation marker of megakaryocytes and platelets and mediates cell adhesion. Using siDAB2, we have shown previously that DAB2 is involved in the regulation of cell-cell adhesion and MAPK activity (7). The spatial and temporal association of DAB2 and integrin α<sub>IIb</sub>β<sub>3</sub> as revealed in this study further suggests a pivotal role of DAB2 in integrin-mediated cell adhesion. In this study, we have demonstrated for the first time that DAB2 possesses anti-adhesive activity. It negatively regulated megakaryocytic cell adhesion to fibrinogen, an extracellular matrix protein that is crucial for platelet activation and aggregation during blood coagulation. This conclusion is supported by the observation that expression of siDAB2 increased fibrinogen adhesion. Moreover, ectopic expression of DAB2 reversed the siDAB2 effect or, by itself, decreased fibrinogen adhesion of K562 cells. It has been reported that, similar to DAB2, the platelet protein plasminogen activator inhibitor type-1 also possesses anti-adhesive activity and interferes with platelet and megakaryoblastic cell adhesion (39). Due to the fact that DAB2 is highly enriched in platelets, our data imply that the anti-adhesive activity of DAB2 may have its physiological function during platelet activation and aggregation.

In contrast to our observations, DAB2 has been shown to play a role in enhancing integrin-mediated adhesion to collagen IV and laminin and non-integrin-mediated adhesion to poly-D-lysine substrate (6). It is not yet completely understood how DAB2 mediates such distinct effects on integrin-mediated cell adhesion. A recent study reported on the differential interaction of the DAB2 PTB domain with the cytoplasmic tail of various integrin β subunits: strong interaction with β<sub>3</sub> and β<sub>5</sub> and no interaction with β<sub>1</sub> and β<sub>2</sub> (11). This may provide a basis to explain the dual effects of DAB2 on integrin-mediated cell adhesion. In this study, we have provided the first experimental evidence to demonstrate that the binding of integrin β<sub>3</sub> by DAB2 negatively regulates the activation of integrin α<sub>IIb</sub>β<sub>3</sub>. This was revealed by the change in binding of anti-PAC-1 antibody, which recognizes the active form of α<sub>IIb</sub>β<sub>3</sub> that mediates high affinity and avidity fibrinogen binding (35), in the K562 stable line expressing siDAB2. We thus conclude that DAB2 serves as an important negative signaling element in megakaryocytic cells and platelets in the control of the activa-
tion and signaling of $\alpha_{\text{IIb}}\beta_3$. The $\alpha_{\text{IIb}}\beta_3$ activation results from many cellular events that converge in integrin inside-out signaling (40). For example, activation of conventional PKC isoforms, phosphatidylinositol 3-kinase-mediated activation of novel and atypical PKCs, members of the Ras GTPase superfamily, and G-protein-coupled receptor signaling have all been reported to play a role in inside-out signaling and $\alpha_{\text{IIb}}\beta_3$ activation (41, 42). These signals direct the interaction between integrin and regulatory proteins, including Src, Syk, Shc, myosin, Pyk2, integrin-associated protein, CD9, talin, and $\beta_3$-endonexin, that stimulate conformational changes in the receptor to expose binding sites for fibrinogen (43). Of these integrin cytoplasmic tail-interacting proteins, DAB2 binds to Src and myosin and modulates their activity (4, 10). It is plausible that the binding of DAB2 to integrin $\beta_3$ alters the protein complexes associated with the cytoplasmic tail of $\beta_3$. Although the effects of DAB2 on integrin signaling still need to be characterized, our study provides clues to the biological effect of DAB2-integrin $\beta_3$ interaction and may explain the dual functions of DAB2 based on the fact that DAB2 has distinct binding activity for different integrin $\beta$ subunits.

We have extended our study to demonstrate the molecular basis for DAB2 acting as a negative regulator of $\alpha_{\text{IIb}}\beta_3$ activation. The evolutionary conservation of Ser24 and its flanking sequences in DAB2 from mammalian species such as human, rat, and mouse implies that Ser24 is an important amino acid for DAB2. Indeed, TPA/PKC-dependent Ser24 phosphorylation of DAB2 is crucial for inhibiting TPA-induced AP-1 activity (14). We have further revealed in this study that Ser24 phosphorylation of DAB2 is involved in the decrease in integrin-mediated fibrinogen adhesion and TCF transactivation. In addition, Ser24 phosphorylation is critical for membrane translocation of DAB2. These findings are in accord with previous studies that reported the phosphorylation-dependent redistribution of key signaling proteins such as focal adhesion kinase, Ras GTPase-activating protein, and HSP25 (44–46). As a result, the inability of S24A to control $\alpha_{\text{IIb}}\beta_3$ signaling is caused by the restricted distribution of DAB2 in the cytosol and the lack of interaction with integrin $\beta_3$. Ser24 phosphorylation of DAB2 serves as a key step in the negative regulation of integrin $\beta_3$-mediated cell adhesion and signaling. The DAB2 N-terminal deletion mutant $\Delta N$ also acts like wild-type DAB2 in the regulation of TCF transcription. The multiprotein signaling network complexes of integrin $\beta_3$ and DAB2 thus appear to be more complicated than expected.

In addition to acting as a negative regulator of integrin signaling, DAB2 negatively regulates $\beta$-catenin-mediated Wnt signaling. In addition, we have revealed that plakoglobin-mediated Wnt signaling is also diminished by DAB2. By mutational analysis, we found that, in contrast to Ser24 phosphorylation-
dependent regulation of integrin signaling, the inhibition of β-catenin and plakoglobin signaling by DAB2 is Ser24 phosphorylation-independent. These observations indicate that DAB2 participates in the control of cell signaling through multiple mechanisms. We postulate that Ser24 phosphorylation is a key step in determining the DAB2 mode of action. Because DAB2 with Ser24 phosphorylated tends to translocate to the plasma membrane (particulate) fraction, its interaction with transmembrane proteins and receptor protein-tyrosine kinases such as integrin β3 appears to be regulated in a Ser24 phosphorylation-dependent manner. On the other hand, interaction with other cytosolic proteins may be mediated in a Ser24 phosphorylation-independent manner. Hence, the reported interaction between DAB2 and the cytosolic protein Dvl-3, a signaling mediator of the β-catenin/Wnt pathway leading to TCF transactivation (12), appears to be mediated in a Ser24 phosphorylation-independent manner. This could explain why the S24A mutant of DAB2 still elicits inhibitory activity for β-catenin-mediated increases in TCF-luciferase activity. Together, DAB2 has multiple modes of action and elicits distinct regulatory mechanisms in αIIbβ3 and β-catenin/plakoglobin signaling in a Ser24 phosphorylation-dependent and -independent manner, respectively.

In summary, this study shows for the first time the anti-adhesive activity of DAB2 in integrin αIIbβ3-mediated fibrinogen adhesion. In addition, the Ser24 phosphorylation-dependent membrane translocation and the subsequent interaction with integrin β3 provide a mechanism for DAB2 to regulate activation of integrin αIIbβ3 and its downstream signaling, including fibrinogen adhesion and TCF transactivation (Fig. 9). Such information can lead to defining a role and expanding our understanding of DAB2 in megakaryocytic differentiation and platelet function.
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