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Characterization of a Novel Integrin Binding Protein, VPS33B, Which is Important for Platelet Activation and *in vivo* Thrombosis and Hemostasis

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Abstract

Background—Integrins are heterodimeric (α/β) membrane proteins that play fundamental roles in many biological processes, *e.g.* cell adhesion and spreading, which are important for platelet function and hemostasis. The molecular mechanism that regulates integrin activation is not completely understood.

Methods and Results—Here, we show that VPS33B, a member of the Sec1/Munc18 (SM) family and a component of the Vps-C tethering complexes, binds directly to the integrin β subunit. Overexpression of VPS33B in CHO cells potentiated α IIB β 3 outside-in signaling but not inside-out signaling. Platelets, from megakaryocyte- and platelet-specific VPS33B conditional knockout mice, had normal morphology yet their spreading on fibrinogen was impaired and they failed to support clot retraction. Platelet aggregation and ATP secretion in response to low-dose agonists were reduced in the VPS33B knockout mice. α IIB β 3-mediated endocytosis of fibrinogen was also defective. Tail bleeding times and times to occlusion in a FeCl₃-induced thrombosis model were prolonged in the VPS33B knockout mice. Furthermore, VPS33B acted upstream of the RhoA-

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ROCK-MLC and Rac1 dependent pathways that lead to clot retraction and cell spreading, respectively.

Conclusions—Our work demonstrates that vesicular trafficking complexes, containing VPS33B, are a novel class of modifiers of integrin function. Our data also provide insights into the molecular mechanism and treatment of ARC syndrome.

Keywords

Integrin; VPS33B; platelet; platelet aggregation; signal transduction; hemostasis; thrombosis

Introduction

Integrins are cell surface, transmembrane receptors that mediate adhesion to the extracellular matrix^{1, 2}. Integrin function is modulated by bi-directional transmembrane signaling: inside-out and outside-in, which is mediated through the interactions between integrin cytoplasmic tails and intracellular, regulatory proteins. The major integrin of platelets, α IIB β 3, plays an essential role in adhesion, spreading, and aggregation *in vitro* and in thrombosis and hemostasis *in vivo*^{3–5}. Antagonists of α IIB β 3 are potent anti-thrombotic drugs and new inhibitors targeting α IIB β 3 are in preclinical testing or large patient trials to treat acute coronary syndromes^{3, 5}. Normally, α IIB β 3 on circulating platelets exists in a “resting” form. Upon platelet activation α IIB β 3 is activated and its binding of ligand forms adhesive bonds that transmits “outside-in” signals, which induce a series of intracellular events, such as protein phosphorylation, elevation of intracellular Ca²⁺, and cytoskeleton reorganization. This leads to platelet responses such as cell spreading, stabilization of cell adhesion, secretion, irreversible aggregation, and clot retraction^{3, 4}. Inside-out signaling, to activate integrin, is achieved by binding two FERMT domain-containing proteins, talin and kindlin, to the β integrin cytoplasmic domain^{6–8}. Outside-in signaling requires Src family kinases, PKC, and G α ₁₃, which have also been shown to bind to the β integrin cytoplasmic domain^{9–12}.

Since integrin α IIB β 3 function requires binding of proteins, including talin^{6, 13}, kindlins^{8, 14, 15}, Src kinase^{9, 10}, and G α ₁₃¹² to the cytoplasmic domain of its β subunit, we searched for novel integrin binding proteins using receptor pull-down and mass spectrometric analysis to gain more insights into α IIB β 3 regulation. Unexpectedly, VPS33B was found associated with β integrins. VPS33B, a homologue of yeast Vps33p, is known to be involved in multiple endomembrane trafficking steps¹⁶. It is a core component of two sorting complexes, the class C core vacuole/endosome tethering (CORVET) and the homotypic fusion and vacuole protein sorting (HOPS)^{17, 18}. In humans, mutations in either VPS33B or its binding partner, VPS16B, are associated with a recessive disorder called arthrogryposis, renal dysfunction and cholestasis (ARC) syndrome^{19–22}. This is a multisystem disorder in which most patients die within one year of birth. Many develop severe hemorrhages, which is most likely due to a deficiency in α granules, reminiscent of that seen in grey platelet syndrome²³. These data show that at least one role for VPS16B/VPS33B is in α granule biogenesis. In our studies, we showed that the interaction between VPS33B and α IIB β 3 was important for outside-in signaling and a number of platelet functions. Using a platelet factor 4-promoter-driven excision system, we maintained normal

α granule biogenesis and were able to focus on VPS33B's acute effects on platelet functions. Platelets deficient in VPS33B had defective spreading, fibrinogen uptake and were unable to mediate normal hemostasis. Our results are some of the first to establish vesicle targeting complexes as an important novel class of modifiers of integrin function.

Methods

Generation and characterization of VPS33B^{-/-} mice

All animal experiments were approved by the Institutional Animal Care and Use Committee of the University of Kentucky. The protocol used to generate platelet-specific VPS33B conditional knockout mice is described in the Supplemental Methods.

Platelet aggregation and secretion

Mouse platelet preparation, platelet aggregation, and ATP release were prepared as described previously²⁴. The detailed procedures are available in the Supplemental Methods.

Immunoblotting analysis of MLC phosphorylation

MLC phosphorylation was detected by Western blot. The detailed procedures are available in the Data Supplement Methods.

Fibrinogen binding assay

Fibrinogen binding to cells was analyzed by flow cytometry. The detailed procedures are available in the Data Supplement Methods.

Detection of PF4 in platelets

The total amount of PF4 in platelet lysates was measured using the anti-mouse ELISA kit from R & D Systems (Minneapolis, MN) following the manufacturer's instruction.

Integrin expression

Integrin $\beta 3$ or $\alpha \text{IIb}\beta 3$ expression was analyzed by flow cytometry. The detailed procedures are available in the Data Supplement Methods.

Platelet spreading on fibrinogen and Platelet-mediated clot retraction

Platelet spreading and clot retraction was performed as described previously²⁵. The detailed procedures are available in the Data Supplement Methods.

Measurement of mouse platelet serotonin

Serotonin was measured as described previously²⁵. The detailed procedures are available in the Data Supplement Methods.

Co-immunoprecipitation and binding assays

The protocol used to detect VPS33B and integrin interaction by co-immunoprecipitation is described in the Data Supplement Methods.

GST bead pull down analysis

Platelet lysates, lysates from CHO cells expressing FLAG-tagged VPS33B, or purified FLAG-VPS33B were incubated with glutathione beads-bound to GST, GST- α IIBCD, GST- β 1CD, GST- β 3CD, or various GST- β 3CD truncated mutants at 4°C overnight. Bead-bound proteins were analyzed by immunoblotting. The detailed procedures are available in the Data Supplement Methods.

RhoA and Rac1 activity assay

The protocols used to measure RhoA and Rac1 activity are available in the Data Supplement Methods.

Detection of endocytosis of fibrinogen in platelets

Purified human fibrinogen (Sigma) was biotinylated by an EZ-link sulfo-NHS-Biotinylation kit (Fisher) according to the manufacturer's instruction. VPS33B^{+/+} and VPS33B^{-/-} mice (male, 8 ~ 9 week old) were injected retro-orbitally with 5.0 mg/mouse biotinylated fibrinogen. Blood was collected from the animals 24 h after injection of the biotinylated fibrinogen. Platelets were harvested, washed, and solubilized in SDS sample buffer. Biotinylated fibrinogen in platelet lysates was analyzed by immunoblotting with a HRP-conjugated avidin.

Bleeding time and *In vivo* thrombosis

Tail bleeding time assay and *in vivo* thrombosis model was performed as described previously²⁴. The detailed procedures are available in the Data Supplement Methods.

Statistical Analysis

Data were analyzed using unpaired Student *t* test. The nonparametric Mann-Whitney test was used for comparison of tail bleeding times and occlusion times to thrombus formation between 2 groups. Values of *P* < 0.05 were considered significant.

Results

VPS33B binds to the cytoplasmic domain of β integrins

To identify novel binding partners of integrin α IIB β 3, agarose beads conjugated with glutathione S-transferase (GST)- β 3 integrin cytoplasmic domain fusion protein (GST- β 3CD) or GST- α IIB integrin cytoplasmic domain fusion protein (GST- α IIBCD) were incubated with human platelet lysates. Proteins bound to GST- β 3CD or GST- α IIBCD beads were subjected to mass spectrometric analysis. The GST- β 3CD beads successfully precipitated known β 3 cytoplasmic domain binding proteins, *i.e.*, talin 1 and 2^{6, 13}, kindlin 2 and 3^{8, 14, 15}, Src kinase^{9, 10}, and integrin-linked protein kinase^{26, 27}. The GST- β 3CD beads also precipitated several adhesion complex proteins, such as myosin, vinculin, and tubulin. Surprisingly, the GST- β 3CD but not the GST- α IIBCD beads specifically bound the vacuolar protein sorting-associated protein 33B (VPS33B), encoded by the *VPS33B* gene.

To further verify that VPS33B associates with α IIB β 3, we infected Chinese hamster ovary (CHO) cells, stably expressing α IIB β 3 (A5 cells), with an adenovirus containing FLAG epitope-tagged human VPS33B cDNA. Cells were allowed to adhere and spread on fibrinogen and were immunostained with a FITC-labeled anti-FLAG monoclonal antibody and a PE-labeled monoclonal antibody against integrin α IIB. As shown in Figure 1A, FLAG-VPS33B exhibited a significant intracellular colocalization with α IIB β 3. To evaluate the physical interaction between VPS33B and α IIB β 3, A5 cells expressing FLAG-tagged VPS33B were solubilized and anti-FLAG immunoprecipitation was performed. Immunoblotting analysis revealed a specific association of β 3 with FLAG-VPS33B. Comparable levels of β 3 were seen in the total cell lysates with or without FLAG-VPS33B (Figure 1B). Coimmunoprecipitated β 3 was detected by immunoblotting with a mouse anti- β 3 monoclonal antibody, SZ-21^{28, 29}. The specific interaction was confirmed by reciprocal immunoprecipitation using a specific β 3 antibody (Figure 1C). Furthermore, in an *in vitro* binding assay, we were able to recover VPS33B from the lysates of CHO cells overexpressing VPS33B on GST- β 3CD beads (Figure 1D). Agarose beads conjugated with GST- β 1 integrin cytoplasmic domain (GST- β 1CD) fusion protein but not with GST- α IIBCD could also bind VPS33B from the cell lysates. Thus, VPS33B specifically interacts with the cytoplasmic domain of integrin β subunits. The interaction between VPS33B and β subunits of integrin appears to be direct, since we were able to pull down purified FLAG-VPS33B (Supplemental Figure 1a) with GST- β 3CD and GST- β 1CD proteins (Figure 1E).

To map the VPS33B binding site in β 3, we generated GST fusion proteins containing various truncations of the β 3 cytoplasmic domain (Figure 1F). The pull-down assay data showed that the β 3 fragment spanning residues 716~730 bound efficiently to FLAG-VPS33B (Figure 1G). In contrast, the fragments containing either residues 729~762 or residues 744~762 fragments did not bind. There are two talin binding sites in β 3: the conserved NPLY sequence; and another binding site for talin head domain between residues 722~738³⁰. This second site appears to overlap with the VPS33B binding site. To determine whether talin binding to β 3 affects VPS33B binding, we established a stable CHO cell line expressing the talin head domain. Overexpressing the head domain did not inhibit but appear to enhance VPS33B binding to β 3 (Figure 1H). The N-terminal 145 amino acids of VPS33B appeared to be critical for binding for β 3 (Figure 1I).

Lo *et al.* previously reported that VPS33B is only present in megakaryocytes and not in platelets²³; however, recent studies by the same group described the successful detection of VPS33B in platelets using a newly generated antibody³¹. Consistently, VPS33B was readily detectable in human platelet lysates (Figure 1J and 1K). Moreover, VPS33B could be immunoprecipitated with a specific polyclonal antibody and then detected by immunoblotting with a separate monoclonal antibody (Supplemental Figure 1b). Figure 1J shows that, like overexpressed FLAG-VPS33B, platelet VPS33B bound GST- β 3CD and GST- β 1CD but not GST alone or GST- α IIBCD. The interaction with platelet VPS33B was also dependent on residues 716~730 of β 3CD (Supplemental Figures 1k, 1c). Finally, using a rabbit polyclonal antibody against VPS33B, we coimmunoprecipitated endogenous β 3 subunit with VPS33B from human platelet lysate (Figure 1L), indicating the presence of the

complex under physiologic conditions. Binding of VPS33B to $\beta 3$ was reduced when platelets were activated with thrombin (Figure 1M).

VPS33B is required for α IIB β 3 outside-in signaling in CHO cells

To investigate the role of VPS33B in integrin activation, we first used a recombinant, CHO cell model of integrin activation. A5 cells were infected with an empty virus or an adenovirus encoding VPS33B cDNA. Two days after infection, >80% of the cells were infected and were positive for VPS33B. While it did not increase surface expression of α IIB β 3 (Figure 2A), overexpression of VPS33B markedly potentiated cell spreading on fibrinogen (Figure 2B). The surface area of cells expressing VPS33B was increased by >2 folds when compared to empty virus infected cells (Figure 2C). Cell spreading on immobilized fibrinogen is a standard assay for α IIB β 3 outside-in signaling^{32, 33}.

Overexpression of VPS33B did not affect cell spreading on poly-L-lysine (Figure 2B and 2C) suggesting that overexpression of VPS33B specifically potentiates α IIB β 3-dependent cell spreading. To investigate whether VPS33B plays a role in α IIB β 3 inside-out signaling, we used α β Py cells, a CHO cell line that expresses a talin-dependent, constitutively-active, chimeric integrin composed of the extracellular and transmembrane domains of α IIB β 3 and the cytoplasmic tails of α 6 β 1^{34, 35}. Both α β Py cells and A5 cells were infected with an empty virus or an adenovirus containing VPS33B cDNA. Fibrinogen binding to either α β Py or A5 cells was not enhanced by overexpression of VPS33B (Figure 2D). Thus, we identified VPS33B as a specific modulator of the α IIB β 3 outside-in signaling that does not alter integrin expression.

VPS33B is required for α IIB β 3 outside-in signaling in platelets

To investigate the role of VPS33B in integrin activation in platelets, we established a mouse model with megakaryocyte- and platelet-specific deletion of VPS33B. We first generated mice harboring a “floxed” VPS33B allele. In the “floxed” VPS33B mice (VPS33B^{fl/fl}), exons 2 and 3 of the VPS33B gene together with an inserted Neo cassette were flanked with 3 LoxP sites (Figure 3A). Megakaryocyte- and platelet-specific deletion of the floxed region was accomplished by breeding of the VPS33B^{fl/fl} mice with transgenic mice expressing Cre recombinase under the control of a platelet factor 4 promoter (*Pf4*-Cre). Male VPS33B^{fl/fl}/Cre⁺ and female VPS33B^{fl/fl}/Cre⁻ were used as breeders. Immunoblotting demonstrated a complete absence of VPS33B in the platelets from the VPS33B^{fl/fl}/Cre⁺ (VPS33B^{-/-}) mice (Figure 3B). Littermate VPS33B^{fl/fl}/Cre⁻ (VPS33B^{+/+}) mice were used as wild-type controls. The abundance of VPS33B in the platelets isolated from the VPS33B^{+/+} mice was comparable with that from the C57BL/6J mice, indicating that insertion of Neo cassette and LoxP sites had no effect on VPS33B expression. VPS33B^{-/-} mice appeared to develop normally with normal blood counts and without observable changes in platelet number or size (Supplemental Figures 2a, b).

VPS33B^{-/-} and VPS33B^{+/+} platelets had similar levels of surface integrin $\beta 3$ (Figure 3C) or α IIB β 3 complex (Supplemental Figure 2c). Platelets lacking VPS33B were defective in spreading on fibrinogen (Figure 3D and 3E). Spreading of VPS33B knockout platelets on collagen or fibronectin was also partially impaired (Supplemental Figure 3). Clot retraction requires α IIB β 3-dependent outside-in signaling^{36–38}. While the maximal clot retraction of

platelets from the VPS33B^{+/+} mice occurred within 2 hours after addition of thrombin (0.4 U/mL; Figure 4A and 4B), VPS33B^{-/-} platelets failed to support clot retraction even after 21 hours. Thrombin-induced fibrinogen binding to platelets (Figure 4C and 4D and Supplemental Figure 4a) was not affected by the loss of VPS33B. Thrombin-induced binding of JON/A, an antibody selectively binds to the activated mouse integrin α IIb β 3, to platelets was similar between VPS33B^{+/+} and VPS33B^{-/-} platelets (Supplemental Figure 4b,c). Collectively, these results are supportive of an important role for VPS33B in α IIb β 3 outside-in signaling but not for α IIb β 3 inside-out signaling.

VPS33B contributes to α -granule biogenesis

In humans, VPS33B is essential for α -granule biogenesis. The α -granule contents, fibrinogen, VWF, and fibronectin, were considerably lower in the VPS33B^{-/-} platelets than those of the VPS33B^{+/+} platelets (Figure 4E – 4G). In contrast, the expression level of an α -granule membrane protein P-selectin was nearly identical. PF4 levels were only slightly reduced in VPS33B^{-/-} platelets (Figure 4H). Consistently, the number of α granules in VPS33B^{-/-} platelets was similar to that in the VPS33B^{+/+} platelets. Serotonin and β -hexosaminidase, as markers for dense granules and lysosomes, respectively, were not affected by loss of VPS33B (Figure 4I and 4J).

Integrin outside-in signaling is involved in irreversible aggregation and the second wave of platelet secretion. To determine whether VPS33B is involved in agonist-induced platelet activation, we measured aggregation and ATP release in response to thrombin. Platelets lacking VPS33B showed a marked defect in ATP secretion and aggregation in response to low-concentration thrombin or collagen (Figure 5A and 5B), compared with control VPS33B^{fl/fl} platelets. Although platelet aggregation was not reduced in the VPS33B deficient mice when stimulated with high concentrations of agonists, ATP release was still lower in the VPS33B knockout platelets than that of wild-type platelets (Figure 5A and 5B). The expression levels of several elements of the platelet secretory machinery were not overtly affected by loss of VPS33B (Figure 5C and 5D).

VPS33B is required for platelet endocytosis

While some α -granule proteins are endogenously synthesized, others, such as fibrinogen, are derived from plasma by endocytosis. The amounts of fibrinogen and fibronectin were reduced in the platelets lacking VPS33B, suggesting that VPS33B might play a role in endocytosis. To ascertain the role of VPS33B in α IIb β 3-dependent endocytosis, VPS33B^{+/+} and VPS33B^{-/-} mice were intravenously injected with biotin-labeled fibrinogen. After 1 day, the amount of biotin-labeled fibrinogen was dramatically reduced in the platelets isolated from the VPS33B^{-/-} mice as compared to that from the control animals (Figure 5E and 5F).

VPS33B regulates α IIb β 3 outside-in signaling through a RhoA and Rac1 pathway

Previous studies have highlighted the importance of the RhoA-ROCK-MLC pathway in α IIb β 3 outside-in signaling leading to clot retraction³⁹. Indeed, we observed that MLC phosphorylation and RhoA activation elicited by immobilized fibrinogen were markedly reduced in VPS33B^{-/-} platelets (Figure 6A and 6B). Consistently, overexpression of

VPS33B in A5 cells increased immobilized fibrinogen-elicited MLC phosphorylation and RhoA activation (Figure 6C and 6D). Consequently, overexpression of VPS33B in A5 cells dramatically potentiated F-actin formation (Supplemental Figure 5a) following cell adhesion to fibrinogen. Although the RhoA-ROCK-MLC pathway is critical for cell retraction and clot retraction, cell spreading is known to be dependent on the activation of Rac1 and Cdc42 but not RhoA³⁸. We found that potentiation of cell spreading by overexpression of VPS33B was abolished by pretreatment of cells with a selective Rac1 inhibitor NSC23766 (Figure 6E and 6F), but not with a selective Rho-associated protein kinase (ROCK) inhibitor Y-27632 (Supplemental Figure 5b), suggesting that Rac1, but not RhoA, is required for VPS33B-potentiated cell spreading. Rac1 activation induced by immobilized fibrinogen was diminished in the platelets lacking VPS33B (Figure 6G).

VPS33B plays an important role in hemostasis and thrombosis

To investigate whether platelet VPS33B exerts a role in hemostasis, tail-bleeding time tests were performed in 7–8-week-old isoflurane-anesthetized littermates generated from mating of VPS33B^{fl/fl}/Cre male mice with VPS33B^{fl/fl} female mice as breeders. Genotyping of the offspring was subsequently determined by PCR analysis. Tail bleeding times were significantly prolonged in the VPS33B knockout mice than that of wild-type littermates (Figure 7A). To evaluate the role of platelet VPS33B in thrombosis *in vivo*, we compared thrombus formation between megakaryocyte-specific VPS33B-deficient mice and wild-type littermates using the FeCl₃-injured carotid artery thrombosis model. The median of the time to formation of stable thrombi in the platelet-specific VPS33B-deficient mice was prolonged compared with wild-type littermates (Figure 7B). Together, these results suggest that platelet VPS33B plays a role in hemostasis and thrombosis *in vivo*.

Discussion

Here we identify a new integrin β subunit-binding protein, VPS33B, and show that it is essential for α IIB β 3 outside-in signaling. We further show, distinct from Kindlin-3 and talin, VPS33B binds to the proximal domain of integrin β 3 subunit. We demonstrate that VPS33B regulates integrin outside-in signaling by promoting RhoA and Rac1 activation, leading to clot retraction and cell spreading, respectively. In addition, our data indicate that VPS33B plays an important role in integrin-dependent fibrinogen endocytosis and *in vivo* thrombosis and hemostasis.

VPS33B is a homologue of yeast Vps33p, a protein known to be involved in multiple stages of endomembrane trafficking and sorting¹⁶. Mutations in the VPS33B gene are associated with ARC syndrome^{19, 20}. A deficiency in α -granules is often observed in ARC syndrome patients²³, indicating that VPS33B is important for human platelet α -granule biogenesis. However, although some α -granule contents, *i.e.* fibrinogen and VWF, were considerably lower in VPS33B^{-/-} platelets, the expression of α -granule membrane protein P-selectin was unaffected. The number of morphologically identifiable α -granule in VPS33B^{-/-} platelets was similar to wild-type platelets. These results suggest that it is not essential to have VPS33B present for the entire process of platelet biogenesis, at least in mice.

Our data demonstrate that VPS33B binds directly to $\beta 3$ and plays an important role in $\alpha \text{IIb}\beta 3$ outside-in signaling. This role does not involve granule secretion, because in recombinant CHO cells that are not generally thought to undergo regulated exocytosis, overexpression of VPS33B potentiated $\alpha \text{IIb}\beta 3$ -dependent cell spreading. Shen, *et al.* recently reported that the EEE motif of $\beta 3$ integrins (residue 731~733) is required for $\text{G}\alpha_{13}$ binding and $\alpha \text{IIb}\beta 3$ outside-in signaling. We show that the proximal domain of integrin $\beta 3$ subunit, residues 716~730, is critical for binding to VPS33B. Interestingly, unlike $\text{G}\alpha_{13}$, which promotes cell spreading through c-Src-dependent RhoA inhibition and negatively regulates clot retraction, VPS33B is required for both cell spreading and clot retraction, which is independent of Src signaling. Our data suggest that VPS33B regulates cell spreading and clot retraction likely through Rac1 and RhoA, respectively. Integrin outside-in signaling plays an important role for irreversible platelet aggregation and secretion. The findings that platelet aggregation becomes reversible in response to low dose agonists and ATP release is diminished in VPS33B deficient platelets support a role of VPS33B in integrin outside-in signaling. Consistent with the *in vitro* findings, VPS33B is important for *in vivo* thrombosis and hemostasis.

Platelet α -granules are crucial not only for thrombosis and hemostasis, but also for inflammation, atherosclerosis, antimicrobial host defense, wound healing, angiogenesis, and malignancy. Aside from its well-recognized roles in platelet aggregation and adhesion, $\alpha \text{IIb}\beta 3$ also acts as a receptor that mediates endocytosis of fibrinogen⁴⁰⁻⁴² as well as fibronectin⁴³. However, the molecular mechanism underlying the $\alpha \text{IIb}\beta 3$ -mediated endocytosis and subsequent incorporation into α granules has remained unidentified. VPS33B is part of the Class C core with VPS11, 16B, and 18, which form the CORVET/HOPS complex that is important for phagocytosis and early endosome fusion reactions¹⁷. Our data indicate that fibrinogen endocytosis by platelets was diminished upon deletion of VPS33B, suggesting that VPS33B is required either for $\alpha \text{IIb}\beta 3$ -mediated endocytosis and/or subsequent trafficking of fibrinogen to α granules. Despite the lack of a detailed mechanism, the data presented here and the phenotype of the ARC syndrome patients suggest an intersection in the trafficking of *de novo* synthesized and endocytosed α granule cargo at a point controlled by VPS33B. This intersection also appears to contribute to integrin function in platelets. Deletion of fibrinogen affects platelet P-selectin levels⁴². Interestingly, although fibrinogen level in the VPS33B knockout platelets is much lower than that of wild-type platelets, platelet P-selectin levels in the VPS33B knockout mice are normal. It has been reported that plasma fibrinogen and the engagement of fibrinogen γ chain's C-terminus with $\beta 3$ is important for the platelet P-selectin synthesis⁴². In VPS33B knockout mice, although platelet fibrinogen is significantly reduced, plasma fibrinogen level is normal (data not shown) and the interaction between fibrinogen and platelet $\alpha \text{IIb}\beta 3$ is not affected, since deletion of VPS33B did not affect $\alpha \text{IIb}\beta 3$ ligand binding. Besides, unlike the fibrinogen knockout mice, VPS33B knockout platelets still had some fibrinogen. The small amount of fibrinogen might be sufficient for maintenance of P-selectin in platelets.

ARC syndrome patients often suffer recurrent infections⁴⁴. Binding of VPS33B to other integrins such as $\beta 1$ integrins could affect integrin activation in leukocytes. Therefore, our results not only for the first time establish vesicle trafficking proteins as an important novel

class of modifiers of integrin function in platelets and cells, but may also provide insight into the molecular mechanisms of ARC syndrome.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Binding of VPS33B to integrin β subunits. **A**, Colocalization of α IIb β 3 and VPS33B in recombinant CHO cells expressing α IIb β 3 (A5) and FLAG-tagged VPS33B. Cells were stained with a FITC-labeled anti-FLAG monoclonal antibody and a PE-labeled anti- α IIb monoclonal antibody. **B**, Lysates from A5 cells infected with a null virus or an adenovirus-containing FLAG-tagged VPS33B (FLAG-VPS33B+) were immunoprecipitated with anti-FLAG M2 Affinity Gel. Precipitated proteins were immunoblotted with a monoclonal antibody (SZ-21) to $\beta 3$ and a monoclonal antibody to FLAG. **C**, Lysates from A5 cells

infected with a null virus or an adenovirus-containing FLAG-tagged VPS33B were immunoprecipitated with SZ-21 or mouse IgG control. Precipitated proteins were immunoblotted with a polyclonal antibody to $\beta 3$ and a monoclonal antibody to FLAG. **D**, Purified GST, GST- $\beta 3$ CD, GST- $\beta 1$ CD, or GST- α IIb CD bound to glutathione beads was mixed with lysates from CHO cells infected with an adenovirus-containing FLAG-tagged VPS33B. Bound proteins were immunoblotted with an antibody to FLAG. **E**, VPS33B protein purified from CHO cells expressing the FLAG-tagged VPS33B was incubated with GST, GST- $\beta 3$ CD, GST- $\beta 1$ CD, or GST- α IIbCD bound glutathione beads. Bead-bound proteins were immunoblotted with antibody to FLAG. **F**, The sequence of human integrin $\beta 3$ cytoplasmic domain fragments. **G**, Lysates from CHO cells expressing FLAG-tagged VPS33B were incubated with indicated GST- $\beta 3$ CD fragment-bound glutathione beads. Bead-bound proteins were immunoblotted with antibody to FLAG. **H**, A5 cells or A5 cells stably expressing the talin head domain were infected with a null virus or an adenovirus-containing FLAG-tagged VPS33B (FLAG-VPS33B+) and lysed. The lysates were immunoprecipitated with the anti-FLAG M2 Affinity Gel. Precipitated proteins were immunoblotted with a polyclonal antibody against $\beta 3$. **I**, CHO cells were transfected with various HA-tagged VPS33B (aa 1–145 or aa 146–615) and lysed. The lysates were incubated with GST- $\beta 3$ CD fragment-bound glutathione beads. Bead-bound proteins were immunoblotted with an antibody against HA. **J**, Human platelet lysates were incubated with GST, GST- $\beta 3$ CD, GST- $\beta 1$ CD, or GST- α IIbCD bound glutathione beads. Bead-bound proteins were immunoblotted with antibody to VPS33B. **K**, Human platelet lysates were incubated with indicated GST- $\beta 3$ CD fragment-bound glutathione beads. Bead-bound proteins were immunoblotted with a rabbit anti-VPS33B polyclonal antibody. **L**, Human platelet lysates were incubated with a rabbit anti-VPS33B polyclonal antibody. Precipitated proteins were immunoblotted with SZ-21. **M**, Human platelets were stimulated either with or without thrombin (0.1U/ml) for 5 min in aggregometer and were lysed. The lysates were incubated with a rabbit anti-VPS33B polyclonal antibody or a rabbit IgG. Precipitated proteins were immunoblotted with SZ-21.

Figure 2.

The role of VPS33B in integrin α IIb β 3 “outside-in” signaling in CHO cells. **A**, Cell surface expression of α IIb β 3 in A5 cells was determined by flow cytometry with SZ-21 at 48 hours after infection of a null virus or an adenovirus-containing VPS33B cDNA. **B** and **C**, Spreading of A5 cells infected with a null virus or an adenovirus-containing VPS33B cDNA on fibrinogen (50 μ g/ml) or poly-L-lysine. Cell area was normalized to cells infected with a null virus spreading on fibrinogen (mean $\pm$ SEM, n=148–168, * P <0.0001). **D**, Fibrinogen

binding to $\alpha\text{IIb}\alpha6\beta3\beta1$ -expressing $\alpha\beta\text{Py}$ cells or A5 cells infected with a null virus or an adenovirus-containing VPS33B cDNA ($n=4$, $p>0.05$).

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Figure 3.

Generation of platelet-specific VPS33B deficient mice and the role of VPS33B in platelet spreading on fibrinogen. **A**, Targeted disruption of VPS33B gene. Top: structure of VPS33B gene. Upper middle: VPS33B gene targeting vector, containing a pMC-Neo gene that is flanked with VPS33B genomic sequence and PGK-TK that is cloned 3' to the VPS33B sequence. Lower Middle: the mutated locus showing that exons 2 and 3 of VPS33B gene were flanked with LoxP sites. Bottom: pMC-Neo gene and exons 2 and 3 were removed by crossbreeding of the VPS33B flox mice with specific Cre transgenic mice. **B**, Detection of

VPS33B in mouse platelet lysates by Western blot with a polyclonal antibody against VPS33B. CHO cells infected with a null adenovirus or a virus containing a cDNA of mouse VPS33B were used to confirm the specificity of the anti-VPS33B antibody. **C**, Washed platelets from VPS33B^{+/+} and VPS33B^{-/-} mice were incubated with a FITC-labeled rat anti-mouse β 3 monoclonal antibody or FITC-labeled control IgG for 30 min at 22°C and analyzed by flow cytometry. Data shown are representative of three independent experiments. **D** and **E**, Platelet spreading on fibrinogen. Area of platelets from 3 random fields was normalized to wild-type platelets spreading on fibrinogen for 30 min shown in **E** (mean $\pm$ SEM, n=287–321, * P <0.0001).

Figure 4.

The role of VPS33B in clot retraction, integrin inside-out signaling, and granule biosynthesis. **A** and **B**, Platelet-poor plasma was mixed with 3×10^8 /mL washed platelets from VPS33B^{+/+} (WT) and VPS33B^{-/-} mice. Coagulation was induced by adding 0.4 U/mL thrombin, and clots were allowed to retract at 37°C and photographed. Two-dimensional retraction of clots in panel **A** was measured using MetaMorph® microscopy automation & image analysis software and expressed as percent retraction (**B**) (mean $\pm$ SD, $n=3$, * $P < 0.001$ vs WT). **C** and **D**, Thrombin-induced fibrinogen binding to VPS33B^{+/+} and VPS33B^{-/-}

platelets. Quantitative results were expressed as fibrinogen binding indices (median of fluorescence intensity of stimulated platelets (Throm)/median of fluorescence intensity of unstimulated platelets (CON) (mean $\pm$ SD, n=3, p>0.05). **E** and **F**, Detection of fibrinogen, VWF, P-selectin in platelet lysates from VPS33B^{-/-} and VPS33B^{+/+} (WT) mice by Western blot. Densitometry measurements from results in panel E were shown in F. Values were normalized with respect to wild-type control for each immunoblot and are expressed as relative amount (mean SD, n=3, *P<0.001 vs WT). **G**, Detection of fibronectin in platelet lysates from VPS33B^{-/-} and VPS33B^{+/+} mice by Western blot., **H~J**, Total PF4 (**H**), serotonin (**I**), and β -hexosaminidase (**J**) in 1×10^9 VPS33B^{-/-} and VPS33B^{+/+} platelets were measured (mean $\pm$ SD, n=3, p>0.05).

Figure 5.

Agonists-induced platelet secretion and aggregation are attenuated in VPS33B deficient platelets. **A** and **B**, Washed platelets from wild-type (VPS33B^{fl/fl}) or VPS33B deficient mice (VPS33B^{fl/fl}/Cre) were stimulated with various concentrations of thrombin (**A**) or collagen (**B**) in a lumi-aggregometer at 37°C. Real-time ATP secretion and platelet aggregation were recorded simultaneously. **C** and **D**, Platelet extracts from VPS33B^{+/+} and VPS33B^{-/-} mice were solubilized in SDS-PAGE sample buffer and analyzed by SDS-PAGE on 4–15% gradient gels and immunoblotted using the indicated antibodies. Immunoblotting results in

panel **C** were quantitated and normalized with respect to RabGDI. Quantitative results are expressed as ratios (normalized values of VPS33B^{-/-} samples/normalized values of VPS33B^{+/+} samples, mean $\pm$ SD, n=3, p>0.05). **E** and **F**, VPS33B^{-/-} and VPS33B^{+/+} male mice were injected intravenously with 5 mg/mouse biotin-labeled human fibrinogen. Platelets were isolated at 24 hours after injection of fibrinogen. Biotin-labeled fibrinogen in platelet lysates was analyzed by immunoblotting with HRP-conjugated avidin (n=3, p<0.01).

Figure 6.

VPS33B promotes α IIb β 3 outside-in signaling through RhoA- and Rac1-dependent pathways. **A**, Washed platelets from VPS33B^{+/+} (WT) and VPS33B^{-/-} mice were added to polystyrene dishes coated with 50 μ g/mL fibrinogen (Fg) or buffer and incubated at 37°C for 30 or 60 min. Adherent platelets were solubilized and phosphorylation of MLC were analyzed by Western blot analysis. Data represent mean $\pm$ SD of 3 independent experiments, *P < 0.01 versus WT at the same time point. **B**, Washed platelets from VPS33B^{+/+} and VPS33B^{-/-} mice were added to polystyrene dishes coated with 50 μ g/mL fibrinogen and

incubated at 37°C for 15 or 30 min and solubilized. RhoA activity was determined as described in Supplemental Methods. Data represent mean±SD of 3 independent experiments, *P < 0.01 versus WT at the same time point. **C**, A5 cells infected with a null virus or an adenovirus-containing FLAG-tagged VPS33B were added to polystyrene dishes coated with 50 µg/mL fibrinogen (Fg) or buffer and incubated at 37°C for 30, 60, or 90 min. Adherent cells were solubilized and phosphorylation of MLC were analyzed by Western blot analysis. **D**, A5 cells infected with a null virus or an adenovirus-containing FLAG-tagged VPS33B were added to polystyrene dishes coated with 50 µg/mL fibrinogen (Fg) or buffer and incubated at 37°C for 30, 60, or 90 min. RhoA activity was determined as described in Supplemental Methods. **E** and **F**, A5 cells infected with a null virus or an adenovirus-containing VPS33B cDNA were pre-treated with vehicle or NSC23766 (100 µM) and then added to fibrinogen-coated slides. Cells were stained with Rhodamine-Phalloidin and photographed using a fluorescence microscope. Cell area was normalized to cells infected with a null virus spreading on fibrinogen (mean ± SEM, n=147–167, *P<0.0001). **G**, Washed platelets from VPS33B^{+/+} (WT) and VPS33B^{-/-} mice were added to polystyrene dishes coated with 50 µg/mL fibrinogen (Fg) or buffer and incubated at 37°C. Rac1 activity was determined as described in Supplemental Methods. Data represent mean ±SD of 3 independent experiments, *P < 0.01 versus WT at the same time point.

Figure 7.

VPS33B mice have prolonged tail-bleeding times and impaired thrombus formation. **A**, Mouse tail bleeding times were determined using methods described in the Supplemental Methods. Note that although the variance of bleeding times were broad in both wild-type mice ($n = 17$) and VPS33B deficient mice ($n = 16$), the difference between groups is statistically significant ($P < 0.02$). **B**, FeCl₃-induced carotid artery injury was performed and time to occlusive thrombus formation recorded as described in the Supplemental Methods. The bars represent the median occlusion time (539 seconds for VPS33B^{fl/fl}/Cre mice, and 350 seconds for VPS33B^{fl/fl} mice ($n = 23$, $P < 0.01$).