A Disease Mechanism Underlying Bleeding in Wiskott-Aldrich Syndrome

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Abstract: The Wiskott-Aldrich Syndrome (WAS) is an X chromosome-linked immunodeficiency disorder. The most common symptom in WAS is bleeding. Several clinical investigations indicate that low platelet counts and defective platelet aggregation are the major causes of bleeding in WAS patients. However, the molecular bases underlying these defects are unclear. This study focuses on the molecular mechanism of defective platelet aggregation of WAS patients. The gene responsible for WAS encodes WAS protein (WASP). The mutations or deletion of WASP causes various functional defects in hematopoietic cells. We previously showed that binding of WASP to calcium- and integrin-binding protein (CIB) is required for activation of platelet integrin, αIIbβ3. I here demonstrate that blocking WASP binding to CIB reduces binding of talin to the β3 cytoplasmic tail, resulting in impaired activation of αIIbβ3. Impaired αIIbβ3 activation causes defective platelet aggregation, resulting in bleeding. This finding suggests a potential disease mechanism underlying bleeding seen in WAS patients.

Keywords: Wiskott-Aldrich Syndrome, WASP, bleeding, platelet aggregation, αIIbβ3 activation

Introduction

The Wiskott-Aldrich Syndrome (WAS) is an X chromosome-linked immunodeficiency disorder. It is characterized by a low number of platelets, small platelets, eczema, recurrent infections, and increased risk for autoimmunity and malignancy (Aldrich et al. 1954; Nonoyama and Ochs, 1998; Thrasher, 2002; Wiskott, 1937). The gene responsible for this disorder has been identified and it turned out that it encodes a 62 kDa-cytosolic protein, Wiskott-Aldrich Syndrome protein (WASP) (Derry et al. 1994). WASP expression is restricted to hematopoietic cells and WASP plays an important role in the assembly of actin cytoskeleton (Higgs and Pollard, 2000; Miki et al. 1998), in signal transduction (Zeng et al. 2003; Zhang et al. 1999) and in apoptosis (Rawlings et al. 1999; Rengan and Ochs, 2000).

Several distinct phenotypes caused by mutations in WASP gene have been reported, including classic WAS, X-linked thrombocytopenia (XLT) (Nonoyama and Ochs, 1998; Thrasher, 2002), X-linked neutropenia (XLN) (Devriendt et al. 2001) or X-linked myelodysplasia (XLM) (Thrasher, 2002). In classic WAS patients, WASP expression is undetectable and severe bleeding, eczema and recurrent infections are observed. Most XLT patients have missense mutations found in the WASP N-terminus (within the residues 1–137), and express the mutant proteins at a lower level than normal controls. Bleeding is the only symptom seen in XLT patients (Imai et al. 2003; Zhu et al. 1997).

Bleeding is thus the most common symptom seen in all the WAS patients with classic WAS and XLT, except for two families with XLN and XLM. It was thought that a low platelet numbers was a caused of bleeding. However, several clinical observations indicate that the low platelet count is not a sole cause and a functional defect in platelet aggregation is also a major cause of bleeding in WAS (classic WAS and XLT) patients. For instance, after splenectomy, platelet counts increase to almost normal levels in WAS and XLT patients, but bleeding tendency is not completely resolved (Mullen et al. 1993).

We hypothesized that such a defect in platelet aggregation is caused by a functional deficiency of the WASP N-terminal region, as bleeding is observed in both WASP-deficient patients (classic WAS) and XLT patients with missense mutations occurring in the WASP N-terminus. We identified calcium- and integrin-binding protein (CIB) as a protein binding to the WASP N-terminus in platelets and demonstrated that the increase in the affinity of platelet integrin, αIIbβ3 for its ligand (αIIbβ3 activation) was impaired, when WASP binding to CIB was blocked in platelets (Tsuboi et al. 2006). We also

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demonstrated that WASP binding to CIB is reduced due to mutations in WAS and XLT patients and that such reduced binding causes impaired αIIbβ3 activation, resulting in defective platelet aggregation (Tsuboi et al. 2006).

In the present study, I further characterize WASP binding to CIB and show how reduced binding of WASP to CIB causes impaired αIIbβ3 activation in platelets.

Materials and Methods

Platelets

After informed consent was obtained, blood specimen from healthy volunteers were collected. Platelet isolation and fluorescence activated cell sorter (FACS) analysis were performed as previously described (Tsuboi et al. 2006). To permeabilize platelets, platelets were incubated for 10 min at 4 °C with 5–15 U/ml of streptolysin O (SLO) (Sigma, St. Louis, Missouri, USA). Permeabilization was confirmed by incubation with a fluorescein isothiocyanate (FITC)-labeled control protein, soybean trypsin inhibitor (Sigma) and monitoring uptake of labeled protein by FACS analysis. Platelets were stimulated for 15 min at 22 °C with 1.0 U/ml of thrombin without stirring. The binding of FITC-labeled PAC-1, an αIIbβ3-specific and activation-dependent monoclonal antibody (Shattil et al. 1985) (Becton Dickinson Immunocytometry Systems, San Jose, California, USA) was analyzed by FACS analysis in the presence or absence of 100 μM of RGD-containing peptide (GRGDSP) (Invitrogen, Carlsbad, California, USA) to block integrin clustering. Specific binding of PAC-1 to activated αIIbβ3 in platelets was defined as binding inhibited by RGD-containing peptide. The Internal Review Boards of Burnham Institute for Medical Research approved these experiments.

Recombinant proteins

The FLAG-tagged CIB N-terminal fragment (residues 1–113) and a control protein, calcyclin were expressed and prepared as previously described (Tsuboi et al. 2006). As a control, we used calcyclin because it is a small calcium-binding protein, similar to CIB (Murphy et al. 1988) and does not interact with WASP. A full-length FLAG-tagged CIB (CIB-FLAG), its myristoylation site deletion mutant (G2A), a full-length Myc-tagged WASP (Myc-WASP) and its N-terminus deletion mutant (residues 106–502) were expressed in human embryonic kidney (HEK) 293 cells by transfection using SuperFect (QIAGEN, Valencia, California, USA).

Immunoprecipitation

For co-immunoprecipitation of Myc-WASP with CIB-FLAG from HEK293 transfectant cells, cells (2×10^8 cells) were lysed in lysis buffer A (50 mM Tris-HCl, pH7.5, 150 mM NaCl, 1% Triton X-100 and proteinase inhibitors including 1 mM phenylmethyl sulfonyl fluoride (PMSF), 1 μg/ml leupeptin, 1 μg/ml pepstatin A and 1 μg/ml aprotinin). The lysates were centrifuged at 10,000 g at 4 °C for 15 min and the supernatant was incubated with anti-FLAG (M2)-agarose (Sigma, 40 μl of 50% slurry) at 4 °C for 1 h. The agarose resin binding the immune complex was washed with 0.5 ml of lysis buffer A three times and the complex was eluted with 1 × Laemmli's SDS-PAGE sample buffer. The eluted proteins were subjected to SDS-PAGE and analyzed by immunoblotting using anti-FLAG (M2) (Sigma) and anti-Myc (9E10) (Santa Cruz Biotechnology Inc, Santa Cruz, California, USA) monoclonal antibodies. For co-immunoprecipitation of talin with the β3 subunit of αIIbβ3, platelets were lysed by the addition of an equal volume of 2 × lysis buffer B (40 mM Tris-HCl, pH7.5, 300 mM NaCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 0.02% NaN_3, 2% CHAPS and proteinase inhibitors). Anti-β3 subunit polyclonal antibody (A1932) (Chemicon, Temecula, California, USA) and protein A-Sepharose (Pierce, Rockford, Illinois, USA) were used for immunoprecipitation. The resin was washed by washing buffer B (the same as lysis buffer B except the concentration of CHAPS was 0.1%) and the immune complex was eluted with 1 × sample buffer. Anti-β3 subunit polyclonal antibody (N-20) (Santa Cruz Biotechnology Inc.) and anti-talin monoclonal antibody (MAB1676) (Chemicon) were used for immunoblotting. For co-immunoprecipitation of actin with talin, platelets were lysed by the addition of an equal volume of 2 × lysis buffer C (40 mM Tris-HCl, pH7.5, 300 mM NaCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 50 mM n-octyl β-glucopyranoside and proteinase inhibitors). Anti-talin monoclonal antibody (8d4) (Sigma) was used for immunoprecipitation and anti-actin polyclonal antibody (Sigma) was used for immunoblotting. Anti-talin antibodies, MAB1676 and 8d4 recognize with the head

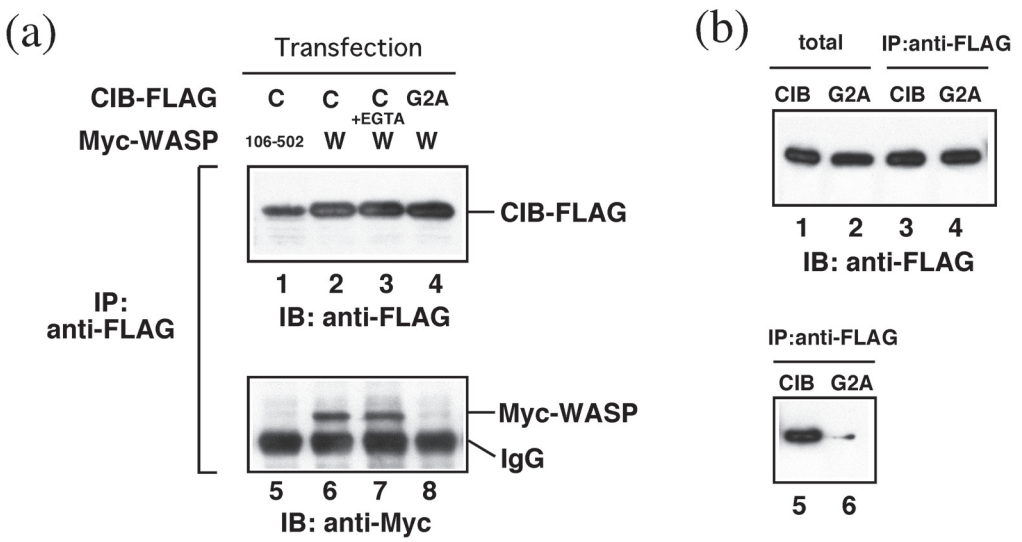

Figure 1. Characterization of binding of WASP to CIB. (**a**) HEK 293 cells were transfected with FLAG-tagged CIB and Myc-tagged WASP constructs. Cells were lysed and subjected to immunoprecipitation with anti-FLAG monoclonal antibody (M2). Proteins were immunoblotted using anti-FLAG (lanes 1–4) and anti-Myc (lanes 5–8) antibodies. C, FLAG-tagged CIB; G2A, myristoylation site deletion mutant of CIB; W, Myc-tagged WASP (full-length); 102–502, N-terminus deletion mutant of Myc-tagged WASP (residues 106–502); +EGTA, in the presence of Ca^{2+} chelator (2 mM of EGTA). (**b**) HEK 293 cells transfected with FLAG-tagged CIB (CIB) and its myristoylation site deletion mutant (G2A) were incubated with ^{3}H-myristate and the cell lysates were subjected to immunoprecipitation with anti-FLAG antibody (lanes 1–4) followed by SDS-PAGE and fluorography (lanes 5 and 6).

and tail group of talin, respectively. ECL plus Western Blotting Detection System (GE Healthcare Lifescience, Piscataway, NJ) was used for detection of the proteins.

Results

A platelet major integrin,αIIbβ3 plays a primary role in platelet aggregation. For platelet aggregation, αIIbβ3 converts its extracellular conformation from a low affinity to a high affinity state for its ligand in response to platelet agonists such as thrombin (αIIbβ3 activation). αIIbβ3 activation is a key process of platelet aggregation. CIB was originally cloned as a protein binding to the cytoplasmic tail of the αIIb subunit of αIIbβ3 (Naik et al. 1997). I demonstrated that CIB activates αIIbβ3 in platelets (Tsuboi, 2002). We also demonstrated that WASP is a binding partner of CIB and binding of WASP to CIB is required for αIIbβ3 activation (Tsuboi et al. 2006). For further characterization of WASP binding to CIB, I used transfectant cells expressing WASP, CIB or their mutant proteins.

HEK293 cells were transfected with the C-terminally FLAG-tagged CIB and N-terminally Myc-tagged WASP constructs (Fig. 1(a)). CIB was immunoprecipitated with anti-FLAG antibody from the lysates of the HEK293 transfectants. The full-length WASP (Myc-WASP) co-immunoprecipitated with CIB (CIB-FLAG) (Fig. 1(a), lanes 2,6), but the N-terminus-deleted WASP (residues 106–502) did not (Fig. 1(a), lanes 1,5), since the CIB binding site of WASP resides in the WASP N-terminal region (residues 1–105) (Tsuboi et al. 2006).

CIB has two calcium binding sites (EF-hand I and II) (Naik et al. 1997). To determine if WASP binding to CIB requires Ca^{2+}, CIB was immunoprecipitated with anti-FLAG monoclonal antibody from the lysates of the transfectant HEK293 cells in the presence of Ca^{2+} chelator (2 mM ethyleneglycol tetra acetic acid (EGTA) (Sigma)) (Fig. 1(a), lane 3). WASP co-immunoprecipitated with CIB in the both presence and absence of the Ca^{2+} chelator (Fig. 1(a), lanes 6, 7), indicating that WASP binding to CIB does not require binding of Ca^{2+} to CIB.

CIB also has a potential myristoylation site at the N-terminus (residue 2, Glycine) (Naik et al. 1997). To determine if the N-terminal myristoylation of CIB is required for binding to WASP, I examined if non-myristoylated CIB bound to WASP in cells. I substituted Glycine (residue 2) with Alanine to make a non-myristoylated CIB (G2A). As FLAG-tagged CIB constructs, I used the C-terminally FLAG-tagged CIB constructs, because the N-terminually tagged FLAG may inhibit efficient N-myristoylation. To confirm that CIB is N-myristoylated in cells, the HEK293 transfectant cells were incubated with ^{3}H-myristate in the presence of cerulenin (Sigma) to

prevent endogenous fatty-acid biosynthesis as described (Cyert and Thorner, 1992). Lysates were prepared and subjected to immunoprecipitation with anti-FLAG antibody (Fig. 1(b), lanes 1–4). The resultant immune complexes were resolved by SDS-PAGE and subjected to fluorography (Cyert and Thorner, 1992). Incorporation of ^{3}H-myristate into CIB was detected (Fig. 1(b), lane 5), but that into G2A was not detected (Fig. 1(b), lane 6). These results confirm that CIB is N-myristoylated in cells but that G2A is not. Substitution of Glycine (residue 2) with Alanine prevents the N-terminal myristoylation of CIB. When non-myristoylated CIB was immunoprecipitated from the transfectant HEK293 cells (Fig. 1(a), lane 4), WASP was not detected in the immune complex (Fig. 1(a), lane 8), indicating that the N-myristoylation of CIB is required for WASP binding to CIB.

We previously showed that blocking binding of WASP to CIB reduced αIIbβ3-mediated cell adhesion (Tsuboi et al. 2006). To mediate cell adhesion, αIIbβ3 converts its extracellular conformation from a low affinity to a high affinity state for its ligand (αIIbβ3 activation). To determine if WASP binding to CIB is required for this conformational change, I examined if αIIbβ3 activation was affected when WASP binding to CIB was blocked in stimulated platelets. To block the binding, I permeabilized platelets and introduced the CIB N-terminal fragment to block WASP binding to CIB as previously described (Tsuboi et al. 2006), and then stimulated platelets with thrombin and examined binding of PAC-1, specific to a high affinity state of αIIbβ3 (Shattil et al. 1985). This method was utilized because platelets are not amenable to transfection. PAC-1 binding to stimulated platelets was inhibited by introduction of the CIB N-terminal fragment into platelets (Fig. 2), indicating that αIIbβ3 activation was impaired when WASP binding to CIB was blocked. This result suggests that WASP binding to CIB plays a critical role in the conformational change of αIIbβ3 to acquire the high affinity state for its ligand.

It has been recently demonstrated that a cytoskeletal protein, talin binds to the β3 cytoplasmic tail and activates αIIbβ3 (Garcia-Alvarez et al. 2003; Tadokoro et al. 2003). To determine the mechanism underlying impairment of αIIbβ3 activation when WASP binding to CIB was blocked, we assayed talin that binds directly to the β3 cytoplasmic tail of αIIbβ3. The β3 subunit was immunoprecipitated with anti-β3 polyclonal antibody (Fig. 3, lanes 9–12). Talin co-immunoprecipitated with the β3 subunit, indicating that talin bound to the β3 cytoplasmic tail in stimulated platelets (Fig. 3, lanes 9,13). SLO-permeabilization did not affect this binding (Fig. 3, lanes 10,14). The amount of talin co-immunoprecipitated with β3 was significantly reduced when SLO-permeabilized platelets were incubated with the CIB N-terminal fragment to block WASP binding to CIB, but not with the control protein (Fig. 3, lanes 15,16). There is no significant difference in the levels of the β3 and talin in total lysates before and after blocking WASP binding to CIB (Fig.3, lanes 1–8). These results, taken together, indicate that talin binding to the β3 tail was reduced by blocking WASP binding to CIB.

CIB, an αIIb cytoplamic tail binding protein, co-immunoprecipitated with the αIIb subunit. We previously showed that there is no significant difference in the amount of co-immunoprecipitated CIB with αIIb before and after blocking WASP binding to CIB (Tsuboi et al. 2006), indicating that CIB binding to the αIIb tail was not affected by blocking WASP binding to CIB. These results suggest that WASP binding to CIB is necessary for talin binding to the β3 cytoplasmic tail but not for CIB binding to the αIIb cytoplasmic tail.

Talin binds to actin to form the linkage between the β3 tail and actin cytoskeleton. I next asked if talin binding to actin was affected when WASP binding to CIB was blocked, as the formation of the linkage between the β3 tail and actin cytoskeleton is also essential for αIIbβ3 activation (Garcia-Alvarez et al. 2003; Tadokoro et al. 2003). Talin was immunoprecipitated with anti-talin monoclonal antibody. Actin co-immunoprecipitated with talin (Fig. 3, lane 21). There is no significant difference in the amount of co-immunoprecipitated actin with talin before and after blocking WASP binding to CIB (Fig. 3, lanes 21–24). Talin binding to actin was not affected by blocking WASP binding to CIB. I also showed that blocking WASP binding to CIB does not affect actin polymerization (Tsuboi et al. 2006). These findings, taken together suggest that blocking WASP binding to CIB reduced talin binding to the β3, resulting in impaired αIIbβ3 activation.

Discussion

In the present study, I found that the N-terminal myristoylation of CIB is required for WASP binding

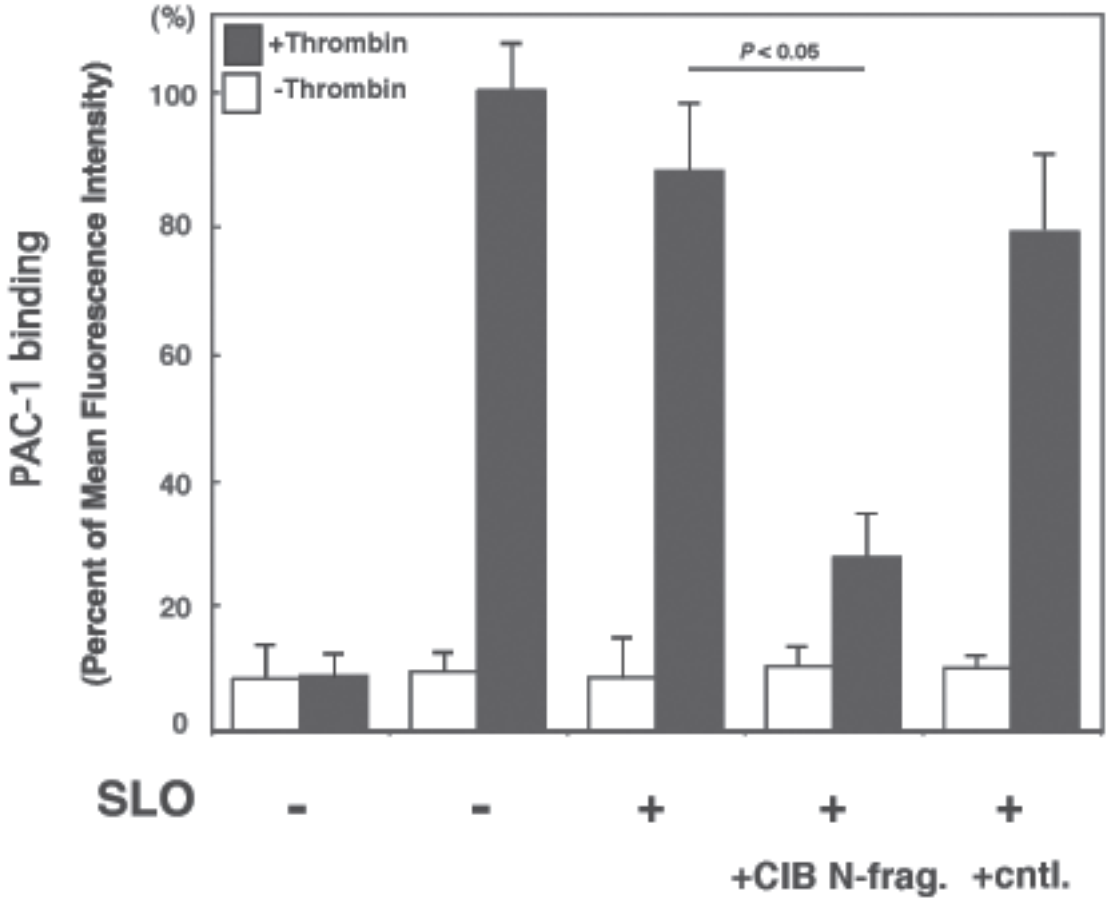

Figure 2. Blocking WASP binding to CIB impairs αIIbβ3 activation. SLO-permeabilized platelets (8×10^7 cells) were incubated with 100 ng of the FLAG-tagged CIB N-terminal fragment or FLAG-tagged calcyclin as a control protein, and then binding of FITC-labeled PAC-1 (a monoclonal antibody specific to high affinity state of αIIbβ3) to platelets with thrombin stimulation (closed bars) or without thrombin stimulation (open bars) was measured by a flow cytometer. As a semi-quantitative estimate of fluorescence, binding was expressed as percent M. F. I. (mean fluorescence intensity). Data represent the mean ± S.D. of triplicate measurements.

to CIB, but Ca^{2+} binding to CIB is not (Fig. 1). These results suggest that WASP recognizes with the myrisoylated N-terminal region of CIB to form the complex. In fact, the WASP binding site of CIB resides in the CIB N-terminus (residues 1–113) (Tsuboi et al. 2006). And also, the CIB N-terminus does not contain Ca^{2+} binding sites, which is consistent with the result that WASP binding to CIB does not require Ca^{2+} binding to CIB.

Furthermore, I demonstrated that blocking WASP binding to CIB reduced talin binding to the β3 cytoplasmic tail that is a key process of αIIbβ3 activation (Fig. 3). These results suggest that the formation of the WASP-CIB complex is necessary for talin binding to the β3 tail in thrombin-stimulated platelets.

Based on these observations, I propose a possible model for the role of the WASP-CIB complex in αIIbβ3 activation (Fig. 4(a)). CIB binds to both the αIIb tail and the WASP N-terminus. The WASP C-terminus binds to the Arp2/3 (actin-related proteins) complex to stimulate actin polymerization (Higgs and Pollard, 2000; Miki et al. 1998). Talin binds to both actin and the β3 cytoplasmic tail to form the linkage between the β3 tail and actin cytoskeleton. Talin binds to the β3 tail and activates αIIbβ3 by disrupting the interaction between the αIIb and β3 cytoplasmic tails (Garcia-Alvarez et al. 2003; Tadokoro et al. 2003). I propose that the role of CIB in αIIbβ3 activation is to tether WASP to the αIIb tail (Fig. 4(a)). WASP tethered to the αIIb tail by CIB interacts with the Arp2/3 complex and mediates the assembly of the actin cytoskeleton at the site of the αIIbβ3 cytoplasmic tails. The actin cytoskeleton is thus recruited to the site of the αIIbβ3 cytoplasmic tails, facilitating talin binding to the β3 cytoplasmic tail. Blocking WASP binding to CIB could change

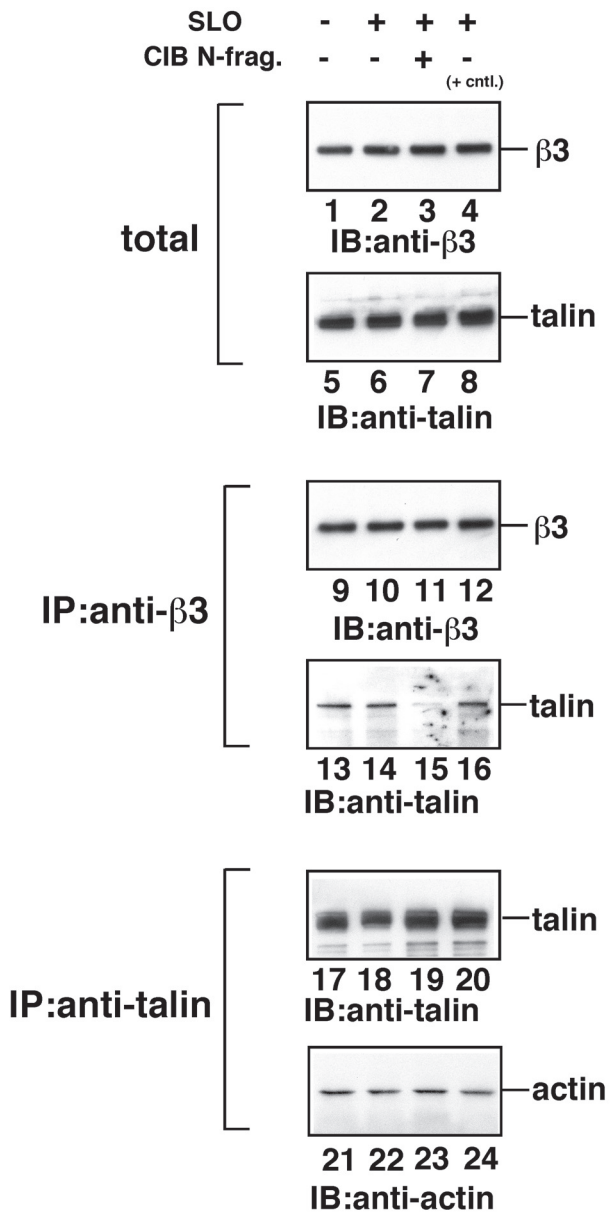

Figure 3. Blocking WASP binding to CIB reduces talin binding to the β3 tail. SLO-permeabilized platelets (2×10^8 cells) were incubated with 200 ng of the CIB N-terminal fragment or control protein (cntl.), and then stimulated with thrombin. The platelets were lysed and the presence of the β3 subunit and talin was detected by immunoblotting in total lysates (lanes 1–8). The β3 subunit was immunoprecipitated with a specific antibody (lanes 9–16). Protein samples prepared from 2×10^7 platelets were analyzed by immunoblotting for β3 (lanes 9–12) and talin (lanes 13–16). Talin was immunoprecipitated with a specific antibody (lanes 17–24) followed by immunoblotting for talin (lanes 17–20) and actin (lanes 21–24).

both localization of WASP and the site of actin polymerization in thrombin-stimulated platelets. Changing the site of actin polymerization may reduce the accessibility of talin to the β3 tail, resulting in reduced binding of talin to the β3 tail (Fig. 3) and impaired αIIbβ3 activation (Fig. 2). CIB plays a key role in this recruitment process by tethering WASP, Arp2/3 complex and actin cytoskeleton to the αIIbβ3 cytoplasmic tails (Fig. 4(a)). Thus, WASP binding to CIB is critical for αIIbβ3 activation.

Yuan et al. proposed a model for the regulation of αIIbβ3 activation involving CIB. Their data suggest that CIB appears to inhibit integrin activation by competing with talin for binding to αIIbβ3 in resting platets (Yuan et al. 2006). However, they provide no evidence that CIB binds to the αIIb cytoplasmic tail in resting platetes. And also, they did not examine if the amount of CIB in platelets are much enough to compete out talin from the β3 tail. The most serious weakness of their study is that they use only a megakaryoblastoid cell line, MEG-01 and that they show no data from the experiments using platelets. Their study is not sufficient for demonstration of what they would like to conclude, since their data are not validated with the experiments using platelets and the megakaryoblastoid cell line, MEG-01 is entirely different from platelets.

On the other hand, just recently Han et al. proposed an interesting model for the regulation of αIIbβ3 activation. Their study established the Rap1-induced formation of an "αIIbβ3 integrin activation complex," containing RIAM, a Rap1 effector and talin (Han et al. 2006). This study did not mention about the involvement of the αIIb cytoplasmic tail in αIIbβ3 activation. I suggested that the formation of the complex containing αIIb, CIB and WASP increases talin accessibility to the β3 cytoplasmic tail (Fig. 4(a)). This complex formation may help the complex containing RIAM and talin to bind to the β3 tail and activate αIIbβ3.

In the previous study, we demonstrated that mutant forms of WASP are expressed at reduced levels or exhibit lower affinities for CIB than wild-type WASP, resulting in impaired αIIbβ3 activation in classic WAS and XLT patients (Tsuboi et al. 2006). Taken together with the present results, I propose a potential disease mechanism underlying bleeding, the most common symptom seen in patients. Reduced levels of mutant WASP expression or lower affinities of mutant WASPs for CIB cause inefficient WASP-CIB complex formation. Inefficient complex formation reduces the accessibility of talin to the β3 tail, resulting in impaired αIIbβ3 activation (Fig. 4(b)). Impaired αIIbβ3 activation accounts for defective platelet aggregation, contributing to the increased and unwanted bleeding in classic WAS and XLT patients.

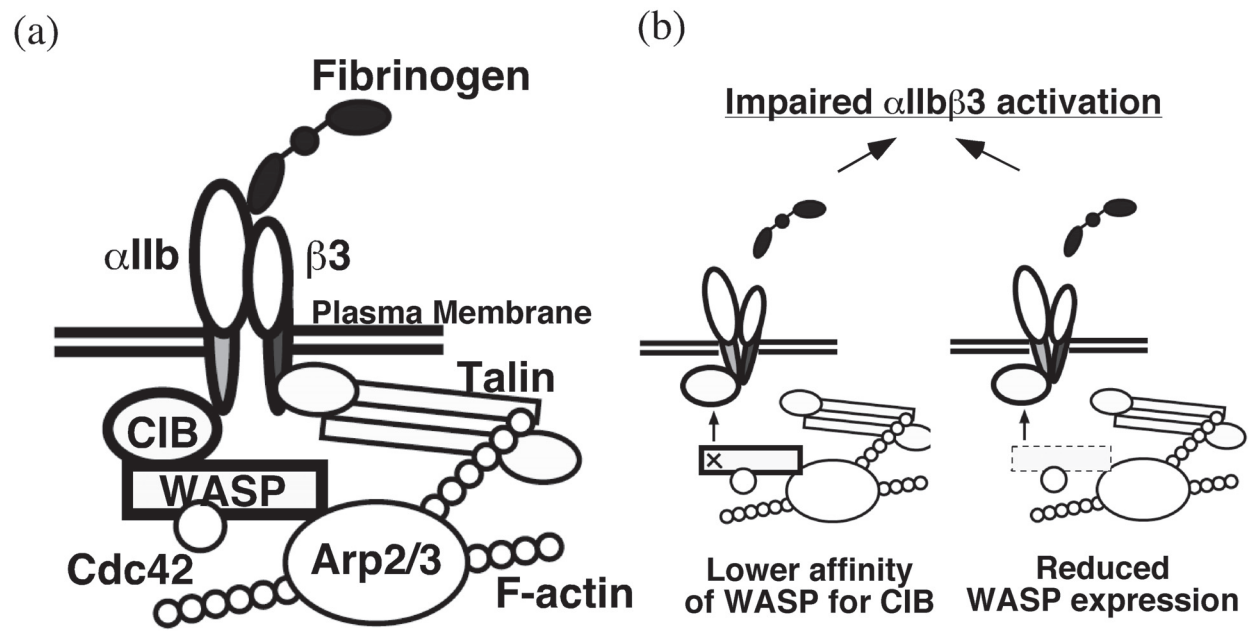

Figure 4. A role of the WASP-CIB complex in αIIbβ3 activation and a disease mechanism of bleeding in WAS. (**a**) A suggested model for the role of the WASP-CIB complex in αIIbβ3 activation. CIB binds to both the αIIb tail and the WASP N-terminus. The WASP C-terminus stimulates actin polymerization. Actin cytoskeleton is recruited to the αIIbβ3 site. The WASP-CIB complex facilitates talin binding to the β3 tail and talin activates αIIbβ3. (**b**) A potential disease mechanism underlying bleeding due to impaired αIIbβ3 activation in WAS patients. Reduced expression of mutant WASPs and/or lower affinity of mutant WASPs for CIB reduce the accessibility of talin to the β3 tail, resulting in impaired αIIbβ3 activation.

In conclusion, I have shown that WASP binding to CIB requires the N-terminal myrisotylation of CIB and that this binding is a critical step leading to the conformational change of αIIbβ3 required for platelet aggregation. Furthermore, I also showed that inefficient formation of the WASP-CIB complex due to mutations reduces talin binding to the β3 tail, resulting in impaired αIIbβ3 activation. The present study not only provides the information on how WASP functions in αIIbβ3 activation but also suggests a potential disease mechanism underlying bleeding in classic WAS and XLT patients.

Acknowledgments

S.T. was supported by National Institute of Health (R01HD42752).

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