SUPPLEMENTARY INFORMATION

SUPPLEMENTARY RESULTS

Direct binding of WASP to CIB

To verify direct interaction of WASP with CIB, we used a surface plasmon resonance-based biosensor, BIACORE 3000 (BIACORE). Binding signals were recorded, when Myc-tagged WASP (Myc-WASP, denoted W in supplementary Fig S1) or the WASP N-terminal fragment (residues 1-105) (WN) was passed over a sensor chip with immobilized FLAG-tagged CIB (CIB-FLAG) (C) as well as with FLAG-tagged CIB N-terminal fragment (residues 1-113) (CN) (supplementary Fig S1). These data indicate that the WASP N-terminus (residues 1-105) binds directly to the CIB N-terminus (residues 1-113).

Binding of WASP to CIB in platelets

CIB was detected only in anti-WASP immunoprecipitates obtained from lysates of stimulated platelets (Fig 1B), indicating that WASP binds CIB in stimulated platelets. To confirm this result, CIB was immunoprecipitated with anti-CIB antibody raised in chickens. WASP was detected only in anti-CIB immunoprecipitates obtained from lysates of stimulated platelets (supplementary Fig S10, lane 6). This result, taken together with Fig 1B confirmed that WASP binds CIB in stimulated platelets upon agonist stimulation.

Time-course of WASP binding to CIB

No significant difference in the expression levels of WASP and CIB between before and after agonists stimulation was observed (supplementary Fig S2A). Platelets were stimulated with ADP or thrombin and lysed by adding an equal volume of 2 x lysis buffer at different time points. WASP was immunoprecipitated from the lysates with anti-WASP antibody followed by immunoblotting for WASP and CIB. The amount of co-immunoprecipitated CIB increased during stimulation in a time-dependent manner (supplementary Fig S2B).

Knockdown of WASP expression in MEG-01 cells

To determine if WASP plays an important role in cell adhesion, WASP expression in MEG-01 cells were silenced by siRNA and its effect on cell adhesion was examined. There are two WASP homologs, WASP and N-WASP expressed in mammalian cells. MEG-01 cells express WASP, but N-WASP expressioin was not detected, indicating that WASP mainly functions in MEG-01 cells (supplementary Fig S11A). MEG-01 cells were co-transfected with siRNA and CIB expression vector. Transfection of siRNA for WASP decreased the expression level of WASP, but barely affect CIB expression (supplementary Fig S11B). When WASP expression was silenced by siRNA in CIB-expressing MEG-01 cells, cell adhesion to fibrinogen was significantly decreased, compared with when transfected with scrambled control

(supplementary Fig S11C). This result indicates that WASP plays an important role in αIIbβ3-mediated cell adhesion.

Blocking WASP-CIB binding affects conformational change of αIIbβ3

We examined binding of a monoclonal antibody, PAC-1, which is specific for the active form of αIIbβ3 to platelets (supplementary Fig S3). Platelets were stimulated with ADP without stirring in the presence (supplementary Fig S3A-F, open histograms) or absence (supplementary Fig S3A-F, closed histograms) of GRGDSP peptide to verify the specificity of PAC-1-binding. Intact platelets became PAC-1-positive upon thrombin stimulation as did most SLO-permeabilized platelets (supplementary Fig S3B, D). When WASP binding to CIB was blocked by the CIB N-terminal fragment, PAC-1 binding to platelets was greatly reduced (supplementary Fig S3E). The control protein, FLAG-calcyclin, barely affected PAC-1 binding (supplementary Fig S3F). These results suggest that formation of the CIB-WASP complex plays an important role in conformational change of αIIbβ3 required to achieve high affinity ligand binding.

Effect of blocking WASP binding to CIB on other cellular events

To test if WASP binding to CIB is involved in other cellular events, we examined activation of another platelet integrin, intracellular calcium mobilization and secretion. We analyzed binding of FITC-labeled collagen to another platelet integrin, α2β1, when WASP binding to CIB was blocked by the CIB N-terminal fragment. Collagen binding to α2β1 in platelets was increased upon thrombin-stimulation (supplementary Fig S4A). Blocking WASP binding to CIB barely affect collagen

binding (supplementary Fig S4B-D). This result suggests that the WASP-CIB complex is not involved in α2β1-mediated cell adhesion, which is consistent with the observation that CIB binds selectively to the αIIb cytoplasmic tail (Naik et al., 1997).

We measured relative calcium concentration in platelets during ADP-stimulation, when WASP binding to CIB was blocked. ADP-induced platelet calcium mobilization was monitored by flowcytometry. Fluo-3 AM-loaded platelets were stimulated with ADP, and Mean fluorescence intensities (MFIs) of Fluo-3 AM were recorded (supplementary Fig S5A). After permeabilization of platelets with SLO and introduction of the CIB N-terminal fragment into platelets, platelets are washed with Tyrode's buffer to remove SLO, and then permeabilized platelets are resealed (Hers et al., 2000). No significant difference in calcium mobilization, when WASP binding to CIB was blocked (supplementary Fig S5A).

To determine if the WASP-CIB complex is involved in platelet secretion, we examined if blocking WASP binding to CIB affects α-granule secretion. Cell surface exposure of P-selectin, an α-granule component was analyzed as an indicator of α-granule secretion. Blocking WASP binding to CIB had no effect on P-selectin exposure (supplementary Fig S5B).

These results suggest that the WASP-CIB complex is not involved in platelet calcium mobilization and secretion.

SUPPLEMENTARY DISCUSSION

Platelets in WASP-deficient mice

In platelets derived from WASP-deficient mice, defective platelet aggregation is not observed (Snapper et al., 1998; Zhang et al., 1999). However, WASP-deficient WAS patients have defective platelet aggregation (Nonoyama and Ochs, 1998). The reason for this difference is likely that in WASP-deficient mice, expression of other WASP-family proteins such as N-WASP and WAVE proteins is high enough to compensate for the absence of WASP, eliminating potential problems with affinity modulation of αIIbβ3. In fact, mouse platelets express N-WASP at approximately 6- to 8-fold higher concentration than human platelets (Falet et al., 2002). Such a compensation for the absence of WASP is the case not only in platelets but also in other hematopoietic cells such as B cells (Snapper et al., 2005).

Effect of blocking WASP binding to CIB on αIIb binding to CIB

No significant difference in the expression levels of αIIb and CIB between before and after platelet stimulation was observed (supplementary Fig S7B, lanes 1-4). And also, no significant difference in the amounts of co-immunoprecipitated CIB with αIIb between before and after platelet stimulation was observed (supplementary Fig S7B, lanes 5-8). These results indicate that the αIIb cytoplasmic tail binds CIB in both resting and stimulated platelets, suggesting that αIIb binding to CIB does not require increase in the intracellular calcium concentration following stimulation. In fact,

recently, several biochemical experiments revealed that binding of CIB to the αIIb cytoplasmic tail is calcium-independent (Yamniuk and Vogel, 2005).

We next asked if inefficient formation of the WASP-CIB complex affects αIIb binding to CIB, since we found WASP binding to CIB is impaired in patients (Fig 5). The most direct way to determine if αIIb binding to CIB is affected by impaired WASP binding to CIB was to examine αIIb binding to CIB in patients' platelets. However, it is not realistic and almost impossible to prepare patients' platelets sufficient for immunoprecipitation because of patients' health condition (We need at least 1 x 10^9 platelets/40 ml peripheral blood/one experiment.). We thus mimicked impaired WASP binding to CIB in patients' platelets by blocking the binding in platelets from normal individuals. WASP binding to CIB was blocked by the CIB N-terminal fragment in platelets, αIIb was immunoprecipitated from the platelets with anti-αIIb antibody followed by immunoblotting for αIIb and CIB. No obvious difference in the amounts of co-immunoprecpitated CIB with αIIb between before and after blocking WASP binding to CIB (supplementary Fig S7C). This result suggests that impaired WASP binding to CIB had no effect on binding of CIB to the αIIb cytoplasmic tail.

Effect of blocking WASP binding to CIB on actin polymerization

The results in Fig 4 suggest that in addition to WASP binding to CIB, the WASP C-terminus stimulating actin polymerization is necessary for cell adhesion, which is consistent with the previous observation that cytochalasins, actin polymerization inhibitors inhibited the increase in the αIIbβ3 affinity for ligands (Bennett et al., 1999; Calderwood et al., 2000; Fox et al., 1996). We examined if

blocking WASP binding to CIB affects actin polymerization. When platelets were stimulated with thrombin, F-actin content was increased (supplementary Fig S7A, top panel, closed histogram). No significant difference in F-actin content in stimulated platelets between before and after blocking WASP binding to CIB was observed (supplementary Fig S7A), indicating that blocking WASP binding to CIB had no effect on actin polymerization in stimulated platelets.

Actin polymerization is not affected by blocking WASP binding to CIB (supplementary Fig S7A). Actin polymerization occurs in platelets from XLT patients (Rengan and Ochs, 2000) and WASP-deficient WAS patients (Falet et al., 2002). Nevertheless, the increase in the αIIbβ3 affinity for ligand is impaired (Figs 3 and 5). These results indicate that actin polymerization stimulated by the WASP C-terminus is not sufficient for αIIbβ3 activation and that in addition to actin polymerization, another important WASP activity residing in the WASP N-terminus is required for αIIbβ3 activation. Our results suggest that the WASP activity is WASP binding to CIB to concentrate and associate actin cytoskeleton to αIIbβ3 sites.

Other proteins binding to the WASP N-terminus

There are three mammalian verprolins identified as the binding partners to the WASP N-terminal region (residues 1-170) termed WIP (WASP interacting protein) (Ramesh et al., 1997), WICH (WIP and CR16 homologous protein) (Kato et al., 2002), WIRE (WIP-related protein) (Aspenstrom, 2002) and CR16 (Ho et al., 2001). WICH is the same protein as WIRE and these two have been identified simultaneously by two independent groups. WIP is crucial for localizing WASP activity both in a vaccinia-

based actin motility system and to the immune synapse (IS) after T-cell receptor ligation (Sasahara et al., 2002). In addition, WIP synergizes with a WASP homologous protein N-WASP (neural WASP) to induce filopodia, when overexpressed in fibroblast (Moreau et al., 2000). The structure of the N-WASP-WIP complex was solved. Based on the structure of the N-WASP-WIP complex, it is proposed that WAS mutations may disrupt the WASP-WIP interaction and disruption of this interaction may result in WAS (Volkman et al., 2002). However, the roles of WASP-WIP interaction in platelets are still unclear and there is no evidence indicating that the inefficient formation of the WASP-WIP complex causes bleeding in WAS patients.

WIP co-immnunoprecipitated with WASP in the lysates from both resting and stimulated platelets (supplementary Fig S9B). This result, taken together with Fig 1B suggests that WASP, CIB and WIP form a complex in stimulated platelets. No significant difference in the amount of co-immunoprecipitated WIP with WASP between before and after blocking WASP binding to CIB by the CIB N-terminal fragment was observed (supplementary Fig S9C, lane 7). This result suggests that the exogenous CIB N-terminal fragment had no effect on WASP binding to WIP.

The CIB binding site is found on the WASP N-terminus (residues 1-105) (Fig 1). WIP binding requires the WASP N-terminal region (residues 1-170), longer than the CIB binding (Ramesh et al., 1997). WASP binding to CIB was blocked by the CIB N-terminal fragment in cells (supplementary Fig S9A, lane 4), but the CIB N-terminal fragment did not affect WASP binding to WIP (supplementary Fig S9A, lane 8). These results, taken together suggest that the reduced $\alpha IIb\beta 3$ affinity for ligand by the CIB N-

terminal fragment (Figs 2,3) is caused by blocking WASP binding to CIB, but not by blocking WASP binding to WIP.

Since there is a similarity between the phenotypes of WASP and WIP-deficient mice (Anton et al., 2002; Snapper et al., 1998; Zhang et al., 1999), WASP and WIP are thought to function as a unit. The WASP-WIP complex might play a role in αIIbβ3-mediated cell adhesion such as actin polymerization. However, we showed that blocking WASP binding to CIB reduced the αIIbβ3 affinity for ligand (Figs 2,3), but that the blocking had no effect on actin polymerization (supplementary Fig S7A) and WASP binding to WIP (supplementary Fig S9C). These results suggest that WASP binding to CIB is independent of actin polymerization and WASP binding to WIP and that WASP binding to CIB is necessary for αIIbβ3-mediated cell adhesion.

SUPPLEMENTARY METHODS

Yeast two-hybrid screening. We screened a human lymphocyte cDNA library (Origene Technlogy Inc.) using the WASP N-terminal domain (residues 1-150) as bait (pGilda (B-C Clontech)) as described previously (Tsuboi, 2002).

Platelet isolation, permeabilization, stimulation and flow cytometric analysis. After informed consent was obtained, 10 ml of peripheral blood from healthy volunteers and from WAS/XLT patients were collected into 0.15 volumes of acid-citrate-dextrose (ACD) (38 mM citric acid, 75 mM sodium citrate and 124 mM dextrose) plus 1 μg/ml prostaglandin E_1 (PGE_1) and then centrifuged at 180 x g for 20 min at 22°C. The

platelets were pelleted through plasma by centrifugation at 750 x g for 10 min. The washed platelets were resuspended in Tyrode's buffer (130 mM NaCl, 10 mM trisodium citrate, 9 mM $NaHCO_3$, 6 mM dextrose, 0.9 mM $MgCl_2$, 0.81 mM KH_2PO_4, 10 mM Tris-HCl, pH7.4) at 2.0 x 10^8 cells/ml. Platelets were incubated for 10 min at 4°C with 5-15 U/ml of streptolysin O (SLO) (Sigma). Permeabilization was confirmed by incubation with FITC-labeled soybean trypsin inhibitor and monitoring uptake of labeled protein by FACS analysis. Platelets were stimulated for 15 min at 22°C with 100 μM of ADP or 1.0 U/ml of thrombin without stirring in the presence or absence of 100 μM of RGD-containing peptide (GRGDSP) (Gibco). The binding of fluorescein isothiocyanate (FITC)-labeled PAC-1, an αIIbβ3-specific and activation-dependent monoclonal antibody (Becton Dickinson Immunocytometry Systems) was analyzed using FACSort flow cytometer (Becton Dickinson). Platelet preparation and analyses of PAC-1 binding to platelets were performed at the site where and on the day when blood was drawn from patients and normal individuals. The Internal Review Boards of The Burnham Institute and University of Washington approved these experiments.

Cell culture and transfection. Human embryonic kidney (HEK) 293 cells and MEG-01 cells were obtained from ATCC and cultured in DME high glucose and RPMI1640 medium, respectively, supplemented with 10% of FCS. Cells were cultured in the presence of 100 units/ml of penicillin and 0.1 mg/ml of streptomycin. HEK293 cells were transfected using SuperFect transfection reagent (QIAGEN) and transfection of MEG-01 cells were performed using amaxa Nucleofector (amaxa Inc.) according to the manufacturer's instructions.

Recombinant proteins. The CIB N-terminal fragment (residues 1-113) and calcyclin were expressed as C-terminally FLAG-tagged proteins in HEK293 cells. Lysates prepared from transfected cells were incubated with anti-FLAG M2 affinity gel (Sigma) and bound FLAG-tagged proteins were eluted with FLAG peptide (50 μg/ml). The WASP, mutant proteins and VCA domain-deficient WASP (WASP-dVCA, WdV, residues 1-435) were also expressed as N-terminally Myc-tagged proteins in HEK cells. Myc-tagged proteins were prepared in an identical manner.

Surface plasmon resonance. A BIACORE 3000 (BIACORE) surface plasmon resonance-based biosensor was used to determine affinity constants for the binding of wild-type WASP and its mutants to CIB. Purified CIB was immobilized onto the carboxymethylated dextran surface of a CM5 sensor chip using the amino-coupling kit (BIACORE). WASP and the mutant forms were diluted in running buffer (10 mM HEPES, pH7.4, 0.15 M NaCl, 3 mM EDTA and 0.005% (v/v) polysorbate 20) and injected onto the sensor chip at varying concentrations. Each analyte was injected at concentrations of 250, 125, 62.5, 37.5, 18.8 and 9.38 nM. Specific interaction data were first subtracted from corresponding controls, zeroed using the BIAevaluation 2.0 software (BIACORE), and then globally fit to a simple bimolecular reaction model using Clamp^{TM} software. The analysis was performed three times independently. The dissociation affinity constants (K_D) of wild-type and mutant WASPs are defined as K_D = koff/kon and estimated using the BIAevaluation 2.0 software.

Anti-CIB antibody. Anti-CIB polyclonal antibody used for some of the experiments in this study (Fig 1B, Fig 3A, Fig 5B and Fig S7C) was raised in chickens using GST-CIB (full-length) as an antigen (Aves Labs, Inc.). Chicken IgY was prepared from eggs and anti-CIB polyclonal antibody was affinity-purified.

Immunoprecipitation. For immunoprecipitation of WASP, platelets (2 x 10^8 cells) were lysed by the addition of an equal volume of 2 x lysis buffer A (30 mM HEPES, pH7.4, 300 mM NaCl, 2 mM phenylmethyl sulfonyl fluoride (PMSF), 2 mM sodium orthovanadate, 2 μg/ml leupeptin, 2 μg/ml pepstatin A, 2 μg/ml aprotinin, 2% Triton X-100). The pellets of MEG-01 cells (2 x 10^7 cells) were lysed in 1 x lysis buffer A. The lysate from platelets or MEG-01 cells was incubated with 2 μg/ml of monoclonal antibody to WASP (Santa Cruz Biotechnology) at 4°C for 2 h. Anti-mouse IgG-agarose (Sigma, 40 μl of 50% slurry) was added and incubated at 4°C for 2 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 1x Laemmi's SDS-PAGE sample buffer. The eluted proteins were subjected to SDS-PAGE and analyzed by immunoblotting using anti-WASP polyclonal antibody (Santa Cruz Biotechnology) and anti-CIB polyclonal antibody raised in chickens. For immunoprecipitation of CIB, platelets (2 x 10^8 cells) were lysed in 1 x lysis buffer A. Anti-CIB polyclonal antibody (chicken IgY) and anti-chicken IgY-agarose (Aves Lab. Inc.) were used. For immunoprecipitation of the αIIb_subunit ofαIIbβ3, platelets were lysed by addition of an equal volume of 2 x lysis buffer B (40 mM Tris-HCl, pH7.5, 300 mM NaCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 50 mM

n-octyl _-glucopyranoside and proteinase inhibitors). The pellets of MEG-01 cells (2 x 10^7 cells) were lysed in 1 x lysis buffer B. Anti-αIIb_subunit monoclonal antibody (M-148) (Santa Cruz) was used for immunoprecipitation and anti-αIIb subunit polyclonal antibody (H-160) (Santa Cruz) was used for immunoblotting.

Calcium mobilization. Platelets (1 x 10^9 cells/ml) were incubated with 5 μM of Fluo-3AM (Molecular Probes Inc.) at 37°C in the dark for 30 min. Fluo-3AM loaded platelets were stimulated with 100 μM of ADP. Mean fluorescence intensities (MIFs) of Fluo-3AM were recorded at different time –points.

Measurement of F-actin. The polymerized actin (F-actin) content in platelets was determined by quantitating FITC-phalloidin (Molecular Probes Inc.) binding to paraformaldehyde-fixed and detergent-permeabilized platelets. Platelets (8 x 10^7 cells) were fixed by the addition of an equal volume of ice-cold 3.7% (w/v) paraformaldehyde (Fluka) in PBS for 30 min. The fixed platelets were permeabilized by adding 0.1 vol of 1.0% Triton X-100 containing 100 μM FITC-phalloidin, and then analyzed using a FACSort flow cytometer after a 60-minute exposure to the FITC-phalloidin.

RNAi. MEG-01 cells were transfected with siRNA for WASP or its scrambled control (Dharmacon Inc.) using amaxa Nucleofector (amaxa Inc.) according to the manufacturer's instructions. The following target sequences were chosen to generate

siRNA for WASP, 5'-CGAGAACCAGCGACTCTTT3' (sense) and 5'-TCCAGACCTTGCAATCGGC-3' (scrambled). The efficiency of siRNA transfection measured using Cy3-labeled control siRNA (siGLO, Dharmacon Inc.) was 60-80%.

REFERENCES TO SUPPLEMENTARY INFORMATION

Anton, I.M., de la Fuente, M.A., Sims, T.N., Freeman, S., Ramesh, N., Hartwig, J.H., Dustin, M.L. and Geha, R.S. (2002) WIP deficiency reveals a differential role for WIP and the actin cytoskeleton in T and B cell activation. *Immunity*, **16**, 193-204.

Aspenstrom, P. (2002) The WASP-binding protein WIRE has a role in the regulation of the actin filament system downstream of the platelet-derived growth factor receptor. *Exp Cell Res*, **279**, 21-33.

Bennett, J.S., Zigmond, S., Vilaire, G., Cunningham, M.E. and Bednar, B. (1999) The platelet cytoskeleton regulates the affinity of the integrin alpha(IIb)beta(3) for fibrinogen. *J Biol Chem*, **274**, 25301-25307.

Calderwood, D.A., Shattil, S.J. and Ginsberg, M.H. (2000) Integrins and actin filaments: reciprocal regulation of cell adhesion and signaling. *J Biol Chem*, **275**, 22607-22610.

Falet, H., Hoffmeister, K.M., Neujahr, R. and Hartwig, J.H. (2002) Normal Arp2/3 complex activation in platelets lacking WASp. *Blood*, **100**, 2113-2122.

Fox, J.E., Shattil, S.J., Kinlough-Rathbone, R.L., Richardson, M., Packham, M.A. and Sanan, D.A. (1996) The platelet cytoskeleton stabilizes the interaction between alphaIIbbeta3 and its ligand and induces selective movements of ligand-occupied integrin. *J Biol Chem*, **271**, 7004-7011.

Hers, I., Donath, J., Litjens, P.E., van Willigen, G. and Akkerman, J.W. (2000) Inhibition of platelet integrin alpha(IIb)beta(3) by peptides that interfere with protein kinases and the beta(3) tail. *Arterioscler Thromb Vasc Biol*, **20**, 1651-1660.

Ho, H.Y., Rohatgi, R., Ma, L. and Kirschner, M.W. (2001) CR16 forms a complex with N-WASP in brain and is a novel member of a conserved proline-rich actin-binding protein family. *Proc Natl Acad Sci U S A*, **98**, 11306-11311.

Kato, M., Miki, H., Kurita, S., Endo, T., Nakagawa, H., Miyamoto, S. and Takenawa, T. (2002) WICH, a novel verprolin homology domain-containing protein that functions cooperatively with N-WASP in actin-microspike formation. *Biochem Biophys Res Commun*, **291**, 41-47.

Moreau, V., Frischknecht, F., Reckmann, I., Vincentelli, R., Rabut, G., Stewart, D. and Way, M. (2000) A complex of N-WASP and WIP integrates signalling cascades that lead to actin polymerization. *Nat Cell Biol*, **2**, 441-448.

Naik, U.P., Patel, P.M. and Parise, L.V. (1997) Identification of a novel calcium-binding protein that interacts with the integrin alphaIIb cytoplasmic domain. *J Biol Chem*, **272**, 4651-4654.

Nonoyama, S. and Ochs, H.D. (1998) Characterization of the Wiskott-Aldrich syndrome protein and its role in the disease. *Curr Opin Immunol*, **10**, 407-412.

Ramesh, N., Anton, I.M., Hartwig, J.H. and Geha, R.S. (1997) WIP, a protein associated with wiskott-aldrich syndrome protein, induces actin polymerization and redistribution in lymphoid cells. *Proc Natl Acad Sci U S A*, **94**, 14671-14676.

Rengan, R. and Ochs, H.D. (2000) Molecular biology of the Wiskott-Aldrich syndrome. *Rev Immunogenet*, **2**, 243-255.

Sasahara, Y., Rachid, R., Byrne, M.J., de la Fuente, M.A., Abraham, R.T., Ramesh, N. and Geha, R.S. (2002) Mechanism of recruitment of WASP to the immunological synapse and of its activation following TCR ligation. *Mol Cell*, **10**, 1269-1281.

Snapper, S.B., Meelu, P., Nguyen, D., Stockton, B.M., Bozza, P., Alt, F.W., Rosen, F.S., von Andrian, U.H. and Klein, C. (2005) WASP deficiency leads to global defects of directed leukocyte migration in vitro and in vivo. *J Leukoc Biol*, **77**, 993-998.

Snapper, S.B., Rosen, F.S., Mizoguchi, E., Cohen, P., Khan, W., Liu, C.H., Hagemann, T.L., Kwan, S.P., Ferrini, R., Davidson, L., Bhan, A.K. and Alt, F.W. (1998) Wiskott-Aldrich syndrome protein-deficient mice reveal a role for WASP in T but not B cell activation. *Immunity*, **9**, 81-91.

Volkman, B.F., Prehoda, K.E., Scott, J.A., Peterson, F.C. and Lim, W.A. (2002) Structure of the N-WASP EVH1 domain-WIP complex: insight into the molecular basis of Wiskott-Aldrich Syndrome. *Cell*, **111**, 565-576.

Yamniuk, A.P. and Vogel, H.J. (2005) Calcium- and magnesium-dependent interactions between calcium- and integrin-binding protein and the integrin alphaIIb cytoplasmic domain. *Protein Sci*, **14**, 1429-1437.

Zhang, J., Shehabeldin, A., da Cruz, L.A., Butler, J., Somani, A.K., McGavin, M., Kozieradzki, I., dos Santos, A.O., Nagy, A., Grinstein, S., Penninger, J.M. and Siminovitch, K.A. (1999) Antigen receptor-induced activation and cytoskeletal rearrangement are impaired in Wiskott-Aldrich syndrome protein-deficient lymphocytes. *J Exp Med*, **190**, 1329-1342.

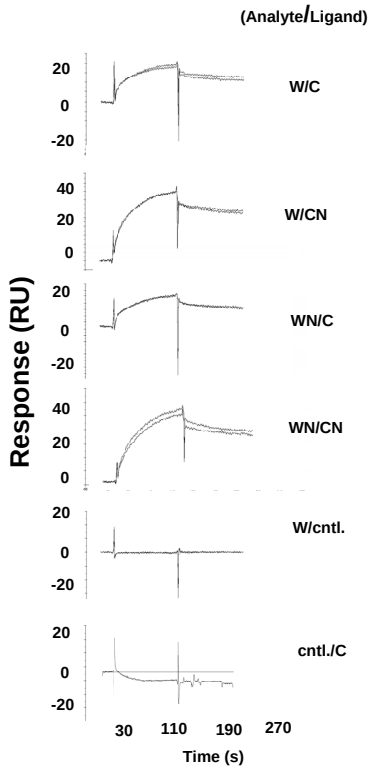

Figure S1. Analysis of direct binding of WASP to CIB by BIACORE 3000. W, Myc-tagged WASP; WN, Myc-WASP N-terminus; C, FALG-tagged CIB; CN, FLAG-CIB N-terminus; Myc-calcyclin and FLAG-calcyclin were used as the control proteins. Each injection of analyte was repeated two times. Results were expressed in resonance units (RU), an arbitrary uint specific for the BIACORE instrument (1000 RU correspond to approximately 1 ng of bound protein/mm2). The analysis was performed three times independently. One representative result is shown.

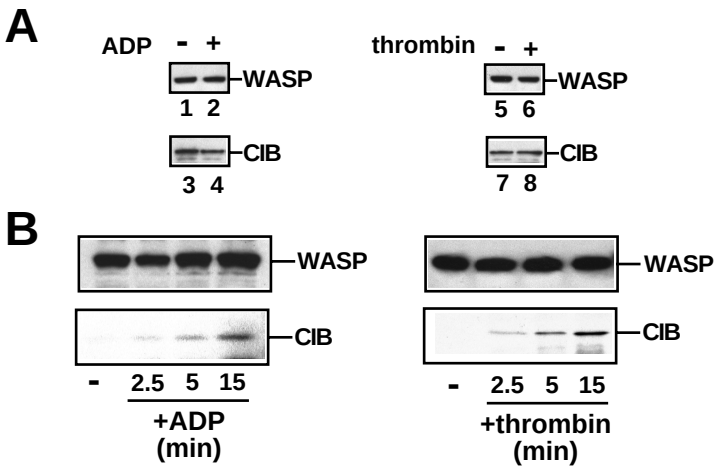

Figure S2. Time-course of WASP binding to CIB in platelets following agonists stimulation. (A) Lysates were prepared from unstimulated platelets (lanes 1,3,5,7) and agonisit-stimulated platelets (lanes 2,4,6,8), and were immunoblotted for WASP (lanes 1,2,5,6) and CIB (lanes 3,4,7,8). Platelets were stimulated with ADP (left panel) or thrombin (right panel). (B) Platelets were stimulated with ADP (left panel) or thrombin (right panel), and lysed by adding 2 x lysis buffer at indicated time-points. WASP was immunoprecipitated from the lysates with anti-WASP antibody followed by immunoblotting for WASP (*top panel*) and CIB (*bottom panel*).

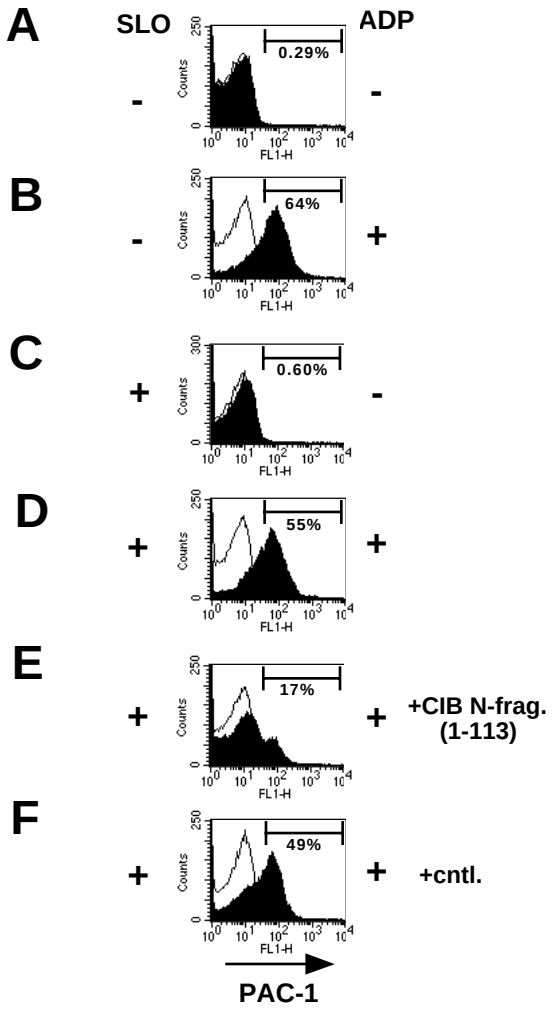

Figure S3. Blocking WASP-CIB binding impairs conformational change of αIIbβ3. (A-F) Platelets were stimulated with ADP without stirring, and then binding of PAC-1 was analyzed with FACSort in the presence (open histograms) or absence (closed histograms) of 100 μM of GRGDSP peptide. (A) Resting platelets. (B) ADP-stimulated platelets. (C) SLO-permeabilized resting platelets. (D) SLO-permeabilized platelets (8×10^7 cells) were stimulated with ADP. (E) SLO-permeabilized platelets were incubated with 100 ng of the CIB N-terminal fragment. (F) SLO-permeabilized platelets were incubated with FLAG-calcyclin as a control protein.

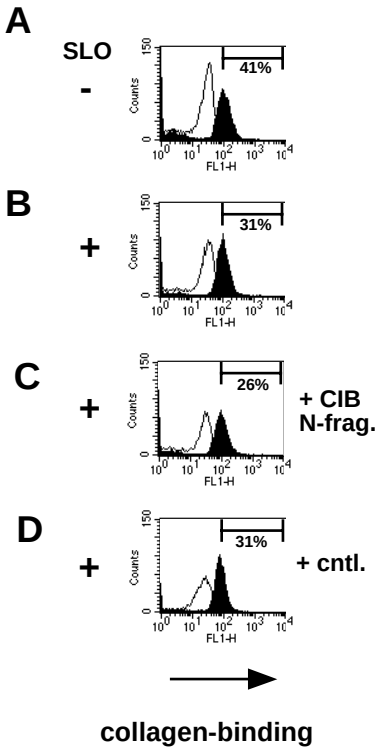

Figure S4. Blocking WASP-CIB binding does not affect $\alpha 2\beta 1$ affinity for its lignad. FITC-labeled collagen binding to platelets with thrombin stimulation (closed histograms) or without thrombin stimulation (open histograms) was measured by a flow cytometer. (A) Intact platelets. (B) SLO-permeabilized platelets. (C) SLO-permeabilized platelets (8 x 10^7 cells) were incubated with 100 ng of the CIB N-terminal fragment. (D) SLO-permeabilized platelets were incubated with the FLAG-calcyclin as a control protein.

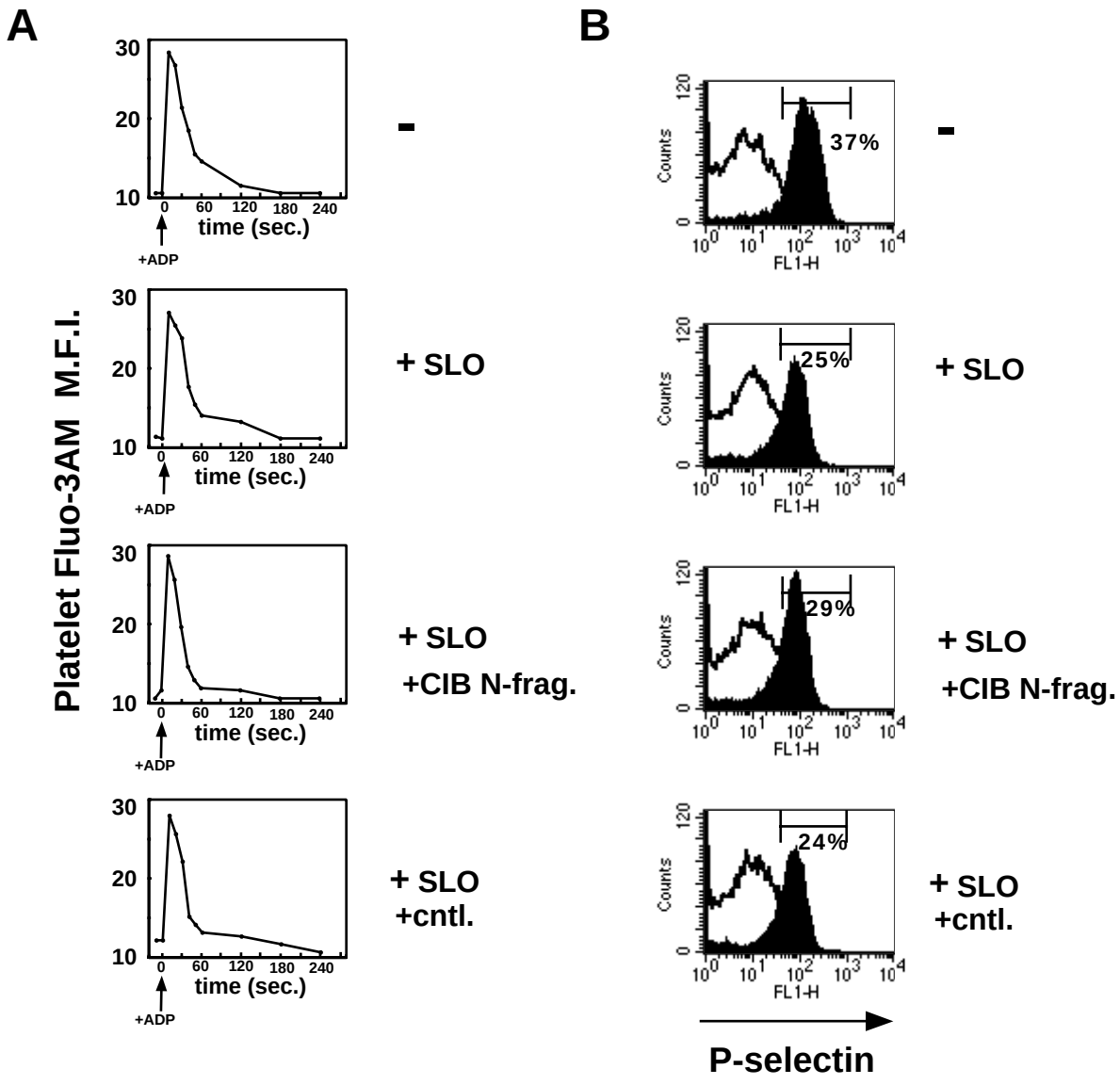

Figure S5. Blocking WASP-CIB binding does not affect calcium mobilization and α-granule secretion. (A) ADP-induced platelet calcium mobilization was monitored by flowcytometry. Fluo-3 AM-loaded platelets were stimulated with ADP, and Mean fluorescence intensities (MFIs) of Fluo-3 AM were recorded at different time-points. (B) Surface exposure of P-selectin was measured as an indication of α-granule secretion. Platelets were stimulated with 0.1 U/ml of thrombin and fixed. Stimulated platelets (closed histograms) or unstimulated platelets (open histograms) were stained with FITC-labeled anti-P-selectin antibody (B-D Pharmingen) and analyzed with a flowcytometer.

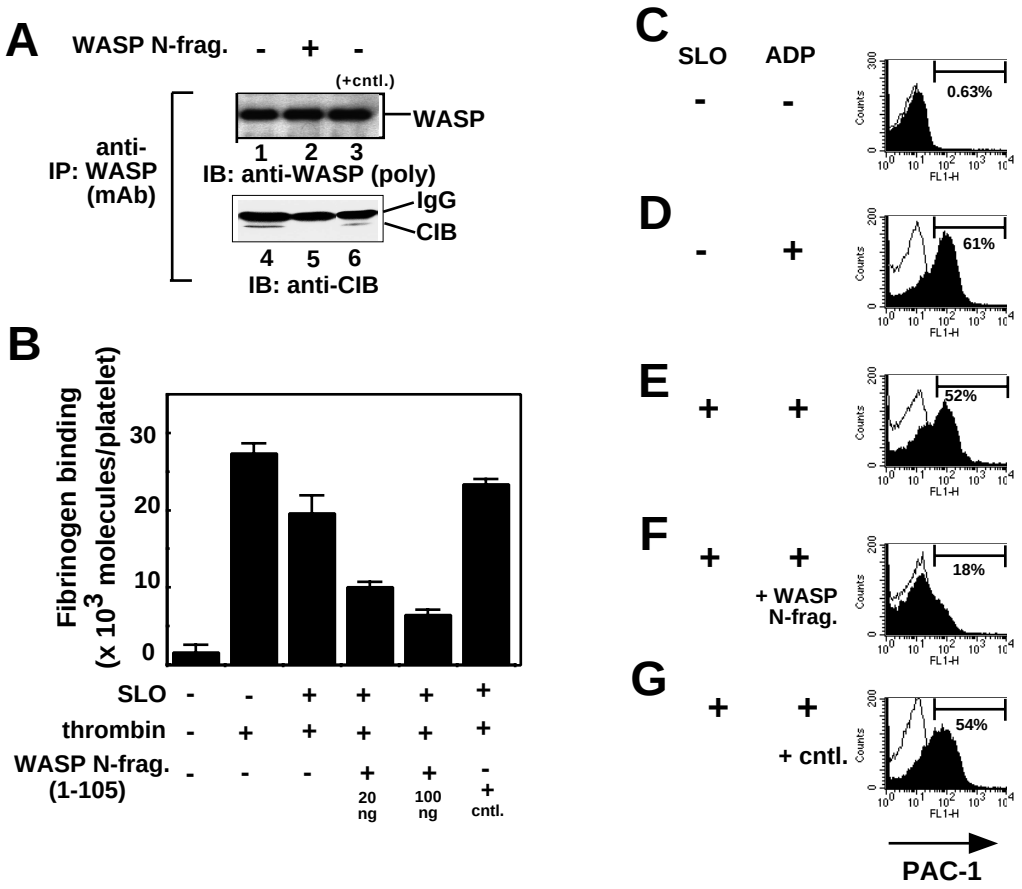

Figure S6. Blocking WASP-CIB binding by the WASP N-terminal fragment reduces αIIbβ3 affinity for its ligand. (A) SLO-permeabilized platelets were incubated with the WASP N-terminal fragment (lanes 2 and 5) or a control protein, calcyclin (lanes 3 and 6) and then stimulated with ADP without stirring in the presence of 100 μM of GRGDSP peptide. WASP was immunoprecipitated from platelet lysates with anti-WASP monoclonal antibody (lanes 1-6) followed by immunoblotting with anti-WASP polyclonal antibody (lanes 1-3) and anti-CIB polyclonal antibody (lanes 4-6). Protein samples prepared from 2.5 x 10^7 platelets were subjected to SDS-PAGE and immunoblotting. (B) SLO-permeabilized platelets (8 x 10^7 cells) were incubated with 20 ng and 100 ng of the WASP N-terminal fragment or 100 ng of calcyclin as a control protein (cntl.) and then stimulated with ADP without stirring. Fibrinogen binding was measured as described in "METHODS". Specific fibribogen binding was defined as that inhibited by GRGDSP peptide. Data are the means ± SEM of three experiments. (C-G) Platelets were stimulated with ADP without stirring, and then binding of PAC-1 was analyzed with FACSort in the presence (open histograms) or absence (closed histograms) of 100 μM of GRGDSP peptide. (C) Resting platelets. (D) ADP-stimulated platelets. (E) SLO-permeabilized platelets (8 x 10^7 cells) were stimulated with ADP. (F) SLO-permeabilized platelets were incubated with 100 ng of the WASP N-terminal fragment. (G) SLO-permeabilized platelets were incubated with calcyclin as a control protein.

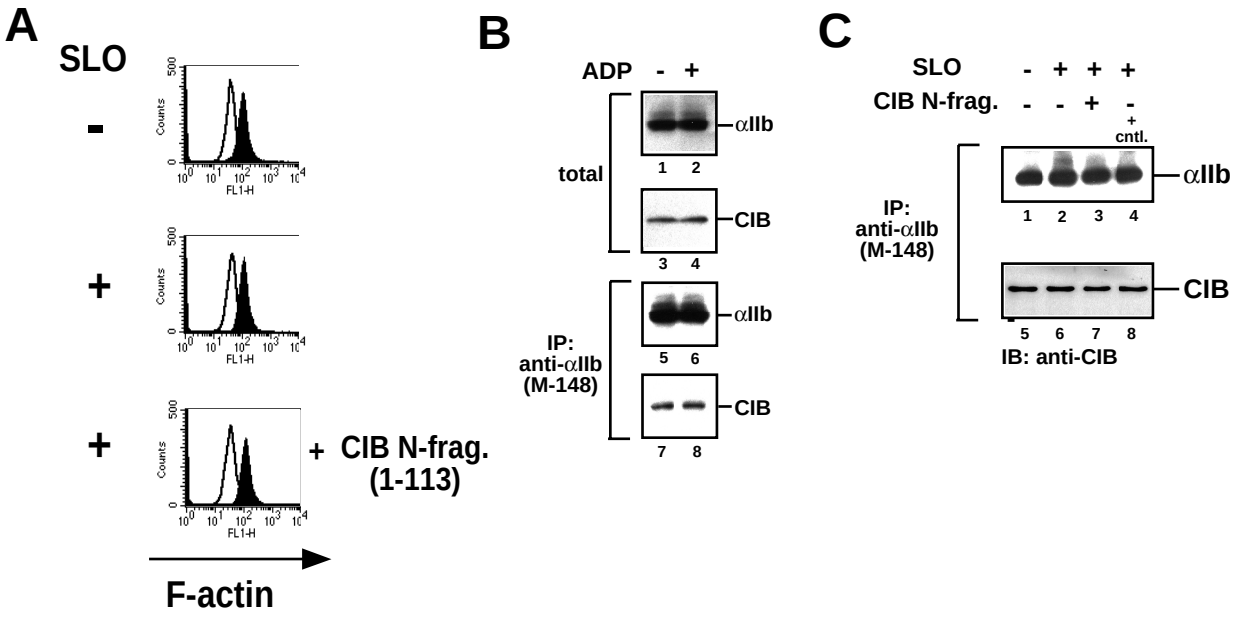

Figure S7. Blocking WASP binding to CIB does not affect actin polymerization and αIIb binding to CIB. (A) F-actin content was measured in platelets with FACSort using FITC-phalloidin. Open and closed histograms indicate resting and ADP-stimulated platelets, respectively. (B) Expression levels of αIIb and CIB in platelets before and after ADP-stimulation were analyzed by immunoblotting (lanes 1-4). αIIb was immunoprecipitated with anti-αIIb monoclonal antibody (M-148, Santa Cruz Biotechnlogy) followed by immunoblotting with anti-αIIb polyclonal antibody (H-160, Santa Cruz Biotechnlogy) and anti-CIB antibody. (C) SLO-permeabilized platelets were incubated with 0.2 μg of CIB N-terminal fragment or control protein and then stimulated with ADP without stirring in the presence of GRGDSP peptide. αIIb was immunoprecipitated followed by immunoblotting for αIIb and CIB.

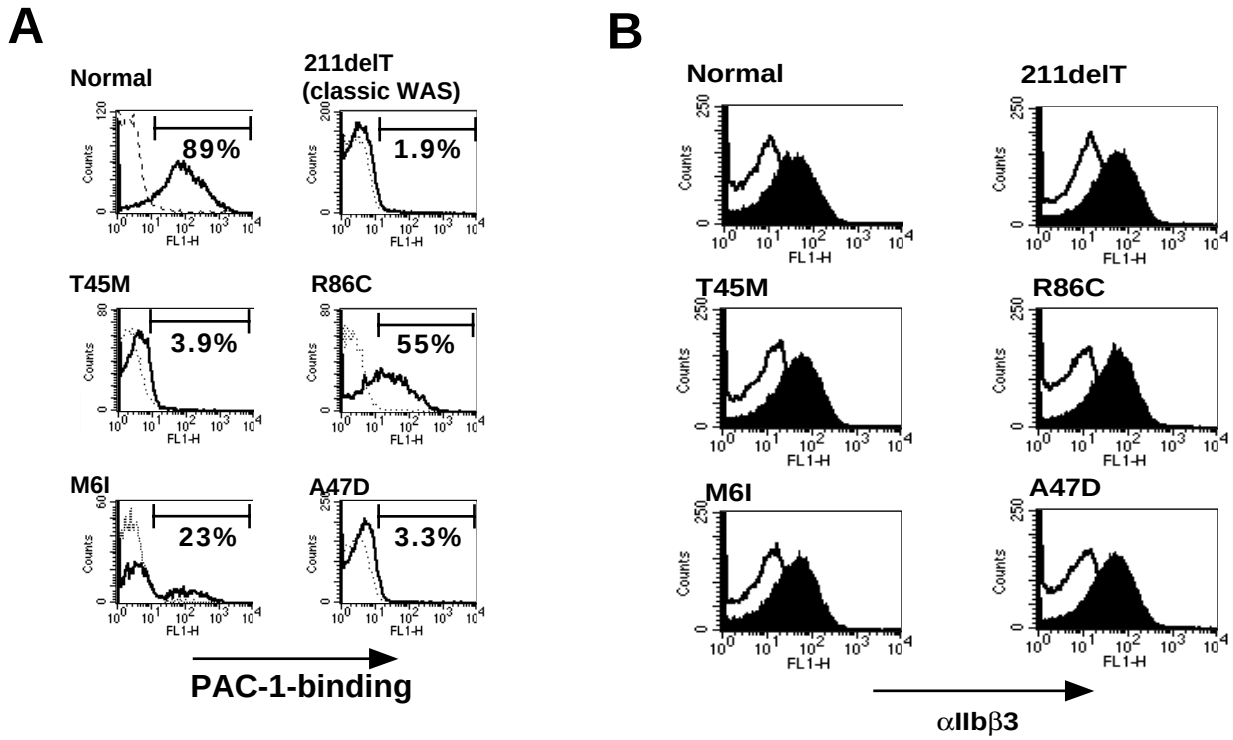

Figure S8. Binding of PAC-1 to platelets from patients. (A) Binding of PAC-1 to platelets from one classic WAS patient (211delT) and four XLT patients (T45M, R85C, M6I and A47D) was analyzed with FACSort. Broken lined histograms and bold lined histograms indicate PAC-1 binding to resting and thrombin-stimulated platelets, respectively. Platelet preparation and analyses of PAC-1 binding to platelets were performed at the site where and on the day when blood was drawn from patients and normal individuals. (B) Surface expression of αIIbβ3 in platelets from normal control, classic WAS (211delT) and XLT (T45M, R86C, M6I and A47D) patients was analyzed using monoclonal antibody to human αIIb subunit (Chemicon, CA3) by FACS analysis. Open histograms and closed histograms indicate staining with isotype-matched control antibody (mouse IgG1) and anti-αIIb, respectively.

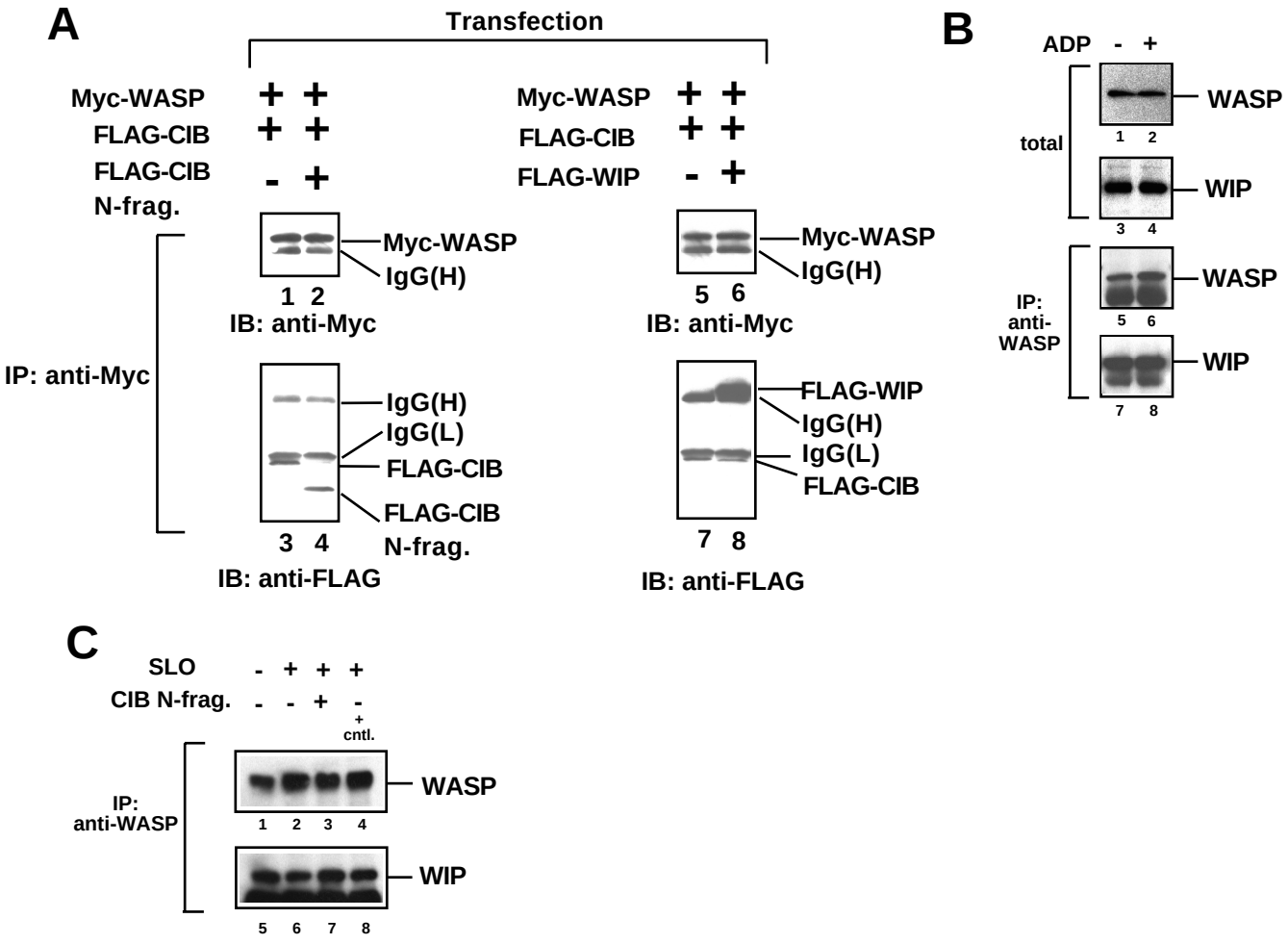

Figure S9. Blocking WASP binding to CIB does not affect WASP binding to WIP. (A) CIB does not compete with WIP for binding to WASP. HEK293 cells were transfected with 5 μg of Myc-tagged WASP (Myc-WASP) and FLAG-tagged CIB (FLAG-CIB) plasmids. Cells were co-transfected with 20 μg of vector (lanes 1,3,5,7), FLAG-CIB N-terminal fragment (residues 1-113) (lanes 2 and 4), or FLAG-WIP (lanes 6 and 8). Myc-WASP was immunoprecipitated with anti-Myc antibody from the cell lysates followed by immunoblotting with anti-Myc (lanes 1,2 5,6) and anti-FLAG (lanes 3,4,7,8) antibodies. (B) Expression levels of WASP and WIP in platelets before and after ADP-stimulation were analyzed by immunoblotting (lanes 1-4). WASP was immunoprecipitated with anti-WASP monoclonal antibody from the cell lysates followed by immunoblotting with anti-WASP polyclonal antibody (Santa Cruz Biotechnlogy) and anti-WIP polyclonal antibody (Santa Cruz Biotechnlogy). (C) SLO-permeabilized platelets were incubated with 0.2 μg of CIB N-terminal fragment or control protein and then stimulated with ADP without stirring in the presence of GRGDSP peptide. WASP was immunoprecipitated from the platelet lysates followed by immunoblotting for WASP and WIP.

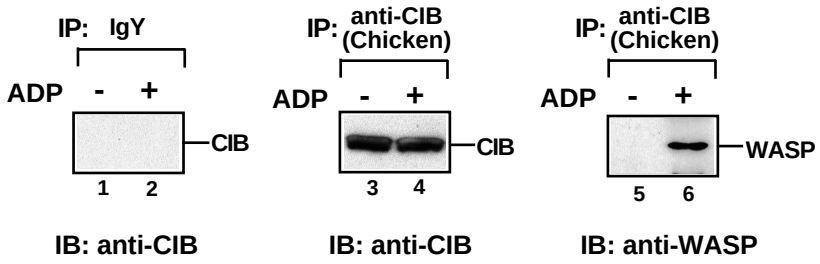

Figure S10. Binding of WASP to CIB in platelets. Platelets were stimulated with 100 μM of ADP. CIB was immunoprecipitated with anti-CIB polyclonal antibody raised in chickens followed by immunoblotting using anti-CIB antibody raised in rabbits (lanes 3, 4) (Tsuboi, 2002) and anti-WASP antibody (lane 5, 6). Chicken IgY was also used for immunoprecipitation as a negative control (lanes 1, 2).

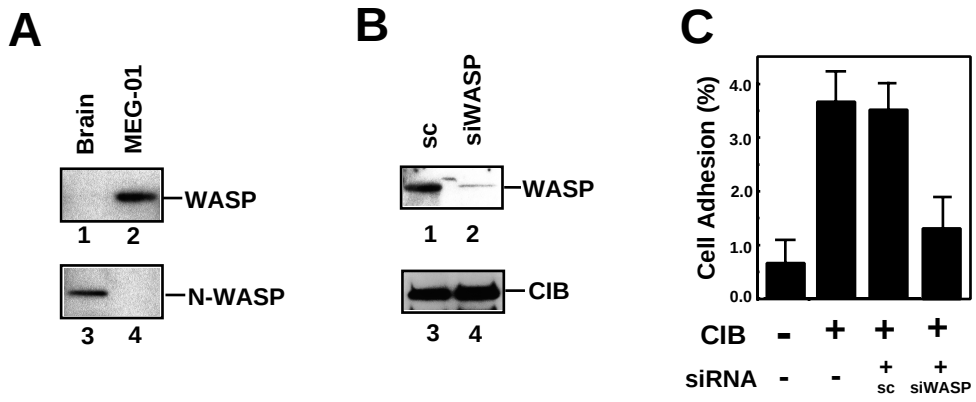

Figure S11. Effect of knockdown of WASP expression on cell adhesion. (A) Expression of two WASP homologs, WASP and N-WASP in MEG-01 cells and human brain was analyzed by immunoblotting. Total cell lysates from MEG-01 cells and human brain (Stratagene) were immunoblotted using anti-WASP polyclonal antibody and anti-N-WASP polyclonal antibody (Santa Cruz Biotechnology). (B) MEG-01 cells were co-transfected with siRNA (siWASP, siRNA for WASP; sc, scrambled control) and CIB expression vector, and then total cell lysates of transfectants were analyzed by immunoblotting using anti-WASP (lanes 1, 2) and anti-CIB (lanes 3, 4) antibodies. (C) Two days after transfection, MEG-01 cells co-transfected with siRNA and CIB were tested for adhesion to fibrinogen. All cell adhesion data are the mean ± S. E. of three experiments.

Patient	Age	Platelet count	Clinical score	Splenectomy
211delT	15	$2.1x10^4$	5	-
T45M	12	$1.0x10^4$	2	-
R86C	24	$1.8x10^4$	5	+
M6I	10	$1.4x10^4$	1	+
A47D	17	$2.0\text{-}5.0x10^4$	2	-

Figure S12. Information on patients. The following information of patients tested in this study, age, platelet count, clinical score and whether or not splenectomized were described. Platelet counts were expressed as number of platelets per one microlitter (μl) of peripheral blood. The severity of WAS/XLT-associated symptoms was estimated and expressed as a score of 1 to 5. A score of 1, assigned to patients with only thrombocytopenia and small platelets, and a score of 2, assigned to patients with additional findings of mild, transient eczema or minor infections, identified XLT patients. Those with treatment-resistant eczema and recurrent infections in spite of optimal treatment received a score of 3 (mild WAS) or 4 (severe WAS). Regardless of the original score, if any patients then had autoimmune disease or malignancy, the score was changed to 5.