Loss of the F-BAR protein CIP4 reduces platelet production by impairing membrane-cytoskeleton remodeling

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Short title: CIP4 in Platelet Biogenesis
Key point summary

1. CIP4 affects the remodeling of both plasma membrane and cortical cytoskeleton in megakaryocytes

2. CIP4 in platelet biogenesis involves involving cortical tension as does WASP, and WASP-independent plasma membrane reorganization

Abstract

Megakaryocytes generate platelets through extensive reorganization of the cytoskeleton and plasma membrane. Cdc42 interacting protein 4 (CIP4) is an F-BAR protein that localizes to membrane phospholipids through its BAR domain and interacts with WASP via its SH3 domain. F-BAR proteins promote actin polymerization and membrane tubulation. To study its function we generated CIP4\(^{-/-}\) mice, which displayed thrombocytopenia similarly to that of WAS\(^{-/-}\) mice. The number of megakaryocytes and their progenitors was not affected. However, the number of proplatelet protrusions was reduced in CIP4\(^{-/-}\), but not WAS\(^{-/-}\), megakaryocytes. Electron micrographs of CIP4\(^{-/-}\) megakaryocytes showed an altered demarcation membrane system. Silencing of CIP4, not WASP, expression resulted in fewer proplatelet-like extensions. Fluorescence anisotropy studies showed that loss of CIP4 resulted in a more rigid membrane. Micropipette aspiration demonstrated decreased cortical actin tension in megakaryocytic cells with reduced CIP4 or WASP protein. These studies support a new biophysical mechanism for platelet biogenesis whereby CIP4 enhances the complex, dynamic reorganization of the plasma membrane (WASP-independent) and actin cortex network (as known for WASP and cortical actin) to reduce the work required for generating proplatelets. CIP4 is a new component in the highly-coordinated system of megakaryocytic membrane and cytoskeletal remodeling affecting platelet production.
Introduction

Megakaryocytes generate platelets through a highly-coordinated process requires membrane and cytoskeletal reorganization.\textsuperscript{1} Important components for cytoskeletal reorganization are the RhoGTPase Cdc42, actin nucleator Wiskott-Aldrich Syndrome Protein (WASP), and actin-associated Arp2/3 complex.\textsuperscript{2} Wiskott-Aldrich Syndrome is characterized by microthrombocytopenia, the mechanism of which is only partially known and may include both autoimmunity\textsuperscript{3} and dysregulated platelet production.\textsuperscript{4,5} Also mysterious is the molecular machinery required for membrane remodeling in megakaryocytes that generate proplatelet protrusions.

The family of F-BAR (Fer/CIP4 homology - Bin, Amphiphysin, Rvs) domain-containing proteins bridges the membrane to the cytoskeleton. BAR domains sense and generate membrane curvature through interaction with membrane phospholipids.\textsuperscript{6} An elongated dimer formed by the antiparallel interaction of two $\alpha$-helical coiled-coils,\textsuperscript{6} the banana-shaped F-BAR domain promotes membrane tubulation of large size (>100 nm).\textsuperscript{6,7} The CIP4 gene encodes a protein with a C-terminal SH3 domain, an N-terminal domain with homology to protein tyrosine kinase Fes/Fer, and a sequence that binds active Cdc42.\textsuperscript{8} We identified CIP4 in a yeast two-hybrid screening with the Src kinase Lyn as bait.\textsuperscript{9} To determine CIP4’s physiological role, we generated CIP4-null mice by disrupting the gene.\textsuperscript{10} The CIP4-null mice appeared grossly normal but displayed decreased endocytosis.\textsuperscript{10}

Through its C-terminal SH3 domain CIP4 binds to WASP, and thus promotes actin cytoskeletal reorganization.\textsuperscript{7,11} Because loss of function in WASP results in thrombocytopenia, we investigated whether deficiency of CIP4 affects platelet biogenesis by remodeling the membrane and the actin cytoskeleton. Indeed, we found that CIP4-null mice displayed thrombocytopenia with a similar severity as that of WASP-null mice. Loss of CIP4 conferred decreased cortical actin tension, similarly to WASP deficiency. However, different than WASP-null mice, loss of CIP4 led to impaired proplatelet formation, reduced megakaryocyte platelet fields, and plasma membrane stiffening. Thus, CIP4 facilitates the cytoskeletal and membrane remodeling that leads
to demarcation membrane system (DMS), known as the reservoir for proplatelet extensions and subsequent platelet release. These studies link platelet biogenesis to membrane biology.

Methods

**CIP4**<sup>-/-</sup> and WAS<sup>-/-</sup> mice. CIP4 KO male C57BL/6 mice aged 3 to 6 months, as previously described. Their ethical use was approved by Northwestern Animal Care Use Committee. The WAS<sup>-/-</sup> mice were genotyped by flow cytometric quantification of WASP in blood lymphocytes. Rabbit anti-WASP polyclonal antibody was prepared against peptide (SSRYRGLPAPGPSPADKK) from murine WASP exon 7.

**Cells.** CHRF-288-11 cells were cultured in IMDM (Gibco) supplemented with Pen/Strep/Glutamax and 10% FBS. Differentiation for proplatelet-like protrusions was obtained by adding PMA (Sigma-Aldrich) or fibronectin (Sigma-Aldrich) as described.

**Subcellular fractionation of platelets.** Platelets were activated with 1 unit/mL thrombin for 5 min, lysed in 1% Triton X100 then centrifuged at 13000g for 15 min at 4°C. The insoluble fraction was resuspended in RIPA, spun at 15400g for 5 min and actin cytoskeleton was collected from supernatant. The soluble fraction was spun at 100000g for 1 hour and the cytosolic fraction was collected. The pellet was resuspended in RIPA buffer, spun at 15400g for 5 min and membrane cytoskeleton was collected from the supernatant.

**Lentiviral shRNA knockdown.** For knockdown in CHRF-288 cells, we used MISSION® shRNA Lentiviral Transduction Particles from Sigma (for CIP4, TRC number TRCN0000063185; for WASP, TRCN0000029822; for TOCA1, TRCN0000129142) provided with Non-Targeting shRNA control transduction particles (SHC002V), according to manufacturer’s instructions.
Complete Blood Counts (CBC) and Histologic staining. CBCs were run on a HemaVet counter. Femoral bone marrow sections were stained with H&E; pictures were taken on an Olympus microscope BX50 with an Olympus DP71.

Megakaryocytic progenitor colonies. Bone marrow was flushed from femurs and tibias, then filtered through a 70 um strainer. For Colony Forming Unit-MK (CFU-MK) counting with the MegaCult-C assay, a total of 2.2X10e6 bone marrow mononuclear cells were used according to the manufacturer's instructions (StemCell Technologies,), with 50 ng/mL human thrombopoietin (TPO) and 10 ng/mL murine IL-3 (PeproTech).

Flow cytometry for ploidy. Bone marrow cells, flushed from femurs and tibias, were washed in PEB (PBS-EDTA-BSA) and incubated with anti-CD41-FITC (BD). Cells were then washed again in PEB and fixed in 0.5% para-formaldehyde. Cells were washed, permeabilized in 70% ice cold methanol, RNAse-treated, incubated with propidium iodide, then run on a BD-LSRII. Data were analyzed using FlowJo 7.6 (Tree Star Inc.).

Proplatelet formation. Megakaryocytes from flushed bone marrows were cultured for 3 days in IMDM (Gibco) with 5% FBS and 50 ng/mL thrombopoietin, separated on a BSA gradient and plated in 96-well plates at a concentration of 3000 cells per well. On day 4, megakaryocytes displaying proplatelets were scored by counting at least 300 cells per well, in at least 3 wells for each condition, using a microscope (Fisher Scientific) with a magnification of 200x. For proplatelet-like protrusion counts and measurements in CHRF-288-11 cells, cells were seeded at a concentration of 30000/mL and then exposed to PMA at 10 ng/mL overnight. Protrusion length was measured with ImageJ software (NeuronJ plugin).

Cell Transfection: CHRF-288-11 cells were transfected with a human CIP4-GFP plasmid (provided by James Goldenring) using the Amaxa Nucleofector II (Lonza) according to manufacturer's instructions, Kit L and program X-005.
**Immunofluorescence and confocal microscopy.** Cells from culture plates were incubated for 2 hours at 37°C on Poly-L-Lysine coated slides (Polysciences) or fibronectin (Sigma, 50 microgram/mL). The cells were then fixed for 20 minutes with 4% paraformaldehyde, permeabilized with Triton-X100 at 0.2% for 3 minutes, washed in PBS, incubated with primary antibody at room temperature for 1 hour or at 4°C overnight, washed in PBS, then incubated with secondary antibody at room temperature for 30 minutes, washed in PBS, and mounted with Vectashield with DAPI. Antibodies were: rabbit anti-vWF antibody (DAKO), mouse anti-CIP4 antibody (BD), mouse anti b-tubulin antibody (Sigma), rabbit anti b-tubulin (Sigma), rabbit anti-WASP antibody (Covance, custom made), Alexa-conjugated secondary antibodies (Alexa-488 or 594, Invitrogen), phalloidin-TRITC or phalloidin-FITC (Sigma). Images were taken on a Leica DM 4000B microscope with a Leica DFC320 camera and analyzed by LAS software (Leica). Confocal Microscopy was performed on a Nikon Eclipse C1Si confocal microscope.

**Electron Microscopy.** Femurs were collected from the mice and bone marrow was flushed into glutaraldehyde 2.5% in PBS. CHRF-288-11 cells were collected and fixed in glutaraldehyde 2.5% in PBS. Fixed samples were kept at 4°C and shipped to the Hospital for Sick Children in Toronto or to the Institut Gustave Roussy in Villejuif, and were further processed as previously described. 5,16

**Western Blot.** Cell extracts were obtained from lysis in Laemmli buffer with β-mercaptoethanol, or lysed in Triton-X100. Proteins were transferred to Immobilon membranes (Millipore) and incubated overnight at 4°C with the primary antibody, then incubated with peroxidase-conjugated secondary antibody. Antibodies used in Western Blot were mouse anti-CIP4 (BD), goat anti-actin antibody (Santa Cruz), mouse anti-WASP (sc-5300 at 1/200), mouse anti-TOCA 1 (a gift from Dr Giorgio Scita, Milan), rabbit anti-FBP17 (Bethyl).

**Fluorescence Anisotropy Studies.** Cells were resuspended in PBS and labeled with 1 uM of 1-(4-(trimethylamino)phenyl)-6-phenylhexa-1,3,5-triene (TMA-DPH, Invitrogen) in
37°C for 10 min\textsuperscript{17} and stimulated with PMA. Alternatively, cells were incubated on fibronectin (Sigma-Aldrich, 50 microgram/mL) coated plates for 2 hours at 37°C then washed in PBS. Endpoint readings were taken to detect changes in plasma membrane fluidity. Each experiment was performed in triplicate. Fluorescence anisotropy values were recorded using a Spectramax M5 microplate reader at the IBNAM facility at Northwestern University. Excitation and emission wavelengths were taken at 360 nm and 430 nm respectively. Polarizers were set at vertical (V) or horizontal (H) positions. Anisotropies (r) were calculated from the intensity maxima using the quantum yield and instrumental correction factor (G-factor) found by measuring cells without TMA-DPH in PBS (r = 0.01) and using the equation: \( G = \frac{I_{HV}}{I_{HH}} \), where \( I_{HV} \) is the intensity when excited with a horizontally polarized light and vertical emission detected, and \( I_{HH} \) is the intensity with horizontally polarized excitation and emission detected in the same horizontal plane\textsuperscript{18}. Assuming \( G = 1 \), steady state fluorescence anisotropy was calculated using the equation: \( r = \frac{(I_{VV} - I_{VH})}{(I_{VV} + 2 \cdot I_{VH})} \).

**Atomistic Molecular Dynamics Simulations.** Molecular dynamics simulations were performed using NAMD 2.8 (http://www.ks.uiuc.edu/Research/namd/) and run on the Texas Advanced Computing Center Ranger supercomputer cluster. The CHARMM 22 force field with CMAP corrections\textsuperscript{19} was used for protein-protein interactions and the CHARMM 36 (Ref in\textsuperscript{20}) force field with CMAP corrections was used for lipid-lipid and protein-lipid interactions. The PIP\textsubscript{2} lipid parameters were used as described.\textsuperscript{21} A square membrane bilayer patch consisting of 55 % POPC, 35 % cholesterol, and 10% PIP\textsubscript{2} was created and relaxed using the CHARMM-GUI\textsuperscript{22} (http://www.charmm-gui.org/). The CIP4 F-BAR domain structure measured by Shimada et al\textsuperscript{23} (PDB ID: 2EFK) was used for the protein-membrane system and manually placed on the membrane bilayer surface. Systems were solvated using VMD 1.9\textsuperscript{24} (http://www.ks.uiuc.edu/Research/vmd/). The temperature was heated to 310 K and held constant.\textsuperscript{25} The pressure was held constant at 1 bar.\textsuperscript{26}

**Measurement of cortical tension.** Cortical tension was measured according to Hochmuth et al.\textsuperscript{27} Observations were made at 40X with a Nikon TiE microscope through a camera (GC1290, Prosilica). CHRF-288-11 cells were injected into an open-sided
chamber. The micropipette aspiration pressure was controlled through a home-made manometer. Cortical actin layer tension the pulls the cell into a spherical shape (with a radius, $R_c$). By finely tuning the suction pressure ($P_p$) relative to the pressure outside the micropipette ($P_0$), the cell is maintained in a spherical shape while the aspirated region is a hemisphere such that the aspiration length ($L_p$) is equal to the pipet radius ($R_p$). Therefore the cortical tension ($T_c$, with units of force per length) can be calculated by the law of Laplace: 

$$T_c = \frac{R_c R_p}{2(R_c - R_p)} P_p.$$ 

**Statistical analysis.** Student’s t-test was used to compare the mean ± S.E.M., with the assumption of normal distribution, unless mentioned otherwise. Experiments were performed with $n=3$ (unless mentioned otherwise).

**Results**

*Genetic ablation of CIP4 results in isolated thrombocytopenia.* By western blot we demonstrated the presence of CIP4 in primary megakaryocytes and the megakaryocytic CHRF-288-11 cells (Figure 1A). The staining of CIP4 was diffuse throughout the cytosol and proplatelets (Figures 1B and 1C). Partial colocalization of CIP4 and WASP occurred in both cytoplasm and the proplatelet swellings (Figure 1C). CIP4 translocated from the membrane cytoskeleton to the actin cytoskeleton, as did WASP (Figure 1D).

We analyzed C57Bl/6 male mice aged 3 to 6 months and found that they displayed thrombocytopenia to a similar degree as C57Bl/6 WAS- male mice (Table 1). CIP4/− mice showed neither lymphocytopenia nor splenomegaly, adenopathy, nor runting. There was no difference in the circulating half-life of *in vivo* Biotin-NHS labeled platelets from wild-type or CIP-null mice (performed as described, 28 data not shown), which argues against immune-mediated destruction of platelets. The platelet size did not differ between CIP4/−, WAS/− mice and their wild-type littermates.

*Loss of CIP4 does not affect megakaryocytic progenitor development.* Histologic sectioning of femurs showed no obvious features for megakaryocytes (Figure 2A). To
study megakaryocyte progenitors, bone marrow cells were cultured from CIP4−/− and WAS−/− mice and control littermates. On day 6, cells were stained with cholinesterase, and the number of CFU-MKs was counted. There was no difference between control and CIP4-null mice (Figure 2B), and no difference in ploidy distribution (Figure 2C). By flow cytometry, the number of CD41+ cells did not differ between CIP4-null and controls (1.7% ± 0.7 v. 1.5% ± 0.3). This indicated that CIP4 does not affect early megakaryocytopoiesis and suggested that the thrombocytopenia more likely reflects defective platelet biogenesis.

Loss of CIP4 decreases proplatelet formation. Bone marrow cells from CIP4-null mice or their wild-type littermates were grown in suspension with thrombopoietin. At day 4, the percentage of proplatelet-forming megakaryocytes was measured (Figure 3A). The fraction of megakaryocytes displaying proplatelet protrusions was decreased, which was not seen in WASP KO megakaryocytes (Figure 3A). The decrease in percentage of CIP4-null megakaryocytes demonstrating proplatelets (40%) correlated with the decrease in platelet number (44%). Since CIP4 was previously shown to interact with tubulin,11,29 we looked at the ability to form tubulin-driven loops at proplatelet tips, which is a critical component for proper proplatelet formation.1 Proplatelet protrusions derived from CIP4-null megakaryocytes appeared normal in structure and stained with tubulin (Figure 3B). We did not notice an abnormal structure in proplatelets from WASP KO megakaryocytes from mice, in line with previous findings.4,5

We confirmed the reduction of proplatelet protrusions with loss of CIP4 in CHRF-288-11, a human megakaryocytic cell line that forms proplatelet-like protrusions with PMA treatment.14 Compared to controls, cells transduced with shRNA to human CIP4 demonstrated marked silencing (79% reduction) of CIP4 (Figure 4A) and a 50% decrease in proplatelet protrusions and in their length (Figure 4B-C-D), which was not observed with knockdown of WASP or TOCA1 (another F-BAR protein highly similar to CIP4).
The demarcation membrane system (DMS) is rich in phosphoinositides, phospholipids that BAR proteins preferentially bind. DMS comprises an intracellular membrane system of branching tubules and flattened cisternae. Because F-BAR proteins polymerize around the lipid tubules, we speculated that loss of CIP4 would not promote extensive tubulation. Transmission electron microscopy (TEM) identifies distinct regions within the cytoplasm of megakaryocytes that have been termed platelet territories, or platelet-regions containing distinct α-granules surrounded by DMS. Although it has been proposed that platelet territories gives rise to platelets via fragmentation, more recent studies suggests that platelets are formed via microtubule containing projections called proplatelets. TEM of bone marrow showed (Figure 5A), reduced to missing platelet territories, suggesting defective DMS formation, in CIP4-null compared to their wild-type littermates (>50 megakaryocytes visualized). DMS membranes separating platelet territories were either poorly defined or missing in CIP4-null megakaryocytes, opposite to what was described for WASP deficiency. Occasionally, TEM revealed some platelet shedding, as had been described for WASP KO mice by Sabri et al. However the abnormal DMS was the predominant abnormality observed, which likely leads to reduced proplatelet formation in CIP4-null megakaryocytes. Thus, in vivo, in vitro, and ultrastructural studies of megakaryocytes suggested that CIP4 affected proplatelet formation. Interestingly, some signs of membrane disruption of the plasma membrane and vacuole membrane were evident in the CHRF cells with knockdown compared with control cells (Figure 5B). Platelets from CIP4-null mice were ultrastructurally not different from their wild-type littermates (data not shown).

Loss of CIP4 promotes membrane rigidity. As CIP4 links membrane and actin cytoskeleton, we hypothesized that loss of CIP4 would affect membrane and cytoskeletal remodeling that leads to proplatelet formation. Cell membrane fluidity is inversely proportional to anisotropy values. We measured anisotropy on cells labeled with TMA-DPH, a molecular probe that localizes specifically in plasma membrane. Treatment with either PMA or fibronectin, agents that promote proplatelet protrusions in CHRF-288-11 cells, induced decreased membrane fluidity. PMA-induced membrane
fluidity increased by 4% (range 2 to 6%) in control cells. However, no effect of PMA was observed in CIP4-deficient cells (Figure 6A). Fibronectin also increased membrane fluidity by 6 ± 1% in control CHRF-288-11 cells. In contrast, CIP4-deficient cells displayed a 3 ± 1% increase in membrane rigidity (Figure 6B). The effect of CIP4 loss was greater on membrane fluidity than that seen with knockdown of WASP. Knockdown of WASP showed less plasma membrane fluidity in response to PMA (the increase in fluidity was only 1.8 ± 0.7%). However, this effect was not found with fibronectin. We also performed simulations based on nuclear magnetic resonance (NMR) deuterium studies. NMR deuterium order parameters, $S_{cd}$, were calculated from atomistic molecular dynamics simulations for the unsaturated phosphatidylcholine acyl lipid tails (Figure 6C, D). The order parameters were calculated for a 12.0 nm by 3.0 nm region of lipids near the CIP4 protein (Figure 6E) and a membrane patch with no protein. The order parameter is a function of the angle formed by a carbon-deuterium bond on the lipid acyl chain and the vector perpendicular to the membrane surface (Figure 6F, G). A lower order parameter value is associated with increased disorder and fluid character of the acyl chains. As seen in Figure 6C, the order parameters of the plateau region are lower for acyl chains near the protein than in the protein-free system; corresponding to an increase in fluid character of the acyl chains near the protein. The order parameter calculations show a 2.34 +/- 0.02% difference between both systems. The simulation results showed that individual F-BAR domains increase lipid disorder in their local region as measured by NMR order parameters. Collectively this behavior can lead to increased membrane fluidity.

**Loss of CIP4 knockdown on cell cortical tension.** Cell cortical rigidity depends on the connections between the actin and the membrane. Through its SH3 domain and recruitment of WASP, F-BAR proteins regulate actin polymerization, a main regulator for cortical rigidity. To measure stiffness of the actin cortex that provides support for the plasma membrane, we performed micropipette aspiration (Figure 7A-F). Compared to control CHRF-288-11 cells, knockdown of CIP4 or WASP resulted in reduced cortical tension in all conditions tested (baseline, PMA or fibronectin, Figure 7B), suggesting that the anchorage of actin fibers with the cellular membrane was weakened. These
studies revealed that the actin-rich cellular cortex is softer (due to decreased actin polymerization) in CIP4 or WASP-deficient cells. These differences were maintained even when cortical tension increased following PMA stimulation. Sometimes, the transparent region turned into blebs (Figure 7C-F). In controls, the transparent region was aspirated farthest into the micropipette and blebbing was rare. With CIP4 knockdown, cell swelling was observed under the isotonic condition, and repeated blebbing was observed in the inner transparent region inside the pipette (measurements shown on Figure 7B were performed before blebbing occurred).

**DISCUSSION**

Our studies demonstrate that loss of CIP4 resulted in thrombocytopenia, which was associated with decreased proplatelet formation. Biophysical studies revealed that loss of CIP4 produced a stiffer membrane and a softer actin cortical tension. Ultrastructural studies revealed poorly defined or missing DMS in CIP4-deficient megakaryocytes. Our findings identify CIP4 as a new component in the molecular machinery that remodels megakaryocyte membrane and generates proplatelets and suggests a new mechanism for the tubulation required for formation of the DMS.

The production of platelets from megakaryocytes requires a series of highly-coordinated processes involving membrane/cytoskeletal remodeling unique in the body.\(^1\) Two events are critical: first, the megakaryocyte gains mass and then the megakaryocyte buds off proplatelets.\(^37\) *In vivo* imaging of megakaryocytes in the bone marrow microenvironment niche demonstrated extension of proplatelets into the vasculature, where blood flow provides shear forces that generate pre-platelet/platelet release into the circulation.\(^38\) To date, only a few molecules have been identified that drive the dynamic remodeling of membrane and cytoplasm in platelet biogenesis. Our studies have now identified a new component, F-BAR protein CIP4, and a new mechanism for proplatelet formation involving CIP4-directed membrane/actin remodeling.

The F-BAR subfamily of proteins is diverse with six subgroups, each possessing
different properties such as tyrosine kinase activity, GTPase function, or phosphatase interaction. The F-BAR protein most closely related to CIP4 is TOCA1. When TOCA1 was silenced by shRNA, no decrease in proplatelet formation was found, suggesting that CIP4 provides a unique function. Other types of BAR proteins exist: N-BAR proteins and I-BAR proteins, such as IRSp53. When platelet biogenesis was studied in IRSp53-deficient cells, no abnormalities were found. As a scaffolding protein, CIP4 interacts with the activated form of Cdc42, inactive state of WASP, Src kinases, and the negatively charged phosphoinositides residing in the inner leaflet of the plasma membrane. The F-BAR domain dimers enable oligomerization and extensive lateral interactions. This tight packing on curved membranes promotes membrane tubulation. In addition, CIP4 brings together actin cortex with these plasma membrane deformations. We recently reported that invadopodia, a form of cellular protrusion, were decreased in breast cancer cells when CIP4 is knocked-down by siRNA. Here, we observed decreased platelet fields in CIP4-null megakaryocytes when analyzing their ultrastructure. Since proplatelet formation depends on the membrane and cytoskeleton of the megakaryocyte, reduced DMS likely impairs proplatelet formation. Therefore not surprisingly did we also observe decreased proplatelet protrusions per megakaryocyte in primary megakaryocytes from CIP4-null mice. We reasoned that loss of CIP4 affected membrane remodeling. Indeed, fluorescence anisotropy and micropipette aspiration assays demonstrated a more rigid plasma membrane and a softer cell cortex. The relationship between 2H-NMR order parameters and elastic properties of the membrane has been experimentally established. As the flexibility of the acyl chain decreases, the membrane adopts a more gel like phase. Interestingly, a previous work showed that TOCA1, a protein closely related to CIP4, binds preferentially to the more gel like/ordered membrane regions; however the gel phase was not an absolute perquisite for TOCA1-induced filopodia-like structure formation. In contrast to spontaneous membrane undulations, a comparatively high level of energy is required to cause membrane bending. Our findings of decreased cortical tension in cells with knockdown of CIP4 or WASP suggest that CIP4 provides a localization cue for engaging WASP-triggered cortex actin network contributing to platelet biogenesis; however besides cortical tension measurements CIP4 behave differently than WASP in regard to
proplatelet formation, DMS formation and effect on plasma membrane order. Even though the difference in plasma membrane order induced by CIP4 depletion was small in our experimental data, it was statistically significant in experimental data and supported by theoretical computer-based simulation. This new finding supports that a change in membrane order at the molecular level will have repercussion in plasma membrane recycling at the cellular level, with eventually perturbing processes as membrane invagination (DMS) and subsequent proplatelet protrusion.

Insights have come from the analyses of hereditary thrombocytopenias – such as Wiskott-Aldrich syndrome, where platelet size is small or those with giant platelet syndromes, including mutations of Myh9, Filamin-A, and beta1-tubulin. Because murine MPV is half that of human MPV, WAS-deficient platelets in mice are already small. Our studies here suggest that loss or mutation in the CIP4 gene would result in a non-X-linked form congenital thrombocytopenia, but the mouse strain cannot predict whether the platelets would be smaller.

Here, we report that gene disruption of CIP4 resulted in thrombocytopenia to a similar degree observed in WAS-null mice. The SH3 domain of CIP4 interacts with WASP. We observed that loss of either CIP4 or WASP resulted in decreased cortical tension in megakaryocytes, suggesting at least one common mechanism in defective platelet biogenesis. While CIP4 contributes to T cell adhesion and migration and to NK cell cytotoxicity, we have not observed in CIP4 mice such a profound immunodeficiency similar to that of WAS-null mice. WAS-null mice and CIP4-null mice also differ in regard to proplatelet and DMS formation in primary megakaryocytes. Thus, we conclude that CIP4’s effects be due to cortical tension-related mechanisms, involving WASP, and also WASP-independent mechanisms on plasma membrane remodeling and membrane recycling. CIP4’s role in megakaryocyte interaction with matrix could be subsequently studied.

In conclusion, we have found that lack of CIP4, a scaffolding protein that interacts with Cdc42, Src kinases, and WASP results in murine thrombocytopenia. F-BAR protein
CIP4 remodels both the plasma membrane and the cortical cytoskeleton. Loss of CIP4 affects both megakaryocyte membrane fluidity and its cortical tension. Recent theoretical models support that modulation of biophysical cortical forces is crucial for platelet formation, with a new finding that depletion of a protein modifying plasma membrane order will result in thrombocytopenia. Our findings should stimulate new investigations into the biophysical events driving cytoskeletal-membrane remodeling as well as new candidate genes in inherited thrombocytopenias.

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Authorship

The authors declare no financial interest.
References


Table 1. Hematological values in WT, CIP4 KO and WASP KO C57Bl/6 mice: CIP4 KO, WASP KO mice are thrombocytopenic. Wbc, white blood cells; PMN, polymorphonuclear leukocytes; lym, lymphocytes; Hb, hemoglobin; MCV, mean corpuscular volume; Plt, platelets; MPV, mean platelet volume. Values shown are Average ± S.E.M. A minimum of 8 mice were analyzed per group. Similar to WASP KO mice, CIP4 KO mice showed thrombocytopenia (p<0.05 by ANOVA).

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<th>WBC (k/uL)</th>
<th>Neutro (k/uL)</th>
<th>Lym (k/uL)</th>
<th>Hb (g/dL)</th>
<th>MCV (fL)</th>
<th>Plt (k/uL)</th>
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<td>WT</td>
<td>16.91 ±1.8</td>
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<td>CIP4 KO</td>
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Figure 1.

CIP4 localizes with WASP and the actin cytoskeleton network in MKs and platelets. (A) CIP4 is present in megakaryocytic cells. Western blotting of human or murine platelets, murine megakaryocytes, or human megakaryocyte cell line CHRF-288-11 demonstrated the presence of CIP4 at the expected 70-80 kD size. A weaker 100 kD band is of unknown nature. (B) CIP4-GFP localizes throughout the cytoplasm and at the plasma membrane. Human CIP4 tagged with GFP was expressed in CHRF-288-11 cells treated with PMA using the Amaxa Nucleofector II. Images were taken with a Leica DM 4000B microscope and analyzed by LAS (Leica Application Suite) software (Leica, Wetzlar, Germany). (Objective 100x/Numerical Aperture 1.3, scale bar = 10 um). (C) CIP4 and WASP colocalize in cultured wild-type megakaryocytes. Confocal microscopy with using a Nikon Eclipse C1Si confocal microscope (Objective 40x/Numerical Aperture 1) and EZ-C1 software (Nikon, Tokyo, Japan) showed the distribution of CIP4 (Alexa-488) or WASP (Alexa-594). When merged, colocalization occurred in both cytoplasm and proplatelets. Upper image: anti-CIP4 staining (secondary antibody conjugated with Alexa-488) Middle image: anti-WASP staining (secondary antibody conjugated with Alexa-594). Lower image: merge and DAPI. (Objective 40x/Numerical Aperture 1, scale bar = 10 um). (D) CIP4 localization to actin cytoskeletal network in platelets. Platelets were activated with thrombin and lysed in 1% Triton X-100. The Triton insoluble fraction was resuspended in RIPA detergent buffer, centrifuged, and the actin cytoskeleton was collected. The Triton X-100 soluble fraction was centrifuged, and the pellet was resuspended in RIPA, centrifuged, and membrane cytoskeleton was collected from the supernatant. Western blot was performed with antibodies directed against either WASP or CIP4, which are found in the cytosol in platelets and translocates from the membrane cytoskeleton to the actin cytoskeleton. WCL: whole cell lysates.
Figure 1A

<table>
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<tr>
<th>MW (kD)</th>
<th>human platelets</th>
<th>mouse platelets</th>
<th>mouse MKs</th>
<th>CHRF-288-11</th>
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<tr>
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Blot CIP4

Blot actin

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Figure 1B
Figure 1C
Figure 1D

<table>
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<tr>
<th>Thrombin treatment</th>
<th>WCL</th>
<th>Cytosol</th>
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<td>-</td>
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**Figure 2. Histologic morphology, colony growth and ploidy of CIP4-null MKs do not differ from wild-type MKs.** (A) Histologic studies of MKs in CIP4 KO v. WT mice. Bone marrow sections were stained with hematoxylin and eosin. There were no consistent morphologic differences between MKs from either CIP-null mice or their wild-type littermates. Photographs were obtained by an Olympus model BX50 with Olympus model DP71 camera and bundled software (Olympus, Tokyo, Japan). Images shown are 1000x; numerical aperture of the objective 1.25. A scale bar corresponding to 50 um is shown. (B) CFU-MK assay. Harvested bone marrow cells were cultured for 6 days in collagen-MegaCult medium with TPO 50 ng/mL and IL3 10 ng/mL. At day 6, colonies were stained and counted. No effect of CIP4 deficiency on CFU-MK was seen. Data are shown as the average ± S.E.M. from 3-8 independent experiments. (C) Distribution of ploidy in CD41+ megakaryocytes from wild-type and CIP4-null mice. Bone marrow cells harvested from femurs were stained with propidium iodine for DNA content and CD41 for megakaryocyte identity, no difference was found. The cells were then analyzed using Becton-Dickinson flow cytometer LSRII and FlowJo 7.6 software (Tree Star Inc). A minimum of 3 mice were analyzed per group.
Figure 2A.
Figure 2B

The graph shows the number of CFU-MKs per 10e5 nucleated cells for WT, CIP4 KO, and WASP KO conditions. The y-axis represents the number of CFU-MKs, while the x-axis categorizes the conditions as WT, CIP4 KO, and WASP KO.
Figure 2C

![Bar chart showing percent of cell population at different ploidy levels for CIP4 +/- and CIP4 -/-]
Figure 3. Impaired proplatelet formation in CIP4-null MKs. (A) CIP4-null MKs form less proplatelets. CIP4-null, WASP-null, and wild-type megakaryocytes were grown in culture in suspension with thrombopoietin. At day 4, the percentage of proplatelet forming MKs was reported by scoring 300 MKs. The data shown are the average ± S.E.M. from three independent experiments, except for WASP KO, which was performed twice. (B) CIP4 KO cells are mechanistically able to extend tubulin loops in their proplatelets tips. The cells were stained with immunofluorescence at day 4 or 5 of culture with TPO for beta-tubulin (Alexa-488 conjugated secondary antibody) to look for ability to form loops, for von Willebrand Factor (Alexa-594 conjugated secondary antibody), to document their megakaryocytic nature, and with DAPI to show the nucleus. The pictures on the top show wild-type megakaryocytes. The pictures on the bottom show CIP4-null megakaryocytes. The pictures on the last column show proplatelets detached from the bodies of megakaryocytes. Pictures were taken with a Leica DM 4000B microscope, visualized with a Leica DFC320 camera and analyzed by LAS software (Leica, Wetzlar, Germany). (Objective 100X/Numerical Aperture 1.3, scale bar = 10 um).
Figure 3A

Percentage of MKs with proplatelets

WT  CIP4 KO  WASP KO
Figure 3B

WT

beta-tubulin

vonWillebrand Factor

DAPI

CIP4 KO

beta-tubulin

vonWillebrand Factor

DAPI
Figure 4. Impaired proplatelet formation in CIP4-deficient human megakaryocytic cell line. (A) Decreased protein expression of CIP4 and WASP following shRNA transduction of the CHRF-288-11 cells. Western blot demonstrated successful knockdown by lentiviral mediated shRNAs of CIP4 (79% reduction compared to control) or WASP (90% reduction compared to control) or TOCA1 (74% reduction compared to control). Control cells were treated with a lentiviral-mediated non-targeting shRNA sequence. B. Morphologic changes of decreased proplatelet protrusion (arrows) and rounding up in CIP4-deficient CHRF-288-11 cells. CHRF-288-11 cells, transduced by control shRNA, on the upper two rows, were compared with CHRF-288-11 cells with CIP4 knockdown on the lower two rows. Pictures were taken on a Nikon Biostation (objective X 20/Numerical Aperture 0.80, scale bar = 10 um, Nikon, Tokyo, Japan) and using the Biostation IM system and dedicated software (Nikon, Tokyo, Japan). C. Decreased proplatelet-like extensions in CHRF-288-11 cells deficient in CIP4. CHRF-288-11 cells with shRNA-CIP4 knockdown or shRNA-WASP knockdown versus control cells with non-targeting shRNA sequence were exposed to PMA at 10 ng/mL overnight. The percentage of cells with proplatelet-like extensions megakaryocytes is reported after scoring 300 cells. Compared with control, the percentage was decreased with shRNA CIP4 (p=0.04) but not for cells with shRNA WASP (p=0.10, t-test) nor with TOCA1 (p = 0.54). The data are shown as the average ± SEM from 3 independent experiments. D. Decreased median length of proplatelet-like extensions in CIP4 deficient CHRF-288-11 cells. Protrusions were measures in at least 25 cells per condition using NeuronJ. The median length of protrusions was decreased in CHRF-288-11 cells with shRNA knockdown compared with control (p=0.049, t-test). Reported is average ± SEM from three independent experiments.
Figure 4A

Control shRNA CIP4

CIP4

Actin

50

37

CTRL shRNA WASP

Blot: WASP

Blot: Actin

CTRL shRNA TOCA1

Blot: TOCA1

Blot: Actin
Figure 4B
Figure 4C

Percentage of cells with proplatelet-like extensions after PMA exposure

Control shRNA CIP4 shRNA WASP shRNA TOCA1
Figure 4D

Median length of proplatelet-like protrusions in CHRF-288-11 cells (microns)

- control
- shCIP4
Figure 5 Ultrastructural features of CIP4-null MKs reveal reduced platelet territories. A. Transmission electron microscopy (JEM 1011, JEOL, Tokyo, Japan) analysis of bone marrow megakaryocytes from WT (left) vs. CIP4 KO (right) mice revealed greatly reduced to completely missing platelet territories in CIP4 KO megakaryocytes. The demarcation membrane system, which are membranes separating platelet territories, were either poorly defined or missing altogether in the CIP4 KO megakaryocytes. A minimum of 3 mice was analyzed for each group. Shown are representative images for each group. Magnification as mentioned on the figure; scale bar=2um. Areas within the boxes are shown in the lower panel at a higher magnification. (B) Ultrastructural features of CHRF-288-11 cells. Signs of membrane disruption of the plasma membrane and vacuole membrane are evident in cells with CIP4 knockdown (bottom images). The plasma membrane is clearly disrupted in the CIP4 KD CHRF-288-11 cell (bottom left 2 images), whereas a vacuole with protruding vesicles is seen in another cell (bottom right 2 images). Areas within the boxes are shown on the right adjacent side at a higher magnification.
Figure 5B

Control

CIP4 knockdown
Figure 6. Loss of CIP4 affects membrane fluidity. A. Decreased membrane fluidity in CIP4-deficient cells by fluorescence anisotropy study with PMA treatment. Fluorescent anisotropy values (r) in response to stimulation by PMA. Results are given as % of time zero values. A lower r value means higher membrane fluidity. Cells with CIP4 knockdown have impaired response in terms of plasma membrane fluidity, when compared with control cells (p < 0.05 when comparing control and shRNA CIP4 cells for 20, 40, and 50 min). B. Decreased membrane fluidity in cells deficient for CIP4 treated with fibronectin. Fluorescent anisotropy value in response to integrin stimulation by fibronectin. Results are % of control (BSA coating) conditions. After labeling, CHRF cells were incubated on a fibronectin- or BSA-coated plate for 2 hours. CIP4 knockdown cells display decreased membrane fluidity compared with control. (p =0.002 for shCIP4 compared, p=0.10 for shRNA WASP). C-G. Simulated NMR deuterium order parameters calculations confirm decreased fluidity in the presence of CIP4 protein. The deuterium order parameters, Scdh, were plotted (C) for each carbon in the unsaturated POPC acyl chain (D) and show a 2.34 +/- 0.02% decrease between the lipids near the protein (E) and the protein free system. The order parameter is a function of the angle formed by a carbon-deuterium bond on the lipid acyl chain and the vector perpendicular to the membrane surface (F and G) and a decrease in order parameter is associated with an increase in disorder of the acyl chain.
Figure 6 A. Abnormal plasma membrane fluidity in cells deficient for CIP4 by fluorescence anisotropy study with TMA-DPH, with PMA treatment.
Figure 6B
Figures 6 C, D, E, F and G.
Figure 7. Loss of CIP4 reduces cortical tension in CHRF cells.  (A) Photomicrograph of micropipette aspiration of a CHRF-288-11 cell. The pressure in buffer ($P_0$), the suction pressure inside the pipette ($P_p$), the inner radius of the pipette ($R_p$), the radius of the spherical portion of the cell outside the pipette ($R_c$), and the length of the cell tongue aspirated inside the pipette ($L_p$) are indicated. Aspiration could cause polarization of cytoplasmic contents. The image shows the condensed region stays away from the pipette orifice where a transparent region is formed nearby. (B) Decreased cortical tension in CHRF cells following reduced levels of CIP4 or WASP either at baseline or after treatment with either PMA or fibronectin. ** $p \leq 0.01$; *** $p \leq 0.001$. (C-D) Representative images of micropipette aspiration of an untreated cell without (C) with (D) CIP4 knockdown. Without CIP4 knockdown, the transparent region was aspirated furthest into the pipette and blebbing was rare. With CIP4 knockdown, cell swelling was observed under the isotonic condition. Repeated blebbing was observed in the inner transparent region inside the pipette. (E-F) Blebbing of CHRF cells. E: Initiation of membrane blebbing. The near-orifice transparent region was pinched off and transformed into a bubble-like protrusion. The dynamic process was driven by the sucking pressure. F: Repeated blebbing. The protruded bleb was snitched out, followed by a newly-extruded immature bleb. (Magnification 40x; scale bar = 5 um). Images were obtained with an inverted microscope with 40x dry lens (Nikon TiE, Nikon, Tokyo, Japan) through a camera (GC1290, Prosilica, Allied Vision Technologies, Augusta Technologie, Munich, Germany) that has a standard video rate (30 frames per second). The acquisition program was home-made.
Figure 7

Panel A: Condensed region

Panel B: Cortical tension (pN/μm)

- **: p < 0.01
- ***: p < 0.001

Panel C: Control

Panel D: WASP KD

Panel E: CIP4 KD

Panel F: Control
Loss of the F-BAR protein CIP4 reduces platelet production by impairing membrane-cytoskeleton remodeling