The GRAF family member oligophrenin1 is a RhoGAP with BAR domain and regulates Rho GTPases in platelets

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Aims
Platelet adhesion and aggregation is essential for haemostasis and thrombosis. Cytoskeletal reorganization of activated platelets plays a crucial role in these processes and implies activation of Rho GTPases. Rho GTPases are regulated by GTPase-activating proteins (GAPs) that stimulate GTP hydrolysis to terminate Rho signalling. In this study, we explored the regulation of Rho GTPases in platelets.

Methods and results
Oligophrenin1 (OPHN1) is a RhoGAP-regulating cytoplasmic protein that has been investigated in patients with X-linked mental retardation. Here, we identified OPHN1 in mouse platelets where it co-localizes to actin-rich regions and Rho GTPases. OPHN1 exhibits strong GTpase-stimulating activity towards RhoA, Cdc42, and Rac1 and regulates cell adhesion and spreading. Furthermore, OPHN1 controls RhoA-mediated stress fibre and focal adhesion formation as well as filopodia and lamellipodia development. The analysis of different domains of OPHN1 revealed distinct functions in Rho hydrolysis. The C-terminus of OPHN1 displays an essential unit for Rho regulation, whereas the PH domain is a regulatory unit of OPHN1 controlling GAP function. The N-terminal BAR (Bin/amphiphysin/Rvs)-like domain is involved in GAP regulation but not in cytoskeleton rearrangements or Rho regulation and acts as a guidance domain to direct this GAP to its substrate.

Conclusion
Our results suggest that OPHN1 is a powerful regulator of Rho GTPase activity in platelets that is critical for the reorganization of the cytoskeleton, which is a major process required for stable platelet adhesion and thrombus formation to occur.

Keywords
OPHN1 • Cytoskeleton • Rho GTPases • Platelets • GTpase-activating proteins

1. Introduction
Platelets play an essential role in haemostasis and thrombosis. After tissue damage, they adhere at the exposed subendothelial matrix and form a haemostatic plug to avoid excessive blood loss. In addition to the important role of platelets in haemostasis, dysfunction of platelet activity is responsible for thrombosis and can lead to myocardial infarction and stroke. Platelet activation and thrombus formation is a multistage process that involves initial platelet adhesion to the injured vessel wall followed by platelet activation leading to shape change, activation of integrins, and secretion. Stable platelet adhesion and aggregation induce thrombus formation mediated mainly by actin-based reorganization of the platelet cytoskeleton that is accompanied by fibrinogen binding to activated integrin αIIbβ3.

Small GTPases of the Rho family, namely RhoA, Cdc42, and Rac1, are key regulators of signalling pathways that regulate actin organization by the formation of stress fibres and focal adhesions, lamellipodia, and filopodia. Furthermore, they play an important role in platelet secretion and activation of phospholipase Cγ2. Rho GTPases are guanine nucleotide-binding proteins and function as molecular switches that cycle between an inactive GDP-bound and an active GTP-bound state. Thus, GTpase activity is spatially and temporally well regulated by different regulatory proteins such as GTpase-activating proteins (GAPs). In the active GTP-bound state, Rho GTPases interact with target proteins until GAPs induce their return back to an inactive state by stimulating GTP hydrolysis. Oligophrenin1 (OPHN1) is a RhoGAP involved in X-linked mental retardation (XLMR) that has been shown to negatively regulate RhoA, Cdc42, and Rac1 in neuronal...
and non-neuronal cells. The cytosolic protein OPHN1 has a defined domain structure that includes an N-terminal BAR (Bin/amphiphysin/Rvs)-like domain followed by a PH domain and a catalytic RhoGAP domain. Recent studies provide evidence that the N-terminal BAR domain of OPHN1 affects its GAP function by acting as a regulator of GAP activity by an auto-inhibitory mechanism.

OPHN1 is involved in neuronal development and function, thereby contributing to XLMR as seen in patients with a loss of function of the OPHN1 gene that leads to constitutive activation of its GTPase targets. Increased activation of GTPases is also seen in contributing to XLMR as seen in patients with a loss of function of the regulatory domain of OPHN1-GAP and guides OPHN1 to its sub-strate, the active Rho GTPase. Although much effort has been made to understand the role of Rho GTPases, their regulation by guanine nucleotide exchange factors (GEFs) and GAPs is only partially understood, and this is complicated by the lack of known GEFs and GAPs. Therefore, a detailed characterization of Rho regulation is necessary to understand activation and inactivation of Rho GTPases critical for cytoskeletal reorganization upon platelet activation and aggregation in processes of thrombosis and haemostasis. In the present study, we show for the first time that OPHN1 is expressed in platelets and co-localizes to actin-rich regions. We have characterized this new platelet GAP with BAR domain as a potent GAP for RhoA, Cdc42, and Rac1 with the same domain structure as that observed in neuronal cells. OPHN1 regulates cell adhesion and spreading while controlling RhoA-mediated stress fibre and focal adhesion formation as well as Rac1-specific lamellipodia development. Additionally, we show that the domains of OPHN1 are differentially involved in Rho hydrolysis. The OPHN1-BAR domain is a regulatory domain of OPHN1-GAP and guides OPHN1 to its sub-strate, the active Rho GTPase.

2. Methods

See Supplementary material online for further details.

2.1 Platelet preparation

Platelets from C57Bl/6 mice were isolated from whole blood taken from the retro-orbital plexus under isoflurane (2%) anaesthesia that was monitored by lack of withdrawal upon hind toe pinching. Animal studies were approved by the local authorities (regional board Tübingen). All animal experiments were performed in accordance with the Guide for the Care and Use of Laboratory animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996), the guidelines for the use of living animals in scientific studies, and the German law for the welfare of animals. Before use, platelets were resuspended in Tyrode's buffer and incubated at 37°C for 30 min.

2.2 Immunoprecipitation

Resting and thrombin stimulated platelets (5 x 10⁸ platelets/mL) were lysed and samples were incubated with appropriate antibody. Protein G-Sepharose was washed and added and samples were rotated overnight at 4°C. The Sepharose pellet was washed before addition of Laemmli sample buffer. Immunoblotting was performed as indicated.

2.3 Real-time quantitative RT–PCR

Real-time RT–PCR analysis was carried out using SYBR Green I as a fluorogenic probe. For the assessment of endogenously expressed OPHN1, total RNA from platelets was extracted. RT reactions were performed using 100 ng of total RNA.

2.4 Cell culture and transfections

AS-CHO cells were cultured in DMEM at 37°C in a 5% CO₂ atmosphere. For transient protein expression, cells were seeded in six-well cell culture plate dishes 24 h prior to transfection. Transfection was carried out using 4 µg plasmid DNA and 10 µL Lipofectamine 2000 per dish.

2.5 Spreading with immunohistology

Cover slips were coated human fibrinogen and platelets were seeded on cover slips and incubated at room temperature for 45 min. Adherent platelets were fixed and permeabilized with PHEM buffer.

AS-CHO cells were seeded on cover slips and incubated at room temperature for 45 min. For ROCK inhibitor experiments, cells were resuspended and incubated with 10 µM ROCK inhibitor at 37°C for 10 min. Adherent cells were fixed and permeabilized. After washing with phosphate-buffered saline (PBS), the slides were stained and fluorescence was detected using a confocal laser scanning microscope.

2.6 G-LISA™ assay

Cells were washed in ice-cold PBS and lysed for 5 min. After centrifugation, supernatant was incubated at 4°C in a Rho-GTP affinity plate. An active GTP-bound Rho protein was then detected by incubation with a specific primary antibody followed by a secondary antibody conjugated to horseshadish peroxidase. The signal was measured at 490 nm using a microplate reader.

2.7 F-actin/G-actin assay

The ratio of F-actin vs. G-actin was determined using a G-actin/F-actin in vivo assay kit. Briefly, cells were lysed, homogenized, and centrifuged at 100 000 g for 60 min at 37°C. The supernatant (G-actin) was separated from the pellet (F-actin) and both were incubated on ice for 60 min. Samples were analysed by western blotting with an actin antibody and the ratio of F-actin vs. G-actin was quantified using ImageJ.

2.8 Statistics

Statistical analysis was performed using Prism Graph software, Student’s t-test, and non-parametric Mann–Whitney test statistics. Results are presented as means ± standard deviations (SD) with P < 0.05 representing significance.

3. Results

3.1 The GAP with BAR domain OPHN1 is expressed in platelets and megakaryocytes and co-localizes to actin-rich regions

The regulation of Rho GTPases in platelets and its impact on platelet activation, adhesion, and cytoskeletal rearrangement are not well understood. To analyse Rho inhibition, we identified OPHN1 in murine and human platelets. We found OPHN1 to be strongly expressed in human and murine platelets as well as megakaryocytes, as shown by western blotting (Figure 1B) and immunohistochemistry (Figure 1C). Furthermore, we detected and quantified mRNA of platelet OPHN1 by quantitative RT–PCR and compared the mRNA level with brain OPHN1 (Figure 1A). The complete cDNA of OPHN1 was cloned from platelet mRNA and sequence analysis revealed 100% homology to brain OPHN1 (GenBank accession number NM_052976.3) (see Supplementary material online, Figure S1A).

For OPHN1 distribution in platelets, we investigated intracellular localization of OPHN1 in platelets spread on a fibrinogen-coated surface in the presence of thrombin (0.008 U/mL) (Figure 1D; see Supplementary material online, Figure S1B). The localization of OPHN1 in

OPHN1 and Rho GTPases in platelets

527
Figure 1  OPHN1 is expressed in platelets and megakaryocytes and co-localizes to F-actin. (A) Detection of Ophn1 mRNA in platelets and brain. Actin was used as a reference gene. RT–PCR products were resolved on agarose gels (left). Quantification of Ophn1 mRNA in platelets compared with brain (right). (B) Western blot analysis of OPHN1 expression. (C) Detection of OPHN1 in megakaryocytes of spleen cryosections using confocal microscopy. Scale bar 50 μm. (D) OPHN1 co-localizes to actin-rich regions in spreading platelets. Platelets spread on fibrinogen were fixed, permeabilized, and stained with appropriate antibodies. Arrows indicate OPHN1 staining at filopodia (i), the central actin ring (ii), and lamellipodia (iii). Scale bar 5 μm. (E) Resting or activated (5 and 15 min) platelets were lysed, immunoprecipitated with OPHN1 antibody, and immunoblotted with actin and α-tubulin antibody.

Figure 2  Time- and activation-dependent co-localization of OPHN1 with small GTPases of the Rho family in platelets. (A) Washed platelets were stimulated with 0.008 U/mL thrombin and allowed to adhere and spread on human fibrinogen (1 mg/mL) for the indicated time points. Platelets spread on fibrinogen were fixed, permeabilized, and stained with OPHN1 (red) and Rac1 (green) antibody. (B) The same experiments were performed using Cdc42 and RhoA antibody (green), respectively, and OPHN1 antibody (red). (C) Resting or activated (5 and 15 min) platelets were lysed, immunoprecipitated with OPHN1 antibody, and immunoblotted with antibodies detecting RhoA, Cdc42, and Rac1. Western blots with antibody against Ran that does not belong to Rho GTPases were performed as a negative control. Scale bar 10 μm (A), 5 μm (A, right panel, B).
OPHN1 contains a Rho-GAP domain and demonstrates co-localization with RhoA, Rac1, and Cdc42 in platelets (Figure 2) and A5-CHO cells (Figure 3B and 6C), suggesting that OPHN1 can interact with all these Rho GTPases. Therefore, we transiently overexpressed OPHN1 full-length protein (OPHN1-FL) in A5-CHO cells and measured Rho GTPase activity with a specific pulldown activation assay called G-LISA (Cytoskeleton, Inc., Denver, CO, USA). We found that OPHN1 decreased global levels of RhoA, Rac1, and Cdc42 with a clear preference for RhoA in all pulldown assays performed using the same conditions (Figure 3A). To study co-localization of OPHN1 and Rho GTPases in activated cells, we overexpressed OPHN1-FL in A5-CHO cells and performed spreading experiments where A5-CHO cells were allowed to spread on a fibrinogen matrix for 60 min. Co-staining of these cells with OPHN1 and Rac1, RhoA, or Cdc42 antibodies demonstrated that Rho GTPases were translocated to the plasma membrane upon OPHN1 overexpression as shown for Rac1 (Figure 3B), RhoA (Figure 6C) and Cdc42 (data not shown), where OPHN1 co-localizes to all these proteins all around the membrane.

3.4 OPHN1 regulates cell adhesion and spreading

The Rho GTPases are known to control many of the changes in the actin cytoskeleton that are triggered when growth factor receptors or integrins bind their ligands. While Rac1 and Cdc42 stimulate the formation of lamellipodia and filopodia, RhoA regulates actin polymerization, contractility, and the assembly of actin stress fibres and focal adhesions. To analyse OPHN1-mediated cytoskeletal re-arrangement, we designed, constructed, and cloned different OPHN1 constructs expressing different OPHN1 domains (Figure 4A) and performed transfection experiments with A5-CHO cells. Different OPHN1 constructs were cloned into the ptdTomatoN1 vector expressing OPHN1 together with a red fluorescent protein. A5-CHO cells were allowed to spread on a fibrinogen matrix for 60 min to induce integrin αIIbβ3 engagement and stained with an antibody for F-actin to visualize the actin cytoskeleton. First, we analysed the total number of adhesive cells and found that OPHN1 overexpression leads to a dramatic reduction in cell adhesion under these conditions. After 60 min, cell adhesion was reduced to 97 ± 5.6 cells per visual field using the full-length protein (OPHN1-FL) and 63.2 ± 8.1 cells per visual field using an OPHN1 construct that expresses only the GAP domain and C-terminus (OPHN1-GAP-Cterm) of the protein (Figure 4B and C). Our results demonstrate that OPHN1 strongly affects cell adhesion, which was more distinct using OPHN1-GAP-Cterm, that lacks the N-terminal domain (BAR domain) known to be involved in regulation of OPHN1-GAP.13

In contrast, A5-CHO cells are able to form filopodia and lamellipodia as described for platelet spreading on fibrinogen.6,22 We therefore investigated filopodia formation and the ability of A5-CHO cells transfected with different OPHN1 constructs to fully spread on a fibrinogen matrix. The analysis of cells that form filopodia revealed no major differences between A5-CHO cells transfected with OPHN1-BAR, OPHN1-FL, or OPHN1-GAP-Cterm (Figure 4D). In contrast, the number of fully spread A5-CHO cells characterized by lamellipodia formation to maximize cell surface demonstrated that only a few A5-CHO cells transfected with OPHN1-FL and OPHN1-GAP-Cterm, respectively, are able to fully spread on fibrinogen compared with control A5-CHO cells (Figure 4B and D). The spreading platelets was dynamic with respect to different phases of platelet activation. To compare the localization of OPHN1 with the cytoskeleton in spreading platelets, we constrained for OPHN1 and polymerized actin using rhodamine-labelled phalloidin. At early phases of platelet adhesion, OPHN1 staining was intense in small unspread platelets. Upon spreading, OPHN1 was localized to filopodia (Figure 1Ei, see arrow), in the central actin ring (Figure 1Eii, see arrow) and lamellipodia (Figure 1Eiii, see arrow) where it co-localizes to actin. In addition, platelet lysates were immunoprecipitated using specific OPHN1 antibody and immunoblotted with actin and α-tubulin antibodies. OPHN1 immunoprecipitates actin and tubulin in resting and activated platelets demonstrating a time- and activation-independent co-localization of these proteins (Figure 1E).

3.2 Time- and activation-dependent co-localization of OPHN1 and small GTPases of the Rho family in platelets

The pattern of OPHN1 distribution in activated platelets suggested that OPHN1 might play a physiological role in cytoskeletal actin assembly by regulating Rho GTPases as shown for neurons. To test this directly, we performed spreading experiments and co-stained platelets with OPHN1 antibody and antibodies against Rho GTPases, namely RhoA, Cdc42, and Rac1. Different time points of spreading (20 and 60 min, respectively) represent different phases of spreading and revealed time-dependent co-localization of Rac1, RhoA, and Cdc42 with OPHN1 (Figure 2A and B). Co-localization of OPHN1 and Rac1 was strong after 60 min of spreading, but only weak after 20 min of platelet spreading where only some platelets were fully spread while others are still in the spreading process and develop filopodia and lamellipodia (see Supplementary material online, Figure S1B). Cdc42 demonstrated a similar co-localization pattern to OPHN1-like Rac1 (Figure 2B). In contrast and independent of time, RhoA co-localization to OPHN1 was observed over the entire spreading process (Figure 2B). Furthermore, immunoprecipitation experiments confirmed activation and time-dependent co-localization of OPHN1 and Rho GTPases. RhoA and OPHN1 co-localization was observed independent of time and activation suggesting sustained RhoA regulation by OPHN1. In contrast, co-localization of OPHN1 with Rac1 and Cdc42 increases with time of activation suggesting enhanced OPHN1-mediated regulation of these Rho GTPases at late time points of platelet activation. In contrast, Ran (RAS-related nuclear protein) (Figure 2C), a member of the Ras superfamily that does not belong to the Rho subfamily,16 did not co-localize with OPHN1. Taken together, these results indicate distinct OPHN1-mediated regulation of Rho GTPases dependent on time of activation and substrate.

3.3 OPHN1 is a GAP of Rho GTPases (RhoA, Cdc42, Rac1) in CHO cells and regulates their translocation to the plasma membrane

Given the fact that platelets are not accessible to transfection studies and no physiological activator of endogenous OPHN1 is known, we decided to analyse GTPase activity and regulation of OPHN1 in cell lines. We made use of a platelet model cell line called A5-CHO cells, a specific CHO cell that stably overexpresses human integrin αIIbβ3.17–20

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Figure 3 OPHN1 is a GAP for Rho GTPases in A5-CHO cells and regulates their translocation to the plasma membrane. (A) GAP activity of OPHN1 overexpressed in A5-CHO cells was analysed. In specific pulldown activation assays (G-LISA, Cytoskeleton), the amount of active, GTP-bound RhoA, Rac1, and Cdc42 was determined using 96-well plates coated with RBD domain of Rho-family effector proteins. The active GTP-bound form of endogenous Rho family protein from A5-CHO cells transfected with OPHN1-FL or control vector that bound to the plate was detected by specific primary antibodies. (B) A5-CHO cells transfected with OPHN1 or control vector were allowed to adhere and spread on human fibrinogen (1 mg/mL) for 60 min and co-stained with OPHN1 (green, left panel) and Rac1 (red, middle panel) antibodies to show cellular localization, overlay (right panel). Scale bar 10 μm. The bar graphs depict mean values ± standard deviation (SD) (n = 3); *p < 0.05, ***p < 0.001.

Figure 4 OPHN1 regulates cell adhesion and spreading. (A) Schematic illustration of generated OPHN1 constructs expressing different domains of OPHN1. (B) A5-CHO cells were transfected with OPHN1-FL, OPHN1-GAP-C term., or control vector expressed in ptdTomatoN1. Cells were allowed to adhere and spread on human fibrinogen (1 mg/mL) for 60 min to induce cytoskeletal reorganization. Different OPHN1 constructs were detected by fluorescence properties of ptdTomato (left panel) and stained with antibodies against F-actin (middle panel), overlay (right panel). (C) Statistical evaluation of cell adhesion and (D) filopodia formation and fully spread cells. The bar graphs depict mean values ± SD (n = 10); **p < 0.01, ***p < 0.001.
majority of these cells developed a round shape and had almost no ability to increase the cell surface as observed in A5-CHO control cells (Figure 4B). In contrast to cell adhesion experiments, we found no major differences between the number of fully spread cells transfected with OPHN1-FL and OPHN1-GAP-Cterm. Transfection experiments with the OPHN1-BAR domain (OPHN1-BAR) revealed that the BAR domain of OPHN1 has no effect on cell adhesion, filopodia formation, or lamellipodia formation (Figure 4C and D).

3.5 OPHN1 controls stress fibre formation, focal adhesions, and actin polymerization

The adhesive character of cells is mediated in part by RhoA and its downstream effector Rho-associated coiled-coil forming protein serine/threonine kinase (ROCK), leading to the formation of stress fibres and focal adhesions.21 To analyse the influence of OPHN1 in these processes, we performed transfection experiments in A5-CHO cells using different OPHN1 constructs. Upon spreading of A5-CHO cells, overexpression of OPHN1 caused a strong reduction in stress fibre formation with 62.3 ± 3.4% number of stress fibres in OPHN1-FL-transfected cells (data not shown) compared with control cells (Figure 3B). The observed defect in stress fibre formation was even stronger in A5-CHO cells overexpressing OPHN1-GAP-Cterm. (Figure 5A and B; 51 ± 1.5% number of stress fibres, P-value = 0.002), demonstrating that the N-terminal BAR domain has a regulatory role in GAP activity of OPHN1.

Additional events triggered by RhoA include the formation of focal adhesions. At cell–extracellular matrix contact points, focal adhesions are formed where bundles of actin filaments are anchored to transmembrane receptors of the integrin family through a multimolecular complex of junctional plaque proteins.23 To test whether OPHN1 influences the formation of focal adhesions, we performed spreading experiments and co-stained spread A5-CHO cells with antibodies against vinculin (red) and actin (green) (Figure 5C). Overexpression of OPHN1-GAP-Cterm. revealed a strong reduction in vinculin staining compared with controls, suggesting strongly reduced formation of focal adhesions upon OPHN1 overexpression. Similar results were obtained using OPHN1-FL. (data not shown). The BAR domain itself showed no influence on stress fibre or focal adhesion formation (data not shown).

Actin cytoskeletal rearrangements also include actin polymerization where all three Rho GTPases are involved.24 To investigate the influence of OPHN1 on actin polymerization, we measured F-actin by flow cytometry. Compared with control cells, F-actin level was markedly reduced in A5-CHO cells overexpressing OPHN1-FL (Figure 5D). Staining of A5-CHO cells with rhodamine-labelled phalloidin confirmed reduced F-actin levels in OPHN1-FL-overexpressing cells measured by flow cytometry (Figure 5D, right panel). The quantification of the ratio of filamentous actin (F-actin) vs. free globular actin (G-actin) in transfected cells confirmed that OPHN1 overexpression leads to reduced actin polymerization (Figure 5E) as observed in flow cytometry experiments.

3.6 OPHN1 regulates cytoskeletal reorganization via RhoA, Rac1, and Cdc42

Previous results indicate that OPHN1 mediates cytoskeletal reorganization mainly by Rac1 and RhoA. To directly analyse OPHN1-mediated Rac1 regulation, we co-transfected A5-CHO cells with OPHN1 expressed in ptdTomato vector and constitutively active Rac1 (EGFP-Rac1Q61L) to investigate the impact of OPHN1 on lamellipodia formation. Overexpression of Rac1 led to the formation of lamellipodia in A5-CHO cells (Figure 5F) confirming recent findings of different groups, indicating that Rac1 is important for the regulation of lamellipodia formation.6 In contrast, co-transfection with OPHN1-FL strongly reduced the number of lamellipodia (Figure 5F and G). An even stronger reduction in lamellipodia formation was observed using OPHN1-GAP-Cterm. that lacks the BAR domain (Figure 5E and G). These results confirm previous results that show an almost stronger effect with OPHN1-GAP-Cterm. in comparison with the full-length protein (OPHN1-FL) (Figure 4B and C).11,13 suggesting that the BAR domain regulates OPHN1-GAP in A5-CHO cells, thereby controlling the effect of OPHN1-GAP on cytoskeletal rearrangements. Furthermore, co-transfection studies using different OPHN1 constructs and constitutively active Cdc42 showed strongly reduced filopodia formation in response to overexpressed OPHN1-FL and an even stronger effect with OPHN1-GAP-Cterm., suggesting GAP regulation by the BAR domain as observed before (see Supplementary material online, Figure S3).

To confirm that OPHN1 mediates its effects on cytoskeletal rearrangement mainly via RhoA, we performed experiments using the ROCK inhibitor Y27632 that inhibits the RhoA effector protein ROCK. We performed spreading experiments and stained the actin cytoskeleton to visualize stress fibres. In line with previous experiments we found reduced stress fibre formation upon ROCK inhibitor treatment (Figure 5H, left panel). In addition, vinculin and actin co-staining demonstrated strongly reduced formation of focal adhesions as observed upon OPHN1 overexpression (Figure 5H, right panel). The analysis of cell adhesion after ROCK inhibitor treatment revealed a dramatic reduction in cell adhesion (Figure 5I) comparable to results obtained from OPHN1 overexpression. Taken together, these results demonstrate that OPHN1-induced reorganization of the cytoskeleton is mediated by RhoA, Rac1, and Cdc42 regulation.

3.7 Distinct function of OPHN1 domains

To characterize the distinct function of different domains of OPHN1, we performed pulldown activation assays (G-LISA) to analyse the impact of different domains in Rho hydrolysis as well as spreading experiments for localization studies.

The work of different groups analysing OPHN1 function in neuronal and non-neuronal cells suggested that the N-terminal fragment of OPHN1, including the BAR domain, interacts directly with the GAP domain and inhibits its activity.11,13 The regulation of the GAP domain by the BAR domain seems to be highly specific, but the mechanism underlying this auto-inhibitory regulation of OPHN1 is currently unclear. In the present study, we were able to confirm a regulatory role for the BAR domain that itself is not involved in Rho regulation (Figure 6A). Different experiments showed a stronger effect of OPHN1 overexpression when the study was performed with OPHN1-GAP-Cterm. compared with the full-length protein (Figure 4B and C, and 5F; see Supplementary material online, Figure S3). G-LISA experiments confirmed these results demonstrating stronger Rho hydrolysis by OPHN1-GAP-Cterm. compared with OPHN1-FL (Figure 6A). Furthermore, we were able to show that the BAR domain itself is not involved in cytoskeleton rearrangements, as shown in adhesion experiments (Figure 4C) as well as filopodia and lamellipodia development experiments (Figure 4D). In line with these results, OPHN1-BAR does not co-localize to actin in A5-CHO cells as observed for OPHN1-FL (see Supplementary material online, Figure S3).
OPHN1 and Rho GTPases in platelets

Figure 5 OPHN1 influences cytoskeletal reorganization. (A) Following transfection, A5-CHO cells were allowed to spread on fibrinogen for 60 min. Spreading-induced formation of stress fibres was analysed by F-actin staining (green) and the number of cells that develop stress fibres was determined. Representative image of control cells (upper panel) and OPHN1-GAP-Cterm-transfected cells (lower panel). (B) Bar graph depicts mean values ± SD (n = 10); *p < 0.001, Scale bar 10 μm (C) Transfected and spread cells were stained for Vinculin (red, left panel) and F-actin (green, overlay, right panel). Scale bar 10 μm. (D) OPHN1-FL-transfected cells were stained with rhodamine-labelled phalloidin. Actin polymerization of transfected cells was quantified by mean fluorescence intensity (MFI) of rhodamine—phalloidin-labelled cells in a flow cytometry assay. Bar graph depicts mean values ± SD, left panel, (n = 3); **p < 0.05. Representative images of control and OPHN1-FL-transfected cells (right panel). Scale bar 5 μm. (E) G-actin (lanes 1—2) and F-actin (lanes 3—4) in A5-CHO cells transfected with control vector (lanes 1 and 3) or OPHN1-FL (lanes 2 and 4) were analysed by western blot with actin antibody and the ratio of F-actin vs. G-actin was quantified. Bars (right panel) represent means ± SD (n = 3). (F) Interaction of different OPHN1 proteins with constitutively active Rac1. A5-CHO cells were co-transfected with different OPHN1 constructs and EGFP-tagged constitutively activate Rac1-Q61L to analyse OPHN1-mediated alterations in lamellipodia formation. (G) Bar graph shows the number of cells developing lamellipodia as a percentage of control (control vector + Rac1-Q61L) and depicts mean values ± SD (n = 10); **p < 0.001. Scale bar 10 μm. (H) The formation of stress fibres (actin, green, left panel) and focal adhesions (actin, green and vinculin, red, right panel) was analysed in control and ROCK inhibitor-treated cells. Scale bar 10 μm. (I) Statistical analysis of adhesive cells per visual field. Bar graph depicts mean values ± SD (n = 10); **p < 0.001.

Figure 2B) Distribution of BAR domain protein was observed around the plasma membrane, but distinct from polymerized actin.

However, it is unlikely that the BAR domain of OPHN1 solely exhibits a regulatory unit for the GAP domain of OPHN1. To study this further, we performed spreading experiments to analyse BAR domain function. In resting cells, the BAR domain protein seems to be distributed all over the cell in a diffuse manner (Figure 6B). Upon cell activation, the BAR domain protein translocated to the plasma membrane. In spreading experiments, overexpression of OPHN1-BAR and OPHN1-FL led to a translocation of the OPHN1 protein to the plasma membrane where OPHN1 co-localizes with Rho GTPases (Figure 6C). In contrast, overexpression of OPHN1-GAP-Cterm. in A5-CHO cells did not show a localization of OPHN1 restricted to the plasma membrane, rather than a more or less diffuse distribution of OPHN1 in the cytosol (Figure 6C, lower panel). Therefore, we speculate that the BAR domain of OPHN1 guides OPHN1 to the plasma membrane where the activated Rho GTPase is localized.

Furthermore, the C-terminus of OPHN1 displayed an essential unit for Rho regulation as seen by overexpression studies with OPHN1-PH-GAP-Cterm. that does not express the C-terminal end of OPHN1 (Figure 6A) leading to RhoA-GTP levels comparable to control experiments. Similar results were obtained with Rac1 and Cdc42 pulldown activation assays (data not shown). In addition, we expected maximal Rho hydrolysis by OPHN1-PH-GAP-Cterm., that lacks the N-terminal BAR domain of OPHN1 (Figure 6A). Surprisingly, OPHN1-PH-GAP-Cterm. showed Rho hydrolysis comparable to OPHN1-FL that contains the GAP regulatory BAR domain. Although the BAR domain as a GAP regulator domain is missing in OPHN1-PH-GAP-Cterm., Rho hydrolysis was reduced, leading to the hypothesis that the PH domain already belongs to the regulatory unit of OPHN1-BAR impeding full Rho hydrolysis achieved by OPHN1-GAP-Cterm. (Figure 6A). This was further confirmed by co-transfection studies using different OPHN1 constructs and constitutively active Cdc42 (see Supplementary material online, Figure S3). Transfection with OPHN1-PH-GAP-Cterm., led to reduction in filopodia formation to the same extent as observed with OPHN1-FL, while an even stronger reduction was detected with OPHN1-GAP-Cterm., that is characterized by the lack of both the PH and the BAR domain.

4. Discussion

In the present study, we have identified and characterized the function of OPHN1 as a new GAP for Rho GTPases in platelets and megakaryocytes. We show that OPHN1 co-localizes to actin-rich regions in platelets as well as small GTPases of the Rho family in a time- and activation-dependent manner. In a platelet cell culture model, OPHN1 regulates cell adhesion and spreading by controlling stress fibre and focal adhesion formation as well as lamellipodia development, leading to the conclusion that OPHN1 influences the cytoskeleton mainly via RhoA and Rac1. Furthermore, the analysis of a different domain function of OPHN1 reveals an involvement of the PH domain in GAP regulation. In addition, the C-terminus of OPHN1 is essential for GAP activity as shown by Rho pull-down activation studies.

The regulation of Rho GTPases in platelets is not well defined. Therefore, a detailed understanding of the mechanisms of regulator proteins is necessary to understand activation and inactivation mechanisms of Rho GTPases and their signalling pathways. GAPs are a group of regulator proteins that accelerate the hydrolysis reaction of Rho-GTP to Rho-GDP by up to five orders of magnitude in vitro because the rate of Rho-mediated GTP hydrolysis is intrinsically slow. 24 Although RhoGAPs represent the major group of GAP proteins (70–80 GAPs), 25–27 to date, only few RhoGAPs are known in platelets, namely IQGAP1 and IQGAP2. 28,29 IQGAP1 regulates procoagulant activity under conditions of shear stress. 28 In contrast, IQGAP2 forms a complex with Arp2/3 and acts as a scaffold by regulating thrombin-induced platelet cytoskeletal actin reorganization. 29 Both GAPs are known to be involved in the regulation of Rac1 and Cdc42. To date, no RhoA-regulating GAP has been described in platelets. We identified OPHN1 as a RhoGAP in platelets with a specific regulatory function for RhoA and Rac1. Immuno precipitation studies suggest that RhoA is permanently regulated by OPHN1 (Figure 2B and C), confirming recent studies of tightly controlled RhoA upon secretion, cell spreading, and cell retraction. 10 However, co-localization of OPHN1 with Rac1 and Cdc42, respectively, suggests enhanced regulation of these Rho proteins at a time of maximal platelet activation.

Spreading experiments revealed a new OPHN1-mediated function in cell adhesion that was reduced by more than 50% upon OPHN1 overexpression. Similar results were obtained with a ROCK inhibitor, strongly suggesting RhoA-mediated effects by OPHN1 that might
strongly terminate RhoA signalling upon overexpression. It is now widely accepted that the adhesive function of integrins is regulated in part by RhoA. Recent studies demonstrated a critical role for RhoA in sustaining stable platelet-matrix interactions under conditions of high shear, pointing to an important role of OPHN1 in these processes.

The importance of OPHN1 for lamellipodia development and stress fibre and focal adhesion formation suggests that OPHN1 is mainly involved in the regulation of RhoA and Rac1. This was further confirmed by co-transfection studies with Rac1- and OPHN1-overexpressing cells as well as inhibitor studies using the ROCK inhibitor Y27632. Recent studies from Khelfaoui et al. have already demonstrated that inhibition of the RhoA pathway rescues the endocytosis defects in an OPHN1 mouse model of XLMR. To date, nothing is known about RhoA regulation in platelets, although RhoA regulation by GAPs is well characterized in other cells. For example, p190RhoGAP is one important RhoGAP that inactivates RhoA in response to nerve growth factor, leading to neurite outgrowth from PC12 cells. DLC1 represents another RhoGAP regulating the activity of RhoA and Cdc42 involved in tumour suppressor function. In addition to OPHN1, GRAF1 (GTPase regulator associated with focal adhesion kinase-1), which belongs to the same subfamily, is known to be involved in RhoA regulation and is a critical component of the integrin signalling pathway. GRAF1 and OPHN1 belong to the GRAF1 subfamily of structurally related regulatory proteins sharing an N-terminal BAR domain, followed by a PH domain and a RhoGAP domain. Although Rho regulation by GAPs is well characterized in different cells, the understanding of GAP regulation remains an open question. Recent studies provide strong evidence that OPHN1-GAP is regulated by its BAR domain in an intramolecular manner that is characterized by an interaction between the BAR-PH and GAP domain. Here, we confirmed these findings in Rho pulldown assays as well as in functional experiments, demonstrating that OPHN1 that lacks the BAR/PH domain is more potent on Rho hydrolysis than OPHN1-BAR. Furthermore, we explored a new function of OPHN1-BAR that is necessary, but not essential to guide OPHN1 to the plasma membrane where it is able to interact with active Rho GTPases, supporting the fact that changes in intracellular localization can count for GAP regulation. In addition, we provide direct evidence that the PH domain is already sufficient to induce GAP regulation, because Rho hydrolysis of OPHN1-PH-GAP-Cterm is comparable to OPHN1-BAR. The regulation of the GAP domain by its PH domain was already shown for ASAP1, an Arf GAP with a PH domain N-terminal to the catalytic Arf-GAP domain that binds to phospholipids of the plasma membrane. Another interesting and important
conclusion was drawn from Rho pulldown assays, demonstrating that the C-terminus of OPHN1 is essential for Rho hydrolysis as seen by G-LISA experiments using OPHN1-PH-GAP that do not show any RhoA regulation (Figure 6A). Studies from other groups do not see these effects.13 A possible explanation is that proteins expressed in bacterial systems often have different binding properties and protein folding than proteins generated from the mammalian expression system used in the present study.

Based on our findings, we hypothesize that GAP regulation is not only achieved by BAR domain-mediated inhibition, as it was already described for DLC1, a GAP that is regulated by different mechanisms.38 Additional regulatory mechanisms of OPHN1-GAP may be phosphorylation of OPHN1. P190RhoGAP was shown to be regulated by phosphorylation mediated by Src kinases39 that may also influence OPHN1-GAP activity. Another possible mechanism for GAP regulation might be the interaction with other proteins that could account for BAR-mediated GAP inhibition. However, it is still under debate how the BAR domain of OPHN1 and GRAF1 binds to the X-linked RhoGAP protein in VLDL-induced mental retardation. Nature 1998;392:923–926.

Further investigations are required to understand OPHN1 regulation and OPHN1-mediated Rho regulation in platelets and immune cells and their impact on disease.

Supplementary material
Supplementary material is available at Cardiovascular Research online.

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References
34. Borkhardt A, Bojesen S, Haas OA, Fuchs U, Bartelheimer D, Loncarevic IF et al. The human GRAF gene is fused to MLL in a unique t(5;11)(q31;q23) and both alleles are disrupted in three cases of myelodysplastic syndrome/acute myeloid leukemia with a deletion 5q. Proc Natl Acad Sci USA 2000;97:9168–9173.


