Characterization of the Rac guanine nucleotide exchange factor P-Rex1 in platelets

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Abstract

**Background:** Blood platelets undergo a carefully regulated change in shape to serve as the primary mediators of hemostasis and thrombosis. These processes manifest through platelet spreading and aggregation and are dependent on platelet actin cytoskeletal changes orchestrated by the Rho GTPase family member Rac1. To elucidate how Rac1 is regulated in platelets, we captured Rac1-interacting proteins from platelets and identified Rac1-associated proteins by mass spectrometry. **Findings:** Here, we demonstrate that Rac1 captures the Rac guanine nucleotide exchange factor P-Rex1 from platelet lysates. Western blotting experiments confirmed that P-Rex1 is expressed in platelets and associated with Rac1. To investigate the functional role of platelet P-Rex1, platelets from $P-Rex1^{-/-}$-deficient mice were treated with platelet agonists or exposed to platelet activating surfaces of fibrinogen, collagen and thrombin. Platelets from $P-Rex1^{-/-}$ mice responded to platelet agonists and activating surfaces similarly to wild type platelets. **Conclusions:** These findings suggest that P-Rex1 is not required for Rac1-
mediated platelet activation and that the GEF activities of P-Rex1 may be more specific to GPCR chemokine receptor mediated processes in immune cells and tumor cells.

Keywords: platelet signaling / cytoskeletal remodeling / GEF / small GTPase

Findings

Upon exposure to agonist signals of vascular injury, platelets spread out on sites of vessel damage to form thrombotic plugs [1, 2]. During this process, platelets undergo an ordered series of shape changes that are determined by a spatial reorganization of the actin cytoskeleton [3]. These geometric changes that occur in the activated platelet are regulated by many of the same proteins that confer motility and regulate the cytoskeleton in nucleated cells, namely the Rho family of GTPases, including Cdc42, Rac1, and RhoA [4]. Accordingly, conditional knock-out mice models deficient in Rac1 do not undergo normal platelet spreading or aggregation and form a weak primary platelet plug over a site of vascular injury [5]. Similarly, constitutive deactivation of RhoA in platelets results in reduced platelet adhesion and an unstable thrombus [6].

Rho family GTPases are regulated in a cyclical manner by different classes of Rho-GTPase binding proteins. When platelets are stimulated to form a plug over the site of vascular injury, guanine nucleotide exchange factors (GEFs) such as Vav1 bind the Rac1 GTPase in its GDP conjugated form and catalyze a nucleotide exchange reaction to form Rac1-GTP [7]. Rac1-GTP is then able to bind downstream effector proteins that regulate cytoskeletal proteins to form actin and myosin filaments. While Vav1 is known to control Rac1-based thrombotic activities in platelets, other well-established Rac1 GEFs have not been explored in regulating thrombosis.

To better understand how Rac1 is activated in platelets, we captured Rac1-associated proteins from platelet lysates and identified potential Rac1 regulatory proteins from thrombin-stimulated platelets by mass spectrometry. Platelets were purified from platelet rich plasma from healthy volunteers with Ficoll-Paque 400 [8] and adjusted to a concentration of 1 x 10^9/ml. Lysates were prepared from resting platelets or platelets activated with 5 U/ml thrombin for 5 minutes. Immobilized Rac1-GST or GDP and GTP-loaded Rac1-GST were added to precleared lysates and incubated for 1 hour at 4°C. Rac1-associated proteins were eluted into Laemelli sample buffer and separated by PAGE. Silver-stained gel slabs from thrombin stimulated Rac1-GST eluates corresponding to 70 – 250 kD (Figure 1A, lanes 6, 7 and 8) were each separately digested with trypsin and resulting peptide fragments were analyzed with a ThermoFinnegan LTQ quadrupole linear ion trap spectrophotometer fitted with an Ion Max nanospray source. Mass
spectra were analyzed with Sequest software (Proteomics Shared Research Center, OHSU) and sequences were compared using Scaffold 2.1 software. Mass spectrometry capture experiments revealed that GTP-loaded Rac1-GST captured the Rac1 GEF P-Rex1 from thrombin-stimulated platelet lysates. Nine unique trypsin-digested P-Rex1 peptides were recovered (Table 1), representing 6% sequence coverage (103/1659 amino acids). Platelet lysates and Rac1-GST eluates were western blotted for the presence of P-Rex1 (Figure 1B), confirming that P-Rex1 is abundant in human platelets (input) and associated with Rac1 in vitro.

P-Rex1 functions as a specific Rac1 and Rac2 activator in neutrophils [9, 10], endothelial cells [11] and breast cancer cells [12]. Intriguingly, the guanine nucleotide exchange activity of P-Rex1 is known to be regulated by both Gβγ and phosphoinositol-3,4,5 phosphate (PIP3) [9, 13, 14], suggesting that P-Rex1 could be involved in regulating G-protein coupled receptor (GPCR) pathways triggered by platelet agonists such as thrombin [15, 16] and ADP [17]. Interestingly, we found that a ternary complex consisting of P-Rex1, Rac1-GTP and Gβγ occurs only in the thrombin-activated platelets (data not shown). P-Rex1 activity is also regulated through mTOR signaling [18], and recent work has described a role for mTOR in the activation of platelet Rac1 through an undetermined mechanism [19]. Accordingly, we hypothesized that P-Rex1 may function as an important Rac activator in response to stimulation of PARs and other platelet GPCRs.

Thrombin markedly upregulated Rac1 activity in platelets from wild type mice as determined by capture of activated Rac1-GTP from quiescent versus stimulated platelet lysates [5] (Figure 1C). Protein capture and western blotting analyses confirmed that P-Rex1 is expressed in mouse platelets and capable of associating with GTP-loaded Rac1 (Figure 1D). To determine if P-Rex1 has a role in GPCR-triggered and Rac1-dependent platelet lamellipodia formation and surface spreading, we isolated platelets from P-Rex1-deficient mice [10] and exposed them to platelet activating surfaces. Washed mouse platelets (2 x 10^7/ml) from wild type (P-Rex1+/+) or P-Rex1−/− mice were placed on 100 µg/ml fibrinogen-coated coverglass in the presence of vehicle, the ADP scavenger apyrase (2 U/ml), or the GPCR agonists thrombin (1 U/ml) or ADP (10 µM) for 45 minutes at 37°C and were examined using differential interference contrast (DIC) microscopy. Platelets from wild type and P-Rex1−/− mice attached to fibrinogen surfaces at the same level (Figure 2A). The addition of the platelet GPCR agonists thrombin or ADP triggered platelet spreading on a surface of fibrinogen to a similar extent in both wild type and P-Rex1−/− platelets (Figure 2A). Deletion of P-Rex1 similarly had no effect on the spreading of platelets on a surface of fibrillar collagen (Figure 2B) or thrombin (Figure 2C).

In conclusion, our study demonstrates that Rac1 interacts with P-Rex1 from platelets, however, the GEF activity of P-Rex1 is not likely essential to PAR and P2Y GPCR- and Rac1-mediated platelet lamellipodia formation and spreading. These results suggest that the activities of P-Rex1 may perhaps be more specific to GPCR chemokine receptor (CXCR)-mediated events in immune cells [10] and tumor cells [12, 20-22]. While P-Rex1 alone does not appear to have a requisite role in activating Rac1 in platelets, recent studies suggest that P-Rex1 can work together with Vav1 to contribute to Rac1 activation [23]. Whether or not P-Rex1 has a secondary role in regulating platelet Rac1 activation and the potential context of such an accessorizing function of P-Rex1 in platelets remains to be determined.
List of Abbreviations

ADP  adenosine 5'-diphosphate
CXCR  chemokine receptor
GEF  guanine nucleotide exchange factor
GST  glutathione S-transferase
GTP  guanosine-5'-triphosphate
GPCR  G-protein coupled receptor
MPER  Mammalian Protein Extraction Reagent
mTOR  mammalian target of rapamycin
PAR  protease activated receptor
PIP3  phosphoinositol-3,4,5 phosphate
P-Rex1  phosphatidylinositol-3,4,5-trisphosphate-dependent Rac exchange factor 1

Competing Interests

The authors have no competing interests to declare.

Author Contributions

All authors designed and carried out experiments. JEA wrote the manuscript. HCW supplied P-Rex1-/- mice. All authors read and approved the final manuscript.

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**Figure Legends**

**Figure 1. Identification of P-Rex1 as a Rac1-associated protein in platelets.** Lysates from quiescent or thrombin stimulated platelets were incubated with glutathione agarose conjugated to GST-tagged Rac1, GDP-loaded Rac1, GTP-loaded Rac1 or GST alone. (A) Captured proteins were eluted into sample buffer and resolved by SDS-PAGE followed by silver staining. (B) Captured protein eluates and whole platelet lysates (input, middle panel) were probed for the presence of P-Rex1 by western blot (WB) with P-Rex1 antisera sc-85805 (Santa Cruz) as previously described [24, 25]. Total Rac1-GST and GST protein inputs for capture experiments are shown by Coomassie stain. (C) Platelets from wild type mice (5 x 10^8/ml) were treated with 1 U/ml of thrombin for 5 minutes and analyzed for Rac1 activation as previously described [5]. (D) Thrombin-stimulated mouse platelets were lysed in MPER buffer as previously described [19] and incubated with glutathione agarose conjugated to GTP-loaded Rac1-GST or GST alone for 1 hour at 4°C. Eluates were probed for mouse P-Rex1 capture by western blot (WB). Total P-Rex1 from mouse platelet lysates is shown as input (10% of total P-Rex1). Total Rac1-GST and GST protein inputs for capture experiments are shown by Coomassie stain.

**Figure 2. Substrate surface spreading of P-Rex1/- platelets.** Washed mouse platelets from wild type (P-Rex1^+/+) or P-Rex1^-/- mice were placed on 100 μg/ml fibrinogen-coated (A), 100 μg/ml fibrillar collagen-coated (B) or 50 μg/ml thrombin-coated (C) coverslips in the presence of vehicle, 2 U/ml apyrase (+apy), apyrase and 1 U/ml thrombin (+apy/+thr) or 10 μM ADP for 45 min at 37°C and imaged by DIC microscopy. The individual surface areas of 300 P-Rex1^+/+ (black line) and 300 P-Rex1^-/- (grey line) platelets were quantified using Image J software and plotted as a frequency distribution. DIC images and platelet surface area histograms are representative of >3 experiments. Scale bar = 10 μm.

**Table 1. Recovered P-Rex1 peptides**

| Peptide (n) | Sequence |
|-------------|----------|

|   | Sequence                           |
|---|-----------------------------------|
| 1 | EIDQDAYLQLFTK                     |
| 2 | LVDWLLAQGDCQTR                    |
| 3 | FLQSAFLHR                         |
| 4 | NQLLLALLK                         |
| 5 | GSLAEVAGLQVGR                      |
| 6 | TTDIPLEGYLLSPIQR                   |
| 7 | IACYQEFAAQLK                      |
| 8 | TTDIPLEGYLLSPIQR                   |
| 9 | LCVLNEIGTER                       |
Figure 1
Figure 2