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RESEARCH ARTICLE

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The Fer tyrosine kinase regulates interactions of Rho GDP-Dissociation Inhibitor α with the small GTPase Rac

Fei Fei^{1†}, Soo-Mi Kweon^{1,2†}, Leena Haataja³, Paulo De Sepulveda⁴, John Groffen^{1,5}, Nora Heisterkamp^{1,5*}

Abstract

Background: RhoGDI proteins are important regulators of the small GTPase Rac, because they shuttle Rac from the cytoplasm to membranes and also protect Rac from activation, deactivation and degradation. How the binding and release of Rac from RhoGDI is regulated is not precisely understood.

Results: We report that the non-receptor tyrosine kinase Fer is able to phosphorylate RhoGDI α and form a direct protein complex with it. This interaction is mediated by the C-terminal end of RhoGDI α . Activation of Fer by reactive oxygen species caused increased phosphorylation of RhoGDI α and pervanadate treatment further augmented this. Tyrosine phosphorylation of RhoGDI α by Fer prevented subsequent binding of Rac to RhoGDI α , but once a RhoGDI α -Rac complex was formed, the Fer kinase was not able to cause Rac release through tyrosine phosphorylation of preformed RhoGDI α -Rac complexes.

Conclusions: These results identify tyrosine phosphorylation of RhoGDI α by Fer as a mechanism to regulate binding of RhoGDI α to Rac.

Background

Rac proteins belong to the larger family of Rho GTPases that act as molecular switches. When bound to GTP, they are in an active conformation. Hydrolysis of GTP to GDP causes an inactivating conformational change. Racs are post-translationally modified by the removal of C-terminal amino acid residues, followed by the attachment of a lipid moiety in the form of a geranyl-geranyl group [1].

Because of this modification, mature Rac proteins are lipophilic and are only present in the cytosol when bound to the chaperone and transport protein RhoGDI. RhoGDI regulates the movement of Rac between the cytosol and membranes. Moreover, a recent study showed that Rho proteins compete for binding to a limited pool of RhoGDI, which, once it has bound them, provides stabilization and protection from degradation [2].

There are only three distinct RhoGDI proteins, of which RhoGDI α is the most ubiquitously expressed. The structure of RhoGDI bound to small GTPases has been elucidated. Rac makes contact with both N- and C-terminal regions in RhoGDI, with its hydrophobic tail fitting into a pocket in the C-terminal part of RhoGDI. However, the exact mechanism by which binding and release of Racs from RhoGDI is mediated is not well known [3,4].

RhoGDI can be phosphorylated on serine S101 and S174 by the serine/threonine kinase Pak [5] and PKC α also phosphorylates RhoGDI α , on residue S96 [6]. Interestingly, constitutively activated oncogenic Src, which has a deregulated tyrosine kinase activity, was shown to phosphorylate RhoGDI α on tyrosine residue Y156 [7]. In the current study we report that a normal cellular tyrosine kinase which is member of the Src family, Fer, is able to specifically tyrosine phosphorylate RhoGDI α and that this can regulate the binding to Rac1.

Results and discussion

The *BCR/ABL* oncogene encodes a chimeric protein that contains most of the Abl tyrosine kinase. Proteomic

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analysis of cells transfected with Bcr/Abl [8] reported changes in phosphorylation of RhoGDI. To investigate if the deregulated Bcr/Abl tyrosine kinase, similar to oncogenic Src [7], is able to modify RhoGDI α , we performed an immunocomplex kinase assay with cells expressing Bcr/Abl or with Fer, a non-receptor tyrosine kinase related to Abl, in the presence of added recombinant GST-RhoGDI α or control GST. As shown in Figure 1A, left panel, the Bcr/Abl protein exhibited strong autophosphorylation activity, as expected. However, there was very little phosphorylated RhoGDI α . In contrast, there was a prominent signal of phosphorylated RhoGDI α in the sample expressing Fer (Figure 1A right panel).

To examine whether Fer and RhoGDI α interact in cells, we transfected COS-1 cells with pEGFP-Fer (wild type, WT) or kinase dead D742R mutant pEGFP-Fer (mutant, MT), with or without RhoGDI α and investigated possible protein complex formation between these by co-immunoprecipitation. As shown in Figure 1B, left panel, Fer-WT clearly co-immunoprecipitated with RhoGDI α , which was phosphorylated on tyrosine, as judged by Western blotting using phosphotyrosine antibodies. In the converse experiment (Figure 1B, right panel, Fer IP) we confirmed the interaction between Fer-WT and RhoGDI α . In the presence of the Fer mutant lacking tyrosine kinase activity, no tyrosine phosphorylated RhoGDI α was detected, indicating that Fer tyrosine kinase activity is directly or indirectly needed to generate tyrosine phosphorylated RhoGDI α . Interestingly, the kinase-defective Fer protein exhibited a reduced ability to form a complex with RhoGDI α (compare lanes in IP Fer, IB for RhoGDI α), suggesting that the binding of these two proteins is facilitated by tyrosine phosphorylation. This is in agreement with the finding that the isolated Fer SH2 domain binds to RhoGDI α on far-Western blots (not shown). Importantly, we also were able to co-immunoprecipitate endogenous Fer with endogenous RhoGDI α in a lysate of the mast cell line RBL-2H3. This indicates that the complex formation between RhoGDI α and Fer also takes place without overexpression (Figure 1C).

Although reactive oxygen species (ROS) were originally identified as a bactericidal mechanism, a role for H₂O₂ as secondary messenger has now been well-established. Many groups suggest that Src may be a primary target of ROS, with some studies reporting activation of Src kinase activity upon ROS exposure [9]. Since constitutively activated oncogenic Src had been shown to phosphorylate RhoGDI α [10,11], we tested if exposure of *c*-Src to H₂O₂ would activate it to phosphorylate RhoGDI α . As shown in Figure 2A, in the presence of *c*-Src and after H₂O₂ exposure, we could detect tyrosine phosphorylated RhoGDI α . Sangrar et al [12] showed that ROS also induce Fer tyrosine autophosphorylation

as well as trans-phosphorylation activity. As shown in Figure 2B, we confirmed Fer activation by H₂O₂ in different types of cells by immunoprecipitation and Western blotting with anti-phosphotyrosine antibodies. In addition, when we co-transfected Fer with RhoGDI α and treated the cells with H₂O₂, increased RhoGDI α tyrosine phosphorylation was detected (Figure 2C). The tyrosine phosphorylated RhoGDI α was visible as two discrete bands, indicating this protein is subject to progressive phosphorylation.

Tyrosine phosphorylation of signal transduction proteins in the absence of an oncogenic, constitutively activated tyrosine kinase may be difficult to detect due to the transient nature of the phosphorylation and the activity of phosphatases. Wang et al. reported that pervanadate treatment allows detection of phosphorylation on RhoGDI α because this inhibits protein-tyrosine phosphatases and traps proteins in their tyrosine-phosphorylated form [13]. Next, we examined if pervanadate would further activate Fer and increase RhoGDI α tyrosine phosphorylation. As shown in Figure 3A, when cells co-expressing RhoGDI α and Fer were exposed to pervanadate for 5 minutes, a marked increase in tyrosine phosphorylation was seen both in Fer and RhoGDI α proteins (Figure 3A). The N-terminal regulatory domain of RhoGDI binds to the switch region of Rac whereas the C-terminal end adopts a conformation that accommodates the hydrophobic tail of Rac [6]. To examine which domain interacts with Fer, we expressed the N- and C-terminus of RhoGDI α as Xpress-tagged proteins, together with Fer, and immunoprecipitated each fragment of RhoGDI α with Xpress antibodies. We could not detect a clear interaction of the N-terminal end with Fer (data not shown). As shown in Figure 3B (RhoGDI α -CT + Fer), in immunoprecipitates with the C-terminal end of RhoGDI α and in the presence of pervanadate, we recovered tyrosine-phosphorylated Fer. Moreover, this domain of RhoGDI α also was tyrosine phosphorylated (Figure 3B). This indicates that Fer interacts with and tyrosine phosphorylates residues in the part of RhoGDI α that includes the domain that binds to the C-terminal end of Rac. We were also able to demonstrate that endogenous RhoGDI α was phosphorylated on tyrosine in response to pervanadate, which activates Fer in RBL-2H3 cells (Figure 3C).

We used purified proteins to examine if tyrosine phosphorylation of RhoGDI α could regulate its ability to bind Rac. Fer was expressed as a GST fusion protein in baculovirus. In Figure 4A (left panel), we first incubated GST-RhoGDI α with Rac to allow these proteins to form a complex. Then GST-Fer or control GST was added with a kinase reaction buffer and the kinase reaction was initiated. As shown in the top panel (pY20 blot), both autophosphorylation of Fer and transphosphorylation of

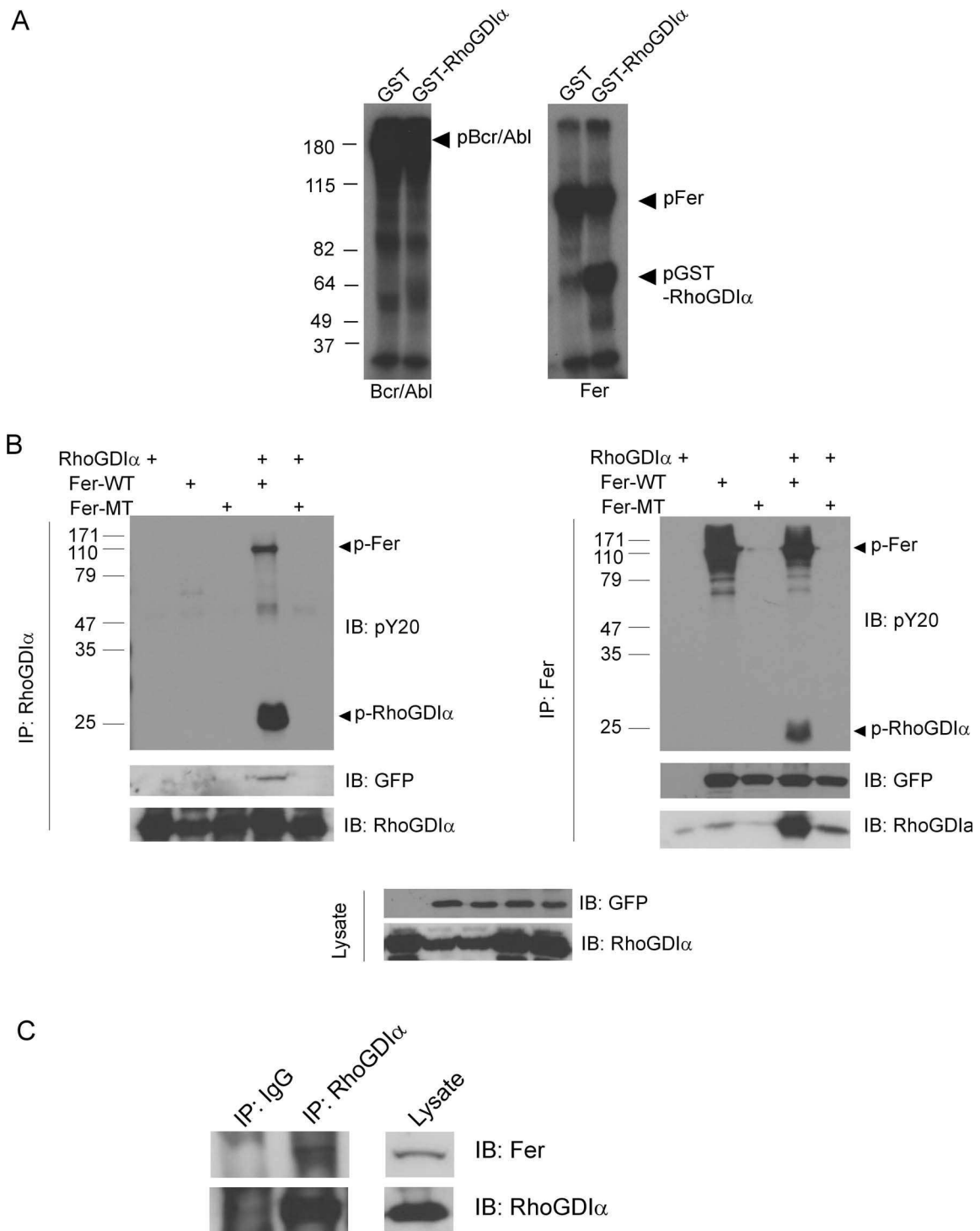


Figure 1 Fer forms a complex with RhoGDIα and phosphorylates it on tyrosine. (A) COS-1 cells were transfected with Bcr/Abl or Fer (pSG5-Fer). Autoradiogram of immune complex kinase reaction performed with immunoprecipitated Bcr/Abl or Fer (CH-6 antibodies) as indicated, with bacterially expressed GST-RhoGDIα or control GST added. (B) Immunoprecipitation (IP) from cells transfected with pEGFP tagged-Fer-WT or Fer-MT alone or together with RhoGDIα using RhoGDIα antibodies (Santa Cruz) (left panel) or Fer monoclonal rat antiserum (right panel). Antibodies used for immunoblot (IB) are indicated below the panels. After reaction with the pY20 antibodies, blots were stripped and reacted with RhoGDIα (Santa Cruz) or GFP antibodies. Arrowheads point to the locations of Fer and RhoGDIα. Molecular weight standards are in kDa. (C) Co-immunoprecipitation of endogenous Fer (using antibodies from Abcam) and RhoGDIα (Santa Cruz antibodies) proteins in RBL-2H3 mast cell lysates. These results are representative of three independent experiments.

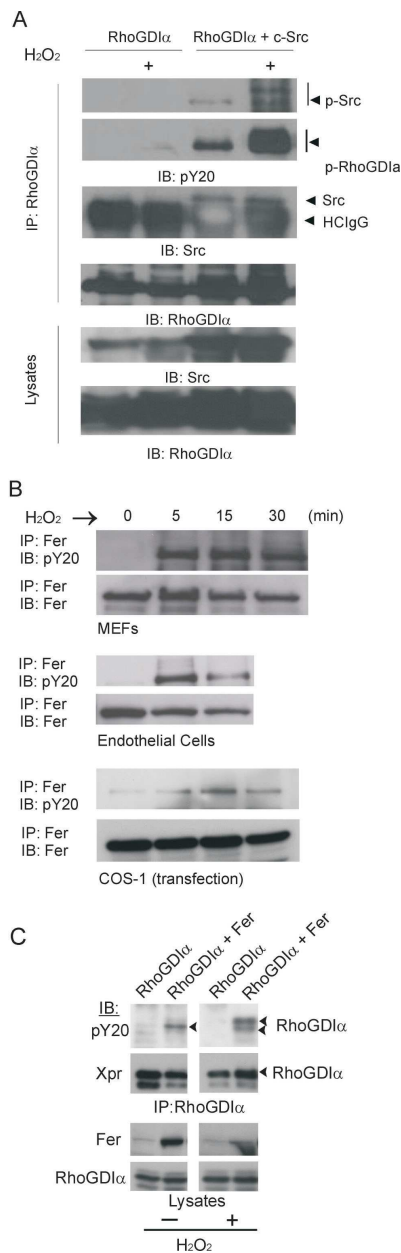


Figure 2 Activation of Fer and non-oncogenic Src. (A) RhoGDIα was expressed alone or with c-Src as indicated above the panel. Cells in lanes marked with a "+" were stimulated with H₂O₂ (10 mM) for 15 minutes and RhoGDIα was immunoprecipitated. Anti-phosphotyrosine (pY20) blots were stripped and re-probed with RhoGDIα antibodies (Santa Cruz). (B) Mouse embryonic fibroblasts (MEFs), mouse endothelial cells and cells transfected with Fer were treated with 5 mM of H₂O₂ for the indicated time. Fer protein was immunoprecipitated from the lysates followed by a pY20 immunoblot to detect activated tyrosine phosphorylated Fer (Abcam). (C) COS-1 cells transfected with Xpress-tagged RhoGDIα were stimulated or not with H₂O₂, as indicated below the panels. RhoGDIα antibodies (Upstate Biotechnology) were used for immunoprecipitation. Antibodies used for Western blotting are indicated to the left of the panels. Fer, CH-6 antibodies. This result is representative of two independent experiments.

RhoGDIα occurred as expected. However, there was no evidence that Rac was tyrosine phosphorylated (not shown). Rac protein was recovered in immunoprecipitates from both Fer-treated and non-Fer treated samples, indicating that the presence or absence of Fer does not affect preformed Rac-RhoGDIα complexes. A different result was obtained when Fer was first incubated with RhoGDIα and a kinase reaction was done. When Rac was subsequently added and allowed to bind, we found that it was not co-immunoprecipitated with RhoGDIα in the Fer-treated sample (Figure 4B). The lack of binding of Rac to RhoGDIα could be because tyrosine-phosphorylated RhoGDIα has decreased affinity for Rac, or because the binding of Fer to RhoGDIα prevented subsequent binding of Rac to RhoGDIα. Interestingly, this effect is similar to that reported for oncogenic Src: when Src phosphorylated RhoGDIα, the affinity of RhoGDIα for Rac was also significantly reduced [7].

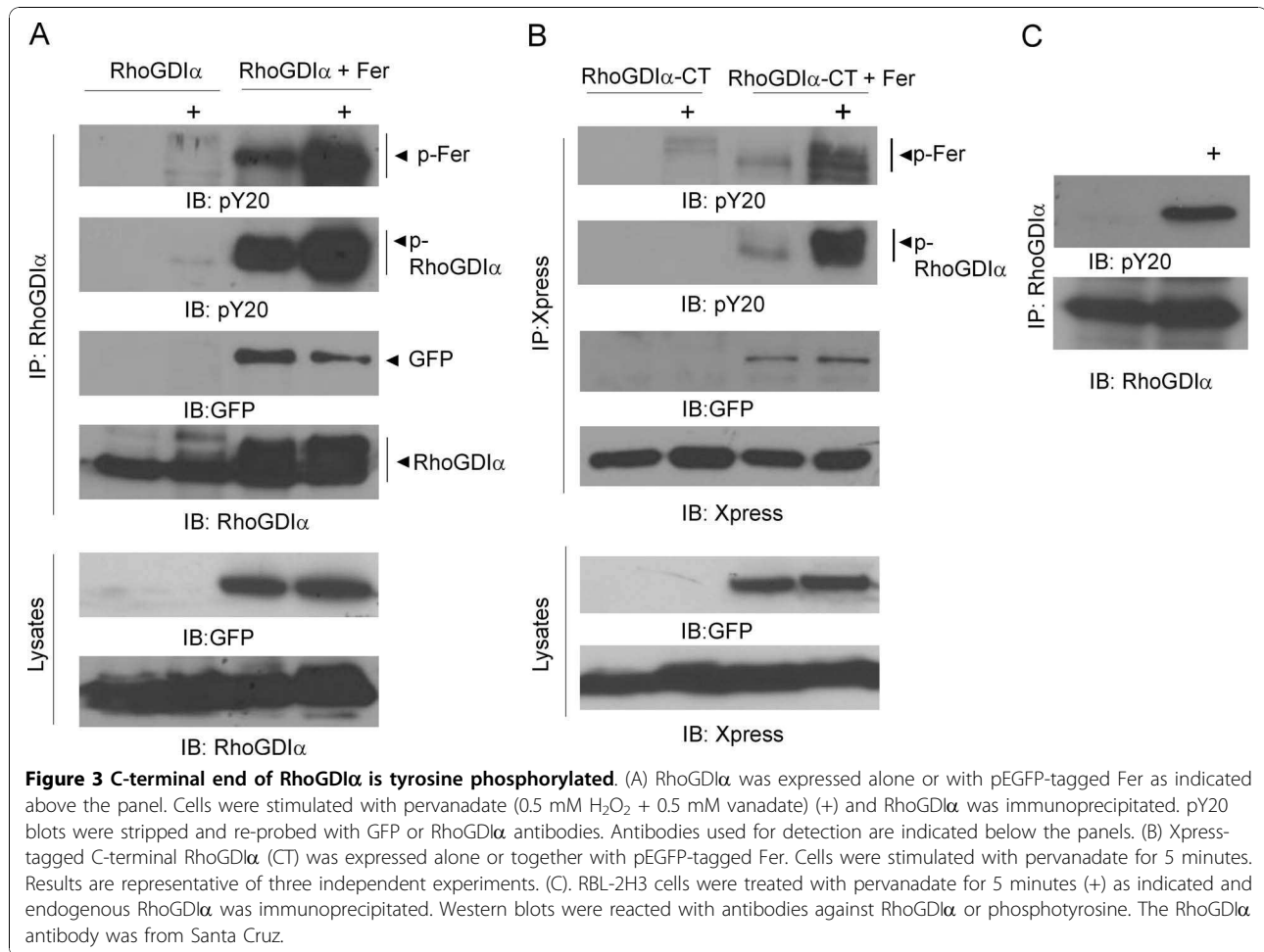
Conclusions

Tyrosine phosphorylation as a mechanism to regulate RhoGDIα has not been widely investigated, although it was recently reported that pervanadate treatment of a pancreatic islet beta cell line caused the appearance of phosphotyrosine on RhoGDIα and was accompanied by lack of Cdc42 binding [13]. There is also increasing evidence that H₂O₂ regulates the balance of tyrosine phosphorylation and de-phosphorylation in cells. The current study shows that non-oncogenic tyrosine kinases are likely to play a role in regulating the binding of Rac GTPases to RhoGDIα and through this, the cellular pool of Rac.

Methods

Antisera and DNA constructs

The Fer polyclonal rabbit anti-Fer CH-6 antiserum [14,15] and anti-Fer monoclonal rat antiserum [16] have been previously described and were used for immunoprecipitation. RhoGDIα antibodies were obtained from Upstate Biotechnology (Temecula, CA) or Santa Cruz (Santa Cruz, CA). Antibodies against Fer, GFP, Xpress and GST were from Abcam (San Francisco, CA), Santa Cruz, Invitrogen (Carlsbad, CA) and Santa Cruz, respectively. Antibodies against pY20 and Rac1 were purchased from BD Bioscience (Franklin Lakes, NJ). pSG5-Fer was constructed by subcloning a 2.9 kb BamHI fragment from human Fer clone L3, encompassing the entire coding region and small 5' and 3' untranslated regions, into the BamHI site of pSG5. pEGFP-Fer (WT), pEGFP-Fer (MT D742R) and RhoGDIα have been previously described [17,18]. The N- and C-terminal fragments of RhoGDIα were subcloned from pEGFP-RhoGDIα-NT and pEGFP-RhoGDIα-CT into pcDNA3.1/HisB by digestion with EcoRI/XbaI and have



been previously described [18]. p-LNCX mouse c-Src was obtained from Addgene (Cambridge, MA).

Recombinant proteins

A fragment containing the start codon of human Fer (5'-gggatccatgggggtttgggagtgcactg) was used to introduce a BamHI site immediately 5' to the Fer ATG. A 155 bp fragment amplified with this primer and a 3' oligonucleotide was digested with BamHI \times NsiI and the 5' BamHI-NsiI fragment ligated with a 3' 1.2 kb NsiI-KpnI fragment isolated from plasmid Fer SV-7-2 [19] into pSK digested with BamHI \times KpnI. A 1.4 kb BamHI-KpnI fragment from this plasmid was ligated with a 1.3 kb KpnI-EcoRI fragment into the pAcG2T baculovirus transfer vector digested with BamHI \times EcoRI. *S. frugiperda* Sf9 cells were grown in Grace's Insect medium (Invitrogen) with 10% FBS (heat-inactivated) and 10 μ g/ml Gentamycin (Invitrogen). DNA transfections used 0.5 μ g BaculoGold viral DNA and 2 μ g of recombinant baculovirus transfer vector. Three rounds of viral amplification were performed and the third amplification used for large-scale infection and baculovirus production. GST

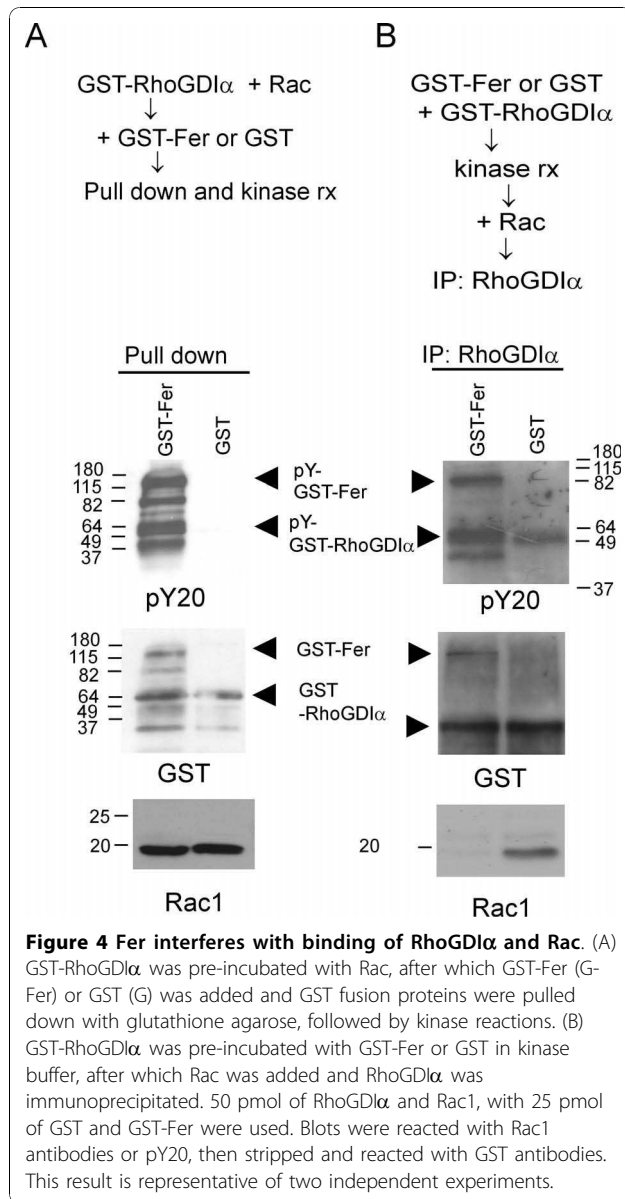
fusion proteins were purified by glutathione-agarose. GST-Fer was washed and concentrated using a Centricon-10 (Amicon) filter. Bacterially expressed RhoGDI α and Rac1 were prepared as previously described, with the GST tag removed from the Rac1 protein by thrombin cleavage [18].

Cell lines and immunoprecipitations

COS-1 cells and rat basophilic leukemia/mast cell RBL-2H3 cells were obtained from the ATCC (Rockville, MD). All tissue culture media and supplements were from Invitrogen (Carlsbad, CA). COS-1 cells were cultured in DMEM or RPMI containing 10% fetal bovine serum (FBS), penicillin (100 U/ml), streptomycin (100 μ g/ml), L-glutamine and sodium pyruvate (1 mM). RBL-2H3 cells were cultured in MEM containing 15% FBS, 1% penicillin/streptomycin. Immunoprecipitations and immunoblotting were performed as described before [18].

Immunocomplex kinase assay

The kinase activity of Fer or Bcr/Abl was measured as described previously [18]. Briefly, Fer-transfected COS-1



cells were lysed with 1 \times RIPA buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Igepal, 0.5% deoxycholate, 0.1% SDS, 5 mM EDTA) containing PMSF, leupeptin, and aprotinin and then incubated with Fer antibody, CH-6 for 2 h at 4 $^{\circ}$ C. After incubation with protein A agarose for 30 min at 4 $^{\circ}$ C, the affinity complex was washed with ice-cold high salt HNTG buffer (80 mM HEPES, pH 7.5, 0.1% Triton-X100, 500 mM NaCl and 10% glycerol) and then with ice-cold low salt HNTG buffer (80 mM HEPES, pH 7.5, 0.1% Triton-X100, 150 mM NaCl and 10% glycerol). For kinase reactions, 10 μ l of 40 mM PIPES and 10 μ l of 40 mM MnCl $_2$ were added as well as 0.5 μ g of GST-RhoGDI α protein or GST protein. The reactions were initiated

by addition of 10 μ Ci of [γ - 32 P]ATP (5000 Ci/mmol) (Amersham). After 40 min on ice, reactions were terminated by the addition of 2 \times SDS-sample buffer and boiling for 5 min. Samples were run on 8% SDS-PAGE gels and the radioactively labeled proteins visualized by autoradiography.

In vitro binding assay for interaction of RhoGDI α and Rac1

To investigate possible binding of phosphorylated-RhoGDI α to Rac1, GST-RhoGDI α protein (50 pmol) was first combined with GST-Fer (25 pmol) or GST alone (25 pmol) in 100 μ l of kinase buffer (20 mM HEPES, pH 7.5, 50 mM NaCl, 25 mM MnCl $_2$, 10 mM MgCl $_2$, 1 mM DTT). 20 μ M of ATP was added to the mixture, which was incubated for 30 min at 4 $^{\circ}$ C for the kinase reaction. Next, 400 μ l of binding buffer (1 \times DPBS, 0.1% IgePal, 10% glycerol, 0.5 mM DTT containing the protease inhibitors PMSE, leupeptin, and aprotinin) and 50 pmol Rac1 protein were added, followed by incubation for 1 h at 4 $^{\circ}$ C. Incubation with antibody against RhoGDI α for 1 h at 4 $^{\circ}$ C was followed by Protein A agarose addition and a second incubation 1 h at 4 $^{\circ}$ C. To investigate if phosphorylation of RhoGDI α by Fer releases Rac1, GST-RhoGDI α protein was incubated with Rac1 in binding buffer for 1 h at 4 $^{\circ}$ C; GST-Fer or GST alone was added, and a pull down with glutathione agarose was done, followed by washing of the precipitate. Kinase reactions were initiated by addition of kinase buffer and ATP for 30 min at 4 $^{\circ}$ C.

List of abbreviations

RhoGDI: Rho GDP-dissociation inhibitor; ROS: reactive oxygen species

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Authors' contributions

FF performed experiments, brought in new ideas and wrote part of the manuscript; S-M K performed experiments and wrote part of the manuscript; LH made the baculovirus Fer protein; PS provided critical reagents; JG designed experiments and provided critical input; NH provided ideas and wrote the manuscript. All authors read and approved the final manuscript.

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