The protein tyrosine phosphatase Shp-2 regulates RhoA activity

Simone M. Schoenwaelder*, Leslie A. Petch†, David Williamson*, Randy Shen‡, Gen-Sheng Feng‡ and Keith Burridge†§

Remodeling of filamentous actin into distinct arrangements is precisely controlled by members of the Rho family of small GTPases [1]. A well characterized member of this family is RhoA, whose activation results in reorganization of the cytoskeleton into thick actin stress fibers terminating in integrin-rich focal adhesions [2]. Regulation of RhoA is required to maintain adhesion in stationary cells, but is also critical for cell spreading and migration [3]. Despite its biological importance, the signaling events leading to RhoA activation are not fully understood. Several independent studies have implicated tyrosine phosphorylation as a critical event upstream of RhoA [4]. Consistent with this, our recent studies have demonstrated the existence of a protein tyrosine phosphatase (PTPase), sensitive to the dipeptide aldehyde calpeptin, acting upstream of RhoA [5]. Here we identify the SH2 (Src homology region 2)-containing PTPase Shp-2 as a calpeptin-sensitive PTPase, and show that calpeptin interferes with the catalytic activity of Shp-2 *in vitro* and with Shp-2 signaling *in vivo*. Finally, we show that perturbation of Shp-2 activity by a variety of genetic manipulations results in raised levels of active RhoA. Together, these studies identify Shp-2 as a PTPase acting upstream of RhoA to regulate its activity and contribute to the coordinated control of cell movement.

Addresses: *Australian Center for Blood Diseases, Monash University Department of Medicine, Melbourne 3128, Australia. †Lineberger Comprehensive Cancer Center, University of North Carolina, Chapel Hill, North Carolina 27599, USA. ‡Burnham Institute, La Jolla, California 92037, USA. §Department of Cell Biology and Anatomy, University of North Carolina, Chapel Hill, North Carolina 27599, USA.

Correspondence: Simone M. Schoenwaelder
E-mail: Simone.Schoenwaelder@med.monash.edu.au

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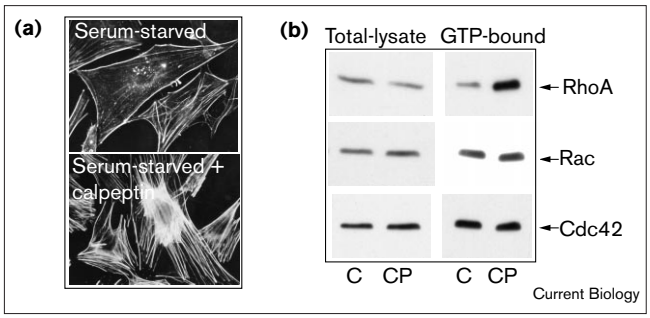
Results and discussion

Activation of the small GTPase RhoA induces a number of morphological events including the promotion of stress fibers, an increase in focal adhesions and cell contractility [2]. Serum-starved Swiss 3T3 fibroblasts treated with calpeptin undergo actin reorganization reminiscent of a RhoA phenotype ([5], and Figure 1a). Our previous studies have shown that calpeptin-mediated cytoskeletal effects are induced through inhibition of one or more PTPase(s) situated upstream of RhoA [5]. To determine if calpeptin-sensitive PTPase(s) regulate RhoA activity, we constructed a glutathione *S*-transferase (GST) fusion protein comprising the RhoA-binding domain (RBD) of Rhotekin (an effector for RhoA), similar to that described previously [6]. This GST fusion protein specifically binds to the active GTP-bound form of RhoA. Analysis of cell lysates treated with calpeptin (100 μg/ml) revealed that this peptide promotes the accumulation of active (GTP-bound) RhoA (Figure 1b), confirming that calpeptin increases the level of active RhoA. Similar studies examining the effect of calpeptin on the activity of other Rho-family GTPases revealed no changes in GTP loading of either Rac or Cdc42 (Figure 1b), demonstrating that calpeptin-sensitive PTPase(s) specifically modulate RhoA activity.

To identify potential calpeptin-sensitive PTPase(s) responsible for regulation of RhoA, we developed an immobilized form of this peptide inhibitor (Figure 2a). Initial analysis of overall PTPase activity precipitated with immobilized calpeptin, using an in-gel PTPase assay, revealed two major PTPases of ~70 and 120–130 kDa (data not shown). Only the PTPase activity migrating at

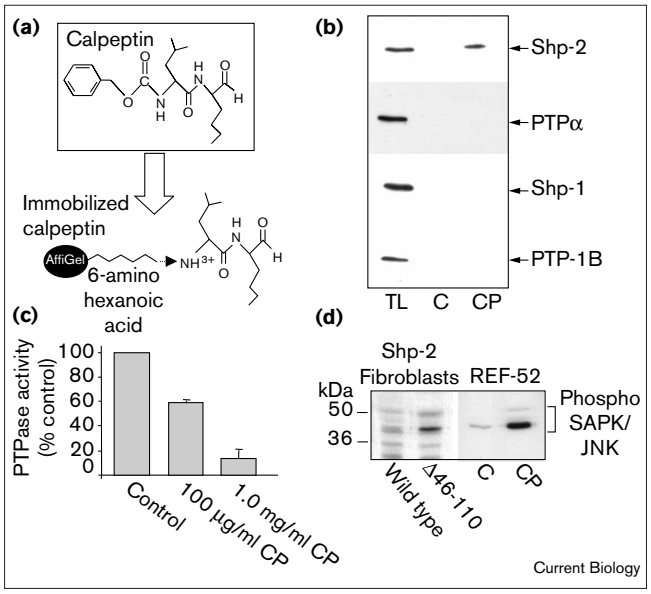
Figure 1



Calpeptin-sensitive PTPase(s) regulate the activity of RhoA. **(a)** Swiss 3T3 fibroblasts were serum-starved for 24 h, to induce disassembly of the cytoskeleton [5]. Cells were incubated with serum-free medium in the presence or absence of calpeptin (100 μg/ml) for 30 min, and stained for actin with rhodamine-conjugated phalloidin. **(b)** The levels of active RhoA, Rac or Cdc42 in REF-52 fibroblasts treated with vehicle alone (C) or 100 μg/ml calpeptin (CP) (30 min) were analyzed by precipitating GTP-bound RhoA with GST–RBD, or GTP-bound Rac and Cdc42 using GST–PBD (see Materials and methods). Proteins precipitating with GST fusion proteins were analyzed by immunoblotting with monoclonal antibodies recognizing RhoA, Rac or Cdc42.

70 kDa specifically bound to immobilized calpeptin, and not to the control resin. Immunoblot analysis demonstrated that the calpeptin-bound activity migrating at approximately 70 kDa was Shp-2, a previously described SH2-containing PTPase [7,8]. Further analysis confirmed that Shp-2 bound to immobilized calpeptin but not to control beads (Figure 2b), and also demonstrated that binding was significantly reduced in the presence of soluble calpeptin (data not shown), confirming the association of Shp-2 with this peptide. Additional immunoblot analysis of calpeptin-binding proteins revealed that immobilized calpeptin did not precipitate PTPα or PTP-1B (Figure 2b). We also assessed the ability of the hematopoietic-specific Shp-1, which belongs to the family of SH2-containing PTPases, to associate with immobilized calpeptin. Despite its similarity to Shp-2, we could not detect any binding of this PTPase to the immobilized peptide (Figure 2b). While these studies do not rule out the possibility that other PTPases may associate with calpeptin, they do identify Shp-2 as a potential calpeptin-sensitive PTPase.

Figure 2

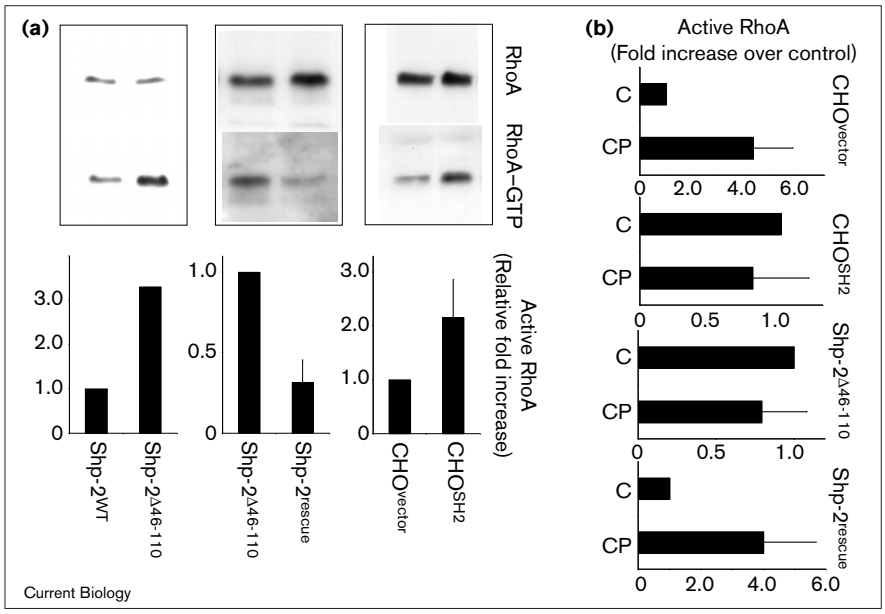


Identification of Shp-2 as a calpeptin-sensitive PTPase. **(a)** The structure of immobilized calpeptin. **(b)** Whole cell lysates (TL) prepared from Jurkat cells were incubated with control resin (C) or immobilized calpeptin (CP), and precipitated proteins analyzed by immunoblotting [5] with monoclonal antibodies directed against Shp-2, PTPα, Shp-1 and PTP-1B. **(c)** The effect of calpeptin on the PTPase activity of Shp-2 was examined by *in vitro* PTPase assays using a GST fusion protein comprising the catalytic domain of Shp-2. Assays were performed in the presence or absence of calpeptin (0.1–1.0 mg/ml), within the linear range with respect to PTPase activity. Phosphatase activity is expressed as a percentage of control (DMSO vehicle alone) and reflects the mean ± SD from three independent experiments. **(d)** REF-52 fibroblasts were treated with vehicle alone (C) or 100 μg/ml calpeptin (CP) (30 min). Alternatively, Shp-2^{WT} (wild type) or Shp-$2^{\Delta 46\text{-}110}$ fibroblasts were left untreated. Whole cell lysates were analyzed for JNK activation by immunoblotting [5] with an activation-specific antibody against SAPK/JNK.

To demonstrate that the binding of calpeptin to Shp-2 has physiological consequences, we analyzed the effect of this inhibitor on Shp-2 signaling. *In vitro* PTPase assays using a GST fusion protein comprising the catalytic domain of Shp-2 were performed in the presence or absence of increasing amounts of calpeptin. We found the effect of calpeptin to be dose-dependent, with 100 μg/ml of calpeptin decreasing activity of this PTPase by approximately 40%, whereas higher concentrations (1.0 mg/ml) inhibited Shp-2 activity by approximately 90% (Figure 2c). The ability of calpeptin to inhibit Shp-2 PTPase activity *in vivo* was also examined, by analyzing the effect of this inhibitor on established Shp-2 signaling pathways. Previous studies have shown that abrogation of this PTPase results in modulation of Jun N-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK) signaling [9]. Examination of calpeptin-treated cells for JNK activation revealed a rise in the phosphorylation of JNK (consistent with its activation), in both HeLa cells (data not shown) and REF-52 fibroblasts (Figure 2d), similar to the elevated JNK activity seen in Shp-2 mutant cells [9]. In contrast to its effect on JNK activity, Shp-2 has been reported to reduce and/or delay the level of ERK activation in response to growth factors or phorbol myristoyl acetate (PMA) [9]. Consistent with this, REF-52 fibroblasts treated with calpeptin show a reduction in sustained ERK activation in response to PMA (see Supplementary material). These findings are consistent with calpeptin's ability to inhibit Shp-2.

The observations presented above raise the possibility that Shp-2 may be the calpeptin-sensitive PTPase involved in the regulation of RhoA activity. To investigate this hypothesis, we analyzed the morphology and level of RhoA activity in embryonic fibroblasts derived from mice with a targeted disruption in exon 3 of the Shp-2 gene (Shp-$2^{\Delta 46\text{-}110}$). These cells express a truncated Shp-2 lacking its amino-terminal SH2 domain, at a greatly reduced level when compared to the full-length wild-type PTPase [9]. Thus far, evidence suggests that the Δ46-110 Shp-2 mutation might behave as a loss-of-function mutant in cells. Comparison of these fibroblasts with wild-type cells revealed an increase in the level of GTP-bound RhoA in the mutant (Shp-$2^{\Delta 46\text{-}110}$)-expressing cells (Figure 3a, left panels), concomitant with an increase in F-actin staining [10]. Re-expression of wild-type Shp-2 in Shp-$2^{\Delta 46\text{-}110}$ mutant cells (Shp-2^{rescue}) decreased RhoA–GTP levels when compared with expression of vector alone (Shp-$2^{\Delta 46\text{-}110}$; Figure 3a, middle panels) and reduced the level of F-actin staining in these cells (see Supplemetary material). Furthermore, Shp-$2^{\Delta 46\text{-}110}$ fibroblasts were less responsive to changes in actin reorganization (see Supplementary material) and Rho–GTP levels (Figure 3b, third and fourth panels) when challenged with calpeptin, an effect which

Figure 3



Shp-2 regulates the level of RhoA activity. **(a)** The level of active RhoA was measured (as described in Materials and methods) in Shp-2^{WT} or Shp-$2^{\Delta 46\text{-}110}$ fibroblasts (left), Shp-$2^{\Delta 46\text{-}110}$ fibroblasts transfected with vector alone (Shp-$2^{\Delta 46\text{-}110}$) or wild type Shp-2 (Shp-2^{rescue}) (middle), or CHO cells transiently transfected with vector alone (CHO^{vector}) or dominant-negative Shp-2 (CHO^{SH2}) (right). Densitometric quantitation of immunoblots corrected for protein loading is presented as relative fold increase (histograms). **(b)** Shp-2 represents a major mediator through which calpeptin induces effects on RhoA activity. First and second panels, CHO cells were transiently transfected with either vector alone (CHO^{vector}) or dominant-negative Shp-2 (CHO^{SH2}). Eighteen to twenty-four hours post-transfection, cells were challenged with vehicle alone (C) or 100 μg/ml calpeptin (CP) for 30 min ($n = 2$). Third and fourth panels, Shp-$2^{\Delta 46\text{-}110}$ or Shp-2^{rescue} fibroblasts were challenged with vehicle alone (C) or 100 μg/ml calpeptin (CP) for 30 min ($n = 3$). Cell lysates from each experiment were prepared and assayed for Rho–GTP levels. Densitometric quantitation of immunoblots corrected for protein loading is presented as fold increase over control-treated cells.

was restored by rescue with wild type Shp-2 (see Supplementary material, and Figure 3b, third and fourth panels). This suggests that Shp-2 inhibition is mediating the calpeptin-induced effects on RhoA activity. We also examined RhoA activity levels in CHO cells transiently transfected with dominant-negative Shp-2 (CHO^{SH2}). Consistent with our previous results in mouse fibroblasts, CHO^{SH2} cells exhibited elevated RhoA-GTP levels (Figure 3a, right panels), when compared with cells transfected with vector alone (CHO^{vector}). As with Shp-$2^{\Delta 46\text{-}110}$ mutant cells, the response of CHO^{SH2} cells to calpeptin was significantly reduced (Figure 3b, first and second panels). These results support our hypothesis that Shp-2 negatively regulates RhoA activity.

The concerted actions of Rho proteins on the arrangement of F-actin influence numerous normal and pathological events including embryonic development, wound healing and tumor metastasis [1]. Many of these processes rely critically on cell migration. RhoA is required for the characteristic formation and maintenance of stress fibers and focal adhesions, but it is also required to maintain a certain level of cell adhesion during cell movement [3]. Concordant with this, our studies show that inhibition of Shp-2 effectively increases the basal level of active RhoA, thereby increasing cell adhesion. Excessive levels of cell adhesion would be expected to negatively affect cell motility, preventing cells from releasing their contact sites as they attempt to migrate across an extracellular matrix. This hypothesis is consistent with several studies that have investigated the biological function of Shp-2 [10–16]. Animals with dysfunctional Shp-2 die at mid-gestation with severe defects in mesodermal patterning and body organization [11]. These mutant embryos fail to undergo morphogenetic movements at gastrulation [15,16]. Cells derived from these embryos exhibit increased focal adhesions, and decreased cell spreading and migration [10], which is also consistent with an increase in RhoA activation. We suggest that abrogation of Shp-2 in these embryos raises the basal level of RhoA activity, thereby impairing migration and contributing to the observed migration-based embryonic defects.

We have shown that Shp-2 is an important regulatory component upstream of the small GTPase RhoA. Although our studies indicate that Shp-2 regulates RhoA, the substrates that are dephosphorylated by Shp-2 are currently under investigation. Interestingly, there is now a precedent for the activation of guanine-nucleotide exchange factors (GEF), which facilitate nucleotide exchange on Rho family proteins, by tyrosine phosphorylation [4]. In addition, tyrosine phosphorylation of GTPase-activating proteins (GAPs), which stimulate the intrinsic GAP activity of small GTPases, have also been reported [17]. With p190RhoGAP, however, increased tyrosine phosphorylation is correlated with increased GAP activity, and consequently decreased RhoA–GTP levels [18].

Ligation of several growth factor receptors results in a loss of stress fibers and focal adhesions, indicative of decreased

RhoA activity [4]. One mechanism contributing to this effect is suggested by our findings here that Shp-2 negatively regulates RhoA activity. We envisage a model wherein recruitment of Shp-2 by growth factor receptors brings this PTPase into close proximity with a critical RhoA GEF (see Supplementary material). Dephosphorylation of the GEF by Shp-2 downregulates its activity, resulting in decreased RhoA–GTP levels, in turn leading to a loss of stress fibers and focal adhesions, and effects on the motile state of the cell.

Materials and methods

Cell lines, antibodies and constructs

Shp-2 wild-type (Shp-2^{WT}), mutant (Shp-$2^{\Delta 46\text{-}110}$) and Shp-$2^{\Delta 46\text{-}110}$ rescued with wild-type Shp-2 (Shp-2^{rescue}) embryonic fibroblast cell lines were established from E9.5 mouse embryos as described ([9,10] and Supplementary material). Transient transfection of CHO cells was carried out using LipofectAMINE PLUS reagent (Gibco BRL), according to manufacturers instructions (see Supplementary material). The plasmid containing cDNA encoding Shp-2 SH2 domains (dominant negative) was generated as previously described [19]. Activation-specific anti-SAPK/JNK polyclonal antibody was from New England BioLabs. Phospho-specific anti-ERK monoclonal antibody was from UBI. All other monoclonal antibodies were purchased from Transduction Laboratories.

Preparation and use of immobilized calpeptin

The reactive portion of calpeptin (Leu–Nle–CHO) was synthesized commercially (SynPep Corporation), and coupled to Affi-Gel10 resin (0.5 mg peptide per ml resin) (BioRad). Affi-Gel10 control resin was prepared by blocking active esters, according to the manufacturer's instructions. Cells lysed in a Triton X-100-containing lysis buffer (10 mM Tris pH 7.6, 150 mM NaCl, 1% Triton X-100, 2 mM $MgCl_2$, 2 mM EGTA, 0.1 mM DTT, 10 μg/ml aprotinin) were incubated with immobilized calpeptin or control resin for 2 h at 4°C, and beads washed with lysis buffer. Precipitated proteins were analyzed by SDS–PAGE and immunoblot analysis.

In vitro *PTPase assays*

The catalytic domain of wild-type Shp-2 was generated by PCR amplification of a cDNA fragment coding for amino acids 217–585 of Shp-2 and cloning into pGex2T vector via *Bam*H1 and *Eco*R1. The resulting GST fusion protein was expressed in BL21 cells and purified as described previously [5]. PTPase assays were performed essentially as described [20], using *p*-nitrophenyl phosphate as a substrate.

Assay for GTP-bound RhoA, Rac and Cdc42

The RhoA-binding domain (RBD) of Rhotekin (amino acids 7–89) was amplified from cDNA prepared from BALB 3T3 mRNA, and cloned into the pGEX-2T vector. The Rac/Cdc42-binding domain of PAK (PBD) was a kind gift from R.A. Cerione (Cornell University, New York). Both RBD and PBD were expressed as GST fusion proteins in BL21 cells. The levels of GTP-bound RhoA, Rac and Cdc42 in cell lysates were measured as described previously [6,21].

Supplementary material

Supplementary material including production of cell lines, figures depicting the effect of calpeptin on ERK activity and cell morphology and a proposed model for Shp-2-mediated regulation of RhoA, is available at http://current-biology.com/supmat/supmatin.htm.

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