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Specific SHP-2 partitioning in raft domains triggers integrin-mediated signaling via Rho activation

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Cell signaling does not occur randomly over the cell surface, but is integrated within cholesterol-enriched membrane domains, termed rafts. By targeting SHP-2 to raft domains or to a non-raft plasma membrane fraction, we studied the functional role of rafts in signaling. Serum-depleted, nonattached cells expressing the raft SHP-2 form, but not non-raft SHP-2, display signaling events resembling those observed after fibronectin attachment, such as β_1 integrin clustering, ^{397}Y-FAK phosphorylation, and ERK activation, and also increases Rho-GTP levels. Expression of the dominant negative N19Rho abrogates raft-SHP-2–induced signaling, suggesting that Rho activation is a downstream event in SHP-2 signaling. Expression of a catalytic inactive SHP-2 mutant abrogates the adhesion-induced feedback inhibition of Rho activity, suggesting that SHP-2 contributes to adhesion-induced suppression of Rho activity. Because raft recruitment of SHP-2 occurs physiologically after cell attachment, these results provide a mechanism by which SHP-2 may influence cell adhesion and migration by spatially regulating Rho activity.

Introduction

Cell functions such as motility require recognition of positional cues coupled to temporal and spatial reorganization of the actin cytoskeleton (Mañes et al., 2001). This is largely affected by integrins, a family of α/β heterodimeric adhesion receptors that link the cytoskeleton to the extracellular matrix (ECM).* Although these adhesion receptors lack intrinsic signaling capacity, integrins can signal through the cell membrane by assembling ECM proteins, integrins, intracellular molecules, and the cytoskeleton into supramolecular complexes known as focal adhesions. Integrins may be considered both acceptors and donors of signaling (Giancotti and Ruoslahti, 1999), as ECM binding triggers signals that are transmitted into the cell (outside-in signaling), but cell molecules also regulate the extracellular activity of integrins (inside-out signaling).

Integrin-mediated cell adhesion creates a hierarchy of signaling events that can be separated kinetically and topologically. One of the earliest detectable biochemical responses to fibronectin (Fn)-induced integrin engagement is the rapid tyrosine phosphorylation of various intracellular proteins. This is due to activation of two nonreceptor protein tyrosine kinases, the membrane-associated Src family kinases (SFK), and the cytoplasmic p125 focal adhesion kinase (FAK) (Schlaepfer et al., 1999). In accordance with the current hypothesis, FAK is first phosphorylated at ^{397}Y; this serves as a high-affinity site for SFK, which further phosphorylates FAK on ^{407}Y, ^{576}Y, ^{577}Y, ^{861}Y, and ^{925}Y (Schlaepfer et al., 1999). Thus, both FAK and SFK initiate an integrin-induced phosphorylation cascade, although it is unclear how these events regulate the actin cytoskeleton reorganization necessary for focal adhesion assembly. Evidence places tyrosine kinases both up- (Mayer et al., 1999; Arthur et al., 2000) and downstream (Barry et al., 1997; Clark et al., 1998) of Rho GTPases. There is also considerable controversy as to whether activation of other downstream signaling pathways, such as the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) pathway, requires activity of FAK, SFK, or both (Schlaepfer et al., 1999).

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*Abbreviations used in this paper: CD, methyl-β-cyclodextrin; Cho, cholesterol; CTx, cholera toxin β-subunit; DRM, detergent-resistant membrane; ECM, extracellular matrix; ERK, extracellular signal-regulated kinase; FAK, focal adhesion kinase; Fn, fibronectin; FRNK, FAK-related nonkinase; GEF, guanine-nucleotide exchange factors; GAP, GTPase-activating proteins; MAPK, mitogen-activated protein kinase; pNPP, p-nitrophenyl phosphate; PTP, protein tyrosine phosphatase; SFK, Src-family kinase; TfR transferrin receptor.

Key words: integrin signaling; lipid rafts; Rho; SHP-2; FAK

This scenario is further complicated, as both receptor and nonreceptor protein tyrosine phosphatases (PTPs) influence the earliest phases of focal adhesion assembly. The SH2-containing PTP SHP-2 is described as a positive effector in integrin function. To act, SHP-2 must be recruited to the membrane by binding to phosphorylated docking proteins such as SHPS-1/SIRP-α1 (Tsuda et al., 1998), indicating that tyrosine phosphorylation is an upstream event in SHP-2 regulation of integrin signaling. Cells derived from SHP-2 loss-of-function mutant embryos or expressing an SHP-2 dominant negative mutant show decreased integrin-induced MAPK activation, spreading, haptotactic, and chemotactic responses (Tsuda et al., 1998; Yu et al., 1998; Mañes et al., 1999b; Oh et al., 1999). Nonetheless, it has been reported that expression of a dominant SHP-2 mutant increases cell adhesion and spreading on different ECM components (Inagaki et al., 2000a).

There is much confusion as to how SHP-2 regulates integrin function. SHP-2 may operate in integrin signaling by regulating FAK phosphorylation. Cells deficient in SHP-2 activity show FAK hyperphosphorylation (Yu et al., 1998; Mañes et al., 1999b), as well as increased numbers of actin stress fibers and focal contacts (Yu et al., 1998; Inagaki et al., 2000a; Saxton et al., 2000), as reported for FAK-deficient cells (Ilic et al., 1995). Accordingly, embryos bearing an SHP-2 loss-of-function mutant show gastrulation defects similar to those of FAK knockout embryos (Ilic et al., 1995; Saxton et al., 1997). SHP-2 may also regulate FAK function indirectly by inducing SFK activation (Oh et al., 1999). In this pathway, integrins induce weak SFK activation that results in SHP-2 membrane recruitment; once there, SHP-2 further activates SFK by dephosphorylation. It is also unclear how SHP-2 rearranges the actin cytoskeleton. Some reports suggest that SHP-2 inhibits RhoA activity (Kodama et al., 2000; Schoenwaelder et al., 2000), whereas others propose that SHP-2 regulates Rho positively (Inagaki et al., 2000b; O'Reilly et al., 2000).

The mechanisms by which integrin engagement coordinates the activation of these signaling pathways is presently unclear, although both ligand binding and lateral aggregation of integrins are essential steps. The conventional picture of membrane proteins diffusing freely in the bilayer has evolved in recent years, as a growing body of evidence suggests that there is a selective confinement of lipids and proteins to discrete detergent-insoluble glycosphingolipid-, sphingomyelin-, and cholesterol (Cho)-enriched domains, termed rafts (Brown and London, 2000). Although initially proposed as platforms for the selective sorting of membrane proteins, it is recognized that lipid rafts may also impose short- and long-range lateral organization of plasma membrane constituents. This is of utmost relevance for integration of complex signaling processes such as cell migration, as rafts may favor protein interactions in specific cell locations (Mañes et al., 1999a; Gómez-Moutón et al., 2001). Accordingly, a number of signaling molecules, including integrins and integrin-associated proteins, are preferentially activated in rafts (for review see Brown and London, 2000; Pande, 2000). The role of caveolae in integrin function seems to be the opposite of that reported for rafts. Integrins are not generally considered to locate in caveolae (Lisanti et al., 1994; Mineo et al., 1996), and the interaction between caveolin-1 and β1 integrins is reported to occur in a Triton X-100–soluble plasma membrane fraction (Wary et al., 1998). Other authors suggest a scenario in which β1 integrin, caveolin, and SFK complexes exist as signaling units in caveolae, but segregate from them in response to ligand-induced clustering and cytoskeletal reorganization (Wei et al., 1999).

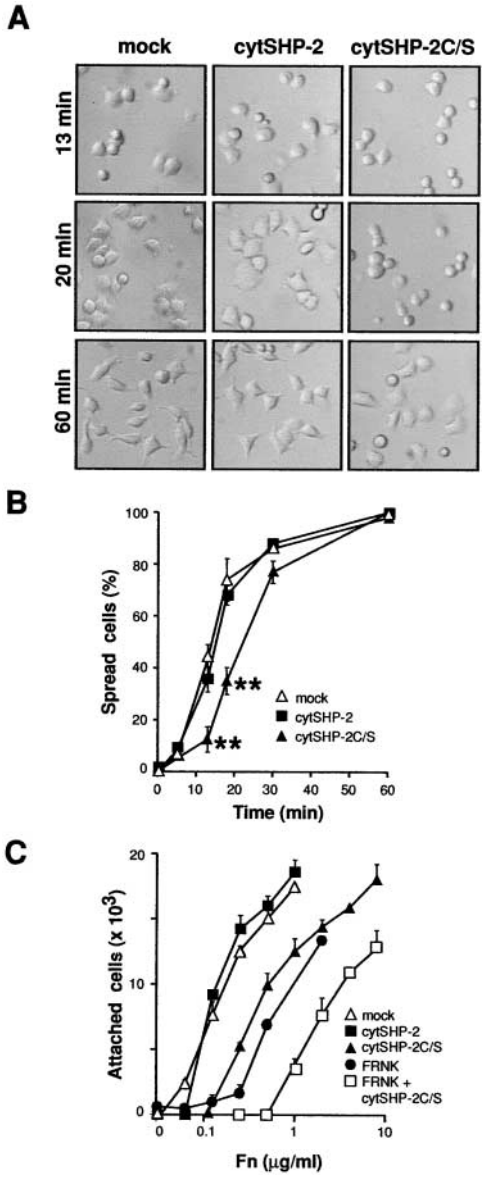

Figure 1. **SHP-2 function is needed for cell adhesion and spreading on Fn.** (A) Serum-starved mock- (△), cytSHP-2– (■), or cytSHP-2C/S–transfected (▲) 293T cells were added to Fn-coated plates. Images were recorded by phase-contrast microscopy (200×) at distinct times after replating. Representative fields are shown ($n = 5$). (B) Quantification of random fields in five experiments in (A). (C) Mock- (△), cytSHP-2– (■), cytSHP-2C/S– (▲), FRNK- (●), or cytSHP-2C/S+FRNK–transfected (□) cells were replated on dishes coated with several Fn concentrations; adhered cells were estimated after 15 min incubation ($n = 4$; **, $P < 0.01$, two-tailed t test).

Here we report that cell attachment to Fn induces recruitment of β1 integrin, SFK, FAK, and SHP-2 to raft domains in wild-type cells, suggesting that integration of these signal-

ing pathways occurs in rafts. Specific SHP-2 targeting to rafts eludes the tyrosine kinase signaling step required for SHP-2 activation after cell attachment, providing a unique model to study SHP-2 function.

Results

SHP-2 activity is required for integrin function

We analyzed the role of SHP-2 downstream of β1 integrins in human embryonic kidney 293T cells by expressing a catalytic inactive dominant mutant of this enzyme (cytSHP-2C/S). SHP-2C/S overexpression drastically delays 293T cell spreading on Fn compared to mock-transfected cells or cells expressing the wild-type SHP-2 (cytSHP-2) form (Fig. 1, A and B). Even at long adhesion times (60 min), SHP-2C/S cells show defects in polarized extension, with a rounded morphology (Fig. 1 A). Cells expressing the mutant also showed decreased adhesion to this ECM substrate (Fig. 1 C). To study the role of SHP-2 in integrin function, we coexpressed SHP-2C/S with the FAK C-terminal domain (FAK-related nonkinase [FRNK]). FRNK overexpression interferes with FAK function in a dominant inhibitory manner, reducing FAK tyrosine phosphorylation and focal adhesion assembly (Schlaepfer et al., 1999). FRNK expression reduces cell adhesion to Fn; FRNK and SHP-2C/S coexpression further decreases cell adhesion to this substrate (Fig. 1 C). These results show that SHP-2 activity is required for integrin function.

SHP-2 is engaged in an Fn-induced signaling complex in membrane rafts

Next, we addressed SHP-2 partitioning in rafts after integrin engagement. Serum-starved cells were replated on Fn-coated dishes for different times; after elimination of nonadhered cells, the Triton X-100–insoluble membrane fraction was isolated in flotation gradients. Raft-associated proteins float in density gradients as detergent-resistant membranes (DRMs), whereas the insoluble complexes formed by association with the cytoskeleton, as well as cytosolic and non-raft membrane proteins, copurify at the gradient bottom. Fn attachment triggers early recruitment of SHP-2, as well as of β_1 integrin and FAK, to the DRM fraction (Fig. 2 A). SFK proteins, visualized with a pan-SRC antibody, are constitutively associated to DRM. Copurification of caveolin in DRM and the full solubilization of non-raft proteins such as the transferrin receptor (TfR) confirm the quality of the preparation.

Flotation gradients probably underestimate the amount of raft-associated FAK and β1, as a fraction of activated FAK and β1 associate with cytoskeleton. Thus, we validated the recruitment of these proteins to rafts in situ by confocal microscopy. Cells were costained with anti–phospho-^{397}Y-FAK or anti-β1 antibodies and with the cholera toxin β-subunit (CTx), which binds specifically to the raft-ganglioside GM1. Cells attached on poly-L-lysine or maintained in suspension do not stain with anti–phospho–^{397}Y-FAK antibody (Fig. 2 B). Plating these cells on Fn resulted in increased ^{397}Y-FAK staining, which colocalizes with CTx (Fig. 2, C and D), indicating that attachment to Fn induces ^{397}Y-FAK and raft clustering at the cell membrane (Fig. 2, C and D). However, β1 integrin appears constitutively associated to rafts, as it colocalizes with CTx in both nonadhered (Fig. 2 E) and adhered cells (Fig. 2, F and G). This result is in contrast with the modest colocalization between β1 and the caveolae marker caveolin-1 reported by others (Wei et al., 1999), again indicating differences between rafts and caveolae in integrin function. Nonetheless, β1 colocalization with CTx contrasts with the non-raft partitioning of β1 integrin in nonattached cell flotation gradients. The difference between these techniques may reflect weak association of β1 with lipid rafts in nonadhered cells, which is stabilized after cell attachment.

Identification of integrins and integrin-associated proteins in the DRM fraction indicates that early integrin signaling is

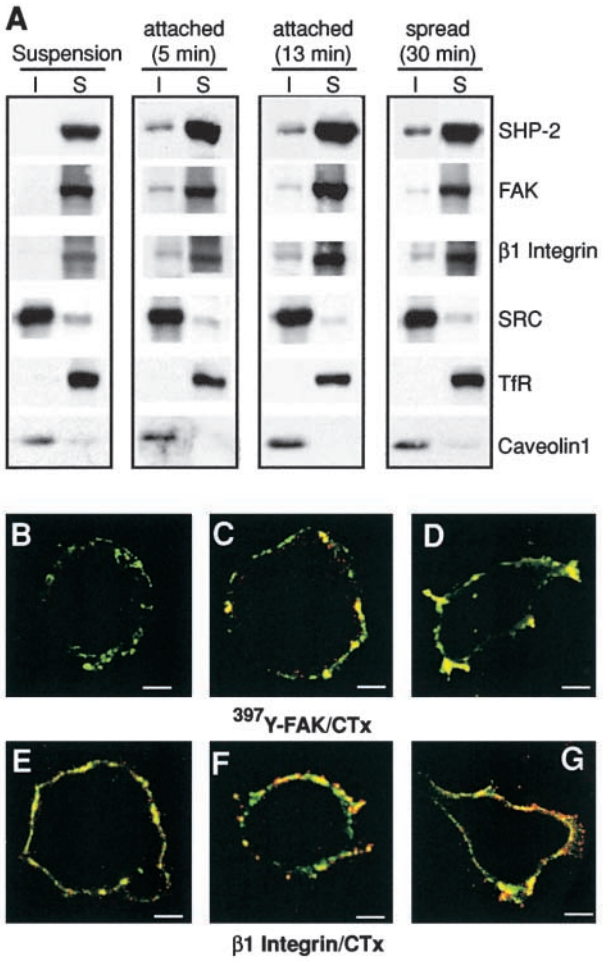

Figure 2. **Early integrin signaling is engaged in rafts.** (A) Serum-starved 293T cells maintained in suspension or replated on Fn-coated plates were Triton X-100 extracted and fractionated in Optiprep gradients. Fractions were collected from top to bottom of the gradient and analyzed by Western blot with the indicated antibodies. Only the first (I, DRM-enriched) and last fractions (S, Triton X-100–soluble material) are shown. Data are representative of four independent experiments. (B–G) Nonattached (B and E) or Fn-adhered cells for 5 (C and F) or 30 min (D and G) were costained with FITC-CTx (green) and anti–^{397}Y-FAK (B–D) or anti-β1 (E–G), followed by a Cy3-labeled second antibody (red), and analyzed by confocal microscopy. Single-color images are available online at http://www.jcb.org/cgi/content/full/jcb.200109031/DC1. Bar, 5 μm.

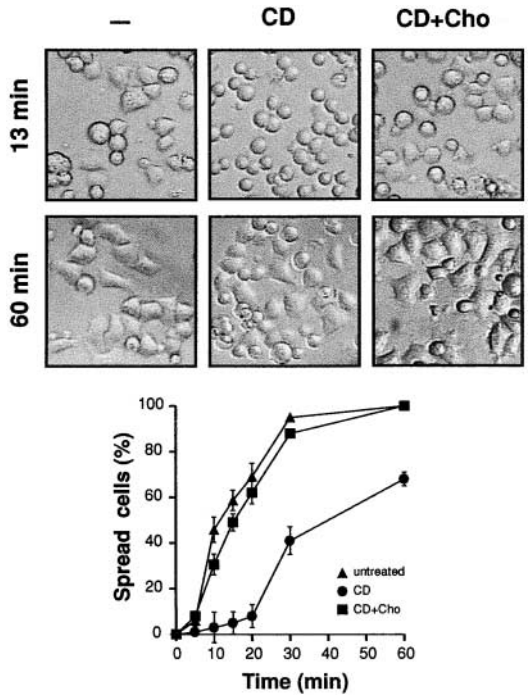

Figure 3. **Cho depletion inhibits cell spreading.** Serum-starved cells were untreated (−, ▲), CD-treated (●), or replenished with Cho after CD treatment (CD + Cho, ■). Phase-contrast images were recorded at different times to evaluate cell spreading by direct counting. Representative fields and quantification are shown ($n = 5$).

engaged in rafts. In addition, depletion of membrane Cho with the drug methyl-β-cyclodextrin (CD) delays 293T cell spreading on Fn, which is restored by adding free Cho to CD-treated cells (Fig. 3); this suggests that cell spreading is dependent on levels of membrane Cho, a lipid particularly abundant in rafts.

Raft partitioning of SHP-2 accelerates integrin signaling

If integrin signaling takes places in rafts, targeting these intermediates to rafts would provide a kinetic advantage to integrin signaling by increasing the interaction efficiency of downstream targets. Double palmitoylation signals of p56LCK are a major determinant in targeting this protein to rafts (Kabouridis et al., 1997). We constructed a SHP-2 chimera by adding the 12 amino acids corresponding to the Lck unique domain, which bears the double *S*-acylation signal, to the SHP-2 N terminus. A membrane-bound SHP-2 chimera with the human c-SRC myristoylation tag was also used (Zhao and Zhao, 1999). Confocal analysis showed that whereas LCK-SHP2 and SRC-SHP2 are targeted to the plasma membrane, only LCK-SHP2 shows extensive colocalization with the raft marker GM1 (Fig. 4 A). In agreement, flotation gradients showed that only LCK-SHP2 partitions constitutively in DRM, whereas SRC-SHP2 recruitment to DRM requires integrin engagement (Fig. 4 B).

We analyzed the in vitro PTP activity of the SHP-2 chimeras. Lysates from serum-starved 293T cells expressing the distinct SHP-2 constructs were immunoprecipitated using an anti-6His tag antibody, and PTP activity in the pellet was

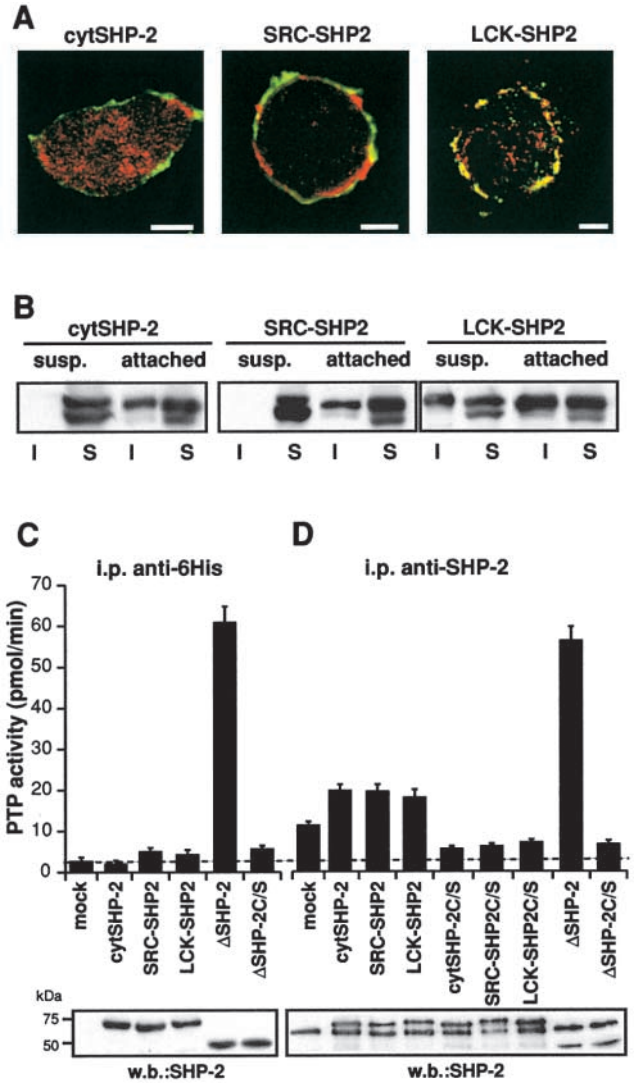

Figure 4. **Membrane targeting of SHP-2.** (A) SHP-2 and GM1 distribution was analyzed by confocal microscopy in nonattached cytSHP-2, SRC-SHP2, or LCK-SHP2 cells by costaining with FITC-CTx (green) and anti–SHP-2 antibody (red); yellow indicates SHP-2 and GM1 colocalization. Single-color images are available online at http://www.jcb.org/cgi/content/full/jcb.200109031/DC1. Bar, 5 μm. (B) cytSHP-2, SRC-SHP2, or LCK-SHP2 cells maintained in suspension or replated on Fn-coated dishes for 5 min were fractionated to analyze DRM partitioning of SHP-2 (DRM [I] and soluble [S] fractions). The lower band corresponds to endogenous SHP-2. Representative data are shown ($n = 4$). (C) Lysates of mock, cytSHP-2, SRC-SHP2, LCK-SHP2, SH2-truncated ΔSHP-2, or ΔSHP-2C/S cells were immunoprecipitated with anti-6His antibody and PTP activity determined in pellets using pNPP as substrate. Values are expressed as picomoles of phosphate released per minute. Background level is indicated with a dashed line. As a control of protein quantity, part of the immunoprecipitate was analyzed in Western blot using anti–SHP-2 antibody. (D) Lysates from serum-starved 293T cells expressing the catalytic active or inactive SHP-2 chimeras indicated were immunoprecipitated with a polyclonal anti–SHP-2 antibody and the pellets used to analyze PTP activity as in (C). Western blot analysis of pellets with anti–SHP-2 antibody is shown.

assayed using pNPP as substrate. Because the C-terminal 6His tag is distant from the SH2 domains, the immunoprecipitation step should not result in SHP-2 activation. We

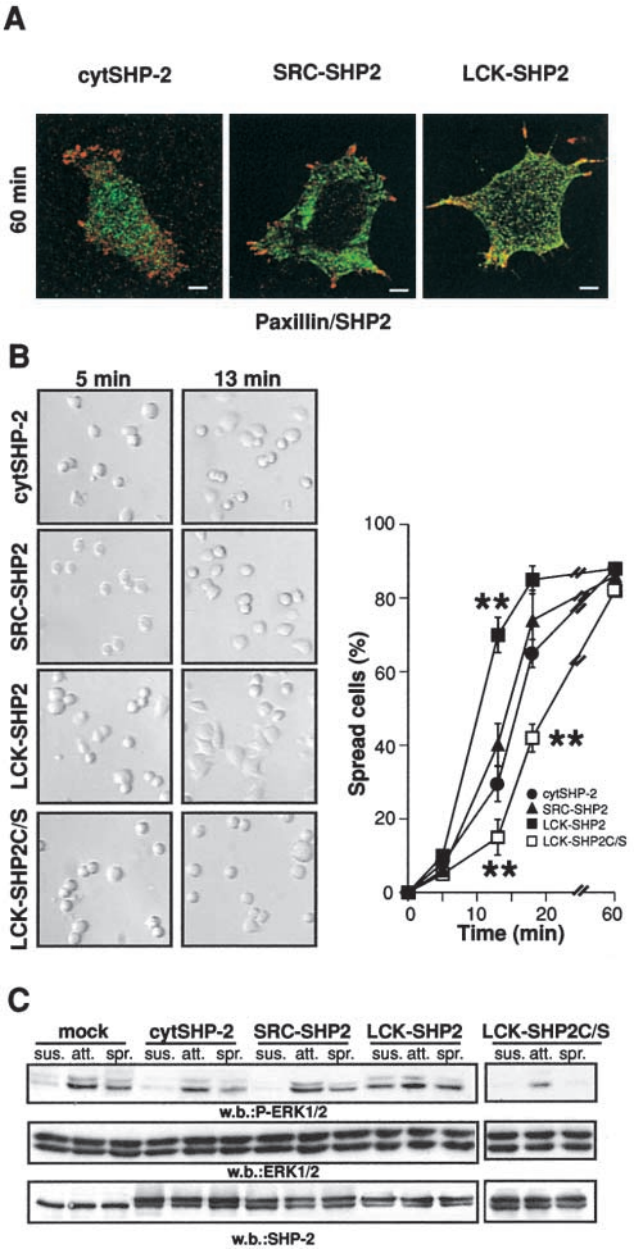

Figure 5. **Partition of LCK-SHP2 into rafts enhances SHP-2 signaling.** (A) Raft-associated LCK-SHP2 is targeted to focal adhesions. CytSHP-2–, SRC-SHP2– and LCK-SHP2–expressing cells were plated onto Fn for 120 min, then costained with anti-paxillin (red) and anti–SHP-2 (green) antibodies. Colocalization (yellow) of markers was analyzed by confocal microscopy. Single-color images are available online at http://www.jcb.org/cgi/content/full/jcb.200109031/DC1. Bar, 5 μm. (B) Raft-targeted LCK-SHP2 accelerates cell spreading. cytSHP-2 (●), SRC-SHP2 (▲), LCK-SHP2 (■), or raft-targeted dominant negative LCK-SHP2C/S mutant (□) cells were trypsinized and replated on Fn-coated plates. Phase-contrast images were recorded at different times to evaluate cell spreading. Quantification of four experiments is shown at the right (**, $P < 0.01$, two-tailed t test). (C) Raft-targeted LCK-SHP2 triggers ERK activation in unstimulated cells. Lysates from serum-starved mock, cytSHP-2, SRC-SHP2, LCK-SHP2 or LCK-SHP2C/S cells maintained in suspension (sus) or replated on Fn for 5 (att) or 30 min (spr) were analyzed by Western blot using anti–phospho-ERK antibody. Filters were rehybridized with anti-ERK antibody as a protein loading control and with anti–SHP-2 to show SHP-2 expression. Results represent four similar experiments.

observed near-background PTP activity levels in anti-6His pellets from cytSHP-2–, SRC-SHP2–, and LCK-SHP2–expressing cells (Fig. 4 C). PTP activity was prominent in anti-6His pellets of cells expressing an SH2-truncated SHP-2 mutant (ΔSHP-2) described to be constitutively active (Zhao and Zhao, 1999). PTP activity was also measured in immunoprecipitates using anti–SHP-2 antibody. Anti–SHP-2 immunoprecipitates from mock-, cytSHP-2–, SRC-SHP2–, or LCK-SHP2–expressing cells showed significant PTP activity, which was not detected in anti–SHP-2 pellets from catalytically inactive C/S mutant cells (Fig. 4 D). The results suggest partial, specific SHP-2 PTP activation after anti–SHP-2 antibody binding. Comparable cytSHP-2, SRC-SHP2, and LCK-SHP2 protein levels showed similar PTP activity, thus suggesting that addition of SRC or LCK tags to the N terminus of SHP-2 does not promote enzyme activation.

As rafts are the sites at which integrins are clustered and FAK is activated (Fig. 2, B–G), one would predict that SHP-2 targeting to rafts would enhance its presence in focal adhesions. We found that a fraction LCK-SHP2 colocalizes with paxillin in well-spread cells, whereas staining of paxillin and non-raft SRC-SHP2 or cytSHP-2 is completely independent (Fig. 5 A). We tested whether SHP-2 partitioning affects integrin function. Membrane targeting of SHP-2 to a non-raft location does not enhance integrin function, as spreading of cells expressing SRC-SHP2 is indistinguishable from that of cells overexpressing the cytosolic enzyme (cytSHP-2) (Fig. 5 B) or transfected with an empty vector (unpublished data). Nonetheless, cells expressing the raft-associated LCK-SHP2 chimera spread more rapidly than SRC-SHP2 or cytSHP-2 cells on Fn. Thus, raft targeting of LCK-SHP2 provides a kinetic advantage, concurring with the idea that integrin signaling is organized in rafts. This is not the consequence of a docking effect, but requires SHP-2 catalytic activity, as expression of a raft-located catalytically inactive SHP-2 mutant (LCK-SHP2C/S) delays cell spreading on Fn (Fig. 5 B).

Raft targeting of SHP-2 activates ERKs

We measured the functional consequences of SHP-2 partitioning on integrin signaling. SHP-2 positively regulates ERK1/2 downstream of β1 integrins; indeed, SHP-2C/S overexpression inhibits Fn-induced MAPK activation. Western blot with antibodies to the dual-phosphorylated active ERK1/2 forms shows that there is little or no active ERK1/2 in nonattached mock-, cytSHP-2–, or SRC-SHP2–transfected cells (Fig. 5 C). Cell attachment to Fn promotes transient ERK1/2 activation that is comparable among the three cell types (Fig. 5 C), indicating that SHP-2 overexpression does not affect integrin signaling. However, in LCK-SHP2 cells, ERK1/2 are activated in the absence of Fn stimulation (Fig. 5 C). Despite this basal activation, Fn-induced ERK activation in LCK-SHP2 cells reaches levels similar to those of SRC-SHP2 or cytSHP-2 cells.

SHP-2 may activate ERK by triggering direct or indirect ^{527}Y-SFK dephosphorylation after integrin engagement, resulting in SFK activation (Oh et al., 1999). Nonetheless, mock-transfected (unpublished data), cytSHP-2, SRC-SHP2, or LCK-SHP2 cells show comparable adhesion-induced SFK phosphorylation in the DRM fraction (Fig. 6 A),

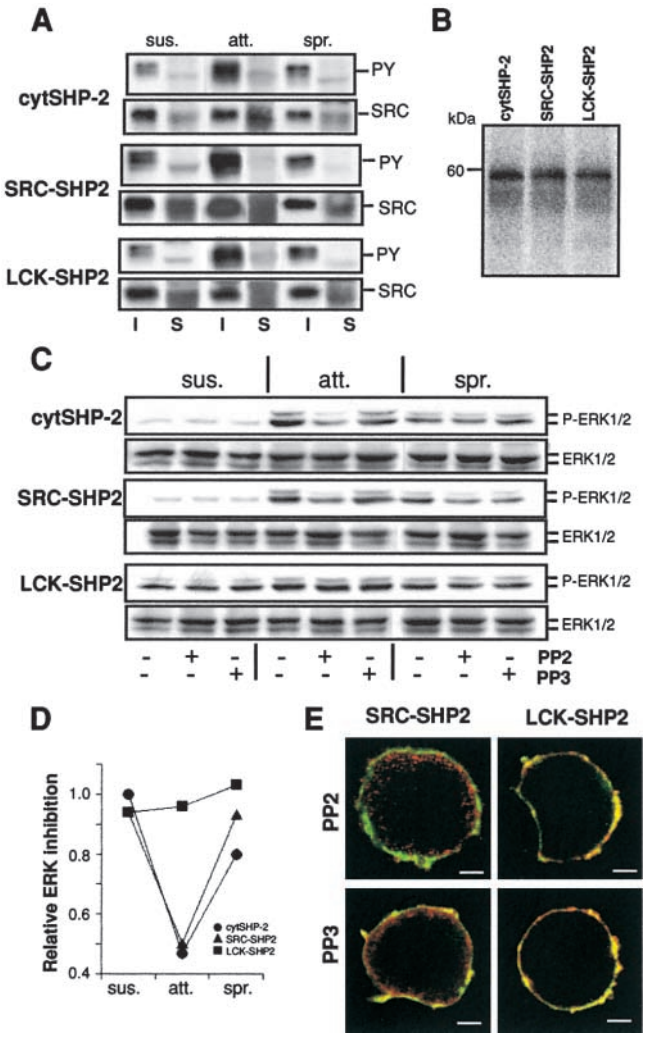

Figure 6. **SFK acts upstream, but not downstream, of SHP-2 in integrin signaling.** (A) Optiprep gradients were prepared from suspended or Fn-attached (5 or 30 min) cytSHP-2, SRC-SHP2, or LCK-SHP2 cells after TX-100 extraction, and fractions were analyzed in Western blot using anti-PY and anti-pan SRC antibodies. Bands correspond to tyrosine phosphorylated (top) and total SFK (bottom) in the DRM (I) and soluble (S) fractions. Data are representative of three experiments. (B) Serum-starved cytSHP-2, SRC-SHP2 or LCK-SHP2 cells were trypsinized and maintained in suspension. SFK in lysates were precipitated with pan-Src antibody and assayed for in vitro kinase activity. Results are representative of three experiments. (C) Serum-starved cytSHP-2, SRC-SHP2, or LCK-SHP2 cells were untreated (−) or treated (+) with PP2 or PP3 as a control. Equal amounts of extracts from cells in suspension (sus) or Fn-adhered for 5 (att) or 30 min (spr) were blotted with anti–phospho-ERK antibody (top panels). Protein loading was controlled using an anti-ERK antibody (bottom panels). Data are representative of three independent experiments. (D) The phospho-ERK2 band from (C) was measured by densitometry and the signal ratio between PP2- and PP3-treated cells calculated; cytSHP-2 (●), SRC-SHP2 (▲), and LCK-SHP2 (■). (E) SRC-SHP2 or LCK-SHP2 cells were treated with PP2 or PP3 as indicated, plated on Fn-coated chambers for 5 min, and costained with FITC-CTx (green) and SHP-2 (red). Colocalization (yellow) of both markers was analyzed by confocal microscopy. Single-color images are available online at http://www.jcb.org/cgi/content/full/jcb.200109031/DC1. Bar, 5 μm.

as well as in vitro SFK activity in nonattached cells (Fig. 6 B), suggesting that the differential partition of SHP-2 does not modify SFK activity. ^{527}Y-Src phosphorylation is unaffected by SHP-2 partition (unpublished data). Pretreatment of cytSHP-2 or SRC-SHP2 cells with the SFK inhibitor PP2 reduces Fn-induced ERK1/2 activation, although PP2 affects neither basal nor Fn-induced ERK1/2 activation in LCK-SHP2 cells (Fig. 6, C and D). Thus, specific SHP-2 targeting to rafts, but not to other membrane regions, bypasses the requirement for SFK activity in Fn-induced ERK activation. PP2 treatment also significantly reduced Fn-induced SRC-SHP2 recruitment to rafts, whereas LCK-SHP2 partitioning in these domains is unaffected (Fig. 6 E). The results indicate that after integrin engagement, one SFK function is to recruit SHP-2 to rafts, which may then activate ERK by an SFK-independent mechanism.

Raft-targeted SHP-2 triggers ^{397}Y-FAK phosphorylation

FAK phosphorylation analysis using anti-phosphospecific antibodies showed a significant ^{397}Y-FAK fraction in nonattached LCK-SHP2 cells, but not in cytSHP-2 or SRC-SHP2 cells (Fig. 7 A). FAK phosphorylation kinetics in mock-transfected cells is similar to that in cytSHP-2 or SRC-SHP2, again indicating that the effect is not due to SHP-2 overexpression. LCK-SHP2C/S expression abrogates ^{397}Y-FAK phosphorylation in nonattached cells (Fig. 7 A), indicating that the LCK-SHP2 effect requires PTP activity. Basal FAK phosphorylation in LCK-SHP2 cells is ^{397}Y-specific, as ^{861}Y-FAK is not observed (Fig. 7 A), consistent with the conclusion that SHP-2 partitioning does not affect SFK activity.

FRNK expression inhibits FAK phosphorylation in nonattached and Fn-adhered LCK-SHP2 cells (Fig. 7 B). Basal ^{397}Y-FAK phosphorylation in nonattached LCK-SHP2 cells is probably the consequence of FAK recruitment to the cell membrane. FRNK also abolishes accelerated spreading in LCK-SHP2 cells (unpublished data), and inhibits Fn-induced ERK activation independently of the SHP-2 form coexpressed. In addition, FRNK abrogates basal ERK activation in nonattached LCK-SHP2 cells (Fig. 7 B), suggesting that SHP-2 requires functional FAK for ERK activation.

SHP-2 regulates Rho activity

Next, we studied the mechanism by which LCK-SHP2 regulates FAK phosphorylation. LCK-SHP2 does not affect β1 affinity, as analyzed with the anti-β1 antibody HUTS-21, which binds specifically to activated β1 integrins (unpublished data). Confocal analysis nonetheless indicates large β1 integrin patches on the surface of suspension LCK-SHP2 cells, compared with diffuse β1 staining in cytSHP2- (unpublished data) or SRC-SHP2 cells (Fig. 8 A). Latrunculin-B disruption of actin cytoskeleton inhibits ^{397}Y-FAK phosphorylation of nonattached LCK-SHP2 cells (Fig. 8 B), suggesting that basal LCK-SHP2–induced signaling is achieved by regulating the actin cytoskeleton. Cytoskeleton rearrangements are controlled by Rho GTPase family members (Kjoller and Hall, 1999), of which Rho is particularly prominent, as it influences integrin-induced ERK activation and FAK phosphorylation (Clark et al., 1998). To analyze whether Rho function is needed for LCK-SHP2 signaling in nonattached cells, we coexpressed a green fluorescent protein (GFP)-tagged N19Rho dominant negative mutant with LCK-SHP2. We found

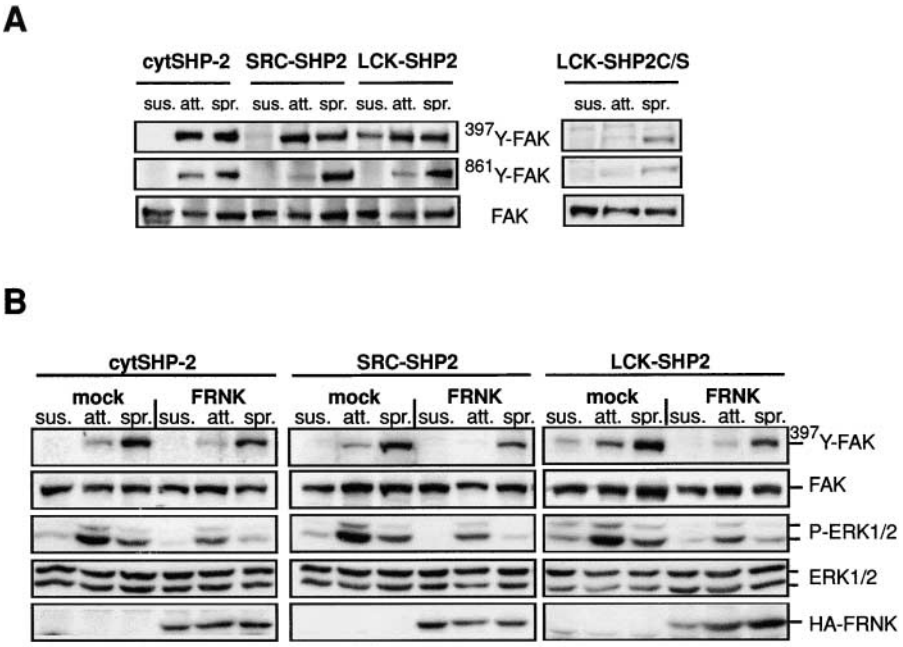

Figure 7. **Raft targeting of LCK-SHP2 triggers ^{397}Y-FAK phosphorylation in nonadhered cells.** (A) Equal amounts of lysates from serum-starved cytSHP-2, SRC-SHP2, LCK-SHP2, or LCK-SHP2C/S cells detached (sus) and replated on Fn for 5 (att) or 30 min (spr) were precipitated with anti-FAK antibody. Pellets were probed with anti–^{397}Y-FAK, –^{861}Y-FAK and –FAK antibodies. (B) cytSHP-2, SRC-SHP2, or LCK-SHP2 were transfected in 293T cells or in 293T cells stably expressing FRNK. Equal amounts of lysates from nonattached (sus) and Fn-replated for 5 (att) or 30 min (spr) were probed with anti–^{397}Y-FAK, -FAK, –phospho-ERK and -ERK antibodies. Anti-HA antibody was used to control FRNK expression.

that N19Rho, but not GFP-N17Rac, inhibits ^{397}Y-FAK phosphorylation in serum-depleted, nonattached LCK-SHP2 cells (Fig. 9 A).

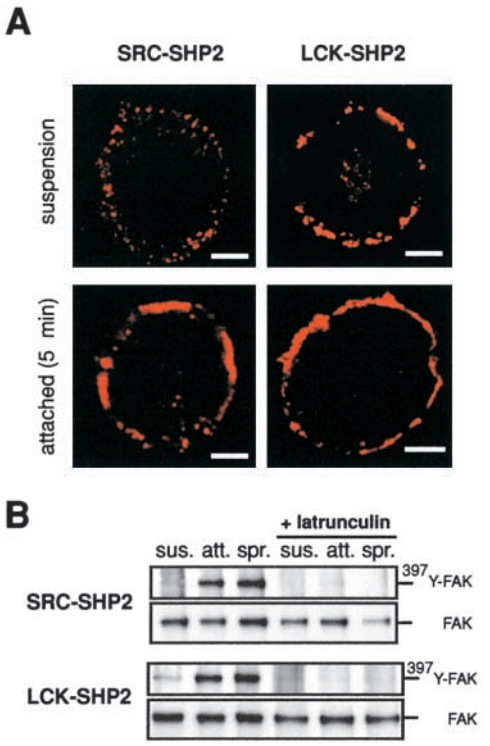

Figure 8. **Raft targeting of LCK-SHP2 regulates actin cytoskeleton in nonattached cells.** (A) Indirect immunofluorescence with anti-β1 antibody of nonattached or Fn-attached SRC-SHP2 or LCK-SHP2 cells, as indicated. Bar, 5 μm. (B) Serum-depleted SRC-SHP2 or LCK-SHP2 cells were latrunculin-B-treated, then left in suspension (sus) or Fn-replated for 5 (att) or 30 min (spr). FAK was precipitated and probed with anti–^{397}Y-FAK and -FAK antibodies (n = 3).

The N19Rho results suggest that Rho may be downstream of SHP-2; thus, we examined whether SHP-2 partitioning affects Rho activity. Serum-depleted, nonattached LCK-SHP2 cells show higher active Rho levels than those of non-raft SRC-SHP2 or cytSHP-2 cells (Fig. 9, B and C). LCK-SHP2C/S expression does not increase, and even reduces active Rho levels in nonattached cells (Fig. 9 B). Expression of neither LCK-SHP2 nor the inhibitor LCK-SHP2C/S affects Rac activity in nonattached cells (Fig. 9, D and E); this suggests that SHP-2 is a specific effector for Rho and that this regulation occurs specifically in rafts. After cell attachment, active Rho levels follow a biphasic curve, with an initial decline followed by an increase in Rho-GTP levels. The initial adhesion-induced Rho inhibition is conserved independently of the SHP-2 form expressed, but LCK-SHP2 cells show a more rapid, pronounced recovery of Rho activity than SRC-SHP2 or cytSHP-2 cells (Fig. 9, B and C), concurring with the hypothesis that SHP-2 regulates Rho activity in rafts. LCK-SHP2C/S expression abrogates adhesion-induced feedback inhibition of Rho activity, suggesting that SHP-2 also contributes to the negative regulation of Rho activity downstream of integrins. Similar results were obtained in SRC-SHP2C/S and cytSHP-2C/S (unpublished data). Adhesion-induced Rac activation is unaffected in cells expressing either the catalytically active or inactive SHP2 chimeras (Fig. 9 D), suggesting that SHP-2 specifically regulates Rho.

Discussion

Recent evidence suggests that clustering (i.e., avidity) of certain integrins is influenced by their partition into discrete membrane domains termed rafts (Krauss and Altevogt, 1999; Pande, 2000). Integrin-mediated signaling is integrated in rafts, and formation of integrin signaling complexes with integrin-associated protein/CD47 (Green et al., 1999) or CD36 (Thorne et al., 2000) is sensitive to Cho de-

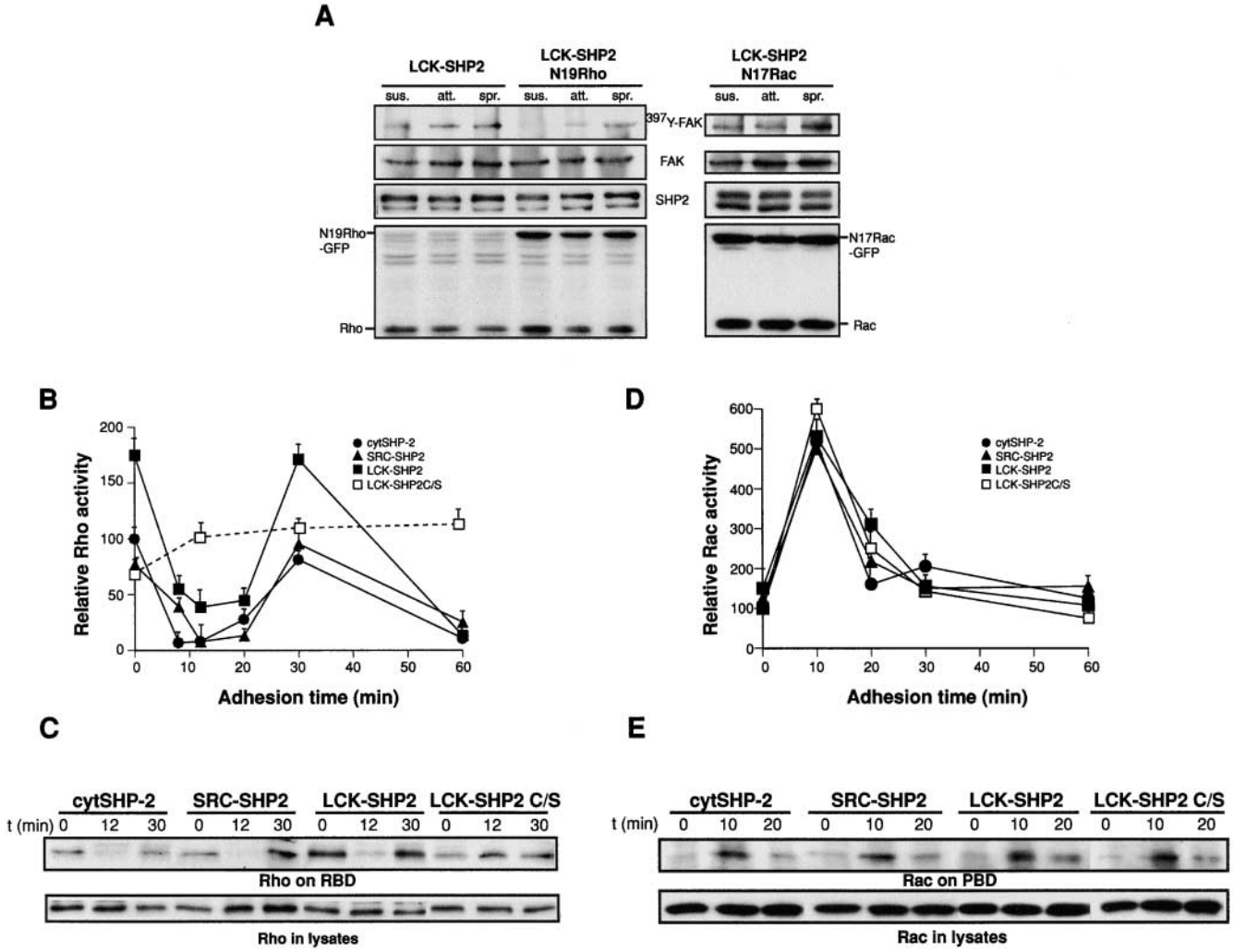

Figure 9. **Raft targeting of LCK-SHP2 triggers Rho activation.** (A) ^{397}Y-FAK phosphorylation was analyzed in lysates of serum-starved LCK-SHP2, LCK-SHP2, and N17Rac or LCK-SHP2 and N19Rho cotransfected cells, in suspension (sus) or Fn-replated for 5 (att) or 30 min (spr). As control, blots were hybridized with anti-FAK, -SHP-2, and -Rho antibodies as indicated ($n = 3$). (B) Serum-starved cytSHP-2 (●), SRC-SHP2 (▲), LCK-SHP2 (■), or LCK-SHP2C/S (□) cells were extracted, and Rho was assayed (Materials and methods). Data are mean ± standard deviations ($n = 3$). (C) Blots are shown in one representative experiment from B. (D) Serum-starved cytSHP-2 (●), SRC-SHP2 (▲), LCK-SHP2 (■), or LCK-SHP2C/S (□) cells were suspended and replated on Fn for the times indicated. Active Rac was precipitated with the agarose-coupled PAK PBD domain. Data are mean ± standard deviations ($n = 3$). (E) Blots are shown in one representative experiment from (D). As a loading control, Rac was also determined in the corresponding cell lysates.

pletion. We report that several proteins that must interact to function, such as β1 integrin, FAK, SFK, and SHP-2, all redistribute to rafts as a consequence of Fn adhesion. Membrane Cho sequestration abrogates Fn-induced cell spreading, implying a functional role for rafts in integrin signaling complex assembly.

Reinforcing this hypothesis, raft-targeted LCK-SHP2 accelerates integrin-mediated cell spreading compared to nonraft SRC-SHP2, cytSHP-2, or mock-transfected cells. For most cell geometry, assuming homogeneous protein distribution, forced membrane expression of a protein should not significantly increase first-encounter rates with other partners, as diffusion in the membrane is lower than in the cytosol (Kholodenko et al., 2000). This situation shifts when all elements of a signaling cascade are concentrated within raft domains of limited area. Thus, targeting LCK-SHP2 to rafts provides a kinetic advantage (i.e., rapid spreading), as other integrin signaling elements are recruited to these domains. These results highlight the role of raft domains as specific platforms for the coordination of integrin signaling.

SHP-2 signaling induced by specific raft partitioning

SHP-2 activation is a combination of compartmentalization and conformational changes. Distinct tyrosine-phosphorylated docking proteins and tyrosine kinase receptors are implicated in membrane recruitment and activation of SHP-2, although membrane location alone is insufficient to trigger its phosphatase activity. SHP-2 contains two SH2 domains, a catalytic (PTP) domain and a C-terminal tail of unknown function (van Vactor et al., 1998). In the absence of an appropriate phosphotyrosyl peptide ligand, the N-SH2 domain binds to the active site of the PTP domain, inactivating the enzyme (Hof et al., 1998). N-SH2 binding to a phosphorylated membrane ligand prevents its union to the

PTP domain, thereby activating the enzyme. However, in unstimulated cells, LCK-SHP2 triggers FAK phosphorylation, integrin clustering and ERK activation by a mechanism that requires PTP activity. This signaling is not a consequence of membrane partitioning, as the non-raft SRC-SHP2 chimera does not signal in unstimulated cells. Moreover, all the chimeras are equally active in vitro, suggesting that addition of the distinct N-terminal tags does not activate PTP. Thus, LCK-SHP2 signals in the absence of stimulation provide a unique model for assessment of downstream consequences of SHP-2 action.

The most striking observation in cells expressing raft-targeted LCK-SHP2 is the specific Rho activation in the absence of cell stimulation. This basal Rho activation requires SHP-2 PTP activity, as the catalytic inactive LCK-SHP2C/S mutant does not show increased Rho activation. LCK-SHP2C/S does not reduce active Rho levels in non-attached cells compared to cells expressing cytSHP-2 or the non-raft SRC-SHP2 chimera, suggesting that SHP-2 PTP activity is important in activating a regulatory component upstream of Rho. The substrates dephosphorylated by SHP-2 to control Rho activity are currently under study. Both guanine-nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs) are regulated by tyrosine phosphorylation (Kjoller and Hall, 1999). p190RhoGAP is an interesting candidate, as increased p190RhoGAP tyrosine phosphorylation correlates with increased GAP activity (Roof et al., 1998); moreover, p190RhoGAP inhibition is sufficient to induce Rho-mediated actin reorganization (Vincent and Settleman, 1999). Thus, SHP-2–induced p190RhoGAP dephosphorylation may inhibit GAP activity and increase Rho-GTP levels. Although control of Rho-GTP by dephosphorylating p190RhoGAP fits well with our data in nonattached cells, it does not explain how SHP-2 suppresses Rho activity after attachment. A role is proposed for Src-induced p190RhoGAP phosphorylation in the initial adhesion-dependent Rho inhibition (Arthur et al., 2000); this raises the possibility that SHP-2 acts directly on different RhoGEFs or GAPs, or indirectly on a third partner that controls the activity of these exchangers (Kodama et al., 2000).

Increased Rho activation in nonattached LCK-SHP2 cells may be responsible for signaling observed in these cells; i.e., FAK phosphorylation and ERK activation. Evidence supporting this conclusion is that N19Rho, but not N17Rac expression inhibits ^{397}Y-FAK phosphorylation in these cells. FAK is phosphorylated on multiple sites, some regulated by autocatalytic activity and others by SFK (Schlaepfer et al., 1999). LCK-SHP2–induced FAK phosphorylation is specific for ^{397}Y, whereas ^{861}Y is not affected in nonattached LCK-SHP2 cells; this suggests that LCK-SHP2–induced FAK phosphorylation is not mediated by SFK activation. Several reports indicate that Rho activity may specifically regulate ^{397}Y-FAK phosphorylation (Barry et al., 1997; Clark et al., 1998). This effect has been explained by Rho-induced acto-myosin contraction, which results in adhesion complex clustering (Chrzanowska-Wodnicka and Burridge, 1996). Interestingly, LCK-SHP2 expression induces β1 integrin clustering, and LCK-SHP2–induced FAK phosphorylation requires an intact actin cytoskeleton. Taken together, the results suggest that raft-targeted LCK-SHP2, acting upstream of Rho, induces actin cytoskeleton rearrangements that culminate in clustering of integrin-bound complexes, resulting in FAK transphosphorylation.

This model may also explain LCK-SHP2–induced ERK activation in nonattached cells, as FRNK inhibits both ^{397}Y-FAK phosphorylation and ERK activation in nonattached LCK-SHP2 cells. The results indicate that SHP-2 requires ^{397}Y-FAK phosphorylation to activate ERKs. SFK activity appears not to be involved in LCK-SHP2–induced ERK activation; indeed, the SFK inhibitor PP2 does not block ERK activation in nonattached LCK-SHP2 cells. Concurrently, LCK-SHP2 expression does not affect ^{527}Y-Src phosphorylation or SFK activity. These data, together with the absence of ^{861}Y-FAK phosphorylation in nonattached LCK-SHP2 cells, indicate that SFK are not activated by LCK-SHP2. As discussed below, SFK may operate upstream of SHP-2 in recruiting this phosphatase to raft domains. Analysis of signaling in nonattached LCK-SHP2–expressing cells predicts a sequence of downstream targets for SHP-2, which may resemble that found after integrin engagement.

Effects of SHP-2 partitioning in integrin signaling

Partitioning SHP-2 to different cell locations may help determine its position in the integrin signaling cascade. Two major differences in integrin-induced events were observed by targeting LCK-SHP2 to rafts. One is that integrin-induced ERK activation in LCK-SHP2 cells is not inhibited by PP2. In the current model, integrin engagement induces tyrosyl phosphorylation of the SHP substrate 1 (SHPS-1/SIRP-1α) through an SFK-dependent mechanism (Tsuda et al., 1998; Oh et al., 1999). This recruits and activates SHP-2, inducing further SFK activation, and leading in turn to ERK signaling. This model suggests that SHP-2 acts both up- and downstream of SFK in Fn-induced signaling to ERK. We observed that, whereas PP2 inhibits the initial peak of Fn-induced ERK activation in cytSHP-2- or the non-raft SRC-SHP2–expressing cells, LCK-SHP2 cells are refractory to PP2-induced ERK inhibition. Because raft targeting of SHP-2 overcomes the PP2 inhibitory effect, we conclude that once in rafts, SHP-2 may activate ERK by acting on substrates other than SFK. As raft location of SHP-2 is important for ERK activation, SFK activation may be critical in SHP-2 recruitment to specific membrane locations. Concurring with this hypothesis, we observed that PP2 treatment inhibits the adhesion-induced recruitment of non-raft SRC-SHP2 or cytSHP-2 to rafts. These results indicate that SFK acts upstream of SHP-2, inducing phosphatase recruitment to raft domains after integrin engagement. SHP-2 location in rafts seems not be involved in SFK activation, as ^{527}Y-SFK dephosphorylation or ^{861}Y-FAK phosphorylation are unaffected by expressing the raft-targeted LCK-SHP2 chimera.

The second major difference detected by raft targeting of SHP-2 is a variation in integrin-induced Rho activation. Integrin engagement to Fn induces transient Rho inhibition, followed by a phase of Rho activation. Feedback regulation of Rho is maintained in LCK-SHP2 expressing cells, but baseline and adhesion-dependent Rho activation are significantly enhanced in LCK-SHP2 cells compared to those expressing SRC-SHP2 or cytSHP-2. This concurs with in-

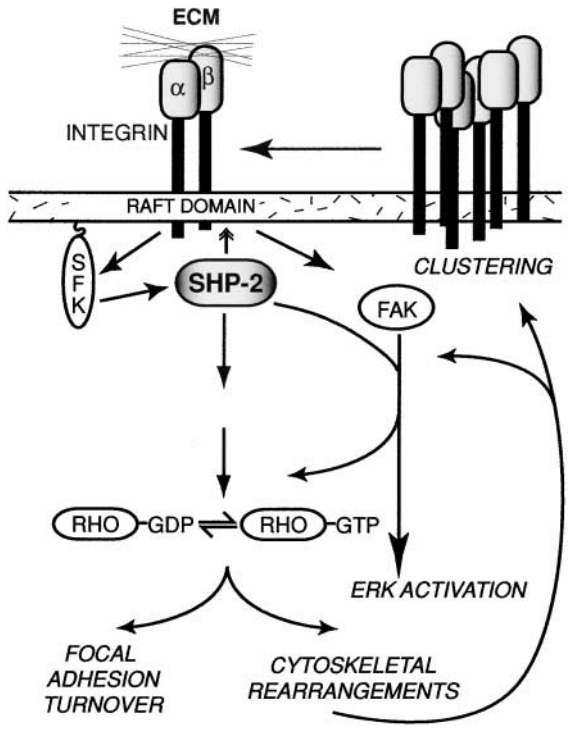

Figure 10. **Model of SHP-2 function downstream of integrins.** Schematic representation of the SHP-2 signaling pathways downstream of integrins. See Discussion for details.

creased Rho-GTP levels in nonattached LCK-SHP2 cells, and reinforces the hypothesis that SHP-2 is a regulator of Rho. However, expression of the inactive LCK-SHP2C/S mutant abrogates the initial adhesion-induced Rho inhibition, suggesting that SHP-2 is also involved in integrin-induced Rho suppression. It is important to note that Rho-GTP levels in LCK-SHP2C/S cells do not reach those observed in cells expressing the active LCK-SHP2 chimera. One interpretation is that the enhanced active Rho levels in LCK-SHP2 cells are the result of SHP-2 enzymatic activity and not the sole consequence of raft partitioning, supporting a physiological role for SHP-2 in Rho activation. Nonetheless, raft targeting of catalytically active or inactive SHP-2 forms does not affect Rac activation downstream of integrins. Together, the results indicate that SHP-2 takes part in specifically regulating Rho activity.

We integrate our data in a model for SHP-2–induced ERK activation and cytoskeletal rearrangement downstream of integrins (Fig. 10). The scheme is partial, as integrins trigger distinct signaling pathways simultaneously. Indeed, we observed no more than 50% ERK inhibition by blocking individual signaling pathways (i.e., SHP-2C/S for SHP-2, PP2 for SFK, FRNK for FAK), suggesting that integrins trigger several independent pathways that converge at ERK activation (Barberis et al., 2000). We propose that integrin binding to the ECM substrate promotes SFK activation, which phosphorylates substrates that recruit SHP-2 to rafts. Activated SHP-2 in rafts is essential for regulating Rho activity downstream of integrins, controlling cytoskeletal rearrangements that culminate in integrin clustering and ^{397}Y-FAK phosphorylation. Both SHP-2C/S and N19Rho expression inhibit basal and integrin-induced ^{397}Y-FAK phosphorylation, suggesting that FAK is downstream of these molecules. Activated FAK, alone or in combination with SHP-2 and SFK, participates in turn in adhesion-dependent Rho inhibition, modulating the focal adhesion turnover required for cell spreading (Ren et al., 1999).

Our observation that FRNK inhibits 50% of integrin-induced ERK activation, independently of the coexpressed SHP-2 form, supports the ^{397}Y-FAK phosphorylation dependence of integrin-induced ERK activation (Schlaepfer and Hunter, 1997). FRNK also inhibits ERK activation in nonattached LCK-SHP2 cells. The simplest interpretation of these results is that FAK is downstream of SHP-2; accordingly, SHP-2C/S expression inhibits ERK and delays ^{397}Y-FAK phosphorylation. Thus, a possible pathway from SHP-2 to ERK may be mediated via FAK.

Despite these correlations, FAK and SHP-2 may activate ERK independently; indeed, maximum ERK activation precedes maximum ^{397}Y-FAK phosphorylation. This uncoupling of FAK phosphorylation and ERK activation suggests the existence of a FAK-independent pathway, which may nonetheless be regulated by SHP-2. We do not observe SHP-2 phosphorylation in unstimulated or Fn-attached cells (unpublished data), indicating that the SHP-2/Grb2/SOS pathway (Bennett et al., 1994) is not relevant for SHP-2–induced ERK activation downstream of integrins. An intriguing possibility is that SHP-2 cooperates with a major integrin-stimulated pathway to further enhance ERK activation. SHP-2 and its *Drosophila* homologue, Corkscrew, regulate RasGAP targeting and activation downstream of the platelet-derived growth factor receptor and Torso, respectively, abrogating Ras inactivation (Klinghoffer and Kazlauskas, 1995; Cleghon et al., 1996). It is attractive to speculate that SHP-2 may regulate both Rho and p120RasGAP activity by controlling p190RhoGAP phosphorylation, a GAP for Rho and a binding partner for RasGAP (Kulkarni et al., 2000).

SHP-2 is a specific regulator of Rho

The link between SHP-2 and Rho is particularly interesting, as both are implicated in cell migration. Previous results placed SHP-2 upstream of Rho, although there is controversy as to whether SHP-2 regulates Rho activity positively (Inagaki et al., 2000b; O'Reilly et al., 2000) or negatively (Kodama et al., 2000; Schoenwaelder et al., 2000). SHP-2–induced Rho inhibition is consistent with the larger numbers of focal contacts and actin stress fibers in cells expressing loss-of-function SHP-2 or SHP-2C/S. Thus, the LCK-SHP2C/S cell phenotype resembles that of FAK$^{-/-}$ and SYF (Src$^{-/-}$Yes$^{-/-}$Fyn$^{-/-}$) cells, which fail to inhibit Rho after adhesion (Arthur et al., 2000; Ren et al., 2000); thus, aberrant adhesion to substrate may cause the migration defects seen in these cells (Yu et al., 1998; Mañes et al., 1999b). Nonetheless, the model of SHP-2 as a constitutive negative regulator of Rho is inconsistent with the defects in polarized extension and spreading of SHP-2–deficient cells.

Our results suggest that SHP-2 functions as a positive or negative regulator of Rho activity, depending on cellular context. Fibronectin replating results indicate that functional SHP-2 is required for 293T adhesion (Fig. 1 B), and that the active LCK-SHP2 form is in fact a positive Rho

modulator. This experiment may mimic integrin-initiated signaling events at the leading edge of migrating cells. Nonetheless, SHP-2 is also essential for Rho inactivation after adhesion occurs. SHP-2–induced Rho inactivation may occur directly by activating Rho-GAP or inactivating Rho-GEF, or indirectly by regulating FAK phosphorylation. Indeed, FAK activity is necessary for Rho inactivation after adhesion (Ren et al., 2000); concurring with other results (Oh et al., 1999), we found that SHP-2 activity is required for FAK activation. As Rho activity is also involved in FAK activation, SHP-2 may be part of the negative feedback mechanism that controls focal adhesion turnover by regulating Rho and/or FAK signaling pathways. This mechanism operates in rafts, providing spatial organization for these signaling networks. The importance of positive and negative Rho regulation is underscored, as both constitutive activation and inhibition of Rho suppress migration. Thus, dual regulation of Rho by SHP-2 may explain the physiological role of this phosphatase in regulating focal adhesion turnover and, hence, in modulating the spreading and motile status of cells.

Materials and methods

Materials

Surfact-Amps X-100 containing 10% Triton X-100 was from Pierce Chemical Co; Optiprep gradient medium was from Nycomed Pharma; fibronectin, methyl-β-cyclodextrin, p-nitrophenyl phosphate (pNPP), water-soluble Cho, poly-L-lysine, BSA, and biotin- and FITC-labeled CTx B subunit were from Sigma-Aldrich; Src inhibitor PP2, PP3, and latrunculin-B were from Calbiochem; and ECL and [γ-^{32}P]ATP were from Amersham Pharmacia Biotech.

Antibodies used included the following: anti–SHP-2, -caveolin-1, -FAK, and anti-pan Src (Santa Cruz Biotechnology); anti-β1 integrin (Chemicon International); anti-TfR and -ERK1/2 (Zymed Labs); anti–phospho-ERK1/2 (Cell Signaling Technology); anti–phospho-FAK (^{397}Y and ^{861}Y) and –phospho-Src (^{527}Y) (Biosource); anti-6His (CLONTECH Laboratories, Inc.); anti-FAK and -paxillin (Transduction Labs); and anti-phosphotyrosine (PY) 4G10, -Rho, -Rac, and the Rho and Rac activation kits were from Upstate Biotechnology. Peroxidase- and Cy3-labeled secondary antibodies were from Dako and Jackson ImmunoResearch, respectively. Anti-HA antibody was from Babco.

Cloning and expression constructs

cDNA coding for cytShp-2 (wt), cytShp-2C/S, the ΔShp-2, and ΔShp-2C/S mutants lacking the two SH2 domains, and the Src-Shp2 and Src-Shp2C/S chimeras containing 14 amino acids from the c-Src N terminus (MGSNKSKPKDASQR) (Zhao and Zhao, 1999) were a gift of Dr. Z.J. Zhao (Vanderbilt University, Nashville, TN). The Lck-Shp2 chimera was obtained by subcloning the first 12 amino acids from Lck (MGCGCSSHPEDD) at the wt Shp2 N-terminal. Before transfection, all constructs were subcloned in pcDNA3.1/Myc-His (Invitrogen) using standard procedures. HA-tagged FRNK and GFP-tagged N19Rho, and N17Rac were gifts of Drs. D. Schlaepfer (Scripps Research Institute, La Jolla, CA) and F. Sánchez-Madrid (Hospital de la Princesa, Madrid, Spain), respectively.

Cell culture

HEK-293T human embryonic kidney cells (American Type Culture Collection) were maintained in DME with 10% FCS, L-glutamine, sodium pyruvate, and antibiotics. Cells were transiently transfected with the plasmids using the standard calcium phosphate method. Transfection efficiency, determined after 48 h, was 60–70% for SHP-2 constructs, 55% for N17Rac-GFP, and 30% for N19Rho-GFP. 293T cells stably expressing FRNK were selected in hygromycin (75 μg/ml), and expression examined by Western blot using anti-HA antibody.

Adhesion and spreading assays

For adhesion experiments, 96-well plates were coated (16 h, 4°C) with Fn (10-0.01 μg/ml) or poly-L-lysine (0.5 mg/ml). 293T or 293T-FRNK cells expressing SHP-2 chimeras were serum starved in DME/0.1% BSA (4 h, 37°C); after trypsinization, 3×10^4 cells/well were plated (15 min, 37°C). After washing, adhered cells were estimated by MTS (Promega) conversion. Each condition was assayed in quadruplicate.

For spreading analysis, serum-starved 293T- or 293T-FRNK cells expressing the indicated SHP-2 constructs (10^6/ml) were trypsinized, resuspended in DMEM/BSA and plated on Fn-coated (10 μg/ml) dishes for indicated times. Spread cells were determined by dark-phase counting (200×) from eight fields. To analyze the effect of Cho depletion, 293T cells were treated with 10 mM CD (30 min, 37°C); after washing, some CD-treated cells were incubated (30 min, 37°C) in medium containing 30 μg/ml free Cho. Cho was removed by washing with DMEM/BSA and untreated-, CD-treated and Cho-replenished (CD+Cho) cells were then replated on Fn as above.

Western blotting and immunoprecipitation

In general, serum-starved 293T or 293T-FRNK cells expressing the SHP-2 forms were trypsinized; part were maintained in suspension (10 min, 37°C), washed twice in phenol red-free DME/BSA, and the pellet was lysed as below. The remaining cells were plated on Fn (10 μg/ml) for the times indicated. After washing to remove nonadhered cells, cells were lysed in modified RIPA buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100) with a protease and phosphatase inhibitor cocktail. In some experiments, suspended cells were treated with PP2, PP3, or latrunculin-B (in all cases 10 μM, 30 min, 37°C) with continuous shaking before replating on Fn.

Protein concentration was determined using the Micro BCA protein assay (Pierce Chemical Co.). For immunoprecipitation, lysates (200 μg) were diluted with 200 μl of lysis buffer containing the appropriate antibody at the concentration indicated by the manufacturer; after incubation (2 h, 4°C), antibodies were precipitated with anti–mouse IgG or protein G-Sepharose beads. Total cell lysates (20 μg/lane) or immunoprecipitated proteins were separated by SDS-PAGE and probed with the indicated antibodies, followed by PO-labeled second antibody and ECL.

SRC in vitro kinase assay

Cell lysates were precipitated with anti-pan SRC antibody (2 h, 4°C) and the pellet washed with ice-cold 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10 mM $MnCl_2$. Pellets were resuspended in 50 μl of kinase buffer (50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM Na_2VO_4, 1 μM ATP, and 10 μCi [γ-^{32}P]ATP) and incubated (10 min, 20°C) before adding 4× sample buffer to terminate the reaction. Radioactivity was quantified after SDS-PAGE with Storm 840 Phosphorimager/Image-QuaNT software (Molecular Dynamics).

SHP-2 in vitro phosphatase assay

Cell lysates from serum-starved 293T cells expressing the distinct SHP-2 mutants were immunoprecipitated with anti–SHP-2-agarose or anti-6His antibodies (2 h, 4°C). The beads capturing total (anti–SHP-2) or expressed SHP-2 chimeras (anti-6His) were suspended in 50 μl of assay buffer (20 mM Tris-HCl, pH 7.4, 100 mM NaCl, 1 mM DTT and 1 mM EDTA), and the reaction was initiated by adding 50 μl of assay buffer containing 24 mM pNPP. After incubation (1 h, 37°C), the reaction was terminated with 10 μl of 5 N NaOH, and the amount of p-nitrophenol released was determined by absorbance at 405 nm; under these conditions, the reaction proceeded in a dose- and time-dependent manner.

Triton X-100 flotation experiments

For flotation gradients, serum-starved 293T cells were plated on Fn (10 μg/ml), and after the indicated times, plates were washed with ice-cold PBS and scraped into 300 μl TXNE buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.2% Triton X-100) containing a protease and phosphatase inhibitor cocktail. Extracted cells were brought to 35% Optiprep and overlaid with 3.5 ml 30% Optiprep in TXNE and 200 μl TXNE in an SW60 tube. After centrifugation (4 h, 170,000 *g*, 4°C), five fractions were collected from top to bottom of the gradient, and normalized protein amounts for each fraction were analyzed in Western blot.

Immunofluorescence

Serum-starved cells were trypsinized and then maintained in suspension or plated on Fn-coated 8-well chamber glass slides (Nunc). After fixing (1% paraformaldehyde, 5 min, 4°C), cells were incubated with FITC-CTx (6 μg/ml, 10 min, 12°C) and blocked with 5% goat serum/0.2% BSA before addition of anti–^{397}Y-FAK, -paxillin, -SHP-2, or -β1 antibody diluted in PBS/BSA/1% goat serum. When antibodies to intracellular proteins were used, cells were permeabilized with 0.1% Triton X-100 (10 min, 4°C). Finally, samples were incubated with the appropriate Cy3- or Cy2-conjugated second antibody (1 h, 4°C), and slides were mounted in Vectashield medium

containing DAPI (Vector Labs) for confocal microscopy analysis (Leica). When two colors were used, signal intensities for both fluorophors were covered by a linear pixel scale. Digital images were processed using Photoshop (Adobe Systems).

Rho and Rac activation assay

Starved cells (2×10^7) were maintained in suspension or plated on Fn for the times indicated. Cell lysates were prepared using the reagents in the Rho or Rac activation assay kit; GTP-bound Rho was precipitated with RBD-agarose beads and GTP-Rac with PBD-agarose beads, according to manufacturer's instructions. The amount of Rho and Rac in the pellets was determined by Western blot with specific antibodies, using crude cell extracts for normalization. Densitometry was performed using NIH Image software.

Online supplemental material

Online supplemental Figs. S1–4 (available at http://www.jcb.org/cgi/content/full/jcb.200109031/DC1) are the single color images from Figs. 2, B–G, 4 A, 5 A, and 6 E, respectively.

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