Materials and methods

Cell lines, plasmid constructs, and antibodies

Cos-7 and HEK 293 cells were from the American Type Culture Collection. Mouse NIH 3T3 cells were provided by Drs. Tony Hunter and Pamela Woodring (The Salk Institute, La Jolla, CA). Bcr/Abl-transformed 3T3 cells were from Dr. Miguel Angel Del Pozo (The Scripps Research Institute, La Jolla, CA) and Dr. Jean Wang (University of California, San Diego, San Diego, CA). The stable Abl−/−Arg−/− (double knock-out) cell line was from Dr. Anthony Koleske (Yale University, New Haven, CT). Reconstitution of Abl−/−Arg−/− cells was induced through retroviral mediated gene transfer using Bosc cell–generated retrovirus according to published procedures (Kain et al., 2003). The HEK 293 cells stably expressing Lasp-1 were generated by calcium phosphate transfection of Lasp-1 in pcDNA3.1/neo (Invitrogen) and selected by G418 (GIBCO BRL). Full-length LaspΔC (aa 1–145) and Lasp SH3 (aa 196–261) genes were amplified by PCR using primers containing appropriate restriction sites and pCMVLasp-1 (Schreiber et al., 1998) as a template. The fragments were cloned into pcDNA 3.1/neo (Invitrogen), pRK5HA, pEGFP C1 (CLONTECH Laboratories, Inc.), and pGEX 6P-2 (Amersham Biosciences), respectively. AblΔPro was a gift from Dr. Bruce Mayer (Smith et al., 1999). LaspY171F mutants in pcDNA and pEGFP vectors were generated using the QuikChange® site-directed mutagenesis kit (Stratagene) and DNA sequencing was performed to confirm mutations. pMyr-Lasp and pMyr-LaspΔC (myristylated forms) were generated by fusing a PCR-amplified DNA fragment encoding the first 14 amino acids of p60src to the NH2 terminus of Lasp-1 and LaspΔC in their respective expressing vectors. Rabbit polyclonal Lasp-1 antibodies were from CHEMICON International (A68990). Anti-phosphotyrosine (4G10; Upstate Biotechnology), anti-Abl 8E9 (BD Biosciences), anti-HA (Roche), anti-c-fos, anti-β2 integrin, anti-vinculin, anti-actin (Sigma-Aldrich), and Alexa Fluor® 568-conjugated goat anti–mouse IgG antibodies (Molecular Probes, Inc.), goat anti-rabbit and anti–mouse antibodies (Bio-Rad Laboratories), and rabbit antibodies to the 89-kD cleaved/activated form of PARP (Cell Signaling Technology) were purchased from commercial sources as indicated. H2O2, LPA, insulin, and cisplatin were obtained from Sigma-Aldrich. STI 571 was obtained from Novartis and PDGF-BB was purchased from Genzyme. Trichostatin A was obtained from Sigma-Aldrich.

Migration, adhesion, apoptosis, and wound assays

Migration assays were performed as described previously (Klemke et al., 1998). In brief, Boyden chambers containing polycarbonate membranes (tissue culture treated, 6.5-mm diam, 10-μm thickness, 8-μm pores; Transwell®, Costar Corp. or CHEMICON International) were either coated on the bottom (haptotaxis) or on the top and bottom (chemotaxis) with either rat tail collagen type I (Upstate Biotechnology, Inc.) or human fibronectin (Oxford Biomedical Products) for 2 h at 37°C. Serum-starved cells were allowed to migrate 3–5 h, were fixed with ethanol, and then were stained with crystal violet. In some cases, migratory cells transfected with Lasp-1 constructs were cotransfected along with the reporter vector pCMVSPORT β-galactosidase (Invitrogen) and developed using X-gal as a substrate according to the manufacturer’s recommendation (Promega) and as described previously (Klemke et al., 1998). For cell adhesion assays, an aliquot of cells used in the migration experiments was allowed to attach for the indicated times to fibronectin- or collagen (10 μg/ml)-coated cell culture wells at 37°C and was stained with crystal violet or X-gal reagent. For wound migration, GFP Lasp–transfected NIH 3T3 cells were plated at 150,000 cells per well on a glass coverslip in media containing 0.5% FBS. Cells were incubated overnight to form a confluent monolayer of cells. A wound was introduced into the monolayer by scraping with a clean micropipette tip and the detached cells were removed into the wound at 37°C in 0.5% FBS for 18 h, and then were rinsed with PBS and fixed in 4% PFA. Vinculin-positive focal adhesion, GFP Lasp, and the actin cytoskeleton were visualized as described above. Standard apoptosis assays using propidium iodide staining were performed as previously described, except cells were maintained in 0.5% FBS to reduce serum-deprived cell death responses (Kain et al., 2003).

Transient transfection

Transient transfection of Cos-7 cells for biochemical and migration assays was performed as described previously (Klemke et al., 1998). For transfections of mouse NIH 3T3 cells and Bcr/Abl-transformed 3T3 cells, Effectene™ (QIAGEN) was used. 300,000 cells were plated per 100-mm dish the night before transfection. 1.5 μg of DNA was used and serum-starved cells (0.5% FBS for 24 h) were lysed 48 h later. Transfection of abl−/−arg−/− and abl−/−arg-fibroblast cells (Koleske et al., 1998) was performed as for the Cos-7 cells, except 300,000 cells were plated in a 100-mm plate and then incubated with DNA and LipofectAMINE™ for 4 h.
Targeting of Lasp-1 to plasma membrane
pMyc-Lasp and pMyc-LaspΔC vectors along with GFP reporter construct were transfected into Cos-7 cells. Cells were fixed after 16 h of transfection. Blebbing cells were counted and deconvolution images were taken as described in the Materials and methods section in the manuscript text.

Immunoblotting, GST purification, and immunoprecipitation
Immunoblotting and immunoprecipitation of proteins were conducted according to published procedures (Klemke et al., 1998). In brief, cells were rinsed with PBS and lysed in Triton X-100 buffer (50 mM NaCl, 1 mM EDTA, 50 mM Hepes, 1 mM EGTA, 1% Triton X-100, 2 mM sodium orthovanadate, and 1 mM sodium fluoride, pH 8.0) on ice for 2 h.

In vitro kinase assay
50 U purified Abl (Cell Signaling Technology) was incubated with 10 ng pGEXLasp purified from *Escherichia coli* in the presence of 200 μM ATP at 30°C for 30 min or with 1 μg purified GST alone. The reaction was stopped by SDS-PAGE sample buffer and analyzed for tyrosine phosphorylation by Western blotting with anti-phosphotyrosine, GST, or Lasp-1 antibodies.

References