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AP-1 Differentially Expressed Proteins Krp1 and Fibronectin Cooperatively Enhance Rho-ROCK-Independent Mesenchymal Invasion by Altering the Function, Localization, and Activity of Nondifferentially Expressed Proteins†

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The transcription factor AP-1, which is composed of Fos and Jun family proteins, plays an essential role in tumor cell invasion by altering gene expression. We report here that Krp1, the AP-1 up-regulated protein that has a role in pseudopodial elongation in v-Fos-transformed rat fibroblast cells, forms a novel interaction with the nondifferentially expressed actin binding protein Lasp-1. Krp1 and Lasp-1 colocalize with actin at the tips of pseudopodia, and this localization is maintained by continued AP-1 mediated down-regulation of fibronectin that in turn suppresses integrin and Rho-ROCK signaling and allows pseudopodial protrusion and mesenchyme-like invasion. Mutation analysis of Lasp-1 demonstrates that its SH3 domain is necessary for pseudopodial extension and invasion. The results support the concept of an AP-1-regulated multigenic invasion program in which proteins encoded by differentially expressed genes direct the function, localization, and activity of proteins that are not differentially expressed to enhance the invasiveness of cells.

For a tumor cell to become metastatic it has to be capable of invading through tissues. The ability to invade is a complex process that involves alterations in the following: cell-cell interactions, cell-extracellular matrix adhesions, the cell cytoskeleton, and cell motility (35, 59). It has been proposed that the transcription factor AP-1, which is activated by many oncogenic signaling pathways, regulates the changes in gene expression that allow these processes to occur (4, 21, 22, 26, 30, 31, 37, 39, 40, 46, 47, 52–54, 64).

AP-1 is composed primarily of heterodimers of various proteins of the Fos and Jun families, providing flexibility for it to activate or repress the expression of genes (9, 19, 27). The identification of the prototypes of Fos and Jun as retroviral oncogenes highlights the role of AP-1 in tumorigenesis, with v-Fos-induced tumors being locally invasive (17, 21). Animal models have also highlighted the role of AP-1 in invasion (48, 63). For example, in c-*fos*-null mice, premalignant papillomas do not progress to malignant skin carcinomas when subjected to chemical carcinogens or an activated Ras oncogene (48). Human squamous cell carcinomas have also been shown to depend on AP-1 for invasion, since expression of a dominant-negative form of c-Jun, TAM67, reduced the invasiveness of the cells (37). The AP-1 family member Fra-1 is up-regulated in invasive tumors and stimulates invasion in carcinoma-derived cells (7, 29). In addition, inhibition of Fra-1 causes a reduction in the invasiveness of cells (58).

Studies to identify AP-1 target genes in oncogenically transformed cells have also highlighted the role of AP-1 in invasion. Ablation of expression or function of individual up-regulated genes reduces the invasiveness of cells, while reexpression of down-regulated genes suppresses invasion. One of the model systems used to identify AP-1-regulated genes is a v-Fos-transformed rat fibroblast (FBR) cell line (4, 5, 18, 22, 26, 30, 31, 39, 44, 45, 47, 53). Upon transformation of the rat fibroblast cell line 208F with v-Fos, the cells undergo a dramatic reorganization of their actin cytoskeletons and become invasive in an in vitro three-dimensional (3D) invasion assay (22). The cells change from being flat with many prominent F-actin stress fibers and focal adhesions to being bipolar spindle-like with long pseudopodia and a much-reduced number of obvious stress fibers and focal adhesions. The cells' mode of 2D motility is also altered. They no longer move by extending lamellipodia but instead extend long pseudopodia. Gene expression profiling has successfully identified genes expressed differentially between the untransformed 208F cells and the FBR cells (5, 22, 26, 45, 53). One of the genes which is down-regulated in FBR cells encodes fibronectin (FN), which is also down-regu-

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lated in a variety of tumors and oncogene-transformed cells (39). Reexpression of FN is capable of inducing reversion of transformed cells to an untransformed normal morphology (2, 3, 8, 15, 24, 38, 43). Two up-regulated genes which encode CD44 and ezrin have been shown to have a role in invasion (30, 31). CD44 is a cell surface hyaluronan receptor that accumulates at the tips of pseudopodia in FBR cells. Depletion of CD44 from FBR cells reduces their invasiveness. Ezrin, an ERM family member, is also localized to the tips of pseudopodia in FBR cells, where it is thought to link CD44 to the cell cytoskeleton (32, 57). Ablation of ezrin from the tips of pseudopodia resulted in a collapse in the pseudopodial structures, suggesting that ezrin has a role in the formation of pseudopodia.

A novel up-regulated gene encodes kelch-related protein 1 (Krp1), a member of the kelch family of proteins (1, 53). kelch family members are characterized by having a BTB/POZ domain at their N terminus and kelch repeats at their C terminus (1, 62). Many of the kelch family members have been shown to be cytoskeleton-interacting proteins. We have previously reported that Krp1 in FBR cells localizes to the tips of pseudopodia. Through overexpression of wild-type Krp1 and dominant-negative Krp1 mutants in FBR cells, we have demonstrated that Krp1 has a role in elongation of pseudopodia, which are structures that are required for FBR cells to invade.

We report here a novel interaction between Krp1 and the nondifferentially expressed protein Lasp-1. Lasp-1 consists of a LIM domain at its N terminus, two nebulin repeat domains, and an SH3 domain at its C terminus (55). Lasp-1 has been shown to be an actin binding protein and to localize to focal adhesions, membrane ruffles, and pseudopodia (11, 13, 33, 34, 51). We demonstrate that Lasp-1, like Krp1, ezrin, and CD44, has a role in pseudopodial elongation and invasion. In addition, we also show that the colocalization of Krp1 and Lasp-1 at the tips of pseudopodia and FBR cell invasion are independent of FN, integrin, and Rho-ROCK signaling. These data suggest not only that AP-1 regulates genes which are involved in invasion but that these genes encode proteins that affect nondifferentially regulated proteins so that they enhance invasion.

MATERIALS AND METHODS

Cell lines. The 208F cell line is a subclone of the Rat-1 fibroblast cell line originally obtained from K. Quade. FBR cell lines were originally obtained from Tom Curran and are transformed nonproducer 208Fs infected with FBR-MuSV. All cell lines were routinely passaged before confluence and maintained in Dulbecco's modified Eagle medium (Invitrogen) supplemented with 10% fetal calf serum at 37°C in 5% CO_2.

Plasmids. The Krp1-myc construct was derived previously in our laboratory (53). For glutathione *S*-transferase (GST) pull-down experiments, full-length Krp1 and Lasp-1 DNA fragments were derived by PCR and cloned into pGEX-2TK. The Lasp-1–hemagglutinin (HA), Lasp-1–green fluorescent protein (GFP), and Lasp-1ΔSH3 constructs are described in references 11 and 13. For the yeast two-hybrid screen, the Clontech pretransformed matchmaker skeletal muscle library was used. Krp1 kelch repeats were used as the bait via PCR and cloning of the PCR product into the bait vector pGBKT7.

GST pull-down assays. GST fusion proteins were expressed in *Escherichia coli* strain BL21 by induction with 0.1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) for 2 h. The bacteria were collected by centrifugation and the pellet was resuspended in bacterial lysis buffer (50 mM Tris [pH 7.5], 1 mM EDTA, 100 mM NaCl, 5% glycerol, 0.1% Triton X-100, 1 mM dithiothreitol). After sonication, the lysate was centrifuged and the pellet was resuspended in bacterial lysis buffer. The fusion proteins were bound to the glutathione beads by rotating the bacterial lysate with 100 μl of 50% glutathione-Sepharose for 30 min at 4°C, followed by three washes with GST-FISH buffer (10% glycerol, 50 mM Tris [pH 7.4], 100 mM NaCl, 1% NP-40, 2 mM $MgCl_2$, 10 μg/ml each aprotinin and leupeptin, and 1 mM PMSF [phenylmethylsulfonyl fluoride]). The GST-fusion protein bound to beads was then resuspended in 100 μl of GST-FISH buffer. COS-7 cells expressing the protein of interest were lysed in GST-FISH buffer, and 1 mg of lysate was incubated with 20 μl of GST-fusion protein bound to the glutathione-Sepharose beads for 2 h. The nebulin DNA fragment was subjected to in vitro transcription/translation using the TNT coupled reticulocyte system (Promega) using the supplier's instructions, and the lysate was added to 20 μl of the GST-fusion protein bound to the glutathione-Sepharose beads for 2 h. The beads were then washed three times in GST-FISH buffer, and the bound proteins were eluted in sodium dodecyl sulfate (SDS) sample buffer. Western blot analysis was carried out as above or, in the in vitro transcription/translation experiment, the gel was dried and then exposed for autoradiography.

Immunoprecipitations. Cell lysates were prepared in radioimmunoprecipitation assay (RIPA) lysis buffer (50 mM Tris [pH 7.4], 150 mM NaCl, 5 mM EGTA, 0.1% SDS, 1% Triton X-100, 10 μg/ml each aprotinin and leupeptin, and 1 mM PMSF) and coimmunoprecipitations were performed overnight at 4°C with either anti-c-*myc*–agarose (Sigma) or anti-HA–agarose (Sigma). Three washes were then performed with RIPA buffer and the immune complexes were solubilized in SDS sample buffer prior to electrophoresis, transfer, and Western blotting.

siRNA. FBR cells were seeded at 1.2×10^5 in 35-mm-diameter tissue culture wells and were transfected with small interfering RNA (siRNA) at a final concentration of 10 nM, using HiPerFect (QIAGEN) according to QIAGEN's "Fast-Forward Transfection of Adherent Cells" protocol, on days 0, 2, and 4. Cells were then used in experiments on day 5. siRNAs used were as follows: Krp1, HP GenomeWide siRNA Rn_Kbtbd10_2_HP (QIAGEN); Lasp-1, siGENOME SMARTpool reagent M-088943-00-0010 (Dharmacon); and siGLO cyclophilin B (human/mouse) D-001610-01-20 (Dharmacon).

Confocal microscopy. Cells were plated onto uncoated or FN-coated coverslips at a concentration of 10 μg/ml. Cells were transfected with the relevant plasmids with Fugene 6 (Roche) and after 24 h the cells were processed for confocal microscopy. Alternatively, transient transfections were performed by nucleofection using the Amaxa Nucleofector system (39) and then plated onto uncoated or FN-coated coverslips. Cells were either prepermeablized in 0.02% Triton X-100–phosphate-buffered saline (PBS) for 1 min and then fixed with 4% paraformaldehyde for 15 min at 37°C, or they were fixed without permeablization. After fixing, the cells were washed extensively in PBS and permeablized in PBS–20 mM glycine–0.05% Triton X-100 for 5 min, if they had not been prepermeablized. Blocking of nonspecific binding was performed for 20 min in blocking buffer (PBS-0.5% bovine serum albumin [BSA]-10% fetal calf serum [FCS]), followed by a 1-h incubation at room temperature with the relevant primary antibody diluted in blocking buffer: anti-Krp1, 1:400; anti-myc (9E10), 1:100; anti-HA, 1:100; anti-Lasp-1, 1:50; and antipaxillin, 1:100. After extensive washing in blocking buffer, the relevant fluorescence-conjugated secondary antibody (Jackson Immunoresearch Laboratories) was added in blocking buffer and, when needed, either tetramethyl rhodamine isothiocyanate (TRITC) or fluorescein isothiocyanate phalloidin was added and the solution was incubated for 45 min at room temperature. Three final washes in PBS were performed and the cells were mounted using Vectashield (Vector Laboratories). Cells were visualized using a Leica confocal microscope (SP2MP) and images constructed using Leica software.

Western blot analysis. Protein lysates were electrophoresed on the appropriate concentration of polyacrylamide gels using standard methods. Proteins were transferred to polyvinylidene difluoride membranes (Immunobilon P; Millipore) with a Trans-Blot wet blotting apparatus (Bio-Rad). Membranes were blocked in blocking buffer (PBS containing 0.1% Tween 20 and 5% nonfat milk) for 1 h and probed with the following antibodies at the given dilution in blocking buffer: anti-Krp1, 1:2,500; anti-Lasp-1, 1:2,000; anti-myc (9E10; Invitrogen), 1:1,000; anti-HA (Cancer Research UK), 1:1,000; anti-Rho (Santa Cruz Biotechnology), 1:500; anti-ROCK 1 (BD Biosciences), 1:1,000; anti-ROCK 2 (BD Biosciences), 1:1,000; anticofilin (BD Biosciences), 1:500; and anti-phospho-Ser cofilin (Santa Cruz), 1:500. Membranes were then washed three times in blocking buffer followed by a 1-h incubation with the secondary antibodies, either peroxidase-labeled goat antirabbit or peroxidase-labeled goat anti-mouse antibody at a 1:10,000 dilution in blocking buffer. Three final washes were performed in PBS containing 0.1% Tween 20, and the blots were visualized using ECL (Amersham).

Active Rho pull-down assay. Active Rho pull-down assays were performed using the Upstate active Rho assay kit. FBR cells were seeded onto either

uncoated or 10-μg/ml FN-coated 10-cm tissue culture dishes. The cells were incubated for 3 days and then washed twice in ice-cold PBS. The cells were then lysed in Upstate lysis buffer (provided with the active Rho assay kit) and 1 mg of lysate was incubated with Upstate Rhotekin RGD-agarose for 45 min at 4°C. The beads were then washed three times with Upstate lysis buffer and the bound proteins were eluted in SDS sample buffer. Western blotting was then performed as described above.

Yeast two-hybrid screen. A yeast two-hybrid screen was performed with the Clontech pretransformed matchmaker skeletal muscle library as the prey and Krp1 kelch repeats as the bait. Approximately 2×10^6 clones were screened, and the positive plasmids were isolated using the supplier's instructions.

Invasion assays. Invasion assays were carried out as previously described (22, 39, 52) using complete matrigel. When ROCK inhibitor Y27632 and FN were added to the invasion assay they were added to the matrigel and to the medium above and below the matrigel at the concentrations stated in the results.

RESULTS

Krp1 interacts with actin binding protein Lasp-1. To identify Krp1-interacting proteins, a yeast two-hybrid screen was performed with the kelch repeats of Krp1 as the bait and a skeletal muscle library as the prey. Approximately 2×10^6 clones were screened and 30 positives were isolated. Two of these represented the last five nebulin repeats of the large actin binding protein, nebulin (25). To substantiate the yeast two-hybrid interaction, the nebulin cDNA clones were subjected to in vitro transcription/translation and these lysates were used in GST pull-down experiments with GST alone and Krp1-GST. GST-Krp1 was able to pull down the nebulin repeats, confirming that Krp1 is able to interact with nebulin (Fig. 1A).

Nebulin repeats are found not only in nebulin proteins but also in NRAP, nebulette, and Lasp-1 (41). It has recently been shown that Krp1 can interact with the nebulin repeats present within NRAP (36). The nebulin repeat-containing protein, Lasp-1, has been shown to be up-regulated in some breast cancer cell lines and, as we have proposed that Krp1 has a role in invasion, we investigated whether Krp1 can interact with Lasp-1 via its nebulin repeats (55). An HA-tagged Lasp-1 construct was expressed in COS-7 cells, and the cell lysate was subjected to GST pull-downs with GST alone and GST-Krp1. GST-Krp1 recognized HA-tagged Lasp-1, confirming that Krp1 can interact with Lasp-1 (Fig. 1B). The reciprocal pull-down was also performed by expressing a myc-tagged Krp1 construct in COS-7 cells and subjecting the cell lysate to a pull-down with GST and GST–Lasp-1 (Fig. 1C). GST–Lasp-1 pulled down the myc-tagged Krp1 protein, confirming the interaction. In addition to GST pull-down experiments, coimmunoprecipitation experiments were performed with FBR cell lysates from cells expressing Krp1-myc. Immunoprecipitation with a myc antibody revealed coimmunoprecipitation of endogenous Lasp-1 (Fig. 1D). The reciprocal coimmunoprecipitation with FBR cell lysate expressing HA-tagged Lasp-1 with an HA antibody revealed coimmunoprecipitation of endogenous Krp1 (Fig. 1E).

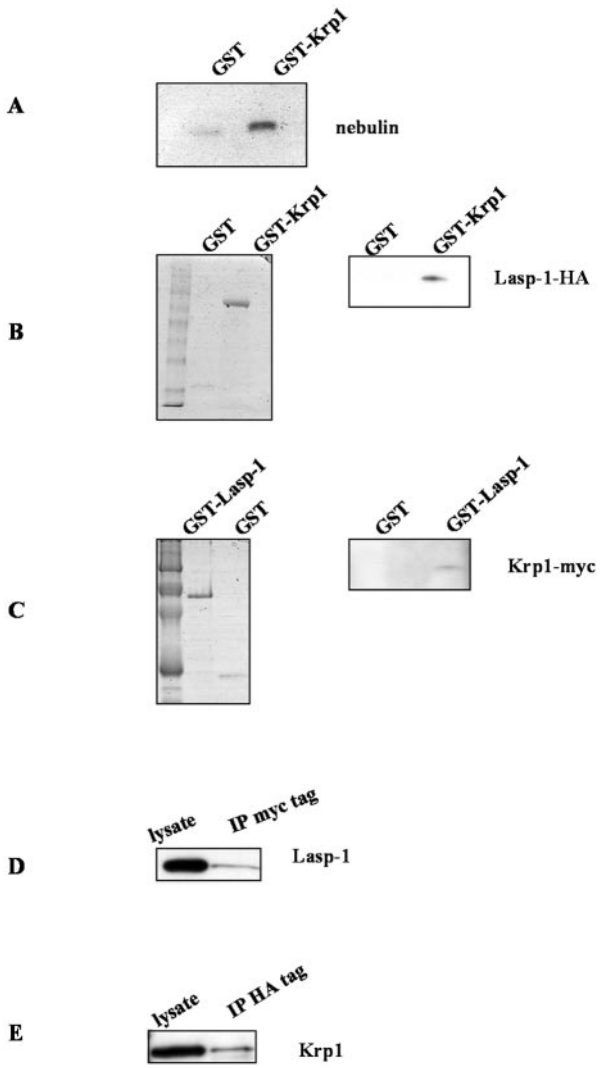


FIG. 1. Krp1 can interact with nebulin repeat structures present in nebulin and Lasp-1. (A) Nebulin repeats of nebulin were subjected to in vitro transcription/translation, and GST pull-downs were performed with GST alone and GST-Krp1. (B) COS-7 cells were transfected with a Lasp-1–HA construct and the total cell lysate was used in a GST pull-down experiment with GST and GST-Krp1. Shown is a Coomassie-stained gel of purified GST and GST-Krp1 proteins and the Western blot of the pull-down which was probed with an HA antibody. (C) The reciprocal GST pull-down was performed by transfecting COS-7 cells with a Krp1-myc construct and the pull-down was performed with GST and GST Lasp-1. Shown is a Coomassie-stained gel of purified GST and GST–Lasp-1 proteins and the Western blot of the pull-down which was probed with a myc antibody. (D and E) FBR cells were transfected with a myc-tagged Krp1 construct (D) and with a Lasp-1–HA construct (E), and the cell lysates were used in coimmunoprecipitation with an anti-c-*myc* agarose (D) and anti-HA agarose (E). Shown is the Western blot analysis, which was probed with anti-Lasp-1 (D) and anti-Krp1 (E). IP, immunoprecipitation.

Krp1 and Lasp1 colocalize to the tips of pseudopodia in FBR cells. To determine the localization of Lasp-1 in FBR cells and 208F cells, a Lasp-1–GFP construct was transiently transfected into both cell lines and visualized by confocal microscopy. In FBR cells, Lasp-1 has a cytoplasmic localization and accumulates at the tips of pseudopodia, where it colocalized with F-actin (Fig. 2A). In 208F cells, Lasp-1–GFP was found in focal adhesions, filopodia, and the cytoplasm (Fig. 2B). A similar localization was observed with an antibody to Lasp-1 (data not shown). To verify that Lasp-1 remains localized at the tips of extending pseudopodia during 3D migration through extracellular matrix, we performed invasion assays with FBR cells transiently expressing Lasp-1–GFP. The invasion assay was allowed to progress for 24 h, and then, using the

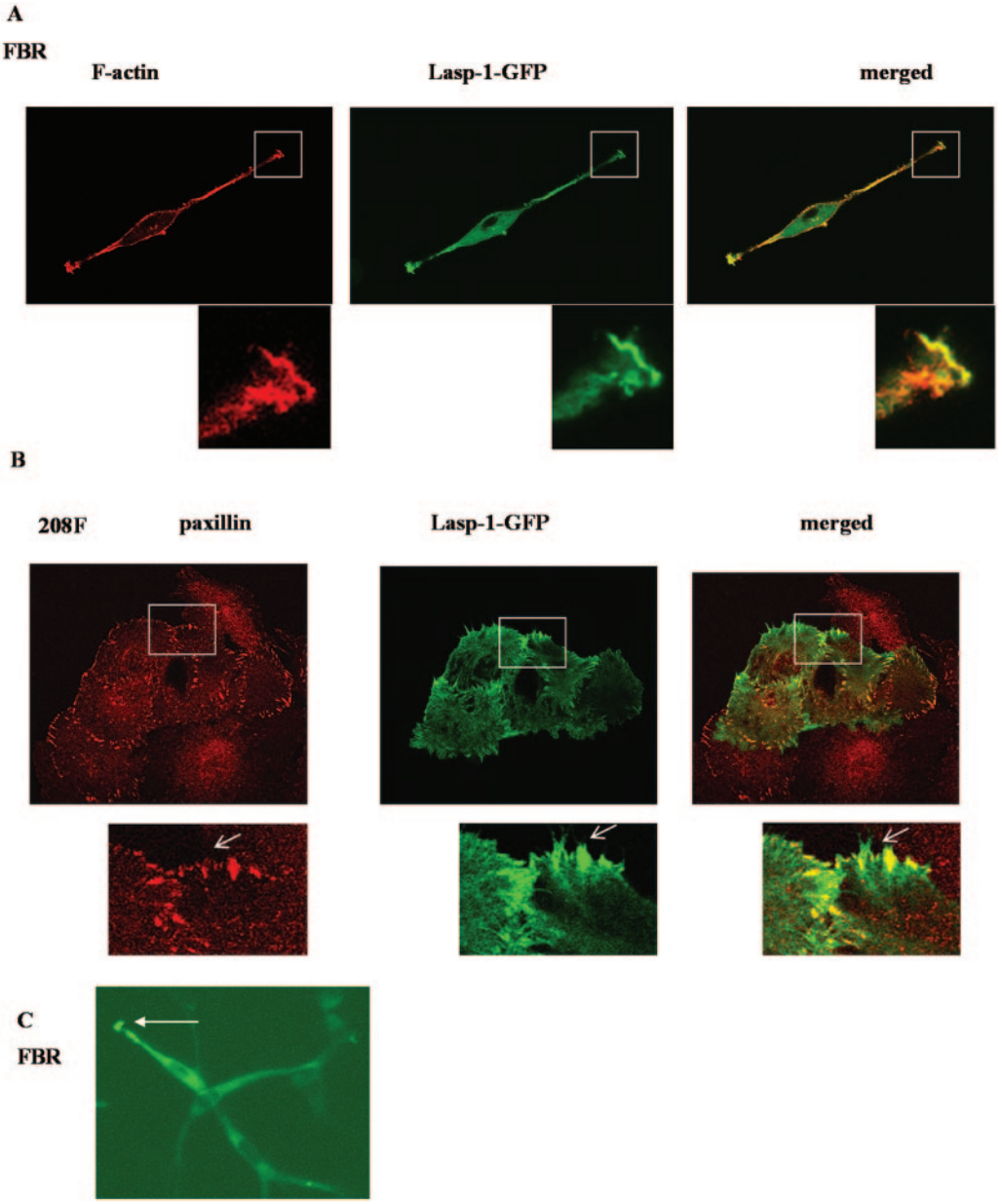


FIG. 2. Localization of Lasp-1 in FBR and 208F cells. FBR (A) and 208F (B) cells were transiently transfected with a Lasp-1–GFP construct. (A) Confocal microscopy with Lasp-1–GFP and TRITC-phalloidin, to visualize F-actin. Boxed area shows the region that was enlarged to highlight the tips of the pseudopodia. (B) Confocal microscopy with Lasp-1–GFP and antipaxillin (red). Boxed area shows the region that was enlarged to highlight the localization of Lasp-1–GFP in focal adhesions and filopodia. (C) FBR cells transiently transfected with Lasp-1–GFP were incubated in a 3D invasion assay for 24 h; the figure shows the localization of Lasp-1–GFP after 24 h. Arrow highlights the accumulation of Lasp-1–GFP at the tips of pseudopodia.

confocal microscope, we captured z-sections of cells invading. Lasp-1 has a cytoplasmic localization and also accumulates at the tips of pseudopodia as the cells invade (Fig. 2C) (see Movie S1 in the supplemental material).

To verify that Lasp-1 and Krp1 colocalize at the tips of pseudopodia, Lasp-1–GFP was transiently expressed in FBR cells and Krp1 was detected with Krp1-specific antisera. Both Lasp-1 and Krp1 have a cytoplasmic localization and accumulate at the tips of pseudopodia (Fig. 3A). Pretreatment of the FBR cells with 0.02% Triton X-100 before fixation, which extracts cytoplasmic proteins apart from the cell cytoskeleton and cytoskeleton-interacting proteins, revealed that Krp1 and Lasp-1 colocalized with F-actin at the tips of pseudopodia (Fig. 3B).

Lasp-1 has a role in pseudopodial elongation. Expression of dominant-negative Krp1 constructs comprised of BTB/POZ domain or the kelch repeats alone in FBR cells resulted in shortened pseudopodia (53). To determine whether Lasp-1 had a similar role in pseudopodial elongation, a Lasp-1 SH3 deletion mutant (Lasp-1ΔSH3) was transiently expressed in

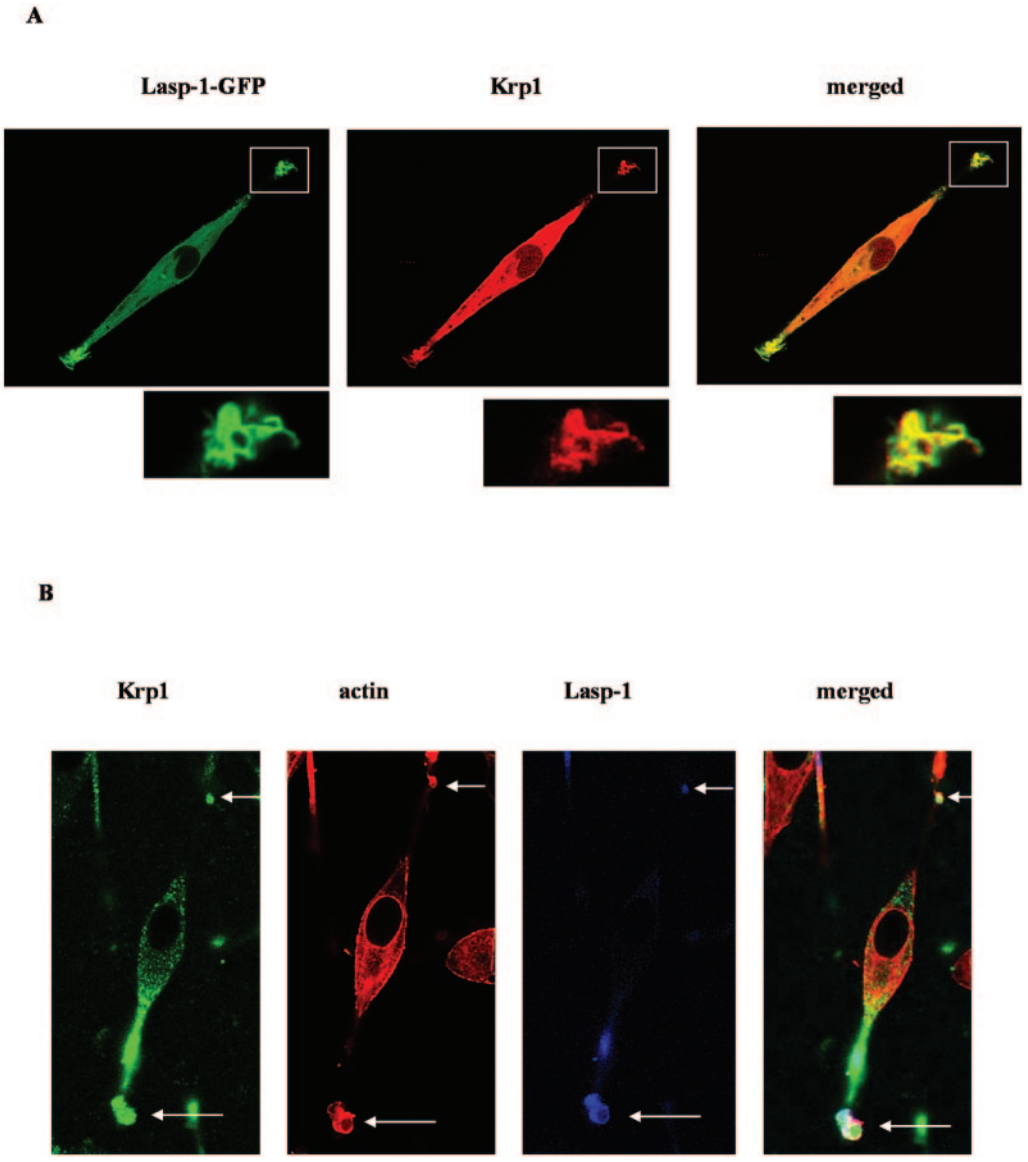


FIG. 3. Krp1 and Lasp-1 colocalize at the actin-rich membrane rufflelike structures at the tips of pseudopodia. (A) FBR cells were transiently transfected with Lasp-1–GFP. Shown are the confocal microscopy images with Lasp-1–GFP and anti-Krp1 (red). The boxed area shows the region that was enlarged to highlight the tips of pseudopodia. (B) Cells were permeablized in 0.02% Triton X-100 for 1 min and then fixed. (B) Confocal microscopy with anti-Krp1 (Green), TRITC-phalloidin, and anti-Lasp-1 (blue). Arrows highlight the tips of pseudopodia.

FBR cells (Fig. 4A and B). Lasp-1ΔSH3 expression resulted in truncated pseudopodia, suggesting that, like Krp1, Lasp-1 has a role in pseudopodial elongation. Although the pseudopodia were truncated, in the presence of Lasp-1ΔSH3, Krp1 and Lasp-1ΔSH3 remained colocalized with F-actin-rich structures at the tips of shortened pseudopodia (Fig. 4A). These data suggest that the SH3 domain of Lasp-1 plays a major role in pseudopodial elongation.

Lasp-1 has a role in the invasion of FBR cells. The mesenchymal mode of invasion is characterized by the extension of long pseudopodia into the extracellular matrix during invasion. Since FBR cells expressing Lasp-1ΔSH3 cannot extend their pseudopodia, in vitro invasion assays were performed on FBR cells transiently expressing Krp1, Lasp-1, Lasp-1ΔSH3, and GFP. The cells expressing Lasp-1ΔSH3 were significantly less invasive (Fig. 4C). These data demonstrate that Lasp-1 has an important role in the invasion of FBR cells. Although the FBR cells expressing Lasp-1ΔSH3 were less invasive, the cells were still motile as they moved through the filter as efficiently as cells expressing GFP, Krp1, and Lasp-1 (data not shown).

siRNAs to Krp1 and Lasp-1 verify that these proteins have a role in pseudopodial elongation. To verify the role of Krp1 and Lasp-1 in pseudopodial elongation, siRNA experiments were performed. Using siRNAs specific to Krp1 and Lasp-1, we were able to see a reduction in both endogenous proteins by Western blotting and confocal microscopy (Fig. 5A and C). SiRNAs to Krp1 and Lasp-1 resulted in shortening of the pseudopodia, while the control nonspecific siRNA did not

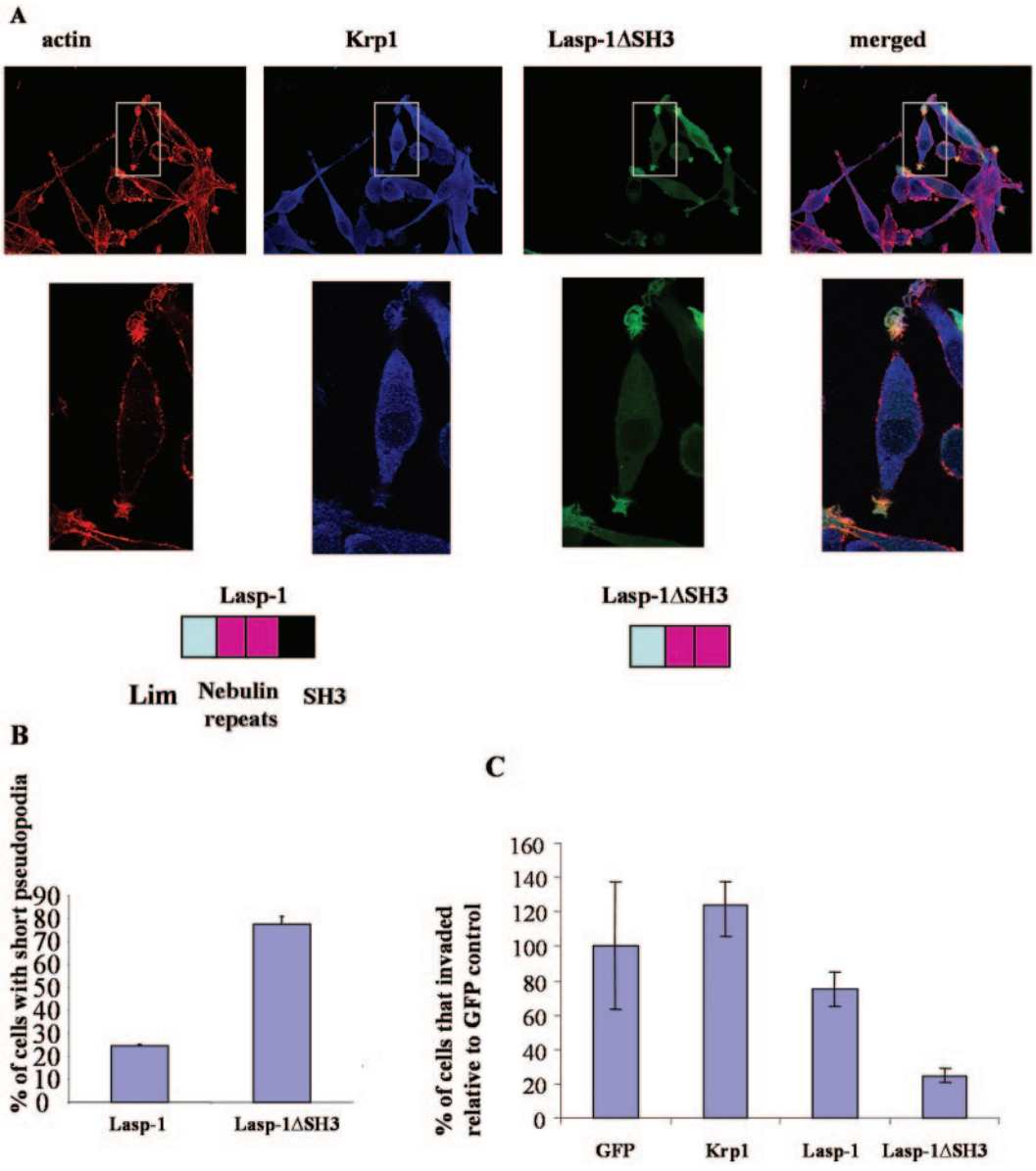


FIG. 4. Expression of the dominant-negative Lasp-1 construct results in truncated pseudopodia and a decrease in the invasiveness of FBR cells in a 3D invasion assay. (A) FBR cells were transfected with Lasp-1ΔSH3–GFP construct; shown are confocal microscopy images with TRITC-phalloidin, anti-Krp1 (blue), and Lasp-1–GFP (green). The boxed region shows the area that was enlarged. (B) Quantitative analysis showing the percentage of transiently transfected cells that show a reduction in pseudopodium length. The experiment was repeated on three occasions. (C) FBR cells were transiently transfected with the following constructs: GFP, Krp1, Lasp-1, and Lasp-1ΔSH3 domain. The cells were allowed to invade in an in vitro 3D invasion assay for 3 days. Shown in the bar chart is the percentage of cells that have invaded. The experiment was repeated on three separate occasions.

cause a decrease in pseudopodium length (Fig. 5B and C). These results confirm the role of Krp1 and Lasp-1 in pseudopodial elongation.

Krp1 and Lasp-1 dissociate when FBR cells revert to an untransformed morphology, as a result of exposing the cells to FN. To dissect the signaling pathways that result in the localization of Krp1 and Lasp-1 at the tips of pseudopodia and allow FBR cells to invade, we used a system where we reverted the FBR cells to an untransformed morphology. This was achieved by reintroducing FBR cells to FN, which we have shown to be down-regulated in these cells (39). FN has been shown to be down-regulated in a variety of oncogene-transformed cells, and exposure of those cells to FN results in a morphological reversion (2, 3, 8, 15, 24, 38, 43). FBR cells were grown for 3 days on FN-coated coverslips, which resulted in a morphological reversion of the bipolar spindle-like morphology to that of 208F cells, with an increase in focal adhesions and actin stress fibers (Fig. 6A). The cells no longer move by extending long pseudopodia, but instead extend broad lamellipodia (see Movie S2 in the supplemental material). These changes are thought to be due to the engagement of integrins by FN that activates Rho-ROCK signaling which is necessary

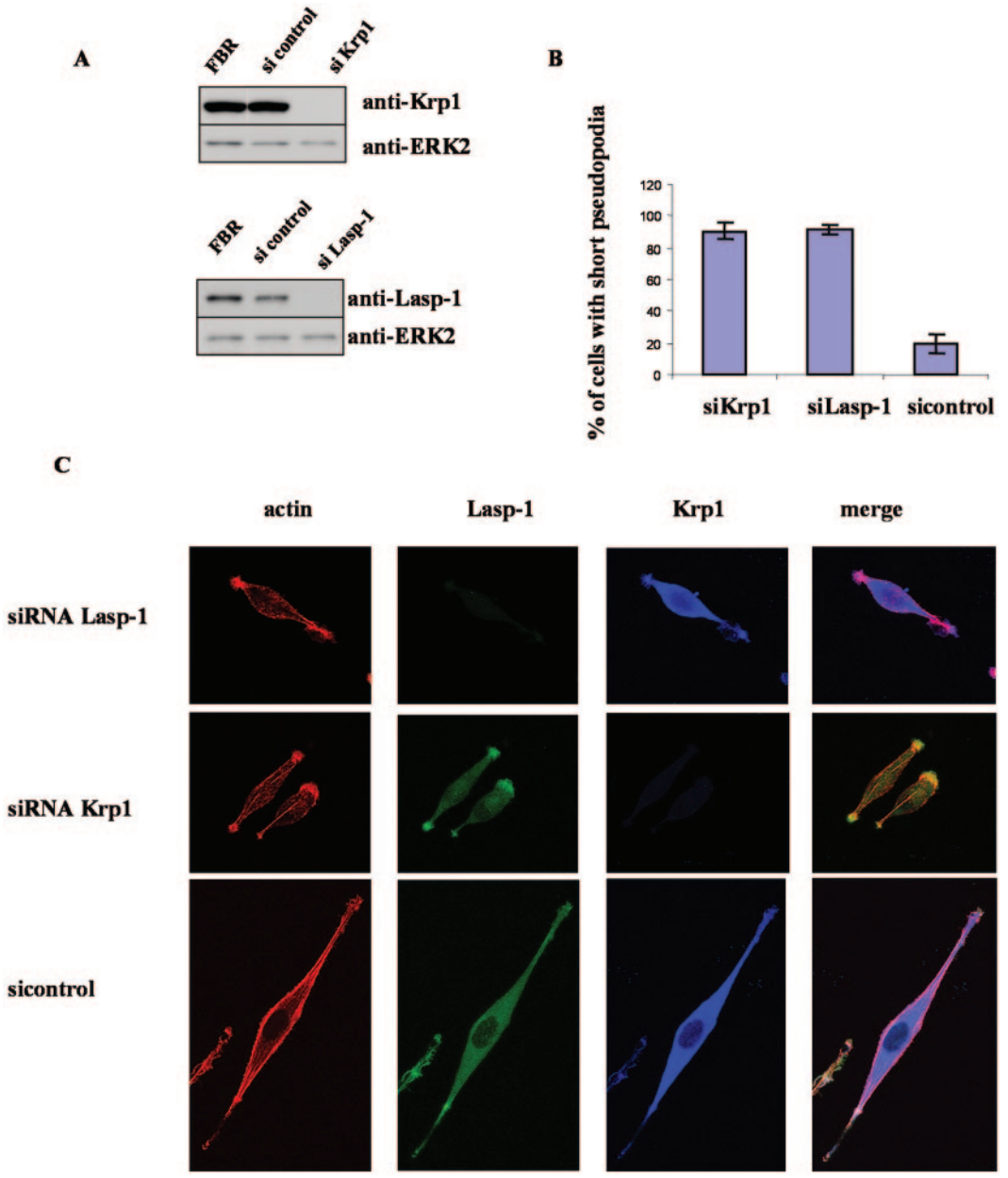


FIG. 5. siRNAs to Krp1 and Lasp-1 result in truncated pseudopodia. (A) Western blot analysis of FBR cells subjected to si control, siKrp1, and siLasp-1. (B) Quantitative analysis of the number of cells showing short pseudopodia when transfected with siKrp1, siLasp-1, and si control. (C) Cells were transfected with siKrp1, siLasp-1, and si control, and confocal microscopy was performed with TRITC-phalloidin, anti-Lasp-1 (green), and anti-Krp1 (blue).

for the subsequent actin rearrangements (42). This suggests that the FBR cells suppress integrin activation and subsequent stress fiber formation by down-regulating FN expression. It is known that integrin activation stimulates autophosphorylation of focal adhesion kinase (FAK), followed by activation of Rho and ROCK and subsequent formation of stress fibers and focal adhesions (10, 64). To determine the localization of FAK when FBR cells are plated on FN, we performed transient transfections of yellow fluorescent protein (YFP)-FAK into FBR cells plated on uncoated and FN-coated coverslips. YFP-FAK had a cytoplasmic localization with an accumulation at the ends of pseudopodia when the FBR cells were plated on uncoated coverslips, while on FN, it localized to focal adhesions (Fig. 6B). These data confirm that when FBR cells are plated on FN, the cells form focal adhesions. We then determined the localization of Krp1 and Lasp-1 in FBR cells cultured on FN. Krp1 is excluded from membrane sites of dynamic actin rearrangements and no longer colocalizes with Lasp-1 or F-actin, while Lasp-1 localizes to membrane ruffles and focal adhesions (Fig. 6C) (see Movie S2 in the supplemental material). Therefore, the colocalization of Krp1 and Lasp-1 at actin-rich structures is disrupted when the cells are plated on FN. To verify these

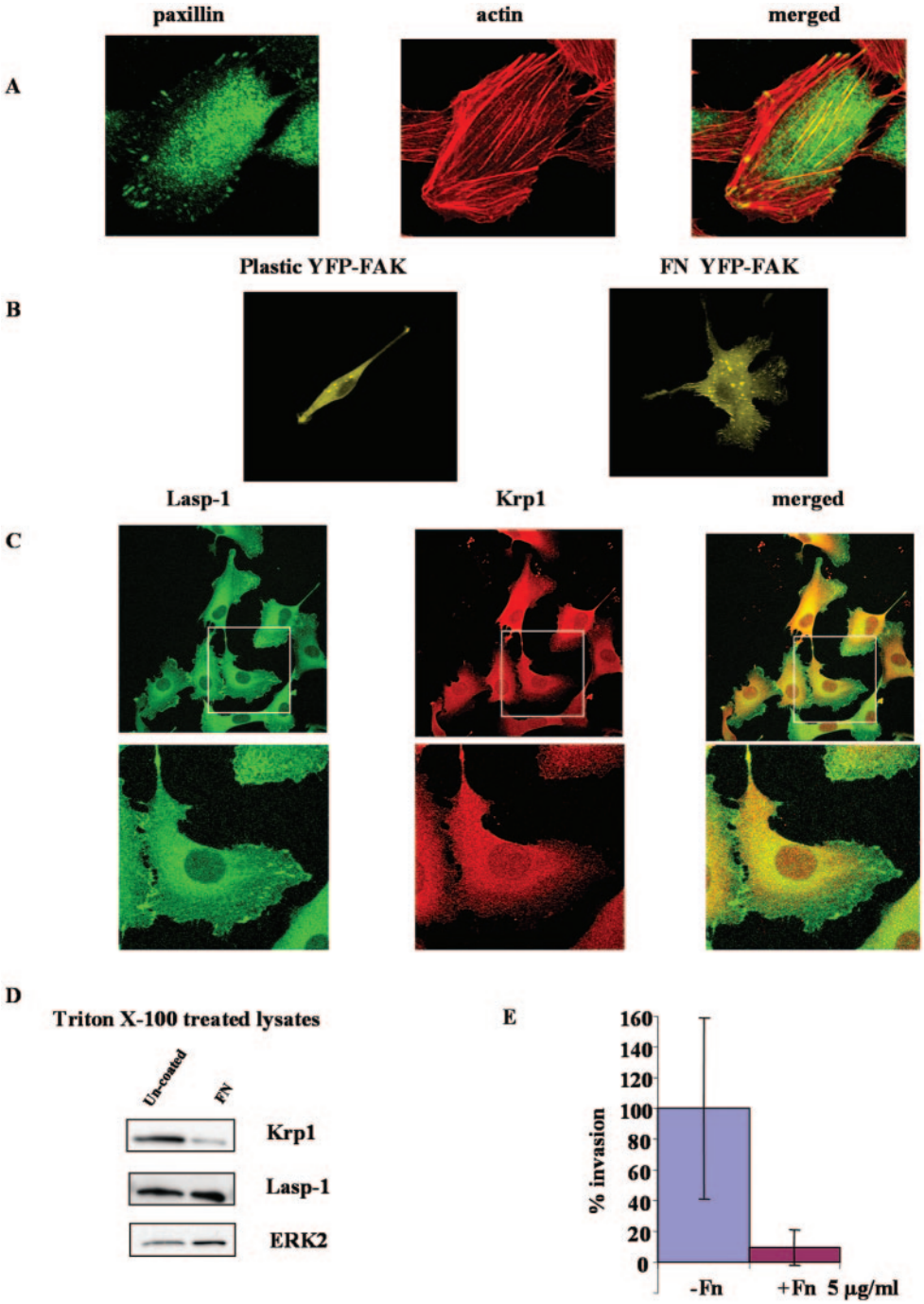


FIG. 6. Plating FBR cells on FN results in a loss of pseudopodium formation and the dissociation of Krp1 and Lasp-1. In addition, the presence of FN in a 3D invasion assay reduces the invasiveness of the cells. (A) FBR cells were plated on FN for 3 days; shown are confocal microscopy images with antipaxillin (green) and TRITC-phalloidin. (B) Confocal microscopy images of FBR cells grown on uncoated or FN-coated coverslips for 2 days and then transiently transfected with YFP-FAK. (C) FBR cells were plated on FN for 3 days, and shown are the confocal microscopy images of anti-Lasp-1 (green) and anti-Krp1 (red). The boxed region represents the cell which was enlarged. (D) Western blot analyses of FBR cells grown on uncoated and FN-coated coverslips that were subjected to treatment with 0.02% Triton X-100 before lysis. The Western blots were probed with anti-Krp1, anti-Lasp-1, and anti-extracellular regulated kinase 2 (anti-ERK2) as a loading control. (E) In vitro 3D invasion assay was performed with FBR cells with and without FN in the matrigel. Shown are the percentages of cells that invaded with and without FN in the matrigel. −Fn, without fibronectin, +Fn, with fibronectin.

findings biochemically, FBR cells were grown on uncoated and FN-coated tissue culture plates and then subjected to 0.02% Triton X-100 for 1 min to release cytoplasmic proteins apart from the cell cytoskeleton and cytoskeleton-interacting proteins. Lysates were then prepared and Western blotting was performed, and the blots were probed with antibodies to Krp1 and Lasp-1 (Fig. 6D). Both Krp1 and Lasp-1 were found to be present on the cells grown on uncoated plates, but there was a

significant reduction in the amount of Krp1 present when the cells were grown on FN. These data confirm that Lasp-1 remains associated with the actin cytoskeleton when the cells are plated on FN, while there is a reduction in the amount of Krp1 that remains associated with the actin cytoskeleton when the cells are grown on FN compared to uncoated plates. These data suggest that we can use this system to dissect the signaling pathways which allow the colocalization of Krp1 and Lasp-1 at the tips of pseudopodia in invasive cells.

FBR cells invade by extending long pseudopodia. Here, we have shown that when FBR cells are grown on FN, they no longer produce pseudopodia. To determine whether FN blocks the invasion of FBR cells, we performed invasion assays with matrigel spiked with FN. The invasiveness of the FBR cells was decreased in the presence of FN-spiked matrigel (Fig. 6E). These data suggest that FBR cells require pseudopodia to invade.

Fibronectin stimulates integrin-mediated stress fiber formation. To determine whether the formation of stress fibers in FBR cells plated on FN is instigated by the engagement of integrins, we performed experiments with an RGD-containing peptide that competitively inhibits binding via $\alpha5\beta1$ or $\alpha v\beta3$ integrins (23). FBR cells were plated onto FN in the presence of the inhibitory peptide (GRGDS) and the noninhibitory peptide (GRADSP). After 40 min, confocal microscopy was performed with an antibody to paxillin and F-actin was detected with TRITC-phalloidin to determine whether cells had attached and spread to form stress fibers and focal adhesions (Fig. 7A). In the presence of either peptide, the cells had attached, but with the inhibitory RGD-containing peptide, the cells did not spread and there were few focal adhesions and stress fibers. To determine whether integrin signaling was necessary for the maintenance of stress fibers and focal adhesions, the peptides were added to cells that had been exposed to FN for 3 days (Fig. 7B). After 2 hours in the presence of the inhibitory peptide, stress fibers and focal adhesions were disrupted, while no effect was seen with the noninhibitory peptide (Fig. 7B). These experiments suggest that the interaction of the cell with FN is via integrins.

The formation of pseudopodia and the localization of Krp1 and Lasp-1 at the tips of pseudopodia are Rho independent. The effect of FN on FBR cells demonstrates that the consequences of integrin–Rho-ROCK signaling are as follows: the rearrangement of the actin cytoskeleton, the disruption of the colocalization of Krp1 with Lasp-1, and the loss of pseudopodia. This implies that pseudopodium formation is independent of Rho-ROCK signaling. To investigate this hypothesis, a dominant-negative mutant of Rho (RhoN19-myc) was transiently expressed in FBR cells plated on uncoated coverslips. No effect on pseudopodium formation was observed, suggesting that the localization of Krp1 and Lasp-1 at these structures is Rho independent (Fig. 8A). In addition, FBR cells grown on either uncoated or FN-coated coverslips were subjected to the Rho inhibitor TAT-C3 for 12 h. The inhibitor had no effect on the cells grown on the uncoated coverslips, but the cells on FN lost stress fibers and formed long pseudopodium-like protrusions, and Krp1 and Lasp-1 accumulated at the tips of pseudopodium-like structures (Fig. 8B). We then introduced constitutively active Rho (Rho V14) into FBR cells grown on uncoated coverslips, which resulted in the formation of stress fibers and the collapse of pseudopodia (Fig. 8C). Expression of Rho-GFP in FBR cells gave the same result (data not shown). To determine the localization of FAK in cells expressing active Rho, YFP-FAK was cotransfected with an active Rho construct, which resulted in YFP-FAK being recruited to focal adhesions (Fig. 8D). In addition, active Rho caused Krp1 to become totally cytoplasmic and Lasp-1 to move into focal adhesions and membrane ruffles (Fig. 8E). These data indicate that the formation of pseudopodia and the localization of Krp1 and Lasp-1 at the tips of pseudopodia are Rho independent.

The above data suggest that Rho-ROCK signaling is suppressed in FBR cells when grown on uncoated coverslips and is stimulated when the cells are exposed to FN-coated coverslips. However, the levels of activated Rho, as measured by GST-Rhotekin pull-down of Rho-GTP from cell extracts derived from FBR cells grown for 3 days on either uncoated or FN-coated tissue culture plates, were equal (Fig. 8F). This suggested that the difference in Rho-ROCK signaling was downstream of the formation of Rho-GTP. One possibility is that the levels of ROCK protein are suppressed in FBR cells, as occurs in Swiss 3T3 cells transformed by the Ras oncogene (50). However, Western blot analysis of cell extracts of FBR cells maintained for 3 days on either uncoated or FN-coated tissue culture plates revealed that the levels of ROCK1 and ROCK2 were equal (Fig. 8G). Another possibility is that there is insufficient FN-mediated integrin activation to allow the translation of Rho-GTP signals into rearrangements of the actin cytoskeleton, as is the case for Rac signaling, when normally adherent cells are grown in suspension and stimulated with growth factors (20). To determine whether there was a difference in the levels of phosphorylation of the downstream effectors of ROCK when the cells are plated in the absence or presence of FN, we measured the levels of phosphorylation on the actin-severing protein cofilin. Activated ROCK phosphorylates and activates LIM kinase that phosphorylates cofilin, thereby suppressing its F-actin severing activity to enhance the stability of F-actin. Western blot analysis of cell extracts from FBR cells maintained on uncoated or FN-coated tissue culture plates for 3 days revealed a significant increase in the level of phospho-cofilin in cells cultured on FN over that found in cells maintained on uncoated tissue culture plates (Fig. 8H). To verify that the increase in phospho-cofilin is a result of activation of the FN–integrin–Rho-ROCK signaling pathway, the cells were subjected to the ROCK inhibitor Y27632. In the presence of ROCK inhibitor there was less phospho-cofilin present on the cells grown on FN compared to cells on FN without the inhibitor, implying that it is the activation of the Rho-ROCK pathway that is responsible for the phosphorylation of cofilin. These data indicate that ROCK signaling in FBR cells is suppressed in the absence of FN, which allows pseudopodial elongation and invasion of these cells.

Pseudopodium formation and invasion of FBR cells is ROCK independent. To determine further the role of ROCK in the formation of stress fibers when the FBR cells are plated on FN, the ROCK inhibitor Y27632 was added to cells grown on uncoated and FN-coated coverslips. The inhibitor did not affect the actin cytoskeleton of cells grown on the uncoated coverslips (data not shown), but for cells grown on FN, the inhibitor disrupted the actin stress fibers and focal adhesions, resulting in the formation of long pseudopodia with Krp1 and

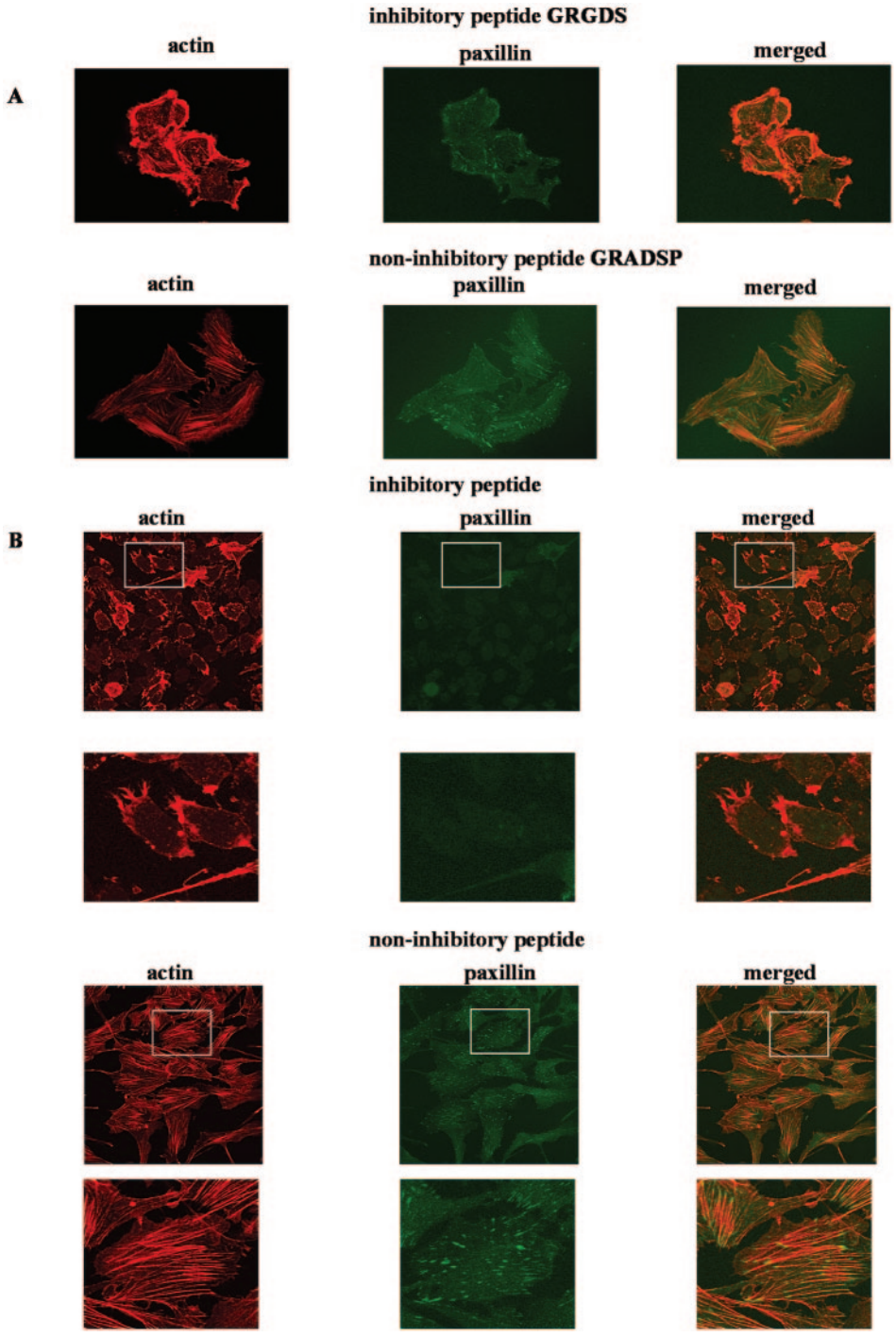
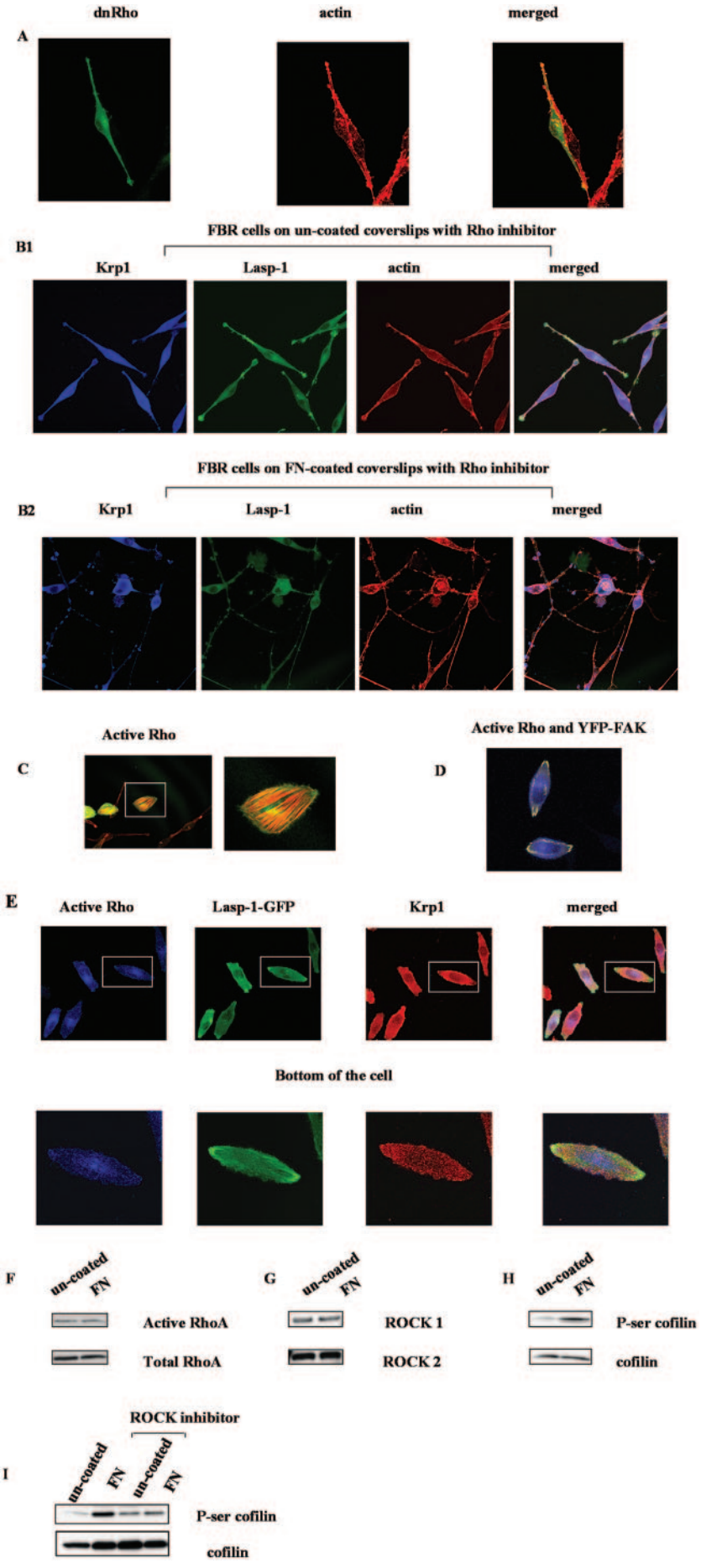


FIG. 7. RGD-containing inhibitory peptide blocks FBR cells spreading and stress fiber formation on FN. (A) FBR cells were plated onto FN-coated coverslips for 40 min in the presence of the inhibitory peptide (GRGDS) and the noninhibitory peptide (GRADSP); shown are the confocal microscopy images of antipaxillin (green) and TRITC-phalloidin (red). (B) FBR cells were plated onto FN-coated coverslips for 3 days and then subjected to either the GRGDS or the GRADSP peptides for 2 h; shown are the confocal microscopy images with antipaxillin (green) and TRITC-phalloidin (red). The boxed region represents the enlarged area.

Lasp-1 detected at their tips (Fig. 9A). In addition, stable FBR cell lines were derived, expressing either an active or a kinase-dead ROCK construct, both of which were fused to the estrogen receptor hormone-binding domain (16). Treatment of the FBR cells with 4-hydroxy-tamoxifen for 24 h resulted in the ROCK-estrogen receptor (ER)-expressing cells forming stress fibers and shortening their pseudopodia, while the kinase-dead ROCK-ER construct resulted in no stress fiber formation and pseudopodia that remained intact (Fig. 9B, C, and D). We then determined the localization of Krp1 and Lasp-1 in these cells.


dnRho
actin
merged
A
FBR cells on un-coated coverslips with Rho inhibitor
B1
Krp1
Lasp-1
actin
merged
FBR cells on FN-coated coverslips with Rho inhibitor
B2
Krp1
Lasp-1
actin
merged
Active Rho
C
Active Rho and YFP-FAK
D
E
Active Rho
Lasp-1-GFP
Krp1
merged
Bottom of the cell
F
un-coated
FN
Active RhoA
Total RhoA
G
un-coated
FN
ROCK 1
ROCK 2
H
un-coated
FN
P-ser cofilin
cofilin
ROCK inhibitor
I
un-coated
FN
un-coated
FN
P-ser cofilin
cofilin

In the ROCK-ER cells, both Krp1 and Lasp-1 displayed diffuse staining (Fig. 9C), while in the kinase-dead ROCK-ER construct, Krp1 and Lasp-1 remained colocalized at the tips of pseudopodia (data not shown). These data confirm that pseudopodium formation in FBR cells is ROCK independent. To determine the phosphorylation status of cofilin in the FBR cells expressing ROCK-ER and the kinase-dead ROCK-ER, Western blot analysis was performed on cell lysates from these cell lines after treatment with 4-hydroxy-tamoxifen for 24 h. There was an increase in the levels of phospho-cofilin in the cells expressing ROCK-ER compared to the kinase-dead ROCK-ER stable cell line (Fig. 9E).

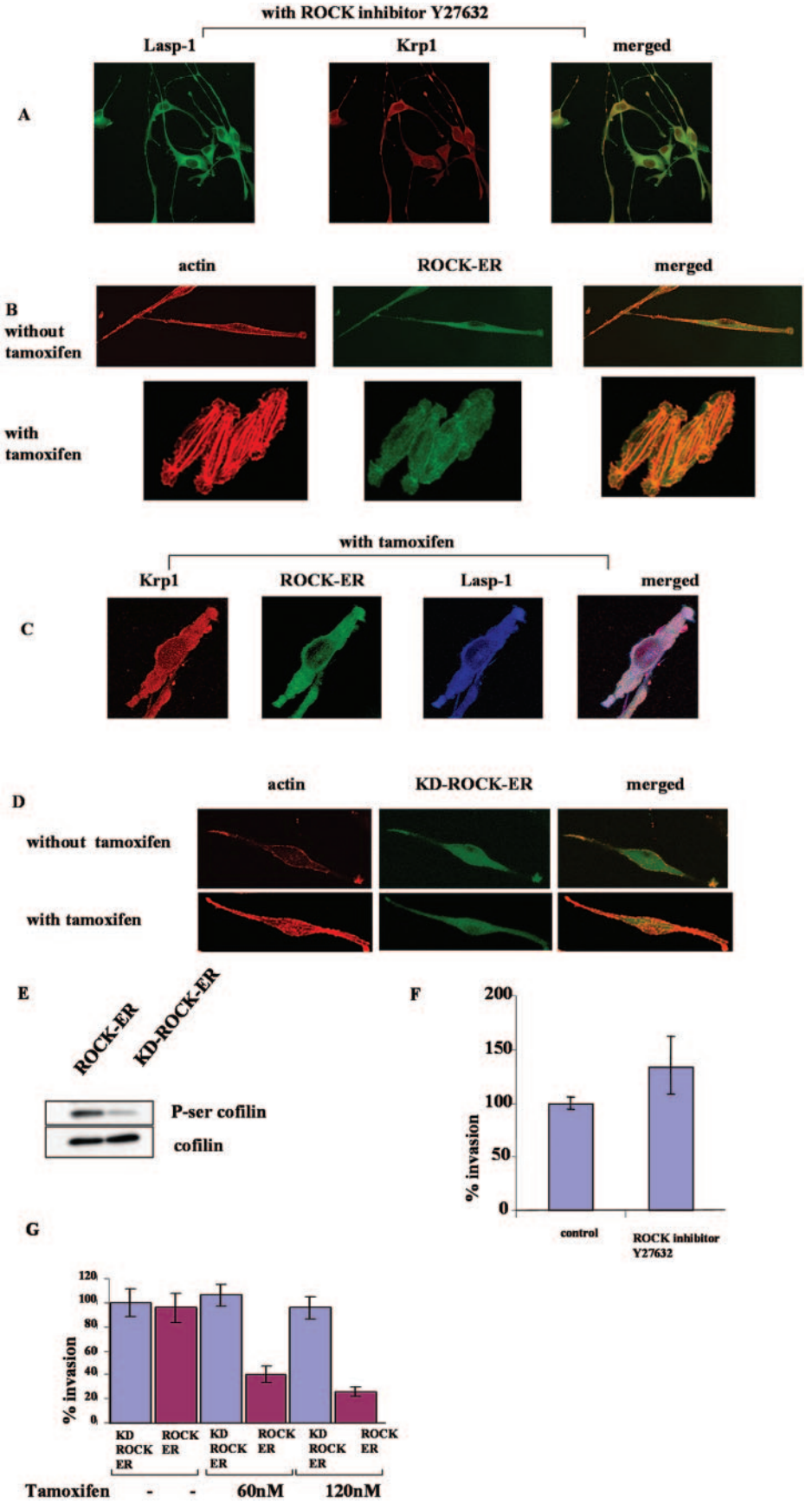
Moreover, to test whether FBR cell invasion was also independent of ROCK, invasion assays were performed in the presence or absence of the ROCK inhibitor Y27362. The presence of the inhibitor did not affect the invasiveness of FBR cells (Fig. 9F). To determine whether activation of ROCK could suppress invasion, assays were performed using stable FBR cell lines expressing either ROCK-ER or kinase-dead ROCK-ER, in the presence or absence of 4-hydroxy-tamoxifen. All the cells invaded in the absence of the drug. In the presence of the drug, invasion of ROCK-ER- but not kinase-dead ROCK-ER-expressing cells was inhibited (Fig. 9G). This confirms that FBR cells invade using a mesenchymal mode of migration.

DISCUSSION

Based upon the combination of gene expression profiling to identify genes differentially expressed as a consequence of the v-*fos* oncogene in both rat and human fibroblasts and functional analysis of differentially expressed genes, we proposed that AP-1 stimulates invasion by activating a multigenic invasion program, in which the up- and down-regulated genes represent effectors and suppressors, respectively, of invasion (22, 26, 30, 31, 37, 39, 52–54). The results presented here further support the concept of an invasion program by demonstrating that up- and down-regulated genes cooperate to enhance pseudopodial elongation and invasion, in part by altering the activity of proteins that are not differentially expressed. Krp1, a novel protein of unknown function, was first investigated in the context of v-Fos transformation, as a test for the concept that up-regulated genes are effectors of invasion (53). Increased expression of full-length Krp1 enhanced pseudopodial elongation in transformed cells, while expression of truncated forms of Krp1 functioned as dominant-negative mutants and inhibited pseudopodial elongation. Here, we confirmed the role of Krp1 in pseudopodial elongation by suppressing its expression with siRNA. The siRNA result further substantiates that Krp1, like another up-regulated protein, ezrin, enhances pseudopodial elongation (31).

The structure of Krp1 suggests that it functions as a scaffold protein (53). This was confirmed by the finding that it interacts with nebulin repeats within the actin binding proteins, N-Rap, and extended here to Lasp-1 and nebulin (36). The exact function of Lasp-1 is not known, but it has previously been reported to localize within multiple sites of dynamic actin assembly such as focal contacts, focal adhesions, lamellipodia, membrane ruffles, and pseudopodia. Its localization and function are regulated by phosphorylation on serine/threonine and tyrosine residues (11–13, 28, 34, 55). It has also been shown that the SH3 domain of Lasp-1 interacts with the extreme N terminus of the focal adhesion molecule zyxin and Ableson tyrosine kinase (33, 34). Here, we demonstrated that the transformation-specific interaction of Krp1 and Lasp-1 results in their colocalization with F-actin at the tips of extending pseudopodia. Inactivation of endogenous Lasp-1, either by a dominant-negative mutant, Lasp-1ΔSH3, or by siRNA, resulted in a reduction in the length of pseudopodia, mimicking similar experiments targeting Krp1. Thus, like Krp1, Lasp-1 has a role in pseudopodial elongation. In addition, Lasp-1ΔSH3 caused a reduction in the invasiveness of the FBR cells. Thus, we have proposed that the novel interaction of Krp1 and Lasp-1 is required for pseudopodial elongation, which is necessary for invasion. The inability of the cells expressing Lasp-1ΔSH3 to extend pseudopodia and to invade highlights the importance of the SH3 domain in pseudopodial elongation and invasion. The continued localization of Lasp-1ΔSH3 to membrane sites, where there is dynamic actin rearrangement in FBR cells, is consistent with its localization to membrane ruffles in NIH 3T3 cells (34). It remains to be determined what role the SH3 domain of Lasp-1 plays in invasion and whether there are other binding partners for this SH3 domain that are important for pseudopodial elongation and invasion. Of the two proteins that have been shown to bind to the SH3 domain of Lasp-1, zyxin would appear to be the most likely candidate for involvement in pseudopodial elongation and invasion. c-Abl kinase is associated with the loss of Lasp-1 from focal adhesions and the initiation of cell death, while zyxin is associated with highly dynamic structures at the

FIG. 8. The association of Krp1 and Lasp-1 and pseudopodium formation are Rho independent. FBR cells were grown on uncoated coverslips (A, B1, C, D, and E) and on FN-coated coverslips (B2). (A) Cells were transiently transfected with dominant-negative Rho (RhoN19-myc); shown are the confocal microscopy images with anti-myc (green) and TRITC-phalloidin (red). (B1 and 2) Cells were subjected to the Rho inhibitor C3 for 12 h; shown are the confocal microscopy images with anti-Krp1 (blue), anti-Lasp-1 (green), and TRITC-phalloidin. Cells grown on FN-coated coverslips without Rho inhibitor are shown in Fig. 6A and C. (C) Cells were transiently transfected with active Rho (RhoV14-myc) and shown are the confocal microscopy images with anti-myc (green) and TRITC-phalloidin. The boxed region represents the cell which was enlarged. Only the merged image is shown. (D) Cells were transiently transfected with active Rho (RhoV14-myc) and YFP-FAK; confocal microscopy images are shown with anti-myc (blue) and YFP-FAK (yellow). Only the merged image is shown. (E) Cells were transiently transfected with active Rho (RhoV14-myc) and Lasp-1–GFP; shown are the confocal microscopy images of anti-myc (blue), Lasp-1–GFP (green), and anti-Krp1 (red). The boxed region shows the cell that was enlarged. (F) GST-rhotekin pull-down assays were performed with lysates from FBR cells plated on uncoated and FN-coated tissue culture plates. Also shown is the input of Rho from each assay. Western blots were probed with anti-Rho A. (G and H) Western blot analysis of FBR cell lysates isolated from plating cells on FN and uncoated tissue culture plates. Western blots were probed with antibodies to ROCK 1 and 2 (G) and phospho-Ser cofilin (H). (I) FBR cells were grown on FN and uncoated coverslips with and without 10 μM of the ROCK inhibitor Y27632. Lysates were used for Western blotting; shown is the Western blot probed with anticofilin and anti-phospho-Ser cofilin.


with ROCK inhibitor Y27632
Lasp-1
Krp1
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actin
ROCK-ER
merged
B
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Lasp-1
merged
C
actin
KD-ROCK-ER
merged
D
without tamoxifen
with tamoxifen
E
ROCK-ER
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P-ser cofilin
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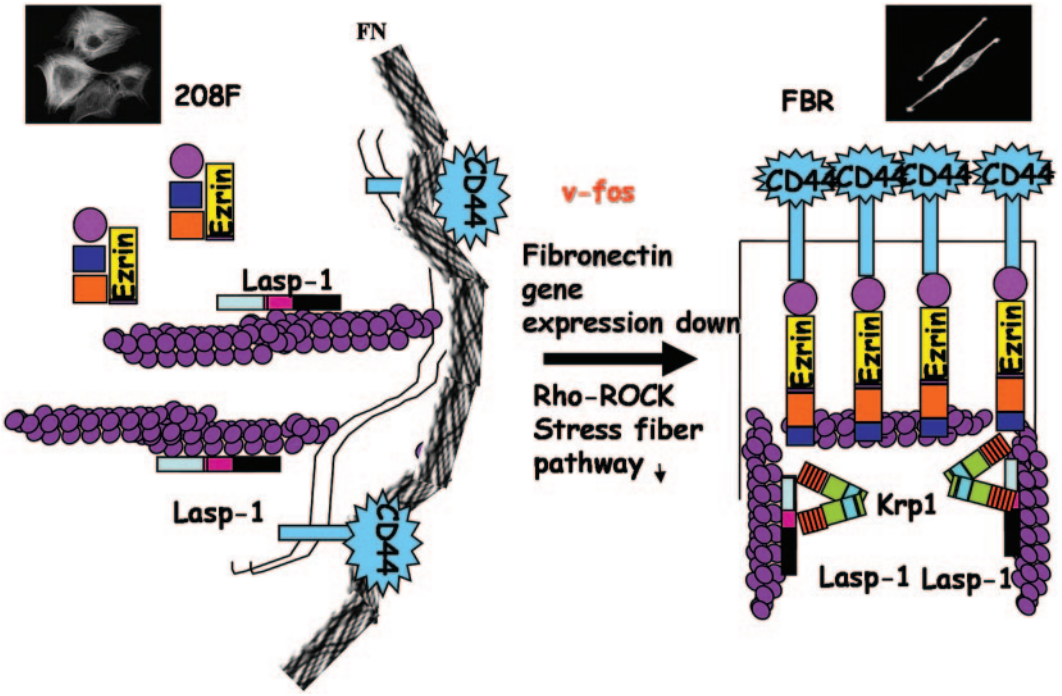

FIG. 10. Model showing how AP-1 mediates a multigenic invasion program.

leading edge of cells in which ROCK has been inhibited. In these cells, the zyxin-containing focal contacts at the leading edge are transient and do not develop into mature focal adhesions. In addition, inhibition of ROCK activity enhances membrane protrusion and increases the rate and persistence of motility (56). This is consistent with the low level of Rho-ROCK signaling in FBR cells as evidenced by the low level of phospho-cofilin, lack of focal adhesions and F-actin stress fibers, and increased rate and persistence of motility of FBR cells compared here to FBR cells maintained on FN or to their parental 208F cells.

The transformation-dependent localization of Krp1 to sites of membrane actin protrusions was confirmed by its relocalization in FBR cells on FN, which induced the reversion to their untransformed morphology. FN not only induced the loss of pseudopodia, formation of F-actin stress fibers, and focal adhesion, it also induced the dissociation of Krp1 and Lasp-1 and inhibited invasion. It is notable that Lasp-1 returns to its normal localization as found in 208F fibroblasts. These results strongly indicate that the formation and elongation of pseudopodia in FBR cells is dependent on the suspension of integrin signaling through the down-regulation of FN and that the interaction of Krp1 with Lasp-1 is inhibited by that signaling.

FN signaling into the cell is mediated by integrins and results in the formation of focal adhesions and F-actin stress fibers that require Rho-ROCK signaling (6, 14). We used the RGD peptide to demonstrate that integrins are involved in the spreading of FBR cells on FN (23). The RGD peptide blocks spreading of FBR cells on FN through decreased focal adhesion and F-actin stress fiber formation. It also disrupts FN-induced focal adhesions and F-actin stress fibers in FBR cells that had been exposed to FN for 3 days. This indicates that the means by which FBR cells avoid the formation of focal adhesions and stress fiber formation is the dramatic down-regulation of FN that reduces outside-in integrin signaling. The down-regulation of FN also renders the cells independent of RhoA and ROCK activity for the organization of the actin cytoskeleton, bipolar spindle-like morphology, and 2D and 3D migration/invasion. Cell migration continues when the Rho pathway is inhibited when the cells are plated on uncoated coverslips or during invasion. This suggests that an alternative pathway to RhoA- and ROCK-mediated contractility is em-

FIG. 9. Pseudopodium formation and invasion of FBR cells is ROCK independent. (A) FBR cells were grown on FN-coated coverslips for 3 days and then treated with the ROCK inhibitor Y27632 for 6 h; shown are the confocal microscopy images with anti-Lasp-1 (green) and anti-Krp1 (red). Cells grown on FN-coated coverslips without ROCK inhibitor are shown in Fig. 6A and C. Stable FBR cell lines expressing ROCK-ER–GFP (B and C) and kinase-dead ROCK-ER–GFP (D) were either treated with 4-hydroxytamoxifen for 24 h or were untreated. (B and D) Shown are the confocal microscopy images with and without tamoxifen with TRITC-phalloidin and ROCK-ER–GFP. (C) Confocal microscopy images with anti-Krp1 (red), ROCK-ER–GFP, and anti-Lasp-1 (blue). (E) Western blot analyses of cell lysates of stable FBR cell lines expressing ROCK-ER and kinase-dead ROCK-ER cells after tamoxifen treatment. Western blots were probed with anticofilin and anti-phospho-Ser cofilin. (F) In vitro 3D invasion assay was performed in the presence of ROCK inhibitor Y27632. The bar graph shows the percentages of cells that invaded in the absence and presence of Y27632. (G) In vitro 3D invasion assays were performed with stable FBR cells lines expressing ROCK-ER and kinase-dead ROCK-ER constructs. Shown are the percentages of cells that invaded after the induction of the constructs with various concentrations of tamoxifen.

ployed by FBR cells. In tumor-derived cells that use the mesenchymal/elongated Rho-ROCK-independent mode of migration, contractility is mainly generated by CDC42-MRCK signaling (60). This also appears to be the case for FBR cells when maintained on plastic.

We have demonstrated that the FBR bipolar spindle-like morphology with elongated pseudopodia is independent of Rho-ROCK signaling, as the Rho inhibitor TAT-C3 or the ROCK inhibitor Y27632 had no effect on cell morphology, the actin cytoskeleton, or pseudopodia. Nor did inhibition of ROCK have any effect on the invasiveness of FBR cells, clearly demonstrating that they invade by using the mesenchymal/elongated mode of invasion that is characterized as being independent of ROCK (49, 61). This is substantiated by the morphology of the invading cells that display elongated pseudopodia with dynamic ruffling structures at their tips, where Krp1 and Lasp-1 colocalize. Expression of active RhoA or activation of ROCK in FBR cells inhibits pseudopodial elongation and stimulates stress fiber and focal adhesion formation but fails to induce cell spreading. This suggests that FN signaling is more complex than the simple activation of Rho-ROCK signaling by outside-in integrin signaling.

Down-regulation of FN has been demonstrated in several types of oncogene-transformed cells and in some tumor-derived cells (2, 3, 8, 15, 24, 38, 43). In cells that have down-regulated FN, exposing them to FN results in a morphological reversion similar to that observed for FBR cells. The analysis of the response of FBR cells to FN demonstrates that the down-regulation of FN is a means of suppressing integrin-mediated activation of Rho-ROCK signaling, leading to focal adhesions and stress fiber formation that would suppress mesenchymal/elongated invasion. The increase in phospho-cofilin when FBR cells are maintained on FN is evidence of an increase in Rho-ROCK signaling leading to focal adhesion and stress fiber formation and is supported by the increase in phospho-cofilin upon tamoxifen stimulation of ROCK-ER expression. Down-regulation of FN is by no means the only mechanism used by tumor cells to suppress Rho-ROCK signaling to facilitate mesenchymal/elongated invasion. In Ras-transformed Swiss-3T3 cells, this is accomplished by promoting the degradation of ROCK (50).

The results presented here support the concept of an AP-1-mediated invasion program in which up-regulated genes, such as that for Krp1, encode proteins that enhance invasion by altering the activity of nondifferentially expressed proteins, such as Lasp-1. The down-regulation of genes that represent invasion suppressors such as FN also enhances invasion, by altering the activity of proteins such as RhoA and ROCK to allow pseudopodial elongation and the association of Krp1 with Lasp-1 that is essential for invasion/3D migration as outlined in the model presented (Fig. 10).

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