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EphrinA1 Activates a Src/Focal Adhesion Kinase-mediated Motility Response Leading to Rho-dependent Actino/Myosin Contractility*

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Matteo Parri[‡], Francesca Buricchi[‡], Elisa Giannoni[‡], Giovanna Grimaldi[‡], Tommaso Mello[§], Giovanni Raugei[‡¶], Giampietro Ramponi[‡¶], and Paola Chiarugi[‡¶1]

From the [‡]Department of Biochemical Sciences, University of Florence, Viale Morgagni 50, 50134 Florence, Italy, [¶]Center for Research, Transfer, and High Education Viale Morgagni 50, 50134 Florence, Italy, and [§]Farmacogenomic Foundation FiorGen, Polo Scientifico Sesto Fiorentino, 50019 Florence, Italy

Eph receptors and ephrin ligands are widely expressed in epithelial cells and mediate cell repulsive motility through heterotypic cell-cell interactions. Several Ephs, including EphA2, are greatly overexpressed in certain tumors, in correlation with poor prognosis and high vascularity in cancer tissues. The ability of several Eph receptors to regulate cell migration and invasion likely contribute to tumor progression and metastasis. We report here that in prostatic carcinoma cells ephrinA1 elicits a repulsive response that is executed through a Rho-dependent actino/myosin contractility activation, ultimately leading to retraction of the cell body. This appears to occur through assembly of an EphA2-associated complex involving the two kinases Src and focal adhesion kinase (FAK). EphrinA1-mediated repulsion leads to the selective phosphorylation of Tyr-576/577 of FAK, enhancing FAK kinase activity. The repulsive response elicited by ephrinA1 in prostatic carcinoma cells is mainly driven by a Rho-mediated phosphorylation of myosin light chain II, in which Src and FAK activation are required steps. Consequently, Src and FAK are upstream regulators of the overall response induced by ephrinA1/EphA2, instructing cells to retract the cell body and to move away, probably facilitating dissemination and tissue invasion of ephrin-sensitive carcinomas.

Eph receptors are the largest subfamily of receptor-tyrosine kinases and are involved in many biological processes including angiogenesis, tissue-border formation, cell migration, axon guidance, and synaptic plasticity. There are 16 known Eph receptors that are divided into EphA and EphB subfamilies according to sequence similarity and ligand binding specificity. The EphA subfamily binds to glycosylphosphatidylinositol-anchored ligands (ephrinA), whereas EphB receptors interact with ligands that have transmembrane domains (ephrinB) (1). Once bound to their ligand, Ephs become phosphorylated on multiple tyrosine residues. This leads to activation of the catalytic activity of the receptor itself and the formation of docking sites for downstream molecules that regulate signaling. Ephrin/Eph interaction is mediated by cell-to-cell contact and propagates through bidirectional signaling. In general, Eph/ephrins transduce a repulsive motile response that requires removal of the receptor ligand complexes from the cell surface by proteolytic cleavage or endocytosis after interaction and adhesion between ephrins and Eph receptors (2–4).

As well as their physiological role, many ephrins and Eph receptors are involved in carcinogenesis. This is indicated by their up-regulation in many tumors and especially in the more aggressive stages of tumor progression (5). EphA2 is up-regulated in breast, liver, and prostate cancer, and strong correlation has been reported with poor prognosis (6–8). In particular, ectopic overexpression of EphA2 gives non-transformed epithelial cells both tumorigenic and metastatic potential (9). In addition, certain Ephs and their ligands are expressed and up-regulated at sites of active neovascularization, *e.g.* in endothelial cells during tumor invasion (10, 11).

Beside being involved in tumor aggressiveness and vascularization, ephrin-Eph interactions have recently been implicated in the coordination of cell migration during several physiological and pathophysiological processes, including development and tissue morphogenesis (12–14) and melanoma cell migration (15). Although the molecular events supporting Eph influence on cell motility are not completely understood, these ligands are known to impair cell attachment to extracellular matrix (ECM)[2] by down-regulating integrin activity. Critical elements of integrin signaling are Src-tyrosine kinase and focal adhesion kinase (FAK); these are two non-receptor-tyrosine kinases regulating focal contact turnover and are both required for efficient cell movement. Recently, Knoll and Drescher (16) have provided evidence of the involvement of Src in EphA-mediated growth cone formation. A role of FAK has been proposed in Eph/ephrin signaling during cell adhesion to ECM and

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[1] To whom correspondence should be addressed: Dept. of Biochemical Sciences, University of Florence, Viale Morgani 50, 50134 Florence, Italy. Tel.: 39-055-4598343; Fax: 39-055-4598905; E-mail: paola.chiarugi@unifi.it.

[2] The abbreviations used are: ECM, extracellular matrix; EGF, epidermal growth factor; EGFP, enhanced green fluorescent protein; FAK, focal adhesion kinase; FRNK, FAK-related non-kinase; GEF, GTP exchange factors; MLC, myosin light chain; HA, hemagglutinin; PBS, phosphate-buffered saline; TBS, Tris-buffered saline; TRITC, tetramethylrhodamine isothiocyanate.

the formation of dendritic spines, although contradictory, results exist over ephrin-dependent phosphorylation and dephosphorylation of FAK (17–19).

Reorganization of cell morphology during adhesion and detachment involves changes in focal adhesion complexes as well as in the actin cytoskeleton and is mainly regulated by signals emanating from clustered cell adhesion receptors. Members of the Rho family of GTPases are important in these pathways (20, 21), and their involvement in Eph-induced cytoskeleton remodeling has been reported (22, 23). Actually, EphA4 stimulation activates Rho through the guanine nucleotide exchange (GEF) factor ephexin, which triggers the retraction of retinal ganglion cell growth cones, and ephrinA5 in melanoma cells induces cell rounding and de-adhesion, again through the activation of RhoA (24). In keeping, the Crk family of adapter proteins (25) are also found involved in Eph signaling, contributing to ephrinB1-induced membrane ruffling of human aortic endothelial cells (26) and in ephrinB2 breast cancer tumorigenicity (27). Below, we show that in prostatic carcinoma cells ephrinA1 elicits a motility response by activating the Src·FAK complex, leading a Rho-dependent actino/myosin contractility activation, finally driving the retraction of the cell body and the re-direction of migration cells.

EXPERIMENTAL PROCEDURES

Materials—Unless specified, all reagents were obtained from Sigma. PC3 cells were from ATCC; recombinant mouse Fc and ephrinA1-Fc chimera were from R&D Systems; antiphosphotyrosine (clone 4G10) and anti EphA2 antibodies were from Upstate Biotechnology, Inc.; anti-P-416, anti-P-527, and anti-RhoA antibodies were from BD Biosciences; anti-FAK, anti-p-FAK (Tyr-576/577 and Tyr-925), anti-HA tag antibodies, and anti-non-muscular myosin regulatory light chain II (MLCII) antibodies were from Santa Cruz Biotechnology; anti-non-muscular-phospho-MLCII (Thr-18/Ser-19) were from Cell Signaling; anti-P-FAK antibodies (Tyr-397, Tyr-407, Tyr-576, Tyr-577, Tyr-861) were from BIOSOURCE; blebbistatin, PP3, PP2, Y-27632, ML-7 inhibitors were from Calbiochem; cell-permeable C3 inhibitor was from Cytoskeleton.

Plasmids and Site-directed Mutagenesis— SrcK− dominant negative Src kinase in pSG5 vector was a gift from A. Graziani (Novara, Italy); dnRhoN19 was a kind gift of K. Defea (Riverside, CA); FAK non-related kinase (FRNK) was subcloned in pTarget vector from the sequence present in pCX4 plasmid (kindly donated by Dr Hajme Yano, Osaka, Japan) using the pTarget™ mammalian expression system from Promega and following the manufacturer's instructions. Human FAK cloned in pRcCMV was kindly provided by Dr. Steve Hanks (Nashville, TN). The Y576F/Y577F-FAK mutant was obtained using the X-L site-directed mutagenesis kit from Qiagen. The pEGFPN1 plasmid encoding EGFP was from Clontech.

Cell Culture, Stimulation, and Protein Overexpression—PC3 cells were cultured in Ham's F-12 medium to which was added 10% fetal calf serum in 5% CO_2 humidified atmosphere. PC3 cells were transiently transfected using Lipofectamine 2000 (Invitrogen). 48 h after transfection the cells were recovered for analysis. For studies using soluble ephrinA1, cells in the logarithmic growth phase were always stimulated with 1 $\mu g \cdot ml^{-1}$ Fc or ephrinA1-Fc for the indicated times.

Immunocytochemistry—To investigate the formation of retraction fibers after washing with PBS, the cells were fixed with 3.7% formaldehyde solution in PBS for 20 min at 4 °C. Then, after extensive washing in PBS, the cells were permeabilized with 0.1% Triton X-100 in PBS and stained with a 50 μg/ml phalloidin-TRITC for 1 h at room temperature followed by several washes with PBS. The cells were mounted with glycerol plastine and observed under a laser-scanning confocal microscope (Leica).

Immunoprecipitation and Western Blot Analysis—1×10^6 cells were lysed for 20 min on ice in 500 μl of complete radioimmune precipitation lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 2 mM EGTA, 1 mM sodium orthovanadate, 1 mM phenylmethanesulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin). Lysates were clarified by centrifuging and were immunoprecipitated for 4 h at 4 °C with 1–2 μg of the specific antibodies. For anti-EphA2 immunoprecipitation we used either anti-EphA2 antibodies or 1 $\mu g \cdot ml^{-1}$ ephrinA1-Fc fusion protein, which precipitates all EphA kinases with similar results. Immune complexes were collected on protein A-Sepharose, separated by SDS/PAGE, and transferred onto nitrocellulose. The immunoblots were incubated in 3% bovine serum albumin, 10 mM Tris/HCl, pH 7.5, 1 mM EDTA, and 0.1% Tween 20 for 1 h at room temperature and were probed first with specific antibodies, then with secondary antibodies. Quantity-One software (Bio-Rad) was used to perform quantitative analyses.

In Vitro Three-dimensional Migration Assay—The *in vitro* motility assays were carried out using the Transwell system of Costar equipped with 8-μm pore polyvinylpyrrolidone-free polycarbonate filters (diameter, 13 mm). Cells were loaded into the upper compartment (5×10^5 cells in 500 μl) in serum-deprived growth medium with or without 1 $\mu g \cdot ml^{-1}$ of ephrinA1. The upper chambers were placed into 6-well culture dishes containing 1 ml of medium with 50 ng/ml human EGF as chemoattractant. After 24 h of incubation at 37 °C, non-invading cells were removed mechanically using cotton swabs, and the micro-porous membrane containing the invaded cells was stained with DiffQuick solution. Chemotaxis was evaluated by counting the cells migrated to the lower surface of the polycarbonate filters. For each filter the number of cells in six randomly chosen fields was determined, and the counts were averaged (mean ± S.D.).

Rho Activity Assay—Rho-binding domain was expressed as glutathione *S*-transferase fusion proteins in BL21 cells. Levels of GTP-bound RhoA in cell lysates were measured as described by Ren *et al.* (28) except that cells were lysed in TBS-Triton X-100, and the NaCl and $MgCl_2$ concentrations were adjusted to 500 and 10 mM, respectively, before the beads were added.

Measurement of Retracted Cells—PC3 cells were plated on FN-coated coverslides. 24 h after starvation the cells were stimulated with ephrinA1-Fc or Fc alone for 15 min and then fixed in 3% paraformaldehyde and stained with phalloidin-TRITC as described above. At least 10 pictures were taken by fluorescence microscopy at 40× magnification, and the mean of the ratio between separated cells that had retracted their cytoplasm and

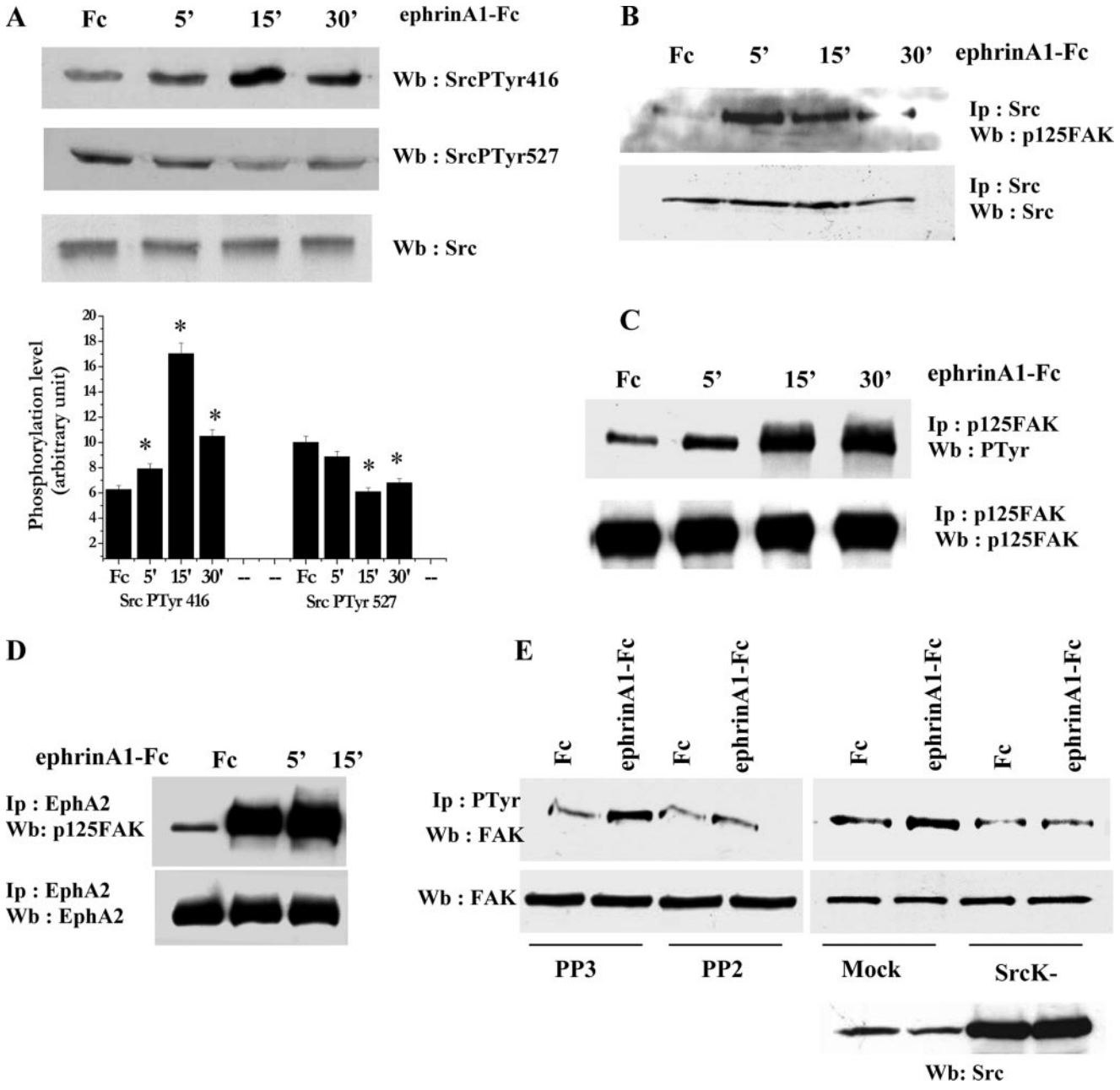

FIGURE 1. **EphrinA1 leads to activation of the Src-FAK pathway.** *A*, PC3 cells were serum-starved for 24 h before stimulating with either Fc or ephrinA1-Fc (1 μg·ml^{-1}) for the indicated times. Cells were then lysed in radioimmune precipitation lysis buffer, and 25 μg of total proteins for each sample were analyzed by anti-Src-Tyr(P)-416 and anti-Src-Tyr(P)-527 immunoblots (*Wb*) were performed. An anti-Src immunoblot was performed for normalization. The *bar graph* indicates the phosphorylation level of the activating (Tyr-416) and inhibitory (Tyr-527) tyrosines of Src; *, $p < 0.001$ *versus* Fc. *B*, PC3 cells were treated as in *A*. Src was immunoprecipitated (*Ip*), and an anti-FAK immunoblot was performed. The blot was then stripped and reprobed with anti-Src antibodies for normalization. *C*, PC3 cells were treated as in *A*. FAK was immunoprecipitated, and an anti-phosphotyrosine immunoblot was performed. The blot was then stripped and reprobed with anti-FAK antibodies for normalization. *D*, PC3 cells were treated as in *A*. EphA2 was immunoprecipitated, and an anti-FAK immunoblot was performed. The blot was then stripped and reprobed with anti-EphA2 antibodies for normalization. *E*, PC3 cells were treated with the Src kinase inhibitor PP2 or its inactive analogue PP3 (3 μM each) for 30 h (*left*) or transfected for 48 h with the dominant negative form of Src, SrcK− (*right*). PC3 cells were serum-starved for 24 h before stimulating with either Fc or ephrinA1-Fc (1 μg·ml^{-1}) for 15 h. An immunoprecipitation anti-phosphotyrosine was performed. The anti-FAK immunoblots are shown. SrcK− overexpression was checked by anti-Src immunoblot. The experiments have been repeated at least three times.

formed retraction fibers and the total number of cells (retracted cells plus spread cells) was calculated by counting cells.

RESULTS

EphrinA1 Leads to Activation of the Src-FAK Pathway—Src family kinases have recently been proposed as possible mediators of EphA receptor signaling during retinal axon guidance (16). It is well known that Src kinase is important in cytoskeletal rearrangement during physiologic and pathological conditions, such as migration and metastasis (29). We hypothesized that cytoskeletal rearrangements induced by repulsive and guidance factors, such as ephrins, are similar to those elicited by chemoattractant motility factors. Indeed, the motility cues induced by guidance factors may play a key role in the directional motility

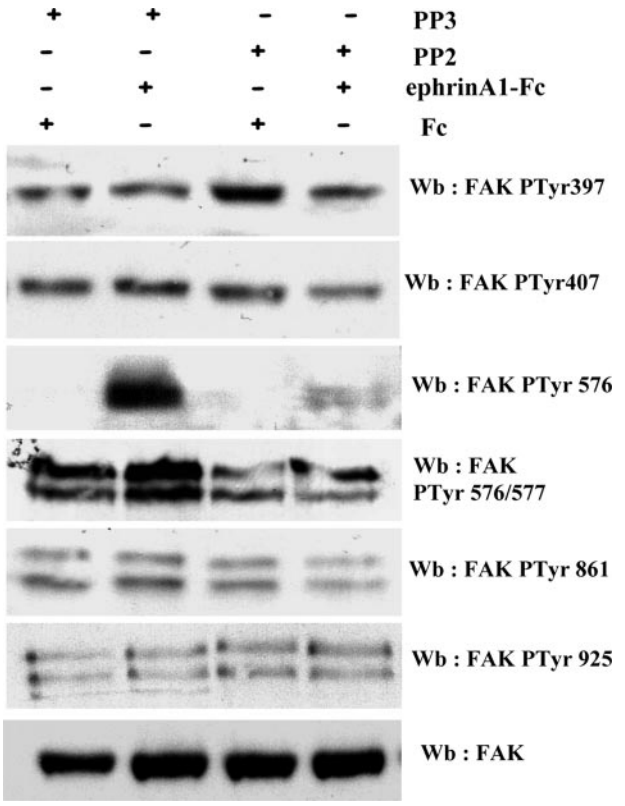

FIGURE 2. **FAK Tyr-576/577 is specifically phosphorylated upon ephrinA1 stimulation.** PC3 cells were serum-starved for 24 h, pretreated with the Src kinase inhibitor PP2 or its inactive analogue PP3 (3 μM each) for 30 h, and then stimulated with either Fc or ephrinA1-Fc (1 μg·ml^{-1}) for 15 h. Cells were then lysed in radioimmune precipitation lysis buffer, and 25 μg of total proteins for each sample were analyzed by anti-phospho-Tyr-397, -407, -576, -576/577, -861, and -925 FAK immunoblots and anti-FAK immunoblot for normalization. The experiments have been repeated at least three times.

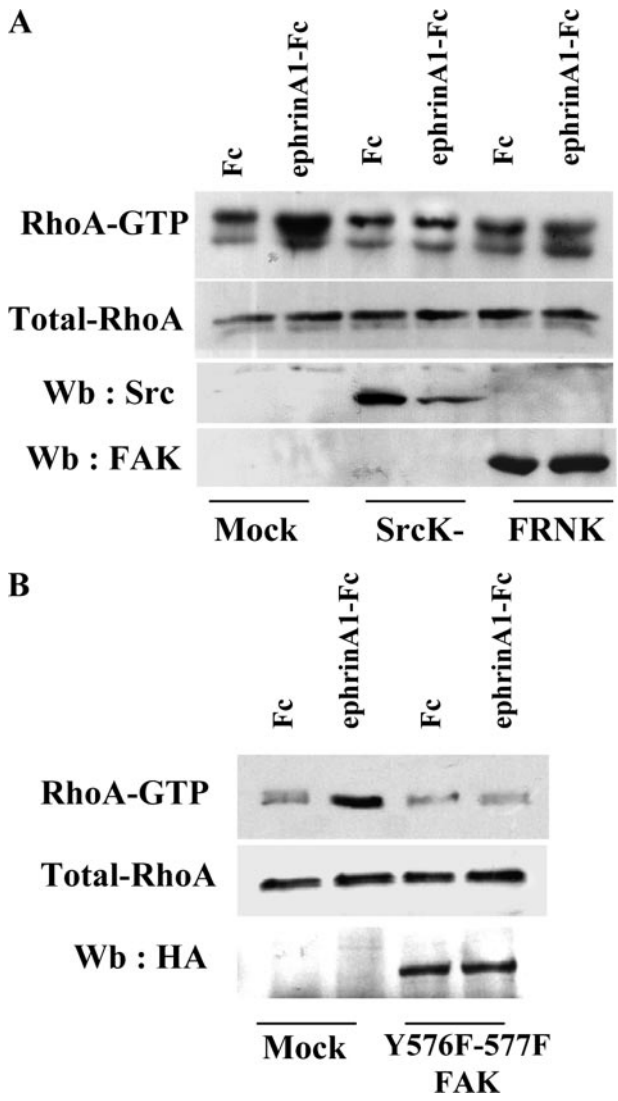

FIGURE 3. **EphrinA1 causes a Src·FAK-dependent RhoA activation.** PC3 cells transfected for 48 h with SrcK− or FRNK (*A*) or transfected with Y576F/Y577F FAK (*B*) were serum-starved for 24 h before stimulating with either Fc or ephrinA1-Fc (1 μg·ml^{-1}) for 15 h. A RhoA activity assay was then performed, and a RhoA immunoblot (*Wb*) was performed for normalization. SrcK−, FRNK, and Y576F/Y577F FAK overexpression were checked by anti-Src, anti-FAK, and anti-HA immunoblots. The experiments have been repeated at least three times.

of disseminating cancers (30). Accordingly, we focused on the role of Src kinase in ephrinA1 elicited signaling, choosing as a model a metastatic prostate carcinoma cell line with high endogenous EphA2 overexpression.

In PC3 cells ephrinA1 is able to induce Src activation, with a maximum observed after 15 min based on the phosphorylation level of Src regulatory tyrosines 416 and 527 (Fig. 1*A*). Activated Src kinase rapidly associates with FAK, forming a bipartite kinase (31). We, therefore, looked at the association between these two molecules in ephrinA1 stimulation (Fig. 1*B*). Upon ephrinA1 stimulation, we find that FAK associates with Src in a ligand-dependent manner. Formation of the Src·FAK complex upon ephrinA1 stimulation leads to enhancement of FAK kinase activity, as demonstrated by phosphorylation of FAK upon ligand engagement (Fig. 1*C*). Involvement of FAK-tyrosine kinase in ephrinA1 signaling is further indicated by its ligand-dependent association with EphA2 receptor (Fig. 1*D*).

To investigate the mechanisms involved in the activation of FAK after ephrinA1 stimulation, we analyzed its phosphorylation level in PC3 cells treated with the Src kinase inhibitor PP2 and in cells transfected with the dominant negative form of Src, SrcK−. The SrcK− mutant is catalytically inactive as it carries a mutation in the ATP binding site, but it is still able to bind substrates. We find that ephrinA1-mediated FAK phosphorylation is Src-dependent since both the treatment with PP2 and the overexpression of SrcK− severely impair FAK activation (Fig. 1*E*).

FAK is a large tyrosine kinase that, upon autophosphorylation on Tyr-397, recruits Src; this in turn phosphorylates FAK on several tyrosine sites, setting up multiple signal transduction pathways (31). Analysis of the specific tyrosines of FAK, such as tyrosine 407, 397, 925, 861, 576, and 577, shows that only Tyr-576 and -577 are specifically phosphorylated upon ephrinA1 stimulation, whereas the phosphorylation level of tyrosines 407, 861, and 397 are not affected (Fig. 2). We also find evidence that the phosphorylation of tyrosines 576 and 577 depends on Src activity, as demonstrated by the use of PP2 (Fig. 2). This tyrosine phosphorylation of FAK was further confirmed in breast carcinoma cells MDA-MB231 and in non-transformed breast epi-

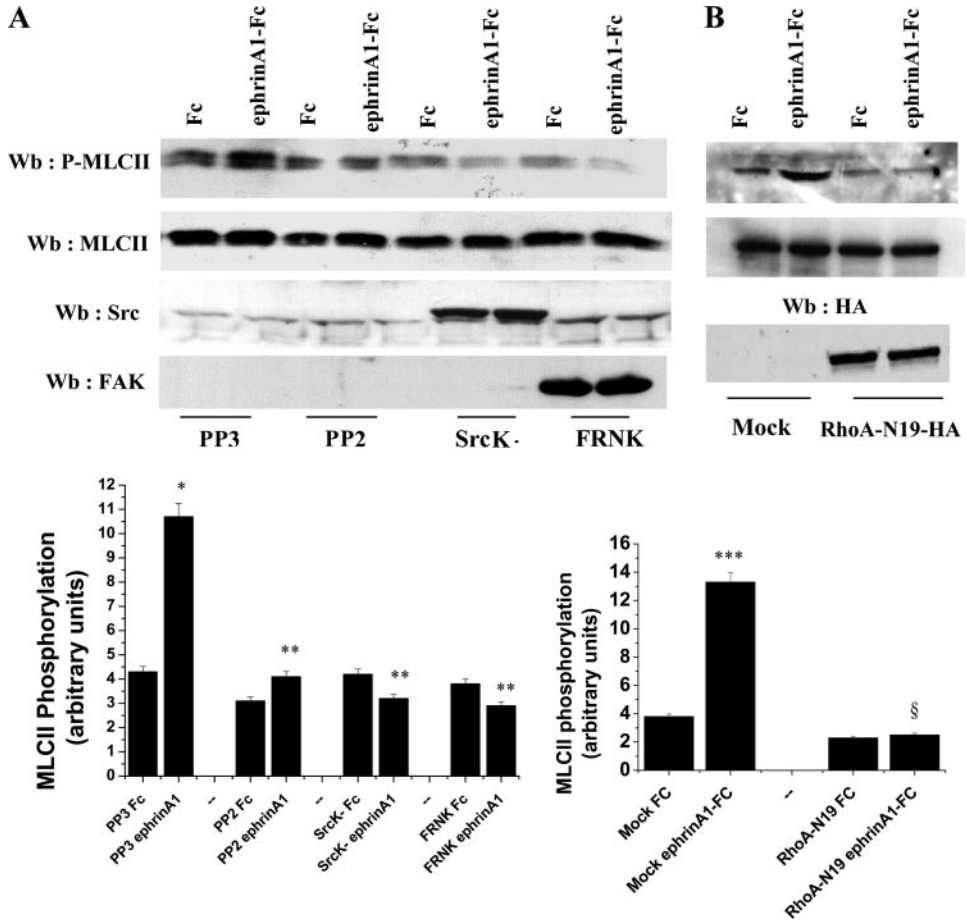

FIGURE 4. **EphrinA1 causes a Src·FAK/RhoA-dependent MLCII phosphorylation.** PC3 cells transfected for 48 h with SrcK− or FRNK or treated with PP2 or its inactive analogue PP3 (3 μM each) (*A*) or transfected with RhoA-N19-HA (*B*) were serum-starved for 24 h before stimulating with either Fc or ephrinA1-Fc (1 μg·ml^{-1}) for 15 h. Cells were then lysed in radioimmune precipitation lysis buffer, and 25 μg of total proteins for each sample were analyzed by anti-phospho (*P*)-MLCII immunoblot (*Wb*). The blots were then stripped and reprobed with anti-MLCII antibodies for normalization. SrcK− and FRNK overexpression were checked by anti-Src and anti-FAK immunoblots. RhoA-N19-HA overexpression was checked by anti-HA immunoblot. The plots indicate the phosphorylation level of MLCII. *, $p < 0.001$ *versus* PP3 Fc. **, $p < 0.001$ *versus* PP3 ephrinA1-Fc. ***, $p < 0.001$ *versus* mock Fc. §, $p < 0.001$ *versus* mock ephrinA1-Fc. The experiments have been repeated at least three times.

thelial cells MCF10A (data not shown). Considering that the phosphorylation of Tyr-576/577 of FAK has been reported to increase the kinase activity of FAK (32), our data provide evidence that ephrinA1 stimulation leads to the recruitment and activation of the Src·FAK complex.

Rho Activation by EphrinA1 Involves Src and FAK Engagement—A recognized way in which Eph receptors affect cellular morphology is by remodeling the actin cytoskeleton and cell shape through the regulation of Rho family GTPases (12). Recently RhoA activation has been reported to affect the ephrinB2-mediated cell migration of melanoma cells (15). In addition, Rho small GTPase has been involved in ephrinA1-mediated angiogenesis or inhibition of integrin-induced cell spreading and dendritic spine retraction (33–35). We, therefore, investigated if Src and FAK activation in response to ephrinA1 plays a role in RhoA regulation in PC3 cells. We used as tools the dominant negative mutants for FAK (FRNK) and Src (SrcK−). The FRNK mutant is a natural dominant negative protein for FAK as a result of its ability to inhibit the formation of the Src·FAK complex (36). Actually FRNK is devoid of kinase activity and contains only the C-terminal region of FAK, maintaining the proline-rich regions responsible for stabilizing Src association through binding of SH3 domains (37). We overexpressed these mutants in ephrinA1-stimulated PC3 cells and analyzed the activation of RhoA by means of glutathione *S*-transferase pulldown. The results indicate that Src activation and the formation of the bipartite kinase Src·FAK are required steps for ephrinA1-induced RhoA activation (Fig. 3*A*). In addition, the phosphorylation of FAK on Tyr-576/577 is a key regulatory step in ephrinA1 RhoA activation, as demonstrated by the overexpression of the ephrin-insensitive Y576F/Y577F FAK mutant (Fig. 3*B*).

EphrinA1 Repulsion Determines Cell Body Retraction Activating Actino/Myosin Contractility—RhoA is acknowledged to affect cell body retraction and rounding through the downstream activation of cytoskeleton contractility. Myosin II is the principal motor protein responsible for cell motility, and the phosphorylation of its MLCII through the Rho-activated kinase is a primary means for activating its motor power and executing cytoskeleton contractility (38). To determine whether the Rho-dependent signal induced by ephrinA1 involves actino/myosin contractility, we investigated the phosphorylation level of MLCII in response to ephrinA1 (Fig. 4*A*). The results show that ephrinA1 elicits a strong activation of MLCII phosphorylation in PC3 cells and that this event is downstream with respect to Src and FAK activation/association. We confirmed the direct involvement of RhoA in MLCII activation by ephrinA1 through overexpression of its dominant negative RhoA-N19 (Fig. 4*B*).

Down-regulation of cell-cell contacts and cell shape changes could be critical during neurite outgrowth, synapse remodeling, vascular sprouting, and in initiation of epithelial cell migration during development and metastasis dissemination. Upon ephrinA1 treatment, cells appear to disrupt cell-cell interactions to repel each other and to retract the cell body. We examined the extent of cell-cell contacts and found that ephrinA1 stimulation causes a strong decrease in these contacts (Fig. 5*A*). In addition we attempted to determine if the activation of the RhoA/Rho kinase/MLCII/myosinII pathway is required for retraction of carcinoma cell body in response to ephrinA1. To this aim we used several

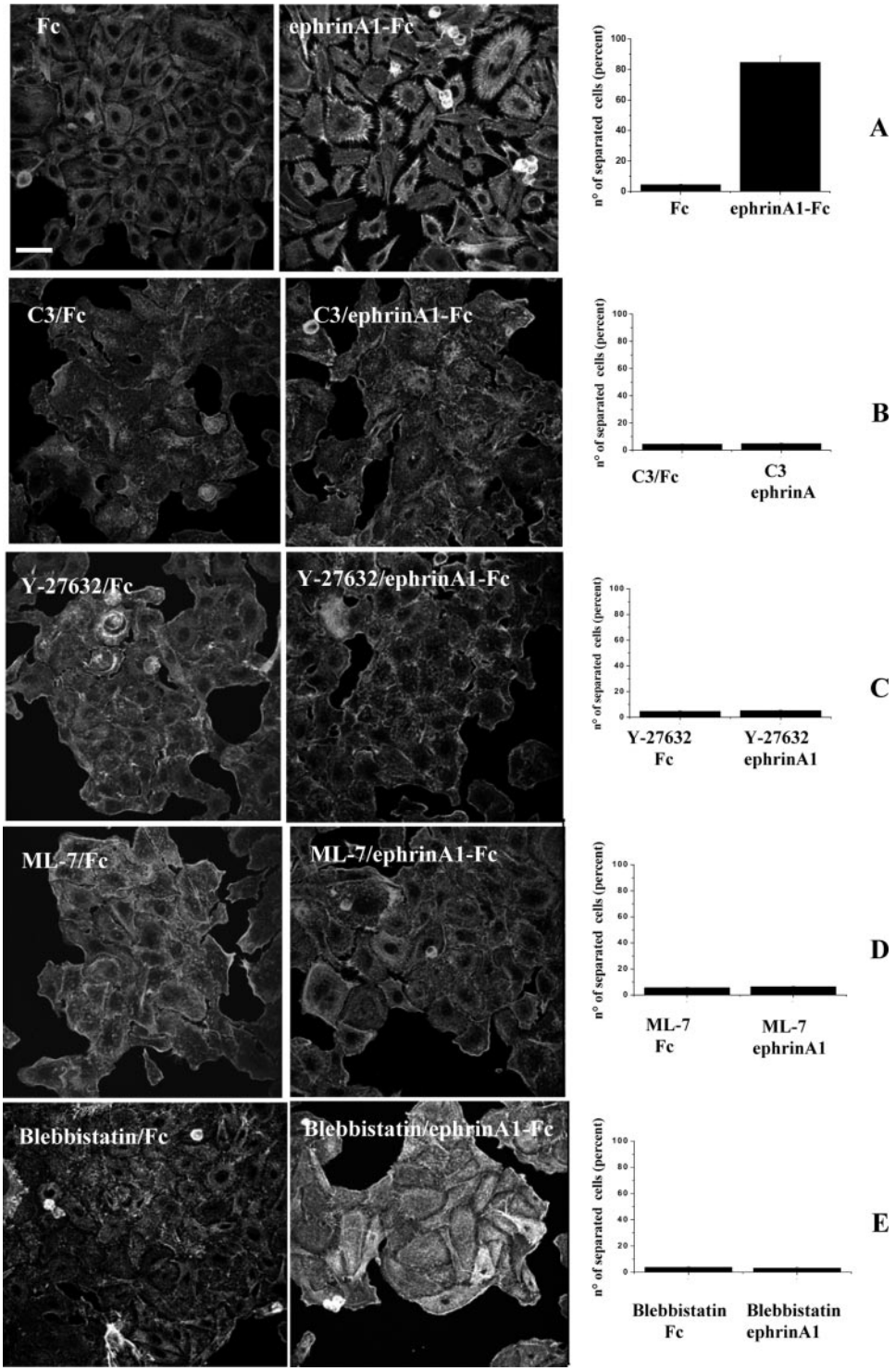

FIGURE 5. **EphrinA1 causes cell body retraction by activating RhoA-dependent actino/myosin contractility.** *A*, cells were seeded onto collagen-coated coverslips, and adhesion was permitted for 24 h. Cells were serum-starved for 24 h before stimulating with either Fc or ephrinA1-Fc (1 $\mu g \cdot ml^{-1}$) for 15 h. In *B*, *C*, *D*, and *E*, the cells were pretreated for 30 h with the cell-permeable Rho inhibitor C3 (*B*, 50 μM), the Rho kinase inhibitor Y-27632 (*C*, 10 μM), the myosin light chain kinase inhibitor ML-7 (*D*, 10 μM), or the myosinII inhibitor blebbistatin (*E*, 50 μM), and then stimulated with ephrinA1-Fc or Fc (1 $\mu g \cdot ml^{-1}$) for 15 h. Confocal microscope analysis after phalloidin-TRITC immunostaining was shown. *Bar*, 40 μm. The average separated cell number, calculated in six randomly chosen fields, is reported in the *bar graphs*, and the mean ± S.D. is indicated. The experiments have been repeated at least three times.

targeted inhibitors of the pathway, including the cell-permeable C3 RhoA inhibitor, the Y27632 Rho kinase inhibitor, the ML7 MLC kinase inhibitor, and the myosinII inhibitor blebbistatin. The analysis of retracted cells (Fig. 5*B*) reveals that the Rho-dependent pathway, converging on myosinII activation, plays a pivotal role in ephrinA1-elicited cell body retraction and prompts cytoskeleton contractility to produce the force necessary for the cell to retract its cytoplasm.

EphrinA1 Repulsive Motility Requires Activation of the Src·FAK Complex—We have previously reported that in PC3 cells ephrinA1 leads to retraction fiber formation as a result of cell body retraction (39). We now show that the activation of both Src and FAK kinases play a specific role in ephrinA1-induced repulsion, as indicated by quantification of cells displaying retraction fibers (Fig. 6). Our tools were again the dominant negative mutant for FAK, Src, and RhoA and the ephrin-signaling insensitive Y576F/Y577F FAK mutant. Inhibition of Src activation through expression of SrcK− stops the ability of ephrinA1 to induce retraction fibers. The ability of FRNK to inhibit retraction fiber formation suggests a decisive role of the complex formed between Src and FAK. Furthermore, the role of ephrin-induced activation of FAK through the up-regulation of Tyr-576/577 phosphorylation is confirmed by overexpression of the Y576F/Y577F FAK mutant, again eliminating the ephrin-elicited response. Finally, RhoA is involved in retraction fiber formation in response to ephrinA1, in keeping with its recognized role of a key player in cytoskeleton rearrangements.

Eph ligands have been reported to affect cell motility, instructing cells to inhibit previously engaged signals toward chemo-attractive molecules and inducing a change of directional motility (14, 40–42). To study the role of the activation of Src and FAK kinases in the motility response induced by ephrinA1, we explored the effect of inhibition of Src, FAK, and RhoA (Fig. 7). Indeed, the overexpression of the dominant negative mutants for Src and FAK, the ephrin-insensitive Y576F/Y577F FAK mutant or the dominant negative RhoA,

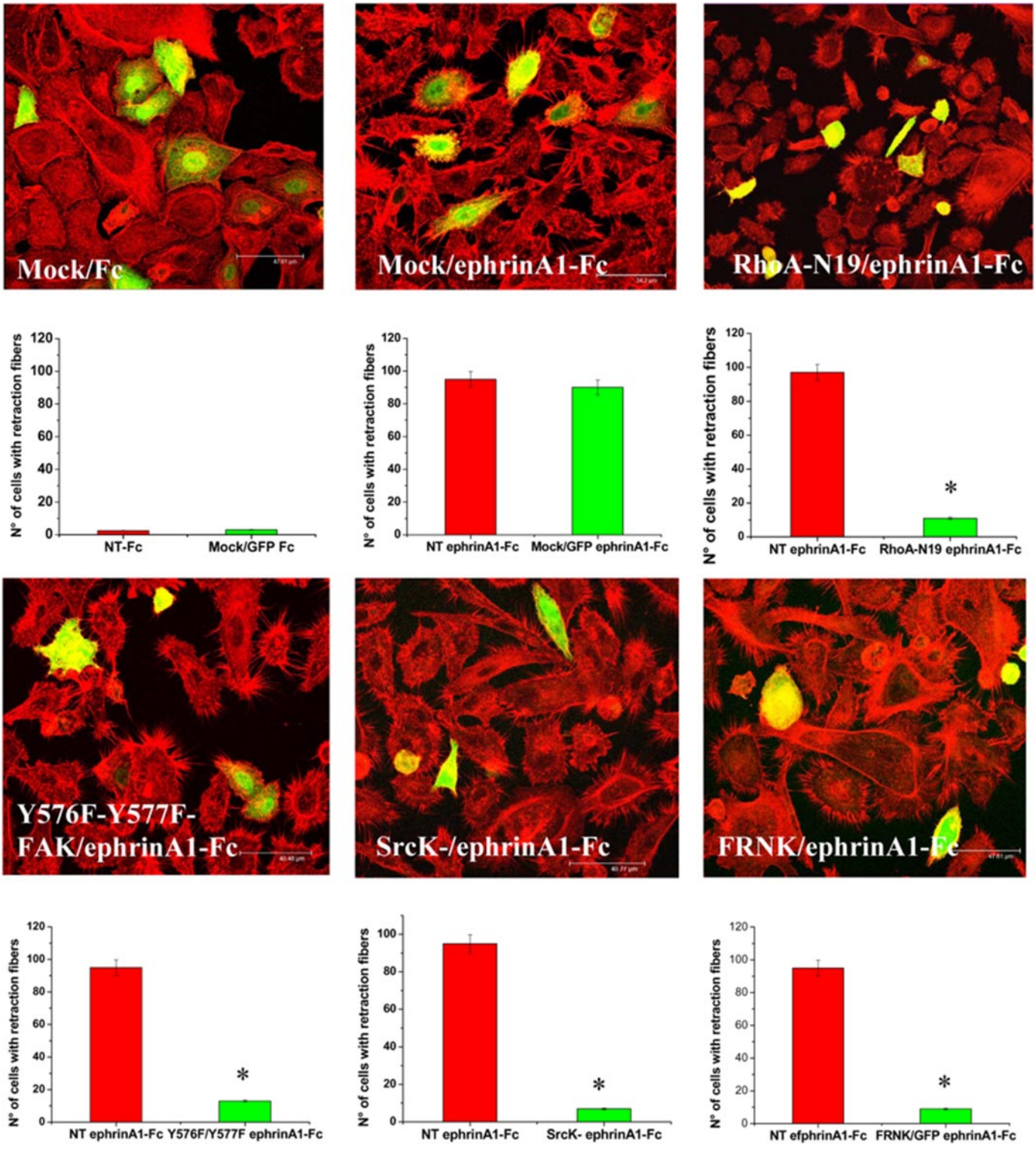

FIGURE 6. **EphrinA1 causes retraction fiber formation through activation of a Src·FAK/RhoA-dependent pathway.** Cells were plated onto collagen-coated coverslips, and adhesion was permitted for 24 h. Cells were then cotransfected for 48 h with EGFP and FRNK, SrcK−, or Y576F/Y577F FAK or RhoA-N19 (in a ratio of 1:10 EGFP:plasmids) before stimulating with ephrinA1-Fc or Fc for 15 h. Confocal microscope analysis was shown; *red* shows fluorescence of F-actin stained with anti-phalloidin-TRITC (non-transfected cells (*NT*)), and *green* shows fluorescence of EGFP (cotransfected with SrcK−, FRNK, Y576F/Y577F FAK mutant, or RhoA-N19). *Bar*, 40 μm. Cells displaying retraction fibers were counted in six randomly chosen fields, and the number of green and red retracted cells is plotted and shown beside. *, $p < 0.001$ *versus* NT. The experiments have been repeated at least three times.

severely impairs the ability of ephrinA1 to inhibit chemotaxis toward EGF. These results suggest that the Src·FAK /RhoA pathway is a key determinant of the physiological response induced by ephrinA1 in prostate carcinoma cells, playing a critical function in retraction of cell body and in the control of directional migration.

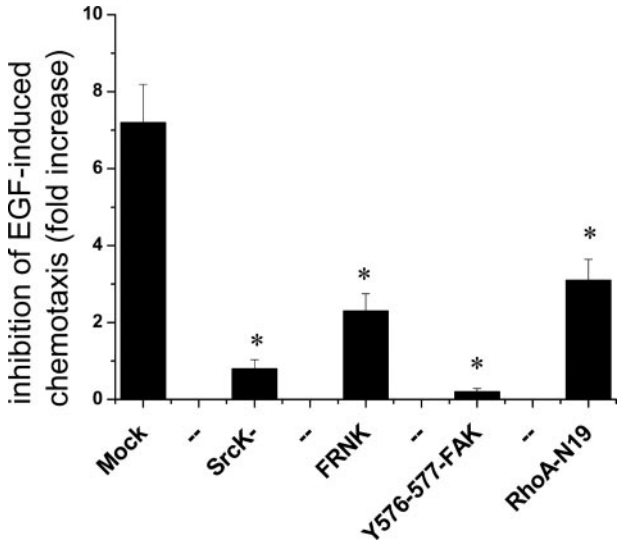

FIGURE 7. **EphrinA1 inhibits EGF-induced chemotaxis through activation of a Src·FAK/RhoA-dependent pathway.** PC3 cells cotransfected for 48 h with EGFP and SrcK−, FRNK, Y576F/Y577F FAK, or RhoA-N19 (in a ratio of 1:10 EGFP:plasmids) after 24 h of serum starvation were seeded into the upper chamber of Transwells. Cells were allowed to migrate for 16 h toward the lower chamber filled with growth medium supplemented with Fc or ephrinA1-Fc (1 μg·ml^{-1}) and EGF 50 ng ml^{-1}. Migration of transfected cells was evaluated by counting green migrated cells, as a measure of transfected cells, in six randomly chosen fields. The normalization of migrated cells has been achieved by DiffQuick staining of the same migration assay chamber and counting cells in six randomly chosen fields. The ratio (-fold increase) of Fc-treated *versus* ephrinA1-Fc-treated migrated cells is reported in the *bar graph*, and the mean ± S.D. is indicated. *, $p < 0.001$ *versus* mock. The experiments have been repeated at least three times.

DISCUSSION

The molecular mechanisms underlying the directional cell motility *in vitro* have come under wide investigation, and this has led to the recognition of several cytoskeletal and signaling proteins implicated in different steps of cell migration. It is likely that *in vivo* a similar setting of mechanisms plays key functions where the migratory movements of cells, as the axonal growth of cones or the dissemination and organ targeting of metastasis, undergo more complex regulation by both attractive and repulsive cues. The mutual effects of these cues decide the timing and exact path of cell motility.

Our data indicate that the activation of a repulsive outcome elicited by ephrinA1/EphA2 leads to the activation of a motile response involving the complex of two tyrosine kinases, Src and FAK. These two kinases are believed to be components of focal adhesion complexes and are involved in cell migration toward chemo-attractants. We report that they are similarly involved in the repulsive motility response, indicating that the features of a moving cell, either elicited by a chemo-attractant or a chemo-repulsive agent, involves some common molecular events. Among the molecular occurrences shared by attraction and repulsion, we include myosin-mediated cytoskeleton rearrangements, again driven through enhancement of Src and FAK activation by ephrinA1. Our studies are in agreement with the recent report by Moeller *et al.* (19), indicating a role for FAK activation in EphB-mediated shortening of dendritic spine precursors, and with the data of Carter *et al.* (17), reporting a role for FAK and p130Cas in EphA2-induced morphological cell changes. However we as well as others (17) were unable to reproduce the data of Miao *et al.* (18) showing a dephosphorylation of FAK in ephrinA1-stimulated carcinoma cells.

Involvement of Src and FAK-tyrosine kinases in cell motility is well known. Src is a multifunctional protein involved in the regulation of a variety of normal and oncogenic processes, including proliferation, differentiation, survival, motility, and angiogenesis (43). Its interaction with several cellular factors, including cell surface receptors as well as FAK, has already been reported (29). The non-receptor-tyrosine kinase FAK is postulated as vital to cell motility and cancer metastasis by modulating the formation and turnover of focal adhesions (44). FAK is widely expressed in different cell types, localizes to nascent focal adhesions, and is typically activated after assembly of integrin-mediated focal adhesions (45). FAK and Src have been shown to form a transient, active complex after integrin engagement by ECM proteins or ligand stimulation of growth factor receptors (46). We now include ephrinA1-mediated repulsion among the stimuli eliciting the association and activation of the Src·FAK bipartite kinase. In keeping with our findings, the Src·FAK complex has been reported to be involved in several motility outcomes, including the invasive capacity of carcinoma cells (36) or chemotactic and haptotactic cell migration (47).

After autophosphorylation on Tyr-397 upon ECM contact, FAK has a double effect on cell signaling. First, it causes tyrosine phosphorylation of several downstream substrate molecules, likely through association with Src and enhancement of its own phosphorylation on Tyr-576/577, leading to kinase "super-activation" (32). Second, activation of the Src·FAK complex leads, through Src-mediated phosphorylation of several FAK tyrosines, to the generation of docking sites for SH2-containing signaling proteins, including the phosphatidylinositol 3-kinase and the mitogen-activating protein kinase activator Grb2 (37). Our results reveal that in prostatic carcinoma cells ephrinA1 signaling elicits phosphorylation of the tyrosines 576 and 577 of FAK but not of the docking sites for SH2 domain-binding proteins (Tyr-397, Tyr-407, Tyr-861, Tyr-925). We speculate that the main consequence of the ephrinA1 repulsive effect, transduced through FAK activation, is related to a Tyr-576/577-mediated increase in FAK kinase activity toward exogenous substrates. On the contrary, ephrinA1-induced FAK activation does not likely serve as a platform for eliciting the activation of intracellular routes through SH2 domain binders as mitogen-activating protein kinase and phosphatidylinositol 3-kinase. These findings are consistent with the claim that adhesive and repulsive cues, although both involving FAK activation, lead to different downstream signaling. Actually adhesive signaling is mediated through a wide phosphorylation of tyrosine residues within the FAK molecule, likely leading to both increase of phosphorylation of exogenous FAK substrates and multiple pathways elicited by SH2 domain binders (37). On the contrary, repulsive ephrin response through FAK is transduced only via phosphorylation of Tyr-576/577, likely affecting only FAK kinase activity toward protein substrates. Furthermore, the non-conventional Tyr-397-independent Src binding to FAK during ephrinA1 signaling suggests an additional difference between adhesive and repulsive signals. We can speculate that in the absence of a phosphorylation of Tyr-397, Src might bind

to FAK proline-rich regions. The association of the two kinases may be either direct or indirectly mediated by an adapter as p130Cas (48, 49). Indeed these two mechanisms have already been proposed to involve two different proline-rich regions of FAK to recruit Src or phosphorylated p130Cas-Src complex to the kinase proximity. In agreement with this hypothesis, ephrinA1 has been reported to phosphorylate p130Cas (17, 48).

It is reasonable that a repulsive response nullifies all previous cell constraints, including adhesion to ECM and to neighboring cells. The effect of ephrin-mediated repulsion on ECM linkage has already been documented. Several ephrins inhibit integrin-mediated cell adhesion, spreading, and migration (18, 33), and in particular, we have recently reported that ephrinA1 strongly inhibits integrin adhesion and signaling in PC3 prostate carcinoma cells.[3] Conversely, the release of constraints from neighboring cells induced by ephrins is an elusive and not fully addressed question. Notably, ephrins are intramembrane ligands and need cell proximity to signal to cognate Eph receptors. Nevertheless, ephrin-mediated cell repulsion requires the two interacting cells to separate and move in opposite directions. Flanagan resolves this paradox by showing that ephrinA2 forms a stable complex with the metalloprotease Kuzbanian, the *Drosophila* homolog of ADAM10 (50). Recently Janes *et al.* (4) show that the ephrin ligand can be proteolytically released from its membrane link by a complex on the opposing cell composed of the ephrin receptor and ADAM10. Beside this mechanism granting cell repulsion, the release of previous cell constraints with both ECM and neighboring cells induced by ephrinA1 can be helped through the initiation of a process of retraction of the cell body and cell rounding. We herein reported the involvement of a RhoA-mediated actino/myosin cytoskeleton contractility in this outcome. In addition, we propose that the formation of the Src·FAK bikinase complex is a prerequisite for ephrin-mediated disruption of cell-cell interactions.

In carcinomas, the interplay between epithelial tumor cells and neighboring stromal cells is mediated by modulation of bidirectional interactions such as adhesion, migration, and homing on target organs. Eph/ephrin signaling is involved in all these processes through transduction of the molecular mechanisms that propel invasive growth and metastasis. The coordinated migration of tumor cells during invasion requires continuous re-organization of the cell cytoskeleton through a series of signaling systems. It is likely that chemo-repulsive factors play a double role in this scenario. First, they cancel previous constraints by inhibiting integrin function and cell-cell contacts. Immediately thereafter the motility response must be engaged, and both the rearrangement of actin cytoskeleton and the contractility of actino/myosin motors are involved. Both Src and FAK kinases have already been involved in cell motility and cancer invasion (37, 45), and we now show that in prostatic carcinoma cells Src and FAK activation sustains the cytoskeleton dynamics and the Rho-mediated activation. As a result the Rho kinase/myosin light chain kinase/myosinII pathway is activated, thus culminating on cytoskeleton contractility and driving the changes of cell shape and the redirection of ephrin-sensitive cells. Indeed, overexpression of RhoA-N19 mimicked the effect of SrcK− and FRNK and blocked the effect of ephrinA1 on retraction fiber formation, cell rounding, and cell migration. In agreement with our data suggesting a key role of FAK in Rho-mediated cytoskeleton rearrangement upon ephrinA1 stimulation, the inhibition of RhoA or loss of FAK has been shown to reduce cell retraction during neurite growth (51, 52). The role of FAK in regulating the retraction of the cell body is further stressed by Rico *et al.* (52), who reported that FAK regulates axonal branching and synapse formation through a Rho-mediated action. Interactions between FAK and Rho are complex as both Rho GEFs, as p190RhoGEF (53), and Rho GTPase activators (GTPase-activating proteins (GAPs)), as Graf (54) or p190RhoGAP (55), have been shown to stimulate Rho activity in cytoskeleton remodeling during axonal branching or growth factor signaling. On the other side, Ephs have been shown to activate Rho GTPases through engagement of several RhoGEFs, including ephexin (22), VmsRhoGAF (56), kalirin (57), and intersectin (58). Although a full characterization of the molecular targets of FAK-mediated RhoA activation in response to ephrins is still lacking, this small GTPase emerges as a key converging point for the mechanisms regulating cell motility of different physiological and pathophysiological settings, likely including carcinoma cells migration. In this context the RhoGEF family appears to acquire a key role as potential Src targets, likely acting as intermediate molecules linking Src·FAK activation to RhoA activation in ephrin-treated cells. In agreement, the RhoGEF ephexin has been reported to be phosphorylated by Src in response to ephrinA1, although the specific role of this modification remains to be elucidated (16).

Although future work is needed to fully characterize the overall response to ephrins, the rising picture describes these factors as wide coordinators of the motility response; they instruct cells to detach from other cells and from ECM, to retract the cell body, and to move away. The obvious implication in relation to this induced behavior is an increase in the potential for invasion for ephrin-sensitive epithelial carcinomas.

[3] Buricchi, F., Giannoni, G., Grimaldi, G., Parri, M., Raugei, G., Ramponi, G., and Chiarugi, P., submitted for publication.

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EphrinA1 Activates a Src/Focal Adhesion Kinase-mediated Motility Response Leading to Rho-dependent Actino/Myosin Contractility

Matteo Parri, Francesca Buricchi, Elisa Giannoni, Giovanna Grimaldi, Tommaso Mello, Giovanni Raugei, Giampietro Ramponi and Paola Chiarugi

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