EphrinA1 Repulsive Response Is Regulated by an EphA2 Tyrosine Phosphatase[*]

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Ephrin kinases and their ephrin ligands transduce repulsion of cells in axon guidance, migration, invasiveness, and tumor growth, exerting a negative signaling on cell proliferation and adhesion. A key role of their kinase activity has been confirmed by mutant kinase inactive receptors that shift the cellular response from repulsion to adhesion. Our present study aimed to investigate the role of low molecular weight protein-tyrosine phosphatase (LMW-PTP) in ephrinA1/EphA2 signaling. LMW-PTP, by means of dephosphorylation of EphA2 kinase, negatively regulates the ephrinA1-mediated repulsive response, cell proliferation, cell adhesion and spreading, and the formation of retraction fibers, thereby confirming the relevance of the net level of tyrosine phosphorylation of Eph receptors. LMW-PTP interferes with ephrin-mediated mitogen-activated protein kinase signaling likely through inhibition of p120RasGAP binding to the activated EphA2 kinase, thereby confirming the key role of mitogen-activated protein kinase inhibition by ephrinA1 repulsive signaling. We conclude that LMW-PTP acts as a terminator of EphA2 signaling causing an efficient negative feedback loop on the biological response mediated by ephrinA1 and pointing on tyrosine phosphorylation as the main event orchestrating the repulsive response.

The Eph family of receptor tyrosine kinases and their ligands, ephrins, constitute the largest receptor tyrosine kinase subfamily, with at least 16 receptors and 9 ligands (1, 2) (cbweb.med.harvard.edu/eph-nomenclature). So far, several biological functions have been attributed to Eph receptors including cell positioning, vascular development, tissue border formation, branching morphogenesis, cell migration, and axon guidance (2). In any case, the influence on cell behavior of ligand-induced Eph receptor activation can generally be related to repulsion of neighboring cells or of cellular processes, such as neuronal growth cone (3) or vasculogenesis (4).

Engagement with an ephrin induces a conformational change in the cytoplasmic portion of the Eph receptor triggered by phosphorylation of two juxtamembrane tyrosines (5), which relieves kinase domain inhibition (6). Occupancy of the juxtamembrane binding sites and additional phosphorylation may further stabilize the active conformation of the Eph receptor, thereby stimulating the activity of the kinase domain. Furthermore, receptor tyrosine phosphorylation creates docking sites for proteins with SH2 [3] or phosphotyrosine binding domains, leading to the recruitment of cytoplasmic targets that regulate downstream signaling (7).

Both Eph kinases and ephrins have been implicated in tumor growth and angiogenesis. They have been found to be overexpressed in many human tumors (8–11), and the higher expression levels correlated with the more aggressive and metastatic tumors (10, 12). Elevated EphA2 levels are observed in many dissimilar types of cancers, including breast, prostate, and colon carcinomas, as well as in aggressive melanomas (13–15). In addition, ectopic overexpression of EphA2 is sufficient to grant tumorigenic and metastatic potential upon nontransformed epithelial cells (14).

Notably, the presence within the same cell of kinase-inactive in concert with wild-type Eph receptors transforms its outcome to ephrins from repulsion to adhesion (16), suggesting that net levels of Eph kinase tyrosine phosphorylation determine the response to ephrin contact. Although phosphorylation is thought to be an important event in the Eph receptor signaling process, the specific role of Eph kinase activity and of associated phosphatases remains to be elucidated. Emerging evidence suggests that protein-tyrosine phosphatases (PTPs) are involved in regulating Eph-mediated responses (17–20). Recruitment of low molecular weight (LMW)-PTP to ephrinB1-activated EphB2 is important for endothelial capillary-like assembly and adhesion (21). In addition, LMW-PTP is frequently overexpressed in transformed cells, and its ectopic overexpression is sufficient to confer transformation in epithelial cells (22), thus suggesting a role for LMW-PTP in transformation progression. Although recent data suggest that LMW-PTP *in vivo* is a positive regulator of both tumor onset and development and that the oncogenic potential of the phosphatase is linked to EphA2 (17), the specific role of this phosphatase in the modulation of the ephrin-mediated cellular response is still unknown.

Herein we demonstrate that LMW-PTP negatively regulates the repulsive response elicited by ephrinA1 through dephosphorylation of EphA2 kinase, thereby confirming the relevance of the net level of tyrosine phosphorylation of Eph receptors. Our results identify LMW-PTP as a negative regulator of the activated EphA2 kinase, ultimately interfering with ephrin-mediated mitogen-activated protein kinase (MAPK) signaling through a negative feedback loop.

[3] The abbreviations used are: SH2, Src homology 2; CMV, cytomegalovirus; EGF, epidermal growth factor; EGFP, enhanced green fluorescent protein; GAP, GTPase-activating protein; HEK, human embryonic kidney; LMW-PTP, low molecular weight PTP; MAPK, mitogen-activated protein kinase; MEF, mouse embryonic fibroblast; PBS, phosphate-buffered saline; PTP, protein-tyrosine phosphatase; RIPA, radioimmune precipitation assay; siRNA, short interfering RNA; wt, wild-type.

EXPERIMENTAL PROCEDURES

Materials—Unless otherwise specified all reagents were obtained from Sigma. PC3 and HEK-293T cells were purchased from ATCC. Recombinant mouse Fc and ephrinA1-Fc chimera from were from R&D Systems. Anti-MAPK, antiphosphotyrosine (clone 4G10), and anti-EphA2 antibodies were from Upstate Biotechnology Inc. Anti-phospho-MAPK antibodies were from Cell Signaling Technology, and anti-p120RasGAP was from Santa Cruz Biotechnology. Mouse embryonic fibroblasts (MEFs) that are wild type or null for p120RasGAP were kindly supplied by Tony Pawson (Toronto, Canada).

Cell Culture and Stimulation—PC3 cells were cultured in Ham's F-12 medium; MEFs and HEK-293T cells were cultured in Dulbecco's modified Eagle's medium. 10% fetal calf serum was added to all media, and all cells were maintained in 5% CO_2 humidified atmosphere. For studies using soluble ephrinA1, cells in logarithmic growth phase were always stimulated with 1 μg ml^{-1} Fc or ephrinA1-Fc for the indicated times.

Cloning of Human Eph Receptor—On the basis of the human EphA2 BLAST sequence (M59371) we synthesized the primers for reverse transcription-PCR: upstream primer, 5′-ATGGAGCTCCAGGCAGC-CCG-3′; and downstream primer, 5′-TCAGATGGGGATCCCCA-CAGT-3′. Total RNA was isolated from PC3 cells with TRIzol reagent (Invitrogen), and the amplification was performed using SuperScript One-step reverse transcription-PCR for long templates (Invitrogen) according to the manufacturer's instructions. The amplified sequence was subcloned into pLargeT vector (Promega). The double mutant EphA2 receptor was obtained using a QuikChange XL Site-directed Mutagenesis kit (Stratagene). The original tyrosines in positions 588 and 594 have been replaced with phenylalanine.

DNA Transfection— 8 μg of pRcCMV-wtLMW-PTP or pEGFP-wtLMW-PTP, in which wild-type LMW-PTP has been cloned upstream of the ATG-deleted EGFP gene of the pEGFP-N1 vector (23), or the wild-type and mutant EphA2 kinase in pLargeT vector, were transiently transfected in PC3 or in HEK-293T cells using Lipofectamine 2000 (Invitrogen). 48 h after transfection cells were recovered for analyses.

Short Interfering RNA (siRNA) Transfections—LMW-PTP siRNA (5′-AAGTCCGTGCTGTTTGTGTGT-3′), p120RasGAP siRNA (5′-AAACTGCCCACTTCGTTGCTT-3′), and a negative control siRNA sequence corresponding to nucleotides 695–715 of the firefly luciferase (U31240) were obtained from Qiagen. PC3 cells plated at 3 × 10^5/dish were transfected with siRNAs using Lipofectamine 2000 as described by the manufacturer. Cells were transfected with 4 μg of siRNA/60-mm dish. siRNA efficiency was tested by anti-LMW-PTP immunoblot; although at 48 h we detected an appreciable inhibition of LMW-PTP expression, the best efficiency was observed 72 h after transfection.

Cell Adhesion onto EphrinA1-Fc-coated Slides—Coverslips were treated with 2% collagen in PBS for 15 min and then washed extensively with PBS and air dried. After that, the slides were coated with 1 μg/ml ephrinA1-Fc for 1 h at room temperature and then washed twice with 2% bovine serum albumin in Dulbecco's modified Eagle's medium. 1.2 × 10^5 cells were seeded in each coverslip, and adhesion was permitted for 15 min, 30 min, and 1 h. Negative controls were performed by seeding cells onto collagen-coated and ephrinA1 noncoated coverslips. Afterward an immunohistochemistry assay was performed using phalloidin-TRITC.

Clonal Cell Growth Assay—200 cells were plated in triplicate directly onto 6- or 12-well cell culture dishes in the presence of Fc or ephrinA1-Fc; media were changed every 3 days. Cellular growth was stopped after 7–10 days in culture by removing the medium and by the addition of a 0.5% crystal violet solution in 20% methanol. After staining for 5 min the fixed cells were washed with PBS and solubilized with 200 μl/well 0.1 M sodium citrate, pH 4.2. The absorbance at 595 nm was evaluated using a microplate reader.

In Vitro Three-dimensional Migration Assay—The in vitro motility assays were carried out with the Costar Transwell system, equipped with 8-μm pore polyvinylpyrrolidone-free polycarbonate filters (diameter, 13 mm). The Matrigel (BD Biosciences) was diluted (30 μg in 100 μl of H_2O), added to the top chamber, allowed to gel for 1 h at 37 °C, air dried for 16 h, and the Matrigel barrier was reconstituted with 100 μl of medium for 2 h at 37 °C before use. Cells were loaded to the upper compartment (5 × 10^4 cells in 300 μl) in serum-deprived growth medium with or without 1 μg ml^{-1} ephrinA1. The Matrigel invasion chambers were placed into 6-well culture dishes containing 1 ml of medium with 20% serum as chemoattractant. After 24 h of incubation at 37 °C, noninvading cells and the Matrigel layer were removed mechanically using cotton swabs, and the microporous membrane containing the invaded cells was fixed in 96% methanol and stained with a crystal violet. 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.).

*Wound Healing Migration Assay*—Confluent PC3 cells were serum starved for 24 h, and the dishes were scored with a sterile 200-μl micropipette tip and photographed. Thereafter cells were treated with 50 ng/ml epidermal growth factor (EGF) and 1 μg/ml of either Fc or ephrin-Fc After 24 h the wounds were photographed again to visualize incoming cells.

Immunocytochemistry—After washing with PBS, cells were fixed with 3.7% formaldehyde solution in PBS for 20 min at 4 °C. Then, after extensive washes in PBS, cells were permeabilized with 0.1% Triton X-100 in PBS and then stained with a 50 μg/ml fluorescent phalloidin conjugate solution in PBS, phalloidin-TRITC, for 1 h at room temperature. After several washes with PBS to remove unbound phalloidin conjugate, the coverslides were mounted with glycerol plasticine and then observed under a confocal fluorescence microscope.

Immunoprecipitation and Western Blot Analysis—1 × 10^6 cells were lysed for 20 min. on ice in 500 μl of complete RIPA 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 centrifugation and 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 (Upstate Biotechnologies) or 1 μg 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. 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, probed first with specific antibodies and then with secondary antibodies. Quantity-One software (Bio-Rad) was used to perform quantitative analyses.

RESULTS

EphA2 Inhibits Cell Proliferation and Cell Migration and Induces Cell Rounding and Retraction Fiber Formation—To study the role and regulation of tyrosine phosphorylation of EphA2 receptor we chose PC3 carcinoma cells, which have been reported to express mainly EphA2 kinase (24). Herein we report that the stimulation of this receptor by its cognate ligand ephrinA1 elicits a growth arrest response together with inhibition of cell migration. PC3 cells were seeded onto collagen-coated
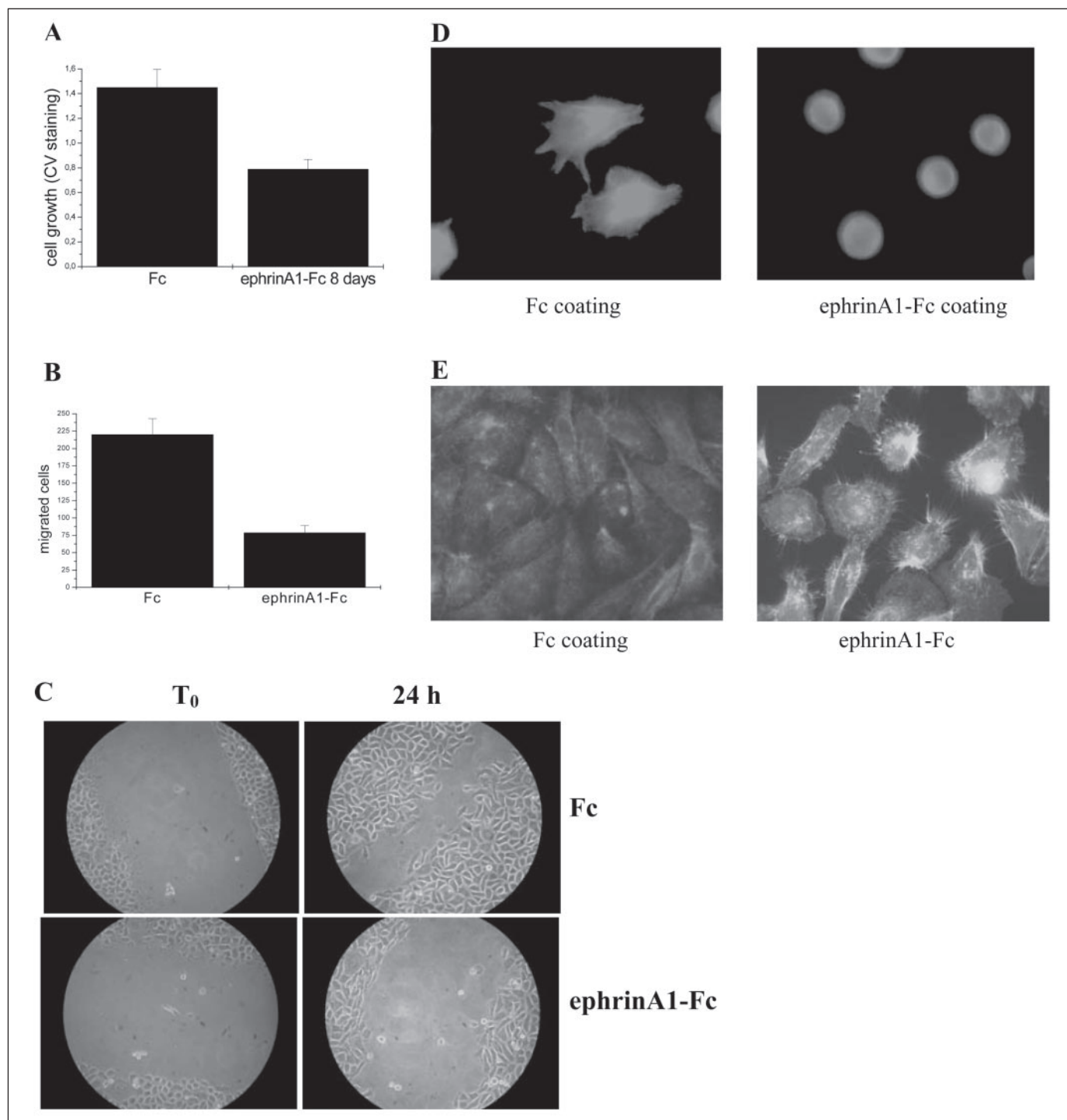
FIGURE 1. **EphA2 inhibits cell proliferation and cell migration and induces cell rounding and retraction fiber formation.** *A*, cell growth assay. PC3 cells were plated in complete medium in the presence of Fc or ephrinA1-Fc. Cellular growth was stopped after 7–10 days, and crystal violet (*CV*)-stained cells were evaluated and reported in the plot. The mean ± S.D. are indicated. *B*, three-dimensional cell migration assay. PC3 cells, after 24 h of serum starvation, were seeded into the upper chamber of Transwell precoated with a matrigel layer and treated with Fc or ephrinA1-Fc. Cell were allowed to migrate for 24 h toward the lower chamber filled with growth medium supplemented with 20% fetal calf serum. Cell migration was evaluated after crystal violet staining by counting cells in six randomly chosen fields. The average cell number is reported in the histogram, and the mean ± S.D. are indicated. *C*, wound healing migration assay. Confluent PC3 were serum starved for 24 h, scratched with a tip, and a photograph was taken. 50 ng/ml EGF was added to induce migration together with 1 μg/ml Fc or ephrinA1-Fc, and after 24 h another photograph was taken. The experiment was repeated four times with similar results. *D*, cell adhesion onto ephrinA1-Fc-coated slides. Cells were seeded onto Fc- or ephrinA1-Fc-coated coverslips, and adhesion was permitted for 1 h. Confocal microscope analysis after phalloidin-TRITC immunostaining is shown. *E*, formation of ephrinA1-induced retraction fibers. Cells were seeded onto collagen-coated coverslips, and adhesion was permitted for 24 h. Thereafter ephrinA1-Fc or Fc stimulation was carried out for 15 min. Confocal microscope analysis after phalloidin-TRITC immunostaining is shown.

dishes, and a clonal growth assay was carried out (Fig. 1A). Moreover, ephrinA1 treatment of PC3 cells inhibits their EGF-induced migration in a three-dimensional motility assay (Fig. 1B). The inhibition of ephrinA1 on cell motility was assessed further by a wound healing migra-tion assay. Confluent PC3 cells were wounded, and their EGF-induced motility was measured in the presence of ephrinA1 (Fig. 1C). Again, ephrinA1 is able to inhibit the cell motility strongly in response to EGF as a chemoattractant.
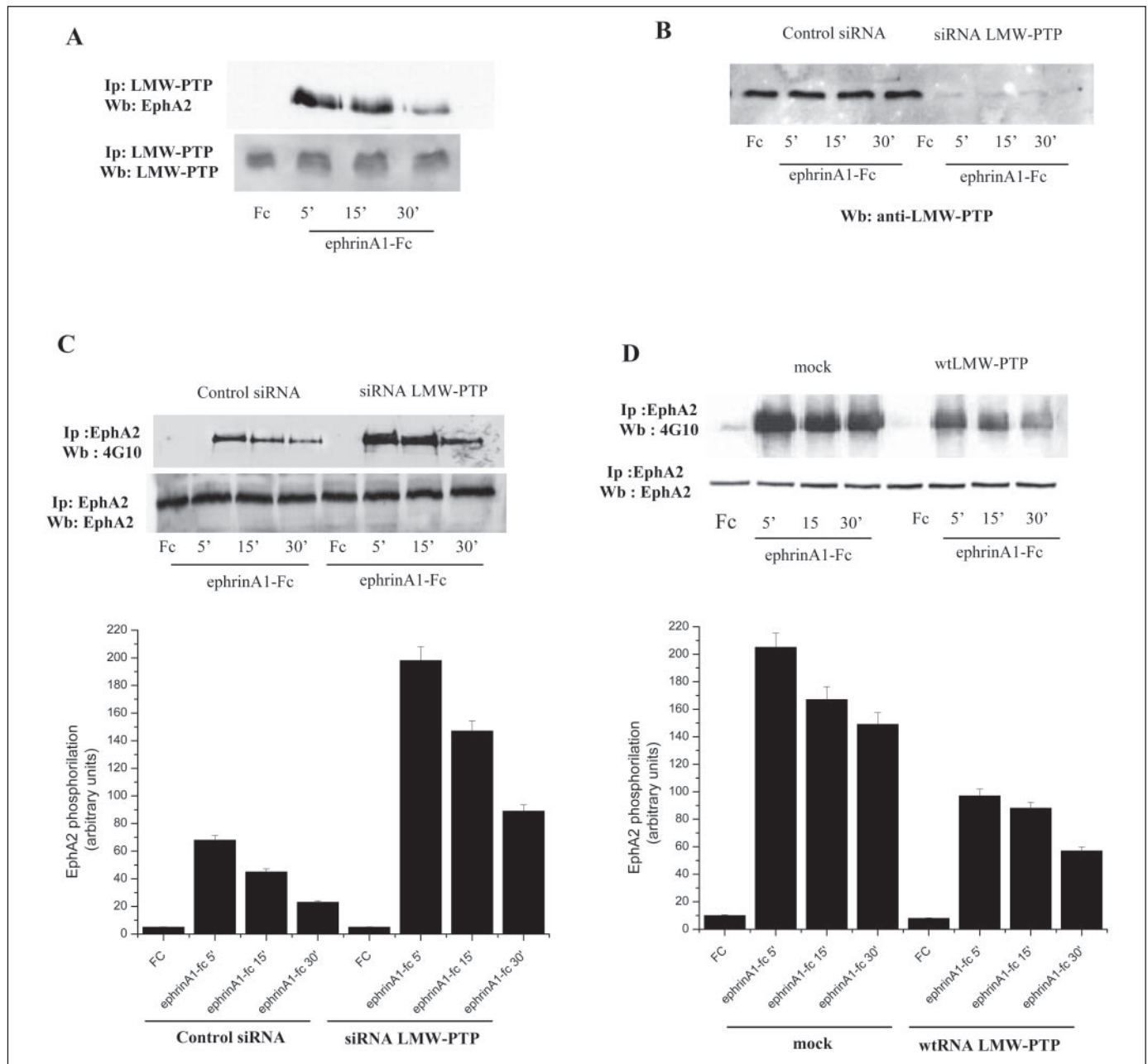
FIGURE 2. **EphA2 receptor is regulated by LMW-PTP.** *A*, association of LMW-PTP with activated EphA2 receptor. PC3 cells were serum starved for 24 h before stimulating with either Fc or ephrinA1-Fc for the indicated times. LMW-PTP was immunoprecipitated (*Ip*), and an anti-EphA2 immunoblot (*Wb*) was performed. The blot was then stripped and reprobed with anti-LMW-PTP antibodies for normalization. *B*, LMW-PTP silencing using siRNA. PC3 cells were treated as in *A* and then lysed in RIPA lysis buffer. 25 μg of total proteins for each sample was analyzed by anti-LMW-PTP immunoblot. *C* and *D*, LMW-PTP controls the EphA2 tyrosine phosphorylation. PC3 cells, in which LMW-PTP was either silenced (*C*) or overexpressed (*D*) for 72 h, were treated as in *A*. LMW-PTP silencing and overexpression were checked by anti-LMW-PTP immunoblot. EphA2 receptor was immunoprecipitated, and its tyrosine phosphorylation level was evaluated by antiphosphotyrosine (4G10 clone) immunoblot. The blot was then stripped and reprobed with anti-EphA2 antibodies for normalization. The graph obtained with data from densitometry analysis of replicate experiments plotted relative to loading controls is shown below.

Many findings have reported that EphA2 activation leads to dramatic changes in cell morphology (3, 19, 25, 26). We observed that in PC3 cells EphA2 activation inhibits the spreading and adhesion of cells onto collagen-treated dishes (Fig. 1*D*), suggesting a dramatic delay, after contact with the extracellular matrix, in the construction of cytoskeleton architecture, because of ephrinA1 stimulation. In addition, ephrinA1 stimulation, while inducing cell retraction from the original spread morphology, provokes the formation of actin retraction fibers (Fig. 1*E*), resulting from retraction of extending membranes of rounding cells. Retraction fibers have been reported to form in several circumstances in which cells need to round up, as in preparation for cell division (27) and during

neurite retraction (28). These fibers are not microspikes/filopodial-like projections but simple residual contact sites that have not yet been released from the substrate. As far as we know this is the first evidence that during the retraction of the cell body, EphA2 receptor activation elicits the formation of branching actin structures, similar to retraction fibers.

EphA2 Receptor Is Regulated by LMW-PTP—A role of LMW-PTP in the dephosphorylation of EphA2 has already been postulated, first in vitro (22) and *in vivo* (17). Our current interest was in investigating the role of LMW-PTP in ephrinA1 signaling. Immunoprecipitation analysis in PC3 cells reveals that EphA2 receptor associates with LMW-PTP and
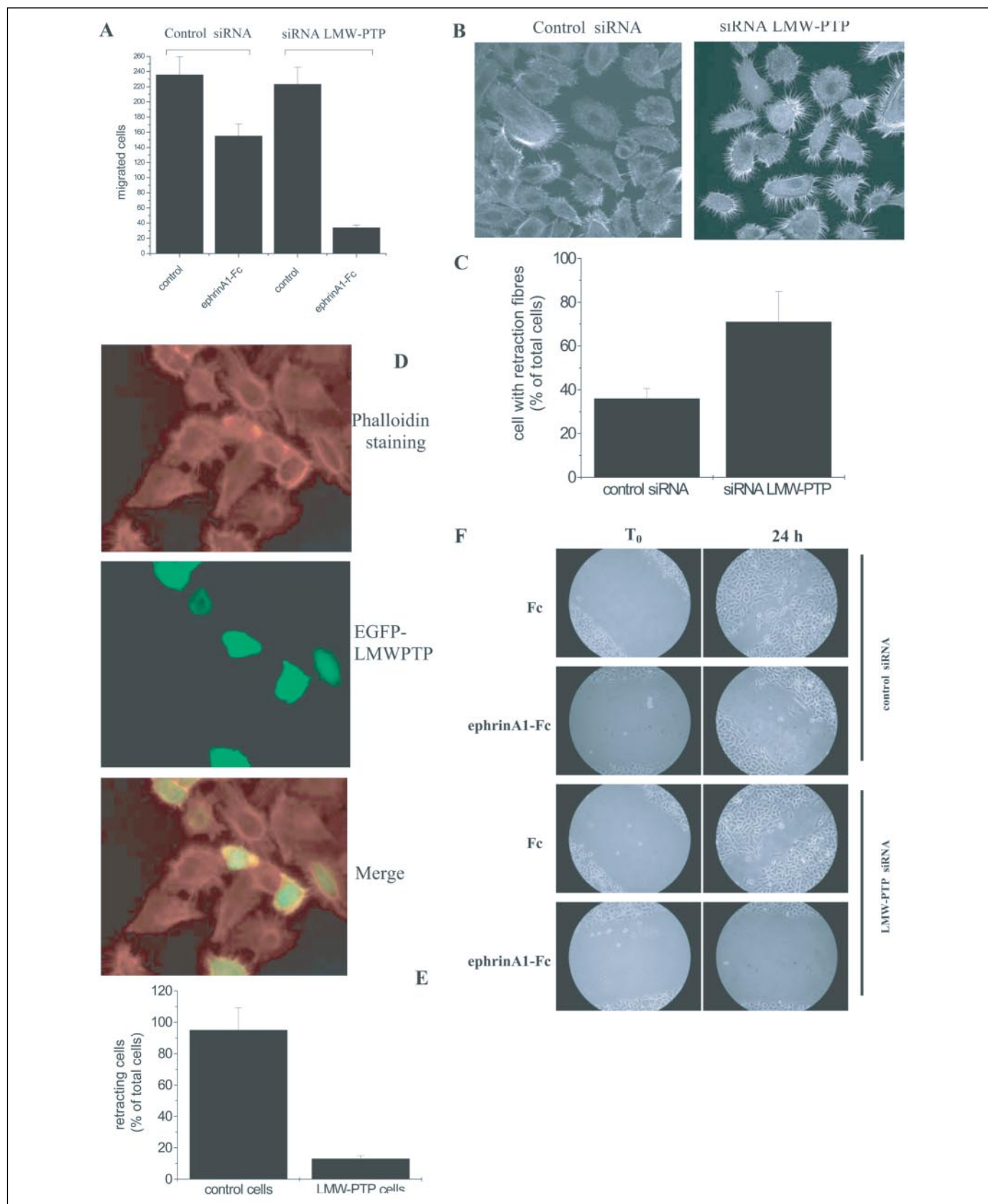
FIGURE 3. **EphrinA1 repulsive outcome is regulated by LMW-PTP.** *A*, three-dimensional cell migration assay. PC3 cells, in which LMW-PTP was silenced for 72 h, after 24 of serum starvation, were seeded with Fc or ephrinA1-Fc, into the upper chamber of a Transwell precoated with a matrigel layer. Cell were allowed to migrate for 24 h toward the lower chamber filled with growth medium supplemented with 20% fetal calf serum. Cell migration was evaluated after crystal violet staining by counting cells in six randomly chosen fields. The average cell number ± S.D. is reported in the *bar graph*. LMW-PTP silencing was checked by anti-LMW-PTP immunoblot. *B*, LMW-PTP is involved in cell rounding and retraction fiber formation. Cells were seeded onto collagen-coated coverslips and were treated as in *B* except that F-actin was stained by anti-phalloidin-TRITC treatment. Confocal microscope analysis is shown. *C*, as a quantification of the phenomenon, cells with actin retraction fibers longer than half the diameter of the cell body were counted. Values represent the

that this association is strictly ligand-dependent (Fig. 2*A*). To verify that LMW-PTP is able to dephosphorylate EphA2 in PC3 cells, we first used the siRNA-based silencing approach. We initially verified that 48 and 72 h after siRNA transfection the expression of LMW-PTP was almost undetectable and that the effect of siRNA-mediated LMW-PTP silencing was not affected by ephrinA1 stimulation (Fig. 2*B*). LMW-PTP silencing did not affect either the EphA2 expression or the overall cell morphology (data not shown). Further analysis with LMW-PTP-silenced PC3 cells revealed that the tyrosine phosphorylation level of EphA2 kinase upon ligand binding was greatly affected by LMW-PTP silencing (Fig. 2*C*). These data suggest that LMW-PTP retains a key role in the dephosphorylation of the receptor kinase, although other phosphatases may be involved in EphA2 signaling termination. Overexpression of wtLMW-PTP in PC3 cells confirms that LMW-PTP is able to dephosphorylate the EphA2 kinase, leading to a significant decrease in EphA2 kinase phosphorylation level (Fig. 2*D*).

EphA2 Function Is Regulated by LMW-PTP—Recent data indicate that the presence of kinase-inactive, together with active Eph receptors within the same cell, changes its response to ligands from repulsion to adhesion (16, 29), indicating a key role for Eph kinase phosphorylation levels. Consequently, we hypothesize that ephrinA1 cellular responses are controlled by the activity of the associated LMW-PTP. To verify that LMW-PTP is able to contrast ephrinA1 effects in PC3 cells, we first analyzed the effect of modulation of LMW-PTP expression on ephrin-mediated migration responses. Our results showed that LMW-PTP silencing causes a dramatic increase in the effect elicited by ephrinA1 in the three-dimensional motility assay, leading to a greater inhibition of serum-induced cell migration (Fig. 3A).

Furthermore, we analyzed the effect of LMW-PTP silencing on the ephrin-mediated formation of retraction fibers, observed during cell repulsion. Remarkably, both the repulsion between ephrinA1 cells and the formation of retraction fibers appear to be enhanced (at least 65%) in LMW-PTP-silenced cells (Fig. 3, *B* and *C*). In addition, to confirm the causal role of LMW-PTP in cell rounding and retraction fiber formation, we overexpressed the EGFP-fused wtLMW-PTP in PC3 cells during ephrinA1 stimulation. We checked that the overexpression of LMW-PTP had not influenced the overall cell rounding and retraction fiber formation in PC3 cells and that the fusion of LMW-PTP with EGFP had not interfered with its enzymatic activity (data not shown). Fig. 3*D* shows the superimposing of green fluorescence of EGFP (cells that express EGFP-wtLMW-PTP) and of red fluorescence from actin labeling. Upon stimulation with EphA1 all of the cells started to retract the cell body at 5 min. By 15 min, nearly all of the cells retracted the membrane protrusions, and the actin retraction fibers were evident. In EGFP-wtLMW-PTP-expressing cells the formation of retraction fibers was strongly inhibited, whereas in adjacent nonexpressing cells the formation of retraction filaments was normal (Fig. 3*D*). Fig. 3*E* shows a quantitative analysis of the morphological changes, namely, loss of membrane extensions and formation of retraction fibers in both wtLMW-PTP and control cells. Finally, the effect of LMW-PTP silencing was assessed in a wound-healing EGF-induced motility assay (Fig. 3*F*), confirming again that the inhibition of expression of LMW-PTP

considerably increases the decline in cell motility induced by ephrinA1.

Thus, taken together our data indicate that LMW-PTP has a dramatic effect on the overall repulsive outcome induced by ephrinA1 ligation.

LMW-PTP Interferes with Ephrin-mediated MAPK Regulation—MAPKs play an essential role in a variety of cellular processes such as proliferation, migration, and development and were recently found to be linked to ephrin signaling (24, 26, 30–32). Although the effect of ephrins on MAPKs is still debated, in ephrinA1-stimulated PC3 cells we detected a strong and clear inhibition of this signaling pathway. The effect of ephrinA1 on the basal MAPK activation level was first analyzed and found to be mainly integrin- and cell adhesion-dependent. The results are consistent with previously reported data (24) and show that ephrinA1 strongly inhibits basal MAPK activity (Fig. 4A). Next, to elicit a strong MAPK activation, we stimulated PC3 cells with EGF. Again, ephrinA1 was able to inhibit strongly the EGF-induced MAPK activation (Fig. 4*B*), confirming that cell repulsion is associated with the repression of MAPKs elicited by both adhesive molecules and by soluble mitogens.

To investigate the role of LMW-PTP in EphA2-mediated inhibition of MAPK signaling, we either silenced LMW-PTP expression or overexpressed wtLMW-PTP, before MAPK assaying. We show that the inhibition of LMW-PTP expression induces a stronger inhibition of MAPK after ephrinA1 stimulation, likely because of EphA2 receptor hyperphosphorylation (Fig. 4*C*). Likewise, the overexpression of active LMW-PTP leads to the abolishment of the inhibitory effect of ephrinA1 on MAPK activation, likely because of receptor dephosphorylation (Fig. 2*D*). Taken together, our results validate that LMW-PTP greatly affects EphA2-dependent signaling, contrasting ephrin-dependent MAPK inhibition.

LMW-PTP Inhibits p120RasGAP Binding to EphA2 Kinase—p120Ras-GAP has been indicated among SH2 domain-containing proteins that, upon ligand binding, bind to Eph kinases (33). The recruitment to cell membrane of the Ras small GTPase GAP leads to activation of its GTPase activity and to a final down-regulation of MAPK signaling (34). We hypothesized that the LMW-PTP effect on ephrinA1-mediated MAPK signaling may be caused by the elimination of a docking site (either single or multiple) for a MAPK regulator containing an SH2 domain as p120RasGAP. To verify our hypothesis we analyzed the binding of p120RasGAP to EphA2 kinase in LMW-PTP silenced cells. Our results indicate that killing of LMW-PTP enhances p120RasGAP binding to EphA2 receptor (Fig. 5A), suggesting a role for this phosphatase in the dephosphorylation of p120 binding site(s) on the activated receptor. Accordingly, in PC3 cells overexpressing wtLMW-PTP we reported a decrease in the amount of p120RasGAP bound to EphA2 kinase upon ligand stimulation (Fig. 5*B*).

The involvement of p120RasGAP in the down-regulation of MAPK in response to Eph receptors has already been proposed (32). To confirm the key role of p120RasGAP in ephrinA1 signaling, we investigated the activation level of MAPK in wild-type cells and in cells in which p120RasGAP has been silenced by RNA interfering. Stimulation of cells with ephrinA1 leads to a dramatic down-regulation of EGF-activated MAPK, whereas no change in MAPK activation was detected in p120RasGAP-silenced cells (Fig. 6*A*). In addition we analyzed the inhibition of MAPK by ephrinA1 in p120RasGAP-null MEFs (Fig. 6*B*), again demonstrating that the absence of this GAP completely abolishes the

mean ±S.D. from six randomly selected fields. *D*, cells were coated onto collagen-coated coverslips and were treated as in *B* and *C* except that EGFP-LMW-PTP was overexpressed 72 h before ephrinA1-Fc and Fc alone treatment for 15 min. Confocal microscope analysis is shown: *top panel*, red fluorescence of F-actin stained with anti-phalloidin-TRITC; *middle panel*, green fluorescence of EGFP; and *bottom panel*, superimposition of both fluorescences. *E*, the number of cells that retract membrane extensions in which actin retraction fibers are evident, together with total number of cells per field, were counted in six randomly chosen fields. Values represent the mean percentage ±S.D. from six different positions. *F*, wound healing migration assay. Confluent PC3 cells, in which LMW-PTP was silenced for 72 h, were serum starved for 24 h, scratched with a tip, and a photograph was taken. 50 ng/ml EGF was added to induce migration together with 1 μg/ml Fc or ephrinA1-Fc, and after 24 h another photograph was taken. LMW-PTP silencing was checked by anti-LMW-PTP immunoblot. The experiment was repeated four times with similar results.
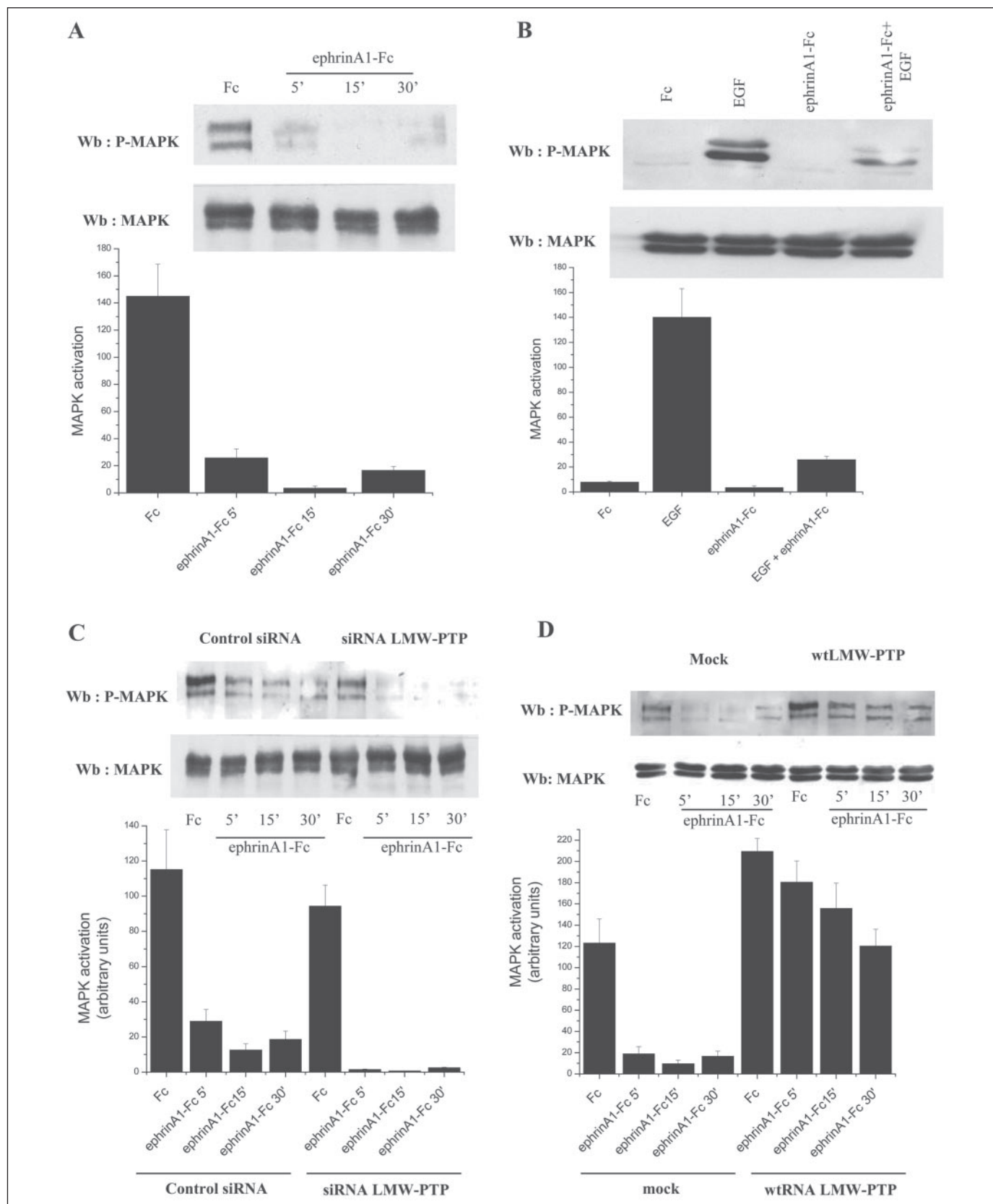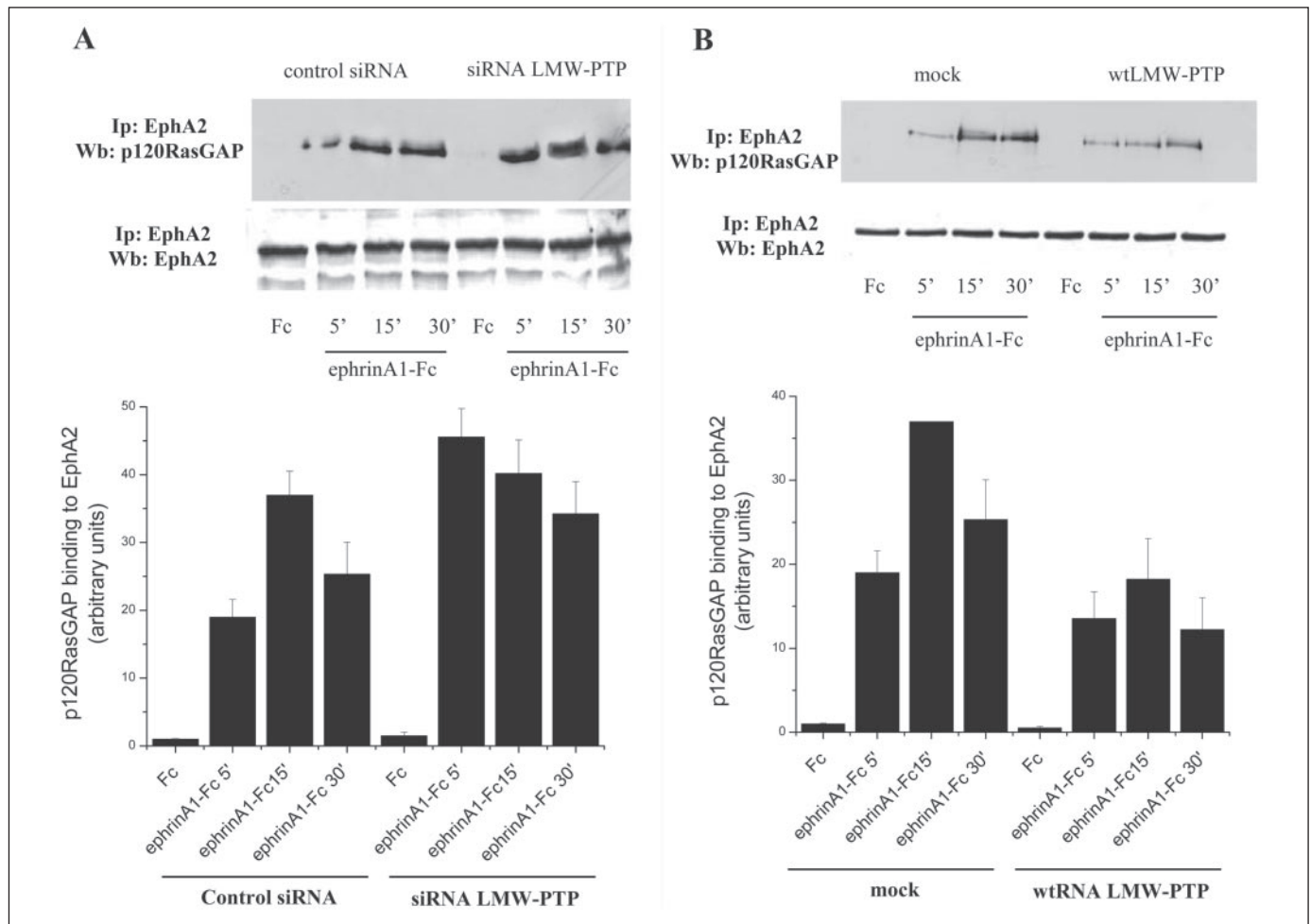
FIGURE 4. **LMW-PTP interferes with ephrin-mediated MAPK regulation.** *A*, ephrinA1 inhibits basal MAPK activity. PC3 cells were serum starved for 24 h before stimulating with either Fc or ephrinA1-Fc for the indicated times. Cells were then lysed in RIPA lysis buffer, and 25 μg of total proteins for each sample was analyzed by anti-phospho-MAPK immunoblot (*Wb*). The blot was then stripped and reprobed with anti-MAPK antibodies for normalization. *B*, ephrinA1 inhibits EGF-induced MAPK activity. Cells were treated as in *A* except that they were stimulated with 50 ng   l EGF with or without ephrinA1-Fc. MAPK activation was evaluated as in *A*. *C* and *D*, LMW-PTP involvement in MAPK regulation by ephrinA1. Cells were treated as in *A* except that LMW-PTP was either silenced (*C*) or overexpressed (*D*) for 72 h. LMW-PTP silencing and overexpression were checked by anti-LMW-PTP immunoblot. MAPK activation was evaluated as in *A*. The *graph* obtained with data from densitometry analysis of replicate experiments plotted relative to loading controls is shown below.

FIGURE 5. **LMW-PTP inhibits p120RasGAP binding to EphA2 kinase.** PC3 cells were serum starved for 24 h before stimulating with either Fc or ephrinA1-Fc for the indicated times. LMW-PTP was either silenced (*A*) or overexpressed (*B*) for 72 h. EphA2 was immunoprecipitated, and an anti-p120RasGAP immunoblot was performed. The blot was then stripped and reprobed with anti-EphA2 antibodies for normalization. LMW-PTP silencing and overexpression were checked by anti-LMW-PTP immunoblot. The *graph* obtained with data from densitometry analysis of replicate experiments plotted relative to loading controls is shown below.

ephrin-mediated MAPK inhibition. Furthermore, the overexpression of wtLMW-PTP in wild-type MEFs powerfully impedes the inhibition of MAPK, strongly resembling the p120RasGAP-null phenotype (Fig. 6*B*). Taken together, these data suggest that the ability of ephrinA1 to down-regulate the MAPK pathway mainly depends on the expression of its negative regulator, namely p120RasGAP and that LMW-PTP is involved in this signaling pathway.

The binding of p120RasGAP to two juxtamembrane sites in EphB2 receptors has already been reported (33). To confirm the role of p120RasGAP and LMW-PTP in EphA2 signaling we analyzed the binding sites of both of these molecules to EphA2 kinase. Sequence comparison between EphA2 and EphB2 indicates Tyr-588 and Tyr-594 in EphA2 kinase as possible p120RasGAP binding sites. We mutated both of these sites and transiently overexpressed wild-type and double mutant EphA2 kinase in HEK-293T cells, which show very low endogenous EphA2 level. Our results show that the binding of both p120RasGAP and LMW-PTP is greatly impaired by Tyr-588 and Tyr-594 mutations, suggesting that these two tyrosines behave as ligand-induced binding sites for both p120RasGAP and LMW-PTP (Fig. 6, *C* and *D*).

Taken together these data suggest that LMW-PTP may inhibit the binding of p120RasGAP to activated EphA2 receptor, likely because of dephosphorylation of the docking site(s) on the stimulated receptor.

The competition for the same site between LMW-PTP and p120RasGAP likely leads to the abolishment of the inhibitory effect on MAPK elicited by ephrinA1.

DISCUSSION

Data presented herein indicate that LMW-PTP negatively regulates the repulsive response elicited by ephrinA1 and interferes with the ephrin-mediated regulation of proliferation, motility outcome, cell adhesion, and spreading, as well as the formation of retraction fibers. LMW-PTP acts as a terminator of ephrinA1 signaling through dephosphorylation of the EphA2 kinase, particularly interfering with ephrin-mediated MAPK signaling through p120RasGAP binding. These data confirm the relevance of the net level of tyrosine phosphorylation of Eph receptors and their key role in the inhibition of MAPK signaling through p120RasGAP recruitment, in the regulation of the motility outcome elicited by the ephrin ligand during epithelial cell communications.

Our data point to the tyrosine phosphorylation level of EphA2 as the molecular motor of the repulsive response elicited by the ligand. Previous data indicated the ability of LMW-PTP to dephosphorylate the EphA2 kinase (17, 22). The dephosphorylation of EphA2 kinase by LMW-PTP was found to be linked to the phosphatase tumorigenic potential because LMW-PTP overexpression is sufficient to induce
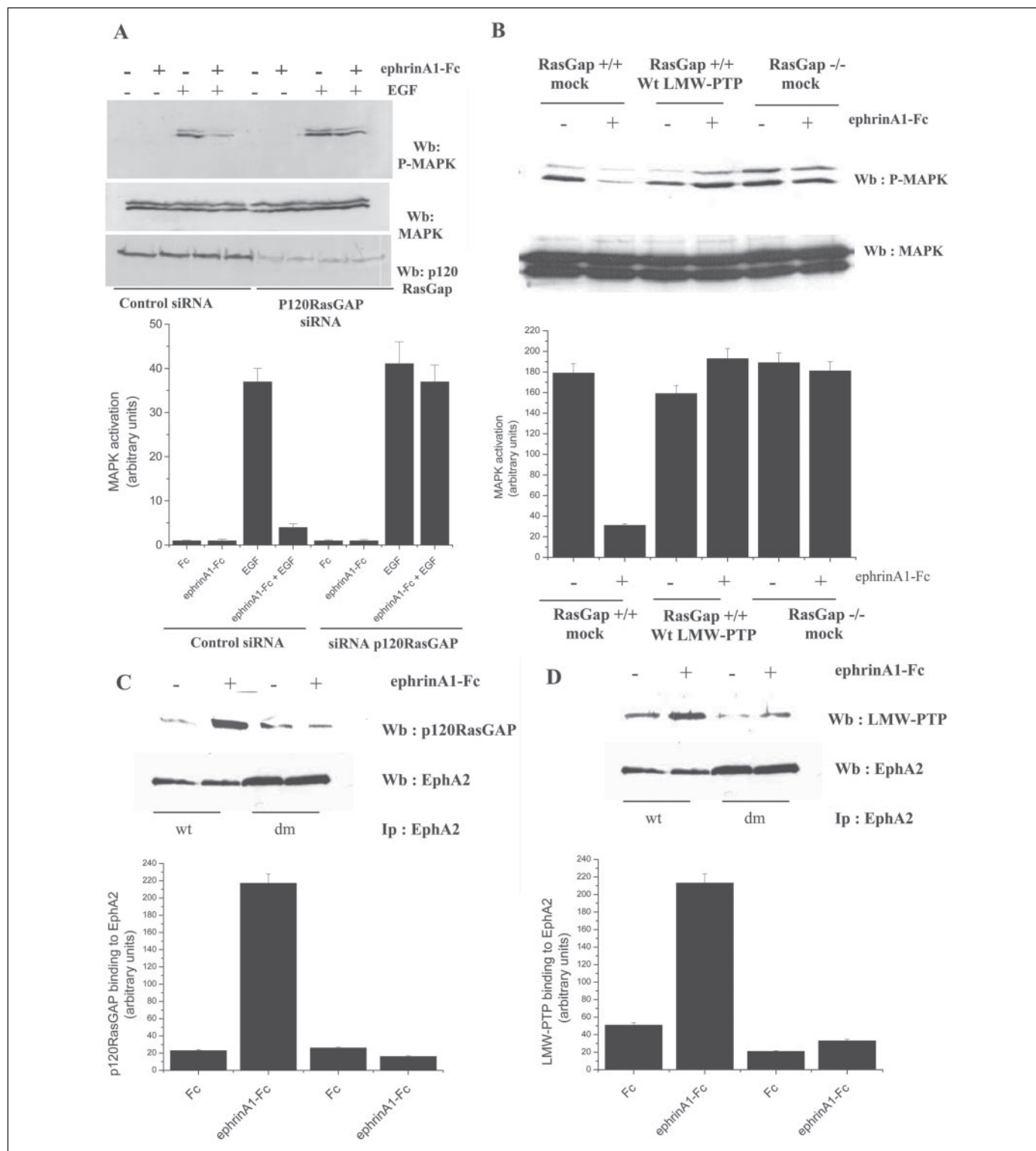
FIGURE 6. **Role of p120RasGAP and LMW-PTP in ephrinA1-mediated MAPK down-regulation.** *A*, PC3 cells, in which p120RasGAP was silenced for 72 h or not, were serum starved for 24 h before stimulating with 50 ng/ml EGF with or without ephrinA1-Fc for 15 min. Cells were then lysed in RIPA lysis buffer, and 25 μg of total proteins for each sample was analyzed by anti-phospho-MAPK immunoblot (*Wb*). The blot was then stripped and reprobed with anti-MAPK antibodies for normalization. *B*, MEFs that are wild type or null for p120RasGAP were serum starved for 24 h before stimulating with ephrinA1-Fc for 15 min. Cells were then lysed and samples treated as in *A*. Where indicated, wtLMW-PTP was transiently overexpressed for 72 h. *C* and *D*, HEK-293T cells were transiently transfected with wild type (*wt*) and double mutant (*dm*) EphA2 kinase for 72 h, serum starved for 24 h, and then stimulated either with ephrinA1-Fc or Fc alone for 15 min. EphA2 was immunoprecipitated (*Ip*), the samples were blotted onto nitrocellulose, and the filter was cut. The upper part was used for an anti-p120RasGAP immunoblot (*C*) and the lower part was used for an anti-LMW-PTP immunoblot (*D*). The upper part of the blot was then stripped and reprobed with anti-EphA2 antibodies for normalization. The *graph* obtained with data from densitometry analysis of replicate experiments plotted relative to loading controls is shown below.

transformation upon nontransformed epithelial cells and greatly enhances the onset and the proliferation rate of solid tumors in nude mice (17, 22). In keeping with these findings we report herein that both overexpression and silencing of LMW-PTP affect the tyrosine phosphorylation level of EphA2 kinase. Hence, in addition to platelet-derived growth factor, EGF, fibroblast growth factor, and insulin receptors, EphA2 kinase has to be included among LMW-PTP-regulated receptor tyrosine kinases. Therefore, for both proliferative and repulsive signals elicited by receptor tyrosine kinase ligands PTP-mediated termination of the signal represents a conserved component of the biochemical machinery triggered by ligands.

LMW-PTP silencing leads to a strong decrease of dephosphorylation although not to a constitutive activation of EphA2 kinase. Because the activation level of a kinase is a balance between the autophosphorylation activity and the counteracting activity of associated PTPs, this effect may be the result of both internalization/degradation of activated EphA2 receptors upon ligand binding or other PTPs next to LMW-PTP. Other PTPs implicated in Eph/ephrin signaling include Src homology phosphatase-2, PTP-basophil-like and PTP-3 (19, 20, 35). Src homology phosphatase-2 is rapidly and transiently recruited to ephrinA1-activated EphA2 and is implicated in the loss of integrin-mediated cell-adhesion (19). Interestingly, also EphB receptor signaling may be negatively regulated by PTP activity as indicated by the ability of PTP-basophil-like, recruited to the ephrinB PDZ targeting site, to dephosphorylate ephrinB1.

Literature on tyrosine phosphorylation of Eph receptors is largely unclear. Tyrosine phosphorylation of EphA2 has been reported to regulate receptor protein stability and is responsible for the recruitment of c-Cbl adapter protein associated with receptor internalization (37). In addition, Holmberg *et al.* (16) reported that the expression of kinase-defective Eph receptor dominates the repulsive response of the full-length receptor, thus abolishing the response to ephrins and suggesting that the level of tyrosine phosphorylation can determine whether a cell responds to its ligand with repulsion or not. On the other hand, recent data revealed that Ephs and/or ephrins retain some tyrosine phosphorylation-independent functions, keeping the debate alive about the role of tyrosine phosphorylation in ephrin signaling (38–40). In the present study we report that the down-regulation of the tyrosine phosphorylation level of EphA2 by LMW-PTP is followed by the inhibition of the cellular outcomes induced by ephrinA1. In particular we report that LMW-PTP interferes with all of the main phenotypic effects elicited by ephrinA1, namely with the formation of retraction fibers, with the inhibition of the cytoskeleton organization during cell adhesion and spreading, and with the inhibition of cell proliferation. Our findings strongly suggest that all of these outcomes elicited by ephrinA1 are guarded by the phosphorylation of EphA2 kinase.

Herein we reported that LMW-PTP dephosphorylation of EphA2 kinase leads to the abolishment of MAPK inhibition exerted by ephrinA1, and we suggest that this effect is likely achieved through the elimination of a binding site for p120RasGAP on the activated EphA2 kinase. The effect of Eph on MAPKs is debated. Although there are few manuscripts showing an increase in MAPK activity following ephrinA1 stimulation (31, 41), there is accumulating evidence that ephrinA1 causes the inhibition of MAPK activity in epithelial cells during branching morphogenesis (26) and neurite collapse (30). Our present data, showing that in PC3 cells ephrinA1 causes the inhibition of both basal and EGF-induced MAPK activity, are in agreement with the last evidence, thus contributing to the depiction of ephrins as negative modulators of MAPK-dependent outcomes. Notably, the data on ephrin-mediated inhibition of EGF-induced MAPKs suggest that the power of

repulsive factors exceeds the proliferative stimulus induced by a growth factor. Thus, when cells respond to multiple signals, which is the most frequent situation, the repulsive/antiproliferative signal is dominant upon the mitotic/motile signal and the site of the conflict is MAPK activity.

The opposite effects of Eph activation on MAPK activity described in literature may be partially explained by the specificity of binding to inhibitors and activators of the MAPK pathway. In most cases, the activating effect of Eph receptors is mediated by direct or indirect employment of the Grb2·Sos1 complex, which acts as a Ras-specific GTP exchange factor (31, 41). Activated EphA2 and B2 receptors down-regulate the Ras/MAPK pathway in neuronal and endothelial cells by direct recruitment of p120RasGAP, a negative regulator of Ras (30, 32, 36, 42). In addition, EphB1 activation does not inhibit MAPK, and this may be explained by its strong association with Grb2 and inability to bind p120RasGAP (24). On the other hand, activated EphB2 induces down-regulation of MAPK activity because it recruits p120RasGAP but not Grb2 (32). These divergent pathways activated by different Eph receptors may account for cell type-specific cellular responses.

In this context we propose that LMW-PTP interferes with the ephrin-mediated signal toward MAPKs, acting on specific tyrosine-phosphorylated sites of EphA2 kinase. LMW-PTP is here reported to regulate negatively the binding to activated EphA2 of p120RasGAP, and we suggest that this causes the block of MAPK inhibition. The binding of p120RasGAP has been described for the EphB2 receptor during axonal guidance, and the two juxtamembrane phosphotyrosines have been proposed as docking sites for its SH2 domain (33). We now confirm that p120RasGAP actually binds to EphA2 receptor as well. Our data propose p120RasGAP as a signal transducer of EphA2 kinase during epithelial cell retraction response, demonstrating that it is mainly responsible for the EphA2-mediated MAPK inhibition, indicating this SH2 containing adapter as a general mediator of ephrin-dependent motility control. Evidence shows that the binding of both p120RasGAP and LMW-PTP to EphA2 is limited to ligand-activated receptors and guarded mainly by the two juxtamembrane tyrosines, namely Tyr-588 and Tyr-594. Thus, upon ligand binding, the competition of LMW-PTP and p120RasGAP for the same phosphorylated tyrosines may lead to dephosphorylation of these sites, to the decrease of p120RasGAP binding to activated receptor, and to the abolishment of the ephrin-mediated inhibition in MAPK signaling.

In conclusion, our present findings indicate that LMW-PTP has a key role in the modulation of ephrinA1 repulsive outcome and that this phosphatase belongs to the biochemical machinery functionally engaged by the ligand to guarantee the completion of the repulsive response. We underline that although it ephrins are thought to be not merely ligands but to have biological activity independent of the kinase activity of their cognate Eph receptor, our findings merge the accumulating evidence that the biological responses mediated by the ephA/EphA system are essentially played by the Eph receptor via signaling pathways actually involving intracellular tyrosine phosphorylation.

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