

PRL Tyrosine Phosphatases Regulate Rho Family GTPases to Promote Invasion and Motility

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Abstract

Phosphatase found in regenerating liver (PRL)-1, PRL-2, and PRL-3 [also known as PTP4A1, PTP4A2, and PTP4A3, respectively] constitute a unique family of putative protein tyrosine phosphatases (PTPs) modified by farnesylation. PRL-3 is amplified and its message is up-regulated in colorectal carcinoma metastases. Its ectopic expression promotes invasive and metastatic properties, supporting a causal link between PRL-3 and late-stage cancer development. However, neither PRL phosphatase substrates nor their signaling pathways have been defined. To address possible mechanisms for the biological activity of PRL-3, we sought to identify its downstream targets, reasoning that regulators of motility and invasion, such as the Rho family of small GTPases, might be logical candidates. We found that levels of active RhoA and RhoC were increased 4- to 7-fold in SW480 colorectal carcinoma cells expressing exogenous PRL-1 and PRL-3, and that PRL-mediated motility and Matrigel invasion were blocked by pharmacologic inhibition of Rho kinase (ROCK), a key Rho effector. In contrast, the activity of Rac was reduced by PRL PTPs, whereas Cdc42 activity was unaffected. PRL-3 stimulated transcription driven by the serum response element in a Rho-dependent manner. We also confirmed that the ability of PRL PTPs to induce invasion and motility is dependent on farnesylation. Catalytic PRL-3 mutants (C104A or D72A) were impaired in PRL-3-induced invasion and Rho activation, indicating that these properties require phosphatase activity. We conclude that PRL PTPs stimulate Rho signaling pathways to promote motility and invasion. Characterization of PRL activity and regulatory pathways should enhance efforts to understand and interfere with PRL-mediated events in invasion and metastasis. (Cancer Res 2006; 66(6): 3153-61)

Introduction

Reversible phosphorylation of proteins on tyrosine residues is a fundamental mechanism by which cells regulate vital signaling pathways controlling virtually all aspects of cell physiology, including proliferation, differentiation, survival/apoptosis, as well as adhesion and motility (1, 2). The proper balance between tyrosine phosphorylation and dephosphorylation is maintained by a large family of protein tyrosine kinases, and an equally large, but much less well-studied family of protein tyrosine phosphatases (PTP; reviewed in ref. 1). Aberrant expression and activation of many receptor tyrosine kinases, such as the epidermal growth

factor receptor, and the proto-oncogene nonreceptor tyrosine kinases Src and Abl, are causative factors in cancer (3). However, dephosphorylation events due to PTP activity are also associated with cancer, suggesting that selective PTP inhibitors might have therapeutic value (reviewed in ref. 4).

The focus of our work is the phosphatase found in regenerating liver (PRL) family of PTPs (also known as PTP4A or PTP-CAAX; reviewed in ref. 5). PRL-1, PRL-2, and PRL-3 constitute a novel family of small ($\cong 19$ kDa), highly homologous PTPs that contain a characteristic PTP motif and catalytic residues. The first PRL to be identified, PRL-1, was discovered as a protein whose expression is rapidly up-regulated in liver following resection (6). The PRL PTPs also contain a carboxyl-terminal prenylation motif (CAAX) similar to those found in Ras family small GTPases, which causes PRL proteins to be modified by farnesylation (7). This feature is unique among PTPs, also suggesting that they may have unique functions compared with other PTPs. Unfortunately, PRL PTPs conspicuously lack other defined structural or functional domains, making it difficult to infer how they might function mechanistically to influence cellular biochemistry and biology.

Considerable evidence has now accumulated that one of these PTPs, PRL-3, is associated with colon tumor invasion and metastasis, suggesting that it may also be an important target for anticancer therapeutics (5). Indeed, several studies have identified PRL-3 as a molecular marker for metastatic tumor cells (8–10). Moreover, the *PRL-3* gene was found to be amplified and its mRNA increased in colorectal cancer metastases versus the corresponding primary tumors (8, 11), and PRL-3 message was found to be generally correlated with colorectal carcinoma tumor progression (10). There is also evidence that PRL-3 expression positively correlates with the progression of other tumor types (5), including gastric tumors, melanomas, prostate tumors, pancreatic tumors, ovarian tumors, and Hodgkin's lymphoma. Moreover, a cause-and-effect relationship between PRL PTPs and tumor metastasis is supported by several recent observations in model systems. First, PRL PTPs promote both cell motility and invasion in cell culture systems *in vitro* (12). Second, they promote metastasis in mouse model systems (12, 13). Third, and most importantly, ablation of endogenous PRL-3 expression by interfering RNA blocked the ability of a naturally metastatic colorectal carcinoma cell line (DLD-1) to form metastasis-like tumors in mice (14). Collectively, these observations strongly support a role for PRL PTPs, especially PRL-3, in tumor progression, and particularly in the metastatic process. As a consequence, PRL-3 might make a tractable target for small-molecule inhibitors designed to prevent and/or treat metastases.

However, the mechanistic basis for PRL function has remained unaddressed. The signaling pathways regulated by PRL PTPs have not been defined, nor have physiologic substrates been convincingly identified *in vivo*. This represents a major gap in our understanding of a novel and potentially important mediator of tumor metastasis. The data presented here begin to define the mechanism by which

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PRL PTPs promote invasion and metastasis. Using a human colon cancer-derived cell system, we show that both PRL-1 and PRL-3 promote the activation of the Rho GTPases RhoA and RhoC, and that signaling through the Rho effector Rho kinase (ROCK) is necessary for PRL-3-induced invasion and motility. In contrast, PRL PTPs reduce the activity of Rac, but have no effect on Cdc42. We also show that PRL-3 is able to activate the serum response element (SRE) transcription factor in a Rho-dependent manner, suggesting that PRL PTPs may regulate motility, invasion, and metastasis both through direct effects on the actin cytoskeleton and through transcriptional regulation of target genes. Our results contribute significantly to the understanding of the role of PRL PTPs in late-stage tumor progression, while also focusing greater attention on the underappreciated role of PTPs in cancer.

Materials and Methods

Plasmid generation. Coding sequences for PRL-1 and PRL-3 (gifts from Silvio Gutkind, NIH, Bethesda, MD) were amplified by PCR and subcloned into either the mammalian expression vector pBABE-HAII-puromycin (15) or pEGFP-C1 (BD Biosciences/Clontech, Mountain View, CA). The parental PRL-3 sequence (PTP4A3v2, NP_009010) was a shorter isoform, which lacks 25 amino acids COOH-terminal to the phosphatase domain, corresponding to amino acids 111 to 135 in the full-length sequence (PTP4A3v1, NP_116000). The biological significance of this form is not clear, but it seems to be inactive and unstable (16). To generate full-length PRL-3, we performed a two-step PCR using primers designed to include the missing amino acids. PRL-3 catalytic point mutants (C104A and D72A) and PRL-1 and PRL-3 SAAX mutants (C170S) were generated using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The correct coding regions of all plasmids were confirmed by sequencing (University of North Carolina-Chapel Hill Genome Analysis Facility).

Transfection of SW480 cells. SW480 colon adenocarcinoma cells (University of North Carolina-Chapel Hill Tissue Culture Facility) were maintained in Leibovitz L-15 medium with glutamine (Mediatech, Inc., Herndon, VA), supplemented with 10% fetal bovine serum (FBS, Sigma, St. Louis, MO) and antibiotics, and grown at 37°C in the absence of CO₂. To generate stable cell lines, SW480 cells were transfected with pBABE-HAII-PRL-1, pBABE-HAII-PRL-3, pBABE-HAII-PRL-3 catalytic mutants (C104A or D72A) or SAAX mutants (C170S), or empty vector using LipofectAMINE Plus (Invitrogen, Carlsbad, CA). At 48 hours after transfection, cells were passaged into and maintained in selective medium containing 1 µg/mL puromycin until colonies formed. At least 40 colonies were pooled for each cell line. Protein expression was confirmed by Western blot analysis for the hemagglutinin (HA) epitope tag (see below). To analyze PRL subcellular localization, SW480 cells were transiently transfected with pEGFP-PRL-1, pEGFP-PRL-3, or corresponding SAAX mutants (C170S) using LipofectAMINE and Plus reagent. Cells were treated for 48 hours with 5 µmol/L FTI-2153 [a gift from Saïd Sebtı (University of South Florida, Tampa, FL) and Andy Hamilton (Yale University, New Haven, CT)], and live cells were visualized and photographed using a Zeiss Axioskop fluorescence microscope equipped with a FITC filter.

Antibodies for Western blot analysis. The primary antibodies used were as follows: mouse monoclonal anti-HA (Covance, Berkeley, CA), rabbit polyclonal anti-RhoA (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit polyclonal anti-RhoC (gift from Kenneth van Golen, University of Michigan, Ann Arbor, MI; ref. 17), mouse monoclonal anti-Rac1 (clone 32A8; Upstate Biotechnology, Lake Placid, NY), and mouse monoclonal anti-Cdc42 (Santa Cruz Biotechnology, Santa Cruz, CA). All primary antibodies were used at 1:1,000 dilution in TBS + 0.1% Tween 20 (TBS-T) and incubated overnight at 4°C. Incubation in horseradish peroxidase-conjugated secondary antibodies (Amersham-Pharmacia, Piscataway, NJ) was for 1 hour at room temperature. All antibody dilutions and washes were done in TBS-T.

Matrigel invasion assays. To evaluate the invasiveness of SW480 cells stably expressing PRL PTPs, BioCoat Matrigel invasion chambers (Becton Dickinson Labware, Bedford, MA) were used according to the protocol of

the manufacturer. Briefly, 5×10^4 cells per cell line were trypsinized, washed, and resuspended in serum-free medium (Leibovitz L-15 + glutamine) and placed in the top portion of the invasion chamber. The lower portion of the chamber contained 10% FBS as a chemoattractant. After 72 hours, cells that had invaded through the Matrigel and migrated to the bottom chamber were fixed in 100% methanol, stained with 1% methylene blue, photographed, and counted. Assays in which cells were exposed to FTI-2153 or a ROCK inhibitor (Y27632, EMD Biosciences/Calbiochem, San Diego, CA) were done as described except that both the top and bottom chambers contained either 5 µmol/L farnesyltransferase inhibitor (FTI) or 5 µmol/L Y27632 throughout the assay.

Scratch wound-healing motility assays. SW480-PRL cells were seeded on 60 mm plates and allowed to grow to confluence. Confluent monolayers were scratched with a pipette tip and maintained under standard conditions for 24 to 48 hours. Plates were washed once with fresh medium to remove nonadherent cells and photographed. For FTI treatment, cells were treated with 5 µmol/L FTI-2153 for 48 hours before wounding and throughout the assay period. Cells were exposed to ROCK inhibitor (5 µmol/L Y27632) immediately after wounding and throughout the assay period.

Rho family GTPase activity assays. SW480 cells stably expressing pBABE-HAII-PRL-1, pBABE-HAII-PRL-3, pBABE-HAII-PRL-3-C104A, pBABE-HAII-PRL-3-D72A, or empty vector were plated at $2 \times 10^6/150$ mm plate and allowed to grow to ~80% confluence. Cells were serum-starved (0%) for 2 hours before lysis, washed once with ice-cold PBS (pH 7.4), lysed for 2 minutes in 1 mL cold lysis buffer [50 mmol/L Tris (pH 7.5), 500 mmol/L sodium chloride, 10 mmol/L magnesium chloride, 1% Triton-X-100, and 10% glycerol] plus protease inhibitors (BD PharMingen), scraped into microfuge tubes, and pelleted at $16,000 \times g$ for 5 minutes. Equal amounts of protein were removed for analysis and brought to equal volumes (500-1,000 µL) with lysis buffer. For RhoA, Rac1, and Cdc42, 200 to 500 µg total protein were used, whereas 2.5 mg was used for RhoC. To each SW480 lysis sample, glutathione-agarose beads containing 30 to 40 µg of glutathione *S*-transferase (GST-Rhotekin (for RhoA and RhoC) or GST-p21-activated kinase (PAK; for Rac and Cdc42) fusion proteins were added and incubated at 4°C for 30 to 60 minutes with rocking. Agarose-GST-Rhotekin or GST-PAK and associated Rho family proteins were pelleted and washed four times with 500 µL wash buffer [25 mmol/L Tris (pH 7.5), 40 mmol/L sodium chloride, and 30 mmol/L magnesium chloride]. Final pellets were resuspended in 1× protein gel sample buffer.

Transcriptional transactivation SRE-luciferase reporter assays. NIH 3T3 fibroblasts were maintained in DMEM-H (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% calf serum (Life Technologies) and antibiotics, and grown at 37°C in 10% CO₂. 1×10^5 cells/35 mm well were transfected using LipofectAMINE and Plus reagent (Invitrogen). For each well, 1 µg pBABE-HAII-PRL-1, pBABE-HAII-PRL-3, pBABE-HAII-PRL-3-C104A, pBABE-HAII-PRL-3-D72A, or empty vector was transfected along with 250 ng SRE-driven luciferase reporter plasmid p(SREm2)-Luc as we have described (18). After 4 hours, medium was replaced with DMEM-H containing 0.2% serum. All assays were done in duplicate. The next day, cells were lysed in luciferase assay lysis buffer (BD Biosciences/PharMingen, San Jose, CA). Luciferase activity was measured with enhanced chemiluminescence reagents (BD Biosciences/PharMingen) in a Monolight 2010 luminometer (Analytical Luminescence, San Diego, CA). To determine whether dominant negative RhoA inhibited PRL-induced SRE activation, luciferase assays were done as described, except that 500 ng/well pBABE-HAII-PRL plasmids (or empty vector) were cotransfected with 0, 250, or 750 ng pZIP-RhoA19N (dominant negative RhoA), and with 250 ng SRE-luciferase reporter plasmid. The total amounts of DNA per well were equalized by the addition of empty pZIPneo vector.

Results

PRL-1 and PRL-3 localize to endomembranes in SW480 colon adenocarcinoma cells in a farnesylation-dependent manner. Increased PRL-3 expression has been observed in a high percentage of colon tumor metastases compared with primary tumors, suggesting that it may contribute to the metastatic process

(8, 11). Supporting this hypothesis, both PRL-1 and PRL-3 promote cell motility, invasion, and metastasis in a variety of model systems (5). However, the mechanisms by which PRL PTPs promote these phenotypes have not yet been elucidated. To begin to define PRL-mediated signaling pathways, we chose to use SW480 colon adenocarcinoma cells, which are derived from a primary, premetastatic colon tumor. We reasoned that introduction of PRL PTPs into these cells might promote an invasive phenotype by mimicking the natural overexpression of PRL-3 in human colon tumor metastases, and might provide an appropriate model system for studying the biochemical mechanism of PRL-induced motility and invasion. To test these hypotheses, it was first necessary to determine whether exogenous PRL proteins were expressed and distributed appropriately within these cells. Figure 1 shows SW480 cells transiently transfected with plasmids coding for either WT PRL-1 or PRL-3 fused to enhanced green fluorescent protein (EGFP) and visualized live by fluorescence microscopy. In live SW480 cells, both PRL-1 and PRL-3 localized primarily to internal punctate structures, possibly endosomes or endoplasmic reticulum, with little staining at the plasma membrane or in the nucleus. This distribution is consistent with some previous reports (19, 20), but not others (6, 8), perhaps due to cell type-specific or cell cycle context-dependent differences.

PRL PTPs are the only tyrosine phosphatases identified to date that are farnesylated (1), and this modification has been shown to be required for their cellular distribution (19, 20). Treatment of SW480-PRL cells with 5 $\mu\text{mol/L}$ FTI-2153 (FTI) for 48 hours caused a complete loss of both PRL-1 and PRL-3 from internal structures and accumulation in the nucleus (Fig. 1) that is characteristic of EGFP-tagged prenylated proteins upon inhibition of prenylation (18). Redistribution of PRL PTPs by FTI is identical to that seen with mutants of PRL-1 and PRL-3 in which farnesylation of the CAAX cysteine is prevented by a cysteine-to-serine mutation (C170S; Fig. 1, SAAX). The dependence of PRL-1 and PRL-3 subcellular distribution on farnesylation in SW480 cells is

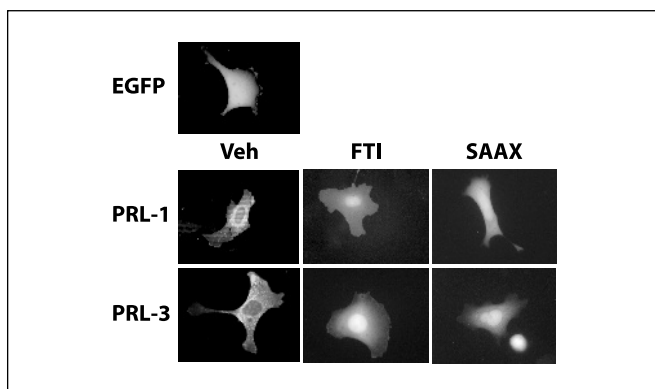


Figure 1. PRL-1 and PRL-3 localize to endomembranes in SW480 colon adenocarcinoma cells in a farnesylation-dependent manner. SW480 cells were transiently transfected with WT PRL-1 or PRL-3, or nonprenylatable PRL-1 and PRL-3 SAAX mutants (C170S), fused to EGFP, treated for 48 hours with either 5 $\mu\text{mol/L}$ FTI-2153 or DMSO (*Veh*), and visualized live by fluorescence microscopy. Consistent with previous reports, vehicle-treated PRL-1 and PRL-3 localize to discrete structures in the cytoplasm, possibly endosomes or endoplasmic reticulum, and are excluded from the nucleus. Treatment with FTI resulted in the loss of PRL-1 and PRL-3 from endomembranes and a characteristic accumulation in the nucleus, presumably by preventing farnesylation. Nuclear accumulation of PRL upon FTI treatment was identical to that seen with PRL SAAX mutants. Together, these observations show a requirement for farnesylation in the distribution of PRL-1 and PRL-3 in SW480 cells.

consistent with previous reports showing inhibition of PRL localization and function by FTI in other cell types (19, 20). From these observations, we conclude that PRL-1 and PRL-3 can be expressed and properly localized within SW480 cells and that these cells may constitute an appropriate system for beginning to delineate the signaling pathways used by PRL PTPs to promote motility, invasion, and metastasis.

PRL-1 and PRL-3 promote farnesylation-dependent invasion and motility in SW480 cells. It was necessary next to determine whether PRL PTPs would promote the invasiveness and motility of these cells as predicted by results from other cell systems (12–14, 21). SW480 cells stably expressing PRL-1, PRL-3, or empty vector were subjected either to Matrigel invasion assays (Fig. 2A) or to scratch wound-healing motility assays (Fig. 2B). As shown by the representative fields above each graph, both PRL-1 and PRL-3 stimulated similarly large increases in the number of cells that invade through Matrigel (5–6-fold) or move into a wound (3–4-fold). Western blot analysis confirmed the approximately equal levels of expression of PRL-1 and PRL-3 (Fig. 2C). Consistent with the farnesylation of PRL PTPs, treatment of PRL-1- or PRL-3-expressing SW480 cells with FTI-2153 completely inhibited both invasion and motility, as did nonfarnesylated SAAX mutants of PRL-1 and PRL-3. Together, these studies show that PRL-1 or PRL-3 promote the farnesylation-dependent invasion and motility in SW480 colon adenocarcinoma cells and further validate the use of these cells as an appropriate and robust model system for analyzing the mechanism of PRL signaling.

PRL PTPs activate RhoA and RhoC, inactivate Rac, and have no effect on Cdc42. Rho family GTPases are key regulators of actin cytoskeletal dynamics associated with cell motility and invasion, and, like PRL-3, their expression and activation generally increase with tumor progression (17, 22, 23). RhoA, the best-characterized Rho GTPase, contributes to cell motility by stimulating contractility and the formation of actin stress fibers (24). The highly homologous GTPase RhoC has distinct as well as overlapping functions with RhoA (25, 26), and its overexpression has recently been closely linked with highly invasive and metastatic forms of breast cancer (17, 23) and melanoma (17, 23). Moreover, cells derived from RhoC-deficient mice are less motile and invasive than their counterparts containing WT RhoC (27), further supporting an important role for RhoC in tumor invasion and metastasis. Rac and Cdc42 also play well-established roles in cell motility. Both GTPases promote actin polymerization associated with membrane-protrusive structures (lamellipodia and filopodia, respectively) at the leading edge of a moving cell (24). In addition, Cdc42 is a key regulator of cell polarization and directional cell movement (24). We hypothesized that PRL PTPs might promote motility and invasion in part by regulating the activities of RhoA, RhoC, Rac, or Cdc42. To address this possibility, we evaluated the levels of active, GTP-bound endogenous RhoA, RhoC, Rac, or Cdc42 in SW480 cells stably expressing PRL-1, PRL-3, or empty vector using either GST-Rhotekin (RhoA and RhoC) or GST-PAK (Rac and Cdc42) to selectively pull down active forms of each GTPase. Figure 3A shows representative Western blot analyses of RhoA and RhoC pulldown experiments, indicating significant increases in active RhoA-GTP and RhoC-GTP in cells expressing PRL-1 or PRL-3 compared with vector. Western blots for total RhoA and RhoC from cell lysates before the pulldown show equal levels of each GTPase across all samples. The data show a 4- to 7-fold increase in active RhoA and RhoC levels (normalized to total levels of each Rho protein) in PRL-1- and PRL-3-expressing cells. These observations suggest that PRL

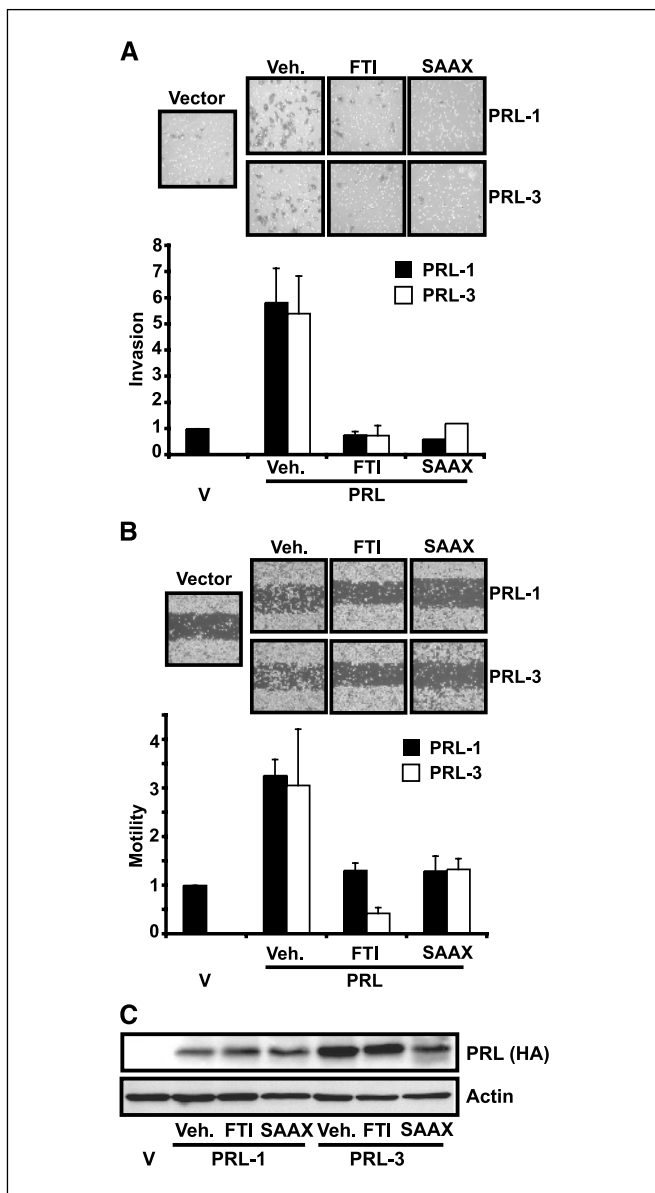


Figure 2. PRL-1 and PRL-3 promote farnesylation-dependent invasion and motility in SW480 cells. SW480 cells stably expressing HA-tagged PRL-1 or PRL-3 were evaluated for invasiveness through Matrigel (A) or motility in a scratch wound-healing assay (B). A, cells (5×10^4) were placed in the top portion of the Matrigel invasion chamber in serum-free medium, whereas the lower portion of the chamber contained 10% FBS as a chemoattractant. Where indicated, 5 $\mu\text{mol/L}$ FTI-2153 was present in both the top and bottom of the chamber for the duration of the assay. After 72 hours, invaded cells were fixed, stained, and counted. Data show a 5- to 6-fold increase in invasion by ectopic expression of PRL-1 or PRL-3, and that invasion was completely blocked by FTI. Consistent with this, PRL-1 and PRL-3 mutants deficient in farnesylation (SAAX) were unable to promote Matrigel invasion; data are shown \pm SD. V, empty vector. B, to evaluate motility, confluent monolayers of SW480-PRL cells were scratched with a pipette tip and cells were allowed to move into the wound for 24 to 48 hours. Where indicated, cells were treated with 5 $\mu\text{mol/L}$ FTI-2153 for 48 hours before scratching and throughout the assay period. As with Matrigel invasion, PRL-1 and PRL-3 stimulate a 3- to 4-fold increase in motility that is blocked by FTI, whereas unfarnesylated PRL SAAX mutants fail to promote motility; data are shown \pm SD. C, anti-HA staining confirmed equivalent expression of exogenous PRL in the cell lines evaluated.

PTPs promote the activation of these Rho GTPases. In contrast, the level of GTP-bound active Rac, which was selectively pulled down using GST-PAK, was reduced 60% to 70% in cells expressing PRL-1 or PRL-3, whereas levels of active Cdc42 were unaffected (Fig. 3B).

That PRL PTPs can produce opposite effects on different Rho family GTPases suggests that they affect cell motility, invasion, and metastasis on multiple levels, and that their regulatory mechanisms are likely to be complex. These important observations shed new light on a biochemical mechanism of PRL function, and potentially identify PRL PTPs as important regulators of Rho family GTPases and cell motility.

PRL-1 and PRL-3 require the Rho effector ROCK to promote invasion and motility. Although activation of RhoA and RhoC downstream of PRL PTPs suggests a functional connection, it does not show that signaling downstream of Rho GTPases is required for

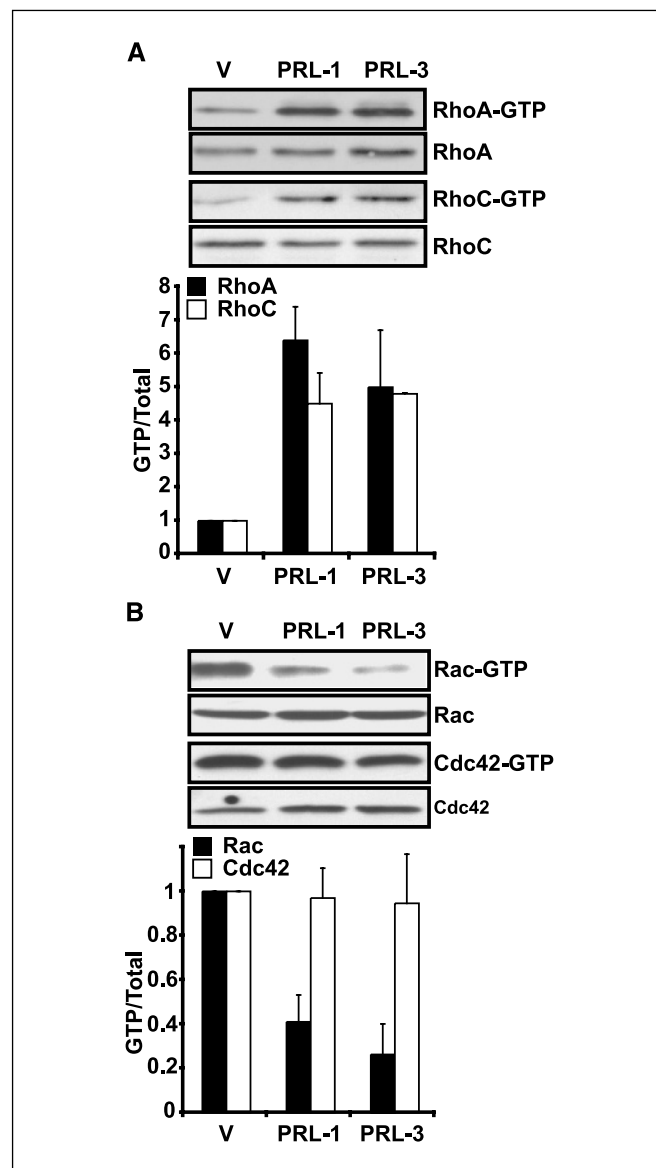


Figure 3. PRL PTPs activate RhoA and RhoC, inactivate Rac, and have no effect on Cdc42. SW480 cells expressing PRL-1, PRL-3, or empty vector were evaluated for levels of active RhoA or RhoC by GST-Rhotekin pull-down, or for active Rac and Cdc42 by GST-PAK pull-down. A, higher levels of active RhoA (RhoA-GTP) and RhoC (RhoC-GTP) were present in PRL-1- and PRL-3-expressing cells compared with empty vector cells. Quantitation of Rho-GTP levels normalized to total Rho in pull-down lysates showed that WT PRL-1 and PRL-3 increased RhoA-GTP and RhoC-GTP levels by 4- to 6-fold over vector. B, in contrast, levels of active Rac-GTP were reduced 60% to 70% in PRL-expressing cells, whereas active Cdc42-GTP levels were unaffected. Data are shown \pm SD.

PRL function. To determine whether signaling through Rho GTPases is necessary for PRL-induced invasion or motility, we evaluated the effect of Y27632, a selective inhibitor of the Rho effector ROCK, on PRL-induced Matrigel invasion and motility. ROCK is a major effector of Rho family GTPases and is activated by both RhoA and RhoC (28). ROCK mediates Rho-dependent effects on stress fibers by phosphorylating other signaling intermediates, such as LIM kinase and myosin light chain phosphatase (28). As shown in Fig. 4, both invasiveness and motility of SW480-PRL-1 and SW480-PRL-3 cells were completely blocked by treatment with 5 $\mu\text{mol/L}$ Y27632, demonstrating that PRL PTPs require ROCK to promote these phenotypes. Together with our observation that PRL-1 and PRL-3 increase RhoA and RhoC activities, this strongly implicates the Rho/ROCK pathway in PRL function.

PRL-1 and PRL-3 activate the SRE transcription factor in a Rho-dependent manner. In addition to their direct effects on the actin cytoskeleton and cell motility, Rho GTPases also regulate gene expression through activation of transcription factors (29). To determine whether PRL-1 or PRL-3 might affect transcriptional regulation through their influence on Rho activity, we assessed their ability to activate the SRE transcription factor, which is activated downstream of Rho GTPases (30). Figure 5A shows that cotransfection of NIH 3T3 fibroblasts with PRL-1 or PRL-3 robustly stimulated SRE, producing 40- to 50-fold activation over vector, suggesting that PRL PTPs may contribute to invasion, motility, and metastasis in part through the regulation of invasion- and motility-related genes.

To determine whether PRL-3 activation of SRE requires signaling through Rho GTPases, we cotransfected PRL-3 with a dominant negative mutant of RhoA (RhoA19N), which irreversibly binds to Rho guanine nucleotide exchange factors (GEF), thereby sequestering them and preventing their activation of WT endogenous Rho GTPases. Figure 5B shows that increasing concentrations of dominant negative RhoA produced a dose-dependent inhibition of PRL-3-induced SRE activation, demonstrating that Rho GTPases function downstream of PRL-3 to activate SRE. Together with the activation of RhoA and RhoC by PRL-3, these observations support a model in which PRL-3 stimulates Rho GTPases to promote SRE activation as well as invasion and motility. Whether the activation of SRE or other transcription factors by PRL-3 directly contributes to its promotion of cell invasion are questions that warrant further investigation.

PRL-3 phosphatase activity is required for Rho family GTPase regulation and Matrigel invasion. Aside from a tyrosine phosphatase domain and a CAAX prenylation sequence, PRL PTPs lack other defined structural or functional motifs, suggesting that they may exert their biological effects primarily through their phosphatase activity. Several studies showing that catalytic PRL mutants lose biological function support this conclusion (13, 20, 21). To determine whether the PRL-dependent phenotypes, especially regulation of Rho family GTPases, that we observed in SW480s could be attributed to phosphatase activity, we generated PRL-3 mutants that are putatively inactive because of substitution of conserved catalytic site residues (C104A or D72A), and evaluated these mutants for invasion, motility, Rho GTPase activity, and SRE activation as already described. Figure 6A shows that catalytically inactive PRL-3 mutants were unable to promote Matrigel invasion, consistent with phosphatase activity being the primary mode of PRL function. Similarly, neither mutant was able to regulate the activities of RhoA or RhoC (Fig. 6B) in contrast to WT PRL-3. Interestingly, the C104A catalytic mutant, but not D72A, reduced

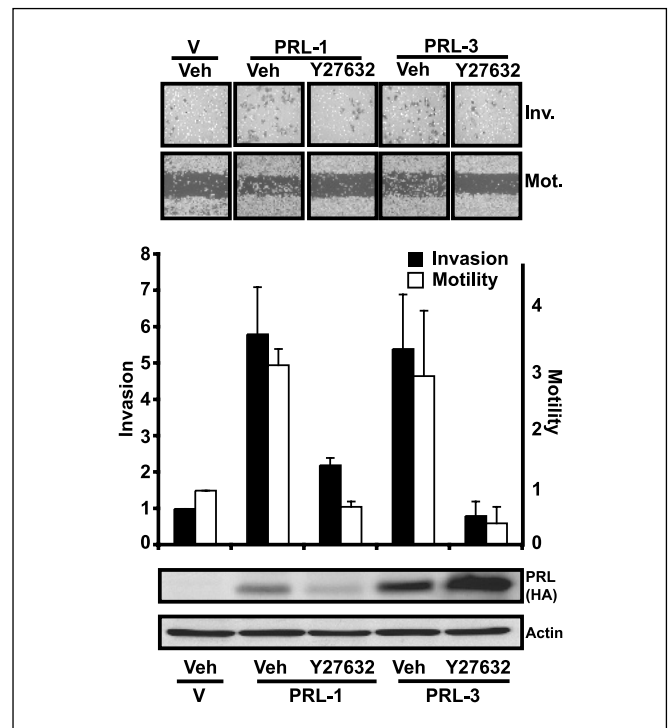


Figure 4. PRL-1 and PRL-3 require the Rho effector ROCK to promote invasion and motility. To determine whether PRL-induced invasion or motility requires signaling downstream of Rho, we evaluated the effect of Y27632, an inhibitor of the Rho effector ROCK, on Matrigel invasion and scratch wound healing of SW480 cells stably expressing PRL-1 or PRL-3. Although both PRL-1 and PRL-3 induced a 5- to 6-fold increase in Matrigel invasion and a 3- to 4-fold increase in motility, treatment with 5 $\mu\text{mol/L}$ Y27632 completely blocked each of these effects; data are shown \pm SD.

the level of active Rac comparably to WT PRL-3 (Fig. 6C). These results suggest that PRL-3 catalytic mutants may retain some functional ability, perhaps scaffolding, and their consequences are functionally distinct. As expected, neither mutant affected Cdc42 activity, consistent with the lack of effect of WT PRL-3. The correlation between PRL-3 catalytic ability and both invasion and Rho family GTPase regulation further support our model that RhoA, RhoC, and Rac are important intermediates in the signaling pathways used by PRL-3 to promote invasion and possibly metastasis.

Interestingly, catalytic activity seems to be at least partly dispensable for the promotion of motility in a wound-healing assay (Fig. 6D) and of SRE activation (Fig. 6E). Both PRL-3-C104A and PRL-3-D72A stimulated SW480 cell motility to the same level as WT PRL-3 (\sim 3-fold). Identical results were observed using a second set of polyclonal SW480 stable cell lines generated at a different time (data not shown), suggesting that this observation was not due simply to peculiarities of the particular cell isolates. Both PRL-3 mutants also retained the ability to promote the activation of SRE (20-30-fold; Fig. 6E), although they did so less strongly than WT PRL-3 (50-fold), suggesting that catalytic activity is partly attenuated by each mutation. Together, these results suggest that PRL-3-induced invasion and Rho GTPase activation may be functionally separable from motility and SRE stimulation, and that the latter may not require PRL-3 phosphatase activity. Alternatively, each of the catalytic mutants may retain a small amount of catalytic activity that is sufficient to promote motility and SRE activation, but is insufficiently robust to support invasion

and regulation of RhoA, RhoC, and Rac activity, at least at the level of detection possible in these assay systems.

Discussion

The majority of cancer deaths result from tumor metastases rather than from primary tumors. Yet, the biochemical mechanisms that regulate invasion and metastasis remain incompletely defined. Recently, the putative PTP PRL-3 has been strongly implicated in colon cancer metastasis, based on several observations. First, PRL-3 is amplified and overexpressed in colorectal cancer metastases compared with primary tumors (8, 10, 11), and its expression correlates with metastasis in other tumor types (5). Second, PRL-3 and the related PTPs, PRL-1 and PRL-2, promote cell motility, invasion, and metastasis in cell culture and mouse model systems (12–14, 21). Third, ablation of endogenous PRL-3 expression blocks the normal invasiveness of a colon tumor cell line (14). Although these observations argue strongly for a causative role for PRL-3 in colorectal carcinoma invasion and metastasis, they do not provide information about the biochemical or signaling mechanism(s) by which PRL-3 promotes these processes.

We hypothesized that PRL PTPs might modulate small GTPases of the Rho family to promote invasion and metastasis. Rho family GTPases, including RhoA, RhoC, Rac, and Cdc42, are critical

regulators of actin organization associated with cell motility, cell cycle progression, and gene expression (26), and there is considerable evidence that aberrant Rho GTPase activation promotes tumor cell growth, invasion, and metastasis (22, 29). Using a human colon cancer–derived cell system, we show here that the PTPs PRL-1 and PRL-3 promote the activation of the Rho family GTPases RhoA and RhoC, reduce the activity of Rac, but have no effect on Cdc42. We also show that signaling through the Rho effector ROCK is necessary for PRL-induced invasion and motility, and that PRL-induced activation of the SRE transcriptional promoter requires Rho. Collectively, these observations strongly imply that Rho family GTPases act downstream of PRL PTPs to promote motility, invasion, and possibly metastasis. We have also shown that invasion, motility, and Rho activation are sensitive to FTIs, which is consistent with the farnesylation of PRL PTPs. Interestingly, although phosphatase activity is crucial for PRL-3-induced invasion and Rho activation, it seems to be at least partly dispensable for motility and SRE activation, suggesting that PRL-3 may have other functions unrelated to dephosphorylation.

RhoA and RhoC are highly homologous both structurally and functionally (reviewed in ref. 25). Both are activated by an overlapping set of GEFs (31), suggesting that they should be activated by many of the same stimuli, including PRL PTPs according to our data. Similarly, they both bind to and activate the same set of downstream effectors (25), although they may do so with different affinities and localization, possibly accounting for their functional differences. Of the numerous effectors shared by RhoA and RhoC, the best studied is the serine/threonine kinase ROCK (reviewed in ref. 28), which regulates actin polymerization associated with motility and invasion through a variety of mechanisms, suggesting that PRL-induced, Rho-dependent invasion and motility would require ROCK activity. We were able to confirm this prediction by showing that inhibition of ROCK blocked these phenotypes. Although our data suggest that PRL signaling through the Rho/ROCK pathway is necessary for invasion and motility, they do not rule out the need for other motility-related Rho effectors, such as mDiaphanous (25).

Our observation that PRL PTPs, which promote motility and invasion, reduce Rac activity was surprising given that Rac promotes motility by stimulating actin polymerization and the formation of lamellipodia (24). However, Rac activity has also been reported to promote E-cadherin-mediated cell-to-cell adhesion, and thereby to antagonize cell invasion (32), supporting our observation that reduced Rac activity correlates with increased invasiveness. Moreover, our observation that Rho and Rac activities are inversely regulated by PRL PTPs is consistent with recent evidence that Rac and Rho can each inhibit the activity of the other (reviewed in ref. 33), and that this opposition may be necessary for coordinating cell motility. Neither PRL-1 nor PRL-3 seems to regulate global Cdc42 activity, suggesting that these PTPs may not influence cell polarity associated with directional cell movement.

Although we have clearly shown an important functional connection between PRL PTPs and Rho GTPases, the nature of this connection remains unclear. Rho family GTPases are active when bound to GTP and inactive when bound to GDP. GEFs promote release of GDP and binding of GTP, leading to activation. GTPase activating proteins (GAP) promote the cleavage of GTP back to GDP, leading to inactivation (26). The stimulation of RhoA and RhoC GTP-loading by PRL PTPs suggests that they either lead to the activation of Rho GEFs or to the inactivation of Rho GAPs, whereas the inactivation of Rac suggests that PRL PTPs may have

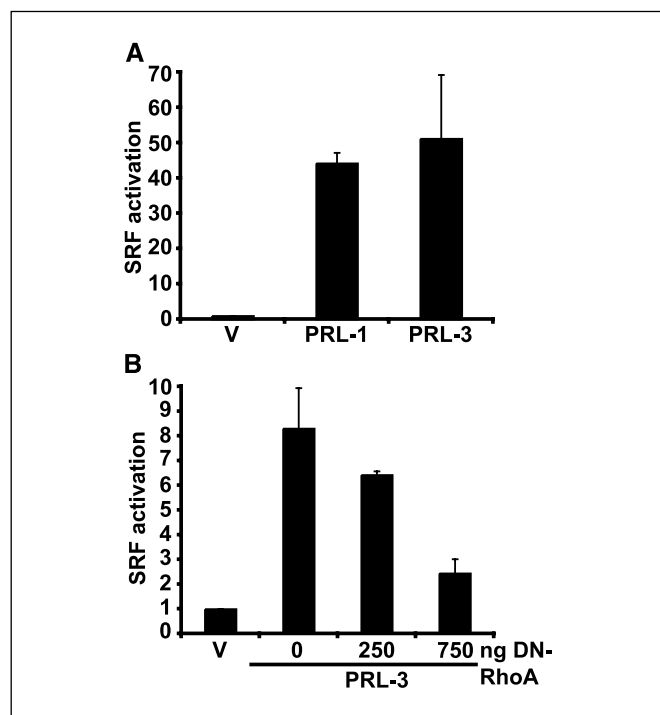
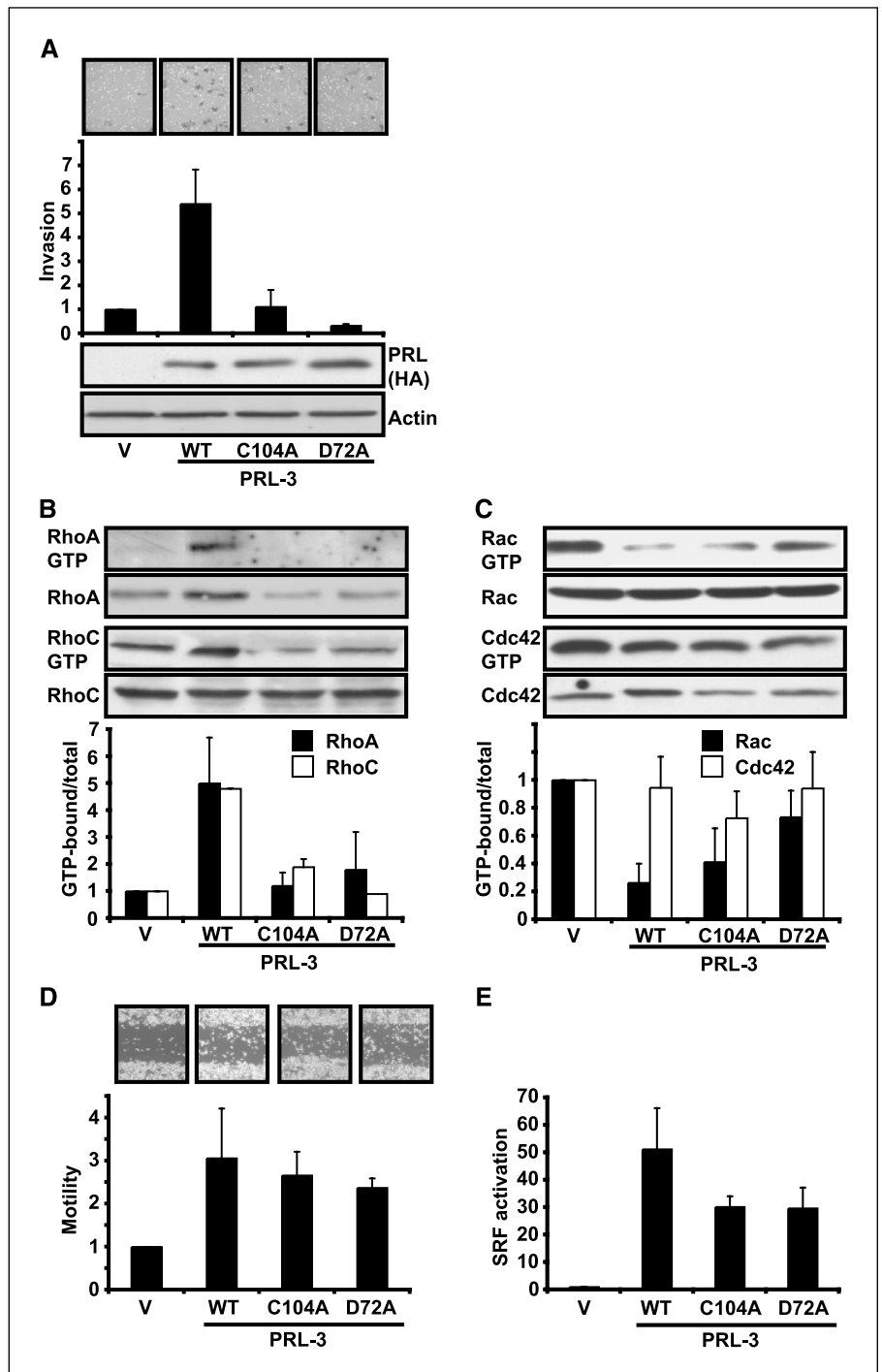


Figure 5. PRL-1 and PRL-3 activate the serum response factor transcription factor in a Rho-dependent manner. To determine whether PRL-1 or PRL-3 may affect gene regulation downstream of Rho GTPases, we evaluated the ability of PRL-1 or PRL-3 to activate serum response factor in a transcriptional transactivation assay. NIH 3T3 fibroblasts were transfected with plasmid coding for PRL-1, PRL-3, or empty vector along with a plasmid in which luciferase expression is controlled by endogenous serum response factor. After 24 hours in low serum (0.2%), cells were lysed and analyzed for luciferase activity using a luminescent substrate. A, both PRL-1 and PRL-3 strongly stimulate serum response factor, producing 40- to 50-fold activation over vector. B, cotransfection of cells with both PRL-3 and increasing concentrations of dominant negative (DN) RhoA(S19N) blocked PRL-3-induced serum response factor activity in a dose-dependent manner. Three independent assays were done. Data are shown \pm SD.

Figure 6. PRL-3 phosphatase activity is required for Rho family GTPase regulation and Matrigel invasion. To determine whether PRL-dependent phenotypes required tyrosine phosphatase activity, PRL-3 mutants lacking putative catalytic residues (C104A and D72A) were evaluated for Rho family GTPase regulation, invasion, motility, and serum response factor activation. *A*, SW480 cells stably expressing PRL-3 or catalytic mutants were subjected to Matrigel invasion assays. Although WT PRL-3 stimulated invasion 5- to 6-fold, phosphatase-dead mutants failed to do so. Data are shown \pm SD. *B*, similarly, PRL-3 mutants failed to activate RhoA and RhoC, whereas WT PRL-3 promoted a 4- to 5-fold increase. *C*, the PRL-3-induced reduction in Rac activity was lost in the PRL-3 D72A mutant, although PRL-3 C104A retained some ability to decrease Rac-GTP levels. Consistent with the inability of WT PRL-3 to modulate Cdc42, neither PRL-3 mutant had any effect on Cdc42 activity. *D*, motility of SW480 cells was evaluated by scratch wound-healing assays, which showed that both WT PRL-3, as well as putatively inactive mutants, stimulate motility, suggesting that PRL-3 may have phosphatase-independent functions. *E*, similarly, both WT PRL-3 and catalytic mutants activate serum response factor in a transcriptional transactivation assay (50- and 30-fold, respectively), although the mutants do so less robustly.



the opposite effect on Rac-specific GEFs and/or GAPs. Whether PRL PTPs regulate Rho GEFs and how they may do so remains to be determined. However, some Rho GEFs are activated by tyrosine phosphorylation by Src family kinases (34–36), suggesting that PRL PTPs, like several other PTPs (37), might dephosphorylate and activate SFKs to regulate Rho GEFs and thereby GTPase activities.

The biochemistry controlling the physical processes of cell motility and invasion involves many proteins, aside from Rho GEFs and GAPs, which are regulated by reversible tyrosine phosphorylation. These include Src family kinases, focal adhesion kinase, paxillin, and p130Cas, suggesting several other points at which PRL

PTPs might exert their influence. Indeed, several PTPs regulate cell motility by modulating actin cytoskeletal dynamics downstream of integrin engagement by extracellular matrix (reviewed in ref. 38). Because no PRL substrates have yet been identified *in vivo*, it is not clear whether PRL PTPs use a set of substrates overlapping with or distinct from other PTPs.

The sensitivity of PRL-3-induced invasion, motility, and Rho activation to farnesylation also suggests that PRL-3 may be a biologically relevant target of FTIs, which are known to exert their anticancer effects in part through the functional inhibition of proteins other than those of the Ras family (39, 40). It has been

reported that FTI reverses the loss of cell-to-cell adhesion associated with invasion, and decreases the metastatic potential of colon, liver, and breast cancer cell lines (41), suggesting that PRL PTPs may promote invasion through FTI-reversible effects on cell-to-cell contacts. Together with the well-established contribution of farnesylated PRL-3 to invasion and metastasis, this observation suggests that inhibition of PRL PTPs by FTIs may help to prevent the development of invasive and metastatic cancers, a possibility that warrants further research. It also suggests that selective small-molecule inhibitors of PRL PTPs may be clinically useful.

We consider it unlikely that residual activity of the PRL-3 mutants may explain their stimulation of motility and SRE activation because both D72 and C104, which correspond structurally to the catalytic residues of other PTPs (42), are thought to participate directly in catalysis. Another possibility is that PRL-3 mutants may influence the activity of their substrates despite failing to dephosphorylate them. Extensive literature on tyrosine phosphatase substrate-trapping mutants (reviewed in ref. 43) analogous to the C104A and D72A PRL mutations used here documents that such substrate-trapping mutants form stable complexes with their phosphorylated substrates allowing for substrate identification. It seems possible that this association may be sufficient to modulate the activity of PRL-3 substrates, resulting in stimulation of some aspects of PRL-3 function. Regardless of the mechanism by which catalytically inactive PRL-3 mutants promote motility and SRF activation, our observations suggest that these phenotypes are biochemically separable from Matrigel invasion and Rho GTPase activation, possibly as a result of PRL-3 regulating distinct sets of substrates in each case. It will be interesting to unravel the specific

substrates used by the PRL phosphatases to regulate motility, invasion, and metastasis.

In light of the increasing interest in PRL-3 and its correlative and functional connections with invasion and metastasis, it is particularly important to clearly define the signaling pathways that PRL PTPs use to promote these phenotypes, as we have begun to do here. Although a complete understanding of the mechanism of PRL function awaits the identification of PRL substrates, our studies provide the first information concerning the signaling mechanisms used by PRL PTPs to promote invasion and metastasis. In characterizing a novel mechanism in motility- and metastasis-related signaling, our studies also contribute to the understanding both of the basic mechanisms underlying normal cell motility, and of the pathology of invasion and metastasis, processes with enormous implications for the treatment of cancer. They also provide new information regarding tyrosine phosphatases, a large and diverse class of enzymes that promises to be crucially important in regulating cell signaling, but whose roles in both normal and aberrant cell physiology are only beginning to be appreciated. Our studies may also have clinical implications insofar as they may further validate PRL-3 as a molecular marker for metastasis and additionally suggest other, as yet unidentified, markers involved in PRL-3 function that could improve early detection of tumors with high metastatic potential.

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PRL Tyrosine Phosphatases Regulate Rho Family GTPases to Promote Invasion and Motility

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