Phosphatase Activity, Trimerization, and the C-terminal Polybasic Region Are All Required for PRL1-mediated Cell Growth and Migration*

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The phosphatase of regenerating liver (PRL) phosphatases are implicated in a number of tumorigenesis and metastasis processes. The PRLs are unique among protein-tyrosine phosphatases in that they have extremely low phosphatase activity, a high propensity for trimer formation, and a polybasic region that precedes the C-terminal prenylation motif. To investigate the functional significance of these distinctive biochemical and structural features, we established a cell-based system in which ectopic PRL1 expression increased cell proliferation and migration, whereas knockdown of endogenous PRL1 abrogated these cellular activities. We showed that the intrinsic PRL1 phosphatase activity is obligatory for its biological function. We provided evidence that trimerization may be a general property for all PRL enzymes, and that PRL1 trimer formation is essential for the PRL1-mediated cell growth and migration. This finding indicates a novel mechanism for phosphatase regulation. We further demonstrated that the conserved C-terminal polybasic region is important for specific phosphoinositide recognition by PRL1. Both the polybasic residues and the adjacent prenylation motif are required for proper PRL1 subcellular localization and full biological activity.

Protein-tyrosine phosphatases (PTPs) constitute a large family of enzymes (>100) that can function either as positive or negative modulators in signal transduction pathways controlling cell proliferation, differentiation, survival/apoptosis, as well as adhesion and motility (1, 2). Defective or inappropriate regulation of PTP activity leads to aberrant tyrosine phosphorylation, which contributes to the development of many human diseases, including cancer (3, 4). The PRL phosphatases represent a novel subfamily of PTPs (5–7), which are implicated in a number of tumorigenesis and metastasis processes (8). The PRLs are unique among the PTPs in that they possess a C-terminal prenylation motif CAAX, where C is cysteine, A is an aliphatic residue, and X is any amino acid. Indeed, all three phosphatases are prenylated at their C terminus, which is important for their localization to the plasma membrane and early endosomal compartments (6, 9, 10).

PRL1 (also known as PTPA1 and PTPCAAX) was originally identified as an immediate early gene in regenerating liver (5). Subsequent studies revealed that PRL1 expression is elevated in several tumor cell lines, and cells expressing high levels of PRL1 exhibit enhanced proliferation and anchorage-independent growth (9, 11–13). Interestingly, up-regulation of the related PRL2 and PRL3 also promotes cell growth and proliferation (6, 13–17). Interestingly, up-regulation of the related PRL2 and PRL3 also promotes cell growth and proliferation (6, 13–17). In addition to a role in cell proliferation, the PRLs are also involved in tumor metastasis. For example, the PRL3 message is amplified in colorectal cancer metastases, whereas its expression in primary tumors and normal colorectal epithelium is undetectable (18). Furthermore, cells stably expressing PRL1 or PRL3 display enhanced motility and invasiveness and induce metastatic tumor formation in mice (16, 17). Finally, alteration of PRL1 expression in a number of cancer cell lines leads to changes in cell adherence and invasive properties (19, 20).

Given their potential involvement in human malignancies, the PRLs have emerged as highly attractive targets for novel anticancer therapy (8). Unfortunately, little is known about the biochemical basis for the PRL-mediated signaling pathway(s). In addition, their physiological substrate(s) are unknown, and the intrinsic phosphatase activity of PRLs toward artificial chromogenic substrates is extremely low (21, 22), raising the question of whether the phosphatase activity is required for PRL function. Furthermore, PRLs are among the smallest PTPs (~170 residues), consisting of a single catalytic domain with no auxiliary docking/regulatory domains other than the prenylation site at the C terminus (Fig. 1). The lack of additional defined structural domains makes it difficult to infer their cellular mechanisms.

To begin to elucidate the roles of the PRL phosphatases in normal cell physiology and in oncogenic processes, a number of structural and mechanistic studies have been initiated (21–23). We solved a number of crystal structures of native PRL1 as well as the catalytically inactive mutant PRL1/C104S in complex with sulfate (22). These structures provided a molecular explanation for the extremely low phosphatase activity and offered a potential mechanism for PRL activation. Surprisingly, the

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4 The abbreviations used are: PTP, protein-tyrosine phosphatase; PRL, phosphatase of regenerating liver; pNPP, p-nitrophenyl phosphate; GFP, green fluorescence protein; HA, hemagglutinin; MTI, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide; RT, reverse transcription; PI, phosphoinositide; PBS, phosphate-buffered saline; DMEM, Dulbecco’s modified Eagle’s medium; GST, glutathione S-transferase; shRNA, short hairpin RNA.
structures revealed that PRL1 exists as a trimer in the crystalline state. Trimerization creates a large, bipartite membrane-binding surface in which the exposed C-terminal basic residues could cooperate with the adjacent prenylated group to anchor PRL1 on the acidic inner membrane. This study was designed to determine the functional significance of these structural observations. We present evidence herein that the phosphatase activity, trimerization, and the C-terminal polybasic region are all required for the PRL1-mediated cell growth and migration. Moreover, we show that the C-terminal polybasic region is responsible for binding distinct phosphoinositides.

**EXPERIMENTAL PROCEDURES**

**Materials**—p-Nitrophenyl phosphate (pNPP) was purchased from Fluka. Zeocin was purchased from Invitrogen. Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum, penicillin, and streptomycin were from Invitrogen. Anti-HA antibody was purchased from Santa Cruz Biotechnology. All other chemicals and reagents were of the highest grade available commercially.

**Constructs and Cell Lines**—For PRL1 expression in mammalian cells, the full-length PRL1 cDNA was subcloned into pcDNA4 vector with an HA tag on the N terminus. PRL1 mutants were generated using QuikChange site-directed mutagenesis kit from Stratagene. Mutations were confirmed by DNA sequencing. For small hairpin RNA interference, a specific 21-oligonucleotide sequence (GTTTAAGGTCGCATTGTGGTGCCCTTCCGTCAATTC-3) targeting the PRL1 mRNA region (571–591) was subcloned into the pSilencer 1.0-U6 construct (Ambion).

**Establishment of Stable Cell Lines**—HEK293 cells were grown at 37°C under an atmosphere of 5% CO2 in DMEM supplemented with 10% fetal bovine serum. Constructs containing PRL1 or its mutants were transfected into HEK293 cells using FuGENE 6 (Roche Applied Science). The day after transfection, Zeocin was added to the culture dishes (500 μg/ml), and drug-resistant cells were allowed to grow for 14 days. Individual Zeocin stable colonies were picked and tested for PRL1 expression level. Clones with PRL1 mRNA at about 1–2-fold over the endogenous level were selected.

**RT-PCR**—Total RNAs were isolated from cultured HEK293 cells by TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. RNA concentration and quality were determined by spectrophotometry. Reverse transcription-PCR was performed with SuperScript one-step RT-PCR kit (Invitrogen) according to the manufacturer’s instructions. Equal amounts of RNA (200 ng) were used as templates in each reaction. The primers used for PRL1 amplification were 5’-CCAGCTCTCTGGAGTCAC-3’ (forward) and 5’-CCATCATCAGGGCAATC-3’ (reverse). As a control, the primers used for 18 S rRNA amplification were 5’-CACAGGGAAGCATTTGGCAAC-3’ (forward) and 5’-GTGGTGCCCTCCGTCAATTC-3’ (reverse).

**Western Blotting**—Immunoblotting—0.5 ml of lysis buffer (50 mM Tris–HCl, pH 7.5, 1% Tween 20, 5 mM EGTA, 150 mM NaCl, 10 mM sodium phosphate, 5 mM iodoacetic acid, 1 mM Na2VO4, 1 mM benzamidine, 10 μg/ml leupeptin, and 10 μg/ml aprotinin) were added to each 10-cm dish once it reached 80% confluency. Cells were scraped with a rubber policeman and lysed on ice for 30 min. During this period, 10 mM dithiothreitol was added to reduce the iodoacetic acid. The cell lysate was then pre-cleared by centrifugation at 10,000 relative centrifugation force for 15 min. Protein concentration of the lysate was estimated using the Bio-Rad protein assay reagent with a series dilution. The Western blotting was done according to standard procedures.

**Immunofluorescence Microscopy**—Cells growing on glass coverslips were washed with PBS and fixed with 4% paraformaldehyde followed by permeabilization with 0.1% Triton X-100. Cells were blocked in 10% normal goat serum/PBS for 1 h at room temperature and incubated with primary anti-HA antibody in blocking buffer at 4°C overnight. Cells were then washed with PBS plus 0.05% Tween 20 and incubated with 4′,6-diamidino-2-phenylindole and goat anti-mouse IgG-conjugated fluorescein isothiocyanate in PBS plus 0.05% Tween 20 with 2% goat serum for 1 h. After washing with PBS, the coverslips were mounted with anti-fade mounting solution. Confocal microscope images were obtained with a Zeiss imaging system.

**MTT Assay**—PRL1 stable cell lines were seeded in a 96-microwell plate (1000 cells/well) containing DMEM, 10% fetal bovine serum. Cell proliferation was then determined over a 120-h period by means of the MTT assay (24), using a multwell spectrophotometer (Victor 2, PerkinElmer Life Sciences). A standard curve of HEK293 cells was plotted to correlate the number of living cells with the absorbance values.

**Cell Motility Assay**—Cell migration was measured as described (16) with some modifications. The assay was performed with Transwells (6.5 mm diameter; 8 μm pore size polycarbonate membrane) obtained from Corning Glass. Cells (3.75 × 105) in 1.5 ml of serum-free medium were placed in the upper chamber, whereas the lower chamber was loaded with 2.6 ml of medium containing 10% fetal bovine serum. After incubation at 37°C with 5% CO2 for 24 h, the total numbers of cells that migrated into the lower chamber was counted with a hemocytometer.

**Subcellular Fractionation**—The subcellular fraction experiment was conducted as described previously (22). Stable clones expressing HA-tagged PRL1 constructs were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum and antibiotics. Cells were washed and scraped down in 1 ml of ice-cold Hepes buffer (10 mM Hepes, pH 7.4, 1 mM EDTA, 0.5 mM phenylmethylsulfonlfy fluoride, protease inhibitor mixture, 250 mM sucrose). Then the cells were passed through a 27-gauge needle 20 times. The resulting cell lysate was centrifuged at 1000 × g for 5 min to

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**FIGURE 1. A schematic diagram of the structure of PRL1.**
pellet the nuclei and intact cells, and the supernatant was again centrifuged at 100,000 × g for 60 min to obtain the S100 soluble fraction and the P100 membrane fraction. Fractions were analyzed by SDS-PAGE and detected by immunoblotting with anti-HA antibody (Santa Cruz Biotechnology).

Kinetic Constants for the PRL1-catalyzed Reaction—Initial rate measurements for the enzyme-catalyzed hydrolysis of pNPP were conducted as described previously (25). All assays were carried out at 25 °C in a pH 7.0 buffer of 50 mM 3,3-dimethylglutarate, containing 2 mM dithiothreitol and 1 mM EDTA, with an ionic strength of 0.15 M, adjusted by addition of NaCl. For the pNPP reaction, assay mixtures of 200 μl in total volume were set up in a 96-well polystyrene plate from Fisher. A substrate concentration range from 0.2 to 5 K_m was used to determine the k_cat and K_m values. Reactions were started by the addition of an appropriate amount of PRL1. The reaction mixtures were quenched with 50 μl of 5 M sodium hydroxide, and the absorbance at 405 nm was read using a plate reader. The steady-state kinetic parameters were determined from a direct fit of the data to the Michaelis-Menten equation using the nonlinear regression program KINETASYST (IntelliKinetics, State College, PA).

Lipid-Membrane Overlay Assays—PIP strips were purchased from Echelon Biosciences. The strips were incubated with 5 μg/ml purified His or GST-tagged PRL1 and mutants in Buffer A containing 10 mM Tris-HCl, pH 7.9, 0.15 M NaCl, 0.1% TBST, and 3% fatty acid-free bovine serum albumin. After extensive washing with Buffer A, PRLs bound to the membrane strips were probed with appropriate anti-His or anti-GST antibodies and then with horseradish peroxidase-conjugated anti-rabbit secondary antibodies. Detection was made by enhanced chemiluminescence reactions followed by exposure to x-ray film. The control experiments were performed without addition of any enzymes or GST tag only under the same conditions.

Statistical Analyses—Cell proliferation was measured eight times, and cell migration measurements were repeated three times. The data were presented as mean ± S.D. Statistical significance was evaluated with Student’s t test. p < 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

PRL1 Promotes HEK293 Cell Growth and Migration—We chose the human embryonic kidney 293 (HEK293) cell line, an epithelium-derived cell line, as a model system to investigate the functional significance of unique structural features observed in previous x-ray crystallographic studies. We first determined the effect of PRL1 down-regulation by transfecting HEK293 cells with a mammalian expression vector pU6 (Biomyn, San Diego) containing a gene-specific insert of a 21-nucleotide sequence targeting the PRL1 mRNA region 571–591. Several clones stably expressing the short hairpin RNA (shRNA) for PRL1 were selected. As shown in Fig. 2, PRL1 mRNA levels in the shRNA-expressing cells were significantly decreased (70–80%) compared with the pU6 vector control. As a control for PRL1 knockdown specificity, we also quantified the mRNA levels of PRL2 and PRL3. The results showed that suppression of PRL1 expression by shRNA did not affect the mRNA levels of PRL2 and PRL3 (Fig. 2). We then measured the effect of PRL1 suppression on cell growth by either direct cell counting or a well established colorimetric microtiter assay using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide or MTT as a substrate. As shown in Fig. 2, the number of viable PRL1 shRNA cells at day 5 was 50% less than the vector control. In addition, the doubling time for the vector control cells was 24 h, similar to that of the parental nontransfected line. In contrast, the doubling times for the PRL1 shRNA clones 1 and 2 were 28 and 29 h, respectively. We also investigated whether a decrease in PRL1 level would have an effect on cell motility. Using a standard Transwell assay to measure cell migration toward serum, we found that the relative migration rates for the PRL1 knockdown cells were ~2-fold slower than that of the vector control cells (Fig. 2). Together, the data indicate that endogenous PRL1 silencing in HEK293 cells inhibited both cell growth and migration.

To further establish the requirement of PRL1 for cell growth and migration, we also determined whether PRL1 overexpression could augment cell growth and migration. A number of stable HEK293 lines ectopically expressing the HA-tagged PRL1 were established. The PRL1 expression levels in the stable clones were ~2-fold higher than that of the empty vector control (Fig. 3). As expected, a rise in PRL1 level increased the cell proliferation by 70%, whereas elevated expression of PRL1 led to an ~3-fold increase in cell motility (Fig. 3). Taken together, the results from both shRNA knockdown and ectopic expression experiments suggest that PRL1 is a positive regulator of both cell proliferation and migration. This conclusion is in complete agreement with previous observations in other cell lines (including Chinese hamster ovary, SW480, D27, and DLD-1) (13, 16, 19, 26) that ectopic expression of PRL1 promotes cell growth and motility, whereas knockdown of PRL1 in DLD-1 or A549 cells by short interfering RNA reduces cell migration and invasive properties (20, 27). This set the stage for evaluating the importance of the intrinsic phosphatase activity,
trimerization and the C-terminal polybasic region for the PRL1-mediated processes using HEK293 cell growth and migration as functional readouts.

**The Intrinsic Phosphatase Activity of PRL1 Is Obligatory for Function**—Although the importance of PRLs in cell growth and migration is well established, there is limited understanding of the structure and function relationship for these unique PTPs. Such knowledge can help develop novel approaches to manipulate the pathways regulated by these enzymes to gain further insight into their roles in signaling and diseases. Unfortunately, the physiological substrates for PRL1 have not been identified. In addition, the activity of PRLs toward the chromogenic substrate pNPP is orders of magnitude lower than those of typical PTPs (21, 22). Like other members of the PTP superfamily, the PTP signature motif in PRL1 (103HCVAGLGR110) forms a phosphate-binding loop at the base of the active site pocket (22, 23). However, the PRL active site motif lacks a conserved hydroxyl group (Ala instead of Ser/Thr at position 111), which is important for the hydrolysis of the phosphoenzymic intermediate (28). PRL1 is also missing an H-bond (a Val at position 113 instead of a Thr) important for the hydrolysis of the phosphoenzyme intermediate (28). PRL1 is also missing an H-bond (a Val at position 113 instead of a Thr) important for the hydrolysis of the phosphoenzyme intermediate (28). PRL1 is also missing an H-bond (a Val at position 113 instead of a Thr) important for the hydrolysis of the phosphoenzyme intermediate (28). PRL1 is also missing an H-bond (a Val at position 113 instead of a Thr) important for the hydrolysis of the phosphoenzyme intermediate (28).

Intracellular migration is well established, there is limited understanding of the conserved active site Arg-110. Arg-110 makes a bidentate hydrogen bond with the phosphoryl group in the substrates and is important for both substrate binding and transition state stabilization in catalysis (28). Consequently, the PRL1/R110S mutant should behave as a true loss-of-function variant. Indeed, replacement of Arg-110 with a Ser not only increased the $K_m$ value by 11-fold but also decreased the $k_{cat}/K_m$ value for the pNPP reaction by 280-fold (Table 1). In addition, we also studied two gain-of-function mutants, PRL1/A111S and PRL1/A111S/V113T, which exhibited $k_{cat}/K_m$ values that were 2.4 and 24-fold higher than that of the wild-type PRL1 (Table 1). We established specific stable cell lines expressing the HA-tagged PRL1/R110S, PRL1/A111S, and PRL1/A111S/V113T mutant PRL1s. Western blot analysis with anti-HA antibodies showed that the protein levels for all mutants were similar to that of the wild-type PRL1 (Fig. 4). In addition, immunofluorescence experiments indicated that the mutants displayed similar subcellular localization as the wild-type enzyme (data not shown). As shown in Fig. 4, introduction of PRL1/R110S into HEK293 cells had no influence on the endogenous PRL1-mediated cell proliferation and migration. This is consistent with the hypothesis that abrogation of Arg-110 side chain results in a loss-of-function phenotype. In accord with the increased catalytic activity for PRL1/A111S and PRL1/A111S/V113T, their expression further enhanced the PRL1-mediated cell growth and migration (Fig. 4). Collectively, these results support the notion that the intrinsic phosphatase activity is required for PRL1 function.

**Trimerization of PRL1 Is Essential for PRL1-mediated Cell Growth and Migration**—One of the most striking features of PRL1 in comparison with other PTPs is that it exists as a trimer in the crystalline state. Our structural work revealed an identical trimeric arrangement of the PRL1 molecules in two different crystallographic space groups (22) for the His$_{6}$-tagged native PRL1 and PRL1/C104S, and C222, for the SeMet-substituted PRL1 (22). Moreover, in the structure reported by Jeong et al. (23), PRL1/C104S also crystallized as a trimer under completely different conditions in the $P_2_1$ space group. These observations indicate that the trimeric state is structurally relevant and unlikely due to crystal packing artifacts. PRL1 trimerization occurs around a 3-fold rotational axis that fixes the C-terminal sequence of each monomer on the same surface of the trimer. Because the C-terminal sequence contains the pre-
The observation that PRL1 exists as a trimer in the crystal is novel among the PTPs. However, recombinant PRL1 appears predominantly as monomers in solution at low concentrations (0.1–3 mg/ml), whereas a mixture of dimers and trimers could be observed by gel filtration and dynamic light scattering measurements at 7 mg/ml of PRL1 (22, 23). Cross-linking experiments with both purified recombinant PRL1 protein (0.05 mg/ml) and membrane fractions derived from HEK293 cells expressing the HA-tagged PRL1 showed that PRL1 is capable of forming trimers in solution and inside the cell (22, 23). Given the structural conservation along the dimer interface among all PRLs, we suspected that trimer formation might be a general property of all PRL enzymes. Indeed, cross-linking of recombinant PRL3 with glutaraldehyde resulted in new bands with molecular weights corresponding to PRL3 dimer and trimer (Fig. 5A). To determine specific association of PRL3 inside the cell, we transfected HEK293 cells with both HA-tagged and green fluorescence protein (GFP)-tagged PRL3. The interaction of the epitope-tagged PRL3 proteins was determined by co-immunoprecipitation (Fig. 5B). The data indicate that HA-PRL3 can associate with GFP-PRL3, which is consistent with trimer formation. Interestingly, deletion of the C-terminal CAAX motif in PRL3 disrupts this association, implicating the importance of prenylation for PRL trimerization.

Taken together, the available data suggest that the PRLs have intrinsic propensity to form trimers. PRL overexpression, as observed in many cancer cell lines, may promote trimer formation. Moreover, C-terminal prenylation will increase the local concentration of PRL in the plasma membrane, which should also enhance PRL trimerization. Thus, it is possible that trimerization may be important for PRL regulation, although its functional significance has not been established. To determine whether trimerization is essential for PRL1 function, we decided to examine the effect of trimer disruption on PRL1-mediated cell growth and motility. If trimerization is important for the biological function of PRL1, we would predict that cells expressing a trimerization-deficient mutant will display phenotypes similar to those of loss-of-function PRL1/R110S mutant or vector control cells. We previously discovered that substitution of Gly-97 in the PRL1 dimer interface by Arg substantially reduced PRL1 trimerization (22). Similarly, Jeong et al. (23) found that replacement of Thr-13 in the dimer interface by Phe also abrogated PRL1 trimerization. As depicted in Fig. 6A, these mutations introduce rather large structural perturbations to the dimer interface, which are expected to disrupt PRL trimerization.

We expressed and purified both PRL1/G97R and PRL1/T13F to homogeneity. Kinetic measurements with pNPP as a substrate indicated that substitutions at residues Gly-97 or Thr-13 had little effect on the catalytic activity of PRL1 (Table 1). We next established stable cell lines expressing either PRL1/G97R or PRL1/T13F that disrupt PRL1 trimer formation. Western blot measurements showed that the PRL1 protein levels were similar in the wild-type and mutant PRL1-expressing cells (Fig. 6B). Cell proliferation and migration were analyzed by the MTT and Transwell assay, respectively (Fig. 6, C and D). In contrast to wild-type PRL1, neither PRL1/G97R nor PRL1/T13F was able to augment the endogenous PRL1-mediated HEK293 cell growth and migration. In fact, the rates of cell proliferation and migration for the PRL1/G97R and PRL1/T13F cells were similar to those of the vector control cells. These results show that disruption of PRL1 trimer formation abolishes the ability of PRL1 to promote cell growth and migration, indicating that PRL1 trimer formation is essential for PRL1 function.
PRL1 Structure and Function

To explore the biochemical basis by which PRL1 is regulated by trimerization, we considered the possibilities that PRL1 trimerization might modulate its cellular localization and/or catalytic activity. In the case of the heterotrimeric G-protein, in addition to the prenylation of one subunit, oligomerization with the others is essential for membrane localization (29). To investigate whether PRL1 trimerization affects its subcellular localization, we transfected the N-terminal HA-tagged PRL1 as well as PRL1/G97R and PRL1/T13F into HEK293 cells and determined their subcellular localization. Both subcellular fractionation and immunofluorescence microscopy experiments were performed using anti-HA antibodies. Similar to wild-type PRL1, the PRL1/G97R and PRL1/T13F mutants reside in the plasma membrane fractions and localize primarily to plasma membrane and early endosome (data not shown). These experiments suggest that mutations at the dimer interface do not significantly affect PRL1 subcellular localization.

We next investigated whether PRL1 trimerization affects its catalytic activity. Oligomerization plays an important role in the regulation of protein-tyrosine kinase activity of growth factor receptors, which often serves as an early step in signal transduction (30, 31). The PRL1 active site points to the outside of the trimer interface and is positioned opposite to the C-terminal polybasic region (22) (Fig. 8), in accord with the known dependence of prenylation on the C-terminal polybasic region in PRL1 subcellular localization and function (3). We then investigated the contribution of the polybasic stretch to PRL1 subcellular localization and function, we made a series PRL1 mutants including PRL1/1–169, which lacks the C-terminal CAAX motif; PRL1/3A-1 and PRL1/3A-2, which have the first (Lys-151, Arg-153, Lys-155, Arg-157, Arg-159, and Lys-161) (6, 9), full-length PRL1, which is prenylated inside the cells, was found predominantly in the membrane fraction, whereas PRL1/1–169 lacking the C-terminal prenylation motif localized entirely to the soluble cytoplasmic fraction (Fig. 7A). As an alternative approach, the subcellular localization of PRL1 was also examined using immunofluorescence microscopy (Fig. 7B). In agreement with the subcellular fractionation data, wild-type PRL1 concentrates on the plasma membrane and early endosome, whereas PRL1/1–169 is localized in the cytoplasm and the nucleus as indicated by the diffused staining. These results confirm the importance of the C-terminal prenylation motif for PRL1 plasma membrane localization. We further demonstrated that deletion of the CAAX motif completely compromised the ability of PRL1 to promote cell growth and migration (Fig. 8), in accord with the known dependence of prenylation for PRL1 cellular activity (9, 12, 19).

We then investigated the contribution of the polybasic region to PRL1 subcellular localization. When all six positively charged residues in the polybasic stretch were replaced with alanines, the resulting mutant PRL1/6A was found completely
in the soluble fraction (Fig. 7A) and localized primarily in the cytoplasm (Fig. 7B). The observation that PRL1/6A failed to localize to the plasma membrane suggests that PRL1 prenylation alone is insufficient for plasma membrane localization and that the C-terminal polybasic region is required for proper membrane targeting of PRL1. The fact that PRL1/6A resides in the cytoplasm and PRL1/1–169 can be found in both the cytoplasm and the nucleus supports the hypothesis that the positively charged residues in the polybasic region may also serve as a nucleus localization signal in the absence of PRL1 prenylation (9, 12, 19). To further characterize the polybasic region, we found that PRL1/3A-2, which lacks the last three basic residues, had similar subcellular fractionation profiles to PRL1/6A, whereas PRL1/3A-1, which misses the first three basic residues, was predominantly in the membrane fraction and enriched in the perinuclear region (Fig. 7, A and B). To further characterize the polybasic region, we found that PRL1/3A-2, which lacks the last three basic residues, had similar subcellular fractionation profiles to PRL1/6A, whereas PRL1/3A-1, which misses the first three basic residues, was predominantly in the membrane fraction and enriched in the perinuclear region (Fig. 7, A and B). Collectively, our results support the notion that proper cellular localization of PRL1 requires both insertion of the farnesyl group into the lipid bilayer and ionic interactions between the polybasic region and the acidic membrane surface. Interestingly, the dual requirement of hydrophobic and electrostatic interactions for correct membrane targeting has also been noted for a number of important signaling proteins, including small G protein K-ras and the Src kinase (36–38).

The results described above indicate that the C-terminal basic residues cooperate with the prenylation event to anchor PRL1 on the plasma membrane. To determine whether PRL1 exhibits any lipid binding specificity, we performed a lipid-membrane overlay assay. The membrane-immobilized lipid strips were incubated with 5 μg/ml of N-terminal His<sub>6</sub>-tagged PRL1, and bound PRL1 was probed with anti-His<sub>6</sub> antibodies. Of the 15 lipids tested (Fig. 7C), PRL1 (and PRL1/1–169) displayed strong binding to several phosphoinositides, including PI(3)P, PI(4)P, and PI(5)P, modest binding to PI(3,4)P<sub>2</sub>, PI(3,5)P<sub>2</sub>, PI(4,5)P<sub>2</sub>, and phosphatidic acid, and weak binding to PI(3,4,5)P<sub>3</sub>. No binding could be detected between PRL1 and two major components of mammalian membrane lipids, phosphatidylcholine and phosphatidylethanolamine, suggesting that the interaction between PRL1 and phosphoinositides is relatively specific. We obtained similar results with recombinant GST-PRL1. GST alone did not bind the lipids. In addition, we had evidence that PRL3 exhibited the same lipid binding specificity as PRL1. Because both PTEN and myotubularin-related phosphatases in the PTP...
superfamily utilize phosphoinositides as substrates, we determined whether the PRL phosphatases are capable of hydrolyzing phospholipids. We found that PRL1 had no lipid phosphatase activity. In fact, our result showed that the PRL1 active site was not involved in phosphoinositide binding, as removal of the active site Arg-110 known to be essential for binding the phospholipids in the substrate (PRL1/R110S) had little effect on the affinity of PRL1 for phospholipids (Fig. 7C).

To identify the phosphoinositide-binding site in PRL1, we determined the effect of mutating the positively charged residues in the C-terminal polybasic region on phospholipid binding. As shown in Fig. 7C, PRL1/6A and PRL1/3A-2 were unable to bind phospholipids, whereas the ability of PRL1/3A-1 to associate with the phospholipids was severely compromised. In addition, deletion of the polybasic region also completely eliminated phosphoinositide binding (data not shown). Taken together, these results suggest that PRL phosphatases are capable of binding monophosphoinositide species, most likely involving the basic residues clustered on the C terminus. This is a rather novel finding as PRLs are unrelated to any known phosphoinositide binding domains. However, it should be pointed out that interactions between phosphoinositides and polybasic motifs have precedence in the literature. For example, the polybasic sequences found in N-WASP (39) and the plant homodomain zinc finger 1 of pf1 (40) are also capable of specific phosphoinositide binding. More recently, the interaction between phosphoinositides and the polybasic residues adjacent to the C-terminal CAAX motif in small GTPases has been demonstrated to be responsible for targeting these signaling molecules to the plasma membrane (41, 42). Because phosphoinositides are often concentrated in specialized membrane microdomains or rafts of unique lipid compositions and function as spatially restricted membrane second messengers in cellular signaling (43–46), one can envision that the coordinated actions of phospholipid binding by PRL1 and its C-terminal prenylation can direct PRL1 to specialized membrane domains.

Finally, to ascertain the functional importance of the polybasic region in PRL1, we established stable cell lines expressing PRL1, PRL1/3A-1, PRL1/3A-2, and PRL1/6A. Cell proliferation and migration were analyzed by the MTT and Transwell assay, respectively. As shown in Fig. 8, mutation of the last three or all six basic residues adjacent to the CAAX motif obliterated the PRL1-mediated cell growth and migration. Interestingly, mutation of the first three basic residues, which induced relocalization of PRL1 from the plasma membrane to the perinuclear region (Fig. 7B), rendered PRL1/3A-1 partially active in promoting cell migration but completely disabled with respect to augmenting cell growth. These results indicate that the PRL1-mediated cell growth and migration depend on the structural integrity of the stretch of basic residues in the C terminus.

In summary, we show that ectopic expression of PRL1 in HEK293 cells can increase cell proliferation and migration, whereas knockdown of endogenous PRL1 in these cells using shRNA can abrogate cell growth and motility. We provide evidence that the intrinsic phosphatase activity of PRL1 is obligatory for cellular processes regulated by PRL1. We find that trimerization may be a general property for all PRL enzymes, and that PRL1 trimer formation is essential for PRL1-mediated cell growth and migration. The functional requirement for PRL trimerization suggests a novel mechanism for PTP regulation. In addition, the trimeric interface presents a unique opportunity for the development of small molecule compounds designed to disrupt PRL trimerization. This offers a distinct advantage compared with the traditional approach to target the active sites, which are highly conserved among the PTPs. We further demonstrate that the conserved C-terminal polybasic region in PRL is important for specific phosphoinositide recognition. Both the polybasic residues and the adjacent CAAX motif are required for proper PRL subcellular localization and full biological activity.

REFERENCES

1. Tonks, N. K., and Neel, B. G. (2001) Curr. Opin. Cell Biol. 13, 182–195
2. Alonso, A., Sasin, J., Bottini, N., Friedberg, I., Friedberg, I., Osterman, A., Godzik, A., Hunter, T., Dixon, J., and Mustelin, T. (2004) Cell 117, 699–711
3. Zhang, Z.-Y. (2001) Curr. Opin. Chem. Biol. 5, 416–423
