The Tyrosine Phosphatase PRL-1 Localizes to the Endoplasmic Reticulum and the Mitotic Spindle and Is Required for Normal Mitosis*

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PRL-1 is one of three closely related protein-tyrosine phosphatases, which are characterized by C-terminal farnesylation. Recent reports suggest that they are involved in the regulation of cell proliferation and transformation. However, their biological function has not yet been determined. Here we show that PRL-1 mRNA is overexpressed in a number of human tumor cell lines, including HeLa cells. Using immunofluorescence we studied the subcellular localization of endogenous PRL-1, and our results demonstrate that PRL-1 exhibits cell cycle-dependent localization; in non-mitotic HeLa cells, PRL-1 is localized to the endoplasmic reticulum in a farnesylation-dependent manner. In mitotic cells PRL-1 relocates to the centrosomes and the spindle apparatus, proximal to the centrosomes, in a farnesylation-independent manner. Conditional expression of a catalytic domain mutant in HeLa cells results in a delay in the progression of cells through mitosis but has no effect on other phases of the cell cycle. Further, expression of a farnesylation site PRL-1 mutant results in mitotic defects, characterized by chromosomal bridges in anaphase and lagging chromosomes, without affecting spindle checkpoint function. Together, these results suggest that PRL-1 function is regulated in a cell cycle-dependent manner and implicate PRL-1 in regulating progression through mitosis, possibly by modulating spindle dynamics.

Protein-tyrosine phosphatases (PTPs)1 have been recognized as important modulators in signal transduction pathways that regulate cell proliferation and cell differentiation, as well as other cellular functions of eukaryotic cells (1, 2). PTPs can function as positive or negative regulators of signal transduction pathways, depending on the particular substrate and the effect of phosphorylation on its activity (3, 4). Like kinases, PTPs have been implicated in various malignancies, including cancer.

The PTP superfamily can be divided into two classes based on sequence similarity and substrate specificity: “classic” PTPs, which dephosphorylate phosphotyrosine residues, and dual specificity phosphatases, which can also use phosphoserine and phosphothreonine residues as substrates. In addition to the conserved catalytic domain, PTPs frequently contain additional domains or sequence motifs important for subcellular localization, substrate recognition, or regulation of their activities (2, 5).

Over the past few years, a new subfamily of PTPs, PRL (also termed PTPCAAX), has been identified (6–8). Although PRL phosphatases contain the PTP signature motif (I/V(HCXXAGXXR/)ST)G at the active site, their sequences are distinct from other PTPs. Overall, their catalytic domain is most similar to that of dual specificity phosphatases. Their closest relatives are CDC14, a mitotic regulator, and the tumor suppressor PTEN (8, 9). To date, three family members (PRL-1, PRL-2, PRL-3) have been identified in mammals. PRL phosphatases are relatively small enzymes with an apparent molecular mass of 22 kDa and contain a C-terminal CAAX motif as the signal for protein farnesylation. All three family members can serve as substrates for farnesytransferases in vitro, and PRL-2 has been shown to be prenylated in vivo (7, 10).

The three enzymes share a high degree of amino acid sequence similarity with each other. For example, PRL-1 shares 87 and 75% sequence identity with PRL-2 and PRL-3, respectively (8). The amino acid sequence similarity of the three family members is also reflected on the cDNA level within the coding region. However, their 5′ non-coding sequences are quite divergent, suggesting possible differences in the regulation of gene expression (8).

PRL-1 was first cloned as an immediate-early gene in rat liver following hepatectomy. More recently, it has been shown that the expression of human PRL-1 is regulated by Egr-1, a growth factor-activated transcription factor (11, 12). Interestingly, overexpression of PRL-1 and PRL-2 in epithelial cells results in a transformed phenotype and the transfected cells are able to form tumors in nude mice (7). Further, stable cell lines overexpressing PRL-1 have enhanced saturation density and a shorter doubling time relative to control cell lines (6, 7). More recently PRL-2 was shown to be overexpressed in prostate cancer (13). Further, Saha et al. (14) demonstrated that the expression of PRL-3 is associated with liver metastasis of colon cancer. Together these data suggest that PRL-1 and PRL-2 may function in the regulation of cell proliferation and that all three members of this subfamily of PTPs may be involved in tumorigenesis. To date, however, little is known about their possible physiological function and how they might be involved in signal transduction processes. In addition, the mechanism of their regulation and the basis of their transforming activity are not understood.

The subcellular localization of a particular protein can sometimes give clues to its physiological function. PRL-1 was originally described as a nuclear protein in transfected cells (6).
More recently, Zeng et al. (10) reported that PRL phosphatases are localized to the plasma membrane and early endosomes and that this localization pattern is dependent on their farnesylation. This study used an overexpression system, which could lead to non-physiological subcellular localization.

By using affinity-purified antibodies against PRL-1, we report that the subcellular localization of endogenous PRL-1 is cell cycle-dependent. In non-mitotic cells PRL-1 localizes to endoplasmic reticulum (ER), whereas in mitotic cells it is associated with the centrosomes and the spindle apparatus. Further, cell cycle analysis of cells expressing a catalytic domain mutant of PRL-1 indicates that the PRL-1 phosphatase activity is required for progression of cells through mitosis. In addition, expression of a farnesylation site mutant of PRL-1 (PRL-1-C170S), which is enriched in the nucleus throughout the cell cycle, results in mitotic defects without compromising spindle checkpoint function. Like wild type PRL-1, PRL-1-C170S localizes to the spindle apparatus in mitotic cells, demonstrating that farnesylation is required for ER but not for microtubule association. Our results demonstrate for the first time a role for PRL-1 in cell cycle regulation and suggest that PRL-1 activity may be required for proper spindle function or spindle dynamics.

MATERIALS AND METHODS

Constructs and Cell Lines—PRL-1 was N-terminally FLAG-tagged by cloning the complete open reading frame into the pMEF vector (15) at the BgII and EcoRI sites. To generate plasmids for inducible expression, cDNAs were subcloned into pRetro-On (Clontech). The expression plasmid for GFP-histone 2B (pBOS-H2BGFP) was from PharMingen. The expression plasmid for GFP-CAAAX was obtained from Martin Oft (DNAX Research Institute). Cell lines were obtained from ATCC and cultured as recommended.

PRL-1-specific antibody was affinity-purified using myelin basic protein (MBP)-PTP1B monoclonal antibody was from Calbiochem; anti-FLAG M2 monoclonal antibody was from Sigma. Purified tubulin (99% purity) was obtained from ICN. Secondary reagents for immunoblot analysis were from Amersham Biosciences. Secondary reagents for immunofluorescence were from Vector Laboratories. The farnesyltransferase inhibitor SCH66336 was a generous gift from Robert Bishop (DNAX Research Institute). Cell lines were obtained from ATCC and cultured as recommended.

Expression Analysis by Real-time Quantitative PCR—For expression of PRLs in HeLa cells, RNA was isolated with RNeasy Mini Kit (Qiagen) according to the protocol from the manufacturer. Three micrograms of RNA were reverse-transcribed with oligo(dT) (Invitrogen) and random hexamer primers (Promega) using standard protocols. mRNA expression was analyzed by quantitative real-time PCR (TaqMan) with 100 ng of cDNA/sample. Gene-specific primers and probes were obtained from PE Biosystems. Primers and probes for the three different PRL genes were tested for specificity using the human PRL cDNAs as templates. No cross-reactivity was detected. All PCR reactions were done in duplicate, and target gene expression was normalized between different samples based on the values of the expression of an internal control (28S rRNA). PRL cDNA levels are expressed as femtograms per 100 ng of total cDNA. For analysis of PRL-1 expression in the tumor cell line panel, quantitative PCR was done as described above with the following modifications: 1 μg of RNA was used for reverse transcription, and 20 ng of the resulting cDNA were used for TaqMan. PRL-1 cDNA levels are expressed as femtograms per 20 ng of total cDNA.

Transfection, Immunoprecipitation, and Western Blotting—A calcium phosphate technique was used for transfection of NIH3T3 cells. HeLa cells were transfected by LipofectAMINE (Life Technologies, Inc.). Cells from a 10-cm plate were lysed in 1 ml of lysis buffer (1% Nonidet P-40, 20 mM Tris-Cl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 2 mM dithiothreitol, 1 μg/ml leupeptin, and protease inhibitors (Roche Molecular Biochemicals). For immunoprecipitation, cell lysates were pre-cleared with 20 μl of protein A/G-agarose beads (Santa Cruz), incubated with the appropriate antibody for 2 h, and then incubated with protein A/G-agarose beads for another 1 h at 4 °C. The immunoprecipitates were washed five times with lysis buffer. Western blotting was done according to standard procedures.

Immunofluorescence and Imaging Acquisition—Cells on glass cover slips were washed with PBS and fixed with 4% paraformaldehyde on ice for 10 min followed by permeabilization with 0.2% Triton X-100. Cells were extracted with ice-cold acetone for 5 min and washed three times with PBS. Cells were blocked in 10% goat serum/FBS for 1 h at room temperature and incubated with primary antibodies in blocking buffer for 4 h at 4 °C overnight. Cells were then washed with PBS plus 0.5% Tween 20, and incubated with goat anti-rabbit IgG conjugated with fluorescein (1:1000) or horse anti-mouse IgG conjugated with Texas Red (1:500) in PBS plus 0.5% Tween 20 with 2% goat serum for 1 h. After washing with PBS, the coverslips were mounted with Vectorshield containing containing 1:1000 DAPI (Vector Laboratories). Confocal microscopy was performed with a Leica imaging system using a 40 × 1.25 numeric aperture objective for all images.

Cell Synchronization and Cell Cycle Analysis—Cells were synchronized by double thymidine block. 2.5 mM thymidine was added to the medium for 20 h. After washing, cells were released in fresh medium for 9 h followed by a second block for 16 h. For cell cycle analysis, cells were trypsinized and washed with PBS plus 1% fetal bovine serum, and fixed with ice-cold 85% ethanol overnight. After washing, the cells were stained with propidium iodide in the presence of RNase for 1 h, followed by fluorescence-activated cell sorting analysis. The data were analyzed with the CellQuest software.

For the determination of mitotic figures and mitotic bridges in anaphase, cells were fixed in methanol:acetone (1:1), stained with Hoechst dye (Sigma), and analyzed by fluorescent microscopy. In each experiment a minimum of 800 cells (per line and condition) were scored in several random microscopic fields.

Phosphatase Assays—For phosphatase assays 5 μg of GST-PRL fusion protein were used per reaction in a total volume of 200 μl. Assays were done at room temperature in a buffer containing 50 mM sodium acetate, pH 5.5, 80 mM NaCl, 10 mM dithiothreitol, and 20% glycerol. Reactions were started by the addition of substrate (o-methylfluorescein phosphate, 10 μM final), and the fluorescent intensity was measured in a fluorometer (LJL Analyst, LJL BioSystems) at regular intervals. For the determination of substrate turnover, standard curves were established from serial dilutions of the corresponding unphosphorylated compound (o-methylfluorescein).

RESULTS

PRL-1 Is Overexpressed in Tumor Cell Lines of Various Tissue Origin—Tissue culture cell lines stably transfected with PRL-1 or PRL-2 showed altered growth characteristics and a transformed phenotype (6, 7), suggesting that overexpression of PRL-1 might be involved in tumorigenesis. To determine whether PRL-1 is overexpressed in tumor cell lines of various tissue origins, we analyzed mRNA expression by quantitative real-time PCR (TaqMan) using gene-specific primers with a large panel of tumor cell line cDNAs. The specificity of the PRL-1 primers was first verified in separate PCR reactions with the three human PRL cDNAs as templates (not shown). The cell line panel consists of 76 different human lines, 9 of which are normal/untransformed controls of various tissue origins. As shown in Fig. 1, the untransformed control cell lines showed relatively consistent expression levels for PRL-1. Compared with the controls, we found high levels of PRL-1 in several tumor cell lines. The highest expression levels were detected in HeLa, HepG2, SK-Lu-1, and several melanoma cell lines. In fact, five of six melanoma cell lines showed increased PRL-1 levels. In three of these, PRL-1 levels are up-regulated greater than 3-fold. In contrast to other tissues, none of the colon carcinoma cell lines shows elevated PRL-1 mRNA levels.

Endogenous PRL-1 Exhibits a Cell Cycle-dependent Localization Pattern—To initiate a functional characterization of the PRL-1 phosphatase, we sought to determine the subcellular localization of the protein. Originally, PRL-1 was shown to localize to the nucleus when overexpressed in NIH3T3 cells (8). More recently, it was reported that PRL-1, along with PRL-2 and PRL-3, is associated with the cytoplasmic membrane and early endosomes when overexpressed in Chinese hamster ovary cells (10). Because PRL proteins are farnesylated in vivo,
overexpression of these proteins may result in nonspecific association with membrane compartments or other proteins. Therefore, we decided to investigate the localization of endogenous PRL-1 protein. For this purpose we chose HeLa cells, which express high levels of PRL-1 mRNA (see Fig. 1), have a distinct morphology, and are widely used for immunofluorescence studies.

We first compared the expression of all three PRL family members in HeLa cells by real-time quantitative PCR (TaqMan). As shown in Fig. 2A, a direct comparison of PRL expression levels shows that PRL-1 is, at least at the mRNA level, the predominant form expressed in asynchronously growing HeLa cells. The expression level of PRL-1 was found to be 8-fold higher than that of PRL-2, and only very low expression could be detected for PRL-3. Because the expression of many genes is cell cycle-regulated, we asked whether the PRL expression profile changes at different stages of the cell cycle. HeLa cells were grown asynchronously (AS) or were synchronized by double thymidine block and harvested at the indicated time points after release. PRL mRNA levels are expressed as femtograms per 100 ng of total cDNA. The results shown are representative of two independent experiments.

We generated an affinity-purified anti-PRL-1 antiserum and first tested the antibody in immunoblot on whole cell lysates from asynchronously growing as well as from synchronized HeLa cells. As shown in Fig. 2B, the purified antibody detected the predominant form expressed in HeLa cells at all stages of the cell cycle.

We generated an affinity-purified anti-PRL-1 antiserum and first tested the antibody in immunoblot on whole cell lysates from asynchronously growing as well as from synchronized HeLa cells. As shown in Fig. 2B, the purified antibody detected
a single protein of 22 kDa, the expected molecular mass of PRL-1. The relative PRL-1 protein levels in synchronized cells were also analyzed by confocal microscopy (Fig. 4G, panel a). As expected, the localization of GFP itself was not affected by FTI treatment (Fig. 4C, panel b). Pre-incubation with recombinant PRL-1 protein blocked the staining with the anti-PRL-1 antibody, but had no effect on the staining with an anti-tubulin antibody, further confirming the specificity of the antibody (Fig. 2C, panels c and d). In addition, the same localization patterns for non-mitotic and mitotic cells were observed when other fixation protocols were used (data not shown). These data indicate that the localization of endogenous PRL-1 is cell cycle-dependent.

**PRL-1 Localizes to the ER in Non-mitotic Cells in a Farnesylation-dependent Manner**—The perinuclear staining pattern of PRL-1 is reminiscent of proteins localized to the ER. The protein-tyrosine phosphatase PTP1B localizes predominantly to the ER and has been used as a marker for ER localization (16, 17). Co-staining of HeLa cells with anti-PRL-1 and anti-PTP1B antibodies demonstrates that PRL-1 and PTP1B localize to the same subcellular compartment, indicating that PRL-1 is also localized to the ER (Fig. 3).

All three human PRL phosphatases as well as their Caenorhabditis elegans and Drosophila orthologs contain a CAAX box at their C termini (Fig. 4A). In PRL-1 the predicted site of prenylation is cysteine residue 170 (C170CID). To test whether farnesylation is required for the localization of PRL-1 to the ER, we exchanged Cys170 to serine (PRL-1-C170S). As control, we also mutated the adjacent cysteine residue, Cys171 (PRL-1-C171S). Mutants as well as wild type PRL-1 were expressed as a single protein of 22 kDa, the expected molecular mass of PRL-1. The relative PRL-1 protein levels in synchronized cells were also analyzed by confocal microscopy (Fig. 4G, panel a). As expected, the localization of GFP itself was not affected by FTI treatment (Fig. 4C, panel b). Pre-incubation with recombinant PRL-1 protein blocked the staining with the anti-PRL-1 antibody, but had no effect on the staining with an anti-tubulin antibody, further confirming the specificity of the antibody (Fig. 2C, panels c and d). In addition, the same localization patterns for non-mitotic and mitotic cells were observed when other fixation protocols were used (data not shown). These data indicate that the localization of endogenous PRL-1 is cell cycle-dependent.

**Function of PRL-1 in Mitosis**—The cell cycle-dependent localization of PRL-1 suggests that PRL-1 translocates from ER to the spindle when cells enter mitosis. To further analyze the mitotic localization of PRL-1, we stained cells with anti-PRL-1 antibodies together with antibodies against γ-tubulin, a component of the centrosomes (Fig. 5A). The cell cycle stages were determined by staining the DNA with DAPI (data not shown). In prometaphase cells we found PRL-1 to be largely localized to the centrosomes (Fig. 5A, panels a–c). At later stages, in metaphase (Fig. 5A, panels d–f) and anaphase (panels g–i), PRL-1 was also found to associate with the spindle microtubules. We
have not observed co-staining of PRL-1 with γ-tubulin before centrosome duplication and separation (data not shown).

To confirm co-localization of PRL-1 with the spindle apparatus, we also stained the microtubules with anti-α-tubulin to visualize the spindle. Co-staining of a cell in prometaphase is shown in Fig. 5B. Interestingly, PRL-1 staining was largely restricted to the base of the spindle. Similar localization patterns were also observed at later stages of mitosis (see also Fig. 7B, bottom row) for a cell in metaphase. We did not detect localization to midbody microtubules in telophase and cytokinesis (data not shown).

Given the localization of PRL-1 to the centrosomes and the spindle in mitotic cells, we hypothesized that PRL-1 directly interacts with tubulin. To test this possibility we performed binding assays with purified tubulin and GST-PRL-1 fusion protein in vitro. As shown in Fig. 6A, α-tubulin readily co-precipitated with GST-PRL-1. No binding of α-tubulin to beads alone, GST, or a GST fusion with another PTP (HePTP) was detected. To test whether the interaction with tubulin was specific for PRL-1 or could also be observed with other PRL family members, we also included GST fusions with PRL-2 and PRL-3 in this experiment. PRL-3, but not PRL-2, was found to bind tubulin in vitro. The inability of PRL-2 to bind to tubulin was confirmed with two independent preparations of GST-PRL-2 fusion protein.

To further examine the association of PRL-1 with tubulin, increasing amounts of GST-PRL-1 were bound to glutathione-agarose beads and then incubated with a constant amount of purified tubulin. Binding of tubulin was then detected by SDS-PAGE, followed by Coomassie staining. The results show that the amount of tubulin precipitated is proportional to the amount of GST-PRL-1 bound to the beads.

The binding of recombinant PRL-1 to tubulin suggested that the spindle localization of PRL-1 might be independent of its farnesylation; therefore, we asked whether PRL-1 was localized to the spindle in FTI-treated cells. To make sure that FTI treatment was effective in preventing farnesylation of PRL-1, we first prepared immunoblots from treated and untreated cells and analyzed the mobility of PRL-1. As shown in Fig. 7A, treatment of HeLa and MCF-7 cells with SCH66336 resulted in a slight decrease in the mobility of PRL-1 on SDS-PAGE, which is indicative of loss of farnesylation (18). We then treated HeLa cells with FTI and analyzed the mitotic localization of PRL-1 by co-staining with anti-PRL-1 and anti-α-tubulin antibodies. As shown in Fig. 7B, FTI treatment did not abolish the spindle localization of PRL-1. To confirm these results we also expressed PRL-1-C170S with an N-terminal FLAG-tag in HeLa cells and stained the cells with a polyclonal anti-FLAG antibody. As expected, the ectopically expressed PRL-1 mutant was found to be concentrated at the spindle in mitotic cells. Together these data demonstrate that PRL-1 localizes to the centrosomes and the mitotic spindle in a farnesylation-independent manner.

**Effect of PRL-1 on Cell Viability and Mitosis—**The localization of PRL-1 to the spindle indicated a potential function for this phosphatase during mitosis. To determine whether PRL-1 phosphatase is required for mitosis, we used dominant negative mutants to interfere with PRL-1 activity.

PTPs and dual specificity phosphatases share a conserved active site in which the cysteine residue is absolutely required for activity (Fig. 8A). Approximately 30–35 residues N-terminal to this cysteine lies a conserved aspartic acid residue (1). PTPs in which the active site cysteine or the aspartic acid residue were mutated to serine and alanine, respectively, have successfully been used as dominant interfering mutants. Within the classic PTPs, the aspartic acid residue is part of the WPD motif. In mammalian PRL phosphatases this region is

**Fig. 5.** PRL-1 localizes to the centrosomes and the spindle in mitotic cells. A, HeLa cells were stained with rabbit anti-PRL-1 (green) and mouse anti-γ-tubulin (red). The mitotic stages of the cells were determined by staining the chromosomes with DAPI (data not shown). In prometaphase PRL-1 is co-localized with γ-tubulin at the centrosomes. In metaphase and anaphase PRL-1 also localizes to the mitotic spindle. B, HeLa cells were stained with anti-PRL-1 (green) and anti-α-tubulin (red). The panel shows a cell in prometaphase. PRL-1 localizes predominantly to the base of the mitotic spindle.

**Fig. 6.** Interaction of PRL-1 with tubulin in vitro. A, binding of PRL-1, PRL-2, and PRL-3 to α-tubulin in vitro (upper panel). 5 μg of each GST-PRL fusion protein was bound to glutathione-agarose beads and then incubated with 5 μg of purified tubulin. Empty beads or beads coupled with GST or GST-HePTP were used as controls. The precipitates were analyzed by immunoblot with anti-α-tubulin antibody (upper panel). For reference, 5% of the tubulin input was run on the same gel. GST-PRL-2a and GST-PRL-2b are two independent preparations of the GST-PRL-1 fusion protein. B, alternatively, increasing amounts of GST-PRL-1 fusion protein (0.1–2.0 μg) were bound to beads and incubated with a constant amount of purified tubulin (3 μg) (bottom panel). Proteins bound to the beads were eluted with 2× SDS loading buffer and visualized by Coomassie staining (bottom panel).
very similar (WPFD) and also contains the tryptophan residue (Fig. 8A). In contrast to human PRLs, Drosophila and C. elegans PRL contain only one aspartic acid residue within this sequence (Fig. 8A). This indicates that the second aspartic acid residue (Asp72 in hPRL-1) within the WPFD motif likely functions as the general acid. We mutated Asp72 (PRL-1-D71A) and Asp72 (PRL-1-D72A), alone or in combination, to alanine. As shown in Fig. 8B, the D71A mutation had no effect on PRL-1 catalytic activity, whereas the D72A mutation resulted in a significant loss of activity. The activity of the double mutant PRL-1-D71A/D72A was similar to PRL-1-D72A, suggesting that the catalytic domain mutant PRL-1-D72A. In stable cell lines, expression of wild type PRL-1 and PRL-1-D72A are expressed in an inducible manner after doxycycline (DOX) treatment (Fig. 9A), catalytic activity of GST-PRL fusion proteins with o-methylfluorescein phosphate as substrate. All reactions were performed in duplicate. The data are representative of three independent experiments. C, overexpression of PRL-1 catalytic domain mutants affects cell viability. Cloning efficiency of NIH3T3 cells after transfection with expression plasmids for wild type and mutant versions of PRL-1. Cells were transfected with 10 μg of plasmid DNA and then selected with G418. Drug-resistant clones were stained and counted. The cloning efficiency of cells transfected with the empty vector was set to 100%. The values represent the mean ± standard deviation of three independent experiments. Each experiment was performed in duplicate.

of the mutants decreased colony formation to below 25% of the vector control. Many of the drug-resistant clones showed altered morphology, had multiple nuclei, and could not be further propagated.

To overcome this problem, we generated stable HeLa cell lines that allow conditional expression of wild type PRL-1 or of the catalytic domain mutant PRL-1-D72A. In stable cell lines, wild type PRL-1 and PRL-1-D72A are expressed in an inducible manner after doxycycline (DOX) treatment (Fig. 9A, top panel). In addition we established a HeLa cell line expressing the farnesylation site mutant PRL-1-C170S (Fig. 9A, bottom panel). None of the HeLa cell clones transfected with PRL-1-C170S showed detectable expression of the transgene (data not shown).

We observed that PRL-1-C170S-expressing cells, but not cells expressing wild type PRL-1 or PRL-1-D72A, show a high frequency of defects in mitosis and cytokinesis. Cells in anaphase often had chromosomal bridges, and lagging chromosomes were found in postmitotic cells. The cells show lagging chromosomes between the separated nuclei. A pair of postmitotic cells is shown in Fig. 9C. Panels a–c show two pairs of cells, which are completing cytokinesis. The cells show lagging chromosomes at the separated nuclei. A pair of postmitotic cells is shown in panels d–f. The well separated cells are connected by a structure containing α-tubulin and lagging chromosomes. To confirm that the lagging DNA is in fact of chromosomal origin, we transfected cells...
with an expression construct for a GFP-histone 2B fusion protein to visualize chromosomal DNA in live cells (19). After transfection the cells were treated with DOX for 48 h to induce expression of PRL-1-C170S. Again, we frequently observed lagging chromosomes (Fig. 9C). The GFP fluorescence between separated nuclei demonstrates that the lagging DNA is of chromosomal origin.

Within the anaphase population we observed occasional cells with chromosomal bridges in the vector control, as well as in cells expressing wild type PRL-1 or PRL-1-D72A. In cells expressing PRL-1-C170S, however, 70% of the cells in anaphase showed chromosomal bridges (Fig. 9D). The increase in chromosomal bridges observed in uninduced PRL-1-C170S cells is likely caused by the somewhat leaky expression in the absence of DOX (see Fig. 8A). Together the data suggest that ectopic expression of PRL-1-C170S, but not of wild type PRL-1 or PRL-1-D72A, interferes with mitotic checkpoint control and/or spindle function, leading to the aberrant separation of sister chromatids.

Phosphatase Activity of PRL-1 Is Required for Progression through Mitosis—To investigate whether PRL-1 activity was required for proper progression through the cell cycle, we synchronized the stable HeLa cell lines by treatment with nocodazole. In this experiment we also included the farnesylation site mutant PRL-1-C170S, to determine whether the observed defects in mitosis and cytokinesis were the result of a defect in the spindle checkpoint. Cells were then released from the block and analyzed for their cell cycle profile as described above. As shown in Fig. 10B, cells expressing wild type PRL-1 progressed through mitosis and returned to G1 phase with kinetics similar to those for the vector control. Again, cells expressing the catalytic domain mutant PRL-1-D72A showed a significant delay in mitosis and progressed into M phase or of a defect in mitosis, we synchronized the stable HeLa cell lines in prophase by treatment with nocodazole. Earlier phases of the cell cycle (S phase and entry into G2) were not affected. This delay was not observed with wild type PRL-1. The delay from G2/M back into G1 was highly reproducible and lasted on average 45.25 (± 14 S.D.) min.

To determine whether the delay observed in PRL-1-D72A-expressing cells was a result of a defect in the transition from G2 into M phase or of a defect in mitosis, we synchronized the stable HeLa cell lines in prophase by treatment with nocodazole. In this experiment we also included the farnesylation site mutant PRL-1-C170S, to determine whether the observed defects in mitosis and cytokinesis were the result of a defect in the spindle checkpoint. Cells were then released from the block and analyzed for their cell cycle profile as described above. As shown in Fig. 10A, lower panel). For 50% of the cells to return to G1, PRL-1-D72A cells were delayed by 86 min, compared with the vector control cells. Earlier phases of the cell cycle (S phase and entry into G2) were not affected. This delay was not observed with wild type PRL-1. The delay from G2/M back into G1 was highly reproducible and lasted on average 45.25 (± 14 S.D.) min.

Cells expressing PRL-1-C170S arrested in G2/M with equal efficiency as the vector control and cells expressing wild type PRL-1 when treated with nocodazole. After drug removal PRL-1-C170S cells progressed normally through mitosis and returned to G1 phase with kinetics similar to those for the control treatments. Together with the data shown in Fig. 9, the results indicate that farnesylation of PRL-1 might be required for proper function during
mitosis and that the mitotic defects of cells expressing PRL-1-C170S are not caused by defects in the spindle checkpoint. The data also demonstrate that expression of the catalytic domain mutant PRL-1-D72A delays transition through mitosis and/or cytokinesis and suggest that PRL-1 phosphatase activity is required for normal mitosis.

**DISCUSSION**

**Expression of PRL-1 in Cancer Cell Lines**—We have surveyed a panel of human cell lines for the expression of PRL family members by Taqman and found high levels of PRL-1 mRNA in a variety of tumor cell lines of different tissue origin. Elevated PRL-1 levels were especially prominent in melanoma cells. These results further point to a possible involvement of PRL-1 in tumorigenesis. However, a proliferative advantage because of PRL-1 overexpression may be cell type- or tissue-specific. It was previously reported that, in various cell types and tissues, PRL-1 expression correlates with cell proliferation (6). In intestine, however, PRL-1 levels correlated with terminal differentiation rather than with proliferation (6). Consistent with this, we did not detect elevated PRL-1 mRNA levels in the eleven colon cancer cell lines tested.

PRL-1 is an immediate early gene in rat regenerating liver, and in arrested fibroblast its expression can be induced by mitogens (6). Our data further extend these findings and demonstrate that, in cycling HeLa cells, PRL-1 mRNA and protein levels are fairly constant and are not subject to further cell cycle-dependent regulation (Fig. 2).

**Cell Cycle-dependent Localization of PRL-1**—Previously, PRL-1 was reported to localize to the nucleus, or to the plasma membrane and early endosomes when overexpressed (6, 10). Here we studied the localization of the endogenous protein. We had generated various anti-PRL-1 antibodies, but found significant cross-reactivity with PRL-2. This is likely explained by the high degree of sequence similarity between the two proteins. However, our Taqman analysis of PRL isoform expression demonstrates that in HeLa cells PRL-1 is the major form expressed, making this cell line a useful system for the analysis of PRL-1. Immunofluorescence with anti-PRL antibodies in HeLa cells should largely reflect the localization of endogenous PRL-1. We verified the specificity of our purified antibody by antigen blocking, which abolished anti-PRL-1 staining but not anti-tubulin staining (Fig. 2C). In addition, the antibody recognizes a single band of 22 kDa in immunoblot of whole cell HeLa extracts (Fig. 2B).

Our results demonstrate that PRL-1 localizes to the ER in interphase cells (Fig. 3). Anti-PRL-1 antibodies stain the same structures as antibodies directed against PTP1B, the first PTP identified to be associated with the ER membrane (16). There was no overlap in the immunofluorescence staining with markers for the nuclear membrane or the Golgi (data not shown). Expression of a GFP-PRL-1 fusion protein resulted in a similar staining pattern, although in this case the fusion protein is also found in other membrane structures, which is most likely the result of overexpression (Fig. 4). The association of PRL-1 with membrane structures is farnesylation-dependent. Mutation of the consensus cysteine residue in the C-terminal CAAX motif as well as treatment of cells with an inhibitor of farnesyltrans-
ferase resulted in a complete loss of membrane association and a diffuse cytoplasmic and nuclear staining. Similar to GFP-PRL-1, a GFP-PRL-2 fusion protein localized to perinuclear membrane structures and to a lesser degree to the plasma membrane (data not shown), and mutation of the predicted farnesylation site of PRL-2 resulted in loss of membrane association and enrichment in the nucleus (data not shown). Our results are consistent with those of Zeng et al. (10), who observed predominantly nuclear localization of PRLs after treatment of transfected cells with an inhibitor of farnesyltransferases (FTI). The enrichment of farnesylative defective PRL-1/2 mutants in the nucleus is possibly mediated by a candidate bipartite nuclear localization signal found in the C termini of PRL phosphatases (Fig. 4A). Alternatively, because of its small molecular weight, PRL-1 could diffuse freely through nuclear pores independent of a nuclear localization signal.

The Association of PRL-1 with the Centrosomes and the Mitotic Spindle—Our results further demonstrate that PRL-1 relocalizes in mitotic cells to the centrosomes and the spindle apparatus (Fig. 5). It has been noted that a farnesyl group provides only modest membrane association and that farnesylated proteins can rapidly dissociate from membrane structures (reviewed in Refs. 20 and 21). Often, farnesylated proteins require additional signals for efficient membrane association. In the case of H-Ras and N-Ras, this is achieved by palmitoylation of cysteine residues N-terminal to the CAAX box. Membrane association of K-RasB, on the other hand, requires a cluster of basic residue near the C box. Membrane association of K-RasB, on the other hand, requires a cluster of basic residue near the C box. PRL-1 and PRL-3, on the other hand, require a cluster of basic residue near the CAAX motif in addition to its farnesylation. One well studied example of reversible membrane association is the Rab proteins, which cycle between membranes and the cytosol. In this case the shuttling is regulated by protein-protein interactions (reviewed in Ref. 21). It is possible that the relocalization of PRL-1 is similarly mediated by protein-protein interactions. Another possibility is that PRL-1 leaves the ER in the process of nuclear envelope breakdown and fragmentation of the ER at the beginning of mitosis.

After being released from the ER at the beginning of mitosis, PRL-1 first co-localizes with γ-tubulin at the separated centrosomes (Fig. 5A). The earliest time point we have observed centrosomal localization of PRL-1 is in prophase (data not shown). In later stages of the cell cycle, PRL-1 is also associated with the spindle microtubules (Fig. 7B). Interestingly, PRL-1 is not evenly distributed along the spindle microtubules but is concentrated at the base of the spindle near the centrosomes. This pattern of localization is similar to the one of human and mouse Aurora A kinase (IAK1, AIK) which localizes to the centrosomes and the spindle poles (22, 23). In contrast to PRL-1, Aurora A also localizes to the midbody during telophase and cytokinesis.

Like PRL-1, the microtubule motor protein CENP-E contains a CAAX motif at the very C terminus and is farnesylated in vivo (24). CENP-E is an essential component of the mitotic spindle checkpoint and binds microtubules as well as kinesin-chores. It was recently demonstrated that unfarnesylated CENP-E, isolated from FTI-treated cells, does not bind microtubules in vitro (24). Our results demonstrate that the localization of PRL-1 to the mitotic spindle is farnesylation-independent. First, we found endogenous PRL-1 localized to spindle microtubules in FTI-treated HeLa cells. Second, recombinant PRL-1, which is not farnesylated, binds tubulin with high affinity in vitro (Fig. 6). Finally, a PRL-1 mutant that cannot be farnesylated (PRL-1-C170S) localizes to the spindle in mitotic HeLa cells (Fig. 7C).

Recently, it was suggested that farnesylation is important for the interaction of the GTP-binding protein K-Ras with tubulin and microtubules (25). Further analysis, however, revealed that the polylysine region within the C terminus of K-Ras is critical for this interaction (26). Interestingly, the C terminus of PRL-1 is also very basic and rich in lysine residues, although in the case of PRL-1 the basic residues are not clustered and are part of the potential bipartite nuclear localization signal (see Fig. 4A). In our in vitro binding assay, we found association of PRL-1 and PRL-3, but not of PRL-2, with tubulin. Thus, the interaction of PRL-1 with tubulin in vitro is likely to be specific and not solely because of the basic nature of the C terminus of the protein. The number and position of most of the basic residues within the C termini of PRL phosphatases are conserved. PRL-2, however, is distinct from PRL-1 and PRL-3 in that it is missing three amino acid residues just before the CAAX box (Fig. 4A). It is possible that this difference may account for the differences in the observed interaction with tubulin.

Although not required for spindle localization of PRL-1, farnesylation could have a regulatory function, which is suggested by the phenotype of cells expressing the farnesylation site mutant PRL-1-C170S. In contrast to HeLa cells overexpressing wild type PRL-1 or the catalytic domain mutant PRL-1-D72A, expression of PRL-1-C170S leads to mitotic defects characterized by mitotic bridges and lagging chromosomes. This defect is not a result of a defect in the spindle checkpoint because cells arrested normally in G2/M when treated with the microtubule destabilizing drug nocodazole (Fig. 10B). Further, after release from nocodazole block, the cells continued progression through mitosis similar to vector control cells. Therefore, it is likely that PRL-1-C170S interferes with proper spindle dynamics rather than with the spindle checkpoint itself.

The Requirement of PRL-1 in Cell Cycle Progression—The localization of PRL-1 to the centrosomes and the spindle suggested a possible function of this enzyme during mitosis. In synchronized HeLa cells, expression of PRL-1-D72A resulted in a significant delay in progression through G2/M, whereas other parts of the cell cycle were not affected (Fig. 10). Wild type PRL-1, on the other hand, had no significant effect on cell cycle progression. With the nocodazole block and release experiments, we further demonstrate that expression of PRL-1-D72A results in a mitotic delay, rather than interfering with the transition from G2 into M phase. The restricted localization to the centrosomes and the spindle poles together with the absence of PRL-1 from the midbody further suggest that the mitotic delay in PRL-1-D72A-expressing cells may not be caused by a defect in cytokinesis. Together, these data suggest that PRL-1 phosphatase activity might be required for normal mitotic progression. Importantly, cells overexpressing PRL-1-D72A or wild type PRL-1 arrested properly in the presence of nocodazole, indicating that this phosphatase is not involved in control of the spindle checkpoint. The observed effect of PRL-1-D72A on mitotic progression is likely the result of a dominant negative effect. PRL-1-D72A possibly competes with endogenous wild type PRL-1 for binding to the spindle microtubules and thus interferes with the dephosphorylation of a critical PRL-1 substrate(s), which might be required for timely progression through mitosis, anaphase initiation, or spindle dynamics in general. In this regard it is interesting to note that many aspects of spindle and centrosome function are regulated by protein phosphorylation, including microtubule motor function (27) and microtubule dynamics (28, 29). In addition, protein phosphatases have been identified to be involved in regulating microtubule and spindle functions (see Refs. 30 and 31 and references therein).

In summary, our data demonstrate that PRL-1 has two discrete localization patterns. In interphase cells it localizes to the ER in a farnesylation-dependent manner, and in mitotic cells it...
localizes to the centrosomes and the spindle independent of its farnesylation. Localization of PRL-1 to centrosomes and the spindle apparatus, delay of mitotic progression by expression of a dominant negative mutant, and the mitotic defects observed in cells expressing a farnesylation-defective PRL-1 all point to a function of this phosphatase in mitosis. The results further suggest that PRL-1 does not regulate the spindle checkpoint but may be involved in regulating spindle dynamics. Our results suggest that PRL-1 plays an important role in mitosis and that this phosphatase is regulated, at least in part, by cell cycle-dependent subcellular localization.

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