PRL-3 Down-regulates PTEN Expression and Signals through PI3K to Promote Epithelial-Mesenchymal Transition

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Abstract

PRL-3 is a metastasis-associated phosphatase. We and others have shown that its overexpression increases cell motility and invasiveness. These phenotypic changes are reminiscent of the epithelial-mesenchymal transition (EMT) that occurs during embryonic development and oncogenesis. The EMT is a complex process that converts epithelia into migratory mesenchymal cells. We here attempt to unravel the underlying mechanistic basis of these phenomena. HeLa cells transiently expressing EGFP-PRL-3 (HeLa-PRL-3) exhibit reduced levels of paxillin. Similarly, Chinese hamster ovary cells stably expressing myc-PRL-3 (CHO-PRL-3) also show marked reductions in paxillin, phosphorylated paxillin-Tyr31, and vinculin at focal adhesion complexes and notable reductions in the levels of RhoA-GTP, Rac1-GTP, and filamentous-actin filaments. DLD-1 human colorectal cancer cells engineered to express EGFP-PRL-3 (DLD-1-PRL-3) underwent changes consistent with EMT. In these cells, PRL-3 activates Akt and inactivates glycogen synthase kinase-3 (GSK3). These cells, PRL-3 activates Akt and inactivates glycogen synthase kinase-3 (GSK3). These changes in these EMT characteristics brought about by PRL-3 can be abrogated by the phosphoinositide-3-kinase (PI3K) inhibitor LY294002, implying that PRL-3 acts upstream of PI3K and could play an initiating role to trigger the EMT switch during cancer metastasis. In addition, PRL-3 can down-regulate phosphatase and tensin homologue deleted on chromosome 10 (PTEN) expression. Further evidence of the involvement of PRL-3 in EMT is provided by the observation that overexpression of PRL-3 in Chinese hamster ovary (CHO) cells promotes cell invasion, invasion, and metastasis (5). A recent study revealed that PRL-3 expression correlates with disease progression in ovarian cancers as higher levels of PRL-3 were detected in advanced (stage III) than in early (stage I) tumors. RNA interference–mediated knockdown of PRL-3 suppressed growth of ovarian cancer cells (6). In view of our finding that PRL-3 was detected in 11% of primary colorectal cancers (7), these results suggest that PRL-3 is a potential anticancer target. PRL-3 is not only associated with cancer metastasis but also with cancer progression. As the catalytic domain of PRL-3 is required in tumor metastasis, the PTP domain of this phosphatase might be useful as a therapeutic target for metastatic tumors (8). Recently, we showed that PRL-3 initiates tumor angiogenesis by recruiting endothelial cells in vitro and in vivo, indicating that PRL-3 may enhance tumor angiogenesis to facilitate cancer metastasis.

Introduction

Cancer metastases, rather than primary tumors, are responsible for most cancer deaths (1). One important insight came from the discovery that cancer cells increased their cell motility and invasiveness in a manner reminiscent of the epithelial-mesenchymal transition (EMT) that occurs during embryonic development and oncogenesis, in which epithelial cells acquire fibroblast-like properties and lose epithelial cell adhesion and cytoskeletal components (2). Several developmentally important genes that induce EMT have been shown to act as suppressors of E-cadherin. The loss of E-cadherin results in disassembly of cell-cell adhesion junctions and increases tumor cell invasiveness in vitro, which contributes to the transition of adenoma to carcinoma in animal models and is a hallmark of EMT (2).

Increasing evidence suggests that PRL-3 plays multiple roles in cancer metastasis. PRL-3 is first linked to metastasis from genomewide transcriptional analysis of colorectal cancer samples (3). PRL-3 mRNA expression is consistently elevated in most metastatic lesions derived from colorectal cancer, regardless of the targeted organs (4). Subsequently, we showed that overexpression of PRL-3 in Chinese hamster ovary (CHO) cells promoted cell migration, invasion, and metastasis (5). A recent study revealed that PRL-3 expression correlates with disease progression in ovarian cancers as higher levels of PRL-3 were detected in advanced (stage III) than in early (stage I) tumors. RNA interference–mediated knockdown of PRL-3 suppressed growth of ovarian cancer cells (6). In view of our finding that PRL-3 was detected in 11% of primary colorectal cancers (7), these results suggest that PRL-3 is a potential anticancer target. PRL-3 is not only associated with cancer metastasis but also with cancer progression. As the catalytic domain of PRL-3 is required in tumor metastasis, the PTP domain of this phosphatase might be useful as a therapeutic target for metastatic tumors (8). Recently, we showed that PRL-3 initiates tumor angiogenesis by recruiting endothelial cells in vitro and in vivo, indicating that PRL-3 may enhance tumor angiogenesis to facilitate cancer metastasis.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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We and others had previously shown that PRL-3 promotes cell motility, invasive ability, and metastatic activity (5), but the underlying molecular mechanisms remain elusive. We have therefore developed an in vitro system to study the effects of PRL-3 on cell adhesion and motility. We transiently transfected HeLa cells with pSTAR-Myc-PRL-3 (5), cultured, and stained for paxillin, phosphorylated paxillin (Tyr 31), and vinculin at focal adhesion complexes as assessed by indirect immunofluorescence. As shown in Figure 1A, PRL-3 reduces the staining of paxillin, phosphorylated paxillin-Tyr 31, and vinculin at focal adhesion complexes when they were <80% confluence and <18 h seeding from previous splitting.

Materials and Methods

Construction of pSTAR-Myc-PRL-3 and pSTAR-β-galactosidase plasmids. PCR fragments of Myc-PRL-3 and β-galactosidase were inserted into the EcoRI and BamHI sites of pSTAR vector (5).

Generation of HeLa cells transiently expressing pSTAR-Myc-PRL-3. HeLa cells from the American Type Culture Collection (ATCC; Manassas, VA) were transiently transfected with pSTAR-Myc-PRL-3 (5), cultured, and selected in RPMI 1640 with 1 mg/mL G418 for 24 to 48 h before analysis.

Generation of stable CHO cell lines expressing pMYC-PRL-3 and β-galactosidase. The detailed steps in generation of stable CHO-K1 from ATCC cell lines expressing pMYC-PRL-3 (CHO-PRL-3) and β-galactosidase (CHO-β-gal) were described in our previous studies (5).

Confocal microscopy. The detailed protocol was described previously (7). The sources of antibodies are as follows: mouse anti-c-Myc antibody 9E10 was from Santa Cruz Biotechnology (Santa Cruz, CA); mouse anti-paxillin and anti-vinculin were from Transduction Laboratory (San Jose, CA); rabbit anti-paxillin pY31 was obtained from Chemicon (Temecula, CA); or TRITC-conjugated Phalloidin was from Molecular Probes (Carlsbad, CA); mouse anti-PRL-3 (7). Confocal imaging was done using Zeiss LSM 510 image browser.

RhoA and Rac1 activity assay. The coding sequences for the Rho-binding domain of Rhotekin (amino acids 7–89) and Rac-binding domain of PAK (amino acids 23–137) were PCR amplified and cloned into the pGEX-4T1 vector to create pGST-RBD and pGST-PBD, respectively. The GST-RBD and GST-PBD fusion proteins were purified with glutathione-Sepharose beads. Activated RhoA or activated Rac1 pull-down assays were done as described elsewhere (10).

Generation of DLD-1 human colorectal cell pools stably expressing EGFP-human PRL-3 and EGFP-human PRL-3 (C104S). Human PRL-3 EST clone was used as PCR template for expression of human PRL-3 NH2-terminally tagged with enhanced green fluorescent protein (EGFP-PRL-3): hR3-5 (5'-gtgaattctatggctcggatgaaccgcccg-3') and hR3-3' (5'-cttgatccacctaaactggagctggg-3') were used to retrieve the human PRL-3 coding region by PCR. To construct pEGFP-PRL-3 (C104S), hR3-mid-3' (5'-cagccgccaagggagtgcagggcctggc-3') and hR3-mid-5' (5'-gtgtgatgaatctgcggcctgggctg-3') were used with similar strategies to create catalytic PRL-3 (C104S). The PRL-3 or mutant PRL-3 PCR fragment was, respectively, inserted into the EcoRI and BamHI sites of pEGFP-C1 vector, and the respective plasmid was transfected into DLD-1rogen carcinoma cells from ATCC CCL-221 using LipofectAMINE 2000 from Invitrogen (Carlsbad, CA).

Assessing DLD-1-PRL-3 and DLD-1- PRL-3 (C104S) cell motility. By plating cells in a confluent monolayer on a coverslip (12 mm), the cell-coated coverslip was then inverted with cell side down to a fresh culture dish (35 mm). Fresh culture medium (2 mL RPMI with 10% fetal bovine serum) was added gently to the dish. Images were taken at 24 h time points (Supplementary Fig. S1).

Western blot analysis. Detailed steps were described in ref. (7). The following antibodies were from Cell Signaling Technology (Beverly, MA): phosphorylated Akt (Ser473), AKT, phosphorylated glycogen synthase kinase-3β (GSK-3β; Ser9), GSK-3β, phosphorylated PTEN (Ser380/Thr382/383), PTEN, E-cadherin, γ-catenin, integrin β3, glyceraldehyde-3-phosphate dehydrogenase. Phosphorylated paxillin (Tyr39) antibody was from Abcam (Cambridge, United Kingdom). The Snail antibody was from AVIVA Systems Biology (San Diego, CA). Anti-fibronectin antibody was from BD (San Jose, CA). PK3 inhibitor LY294002 from Cell Signaling Technology was used at 20 to 50 μmol/L, to treat cells for 6 h at 37°C. We harvested cells when they were <80% confluence and <18 h seeding from previous splitting.

Results and Discussion

PRL-3 reduces paxillin, phosphorylated paxillin-Tyr 31, and vinculin adhesion molecules. The cancer metastatic process consists of a series of molecular events (1). The first important step involves neoplastic epithelial cells losing cell-cell adhesion and gaining motility, which enables them to invade the adjacent tissue. We and others had previously shown that PRL-3 promotes cell motility, invasive ability, and metastatic activity (5), but the underlying molecular mechanisms remain elusive. We have attempted to uncover the molecular basis of the changes associated with PRL-3 overexpression by assessing the effects of PRL-3 on several proteins involved in cell adhesion. HeLa cells transiently expressing EGFP-PRL-3 showed reduced paxillin staining at focal adhesion complexes (Fig. 1A, b and e, white arrows) compared with untransfected HeLa cells (red arrows). CHO cells stably expressing myc-PRL-3 (CHO-PRL-3) also showed reduced staining of paxillin as well as phosphorylated paxillin-Tyr31 (Fig. 1B, d and e). Paxillin is an important cytoskeletal protein capable of interaction with a variety of intracellular signaling molecules. Paxillin binds to vinculin, a key focal adhesion protein in vitro (11), and significantly, we found PRL-3 also reduced the levels of vinculin in focal adhesion complexes (Fig. 1B, f) compared with

Figure 1. PRL-3 reduces the staining of paxillin, phosphorylated paxillin-Tyr31 (p-paxillin), and vinculin at focal adhesion complexes as assessed by indirect immunofluorescence. A, HeLa cells transiently expressing EGFP-PRL-3 (HeLa-PRL-3, a and d) were labeled with rabbit anti-paxillin antibody and then antirabbit IgG conjugated with Texas Red (b and e). White arrows, HeLa-PRL-3 cells. Red arrows, untransfected HeLa cells as internal control. Bar, 20 μm. B, CHO-β-gal cells (a–c) or CHO-PRL-3 cells (d–f) were labeled for paxillin (a and d), phosphorylated paxillin (Tyr31; b and e), and vinculin (c and f) at focal adhesion complexes. Bar, 20 μm. C, lysates prepared from CHO-β-gal, CHO-PRL-3, DLD-1-PRL-3 (C104S), and DLD-PRL-3 cells were examined for the total levels of these adhesion molecules as indicated.
control CHO cells expressing β-gal (Fig. 1B, a–c). To further verify the above observations obtained from indirect immunofluorescence, we also showed an overall decrease in the total cellular levels of these adhesion molecules by Western blot analysis (Fig. 1C). We conclude that overexpression of PRL-3 causes a reduction in phosphorylated paxillin (Tyr31), paxillin, and vinculin levels but, more significantly, a decrease in cellular focal adhesion complexes.

PRL-3 decreases filamentous actin, RhoA-GTP, and Rac1-GTP. Using CHO-β-gal cells as control (Fig. 2A, a), the results of immunofluorescence microscopy show that PRL-3 reduces levels of filamentous-actin (F-actin) bundles in CHO-PRL-3 cells (Fig. 2A, b). Cell movement involves the orchestrated assembly and disassembly of actin filaments. The marked decrease in cytoskeletal F-actin would be consistent with increased cell migration (2). PRL-3 lowered the levels of RhoA-GTP and Rac1-GTP in both CHO-PRL-3 and DLD-1-PRL-3 cells (Fig. 2B, lanes 2 and 4) relative to their respective controls (Fig. 2B, lanes 1 and 3). A decrease in RhoA activation could decrease adherens junction stability (2). RhoA had been shown to increase levels of vinculin present in focal adhesions in Madin-Darby canine kidney cells (12), which could explain our observations of less active RhoA present in PRL-3–expressing cells, which might contribute to decreased vinculin at focal adhesion complexes and might weaken cell-cell junctions. Decreasing Rac1 activity switches cells from a random to directionally persistent migration in that cells continue rapid, directional migration in the same direction without turning (13). Thus, reduced levels of Rac1-GTP in PRL-3–expressing cells might drive more rapid directional migration (5). Notably, in some motile cells, the distribution of PRL-3 was polarized at the structures of membrane protrusions (Fig. 2C), where lamellipodia and lamellae at the leading edge of the cell mediate forward cell movement.

PRL-3 signals through PI3K to promote EMT. When cancer cells acquire motility, they can disseminate from the site of the primary tumor and establish secondary tumors in distant organs. This motile property recapitulates the EMT that is involved in the formation of the body plan, tissue remodeling, and cancer progression. It is increasingly clear that EMT is an integral component of the progression of epithelial derived tumors (2). Because PRL-3–expressing cells acquire enhanced motility, it is reasonable to investigate a possible role of PRL-3 in EMT induction. PRL-3 was investigated with respect to colorectal cancer metastasis. Using the human DLD-1 colorectal cancer cell line, we generated two stable pools expressing either EGFP-PRL-3 or a mutant EGFP-PRL-3 (C104S). DLD-1 cells expressing EGFP-PRL-3 have much enhanced migratory property compared with DLD-1 cells expressing EGFP-PRL-3 (C104S; Supplementary Fig. S1). We used these cells, together with parental DLD-1 cells, to examine possible roles of PRL-3 in the EMT pathway. PRL-3 but not PRL-3 (C104S) activated Akt as assessed by Ser473 phosphorylation (Fig. 3A, lane 3), which in turn inactivated GSK-3β at Ser9 (Fig. 3B, lane 3), resulting in a general down-regulation of the epithelial markers E-cadherin, γ-catenin (plakoglobin), and integrin β3 (Fig. 3C, lane 3). PRL-3 overexpression also enhanced the expression of the mesenchymal markers fibronectin and Snail (Fig. 3D, lane 3). The down-regulation of E-cadherin could be caused by Snail repression (14). Because PI3K/Akt is emerging as a central feature in the EMT pathway (15), we then investigated if PRL-3 signals through PI3K. DLD-1 cells expressing PRL-3 were treated with the PI3K inhibitor LY294002 (20 μmol/L). We noted that the effects of PRL-3 on Akt, GSK-3β, E-cadherin, γ-catenin, integrin β3, fibronectin, and Snail were all blocked by the PI3K inhibitor (Fig. 3A–D, lane 4). These data suggest that PRL-3 might be positioned upstream of, and signal through, the PI3K pathway leading to EMT.

PRL-3 down-regulates PTEN expression. We next examined the effect of PRL-3 on PI3K-negative regulators that would oppose the effects of PI3K activation. PTEN is the most important negative regulator of PI3K/Akt signaling. Using CHO-β-gal cells as a negative control (a), CHO-PRL-3 showed reduced level of F-actin (b) as revealed by indirect immunofluorescence staining with Phalloidin. Bar, 20 μm. B, cell lysates prepared from CHO-β-gal (lane 1), CHO-PRL-3 (lane 2), DLD-1-PRL-3 (C104S; lane 3), and DLD-1-PRL-3 (lane 4) cells were examined for the levels of RhoA-GTP, total RhoA, Rac1-GTP, and total Rac1 by immunoblot. The levels of PRL-3 were also assessed, whereas glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control. C, using PRL-3 monoclonal antibody, PRL-3 was detected in membrane protrusions in some motile cells (a). To-pro-3 iodide was used to stain DNA (b). Bar, 20 μm.
regulator of PI3K that antagonizes the effects of PI3K action. As such, the regulation of PTEN seems to be as important as PI3K during cell survival and migration. The DLD-1 cells stably expressing PRL-3 or PRL-3 (C104S) were examined for PTEN expression levels. PRL-3 but not its phosphatase-dead mutant PRL-3 (C104S) down-regulated phosphorylated PTEN and total PTEN levels (Fig. 4A, lane 3). The down-regulation of PTEN would release its suppressive effect on and thus promote the activities of PI3K (15) and, consequently, indirectly augment the action of PI3K on multiple signaling pathways to facilitate tumor formation and cancer metastasis. The reduction of PTEN protein levels caused by PRL-3 is PI3K independent, as LY294002 does not inhibit this effect (Fig. 4A, lane 4). Reverse transcription-PCR showed that mRNA levels of PTEN were unchanged (data not shown), implying that the down-regulation of PTEN is a posttranscriptional event. Although the basis of this reduction in PTEN protein is unknown, one possibility is that the loss of vinculin-containing cell adhesions suppresses PTEN levels (16). Another possibility is that PTEN is affected by altered RhoA signaling (17); decreased RhoA-GTP might contribute to the down-regulation of PTEN in DLD-1-PRL-3 cells.

**PRL-3 is linked to the PTEN-PI3K pathway.** Our data suggest PRL-3 acts as one of the initiators to orchestrate the EMT event, in which PRL-3 might play an important role to suppress PTEN;
consequently, PI3K and Akt are activated, whereas GSK-3β is inhibited. The latter would relieve inhibition of the mesenchymal marker Snail (14). Up-regulation of Snail, together with other transcriptional events, likely leads to down-regulation of the epithelial markers E-cadherin, γ-catenin, and integrin β3 (Fig. 4B). Thus, cells undergo an epithelial-mesenchymal–like transition. It has been shown that the mitogen-activated protein kinase pathway is involved in EMT (18). PRL-3 has also been shown to enhance the phosphorylation level of extracellular signal-regulated kinase 1/2 (Erk1/2) in 293 cells (19). In our system, we also observed that Erk1/2 was slightly activated by PRL-3 but not PRL-3 (C104S; data not shown) in DLD-1 cells. Therefore, we suggest that PRL-3 plays a role in tumor progression in colorectal cancer through the induction of EMT. As PRL-3 can exhibit a number of activities that contribute to cancer metastasis (20), PRL-3 seems to act upstream of the EMT transition pathway and is able to act as one of the initiators. This study highlights the importance of PRL-3 as a potential therapeutic target. Inhibition of PRL-3 may thus be a useful strategy to impede cancer cell invasion and metastasis.

Acknowledgments

Received 9/27/2006; revised 12/12/2006; accepted 1/24/2007.

Grant support: The Agency of Science, Technology, and Research (A* STAR), Singapore.

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We thank Profs. Alan Porter, Wanjun Hong, and Bor Luen Tang for their critical reading of the article; Dr. Graeme Wistow from NIH for a gift of human EST clone; and Cheng Peow Tan for his technical help.

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