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PTEN modulates GDNF/RET mediated chemotaxis and branching morphogenesis in the developing kidney

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Abstract

The RET receptor tyrosine kinase is activated by GDNF and controls outgrowth and invasion of the ureteric bud epithelia in the developing kidney. In renal epithelial cells and in enteric neuronal precursor cells, activation of RET results in chemotaxis as Ret expressing cells invade the surrounding GDNF expressing tissue. One potential downstream signaling pathway governing RET mediated chemotaxis may require phosphatidylinositol 3-kinase (PI3K), which generates PI(3,4,5) triphosphate. The *PTEN* tumor suppressor gene encodes a protein and lipid phosphatase that regulates cell growth, apoptosis and many other cellular processes. PTEN helps regulate cellular chemotaxis by antagonizing the PI3K signaling pathway through dephosphorylation of phosphotidylinositol triphosphates. In this report, we show that PTEN suppresses RET mediated cell migration and chemotaxis in cell culture assays, that RET activation results in asymmetric localization of inositol triphosphates and that loss of PTEN affects the pattern of branching morphogenesis in developing mouse kidneys. These data suggest a critical role for the PI3K/PTEN axis in shaping the pattern of epithelial branches in response to RET activation.

Keywords: PTEN; RET; GDNF; Chemotaxis; Branching morphogenesis

Introduction

The process of epithelial branching morphogenesis occurs in a variety of developing mammalian tissues, including mammary gland, the prostate, the lung and the kidney, and consists of common mechanistic elements (Costantini, 2006; Lu et al., 2006). Initial outgrowth of an invasive bud is followed by proliferation and cell migration to generate an ampullae that bi- or trifurcates to form new buds that will repeat the process and generate a complex arborized epithelial network. The pattern of arborization depends on positive regulation by growth factors and receptors to promote proliferation and migration and negative regulation to inhibit excessive budding and bifurcation. How potential positive and negative signals are integrated to control cell movement, while retaining the essential character of an invasive epithelia, remains unclear.

In the developing kidney, the ureteric bud diverticulum grows out of the nephric duct and invades a group of adjacent cells, the metanephric mesenchyme (Dressler, 2006). This outgrowth is stimulated by glial derived neurotrophic factor (GDNF), which is expressed in the mesenchyme and activates the RET receptor tyrosine kinase on the ureteric bud epithelia through a co-receptor GFRα1 (Costantini and Shakya, 2006). Loss of RET, GDNF or the co-receptor GFRα1 results in renal agenesis in mice, due to inhibition of ureteric bud growth and branching (Cacalano et al., 1998; Moore et al., 1996; Pichel et al., 1996; Sanchez et al., 1996; Schuchardt et al., 1994). In cell culture, GDNF is a chemoattractant for RET expressing epithelial cells (Tang et al., 1998) and is sufficient to promote ureteric bud outgrowth (Brophy et al., 2001; Sainio et al., 1997). Similarly, the RET/GDNF pathway is required for migration of enteric neuron precursor cells into the gut, also through a chemotactic mechanism (Natarajan et al., 2002).

Chemotaxis in response to extracellular gradients has been well described in *Dictyostelium*, in which asymmetry in the distribution of phosphatidylinositol (3,4,5)-triphosphate, (PI(3,4,5)P_3), can promote directional migration (Funamoto

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et al., 2002; Huang et al., 2003; Iijima and Devreotes, 2002). Coincident with the activation of phosphatidylinositol 3-kinase (PI3K) at the leading edges of migrating cells is the localization of the lipid phosphatase, PTEN, at the trailing ends. *Pten* is a tumor suppressor gene whose loss is detected in a variety of human cancers (Cairns et al., 1998; Li et al., 1997; Steck et al., 1997). PTEN has a dual specificity protein phosphatase and lipid phosphatase activity that can dephosphorylate $PI(3,4,5)P_3$ (Myers et al., 1997, 1998). Thus, PTEN antagonizes PI3K activity to suppress protein kinase B (Akt/PKB) dependent pathways, which can regulate cell migration, proliferation and apoptosis (Stambolic et al., 1998). Akt/PKB contains a pleckstrin homology (PH) domain that mediates binding to $PI(3,4,5)P_3$ to stimulate intracellular signaling pathways (Lemmon and Ferguson, 2000). Furthermore, the protein phosphatase activity of PTEN includes dephosphorylation and inactivation of focal adhesion kinase (FAK), which can modify the interactions between the extracellular matrix and the cytoskeleton (Tamura et al., 1998). PTEN can down-regulate integrin-mediated cell spreading and focal adhesion formation by a phosphatase-dependent manner, suggesting that PTEN functions in controlling cell surface interactions in which integrins, focal adhesion kinase and cell migration are involved (Tamura et al., 1998). Similarly, the lipid phosphatase Ship1, which can also dephosphorylate $PI(3,4,5)P_3$, is essential for neutrophil migration in response to chemotactic gradients (Nishio et al., 2007).

While the RET/GDNF pathway is essential for early kidney development, the intracellular mechanism regulating epithelial cell migration and branching morphogenesis are not entirely clear. Ligand dependent activation of RET results in the autophosphorylation of multiple tyrosine residues of which Y1062 is the most critical for renal development (Wong et al., 2005). Subsequent recruitment of multiple intracellular signaling partners, including Shc, Grb2, p85(PI3K), enigma and Dok-6 (Besset et al., 2000; Crowder et al., 2004; Degl'Innocenti et al., 2004; Durick et al., 1998), has been reported. Previously, we have utilized renal epithelial cells that stably express RET to demonstrate GDNF mediated chemotaxis (Tang et al., 1998). In this system, RET activation increased PI3K activity. Suppression of PI3K by pharmacological inhibitors inhibited GDNF mediated chemotaxis in epithelial cells and ureteric bud outgrowth in embryonic organ cultures (Tang et al., 2002). If the pattern of branching morphogenesis is shaped by PI3K activation in response to RET/GDNF, then the lipid phosphatase PTEN may also be required to down-regulate the signaling responses and fine tune the pattern during development. To address this question, we show that PTEN suppresses GDNF/RET mediated cell migration and chemotaxis in epithelial cell culture. RET activation results in asymmetric distribution of $PI(3,4,5)P_3$ as cells approach a gradient of GDNF. Furthermore, loss of PTEN in the ureteric bud and developing collecting duct system results in aberrant branching patterns, mislocalization of glomeruli and lethality. These data indicate an essential role for PTEN in shaping the pattern of branching morphogenesis in the developing kidney and suggest that $PI(3,4,5)P_3$ is an important signaling mediator for the GDNF/RET pathway.

Materials and methods

Plasmids

PTEN and PTEN-C124S expression plasmids were kindly provided by J. Dixon (UCSD, San Diego, CA). PTEN(C124S) is a mutant form in which serine at 124 is substituted for cysteine and its DNA sequence was confirmed. The fragments of PTEN-IRES-EGFP or PTEN(C124S)-IRES-EGFP from the previous constructs were also subcloned into CMV vector ($CB6^+$) to produce CMV-PTEN-IRES-EGFP and CMV-PTEN(C124S)-IRES-EGFP, respectively, and used for chemotaxis study. Akt/PKB PH domain-GFP fusion (PH-GFP) was kindly provided by T. Meyer (Stanford Univ., Palo Alto, CA).

Stable transfection of MDCK cell line

RET-overexpressing MDCK (Ret9) cells were cotransfected with PGK-Hygro and CMV-PTEN-IRES-EGFP, CMV-PTEN(C124S)-IRES-EGFP or PH-GFP, respectively, and selected for 10 days with 0.3 mg/ml hygromycin in Dulbecco's modified Eagles medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin under humidified 5% CO_2/95% air at 37 °C. At least 3 clones from each construct were obtained and frozen down at −140 °C.

Western blotting

Cells were lysed in PK-lysis buffer (Cai et al., 2002) and protein levels were quantified by the Bio-Rad colorimetric assay (Bio-Rad, Hercules, CA). SDS/PAGE sample buffer was added and samples were boiled for 5 min. Samples were run on a 8% polyacrylamide gel, transferred to PVDF membrane (Perkin-Elmer, Boston, MA) and blocked with 5% non-fat milk in Tris-buffered saline. Membranes were immunoblotted with anti-HA antibody (Covance, Richmond, CA), anti-PTEN antibody (Cell signaling, Beverly, MA) and anti-p-Akt antibody (Cell signaling, Beverly, MA) in 10 mM Tris, pH 7.5, 100 mM NaCl, 0.1% Tween 20, 1% non-fat milk. Secondary antibodies conjugated to horseradish peroxidase were used at a 1:10,000 dilution, and the signal was visualized by chemiluminescence (Amersham, Buckinghamshire, England). For quantitation, fluorescent conjugated secondary antibodies and a Li-Cor Odyssey infrared imager were used to scan the membranes.

Chemoattraction and cell migration assays

For chemoattraction assay, preparation of type I collagen gel and GDNF bead was described by Tang et al. (1998). RET-PTEN, RET-PTEN(C124S) or PH-GFP cells were seeded on the surface of the gel at 40,000 cells/well with DMEM containing 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin, 0.3 mg/ml G418, 0.2 mg/ml hygromycin and supplemented with 250 ng/ml GFRα1. Cells around the beads were photographed over time at 24 h intervals. For cell migration assay, 1×10^4 cells were plated onto the transwell filters containing 8 μm pores (Corning, Inc., Corning, NY) with the same media previously described. GDNF and GFRα1 were added to the bottom chamber or both the bottom and the top chambers at 100 ng/ml and 250 ng/ml, respectively. After a 24-h incubation, cells on the filters were fixed with 4% paraformaldehyde and stained with DAPI. All experiments were done in triplicate and averages presented with one standard deviation from the mean.

PTEN tissue specific mutants

The *pten* floxed allele was obtained from T. Mak and genotyped as described (Suzuki et al., 2001). The HoxB7-Cre transgene was obtained from C. Bates and utilized as described previously (Zhao et al., 2004). For organ cultures, E11.5 kidneys were dissected free and placed on transwell filters in serum free media. After 48 h of culture, organ rudiments were fixed in methanol and stained whole with mouse anti-pan-cytokeratin (Sigma) and rabbit anti-laminin (Sigma).