REGULATION OF THE POLARITY PROTEIN PAR6 BY THE TGFβ RECEPTOR CONTROLS EPITHELIAL CELL PLASTICITY

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INTRODUCTION: Transforming growth factor β (TGFβ) is a key regulator of epithelial-to-mesenchymal transition (EMT) (1). However, the molecular mechanisms that control the dissolution of tight junctions, an early event in EMT, remain elusive. Here we demonstrate that Par6, a regulator of epithelial cell polarity and tight junction assembly, interacts with the TGFβ receptor and is a substrate of TβRII. We show that this phosphorylation of Par6 is required for TGFβ-dependent EMT in mammary gland epithelial cells and functions by controlling the interaction of Par6 with the E3 ubiquitin ligase, Smurf1. Smurf1, in turn, targets the GTPase RhoA for degradation, thereby leading to tight junction loss. Thus, these studies define how an extracellular cue signals to the polarity machinery to control epithelial cell morphology.

METHOD: Par6 was identified as a TβRI interaction partner with the use of a novel luminescence-based in-vivo protein-protein interaction screen. Immunoprecipitation, orthophosphate labeling, tryptic phosphopeptide mapping, and in-vitro kinase assays were used to identify additional interaction partners for Par6 and to demonstrate that Par6 is a substrate of TβRII upon stimulation with TGFβ. Standard immunofluorescence methodologies were used to test the ability of cells to engage in EMT.

RESULTS: The N-terminal 104 amino acids of Par6 were necessary for binding to TβRI. Par6(K19A), a mutation that interferes with the PKCζ binding (2), bound to TGFβ receptors at levels comparable to WT Par6. However, PKCζ association with the receptor complexes was lost in the presence of Par6(K19A). Immunofluorescence analysis revealed that TβRI is localized to tight junctions with Par6 and TGFβ stimulation induces redistribution of cell surface TβRII into tight junctions. Analysis in [32P]phosphate-labeled cells revealed TGFβ-dependent in vivo phosphorylation of TβRI-bound Par6. In-vivo and in-vitro tryptic phosphopeptide mapping revealed that Par6 is a direct substrate of TβRII and is phosphorylated upon TGFβ stimulation. Analysis in NMuMG cell lines revealed that while stable expression of WT Par6 did not affect TGFβ-induced EMT, clones expressing the Par6 phosphorylation mutant displayed stable tight junctions and retained cortical actin staining even after prolonged TGFβ stimulation (Fig. 1). Interestingly, TGFβ stimulated expression of vimentin in Par6 phosphorylation mutant-expressing cells despite the complete retention of tight junctions. We also showed that TGFβ-dependent alteration in cell morphology and tight junction homeostasis during EMT occurs through phosphorylation of Par6; furthermore, this phosphorylation has no effect on Smad signaling (Fig. 2). We hypothesized that in polarized epithelial cells, phosphorylation of Par6 by the TGFβ receptor might regulate tight junction dissolution by controlling Par6-Smurf1.
association and the localized degradation of RhoA. To investigate this, we analyzed the interaction of endogenous Smurfl with either Par6 or Par6 phosphorylation mutants in NMuMG cells. In the absence of TGFβ signaling we detected a basal level of Smurfl co-precipitating with WT Par6 and this was enhanced by TGFβ treatment. In contrast, when we examined Par6 phosphorylation mutants there was little if any detectable association with Smurfl (Fig. 3). This suggested that Smurfl preferentially binds the Par6 phosphoisomer that is phosphorylated by TβRII. Subsequent analysis with Smurfl mutants and siRNA targeting of endogenous Smurfl expression revealed that Smurfl functions as a pro-mesenchymal progression factor during TGFβ–mediated EMT. We next examined whether RhoA might function in this pathway by testing whether RhoA steady-state levels were regulated by TGFβ. In control cells, TGFβ caused a reduction of RhoA by 30% and treatment with the proteasome inhibitor MG132 reversed the decrease. In addition, this TGFβ-dependent decrease in RhoA was not observed in cells expressing the Par6 phosphorylation mutant, suggesting that TGFβ can regulate RhoA levels via Par6. We then identified the lysines on RhoA that serve as acceptors for Smurfl ubiquitination and generated the corresponding RhoA mutants to examine whether Smurfl-resistant RhoA affected dissolution of tight junctions in response to TGFβ. Although WT RhoA had no effect on EMT, Smurfl-resistant RhoA inhibited TGFβ-dependent EMT.

**DISCUSSION:** Altogether, our results demonstrate a direct link between TGFβ receptors, the polarity complex and the regulation of tight junction dissolution during EMT. In this model, cell surface TβRI is localized to tight junctions in polarized epithelial cell sheets. TGFβ, which induces association of TβRI with TβRII, leads to the accumulation of receptor complexes in tight junctions thereby bringing the TβRII receptor kinase to the Par6-TβRI complex. This leads to phosphorylation of Par6, which in turn stimulates binding of Par6 to Smurfl. The Par6-Smurfl complex then mediates the localized ubiquitination of RhoA to enable the TGFβ-dependent dissolution of tight junctions, thereby allowing EMT to progress. Our demonstration that both Smad activation and the induction of mesenchymal gene expression can be dissociated from a loss of tight junctions reveals a bifurcated signaling network at the level of the TGFβ receptor. This dynamic interplay of multiple pathways likely allows the TGFβ receptor system to orchestrate EMT by coordinating dissolution of tight junctions with gene expression programs.

**ACKNOWLEDGMENTS:** This work was supported by grants from the Canadian Institutes of Health Research, National Cancer Institute of Canada, Natural Sciences and Engineering Research Council of Canada and Canadian Cancer Society.

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