Published OnlineFirst January 13, 2009; DOI: 10.1158/0008-5472.CAN-07-6270

Protein Kinase D1–Mediated Phosphorylation and Subcellular Localization of β-Catenin

Cheng Du,[1] Meena Jaggi,[2] Chuanyou Zhang,[1] and K.C. Balaji[1]

[1]Division of Urology, Department of Surgery, University of Massachusetts Medical School, Worcester, Massachusetts and [2]Department of Obstetrics and Gynecology and Basic Biomedical Science, Sanford School of Medicine, The University of South Dakota, Sioux Falls, South Dakota

Abstract

β-Catenin is essential for E-cadherin–mediated cell adhesion in epithelial cells and also acts as a key cofactor for transcription activity. We previously showed that protein kinase D1 (PKD1), founding member of the PKD family of signal transduction proteins, is down-regulated in advanced prostate cancer and interacts with E-cadherin. This study provides evidence that PKD1 interacts with and phosphorylates β-catenin at Thr^{112} and Thr^{120} residues *in vitro* and *in vivo*; mutation of Thr^{112} and Thr^{120} results in increased nuclear localization of β-catenin and is associated with altered β-catenin–mediated transcription activity. It is known that mutation of Thr^{120} residue abolishes binding of β-catenin to α-catenin, which links to cytoskeleton, suggesting that PKD1 phosphorylation of Thr^{120} could be critical for cell-cell adhesion. Overexpression of PKD1 represses β-catenin–mediated transcriptional activity and cell proliferation. Epistatic studies suggest that PKD1 and E-cadherin are within the same signaling pathway. Understanding the molecular basis of PKD1–β-catenin interaction provides a novel strategy to target β-catenin function in cells including prostate cancer. [Cancer Res 2009;69(3):1117–24]

Introduction

β-Catenin was originally identified as a key effector of the Wnt/Wg signaling pathway in *Drosophila* (1). It has also been identified as a component of adherens junctions with the primary cell adhesion molecule E-cadherin (2–4). β-Catenin promotes cell adhesion by binding to the intracellular domain of the cadherin adhesion receptors and linking cadherin to the actin cytoskeleton through the adaptor protein α-catenin (reviewed in ref. 5). This association with the actin-based cytoskeleton is important for the adhesion function of all classic cadherins, as best shown for the E-cadherin-catenin complex in adherens junctions of epithelial cells (6).

β-Catenin can be found in three cell compartments: the plasma membrane, the cytoplasm, and the nucleus. Nuclear β-catenin acts as cofactor for a wide variety of transcription factors, including T-cell factor (TCF), androgen receptor (7), and nuclear factor κB (8). Soluble cytoplasm pool of β-catenin is highly unstable and is stabilized by Wnt signal resulting in nucleus localization. The dynamic balance of β-catenin functions in both cell adhesion and transcription has recently won increasing interest. Properly balancing between these functions is crucial for normal development, and the wrong choice can lead to cancer.

Molecular switches of the dual role of β-catenin are tightly regulated. The structural and functional integrity of the cadherin-catenin complex is mainly regulated by phosphorylation. Casein kinase II phosphorylates β-catenin (9) and E-cadherin (10) on serine and threonine residues. This phosphorylation leads to increased stabilization of the cadherin-catenin complex. Phosphorylation by AKT kinase causes β-catenin disassociation from cell-cell contacts and accumulation in both the cytosol and the nucleus, increases β-catenin transcriptional activity, and promotes tumor cell invasion (11). On the other hand, tyrosine phosphorylation of β-catenin disrupts binding of the E-cadherin/β-catenin/α-catenin complex (12–15). In contrast, activation of protein phosphatases stabilizes the cadherin-catenin complex and results in increased cadherin-mediated cell-cell adhesion (16, 17).

Protein kinase D1 (PKD1), founding member of a new family of serine/threonine protein kinases, lies downstream of the signal transduction pathways initiated by diacylglycerol and protein kinase C. Diacylglycerol regulates the intracellular localization of PKD1 and also activates PKD1 through protein kinase C by phosphorylation. Prior studies show that PKD1 plays a role in cell growth, gene expression, survival, motility, protein trafficking, and lymphocyte biology (18). We initially discovered that PKD1 is down-regulated in advanced prostate cancer and later described its interaction with E-cadherin (19, 20). In this study, we show that PKD1 directly binds to and phosphorylates β-catenin and show the biological consequences of the phosphorylation.

Materials and Methods

Constructs. Human β-catenin clone was obtained from Mammalian Gene Collection. Full-length β-catenin was amplified by PCR and cloned into bacterial expression vector pGEX-5X-1, yeast two-hybrid plasmid pGADT7, and mammalian expression vectors pEGFP-C1 and DsRed2-C1. Another β-catenin expression vector, β-catenin-pCS2$^+$, was kindly provided by Dr. R. Kemler (Department of Molecular Embryology, Max-Planck Institute of Immunology, Freiburg, Germany; ref. 9). Site-specific mutagenesis to change Thr^{102} to isoleucine (T102I), Thr^{112} to arginine (T112R), and Thr^{120} to isoleucine (T120I) was done with a PCR-based method. Primers for mutagenesis: forward (T102I) 5′-GCTGCTATGTTCCCTGAGATCTTAGATGAGGGCATGCAG; (T112R) 5′-GCATGCAGATCCCATCTAGACAGTTTGATGCTGCTCATC; and (T120I) 5′-TTGATGCTGCTCATCCCATTAATGTCCAGCGTTTGGCTG, where the underlined sequences contain mutated sites and new restriction sites to facilitate clone selection.

A COOH-terminal truncated β-catenin in pEGFP-C1 (β-catN/pEGFP) was made by restriction digestion of correspondent full-length vector with deletion of *Plm*I and *Sma*I fragments. The construct includes the first 150 amino acid residues of β-catenin. Expression plasmid for full-length E-cadherin expression vector was constructed by cloning PCR product into pEGFP-N1 vector. shRNA vectors targeting PKD1 (siPKD1) and E-cadherin (siEcad) were described in ref. 21. All constructs were confirmed by sequencing.

Note: C. Du and M. Jaggi contributed equally to this work.

Requests for reprints: Cheng Du or K.C. Balaji, Division of Urology, Department of Surgery, University of Massachusetts Medical School, 55 Lake Avenue, North Worcester, MA 01655. Phone: 508-856-2576; Fax: 508-856-3137; balajik@ummhc.org or cheng.du@umassmed.edu.

doi:10.1158/0008-5472.CAN-07-6270

Published OnlineFirst January 13, 2009; DOI: 10.1158/0008-5472.CAN-07-6270

Yeast two-hybrid assay. The C1a, C1b, pleckstrin homology (PH), and catalytic domains of PKD1 were cloned in pGBKT7 vector as previously described (22). Yeast strain PJ69-4A was used to detect protein interaction according to standard protocol (23).

***In vitro* phosphorylation assay.** Wild-type and mutant β-catenin proteins were expressed and purified from *E. coli*. Preparation of active and inactive PKD1 from 293T cells transfected with PKD1 expression vectors pEGFP-PKD1 and pEGFP-PKD1-KD, respectively (20), by immunoprecipitation was described (24). Briefly, transfected cells were treated with 0.5 μmol/L phorbol 12,13-dibutyrate for 30 min before being lysed. Cells were washed twice in TBS [25 mmol/L Tris (pH 7.5), 0.1 mol/L NaCl], followed by lysis in TBS, 1% Triton X-100, and protease inhibitor cocktail (Sigma Chemicals). Immunoprecipitation used anti-PKD1 antibody (C20, Santa Cruz Biotechnology) and final beads were resuspended in TBS plus 10% glycerol. *In vitro* kinase assay was carried with 5 μL PKD1 kinase, 30 μL purified protein, 5 μL kinase buffer [250 mmol/L Tris (pH 7.5), 100 mmol/L $MgCl_2$, 1 mmol/L ATP], and 1 μL of [^{32}P]ATP (3,000 μCi/mmol) in a 50-μL volume at 37°C for 1 h.

Transient transfection and luciferase assay. Transfection was done by using the Lipofectamine 2000 method (Invitrogen). Luciferase dual-reporter assays with Super8xTopflash (25) and pRL-TK (Promega) and dual-reporter luminofluorescence assay (Promega) were done 48 h after transfection according to the manufacturer's recommendation.

Immunoprecipitation and Western blots. LNCaP cells were cultured in RPMI 1640 plus 10% fetal bovine serum. The cells were lysed in TBST (50 mmol/L Tris, pH 7.2, 100 mmol/L NaCl, 1% Triton X-100) plus protease inhibitor cocktail (Sigma Chemicals). The lysate was used for immunoprecipitation by a 1-h incubation with respective antibodies [2 μg of anti-PKD1 (Santa Cruz) or anti–β-catenin (BD Biosciences)] followed a 1-h incubation with Protein A agarose beads. Immunocomplexes were washed five times with TBS [50 mmol/L Tris (pH 7.2), 100 mmol/L NaCl] and resolved by SDS-PAGE.

The following antibodies were used in Western blot: anti–E-cadherin (Santa Cruz, rabbit; BD Biosciences, mouse), anti–green fluorescent protein (GFP; Santa Cruz, rabbit; Genscript, mouse), anti-PKD1 (Santa Cruz, rabbit), anti–activated PKD1 (pS916, from Cell Signaling), and anti-hemagglutinin (HA) tag (3F10, Roche Applied Science).

Phosphatase treatment. The β-catN/pEGFP vector was cotransfected with PKD1 into NIH 3T3 cells. Bryostatin 1, a marine-derived macrolactone and PKD-selective activator, was added into the culture 1 d after transfection and incubated for 30 min. Cell lysate was incubated with λ phosphatase (New England Biolabs) for 30 min at 30°C. In a control tube, phosphatase inhibitor cocktails (AG Scientific) were preincubated with λ phosphatase before being added to the cell lysate.

Cell lines. Unless mentioned specifically, all cells were cultured in DMEM plus 10% serum. C4-2/PKD1 cells are C4-2 cells that stably express GFP-tagged full-length PKD1 selected by antibiotic Gtgesicqz8 treatment and sorted by fluorescence-activated cell sorting, consisting of stably transfected cells from multiple colonies (20). This cell line was cultured in DMEM plus 10% serum and sodium pyruvate.

Matrigel assay. Full-length wild-type β-catenin and T112R/T120I mutant constructs were transfected into NIH 3T3 fibroblasts. The cells were trypsinized 2 d later and washed. About 200,000 cells were added into a transwell (8 μm) containing Matrigel (BD Biosciences). After 2 d, Matrigel was swapped and transwells were stained with the use of Diff Quick kit. Cell numbers were counted under a light microscope and analyzed.

Results

Ectopic expression of both E-cadherin and PKD1 downregulates β-catenin/TCF activity. Previous studies showed that overexpression of E-cadherin reduces β-catenin–mediated Wnt signaling, as determined by Topflash assay (26–28). The suppression requires the β-catenin binding ability of E-cadherin but is independent of the adhesion function of E-cadherin.

Because E-cadherin forms a protein complex with β-catenin, we tested if PKD1 affects β-catenin transcription activity. Expression of β-catenin was able to increase TCF transcription activity in Topflash assay in NIH 3T3 cells (Fig. 1*A*). Ectopic expression of E-cadherin or PKD1 individually inhibits β-catenin/TCF transcription activity. Expression of a kinase-dead PKD1 (PKD1-KD) has less inhibitory effects than wild-type PKD1. Conversely, expression of shRNAs against PKD1 (siPKD1) or E-cadherin (siEcad) increases β-catenin/TCF transcription activity. Similar results were obtained from the colon cancer SW480 cell line, which is negative for adenomatous polyposis coli and with high levels of β-catenin, and the prostate cancer C4-2 cell line (data not shown). The data strongly suggest that both PKD1 and E-cadherin regulate β-catenin/TCF signaling negatively.

The similar phenotypes of PKD1 and E-cadherin on β-catenin/TCF function led us to study their epistatic relationship. We asked if knockdown of PKD1 could overcome the E-cadherin repression on Topflash activity or vice versa. The siPKD1 was cotransfected with E-cadherin expression vector or siEcad with PKD1 expression vector into mouse 3T3 cells. Knockdown of PKD1 can overcome the inhibition caused by overexpression of E-cadherin and vice versa (Fig. 1*A*), suggesting that PKD1 and E-cadherin regulate β-catenin/TCF activity via the same pathway.

PKD1 colocalizes and associates with β-catenin in prostate cancer cells and human tissue. We examined the subcellular localization of PKD1 and β-catenin in LNCaP prostate cancer cells (Fig. 1*B*). Whereas PKD1 localizes in plasma membrane and cytosol, β-catenin mainly localizes to plasma membrane. Merged image shows that the two proteins colocalize only at cell adherens junctions. Interestingly, PKD1 is not present on cell surfaces that are not in contact with other cells, whereas β-catenin is present on entire cell surface (Fig. 1*B*, *white arrows*). Similar results were obtained from C4-2 cells (data not shown). Furthermore, PKD1 and β-catenin are colocalized on cell membrane in all five normal human prostate tissue paraffin samples examined (Fig. 1*C*). Because E-cadherin anchors the E-cadherin/β-catenin complex on plasma membrane and PKD1 colocalizes with β-catenin, it is interesting to know if E-cadherin mediates PKD1 membrane localization. Using E-cadherin–negative JCA bladder cancer cell line (Fig. 1*D*) and SW480 cells (data not shown), we show that PKD1 colocalizes with β-catenin in cytosol but not at plasma membrane, suggesting that the colocalization can occur in the absence of E-cadherin.

PKD1 binds to and phosphorylates β-catenin. Because PKD1 and β-catenin colocalize together, we explored interaction of the two proteins by immunoprecipitation experiments. Figure 2*A* confirms that PKD1 and β-catenin are present in the same protein complex. To prove that PKD1 and β-catenin can interact directly, yeast two-hybrid analysis was used. PKD1 has two cysteine-rich domains, C1a and C1b, a PH domain, and a catalytic domain. Figure 2*B* shows that β-catenin directly interacts with the catalytic and the C1b domains of PKD1, suggesting that PKD1 can interact directly with β-catenin in the absence of E-cadherin. Because β-catenin interacted strongly with PKD1 kinase domain, we performed an *in vitro* phosphorylation assay, which shows that active PKD1 can phosphorylate β-catenin whereas catalytic inactive PKD1 cannot (Fig. 2*C*).

To map PKD1 phosphorylation site, the *in vitro* phosphorylated β-catenin protein was trypsinized and the resulting peptide fragments were separated by high-performance liquid chromatography. The ^{32}P-containing "hot" peptide was isolated and

Published OnlineFirst January 13, 2009; DOI: 10.1158/0008-5472.CAN-07-6270

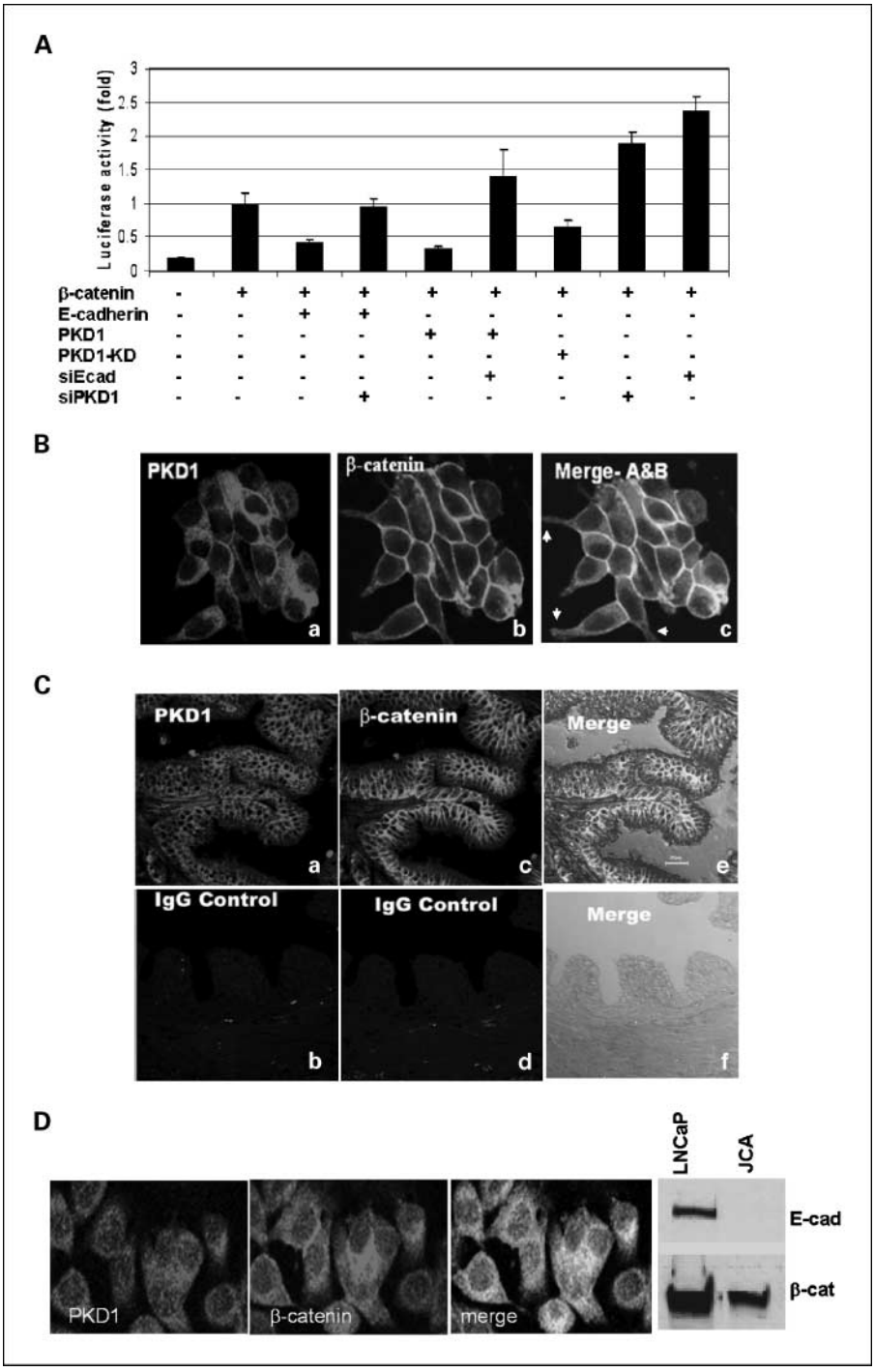

Figure 1. PKD1 associates with β-catenin and negatively regulates β-catenin/TCF transcription activity. *A,* both PKD1 and E-cadherin negatively regulate β-catenin/TCF transcription activity (Topflash assay). Fifty nanograms of Topflash plasmid, 2.5 ng of Renilla luciferase, and 50 ng of wild-type β-catenin were cotransfected with 100 ng of PKD1, kinase-dead PKD1, or E-cadherin constructs, or with 25 ng of shRNA constructs against either E-cadherin (siEcad) or PKD1 (siPKD1) into NIH 3T3 cells. Firefly luciferase activities were measured 48 h after transfection with Promega dual reporter assay reagents and the firefly luciferase activity was normalized by Renilla luciferase activity. Overexpression of PKD1 or E-cadherin inhibits β-catenin transcription activity. In contrast, knockdown of either PKD1 or E-cadherin by siRNA increases β-catenin transcription activity. Knockdown of PKD1 relieves the inhibitory effects caused by overexpression of β-catenin and vice versa. *Bars,* SD based on triplicate samples. The efficacy of these shRNA constructs was shown in ref. 20. *B* to *D,* colocalization and *in vivo* association of PKD1 and β-catenin. *B,* PKD1 and β-catenin mainly colocalize on plasma membrane in LNCaP cells, which are E-cadherin positive. *C,* PKD1 and β-catenin colocalize in normal prostate tissue. *D,* PKD1 and β-catenin mainly colocalize in cytosol in bladder cancer JCA cells, which are E-cadherin negative.

further analyzed by Edman degradation. The first five amino acid residues were RAAMF, which matched to β-catenin amino acid sequence 95 to 99. Because this peptide (amino acid residues 95–123) contains three threonine residues and one serine residues, site-specific mutagenesis was used to modify single or combination of two or three of these residues. Wild-type and mutated β-catenin proteins purified from bacterial expression system were incubated with purified active PKD1 kinase. As shown in Fig. 2*D*, whereas wild-type β-catenin is phosphorylated, mutation of all three threonine (Thr^{102}, Thr^{112}, and Thr^{120}) residues abolishes the phosphorylation, suggesting that the serine residue is not a PKD1 phosphorylation site. When only a single threonine residue is replaced, β-catenin still can be phosphorylated; when Thr^{112} and Thr^{120} were altered, β-catenin is no longer phosphorylated. Therefore, we concluded that Thr^{112} and Thr^{120} are PKD1 phosphorylation sites. These two residues are within α-catenin binding region. Indeed, Thr^{112} was also identified as a CKII phosphorylation site, and mutation of Thr^{112} was implied in decreased affinity to α-catenin (9). Previous study showed that mutation of Thr^{120} abolished α-catenin binding ability, although no specific kinase was identified for Thr^{120} phosphorylation (29).

Published OnlineFirst January 13, 2009; DOI: 10.1158/0008-5472.CAN-07-6270

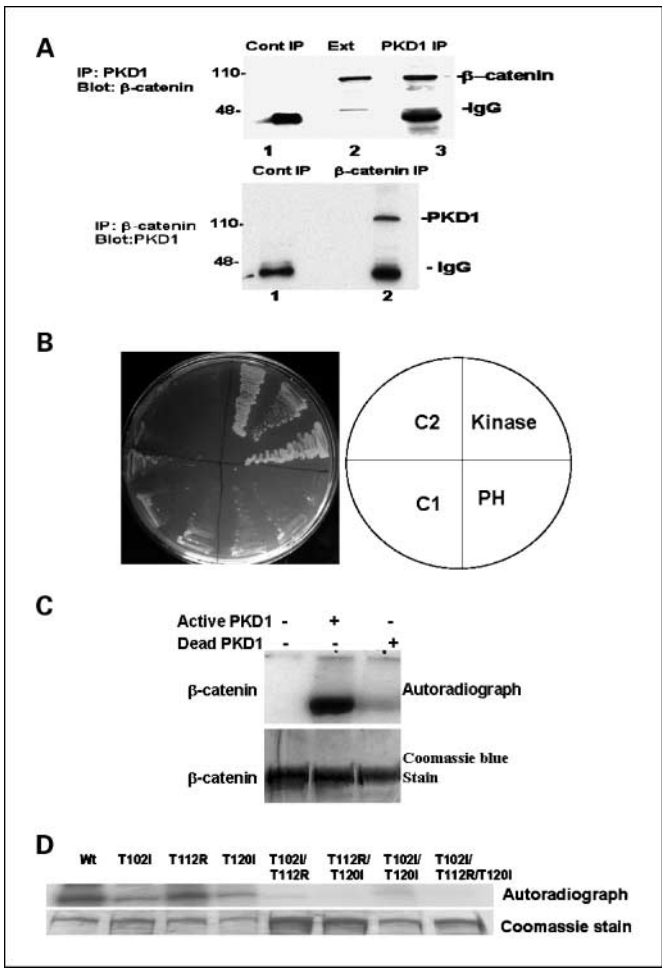

Figure 2. Direct interaction and phosphorylation of β-catenin by PKD1. *A,* reciprocal coimmunoprecipitation of PKD1 and β-catenin from LNCaP cell. *Top,* coimmunoprecipitation with PKD1 antibody and blotting with β-catenin antibody. *Lane 1,* immunoprecipitation with control antibody; *lane 2,* whole cell lysates only; *lane 3,* immunoprecipitation with PKD1 antibody. *Bottom,* coimmunoprecipitation with β-catenin antibody and blotting with PKD1 antibody. *Lane 1,* immunoprecipitation with control antibody; *lane 2,* immunoprecipitation with β-catenin antibody. *B,* yeast two-hybrid test. Three individual colonies were used for testing. Only PKD1 catalytic domain interacts with β-catenin. *C, in vitro* phosphorylation. Purified full-length glutathione *S*-transferase (GST)–tagged β-catenin protein from *E. coli* was incubated with either active or kinase-dead PKD1 in the presence of [γ-^{32}P]ATP. β-Catenin was phosphorylated by active PKD1. *D,* mapping of PKD1 phosphorylation sites on β-catenin *in vitro*. Wild-type and mutated GST-tagged full-length β-catenins were expressed in and purified from *E. coli.* The purified proteins were incubated with purified active PKD1 in the presence of [^{32}P]ATP. After incubation, the proteins were separated by SDS-PAGE, stained with Coomassie blue, and exposed onto films. Refer to Materials and Methods for the mutant nomenclature.

To confirm the phosphorylation *in vivo*, two COOH-terminal truncated β-catenins, wild-type and T112R/T120I counterpart that includes the first 150 amino acid residues of β-catenin, were made. These vectors expressing the NH_2-terminal region of β-catenin were cotransfected into 293T cells with either active or kinase-dead PKD1. Cell lysates were analyzed by immunoblotting. Figure 3*A* shows that the wild-type β-catenin can be phosphorylated as indicated by an electrophoretic mobility shift in the presence of active PKD1, but not T112R/T120I mutant, suggesting that Thr^{112} and Thr^{120} are phosphorylated *in vivo*. In contrast, there is no mobility shift of the wild-type β-catenin NH_2-terminal region when incubated with inactive PKD1 (Fig. 3*A*). To further confirm that the lower molecular weight band is phosphorylated β-catenin, the PKD1-containing β-catenin lysate was incubated with λ phosphatase (Fig. 3*A*). In the presence of phosphatase, the lower molecular weight band disappeared. However, if the phosphatase was preincubated with phosphatase inhibitors, the lower molecular weight band remained, suggesting that the low molecular weight band is phosphorylated β-catenin.

Cellular PKD1 can be activated when treated by bryostatin 1 (30). The activation of PKD1 can be monitored by phosphoserine antibody pS916, which recognizes autophosphorylation of Ser^{916} on PKD1. The cells that were cotransfected with PKD1 and truncated β-catenin were treated with 10 nmol/L bryostatin 1 for various time periods up to 1 h. Cell lysates were analyzed by Western blot. As seen in Fig. 3*B*, active PKD1 accumulated over time along with accumulation of the phosphorylated β-catenin.

To confirm that endogenous β-catenin is phosphorylated by PKD1, we used C4-2 cells known to have much lower PKD1 activity than their parental LNCaP cells (19). The phosphorylation of endogenous β-catenin in C4-2 cells and in C4-2 cells stably expressing PKD1 (C4-2/PKD1) were compared. As seen in Fig. 3*B*, following activation of PKD1 by bryostatin 1, the endogenous β-catenin is phosphorylated in PKD1-transfected cells only.

Phosphorylation by PKD1 alters β-catenin subcellular localization. Because the NH_2-terminal region of β-catenin is involved in regulating its protein stability, the wild-type and the T112R/T120I mutant were studied for their stability. Transiently transfected 3T3 cells were treated with either MG132, a proteosome inhibitor, or cycloheximide, a protein synthesis inhibitor, for 0, 1, and 4 hours. The protein amounts were compared on immunoblots. The wild-type and the mutant displayed very similar patterns in the presence of these inhibitors, suggesting that the nonphosphorylated mutant has similar protein stability as wild-type β-catenin (Fig. 3*C*).

To study subcellular distribution of nonphosphorylation β-catenin, exogenous HA-tagged wild-type and T112R/T120I mutated β-catenin were transiently transfected into C4-2/PKD1 cells and stained with anti–HA tag antibody. Wild-type β-catenin mainly distributes on plasma membrane with a minor portion in cytosol and nucleus (Fig. 3*D*). Although some of the T112R/T120I mutant localizes on plasma membrane, an increasing amount of T112R/T120I was found in nucleus (Fig. 3*D*).

PKD1 modulates β-catenin transcription activity. To evaluate the PKD1 influence on β-catenin transcription activity, we examined the expression of β-catenin target genes, including *cyclin D1* and *c-Myc*, which are under control of β-catenin/TCF, and tumor metastasis suppressor *KAI1* gene, which is inhibited by β-catenin/κB P50 in metastatic prostate cancer cells (8). Compared with parental C4-2 cells, *KAI1* mRNA level was much higher in C4-2 cells that overexpress PKD1 (Fig. 4*A*), but expression of *cyclin D1* and *c-Myc* was higher in parental C4-2 cells, suggesting that PKD1 decreases β-catenin transcription activity. The results of down-regulation of *cyclin D1* and *c-Myc* by PKD1 are consistent with our observation in Topflash reporter assay in Fig. 1 and our prior publication (21). We have also published the corollary, that knockdown of PKD1 by siRNA increases cyclin D1 and c-Myc protein expression, suggesting that PKD1 represses β-catenin transcriptional activity (21).

Wild-type and T112R/T120I mutated β-catenin were tested for their transcription activity by Topflash assay. Surprisingly, single mutations of Thr^{112} and Thr^{120} decrease β-catenin/TCF transcription activities; the nonphosphorylation mutant T112R/T120I has

Published OnlineFirst January 13, 2009; DOI: 10.1158/0008-5472.CAN-07-6270

dramatic reduction of β-catenin/TCF activity as compared with control wild-type β-catenin (Fig. 4*B*). The results are contradictory to our observation in Fig. 1 that PKD1 functionally represses β-catenin/TCF activity. To confirm the finding, we made mutant T112E/T120E, which mimics constitutive phosphorylation and tested on Topflash assay. The T112E/T120E has higher β-catenin/TCF transcription activity than wild-type β-catenin (Fig. 4*C*). Thus, it is possible that PKD1 phosphorylation is required to maintain β-catenin/TCF transcription activity and that PKD1 has multiple roles in regulating β-catenin subcellular localization and transcription functions (see Discussion).

Because both PKD1 and CKII kinases can phosphorylate Thr^{112} of β-catenin, we compared the CKII nonphosphorylation mutant T102I/T112R with PKD1 nonphosphorylation mutant T112R/T120I on β-catenin/TCF transcription activity. The CKII mutant T102I/T112R does not have any detectable influence on β-catenin/TCF activity (Fig. 4*D*). A combined CKII/PKD1 mutant T102I/T112R/T120I partially restores β-catenin/TCF transcription activity compared with the PKD1 nonphosphorylation T112R/T120I mutant (Fig. 4*D*), suggesting that CKII phosphorylation on Thr^{102} can neutralize the PKD1 phosphorylation effect.

Addition of E-cadherin can lower β-catenin transcription activity by sequestering cytoplasm β-catenin (26). The nonphosphorylated β-catenin mutants were also tested for their E-cadherin binding ability on Topflash assays. All β-catenin isoforms tested, including PKD1 and CKII nonphosphorylation mutants, do not affect β-catenin reporter activity, suggesting that binding with E-cadherin was not affected (Fig. 4*D*).

PKD1 phosphorylation regulates β-catenin–mediated cell proliferation and motility. Overexpression of E-cadherin causes cell growth arrest and apoptosis in a dose-dependent manner (26–28). Coexpression of β-catenin with E-cadherin counteracts E-cadherin–mediated cell growth arrest. We tested the ability of mutated β-catenin to overcome cell growth arrest caused by E-cadherin. Experimental conditions were chosen so that the amount of wild-type β-catenin marginally rescued the E-cadherin–mediated inhibition on LNCaP cell growth (Fig. 5*A*). In the presence of ectopic E-cadherin, the addition of β-catenin with a single threonine residue substituted (T112R or T120I) did not relieve cell growth inhibition. However, double nonphosphorylation T112R/T120I β-catenin mutant had higher rescue potential than wild-type β-catenin, suggesting that PKD1 phosphorylation inhibits β-catenin–mediated proliferation function.

Similar to E-cadherin, overexpression of PKD1 can also inhibit cell proliferation (Fig. 5*A*; ref. 21). Cotransfection of siPKD1 with E-cadherin expression vector or cotransfection of siEcad with

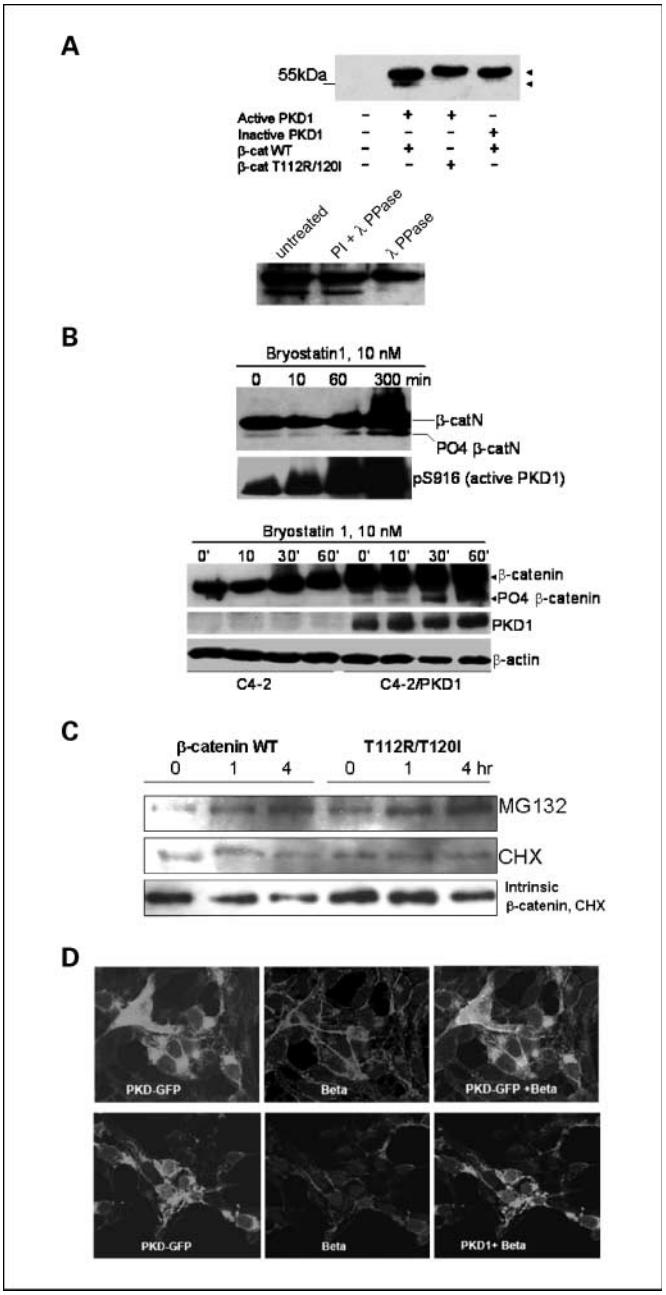

Figure 3. PKD1 phosphorylates β-catenin *in vivo*. *A, top, in vivo* phosphorylation of truncated β-catenin. A COOH-terminal truncated construct that includes the first 150 amino acid residues of β-catenin (*β-catN*) was cotransfected with PKD1 or kinase-dead PKD1 (inactive) into NIH 3T3 cells. Cell lysates were collected 2 d after transfection and immunoblotted with anti-GFP antibody. The wild-type β-catenin has an extra fast-mobile band when incubated with active PKD1, but the band does not appear in the presence of inactive PKD1. When Thr^{112} and Thr^{120} were mutated, the fast-mobile band disappeared even in the presence of active PKD1, indicating that these two threonine sites are necessary for PKD1 phosphorylation. The T112R/T120I moves slower than the wild-type because it carries an extra positive charge on the T112R mutation. *Bottom,* phosphatase treatment. Cells transiently transfected with wild-type β-catN and PKD1 were treated with 10 nmol/L bryostatin 1 for 1 h. The cells were lysed in TBST buffer. The cell lysates were treated with either λ phosphatase or λ phosphatase plus phosphatase inhibitor cocktail (*PI*; from AG Scientific). After λ phosphatase treatment, the low molecular weight band disappeared. *B,* activation of PKD1 increases phosphorylation of β-catenin. *Top,* cells transiently transfected with wild-type β-catN and PKD1 were treated with 10 nmol/L bryostatin 1. The duration of treatment was indicated. The phosphorylated band increased over time. *Bottom,* endogenous β-catenin phosphorylation increases during PKD1 activation. C4-2 has very low of PKD1 activity (31). When stimulated by bryostatin 1, the endogenous β-catenin in C4-2 cells remained mainly unchanged. In contrast, endogenous β-catenin in C4-2 cells that stably express PKD1 became phosphorylated as indicated by the increase of a low molecular weight band. *C,* the nonphosphorylated mutant has similar protein stability as wild-type β-catenin. Full-length wild-type and T112R/T120I mutant were transfected into NIH 3T3 cells for 2 d. The cells were treated with either 2 μmol/L of MG132, a proteosome inhibitor, or 20 μmol/L of cycloheximide, a protein synthesis inhibitor, for the times indicated before collection. Immunoblotting was done with anti–β-catenin. The endogenous β-catenin was used as loading control. There was no detectable difference between wild-type and T112R/T120I mutant in protein stability. *D,* nonphosphorylated β-catenin has altered subcellular location. HA-tagged wild-type and T112R/T120I mutant were individually transfected into C4-2/PKD1 cells. Cells were stained with anti–HA tag antibody. The pattern of mutant β-catenin was different from that of wild-type, with more in nuclei.

Published OnlineFirst January 13, 2009; DOI: 10.1158/0008-5472.CAN-07-6270

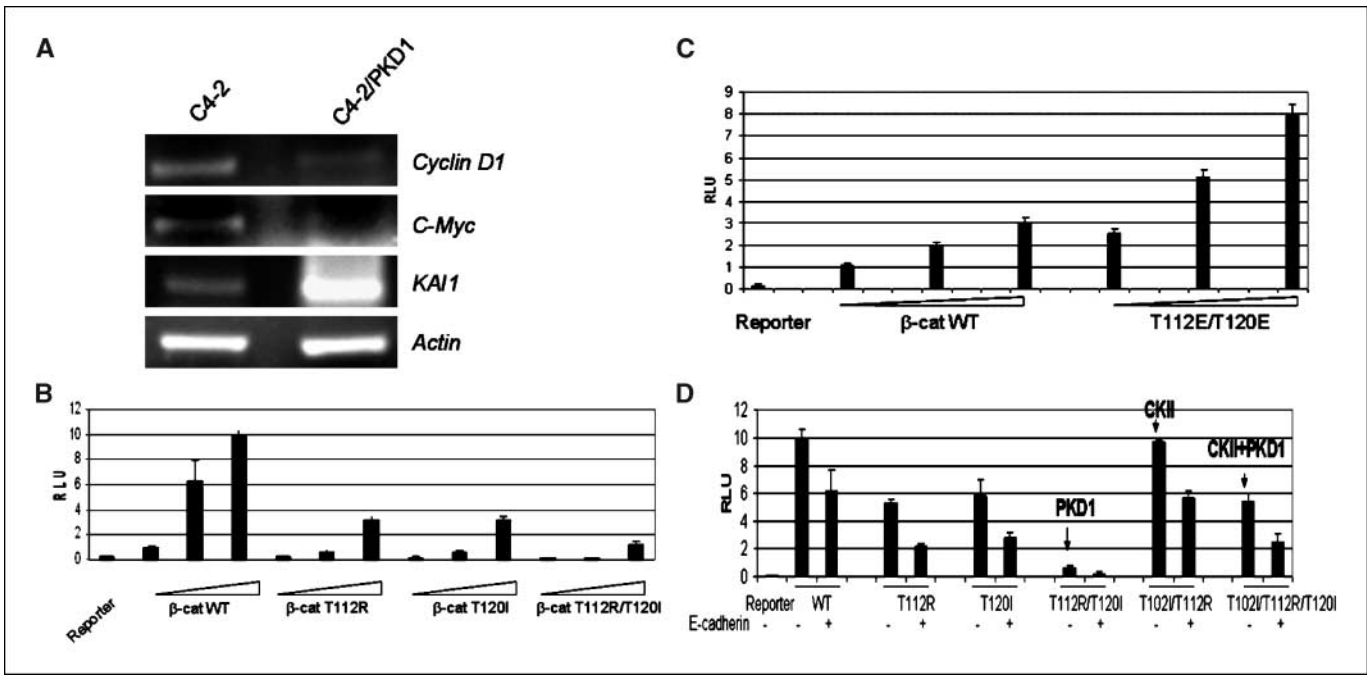

Figure 4. PKD1 modulates β-catenin transcription activity. *A,* expression of β-catenin target genes *cyclin D1, c-Myc*, and *KAI1* in the presence of PKD1. Total RNA from parental C4-2 cells and C4-2/PKD1 cells was amplified by semiquantitative reverse transcription-PCR. Expression of cyclin D1 and c-Myc is higher in C4-2 cells, whereas KAI1 is higher in C4-2/PKD1 cells. *B* to *D,* PKD1 phosphorylation increases β-catenin transcription activity (Topflash assay). *B,* nonphosphorylated β-catenin mutants have decreased transcription activity. Fifty nanograms of Topflash plasmid, 2.5 ng of Renilla luciferase, and 25, 50, and 100 ng of wild-type β-catenin were transfected NIH 3T3 cells. Luciferase assay was carried out 2 d later. *C,* β-catenin mutant that mimics constitutive phosphorylation has increased transcription activity. *D,* comparison of PKD1 and CKII phosphorylation on β-catenin transcription activity. Topflash assays were done with wild-type β-catenin and indicated mutants alone or in the presence of full-length E-cadherin. CKII phosphorylation on Thr^{102}/Thr^{112} does not affect β-catenin transcription activity. E-cadherin can bind to all β-catenin isoforms as indicated by its inhibitory effect on Topflash assay. Data were normalized and all experiments were done in triplicate. *Bars,* SD.

PKD1 expression vector can relieve growth inhibition caused by overexpression of E-cadherin or PKD1 (Fig. 5*A*). The data further support the hypothesis that PKD1 and E-cadherin regulate β-catenin activity in the same signaling pathway.

We also studied the effects of PKD1 phosphorylation on cell motility. Mouse 3T3 cells transfected with either wild-type β-catenin or T112R/T120I mutant were tested by wound healing assay. As seen in Fig. 5*B*, the T112R/T120I mutant has higher mobility than the wild type. Matrigel assay also confirmed that the T112R/T120I mutant had higher invasive ability compared with wild-type β-catenin (Fig. 5*C*).

Discussion

The consensus PKD1 phosphorylation motif is LxRxxS/T (x is any amino acid; refs. 31–33). In this study, we identified Thr^{112} and Thr^{120} in β-catenin as PKD1 phosphorylation sites. The two threonine residues are in context of $_{106}$-GMQIPSpTGFDA and $_{114}$-FDAAHPpTNVQR (pT is phosphorylated). Two recent articles have reported that PKD1 is capable of recognizing and phosphorylating sequences different from the consensus (34, 35). In one report (35), type IIα phosphatidylinositol 4-phosphate kinase was phosphorylated at Thr^{376} in a sequence of $_{370}$-AAHAAKpTVKHG by PKD1. In the other report (34), Ser^{58} in c-Jun was identified as PKD1 substrate in a sequence of $_{52}$-HLRAKNpSDLLT. Taken together, PKD1 may have certain flexibility to recognize phosphorylation sites. It is noted that, in all the four cases with novel sites, the −3 position is an aliphatic amino acid, preferably an alanine.

PKD1 is the first kinase identified to phosphorylate Thr^{120}. The Thr^{120} residue, which is within the α-catenin binding region of β-catenin (amino acids 118–146), is conserved across species (29). In fact, mutation of Thr^{120} of β-catenin to alanine abolishes its ability to bind to α-catenin (29). It has been shown that residues 121 to 1tgesicqz of β-catenin form an α-helix, and this helix interacts with three α-helices in α-catenin by forming a four-helix bundle (36, 37). Molecular modeling suggests that Thr^{120} side chain is close to charged side chains of Asp^{146}, Lys^{149}, Lys^{193}, and Glu^{197} in α-catenin that are outside the identified β-catenin binding domain of 29 amino acids (117–143; ref. 38), strongly suggesting Thr^{120} phosphorylation changes the molecular interaction among the charged side chains. Tyr^{142} (Y142) of β-catenin is essential for α-catenin binding (29). Phosphorylation of Y142 causes dissociation of β-catenin/α-catenin complex (12). Interestingly, Thr^{120} locates immediately upstream whereas Y142 locates immediately downstream of the α-helix of residues 121 to 141 in β-catenin. The phosphorylation on these two residues probably contributes to the dynamic balance between β-catenin and α-catenin by influencing stability.

Single residue mutation on Thr^{112} and Thr^{120} decreases β-catenin transcription activities and combined mutation results in further decrease. This raises an interesting paradigm that although unphosphorylated β-catenin mutant is able to accumulate in the nucleus, it is unable to increase β-catenin/TCF transcription activity. This suggests that unphosphorylated β-catenin may be able to bind to TCF but fails to activate transcription by acting as a dominant negative of wild-type β-catenin. Developing a Thr^{120} phosphothreonine specific antibody will help to distinguish β-catenin biochemical identities and their functionalities. Because the Thr^{112} residue can be phosphorylated by CKII and PKD1, it is not clear what physiologic regulations control the Thr^{112}/Thr^{120}

Published OnlineFirst January 13, 2009; DOI: 10.1158/0008-5472.CAN-07-6270

residue phosphorylation and if the two protein kinases coordinate on the Thr^{112} phosphorylation *in vivo*. However, there are two major differences between the consequences of two kinases phosphorylation: (*a*) CKII phosphorylation on Ser^{29}, Thr^{102}, and Thr^{112} affects β-catenin protein stability (9), which is not sensitive to PKD1 phosphorylation; (*b*) CKII phosphorylation on Thr^{102} and Thr^{112} does not affect β-catenin/TCF activity, although one report showed that CKII also phosphorylated on Thr^{393} of β-catenin and enhanced β-catenin transcription activity (39). However, the regulation of β-catenin activity by serine and threonine phosphorylation is very complex (Ser^{29}, Ser^{33}, Thr^{41}, Ser^{45}, Thr^{102}, Thr^{112}, Thr^{120}, Thr^{393}, etc.) and therefore the influence of PKD1 phosphorylation of β-catenin may be context dependent on other *in vivo* kinases.

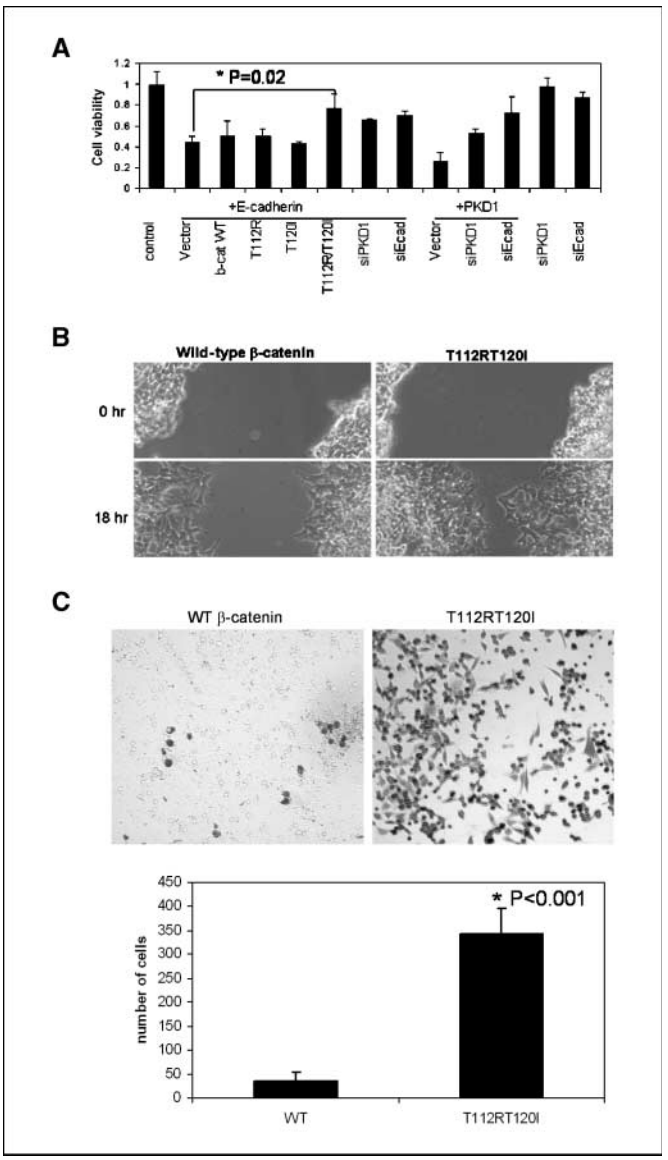

Figure 5. Phosphorylation regulates β-catenin–mediated cell proliferation and motility. *A,* nonphosphorylated β-catenin has more potential to rescue LNCaP cells that overexpress E-cadherin. LNCaP cells were transfected with 100 ng of full-length E-cadherin or PKD1 expression vector. Fifty nanograms of β-catenin constructs (wild-type or mutants as indicated) or 25 ng of siPKD1 or siE-cadherin were individually cotransfected with E-cadherin or PKD1. Cell viability was measured 48 h after transfection by MTS method (Promega). The experimental conditions were optimized so that the wild-type β-catenin construct had marginal rescue power. Cells with T112R/T120I grew faster than those with wild-type β-catenin in the presence of E-cadherin. Knockdown PKD1 by siRNA could restore cell growth inhibited by E-cadherin overexpression and vice versa. *Bars,* SD based on triplicate samples. *P* value was determined by using one-way ANOVA. *B,* nonphosphorylated β-catenin enhances cell migration. Transiently transfected 3T3 cells expressing wild-type or T112R/T120I were subjected to wound healing assay 2 d after transfection. The photograph was taken 18 h after wound was created. *C,* Matrigel assay of cell invasion. Full-length wild-type and T112R/T120I mutant β-catenin expression vectors were transfected into 3T3 cells for 2 d before adding to transwells containing Matrigel. The cells were grown on Matrigel for 2 d. Attached cells were stained using Diff Quick kit (*top*). *Columns,* average number of attached cells per filter based on triplicate samples; *bars,* SD (*bottom*).

In conclusion, we showed that PKD1 represses β-catenin/TCF transcription activity. PKD1 phosphorylates β-catenin *in vitro* and *in vivo*. The β-catenin mutants accumulate in the nucleus and paradoxically decrease β-catenin transcription activity. Epistatic study showed that PKD1 and E-cadherin act in the same signaling pathway to regulate β-catenin function. Understanding PKD1-β-catenin interaction in prostate cancer provides a novel target for further research.

Besides being an important component of the E-cadherin cell-adhesion complex, β-catenin plays a critical role in Wnt signaling. The loss of E-cadherin expression relieves sequestered β-catenin from the E-cadherin/β-catenin complex and induces β-catenin transcriptional activity (26, 40). The β-catenin nonphosphorylation T112R/T120I mutant, which is as stable as wild-type and has more nuclear distribution, can rescue E-cadherin–mediated cell growth arrest in LNCaP cells, yet has much less Wnt transcription activity, suggesting the E-cadherin–mediated cell growth arrest is independent of Wnt signaling at least in LNCaP cells.

β-Catenin nonphosphorylated T112R/T120I mutant shows decreased β-catenin/TCF transcription activity. We postulate that PKD1 phosphorylation is required to maintain β-catenin transcription activity. However, overexpression of PKD1 inhibits β-catenin/TCF transcription activity. The divergent results lead us to propose that PKD1 has multiple functions in regulating β-catenin. Indeed, we observed that PKD1 involved in membrane transport of β-catenin through trans-Golgi network and activation of PKD1 causes more β-catenin moving to plasma membrane (41). Thus, it is possible that overexpression of PKD1 transports more cytoplasm β-catenin to plasma membrane and decreases availability of nuclear β-catenin.

A major event during development of malignant invasive tumors is the loss of cell adhesion (42). E-cadherin has been viewed as a classic suppressor of invasion because E-cadherin is lost concomitantly with tumor progression in most epithelial cancers (43). We previously showed that overexpression of PKD1 in C4-2 cells increased cell aggregation and decreased cell motility (20). In this study, we show that cells with nonphosphorylated β-catenin are more mobile than those with wild-type β-catenin, suggesting that PKD1 influences the function of cadherin-catenin protein complex at multiple levels.

Sequestering cytoplasm β-catenin by E-cadherin has been shown as a key regulator of β-catenin activity (not limited to β-catenin–mediated Wnt pathway). We show that the repressive effect of PKD1 on cell growth and β-catenin transcription activity is coupled with E-cadherin. The phenotypes of overexpression or knockdown of PKD1 highly resemble those of E-cadherin. Using siRNA to knock down one of the two genes can relieve the cell growth inhibition caused by overexpression of the other gene, strongly suggesting that PKD1 and E-cadherin are on the same pathway that regulates β-catenin subcellular localization and activity.

Published OnlineFirst January 13, 2009; DOI: 10.1158/0008-5472.CAN-07-6270

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

Received 11/15/2008; revised 11/20/2008; accepted 11/26/2008.

Grant support: Prostate Cancer Foundation at University of Nebraska Medical Center, Omaha, Nebraska and University of Massachusetts Medical School, Worcester, Massachusetts.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Dr. Laurey Steinke and the Protein facility at University of Nebraska Medical Center for mapping phosphorylation sites, and Drs. R. Kemler, K. Johnson, M. Wheelock, R. Moon, and F.J. Johannes for reagents.

References

1. Nusslein-Volhard C, Wieschaus E. Mutations affecting segment number and polarity in *Drosophila*. Nature 1980;287:795–801.
2. McCrea PD, Turck CW, Gumbiner B. A homolog of the armadillo protein in *Drosophila* (plakoglobin) associated with E-cadherin. Science 1991;254:1359–61.
3. Oda H, Uemura T, Shiomi K, et al. Identification of a *Drosophila* homologue of α-catenin and its association with the armadillo protein. J Cell Biol 1993;121:1133–40.
4. Ozawa M, Baribault H, Kemler R. The cytoplasmic domain of the cell adhesion molecule uvomorulin associates with three independent proteins structurally related in different species. EMBO J 1989;8:1711–7.
5. Gates J, Peifer M. Can 1000 reviews be wrong? Actin, α-catenin, and adherens junctions. Cell 2005;123:769–72.
6. Nelson WJ, Nusse R. Convergence of Wnt, β-catenin, and cadherin pathways. Science 2004;303:1483–7.
7. Yang F, Li X, Sharma M, et al. Linking β-catenin to androgen-signaling pathway. J Biol Chem 2002;277:11336–44.
8. Kim JH, Kim B, Cai L, et al. Transcriptional regulation of a metastasis suppressor gene by Tip60 and β-catenin complexes. Nature 2005;434:921–6.
9. Bek S, Kemler R. Protein kinase CKII regulates the interaction of β-catenin with α-catenin and its protein stability. J Cell Sci 2002;115:4743–53.
10. Lickert H, Bauer A, Kemler R, et al. Casein kinase II phosphorylation of E-cadherin increases E-cadherin/β-catenin interaction and strengthens cell-cell adhesion. J Biol Chem 2000;275:5090–5.
11. Fang D, Hawke D, Zheng Y, et al. Phosphorylation of β-catenin by AKT promotes β-catenin transcriptional activity. J Biol Chem 2007;282:11221–9.
12. Piedra J, Miravet S, Castano J, et al. p120 catenin-associated Fer and Fyn tyrosine kinases regulate β-catenin Tyr-142 phosphorylation and β-catenin-α-catenin interaction. Mol Cell Biol 2003;23:2287–97.
13. Brembeck FH, Schwarz-Romond T, Bakkers J, et al. Essential role of BCL9-2 in the switch between β-catenin's adhesive and transcriptional functions. Genes Dev 2004;18:2225–30.
14. Roura S, Miravet S, Piedra J, et al. Regulation of E-cadherin/catenin association by tyrosine phosphorylation. J Biol Chem 1999;274:36734–40.
15. Piedra J, Martinez D, Castano J, et al. Regulation of β-catenin structure and activity by tyrosine phosphorylation. J Biol Chem 2001;276:20436–43.
16. Hellberg CB, Burden-Gulley SM, Pietz GE, et al. Expression of the receptor protein-tyrosine phosphatase, PTPμ restores E-cadherin-dependent adhesion in human prostate carcinoma cells. J Biol Chem 2002;277:11165–73.
17. Nawroth R, Poell G, Ranft A, et al. VE-PTP and VE-cadherin ectodomains interact to facilitate regulation of phosphorylation and cell contacts. EMBO J 2002;21:4885–95.
18. Jaggi M, Du C, Zhang W, et al. Protein kinase D1: a protein of emerging translational interest. Front Biosci 2007;12:3757–67.
19. Jaggi M, Rao PS, Smith DJ, et al. E-cadherin phosphorylation by protein kinase D1/protein kinase Cμ is associated with altered cellular aggregation and motility in prostate cancer. Cancer Res 2005;65:483–92.
20. Syed V, Mak P, Du C, et al. β-Catenin mediates alteration in cell proliferation, motility and invasion of prostate cancer cells by differential expression of E-cadherin and protein kinase D1. J Cell Biochem 2008;104:82–95.
21. Rao PS, Jaggi M, Smith DJ, et al. Metallothionein 2A interacts with the kinase domain of PKCμ in prostate cancer. Biochem Biophys Res Commun 2003;310:1032–8.
22. James P, Halladay J, Craig EA. Genomic libraries and a host strain designed for highly efficient two-hybrid selection in yeast. Genetics 1996;144:1425–36.
23. Johannessen M, Delghandi MP, Rykx A, et al. Protein kinase D induces transcription through direct phosphorylation of the cAMP response element-binding protein. J Biol Chem 2007;282:14777–87.
24. Veeman MT, Slusarski DC, Kaykas A, et al. Zebrafish prickle, a modulator of noncanonical Wnt/Fz signaling, regulates gastrulation movements. Curr Biol 2003;13:680–5.
25. Stockinger A, Eger A, Wolf J, et al. E-cadherin regulates cell growth by modulating proliferation-dependent β-catenin transcriptional activity. J Cell Biol 2001;154:1185–96.
26. Gottardi CJ, Wong E, Gumbiner BM. E-cadherin suppresses cellular transformation by inhibiting β-catenin signaling in an adhesion-independent manner. J Cell Biol 2001;153:1049–60.
27. Hayashida Y, Honda K, Idogawa M, et al. E-cadherin regulates the association between β-catenin and actinin-4. Cancer Res 2005;65:836–45.
28. Batlle E, Sancho E, Franci C, et al. The transcription factor snail is a repressor of E-cadherin gene expression in epithelial tumour cells. Nat Cell Biol 2000;2:84–9.
29. Aberle H, Schwartz H, Hoschuetzky H, et al. Single amino acid substitutions in proteins of the armadillo gene family abolish their binding to α-catenin. J Biol Chem 1996;271:1520–6.
30. Matthews SA, Pettit GR, Rozengurt E. Bryostatin 1 induces biphasic activation of protein kinase D in intact cells. J Biol Chem 1997;272:20245–50.
31. Jaggi M, Rao PS, Smith DJ, et al. Protein kinase Cμ is down-regulated in androgen-independent prostate cancer. Biochem Biophys Res Commun 2003;307:254–60.
32. Doppler H, Storz P, Li JC, et al. A phosphorylation state-specific antibody recognizes Hsp27, a novel substrate of protein kinase D. J Biol Chem 2005;280:15013–9.
33. Nishikawa K, Toker A, Johannes FJ, et al. Determination of the specific substrate sequence motifs of protein kinase C isozymes. J Biol Chem 1997;272:952–60.
34. Hutti JE, Jarrell ET, Chang JD, et al. A rapid method for determining protein kinase phosphorylation specificity. Nat Methods 2004;1:27–9.
35. Waldron RT, Whitelegge JP, Faull KF, et al. Identification of a novel phosphorylation site in c-jun directly targeted *in vitro* by protein kinase D. Biochem Biophys Res Commun 2007;356:361–7.
36. Hinchliffe KA, Irvine RF. Regulation of type II PIP kinase by PKD phosphorylation. Cell Signal 2006;18:1906–13.
37. Pokutta S, Weis WI. Structure of the dimerization and β-catenin-binding region of α-catenin. Mol Cell 2000;5:533–43.
38. Graham TA, Weaver C, Mao F, et al. Crystal structure of a β-catenin/Tcf complex. Cell 2000;103:885–96.
39. Huber O, Krohn M, Kemler R. A specific domain in α-catenin mediates binding to β-catenin or plakoglobin. J Cell Sci 1997;110:1759–65.
40. Song DH, Dominguez I, Mizuno J, et al. CK2 phosphorylation of the armadillo repeat region of β-catenin potentiates Wnt signaling. J Biol Chem 2003;278:24018–25.
41. Jaggi M, Chauhan SC, Du C, et al. Bryostatin 1 modulates β-catenin subcellular localization and transcription activity through protein kinase D1 activation. Mol Cancer Ther 2008;7:2703–12.
42. Wong AS, Gumbiner BM. Adhesion-independent mechanism for suppression of tumor cell invasion by E-cadherin. J Cell Biol 2003;161:1191–203.
43. Hanahan D, Weinberg RA. The hallmarks of cancer. Cell 2000;100:57–70.

Cancer Research
The Journal of Cancer Research (1916–1930) | The American Journal of Cancer (1931–1940)

AACR American Association for Cancer Research

Protein Kinase D1–Mediated Phosphorylation and Subcellular Localization of β-Catenin

Cheng Du, Meena Jaggi, Chuanyou Zhang, et al.

Cancer Res 2009;69:1117-1124. Published OnlineFirst January 13, 2009.