

E-Cadherin Phosphorylation by Protein Kinase D1/Protein Kinase C μ is Associated with Altered Cellular Aggregation and Motility in Prostate Cancer

Meena Jaggi, Prema S. Rao, David J. Smith, Margaret J. Wheelock, Keith R. Johnson, George P. Hemstreet and K.C. Balaji

University of Nebraska Medical Center, Omaha, Nebraska

Abstract

The cadherin family of transmembrane glycoproteins plays a critical role in cell-to-cell adhesion and cadherin dysregulation is strongly associated with cancer metastasis and progression. In this study, we report a novel interaction between protein kinase D1 [PKD1; formerly known as protein kinase C μ (PKC μ)] and E-cadherin. PKD1 is a serine/threonine-specific kinase known to play a role in multiple cellular processes including apoptosis, cytoskeleton remodeling, and invasion. Our study shows that PKD1 colocalizes with E-cadherin at cell junctions in LNCaP prostate cancer cells and coimmunoprecipitates with E-cadherin from lysates of LNCaP cells. *In vitro* kinase assays have shown that PKD1 phosphorylates E-cadherin. Inhibition of PKD1 activity by the selective inhibitor Gö6976 in LNCaP cells resulted in decreased cellular aggregation and overexpression of PKD1 in C4-2 prostate cancer cells increased cellular aggregation and decreased cellular motility. We also validated the PKD1 and E-cadherin colocalization in human prostate cancer tissue by confocal laser scanning microscopy. Our study has identified E-cadherin as a novel substrate of PKD1, and phosphorylation of E-cadherin by PKD1 is associated with increased cellular aggregation and decreased cellular motility in prostate cancer. Because both E-cadherin and PKD1 are known to be dysregulated in prostate cancer, our study identified an important protein-protein interaction influencing the signal transduction system associated with cell adhesion in prostate cancer. (Cancer Res 2005; 65(2): 483–92)

Introduction

Prostate cancer is the most commonly diagnosed noncutaneous cancer and is the second leading cause of cancer-related deaths in American men (1, 2). Cancer cell metastasis is influenced by the ability to migrate from the primary site and thrive in the microenvironment of a secondary site. Disruption of normal cell-cell adhesion in malignant cells contributes to enhanced tumor cell migration and proliferation, leading to invasion and metastasis. This disruption can be caused by down-regulating the expression of E-cadherin or catenin family members or by activation of signaling pathways that prevent the assembly of adherens junctions (3).

Requests for reprints: K.C. Balaji, Urological Oncology Research, Urological Surgery, 982360, University of Nebraska Medical Center, Omaha, NE 68198-2360. Phone: 402-559-9041; Fax: 402-559-6529; E-mail: kcbalaji@unmc.edu.

©2005 American Association for Cancer Research.

The cadherin family of transmembrane glycoproteins plays a critical role in cell-to-cell adhesion and cadherin dysregulation is strongly associated with prostate cancer progression (4–6). The interepithelial binding of E-cadherin mediates lateral cell-cell adhesion in secretory tissues such as the prostate and mammary gland, resulting in the formation of adherens junctions that are required for tissue morphogenesis and maintenance of the differentiated phenotype (7, 8). E-cadherin is composed of an extracellular domain, responsible for cell-cell interactions, a transmembrane domain and a cytoplasmic domain that is linked to the cytoskeleton. The cytoplasmic domain of E-cadherin is anchored to the actin cytoskeleton via three cytoplasmic proteins: α -catenin, β -catenin, and γ -catenin (plakoglobin; ref. 9). Another catenin, p120, is less tightly associated with E-cadherin (10). The cadherin ectodomains mediate homophilic ligation and adhesive recognition, whereas the highly conserved cytoplasmic tail interacts with proteins capable of linking cadherin adhesion to the actin cytoskeleton (11, 12).

The functional integrity of the cadherin-catenin complex is regulated by phosphorylation (13). In general, activation of tyrosine kinases results in the loss of cadherin-mediated cell-cell adhesion and an increase in the level of cytoplasmic β -catenin, either by direct release of β -catenin into the cytoplasm, or by activating cadherin endocytosis (14). In contrast, activation of protein tyrosine phosphatase stabilizes the cadherin-catenin complex (15). Alternately, serine/threonine phosphorylation of E-cadherin or β -catenin also results in increased stabilization of the cadherin-catenin complex and increased cadherin-mediated cell-cell adhesion (16–18). E-cadherin is known to be phosphorylated within the cytoplasmic domain Ser cluster by casein kinase II and glycogen synthase kinase-3 β . Preventing this phosphorylation not only reduces the interaction between β -catenin and E-cadherin *in vitro* but also reduces E-cadherin-mediated cell-cell adhesion in transfected mouse NIH3T3 fibroblasts (17). The lack of complete inhibition of E-cadherin phosphorylation by casein kinase II and glycogen synthase kinase-3 inhibitors, heparin, and lithium chloride, respectively, suggests that E-cadherin may be phosphorylated by other kinases (17). In this study, we report the interaction and phosphorylation of E-cadherin by protein kinase D1/protein kinase C μ (PKD1/PKC μ) which identifies E-cadherin as a novel substrate of PKD1.

We have previously published that PKD1 (formerly known as PKC μ) localized to cell junctions in LNCaP cells (19). PKD1 was originally described as a novel member of the PKC family of signal transduction proteins. PKD1 has recently been reclassified as a member of the PKD family, a novel subgroup of the calcium/calmodulin-dependent protein kinase (CAMK) family characterized by sequence similarities in the kinase domain (20). The PKD family is comprised of PKD1 (PKC μ /PKD), PKD2, and PKD3 (PKC- ν ; ref. 21). PKD1 is emerging as an important modulator of several kinase signal-transduction pathways such as p42 extracellular signal-regulated kinase (ERK) mitogen-activated protein kinase and growth factor-induced ERK activation (22, 23). PKD1 has also been

shown to phosphorylate Kidins220, a membrane protein abundant in neurite tips and growth cones of PC12 cells (24), pointing to a role for PKD1 in cytoskeleton reorganization (23). PKD1 targeted to the plasma membrane functions as a regulator of signaling traffic by immune receptors and growth factor receptors and also as a regulator of cell shape and tumor cell invasion (23). Although we and others have shown altered expression of PKD1 in prostate cancer, the exact mechanisms or substrates involved in PKD1-mediated effects in prostate cancer remain to be established (19, 25).

The spatial and temporal localization of a signaling molecule, combined with its substrate specificity, lead to specific responses (26). Therefore, we analyzed the subcellular localization of PKD1 and its interaction with E-cadherin, a major adherens junction protein, in LNCaP cells. Our study shows that PKD1 colocalizes with E-cadherin at the cell junctions in LNCaP prostate cancer cells, and interacts with E-cadherin, resulting in phosphorylation of E-cadherin. Altered PKD1 activity in prostate cancer cells influenced cellular aggregation and motility. Because E-cadherin plays a major role in prostate cancer progression, a fundamental understanding of the molecular mechanisms involved in the E-cadherin/PKD1 interaction will provide novel insights into prostate cancer progression.

Materials and Methods

Cell Culture. LNCaP (American Type Culture Collection, Manassas, VA) and C4-2 (Urocor, Oklahoma City, OK) human prostate cancer cells were grown at 37°C, 5% carbon dioxide in RPMI 1640 media with 10% fetal bovine serum (Invitrogen, Carlsbad, CA). Transfections were carried out using LipofectAMINE Plus (Invitrogen). C4-2 cells stably expressing PKD1-GFP, or GFP vector, were selected in the presence of G418 (400 µg/mL; Invitrogen) and fluorescence activated cell sorting was used to select for a clonal population of PKD-GFP expressing transfected cells.

Immunoprecipitation and Immunoblot Analysis. Early passage LNCaP (passage 35–40) monolayers were washed in ice-cold PBS and lysed in 20 mmol MOPS (pH 7), 2 mmol EGTA, 2 mmol EDTA, sodium pyrophosphate 10 mmol, sodium fluoride 30 mmol, orthovanadate 2 mmol, protease inhibitors (Sigma, St. Louis, MO) and NP40 0.5%, or in RIPA buffer. Equal amount of cell extracts from LNCaP and C4-2 cells were immunoprecipitated using the C-20 polyclonal antibody against PKD1/PKC μ (Santa Cruz Biotechnology, Santa Cruz, CA) or with E-cadherin antibody (4A2, has been previously described 27) by gently mixing for 1 hour at 4°C. Fifty microliters of packed anti-mouse or anti-rabbit IgG affinity gel (ICN Pharmaceuticals, Costa Mesa, CA) was added, and mixing continued for 1 hour. Immune complexes were washed thrice in TBST [10 mmol Tris-HCl (pH 7.5), 150 mmol NaCl, 0.05% Tween 20], beads were suspended in 25 µL of 2× Laemmli sample buffer, boiled for 5 minutes, the proteins resolved by SDS-PAGE and electrophoretically transferred to immunoblot polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA). The membranes were blocked in 5% nonfat dry milk in TBST before incubation with specific antibodies for 2 hours at room temperature. Membranes were washed in TBST at room temperature, probed with horseradish peroxidase-conjugated goat anti-rabbit or mouse antibody and antibody binding was detected with an ECL Chemiluminescence Kit (Amersham Pharmacia, United Kingdom).

Metabolic Labeling of LNCaP Cells. LNCaP were grown overnight in a 25 cm² flask in normal growth medium, which was then replaced with RPMI lacking methionine, supplemented with 1% dialyzed fetal bovine serum (both from Biosource International, Camarillo, CA) for 4 hours, after which cells were pulsed with 250 µCi of Tran[³⁵S]-label (ICN Pharmaceuticals) for 90 minutes, lysed with MOPS buffer, subjected to immunoprecipitation with PKD1 antibodies as described previously, separated by SDS-PAGE, transferred onto polyvinylidene difluoride membrane and exposed to autoradiography film for 96 hours.

PKD1/E-Cadherin Immunoprecipitation and Kinase Activity Assay. Cells were lysed in kinase assay lysis buffer [50 mmol Tris-HCl (pH 7.6), 2 mmol EGTA, 2 mmol EDTA, 2 mmol DTT, protease inhibitors, 1% Triton X-100] to dissociate E-cadherin from PKD1. Equal amounts of LNCaP and C4-2 cell extracts were immunoprecipitated using the C-20 against PKC μ /PKD1 (Santa Cruz Biotechnology) or E-cadherin antibody (4A2) overnight at 4°C. The immune complexes were recovered using 20 µL of a mixture of protein A and G coupled to Sepharose (Santa Cruz Biotechnology). The E-cadherin immunocomplexes were suspended in 40 µL of a phosphorylation mixture containing 30 mmol Tris (pH 7.4), 30 mmol MgCl₂, 20 µmol/L diC8 (1,2-dioctanoyl-*sn*-glycerol, Sigma), 100 g/mL PS (dioleoyl phosphotydyl serine, Sigma) and the immunoprecipitated endogenous PKD1. Syntide-2 (2.5 mg/mL, American Peptide Company, Sunnyvale, CA) was used as a PKD1 control substrate. For PKD1 kinase inhibition studies, the PKD1-selective inhibitor, Gö6976 (Calbiochem, La Jolla, CA), was added to assays at 1 mol/L concentration. Phosphorylation was initiated with 200 mol/L (³²P) ATP (specific activity, 600 cpm/pmol, Amersham Pharmacia, Piscataway, NJ) for 30 minutes at 30°C with intermittent mixing to maintain the Sepharose beads in suspension throughout the period of incubation. The reaction was stopped by placing the tubes on ice and 30 L of the assay mixture was spotted onto a p81 paper (Whatman, Maidstone, United Kingdom). Unincorporated labeled nucleotide was separated from labeled substrate by washing the filter paper with 40 mL of 75 mmol H₃PO₄ in a vacuum manifold. The dried papers were then placed in a scintillation counter to determine the radioactivity incorporated into substrates. The parallel samples (immune complexes) were washed thrice in TBST [10 mmol Tris-HCl (pH 7.5), 150 mmol NaCl, 0.05% Tween 20]. After the final wash, the packed beads were suspended in 25 L of 2× Laemmli sample buffer, boiled for 5 minutes and the proteins resolved by SDS-PAGE (4–20% gradient gel, ISC BioExpress, Kaysville, UT) were transferred to polyvinylidene difluoride membrane and exposed to Biomax MR1 film (Kodak, Rochester, NY) at –70°C using intensifying screens (Transcreen LE, Kodak), or phosphorimager screens (Molecular Dynamics-Amersham, Piscataway, NJ) to quantify band intensity. For identification of radiolabeled protein bands, the membranes were immunoblotted with E-cadherin and PKD1 antibodies as described before.

Confocal Immunofluorescence Microscopy. LNCaP cells were cultured on glass coverslips until subconfluence. Coverslips were fixed in HistoChoice for 15 minutes at room temperature and permeabilized with cold methanol at –20°C for 2 minutes. C4-2 and PKD-GFP transfected cells were fixed in 1% paraformaldehyde for 30 minutes, permeabilized with 0.05% Triton X-100, washed in PBS for 5 minutes and incubated in 10% goat serum in PBS at room temperature for 1 hour. The coverslips were incubated with primary antibody in 10% goat serum in PBS. After washing with PBS, coverslips were incubated with fluorescein isothiocyanate, or rhodamine conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA) for 1 hour. The cells were washed again in PBS followed by a brief rinse in distilled water and mounted in aqueous antifade medium (Vector Laboratories, Burlingame, CA) followed by analysis with a Zeiss LSM 410 confocal laser scanning microscope (Goettinger, Germany). Images and serial Z-section of cells at every micrometer were taken using the LSM version 399 software.

Aggregation Assay. Aggregation assays were done on the LNCaP, C4-2, C4-2-GFP vector, and C4-2-PKD-GFP transfected cell lines as described previously (28) with minor modifications. The cells were trypsinized and suspended in the appropriate medium with or without the PKD1-selective inhibitor Gö6976 (1 µmol/L). Five thousand cells in 20 µL were dropped onto the inner surface of the lid of a Petri dish. After 24 hours of incubation, the lid of the Petri dish was inverted and drops of cells were resuspended. Three independent experiments were done with each cell line and images taken through a microscope (Nikon Eclipse E800, Japan).

Motility Assay. Motility assays were carried out in 24-well cell culture plates using cell culture inserts (Transwell) containing filters with pore size of 8 µm, (Becton Dickinson, Franklin Lakes, NJ). For the motility assay 5 × 10³ cells were plated in the top chamber of cell culture inserts, the cells were incubated for 24 hours, and cells that did not migrate were removed with a cotton swab. Cells traversing the membrane through the

pores were stained with the protocol staining set (HEMA-3, Fisher, Pittsburgh, PA). The experiments were done in triplicate and cells were counted and expressed as the average number of cells that migrated through the pores.

Statistical Analysis. ANOVA was used to compare the motility of C4-2, C4-2-GFP vector, and C4-2-PKD1-GFP cells and P values ≤ 0.05 were considered statistically significant.

Results

PKD1 Colocalizes with E-Cadherin in Prostate Cancer Cells.

It has been proposed that PKD1 function is cell context-dependent and may be dependent on the localization of the enzyme inside the cell (29, 30). We have previously reported that PKD1 is localized to cell junctions in LNCaP cells (19). Because E-cadherin is a major cell junction protein, we explored the localization of PKD1 and E-cadherin in LNCaP cells by confocal laser scanning microscopy. We observed perinuclear and junctional localization of PKD1 (Fig. 1C). Serial Z -sections of the LNCaP cells were taken at every micrometer using the LSM version 399 software and a gallery of serial Z -sections was constructed confirming the junctional localization and colocalization of PKD1 with E-cadherin in LNCaP prostate cancer cells. The Z -section images are shown in Fig. 1 to illustrate the localization of PKD1 (Fig. 1C) and E-cadherin (Fig. 1B) in the same confocal plane at cell borders. Merging the images of PKD1 and E-cadherin showed colocalization (yellow) of E-cadherin/PKD1 at cell borders (Fig. 1D) in LNCaP cells, suggesting that these proteins are appropriately localized to interact with one another *in vivo*.

E-Cadherin Coimmunoprecipitates with PKD1 in LNCaP Prostate Cancer Cells. Because E-cadherin and PKD1 colocalize at the cell borders, we sought to determine whether E-cadherin interacts with PKD1 in prostate cancer cells by immunoprecipitation immunoblot assays. PKD1 was immunoprecipitated from

LNCaP cell extracts prepared in MOPS lysis buffer, and resolved by SDS-PAGE. Immunoblot analysis using E-cadherin-specific antibody showed the coimmunoprecipitation of E-cadherin with PKD1 in LNCaP cells [Fig. 2A(1) *E-cad*, lane 2]. The blot was stripped and reprobed with PKD1 antibody to confirm that PKD1 was immunoprecipitated by PKD1-specific antibody and not by IgG [Fig. 2A(1) *PKD1*, lanes 1 and 2]. To provide evidence for specificity of the PKD1/E-cadherin interaction, we probed the PKD1 immunoprecipitation blot with another major membrane-associated protein HER2 using HER2 Neu antibody (c-118, sc-284, Santa Cruz Biotechnology). The presence of HER2 in LNCaP cell extract [Fig. 2A(1) *HER2*, lane 3] and absence in PKD1 immunocomplex [Fig. 2A(1) *HER2*, lane 2] suggests that PKD1/E-cadherin interaction is specific and other non-interacting membrane proteins are not present in the PKD1/E-cadherin immunocomplex. In order to further confirm the interaction of these two proteins, we also did reciprocal immunoprecipitation assays with E-cadherin antibody and immunoblotted for PKD1. PKD1 was also immunoprecipitated with the E-cadherin antibody [Fig. 2A(2) lane 2]. The blot was stripped and reprobed with E-cadherin antibody to confirm that PKD1 was immunoprecipitated by E-cadherin-specific antibody and not by IgG [(Fig. 2A(2) *Reblot E-cad*, lanes 1 and 2)]. Reciprocal immunoprecipitation experiments confirmed that E-cadherin and PKD1 interact in LNCaP prostate cancer cells.

Furthermore, we carried out the immunoprecipitation experiments with [35 S]-labeled methionine and cysteine LNCaP cell protein lysate, followed by analysis of all the immunoprecipitated proteins on SDS-PAGE and autoradiography. Under these conditions, relatively few proteins coimmunoprecipitated [(Fig. 2A(3) lane 1)] which provided additional evidence for specificity of the PKD1/E-cadherin interaction.

PKD1 Phosphorylates E-Cadherin in LNCaP Cells. The studies using an oriented peptide library technique (31, 32) have shown that

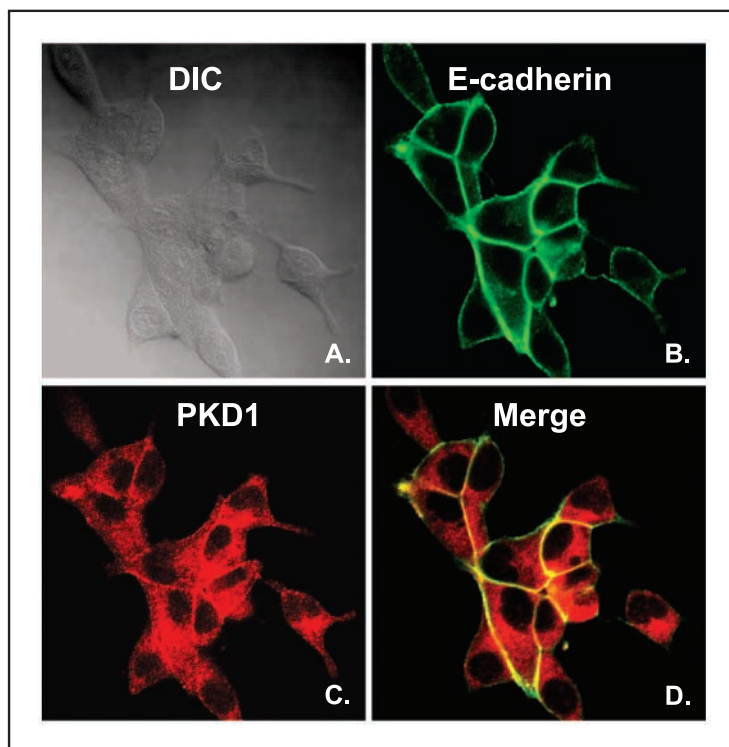


Figure 1. E-cadherin colocalizes with PKD1 at cell junctions in LNCaP prostate cancer cells. Cells were cultured on glass coverslips, probed with PKD1 and E-cadherin antibodies (C-20 and 4A2), incubated with FITC and rhodamine-conjugated secondary antibodies and analyzed by laser scanning confocal microscopy (Carl Zeiss, Germany). A, DIC image of LNCaP cells; B, localization of E-cadherin (green); C, localization of PKD1 (red); D, merged images (from B and C) showing E-cadherin/PKD1 colocalization (yellow) at cell borders in LNCaP cells.

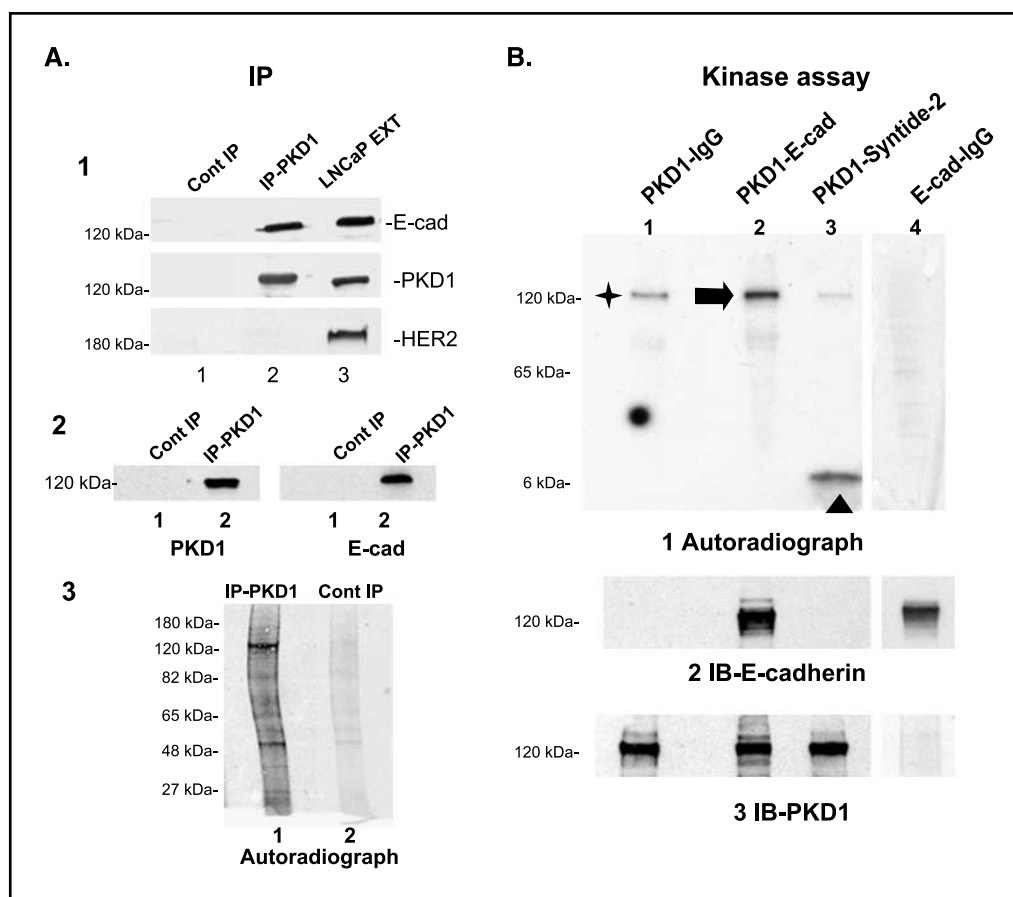


Figure 2. PKD1 interacts and phosphorylates E-cadherin in LNCaP prostate cancer cells. **A**, E-cadherin coimmunoprecipitates with PKD1 in LNCaP prostate cancer cells. (1) Coimmunoprecipitation of E-cadherin with PKD1 in LNCaP cells. Equal amounts of cell extract were incubated with PKD1-specific antibody or IgG (negative control) gently mixed at 4°C for 1 hour. Fifty microliters of packed anti-rabbit IgG affinity gel (ICN Pharmaceuticals) were added, and mixing continued for 30 minutes. Lane 1, immune complexes were washed and resolved by SDS-PAGE, followed by immunoblotting with anti-mouse E-cadherin antibody demonstrating coimmunoprecipitation of E-cadherin with PKD1 in LNCaP cells (lane 2). The blot was stripped and reprobed with PKD1 antibody to show the presence of PKD1 in the immunocomplex. PKD1 immunoprecipitation blot was also probed with another major membrane-associated protein HER2 using HER2 Neu antibody. The presence of HER2 in LNCaP cell extract (lane 3) and absence in PKD1 immunoprecipitation (lane 2) suggests that PKD1/E-cadherin interaction is specific and other non-interacting membrane proteins are not present in the PKD1/E-cadherin immunocomplex (1, HER2; lane 2). Lane 1, LNCaP cell extract immunoprecipitation IgG (negative control); lane 2 LNCaP cell extract immunoprecipitation PKD1 antibody; and lane 3, LNCaP cell extract. (2) Coimmunoprecipitation of PKD1 with E-cadherin in LNCaP cells. Equal amounts of cell extract were immunoprecipitated with E-cadherin antibody or IgG (negative control). Immune complexes were washed and resolved by SDS-PAGE, followed by immunoblotting with PKD1 antibody. Blot was stripped and reprobed with E-cadherin-specific antibody to confirm the presence of E-cadherin in the immunocomplex. Lane 1, LNCaP cell extract immunoprecipitation IgG (negative control), and lane 2, LNCaP cell extract immunoprecipitation E-cadherin antibody. (3) Autoradiograph showing coimmunoprecipitated proteins in [³⁵S]-labeled LNCaP cells. LNCaP cells were grown overnight in a 25 cm² flask in normal growth medium, which was then replaced with RPMI lacking methionine, supplemented with 1% dialyzed fetal bovine serum for 4 hours, after which cells were incubated for 90 minutes in deficient media supplemented with [³⁵S]-labeled methionine and cysteine, then lysed with MOPS buffer, immunoprecipitated with PKD1 antibodies, separated by SDS-PAGE. Membranes were exposed to autoradiography film at -70°C. Under these conditions, relatively few proteins coimmunoprecipitated with PKD1 (E-cadherin, catenins, and some low molecular weight proteins) providing additional evidence of the specificity of the PKD1/E-cadherin interaction. Lane 1, LNCaP immunoprecipitation PKD1 antibody; lane 2, LNCaP immunoprecipitation IgG (negative control). Numbering on the left indicates molecular weight in kilodaltons. **B**, autoradiograph of kinase assay demonstrating phosphorylation of E-cadherin by PKD1. (1) LNCaP cell extracts were made in RIPA buffer in order to disrupt E-cadherin and PKD1 interaction. PKD1 and E-cadherin were isolated from these cell extracts using specific anti-E-cadherin (4A2) and anti-PKD1 (C-20) antibodies. E-cadherin phosphorylation was done as described in MATERIALS AND METHODS. Proteins were resolved on a 4% to 20% gradient gel. [³²P]-labeled proteins were identified by autoradiography. Lane 1, IgG immunoprecipitation as substrate (negative control), weak band at 120 kDa (+) indicates autophosphorylation of PKD1; lane 2 E-cadherin immunoprecipitation as substrate, strong band at 120 kDa (→) indicates phosphorylation of E-cadherin by PKD1 in the presence of [³²P] ATP. Lane 3, Syntide-2 as substrate (positive control). ▲, phosphorylation of Syntide-2 by PKD1 in the presence of [³²P] ATP. Lane 4, E-cadherin as substrate (negative control) in the presence of [³²P] ATP with IgG immunoprecipitation (without PKD1 kinase), the absence of a band at 120 kDa indicates that E-cadherin is not phosphorylated without PKD1 in the kinase assay. Identities of phosphorylated proteins were confirmed by immunoblotting. (2) Immunoblot of E-cadherin expression, and (3) immunoblot of PKD1 expression in the same kinase assay. The immunoblots clearly show that the phosphorylated protein in lane 2 is E-cadherin as expression of PKD1 is identical in all three lanes.

PKD1 is a basophilic kinase and has a unique substrate specificity (LVRQMS). The most critical residue for PKD1 selectivity is a leucine at -5 with respect to the phosphorylatable serine. *In silico* analysis of the E-cadherin amino acid sequence using the NetPhos 2.0 Server (33) revealed the presence of a comparable PKD1 substrate sequences (LSSLNS at serine 850 and LNSSSE at 853) with leucine at -5. Serine residues 850 and 853 are potential

phosphorylation sites with a NetPhos score of 0.96 and 0.99, respectively (a score of > 0.9 indicates a highly likely phosphorylation site). These observations strongly suggest that E-cadherin could be phosphorylated by PKD1. Therefore, we explored whether E-cadherin is a naturally occurring substrate for PKD1 in LNCaP cells by *in vitro* kinase assay. The kinase assay was done as previously described (19) with minor modifications, using

endogenously expressed PKD1 and E-cadherin from LNCaP cells. Analysis of phosphorylation by autoradiography (Fig. 2B) showed strong incorporation of radioactivity in the E-cadherin immunoprecipitate at 120 kDa, demonstrating phosphorylation of E-cadherin by PKD1 [Fig. 2B(1), lane 2)]. When a control E-cadherin phosphorylation assay was done without PKD1 kinase, incorporation of radioactivity was not detected in the E-cadherin immunoprecipitate [Fig. 2B(1) lane 4)] confirming that there are no other kinases capable of phosphorylating E-cadherin present in E-cadherin immunoprecipitation in kinase assays. Because there is no well-established natural substrate for PKD1, we used a synthetic substrate, Syntide-2 (31, 32), as a model substrate to assay the kinase activity of endogenous PKD1 and mouse IgG as a negative control for immunoprecipitation. A weak band at 120 kDa (Fig. 2B(1) lane 1)] indicates autophosphorylation of PKD1 and a strong band in lane 3 below 6 kDa [Fig. 2B(1) lane 3)] indicates Syntide-2 phosphorylation by PKD1. The identity of phosphorylated proteins was confirmed by immunoblotting the same membrane with E-cadherin and reprobing with PKD1 antibodies (Fig. 2B, lanes 2 and 3). Immunoblots showing expression of E-cadherin and PKD1 at 120 kDa in the same kinase assay clearly show that the phosphorylated protein in the autoradiograph [Fig. 2B(1) lane 2)] is E-cadherin, as the amount of PKD1 is identical in all the three lanes.

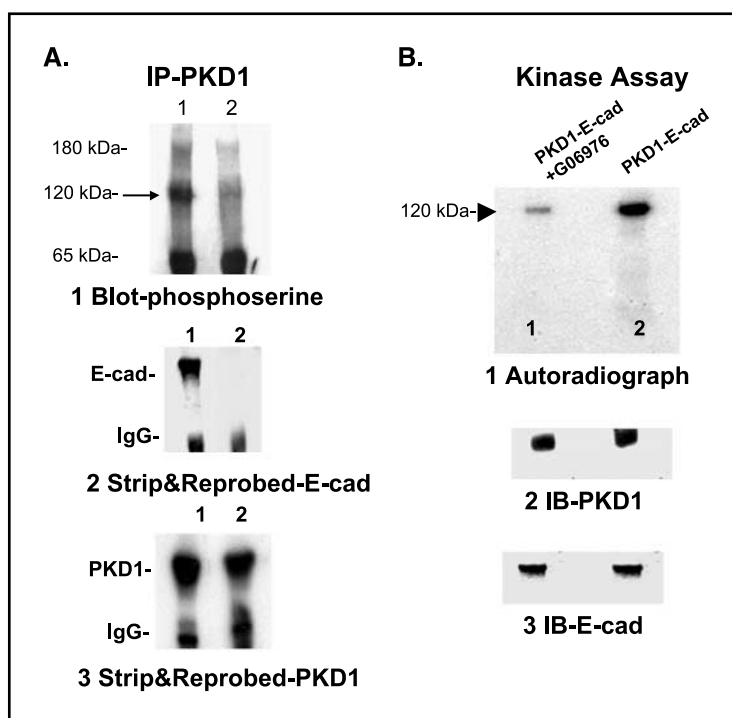
E-Cadherin is Phosphorylated by PKD1 *In vivo*. LNCaP cell extracts were immunoprecipitated with PKD1-specific antibody and immunoblotted with phosphoserine-specific antibodies (Poly-Z-PS1, Zymed Laboratories, Inc., CA) to confirm E-cadherin phosphorylation *in vivo*. Figure 3A shows a serine phosphorylated protein of 120 kDa, suggesting that E-cadherin is phosphorylated (Fig. 3, lane 1). The faint band seen in lane 2 at 120 kDa may represent the autophosphorylated form of PKD1. The same blot was stripped and reprobed with E-cadherin antibody confirming the presence of E-cadherin at 120 kDa [Fig. 3A(2) lane 1)]. As a

negative control, we immunoprecipitated PKD1 from highly stringent RIPA buffer cell extracts which destabilizes the PKD1 and E-cadherin interaction [Fig. 3A(2) lane 2)]. The same blot was re-stripped and reprobed with PKD1 antibody, confirming the equal expression of PKD1 in lanes 1 and 2 [Fig. 3A(3)]. Phosphorylated E-cadherin, which is associated with PKD1, contributes to a strong band in lane 1 at 120 kDa (MOPS lysis buffer preserves the E-cadherin/PKD1 interacting immunocomplex). The presence of a strong 120 kDa band only in MOPS immunoprecipitation (lane 1), which is absent in RIPA immunoprecipitation (lane 2), clearly shows that E-cadherin associated with PKD1 is phosphorylated in LNCaP cells *in vivo*.

PKD1 Selective Inhibitor Gö6976 Inhibits Phosphorylation of E-Cadherin. We did *in vitro* kinase assays using E-cadherin immunoprecipitates and the endogenous PKD1 from the same lysate as E-cadherin, in the presence of PKD1-selective inhibitor Gö6976 (1 mol/L; ref. 34). Autoradiographs of these kinase assays show inhibition of E-cadherin phosphorylation in the presence of Gö6976 at 1 m concentration (Fig. 3B, lane 1, →) compared with vehicle (DMSO) only control (lane 2). Immunoblotting of the kinase assay blot with E-cadherin and PKD1 antibodies confirmed the presence of equal amounts of PKD1 and E-cadherin in lanes 1 and 2 (Fig. 3B, lanes 2 and 3)). The results of these experiments confirmed that E-cadherin phosphorylation by PKD1 can be inhibited by a PKD1-selective inhibitor.

Characterization of PKD1-GFP Expressing C4-2 Cells. We have previously identified and confirmed the down-regulation of PKD1 in the prostate cancer C4-2 cell line (19). Although C4-2 cells are derived from LNCaP cells, C4-2 cells proliferate rapidly, are highly metastatic, and have androgen receptors that are down-regulated by about 2.5-fold compared with LNCaP cells, thereby phenotypically matching the clinical course of advanced prostate cancer in humans (35). Therefore, we transfected C4-2

Figure 3. E-cadherin is phosphorylated by PKD1 *in vivo* and PKD1-selective inhibitor Gö6976 inhibits phosphorylation of E-cadherin (A) E-cadherin is phosphorylated by PKD1 *in vivo*. (1) LNCaP cell extract prepared in MOPS or RIPA lysis buffer were immunoprecipitated with PKD1 and immunoblotted with phosphoserine-specific antibodies. →, a serine phosphorylated protein of 120 kDa, suggesting E-cadherin phosphorylation. Lane 1, LNCaP MOPS lysis buffer extract-immunoprecipitation PKD1 antibody; lane 2, LNCaP RIPA lysis buffer extract-immunoprecipitation PKD1 antibody (negative control). Numbering on the left indicates molecular weight in kilodaltons. (2) The same blot was stripped and reprobed with E-cadherin antibody showing the presence of E-cadherin at the same position and same molecular weight in lane 1 only (MOPS lysis buffer immunoprecipitation) not in lane 2 (RIPA buffer immunoprecipitation). (3) PKD1 immunoblot showing equal amounts of PKD1 in lanes 1 and 2. B, PKD1-selective inhibitor Gö6976 inhibits phosphorylation of E-cadherin. (1) Autoradiograph of kinase assays with or without PKD1-selective inhibitor Gö6976 (1 μmol/L) showing inhibition of phosphorylation of E-cadherin by Gö6976. (2 and 3) Immunoblot of the same kinase assay showing equal loading of PKD1 and E-cadherin.



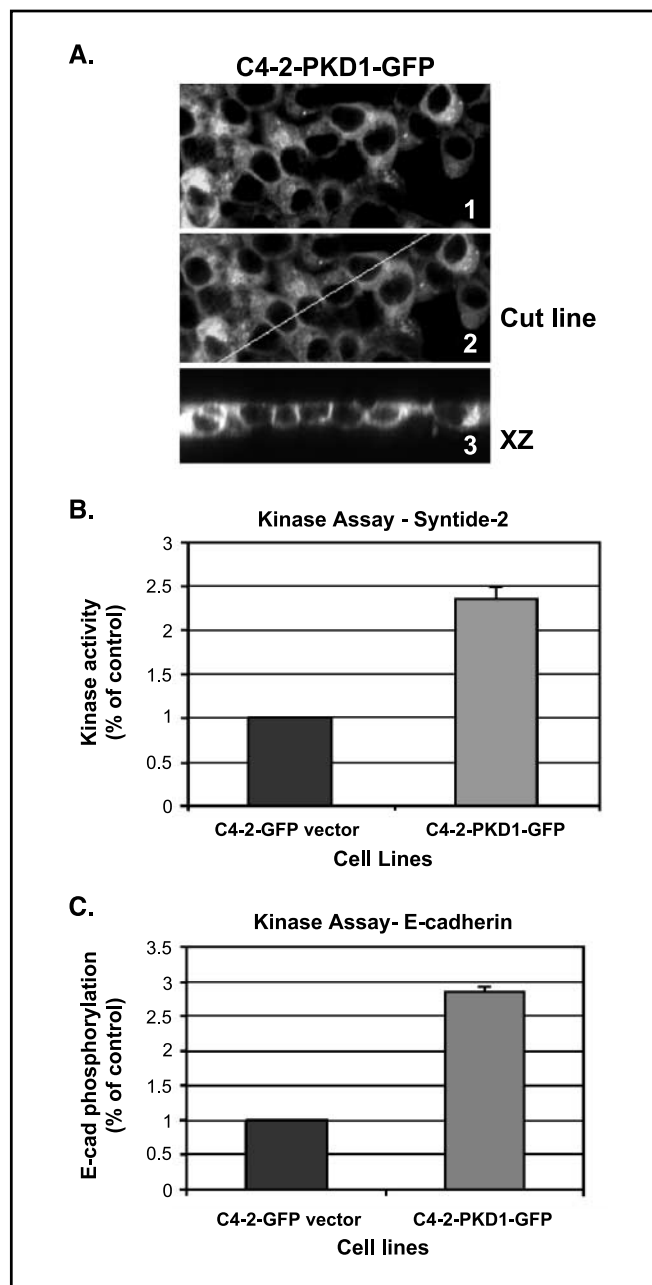


Figure 4. Characterization of C4-2 cells expressing PKD1-GFP fusion protein. A, localization of PKD1 in C4-2 cells expressing PKD1-GFP fusion protein. 1, C4-2-PKD1-GFP cells were cultured on glass coverslips and analyzed by laser scanning confocal microscopy. Note that PKD1 is diffusely distributed throughout the cell and enriched on cell junctions. To further confirm junctional localization, a section image defined by cut line (2) was created. 3, Vertical XZ image of cells shows junctionally localized PKD1. B, PKD1 kinase activity in stable C4-2 transfectants. C4-2 prostate cancer cells were stably transfected with GFP vector only (C4-2-V), GFP fused PKD1 (C4-2-PKD1) and the kinase activity was measured as cpm/mg protein following phosphorylation of Syntide-2 (American Peptide Company) as selective substrate by PKD1 in the presence of [γ ³²P] ATP. The data were plotted as a percentage of control (average of two separate experiments). Bars, SE. C, overexpression of PKD1 increases phosphorylation of E-cadherin in C4-2-PKD1-GFP cells. Kinase assay demonstrating increased phosphorylation of E-cadherin by PKD1 in C4-2-PKD1-GFP cells. E-cadherin and PKD1 were isolated from C4-2-GFP vector and C4-2-PKD1-GFP-expressing cells using specific anti-E-cadherin and anti-PKD1 (4A2 and C-20) antibodies. E-cadherin phosphorylation was done and the radioactivity incorporated into E-cadherin was measured as cpm/mg protein. The data were plotted as a percentage of control (average of two separate experiments). A 2.8-fold increase in E-cadherin phosphorylation was detected in C4-2-PKD1-GFP cells compared with C4-2-GFP vector cells. Bars, SE.

cells with a PKD1 expression construct in order to study localization and the physiologic significance of PKD1 in prostate cancer. We have generated stable C4-2 transfectants with full-length PKD1-GFP, or GFP vector alone. Homogenous PKD1-GFP expressing clones (Fig. 4A) were selected by G418 resistance and fluorescence activated cell sorting. Western blotting confirmed 3-fold increased PKD1 expression in the C4-2-PKD1-GFP cell line (data not shown). We carried out confocal laser scanning analysis on C4-2-PKD1-GFP cells to study the PKD1 localization pattern. C4-2-PKD1-GFP cells were grown on glass coverslips to subconfluence, fixed, and then analyzed by confocal laser scanning microscopy. Interestingly, whereas single or sparsely populated cells showed intense cytoplasmic localization, confluent cells had intense PKD1 localization at cell junctions (Fig. 4A1). To further confirm junctional localization of PKD1-GFP, vertical XZ-section images obtained by LSM-using Software 399 confirmed cytoplasmic and junctional localization of PKD1-GFP in C4-2 cells (Fig. 4A3, XZ).

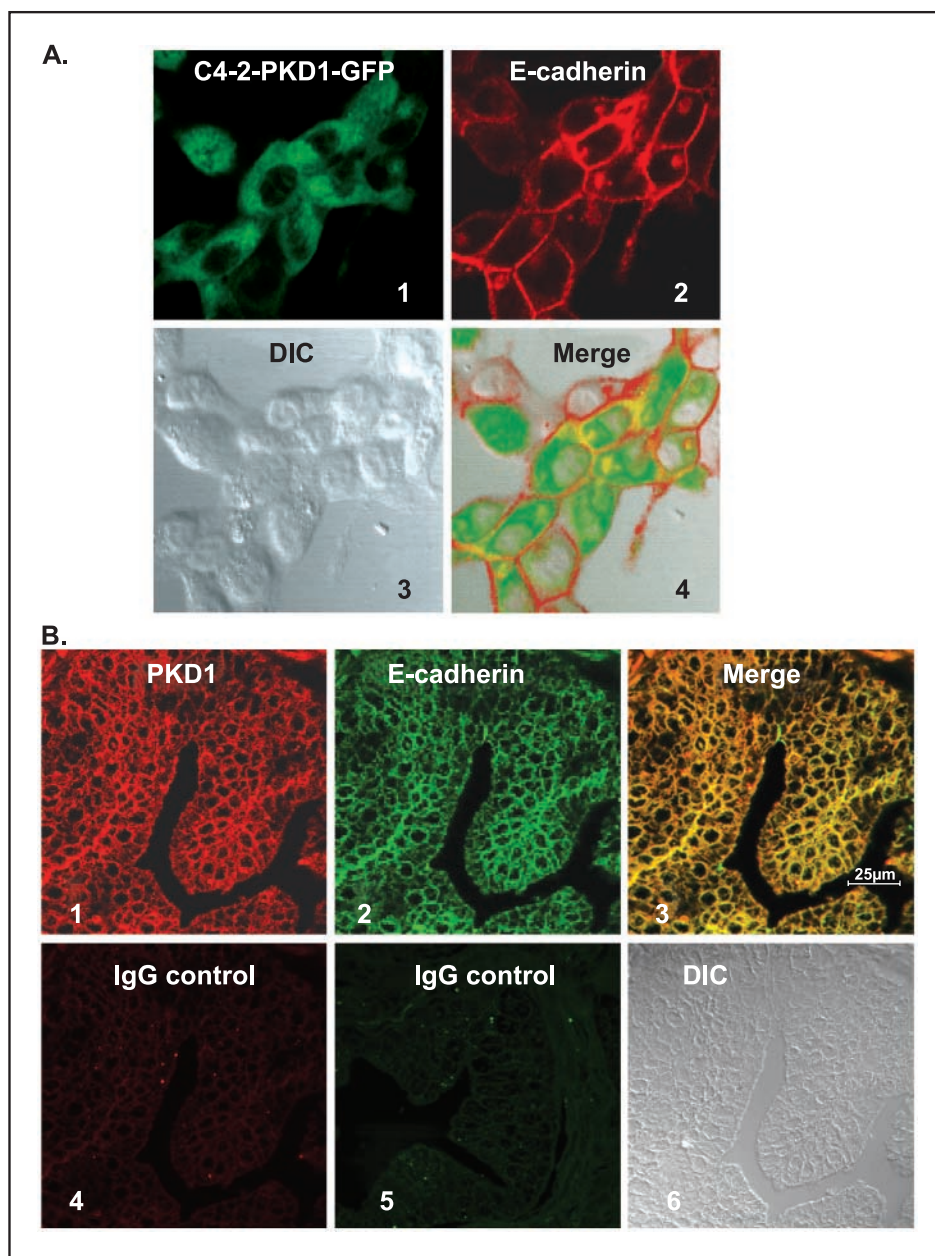
The kinase activity of PKD1 in C4-2, C4-2-GFP vector, and C4-2-PKD1-GFP-expressing cells was determined using Syntide-2 as a model PKD1 substrate (Fig. 4B). The radioactivity incorporated into Syntide-2 was measured and results were plotted as a percentage of control. A 2.4-fold increase in PKD kinase activity was detected in C4-2-PKD1-GFP cells compared with C4-2 cells (Fig. 4B).

Overexpression of PKD1 Increases Phosphorylation of E-Cadherin in C4-2 Cells. The kinase assay was done to explore whether overexpression and increased PKD1 kinase activity increases E-cadherin phosphorylation in C4-2-PKD1-GFP cells. PKD1 and E-cadherin were isolated from C4-2-GFP vector and C4-2-PKD1-GFP cells using specific PKD1 and E-cadherin antibodies. E-cadherin phosphorylation was determined by *in vitro* kinase assay as described previously. The radioactivity incorporated into E-cadherin was measured as cpm/mg protein and results were plotted as a percentage of the controls. A 2.8-fold increase in E-cadherin phosphorylation was detected in C4-2-PKD1-GFP cells compared with C4-2-GFP vector cells (Fig. 4C).

PKD1 Colocalizes with E-Cadherin in C4-2-PKD1-GFP Cells. We explored the colocalization of PKD1 and E-cadherin in C4-2-PKD1-GFP cells to confirm that these proteins are appropriately localized to interact with one another *in vivo*. The cells were cultured on glass coverslips, immunostained with E-cadherin antibody, and analyzed by laser scanning confocal microscope. Figure 5 shows the merging of images taken in *green* (1, PKD1-GFP), *red* (2, E-cadherin), and *DIC* (3, DIC) channels indicating that PKD1 colocalizes with E-cadherin at cell junctions (4, merged; *yellow*).

PKD1 Colocalizes with E-Cadherin in Human Prostate Cancer Tissues. To establish the translational validity of PKD1 and E-cadherin interaction, we studied the E-cadherin/PKD1 subcellular localization in five human prostate cancer tissue samples. All five samples showed colocalization of PKD1 and E-cadherin at cell junctions. Similar to cell lines, PKD1 also showed junctional and cytoplasmic localization (Fig. 5B1) and E-cadherin was localized to cell junctions in human prostate cancer tissue (Fig. 5B2). Merging of images 1 and 2 show that PKD1 and E-cadherin colocalized at the cell junctions of prostatic glandular epithelial cells (Fig. 5B3). Specificity of expression was confirmed by using isotype controls (Fig. 5B4 and 5). Thus we validated the result from our *in vitro* cell line studies in human prostate cancer. Because PKD1 and E-cadherin colocalizes in human prostate cancer epithelial cells, it is highly

Figure 5. Colocalization of PKD1 with E-cadherin at cell junctions in C4-2-PKD1-GFP cells and in human prostate cancer tissue. **A**, colocalization of PKD1 with E-cadherin in C4-2-PKD1-GFP cells. The cells were cultured on glass coverslips and immunostained with E-cadherin antibody and analyzed by laser scanning confocal microscopy (Carl Zeiss). 1, localization of PKD1-GFP (*green*); 2, localization of E-cadherin (*red*); 3, DIC image; and 4, merging of 1, 2 and 3 showing E-cadherin/PKD1 colocalization (*yellow*) at cell junctions. **B**, colocalization of PKD1 with E-cadherin in human prostate cancer tissue. Immunostaining was done on paraffin-embedded prostate tissue sections using FITC-conjugated anti-mouse antibodies for E-cadherin and rhodamine-conjugated anti-rabbit for PKD1. 1, PKD1 localized to perinuclear and cell junctions (*red*). 2, E-cadherin localized to cell junctions (*green*). 3, following merging of images 1 and 2, colocalization of PKD1 and E-cadherin (*yellow*) is confirmed in human prostate glandular epithelium. 6, corresponding DIC image. Specificity of expression was confirmed by using isotype controls (4 and 5).



probable that the PKD1/E-cadherin interaction may also occur in human prostate cancer.

Overexpression of PKD1 and Increased E-Cadherin Phosphorylation in Prostate Cancer Cells Leads to Increased Cellular Aggregation. E-cadherin is known to be involved with altered cellular aggregation and adhesion, both of which are required for a cancer cell to successfully complete the metastatic cascade (4). Because we have shown that PKD1 interacts with and phosphorylates E-cadherin, we sought to determine whether overexpression of PKD1 and increased E-cadherin phosphorylation influences cellular aggregation and motility in prostate cancer cells. C4-2 cells overexpressing PKD1 were tested for their ability to aggregate in hanging drop suspension cultures. Aggregation assays were done on C4-2 cells expressing PKD1-GFP or GFP vector alone as described previously (28). Our experiments showed increased cellular aggregation in C4-2-PKD-GFP cells compared with parental

and GFP vector expressing C4-2 cells (Fig. 6A1-3). To further confirm that PKD1 kinase activity modulates prostate cancer cell aggregation properties, aggregation assays were done on LNCaP cells treated with or without PKD1-specific inhibitor Gö6976 at 1 mol/L concentration. Our experiments showed decreased cellular aggregation in Gö6976 (1 mol/L)-treated cells compared with control LNCaP cells (Fig. 6A, lanes 4 and 5).

Overexpression of PKD1 and Increased E-Cadherin Phosphorylation Leads to Decreased Cellular Motility. The effect of PKD1 expression on motility was studied by *in vitro* motility assays. For this assay, 5×10^3 cells (C4-2, C4-2 GFP vector, and C4-2-PKD1-GFP) were plated into the top chamber of motility inserts. The total number of cells that migrated through the pores was counted. Experiments were repeated four times and results expressed as the average number of cells that migrated through the pores (Fig. 6B). ANOVA was used to compare the motility of C4-2, C4-2-GFP vector

and C4-2-PKD1-GFP cells. A significant difference in cell motility was found between C4-2 and C4-2-PKD1-GFP ($P = 0.021$) and between C4-2-GFP vector and C4-2-PKD1-GFP ($P = 0.0041$). The difference in cell motility between C4-2 and C4-2-GFP vector cells was not significantly different ($P = 0.33$).

Discussion

The cadherin/catenin complex of proteins is a major target of posttranslational modifications such as phosphorylation/dephosphorylation. Tyrosine phosphorylation of β -catenin by the cytoplasmic kinase Fer disrupts binding of β -catenin to α -catenin, whereas phosphorylation by Src or the epidermal growth factor receptor disrupts binding of β -catenin to cadherin (36, 37). It has been shown that E-cadherin is phosphorylated by casein kinase II and glycogen synthase kinase-3 β , and this stabilizes the interaction of E-cadherin and β -catenin. In this study, we report a novel interaction between E-cadherin and PKD1. Our studies show that E-cadherin is phosphorylated by PKD1 and increased kinase activity and overexpression of PKD1 increases cell aggregation and decreases cell motility. E-cadherin belongs to the family of classical cadherins, which also includes placental and neural cadherins as well. Although epithelial tissues do not normally express placental or neural cadherin, abnormal cadherin expression may occur in disease states including cancer (3, 4). Because of the sequence homology between the members of the classical cadherin family, it is possible that PKD1 may interact with other cadherins. Neural

cadherin protein expression was not detected in LNCaP and C4-2 cells. LNCaP cells do not express placental cadherin (38). Therefore, we are currently exploring other cell line models to study the PKD1 interaction with other classical cadherins.

PKD1 is a multisite phosphorylated enzyme with one autophosphorylation and two transphosphorylation sites, capable of multiple biological functions through complex interaction with other cellular molecules (39). Although PKD is similar to PKC at the C1 domain, PKD is markedly divergent from PKC in other structural features: (1) the catalytic domain of PKD/PKC μ is distantly related with and shows little homology with the catalytic domains of the PKC family; (2) PKD possesses a putative transmembrane sequence and a pleckstrin homology domain, both lacking in PKC; and (3) conversely, PKD lacks the pseudosubstrate domain found in the PKCs. In addition, PKD1 is constitutively localized to the cellular membranes such as the Golgi apparatus, unlike other members of the PKC family, which are constitutively cytosolic and localize to membranes only on activation (40, 41). These unique structural characteristics suggest a novel function for PKD1 distinct from other members of the PKC family of proteins.

Studies on PKD1 reveal its role in Golgi function, cell proliferation, and apoptosis (23). The PKD1-mediated cellular responses depend on the nature of the substrates that are phosphorylated by PKD1(42). Only a few proteins to date have been identified to interact with and/or regulate PKD1, including 14-3-3 protein, NF- κ B-related genes, p32, Btk, PKC η , PKC δ , G β

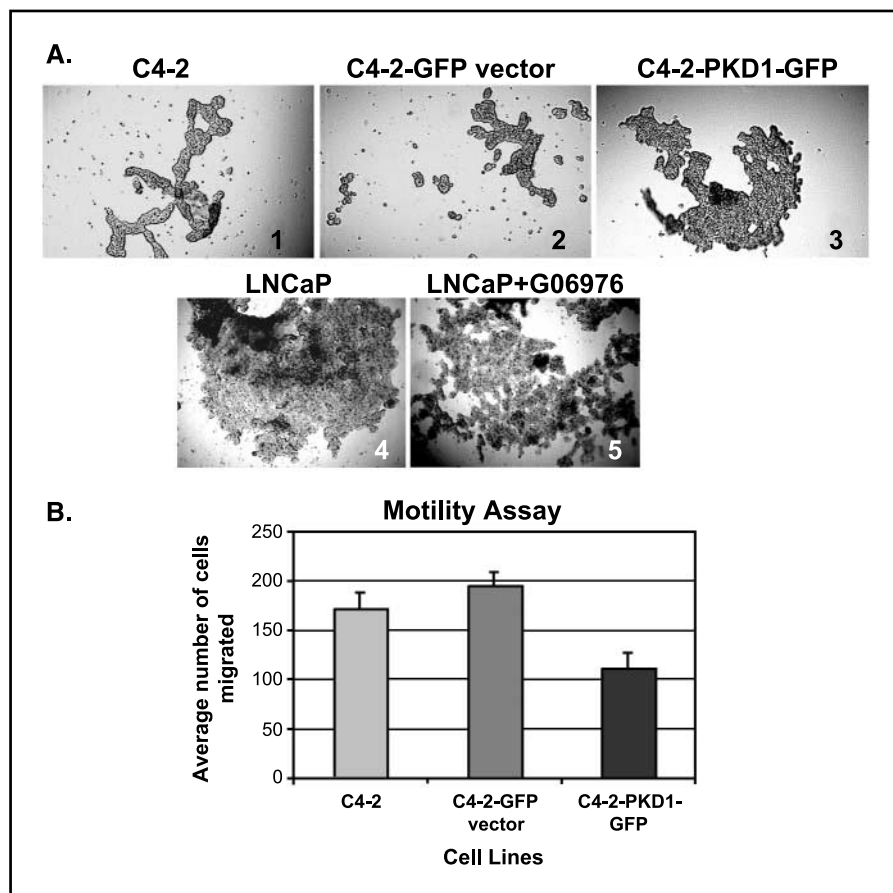


Figure 6. Increased PKD1 kinase activity and increased E-cadherin phosphorylation influences cellular aggregation and motility in prostate cancer cells. **A**, increased PKD1 activity in prostate cancer cells increases cellular aggregation. Five thousand cells (C4-2, C4-2-GFP vector, and C4-2-PKD1-GFP-expressing cells) in 20 μ L were dropped onto the inner surface of the lid of a Petri dish. After 24 hours of incubation, the lid of the Petri dish was inverted and drops of cells were resuspended. Three independent experiments were done and images were taken (Nikon microscope). C4-2 cells expressing a PKD1-GFP fusion protein showed increased cellular aggregation (3) compared with C4-2 cells expressing GFP vector alone (2) or parental C4-2 cells (1). Aggregation assays were done on LNCaP cells treated with or without PKD1-selective inhibitor G06976 (1 μ mol/L). Note decreased cellular aggregation in G06976-treated LNCaP cells (6) compared with control LNCaP cells (5). **B**, increased PKD1 activity in prostate cancer cells decrease cellular motility. C4-2, C4-2-GFP vector, and C4-2-PKD1-GFP-expressing cells were plated on membrane inserts and incubated for 24 hours, and cells that did not migrate through the pores in the membrane were removed by scraping with a cotton swab. The remaining cells were stained, and the number of cells transverse the membrane were determined by counting the cells under a microscope. Graph shows decreased motility of C4-2-PKD1-GFP cells compared with C4-2-GFP vector and C4-2 cells. The data are expressed as the number of cells (average of four separate experiments). Bars, SE. ANOVA was used to compare the motility of C4-2, C4-2-GFP vector, and C4-2-PKD1-GFP cells. A significant difference in cell motility was found between C4-2 and C4-2-PKD1-GFP ($P = 0.021$) and between C4-2-GFP vector and C4-2-PKD1-GFP ($P = 0.0041$). The difference in cell motility between C4-2 and C4-2-GFP vector cells was not significantly different ($P = 0.33$).

proteins, and metallothionein 2A (39, 43–46). In this study, we report a novel substrate for PKD1, E-cadherin, which is a well-studied cell adhesion molecule. Studies have shown that phosphorylation of E-cadherin is concentrated in a short stretch of 30 amino acids in the cytoplasmic domain (47). This region is necessary and sufficient for the interaction with β -catenin, or plakoglobin, and harbors a cluster of eight serine residues. When all the serine residues were substituted by alanines, phosphorylation of E-cadherin *in vivo* was completely abolished (47). The absence of phosphorylation also prevented association between E-cadherin and β -catenin or plakoglobin. Thus, phosphorylation of E-cadherin is a major posttranslational event that seems to significantly influence cadherin-mediated cell-cell adhesion.

The *in silico* analysis of the E-cadherin amino acid sequence revealed the presence of PKD1 comparable substrate sequences (LSSLNS at serine 850 and LNSSES at 853) in the short stretch of 30 amino acids in the cytoplasmic domain (33) which is necessary for its interaction with β -catenin (47). This suggests that E-cadherin interaction with PKD1, a serine threonine kinase, may stabilize E-cadherin binding to catenins, which in turn can modulate cell-cell adhesion. The other unique feature of the PKD1 translocation/activation mechanism is that it translates a short-lived DAG signal into persistent PKD1 activation. The persistent activation of PKD1 potentially leads to long-term cellular effects and explains one mechanism by which brief hormonal stimuli result in long-lasting responses, such as gene transcription or cellular motility (42).

A hallmark of metastatic progression is alteration in intercellular adhesion resulting in cell shedding from primary tumor and migration to other sites (48). Mechanisms of migration have been investigated in carcinoma cell lines as well as in numerous embryonic fibroblasts. Although the common requirement of FAK and Src kinases have been described, there are varied signal transduction pathways including PI3-kinase, mitogen-activated

protein kinase, PKC, and PKA pathways (49–51) in different cell types. In our experimental model, we have determined that PKD1-mediated E-cadherin phosphorylation alters cellular aggregation and motility in prostate cancer. PKD1 has been shown to be activated via a PKC-dependent pathway and is implicated in mitogenic signal cascades (p42 mitogen-activated protein kinase; ref. 52). Therefore, it is conceivable that mitogen-activated protein kinase-dependent or PKC-dependent cellular alterations may be mediated through PKD1/E-cadherin interaction.

In conclusion, we have identified a novel interaction between E-cadherin and PKD1 resulting in E-cadherin phosphorylation, thereby establishing E-cadherin as a novel substrate of PKD1. We have previously shown that PKD1 is down-regulated in advanced human prostate cancer. Based on this and data from the present study, we postulate that decreased expression of PKD1 results in decreased E-cadherin phosphorylation, resulting in destabilization of the cadherin/catenin complex. This may lead to decreased cell aggregation and increased cellular motility, contributing to metastatic progression of prostate cancer. In addition, increased amounts of β -catenin may become available for translocation to the nucleus, which in turn can participate in the Wnt signaling pathway, associated with cellular proliferation.

Acknowledgments

Received 8/9/2004; revised 10/28/2004; accepted 11/10/2004.

Grant support: Supported by a seed grant from the University of Nebraska Foundation, Omaha, NE.

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 the late Dr. F.J. Johannes, Stuttgart, Germany, for generously providing the PKC μ /PKD1 cDNA; Janice Taylor of the Confocal Laser Scanning Microscope Core Facility at University of Nebraska Medical Center, which is supported by the Nebraska Research Initiative, for providing assistance with confocal microscopy; and Lynette M. Smith for statistical analysis.

References

- Hellerstedt BA, Pienta KJ. The current state of hormonal therapy for prostate cancer. *CA Cancer J Clin* 2002;52:154–79.
- Jemal A, Tiwari RC, Murray T, et al. Cancer statistics, 2004. *CA Cancer J Clin* 2004;54:8–29.
- Wheelock MJ, Johnson KR. Cadherins as modulators of cellular phenotype. *Annu Rev Cell Dev Biol* 2003; 19:207–35.
- Yap AS. The morphogenetic role of cadherin cell adhesion molecules in human cancer: a thematic review. *Cancer Invest* 1998;16:252–61.
- Bringuier PP, Umbas R, Schaafsma HE, Karthaus HF, Debruyne FM, Schalken JA. Decreased E-cadherin immunoreactivity correlates with poor survival in patients with bladder tumors. *Cancer Res* 1993;53: 3241–5.
- Umbas R, Isaacs WB, Bringuier PP, et al. Decreased E-cadherin expression is associated with poor prognosis in patients with prostate cancer. *Cancer Res* 1994; 54:3929–33.
- Geiger B, Ayalon O, Ginsberg D, et al. Cytoplasmic control of cell adhesion. *Cold Spring Harb Symp Quant Biol* 1992;57:631–42.
- Takeichi M. Cadherin cell adhesion receptors as a morphogenetic regulator. *Science* 1991;251: 1451–5.
- Nagafuchi A, Takeichi M. Cell binding function of zE-cadherin is regulated by the cytoplasmic domain. *EMBO J* 1988;7:3679–84.
- Reynolds AB, Daniel JM, Mo YY, Wu J, Zhang Z. The novel catenin p120cas binds classical cadherins and induces an unusual morphological phenotype in NIH3T3 fibroblasts. *Exp Cell Res* 1996;225: 328–37.
- Nieset JE, Redfield AR, Jin F, Knudsen KA, Johnson KR, Wheelock MJ. Characterization of the interactions of α -catenin with α -actinin and β -catenin/plakoglobin. *J Cell Sci* 1997;110:1013–22.
- Knudsen KA, Soler AP, Johnson KR, Wheelock MJ. Interaction of α -actinin with the cadherin/catenin cell-cell adhesion complex via α -catenin. *J Cell Biol* 1995;130:67–77.
- Lilien J, Balsamo J, Arregui C, Xu G. Turn-off, drop-out: functional state switching of cadherins. *Dev Dyn* 2002;224:18–29.
- Le TL, Yap AS, Stow JL. Recycling of E-cadherin: a potential mechanism for regulating cadherin dynamics. *J Cell Biol* 1999;146:219–32.
- Hellberg CB, Burden-Gulley SM, Pietz GE, Brady-Kalnay SM. Expression of the receptor protein-tyrosine phosphatase, PTP μ , restores E-cadherin-dependent adhesion in human prostate carcinoma cells. *J Biol Chem* 2002;277:11165–73.
- 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.
- Lickert H, Bauer A, Kemler R, Stappert J. 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.
- Serres M, Filhol O, Lickert H, et al. The disruption of adherens junctions is associated with a decrease of E-cadherin phosphorylation by protein kinase CK2. *Exp Cell Res* 2000;257:255–64.
- Jaggi M, Rao PS, Smith DJ, Hemstreet GP, Balaji KC. Protein kinase C μ is down-regulated in androgen-independent prostate cancer. *Biochem Biophys Res Commun* 2003;307:254–60.
- Manning G, Whyte DB, Martinez R, Hunter T, Sudarsanam S. The protein kinase complement of the human genome. *Science* 2002;298:1912–34.
- Valverde AM, Sinnett-Smith J, Van Lint J, Rozengurt E. Molecular cloning and characterization of protein kinase D: a target for diacylglycerol and phorbol esters with a distinctive catalytic domain. *Proc Natl Acad Sci U S A* 1994;91:8572–6.
- Rykx A, De Kimpe L, Mikhalap S, et al. Protein kinase D: a family affair. *FEBS Lett* 2003;546:81–6.
- Lint JV, Rykx A, Vantus T, Vandenheede JR. Getting to know protein kinase D. *Int J Biochem Cell Biol* 2002; 34:577–81.
- Iglesias T, Cabrera-Poch N, Mitchell MP, Naven TJ, Rozengurt E, Schiavo G. Identification and cloning of Kidins220, a novel neuronal substrate of protein kinase D. *J Biol Chem* 2000;275:40048–56.
- Powell CT, Fair WR, Heston WD. Differential expression of protein kinase C isozyme messenger RNAs in dunning R-3327 rat prostatic tumors. *Cell Growth Differ* 1994;5:143–9.
- Newton AC. Protein kinase C: structure, function, and regulation. *J Biol Chem* 1995;270:28495–8.
- Johnson KR, Lewis JE, Li D, et al. P- and E-cadherin are in separate complexes in cells expressing both cadherins. *Exp Cell Res* 1993;207:252–60.
- Redfield A, Nieman MT, Knudsen KA. Cadherins promote skeletal muscle differentiation in three-dimensional cultures. *J Cell Biol* 1997;138: 1323–31.
- Hurd C, Rozengurt E. Uncoupling of protein kinase D from suppression of EGF-dependent *c-Jun*

- phosphorylation in cancer cells. *Biochem Biophys Res Commun* 2003;302:800-4.
30. Van Lint J, Rykx A, Maeda Y, et al. Protein kinase D: an intracellular traffic regulator on the move. *Trends Cell Biol* 2002;12:193-200.
 31. Songyang Z, Lu KP, Kwon YT, et al. A structural basis for substrate specificities of protein Ser/Thr kinases: primary sequence preference of casein kinases I and II, NIMA, phosphorylase kinase, calmodulin-dependent kinase II, CDK5, and Erk1. *Mol Cell Biol* 1996;16:6486-93.
 32. Nishikawa K, Toker A, Johannes FJ, Songyang Z, Cantley LC. Determination of the specific substrate sequence motifs of protein kinase C isozymes. *J Biol Chem* 1997;272:952-60.
 33. Blom N, Gammeltoft S, Brunak S. Sequence and structure-based prediction of eukaryotic protein phosphorylation sites. *J Mol Biol* 1999;294:1351-62.
 34. Gschwendt M, Dieterich S, Rennecke J, Kittstein W, Mueller HJ, Johannes FJ. Inhibition of protein kinase C μ by various inhibitors. Differentiation from protein kinase C isoenzymes. *FEBS Lett* 1996;392:77-80.
 35. Wu HC, Hsieh JT, Gleave ME, Brown NM, Pathak S, Chung LW. Derivation of androgen-independent human LNCaP prostatic cancer cell sublines: role of bone stromal cells. *Int J Cancer* 1994;57:406-12.
 36. 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.
 37. Roura S, Miravet S, Piedra J, Garcia de Herreros A, Dunach M. Regulation of E-cadherin/catenin association by tyrosine phosphorylation. *J Biol Chem* 1999;274:36734-40.
 38. Bussemakers MJ, Van Bokhoven A, Tomita K, Jansen CF, Schalken JA. Complex cadherin expression in human prostate cancer cells. *Int J Cancer* 2000;85:446-50.
 39. Vertommen D, Rider M, Ni Y, et al. Regulation of protein kinase D by multisite phosphorylation. Identification of phosphorylation sites by mass spectrometry and characterization by site-directed mutagenesis. *J Biol Chem* 2000;275:19567-76.
 40. Prestle J, Pfizenmaier K, Brenner J, Johannes FJ. Protein kinase C μ is located at the Golgi compartment. *J Cell Biol* 1996;134:1401-10.
 41. Powell CT, Brittis NJ, Stec D, Hug H, Heston WD, Fair WR. Persistent membrane translocation of protein kinase C α during 12-O-tetradecanoylphorbol-13-acetate-induced apoptosis of LNCaP human prostate cancer cells. *Cell Growth Differ* 1996;7:419-28.
 42. Oancea E, Bezzerides VJ, Greka A, Clapham DE. Mechanism of persistent protein kinase D1 translocation and activation. *Dev Cell* 2003;4:561-74.
 43. Johannes FJ, Horn J, Link G, et al. Protein kinase C μ downregulation of tumor-necrosis-factor-induced apoptosis correlates with enhanced expression of nuclear factor- κ B-dependent protective genes. *Eur J Biochem* 1998;257:47-54.
 44. Cabell CH, Verghese GM, Rankl NB, Burns DJ, Blackshear PJ. MARCKS phosphorylation by individual protein kinase C isozymes in insect Sf9 cells. *Proc Assoc Am Physicians* 1996;108:37-46.
 45. Hausser A, Storz P, Link G, et al. Protein kinase C μ is negatively regulated by 14-3-3 signal transduction proteins. *J Biol Chem* 1999;274:9258-64.
 46. Rao PS, Jaggi M, Smith DJ, Hemstreet GP, Balaji KC. Metallothionein 2A interacts with the kinase domain of PKC μ in prostate cancer. *Biochem Biophys Res Commun* 2003;310:1032-8.
 47. Stappert J, Kemler R. A short core region of E-cadherin is essential for catenin binding and is highly phosphorylated. *Cell Adhes Commun* 1994;2:319-27.
 48. Enmon RM Jr, O'Connor KC, Song H, Lacks DJ, Schwartz DK. Aggregation kinetics of well and poorly differentiated human prostate cancer cells. *Biotechnol Bioeng* 2002;80:580-8.
 49. Sieg DJ, Hauck CR, Ilic D, et al. FAK integrates growth-factor and integrin signals to promote cell migration. *Nat Cell Biol* 2000;2:249-56.
 50. Klemke RL, Cai S, Giannini AL, Gallagher PJ, de Lanerolle P, Cheresch DA. Regulation of cell motility by mitogen-activated protein kinase. *J Cell Biol* 1997;137:481-92.
 51. You X, Yu HM, Cohen-Gould L, et al. Regulation of migration of primary prostate epithelial cells by secreted factors from prostate stromal cells. *Exp Cell Res* 2003;288:246-56.
 52. Brandlin I, Eiseler T, Salowsky R, Johannes FJ. Protein kinase C(μ) regulation of the JNK pathway is triggered via phosphoinositide-dependent kinase 1 and protein kinase C(ϵ). *J Biol Chem* 2002;277:45451-7.