Published OnlineFirst September 2, 2008; DOI: 10.1158/1535-7163.MCT-08-0119

Bryostatin 1 modulates β-catenin subcellular localization and transcription activity through protein kinase D1 activation

Meena Jaggi,[1] Subhash C. Chauhan,[1] Cheng Du,[2] and K.C. Balaji[2]

[1]Cancer Biology Research Center, Sanford Research/University of South Dakota, Sanford School of Medicine, University of South Dakota and Sanford Hospitals and Health Systems, Sioux Falls, South Dakota and [2]Division of Urology, Department of Surgery, University of Massachusetts Medical School, Worcester, Massachusetts

Abstract

In recent years, the use of natural products for cancer prevention and treatment has received considerable attention. Bryostatin 1 is a natural macrocyclic lactone and a protein kinase D (PKD) modulator with potent antineoplastic properties that has been used to treat human cancers in clinical trials with limited success. Further understanding the mechanistic basis of Bryostatin 1 action may provide opportunities to improve clinical results of treatment with Bryostatin 1. We identified that PKD1, founding member of PKD family of serine/threonine kinases, modulates E-cadherin/β-catenin activity, which plays an important role in cell integrity, polarity, growth, and morphogenesis. An aberrant expression and localization of E-cadherin/β-catenin has been strongly associated with cancer progression and metastasis. In this study, we examined the effect of Bryostatin 1 treatment on PKD1 activation, β-catenin translocation and transcription activity, and malignant phenotype of prostate cancer cells. Initial activation of PKD1 with Bryostatin 1 leads to colocalization of the cytoplasmic pool of β-catenin with PKD1, trans-Golgi network markers, and proteins involved in vesicular trafficking. Activation of PKD1 by Bryostatin 1 decreases nuclear β-catenin expression and β-catenin/TCF transcription activity. Activation of PKD1 alters cellular aggregation and proliferation in prostate cancer cells associated with subcellular redistribution of E-cadherin and β-catenin. For the first time, we have identified that Bryostatin 1 modulates β-catenin signaling through PKD1, which identifies a novel mechanism to improve efficacy of Bryostatin 1 in clinical settings. [Mol Cancer Ther 2008;7(9):2703–12]

Introduction

Bryostatin 1 is a marine-derived macrolactone that has shown antineoplastic activity in several tumors and inhibits growth and/or opposes the effects of TPA. Other possible modes of action include apoptosis modulation, interaction with MDR-1, neutrophil/monocyte activation, T-cell 5 activation, stimulation of normal hematopoiesis, and radioprotection. Bryostatin 1 is in phase II clinical trials for treatment of ovarian cancer, cervical cancer, melanoma, leukemia, lymphoma, and colorectal cancer. In spite of encouraging preclinical studies, phase II studies using Bryostatin 1 in treatment of several human cancers including prostate cancer showed minimal or no tumor response. Therefore, further understanding of mechanism of Bryostatin 1 action is necessary for improving clinical results. Because several cellular processes influenced by Bryostatin 1 such as apoptosis modulation and white cell activation are also known to be associated with protein kinase D (PKD), we explored the effect of Bryostatin 1 on PKD1-mediated function.

PKD1 is a widely distributed protein kinase in eukaryotic cells and is involved in a multitude of functions in normal and diseased states in humans (1). Currently, three kinases have been identified as members of the PKD family: PKD1, PKD2, and PKD3 (PKD*ν*; refs. 2–4). The PKD family members share a similar modular structure, which consists of alanine and proline-rich, cysteine-rich (divided into two subdomains: C1a and C1b), pleckstrin homology, kinase domains, and an acidic rich region (2, 3). The salient features of the PKD subfamily structure and function include a COOH-terminal catalytic kinase domain with a primary sequence and substrate specificity distinct from the protein kinase C (PKC) family of proteins. The catalytic domains of PKD (including PKD1) are distantly related to those of calcium calmodulin-dependent kinases (5). PKD1 has been shown to play a role in proliferation of keratinocytes, endothelial and pancreatic cancer cells (6, 7), cardiac myocyte contraction (8), osteoblast differentiation (9), aggregation of prostate cancer cells (10), Golgi apparatus reorganization, and regulation of the fission of vesicles from the trans-Golgi network (TGN; ref. 11) and several PKD1 functions are domain specific. PKD1 seems to play a central role in signal transduction and has been shown to interact with several proteins including the cadherin-catenin complex of proteins (1).

Received 2/8/08; revised 6/13/08; accepted 6/20/08.

Grant support: Prostate Cancer Foundation, University of Nebraska; University of Massachusetts Medical School; and Cancer Biology Research Center, Sanford Research/University of South Dakota, Sanford School of Medicine, University of South Dakota and Sanford Hospitals and Health Systems. This core facility is supported by the National Center for Research Resources, NIH grant P20 RR17662 [A. Martin Gerdes, Ph.D (Principal Investigator)].

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.

Requests for reprints: Meena Jaggi, Cancer Biology Research Center, Department of OBGYN and Basic Biomedical Science, Sanford Research/University of South Dakota, Sanford School of Medicine, University of South Dakota, 1400 West 22nd Street, Sioux Falls, SD 57105. Phone: 605-357-1552; Fax: 605-357-1409. E-mail: meena.jaggi@usd.edu

doi:10.1158/1535-7163.MCT-08-0119

Published OnlineFirst September 2, 2008; DOI: 10.1158/1535-7163.MCT-08-0119

Cadherin-catenin complexes of proteins play a major role in cell adhesion and proliferation (12). The cytoplasmic domain of E-cadherin is anchored to the actin cytoskeleton via three cytoplasmic proteins: β-, α-, and γ-catenin (plakoglobin; ref. 13). β-Catenin plays a major role in ontogenesis and oncogenesis (14). There are several regulatory mechanisms for the down-regulation of the Wnt/β-catenin signal, perhaps reflecting the pivotal nature of the pathway and the detrimental consequences of inappropriate activation (15). Deregulation of this system, which promotes the transcriptional function of β-catenin at the cost of its function in adhesion, lies at the heart of the development and progression of many human malignancies (16, 17). It has been shown that β-catenin plays a significant role in several cancers. This is most well defined at a molecular level in colon cancer (18). A role of β-catenin in prostate cancer is suggested by down-regulation of β-catenin in prostate cancer compared with benign glands, which correlates with increasing Gleason grade, decreased β-catenin staining in prostatic intraepithelial neoplasia, and increased nuclear staining in high Gleason grade prostate cancer (10, 19, 20). Because subcellular localization of β-catenin can be used as a surrogate marker for its function in cells, distinct nuclear localization of β-catenin in high-grade human prostate cancer suggests a role for β-catenin in prostate cancer progression (15).

It has been reported that β-catenin binds to the cytoplasmic domain of E-cadherin shortly after E-cadherin synthesis, whereas α-catenin joins the complex later, after E-cadherin has reached the cell surface (21, 22). Thus, E-cadherin is sorted and targeted to the basolateral membrane in a stoichiometric complex with a cytosolic protein, β-catenin. Because Bryostatin 1 is a PKD modulator and PKD1 is known to play a role in subcellular trafficking of proteins, we explored the relationship between PKD1 activation by Bryostatin 1, assembly of the E-cadherin/β-catenin complex, and involvement of other vesicular transport proteins at TGN.

Materials and Methods

Cell Culture

LNCaP (American Type Culture Collection), C4-2 human prostate cancer cells (UroCor), C4-2-GFP, and C4-2-PKD1-GFP (23) were grown at 37°C and 5% CO_2 in RPMI 1640 with 10% fetal bovine serum (Life Technologies).

Antibodies

The mouse monoclonal antibody against β-catenin has been described previously (24). Rabbit polyclonal antibodies against PKD1/PKCμ and phospho-PKD 916 (Santa Cruz Biotechnology; Cell Signaling Technology), Golgi and vesicular transport-specific antibodies (Golgi Sampler Kit; BD Transduction Laboratories), horseradish peroxidase, fluorescence-conjugated IgGs (Jackson ImmunoResearch Laboratories), and the phosphoserine-specific antibody, Poly-Z-PS1 (Zymed Laboratories), were obtained commercially.

Immunoprecipitation and Immunoblot Analysis

Early-passage LNCaP (passages 35-40) monolayers were washed twice in ice-cold PBS, lysed in a buffer containing 20 mmol/L MOPS (pH 7), 2 mmol/L EGTA, 2 mmol/L EDTA, 10 mmol/L sodium pyrophosphate, 30 mmol/L sodium fluoride, 2 mmol/L orthovandate, protease inhibitors (Sigma), and 0.5% NP-40, and equal amounts of cell extracts from LNCaP and C4-2 cells were immunoprecipitated using the C-20 polyclonal antibody against PKD1 or with β-catenin antibody by gently mixing for 1 h at 4°C. Immune complexes were washed three times in TBS-Tween 20 and beads were suspended in 25 μL of 2× Laemmli sample buffer; the proteins were resolved by SDS-PAGE and transferred to immunoblot polyvinylidene difluoride membranes (Bio-Rad Laboratories) and antibody binding was detected with an Enhanced Chemiluminescence Kit (Amersham).

Confocal Immunofluorescence Microscopy

LNCaP or C4-2 cells were cultured on glass coverslips until subconfluent. Coverslips were fixed in histochoice for 15 min at room temperature and permeabilized with cold methanol at −20°C for 2 min. C4-2-GFP and PKD-GFP transfected cells were fixed in 1% paraformaldehyde for 30 min and permeabilized with 0.05% Triton X-100. The cells were washed in PBS for 5 min and incubated in 10% goat serum in PBS at room temperature for 1 h. The coverslips were incubated with primary antibody in 10% goat serum in PBS. After washing with PBS, coverslips were incubated with FITC- or rhodamine-conjugated secondary antibodies for 1 h. The cells were washed again in PBS followed by a brief rinse in distilled H_2O and mounted in aqueous antifade medium (Vector Labs) followed by analysis with a Zeiss LSM 410 confocal laser scanning microscope (Carl Zeiss). Images were taken using the LSM version 399 software.

Silencing of PKD1

The PKD1 expression was silenced over 90% by transfecting synthetic small interfering RNA (siRNA; sense 5-GGAAGGAAAUAUCUCAUGAUU, antisense 5-PUCAUGAGAUAUUUCCUUCCUU, and negative control sense 5-UAGCGACUAAACACAUAA; Dharmacon). The C4-2-PKD1-GFP cells were transfected with siRNA duplexes diluted to 25 nmol/L using DharmaFect Transfection Reagent (Dharmacon).

TOPFlash Reporter Assay

To measure TCF reporter activity in the prostate cancer, cultured cells were plated in a 12-well plate 16 h before transient transfection with phosphorylation and the reporter constructs TOPFlash or FOPFlash (gifts from Dr. R. Moon, Washington University) and cotransfected with pRL-TK (*Renilla* luciferase; Promega) for 48 h. Luciferase activities were assayed using the Dual Glo (Dual Reporter Assay; Promega) with a GLOMAX 96 microplate luminometer. Following normalization of firefly luciferase activity to that of *Renilla* luciferase, FOPFlash was subtracted from TOPFlash signal. Experiments were done in triplicate. For comparison of TOPFlash reporter activity between C4-2-PKD1-GFP treated with or without

Published OnlineFirst September 2, 2008; DOI: 10.1158/1535-7163.MCT-08-0119

Bryostatin 1, the nonpaired sample *t* test was used to determine statistical significance to a level of $P < 0.05$.

Cell Proliferation Assay

The effect of Bryostatin 1 on C4-2-PKD1-GFP cell proliferation was determined using CellTiter-Glo Luminescent Cell Viability Assay (Promega). C4-2-PKD1-GFP cells were counted using a Beckman Coulter Particle Counter. In a white 96-well plate, 10,000 cells per well were plated in 50 μL medium. After 24 h of culture, 10 nmol/L Bryostatin 1 or equal volume of DMSO was added. After 48 h, culture plates were equilibrated to room temperature for 30 min, CellTiter-Glo Reagent equal to the volume of medium present in each well was added, and the contents were mixed for 2 min on an orbital rotator. The plates were read using GLOMAX 96 microplate luminometer (Promega) after 10 min incubation at room temperature.

Aggregation Assay

Aggregation assays were done on the Bryostatin 1 activated C4-2-PKD1-GFP cell lines as described previously (23) with minor modifications. The cells were trypsinized and suspended in the appropriate medium with or without the Bryostatin 1 (10 μmol/L). Five thousand cells in 20 μL were dropped onto the inner surface of the lid of a Petri dish. After 24 h 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 were taken through a microscope (Nikon Eclipse E800).

Results

Time- and Dose-Dependent Activation of PKD1 by Bryostatin 1

We have previously identified and confirmed the down-regulation of PKD1 in the prostate cancer C4-2 cell line (25). We have also generated stable C4-2 transfectants with full-length PKD1-GFP or GFP vector alone (23). Treatment of the prostate cancer cells stably transfected with PKD1-GFP with increasing concentrations (10-30 nmol/L) of Bryostatin 1 for 3 h showed increased transphosphorylation of Ser^{738} and Ser^{742} and autophosphorylation of Ser^{910} residues of PKD1 (Fig. 1.1*A* and *B*). The activation of PKD1 is phosphorylation dependent, and Ser^{738} and Ser^{742} residues in human PKD1 (corresponding to Ser^{744} and Ser^{748} in mouse) have been identified as crucial phosphorylation sites. These serine residues are located in the activation loop of the PKD1 catalytic domain. The COOH-terminal Ser^{916} residue has been identified as an autophosphorylation site in PKD1 (26). Phosphorylation of these serine residues affects PKD1 activity and plays a role in modulation of PKD1 function *in vivo*. To exclude cell line-specific effects, we also confirmed that Bryostatin 1 activated and is associated with membrane translocation of PKD1 in androgen-dependent LNCaP cells (data not shown).

Effect of Activation of PKD1 by Bryostatin 1 on E-Cadherin and β-Catenin Subcellular Localization

We have reported previously that PKD1 interacts with E-cadherin in LNCaP and C4-2 cells overexpressing PKD1 (27). Immunobloting of PKD1 immunoprecipitate in C4-2-PKD1-GFP cells revealed the presence of β-catenin in PKD1-E-cadherin immunocomplexes (Fig. 1.2). We analyzed subcellular localization of PKD1, E-cadherin, and β-catenin in Bryostatin 1 activated C4-2-PKD1-GFP cells by confocal microscopy. Bryostatin 1 is also known to modulate activity of other PKC family members. Therefore, to examine PKD1-specific changes in subcellular localization of E-cadherin and β-catenin, we compared E-cadherin and β-catenin localization in Bryostatin 1 activated C4-2-GFP and C4-2-PKD1-GFP cells. In vector-transfected C4-2 cells, we did not detect any change in E-cadherin or β-catenin localization after Bryostatin 1 activation (Fig. 2.1). Our immunofluorescence study clearly revealed perinuclear and membrane localization of PKD1-GFP on activation by Bryostatin 1 (Fig. 2.2). The most striking observation was the colocalization of E-cadherin and β-catenin with PKD1-GFP in Bryostatin 1 activated C4-2-PKD1-GFP cells at perinuclear areas in addition to cell membranes (Fig. 2.2, *arrows*). After 24 h of Bryostatin 1 treatment, strong membrane staining of E-cadherin/β-catenin and some perinuclear staining was also noticed (Fig. 2.3). Although the C4-2-GFP cells do not overexpress PKD1-GFP (Fig. 2.1), they do not show perinuclear localization of E-cadherin/β-catenin. This observation confirms that E-cadherin/β-catenin subcellular distribution is specifically mediated by PKD1 activation and not by other kinases activated by Bryostatin 1.

Perinuclear colocalization of E-cadherin and β-catenin is consistent with other studies showing that the E-cadherin/β-catenin complex is first formed at the endoplasmic reticulum level. The sequence for assembly of other components of the complex and the mechanism of sorting the E-cadherin/β-catenin complex at the Golgi compartment and trafficking are unclear. PKD1 is known to play a pivotal role in regulating the fission of cargo-containing tubular elements from the TGN to the cell surface (28, 29). Therefore, we studied the effect of PKD1 activation on the β-catenin subcellular localization and trafficking component in detail.

Our time-course immunofluorescence study revealed costaining of PKD1 and β-catenin at perinuclear and cell surface areas occurred as early as 15 min of Bryostatin 1 activation, which is associated with initiation of activation of PKD1 (Figs. 1.1 and 3*E* and *F*), and costaining was very prominent after 3 h of activation with Bryostatin 1 consistent with maximal activation of PKD1 at 3 h (Figs. 1.1 and 3*F* and *I, blue arrow*). The cells that lack PKD1-GFP expression did not show strong membrane or perinuclear localization of β-catenin (Fig. 3*F* and *I, white arrow*). This observation confirms that β-catenin subcellular distribution is predominantly mediated by PKD1 activation and not by other kinases activated by Bryostatin 1.

PKD1 and β-Catenin Colocalize at TGN

The colocalization of PKD1 and β-catenin in the perinuclear area is suggestive of localization to Golgi or endoplasmic reticulum (Fig. 3). We carried out immunofluorescence studies using TGN to membrane (p230),

Published OnlineFirst September 2, 2008; DOI: 10.1158/1535-7163.MCT-08-0119

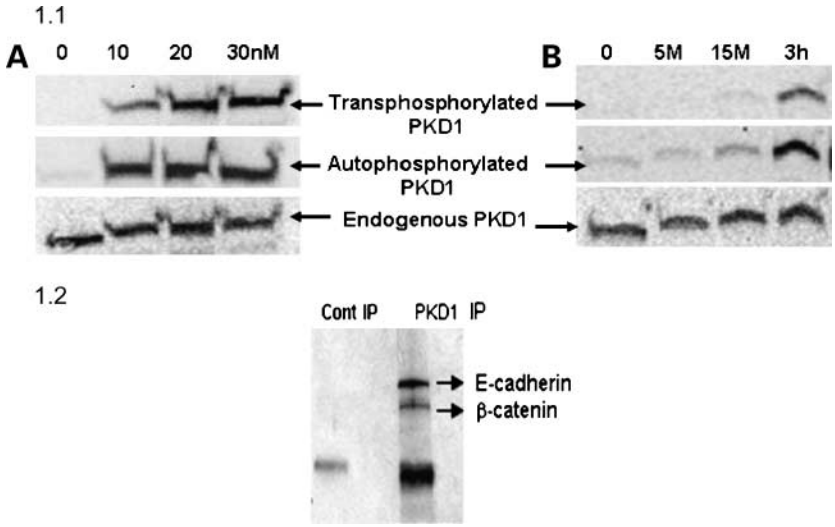

Figure 1. **1.1,** Time-dependent phosphorylation of PKD1 in Bryostatin 1-treated cells, **A,** C4-2 cells stably transfected with GFP-fused PKD1 (C4-2-PKD1-GFP) were treated with 0, 10, 20, and 30 nmol/L Bryostatin 1. Cell extracts were immunoblotted with autophosphorylation and transphosphorylation site-specific antibodies. Note optimum phosphorylation at 10 nmol/L. **B,** C4-2 -PKD1-GFP cells were treated with 10 nmol/L Bryostatin 1 for 0 to 3 h. Cell extracts were immunoblotted with autophosphorylation and transphosphorylation site-specific antibodies. Note maximum phosphorylation at 3 h. **1.2,** coimmunoprecipitation of PKD1 and β-catenin. Equal amounts of cell extract were incubated with PKD1-specific antibody or IgG (negative control) and gently mixed at 4°C for 1 h. Packed anti-rabbit IgG affinity gel (50 μL; ICN Pharmaceuticals) was added and mixing continued for 30 min. Immune complexes were washed and resolved by SDS-PAGE followed by immunoblotting with anti-mouse β-catenin and E-cadherin antibody showing coimmunoprecipitation of β-catenin and E-cadherin with PKD1 in C4-2-PKD1-GFP cells.

endoplasmic reticulum to Golgi (GS15), medial Golgi to TGN (GS27), and between Golgi compartments (GS28) and transport-specific marker antibodies (SNARE proteins). The immunofluorescence images (Fig. 4.1) of Bryostatin 1 activated PKD1-GFP and β-catenin show colocalization of cytoplasmic pool of β-catenin and PKD1 with p230 at the TGN. The p230 protein recycles between the cytosol and the vesicles of TGN membranes and is associated with a specific population of TGN-derived transport vesicles (30). The colocalization of PKD1, β-catenin, and p230 (Fig. 4.1*E, wide arrow*) after Bryostatin 1 activation suggests that PKD1 may be involved in the transport of E-cadherin/β-catenin from TGN to the plasma membrane (Fig. 4.1*E, thin arrow*). Bryostatin 1 activated PKD1-GFP and β-catenin does not colocalize with GS15 or GS27 or GS28, suggesting that PKD1 and β-catenin is not localized at endoplasmic reticulum or medial or *cis*-compartment of Golgi (Fig. 4.2).

Colocalization of PKD1, β-Catenin, and Vesicular Transport Proteins

Protein trafficking involves packing of molecules in membranous vesicles mediated by integral membrane SNARE proteins: V-SNARE (vesicle) and t-SNARE (target; ref. 31). Rab8 proteins regulate vesicular transport from TGN to basolateral plasma membranes in epithelial cells. Using confocal scanning microscopy, we analyzed the localization of several members of the SNARE family of proteins and their colocalization with PKD1 and β-catenin using specific SNARE protein antibodies (Syntaxin 6 and 11, Vtila and B, and Rab8) in Bryostatin 1 activated C4-2-PKD1-GFP cells by immunofluorescence studies. Our results show that Syntaxin 11 (Fig. 4.3) and Vtila (Fig. 4.4) colocalize with PKD1 and β-catenin, but Rab8 does not (Fig. 4.5), suggesting that PKD1 is involved in vesicular transport of β-catenin through SNARE family of proteins.

Effect of PKD1 Inhibition on β-Catenin Subcellular Localization

To further show the specific function of PKD1 in mediating the subcellular redistribution of β-catenin, PKD1 expression was inhibited 90% by using siRNA in C4-2-PKD1-GFP cells activated with Bryostatin 1 (Fig. 5.1). After inhibition of PKD1 expression, cells transfected with nontargeted siRNA were activated with Bryostatin 1, stained for β-catenin and p230 antibody, and analyzed by confocal microscopy. Nontargeted siRNA-transfected and Bryostatin 1 activated cells showed perinuclear localization of PKD1 and β-catenin. Merging of PKD1, β-catenin, and p230 images from these cells (Fig. 5.2*G*) shows colocalization of these three proteins at the perinuclear region and colocalization of PKD1 and β-catenin at the cell junction. Immunofluorescence images of PKD1 siRNA-transfected C4-2-PKD1-GFP cells (Fig. 5.2*D-F* and *H*) shows inhibition of PKD1-GFP (Fig. 5.2*E*), reduced staining of β-catenin at the membrane, and lack of β-catenin localization at TGN (Fig. 5.2*E* and *H*). Merging of PKD1, β-catenin, and p230 images taken at the same confocal level in PKD1 siRNA-transfected C4-2-PKD1-GFP cells (Fig. 5.2*H*) does not show colocalization of the proteins at the perinuclear region. These results suggest that β-catenin subcellular localization is modulated predominantly by activated PKD1 and not by other kinases (PKC isoforms) activated by Bryostatin 1. Interestingly, down-regulation of PKD1 by RNA interference

Published OnlineFirst September 2, 2008; DOI: 10.1158/1535-7163.MCT-08-0119

decreased β-catenin at plasma membrane (Fig. 5.2*E*), which further suggests that PKD1 plays a major role in membrane transport of β-catenin. We have published previously that down-regulation of PKD1 in fact increases total cellular β-catenin. This provides further corroborative evidence for role of PKD1 in membrane trafficking of β-catenin because membrane β-catenin is decreased in spite of increased total levels of cellular β-catenin when PKD1 expression is reduced (32). However, the exact mechanism of regulation of β-catenin expression by PKD1 remains to be investigated.

PKD1 Activation Decreases Nuclear β-Catenin and β-Catenin Transcriptional Activity

In addition to contributing to cell-cell adhesion, β-catenin also contributes to cellular proliferation through the Wnt signaling pathway (as a signaling molecule) by translocating to the nucleus. We studied the expression of β-catenin in the cytoplasm and nucleus of prostate cancer cells at the protein level. Western blot analysis confirmed membranous, nuclear, and cytoplasmic expression of β-catenin in prostate cancer, suggesting a role of β-catenin in the Wnt signaling pathway in addition to cell adhesion (Fig. 6.1*A*). To verify the possible role of PKD1 in modulating β-catenin nuclear expression, we analyzed β-catenin expression in nuclear extracts from PKD1-GFP cells activated with Bryostatin 1. Activation of PKD1 resulted in decreased nuclear expression of β-catenin compared with DMSO-treated control cells (Fig. 6.1*B*).

It has been shown that nuclear β-catenin forms a complex with TCF/lymphoid enhanced transcriptional factors, which transactivates downstream targets that have been implicated in cellular proliferation and tumor development (33, 34). Therefore, we investigated the effect of PKD1 activation on β-catenin-mediated transcriptional activity

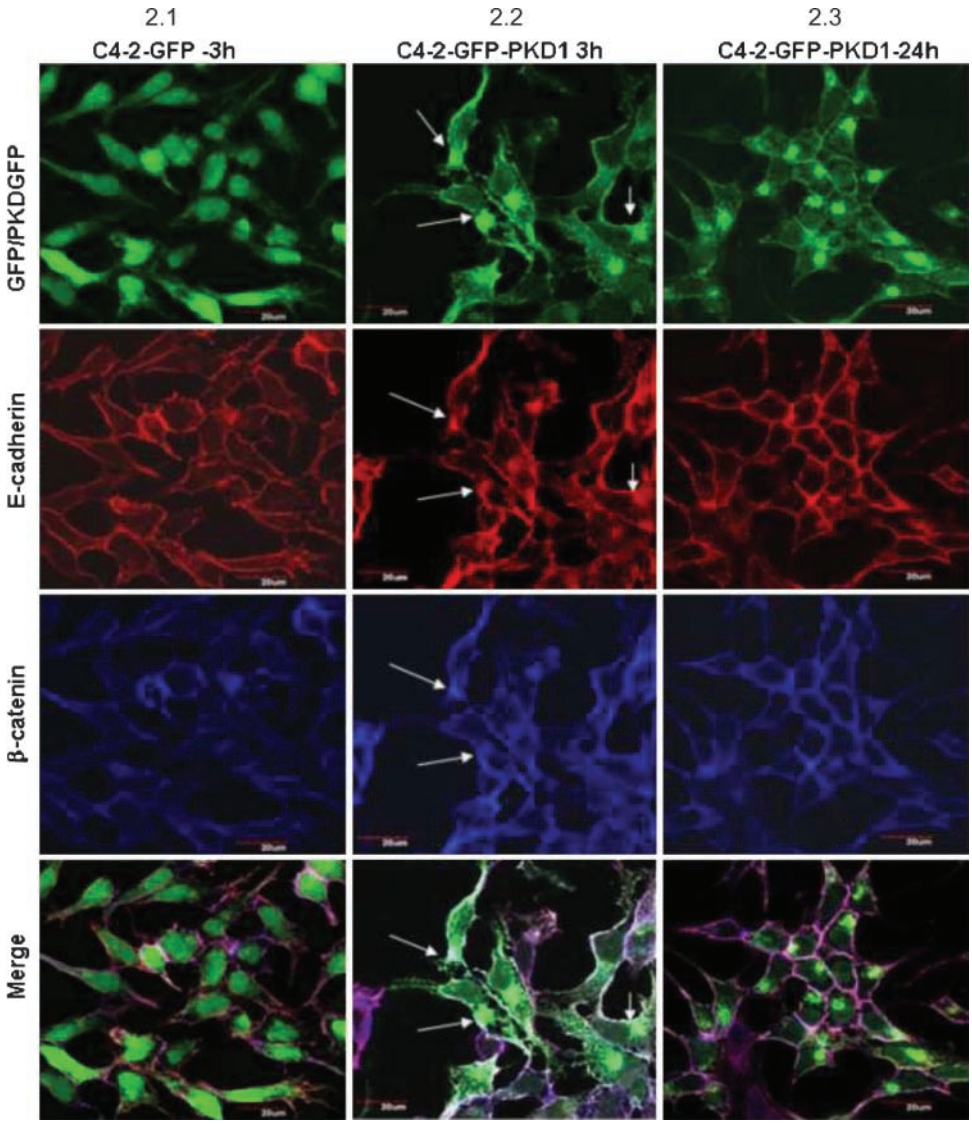

Figure 2. Activation of PKD1 by Bryostatin 1 on E-cadherin and β-catenin subcellular localization. Bryostatin 1 activated C4-2-GFP and C4-2-PKD1-GFP cells were stained for E-cadherin and β-catenin and analyzed by laser scanning microscope. C4-2-GFP (**2.1**) and C4-2-PKD1-GFP (**2.2** and **2.3**) cells show differences in subcellular localization of E-cadherin (*red*) and β-catenin (*blue*). E-cadherin and β-catenin colocalizes with PKD1-GFP at perinuclear areas in addition to cell membranes (**2.2**, *arrows*) in Bryostatin 1 activated C4-2-PKD1-GFP cells (**2.2** and **2.3**) but not in C4-2-GFP cells.

Published OnlineFirst September 2, 2008; DOI: 10.1158/1535-7163.MCT-08-0119

and proliferation in prostate cancer cells. To investigate the effect of PKD1 on β-catenin-mediated transcriptional activation of TCF, we transfected plasmids containing a wild-type TCF-binding site (TOPFlash) or a mutated site as a negative control (FOPFlash) with pRL-TK (*Renilla* luciferase) in C4-2-PKD1-GFP cells activated with Bryostatin 1 or DMSO. The firefly and *Renilla* luciferase activities were measured with the Dual-Luciferase Reporter Assay System. After normalizing firefly luciferase activity to that of *Renilla* luciferase, the FOPFlash reporter plasmid luciferase values were subtracted from the normalized values obtained with the TOPFlash reporter plasmid. Bryostatin 1 activation in C4-2-PKD1-GFP cells led to a significant reduction ($P = 0.019$) in β-catenin reporter activity (Fig. 6.2). The cell proliferation ability of Bryostatin 1 activated C4-2-PKD1-GFP cells was assayed by CellTiter-Glo. Bryostatin 1 activated C4-2-PKD1-GFP cells showed a 40% decrease in cell proliferation compared with DMSO-treated cells (Fig. 6.3).

Effect of PKD1 Activation on Cellular Aggregation

PKD1 is also known to be involved with altered cellular aggregation, which is required for a cancer cell to successfully complete the metastatic cascade (23). Because we have shown that PKD1 activation with Bryostatin 1 is involved in trafficking of β-catenin, we sought to determine the effect of Bryostatin 1 activation on cellular aggregation in C4-2 cells overexpressing PKD1. Aggregation assays were done on C4-2 cells expressing PKD1-GFP as described previously. Our experiments showed increased cellular aggregation in Bryostatin 1 treated C4-2-PKD1-GFP cells compared with vehicle only treated cells (Fig. 6.4).

Discussion

Multiple signal transduction pathways in cells tightly regulate protein function through a complex network of protein-protein interactions. PKD1 also interacts with E-cadherin, which directly binds to β-catenin in cells, and is associated with increased cellular aggregation and decreased motility (23). The interaction of PKD1 with both E-cadherin and β-catenin suggests that PKD1 may play an important role in regulating the function of the E-cadherin-catenin complex of proteins, with subsequent effects on cell

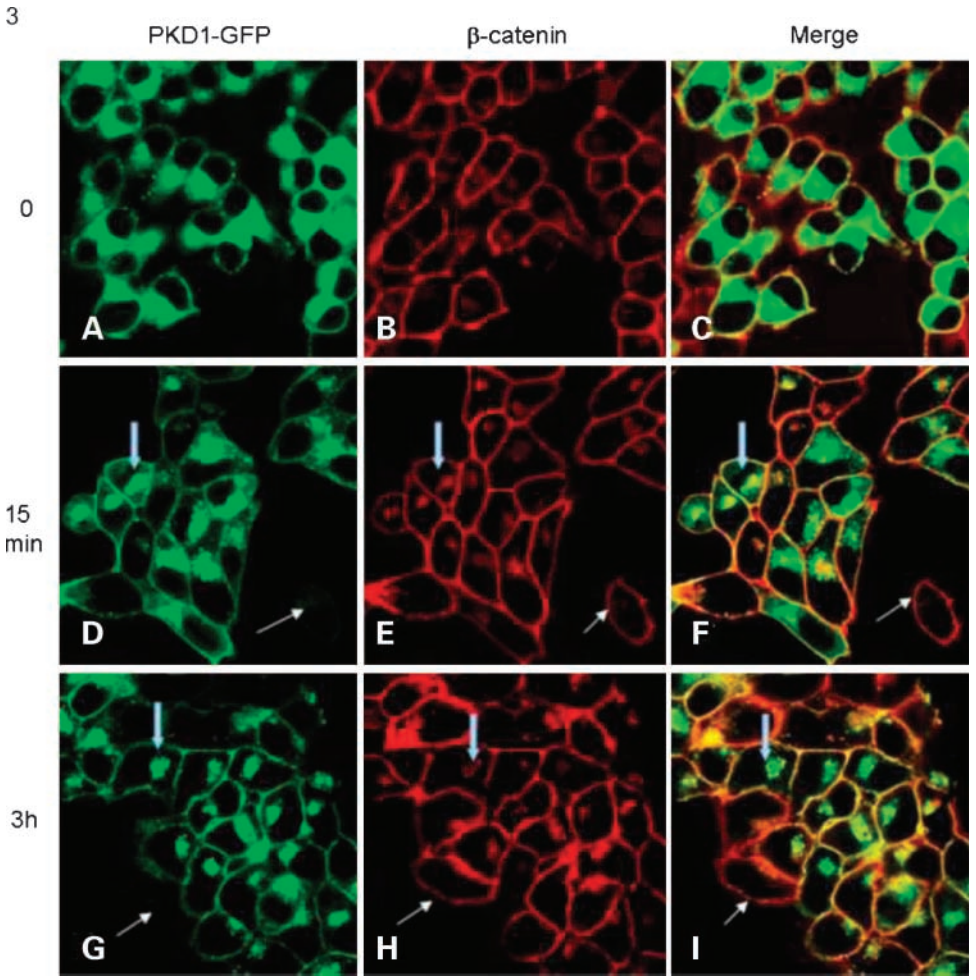

Figure 3. Effect of PKD1 activation on β-catenin subcellular distribution. Time-course experiments were carried out following stimulation of C4-2-PKD1-GFP cells by Bryostatin 1. Cells were stained for E-cadherin and β-catenin and analyzed by laser scanning microscope. Note colocalization of β-catenin and PKD1 at perinuclear area and membrane (*blue arrows*). The cells lacking PKD1-GFP (**D, F, G**, and **I**, *white arrows*) did not show strong membrane or perinuclear localization of β-catenin.

Published OnlineFirst September 2, 2008; DOI: 10.1158/1535-7163.MCT-08-0119

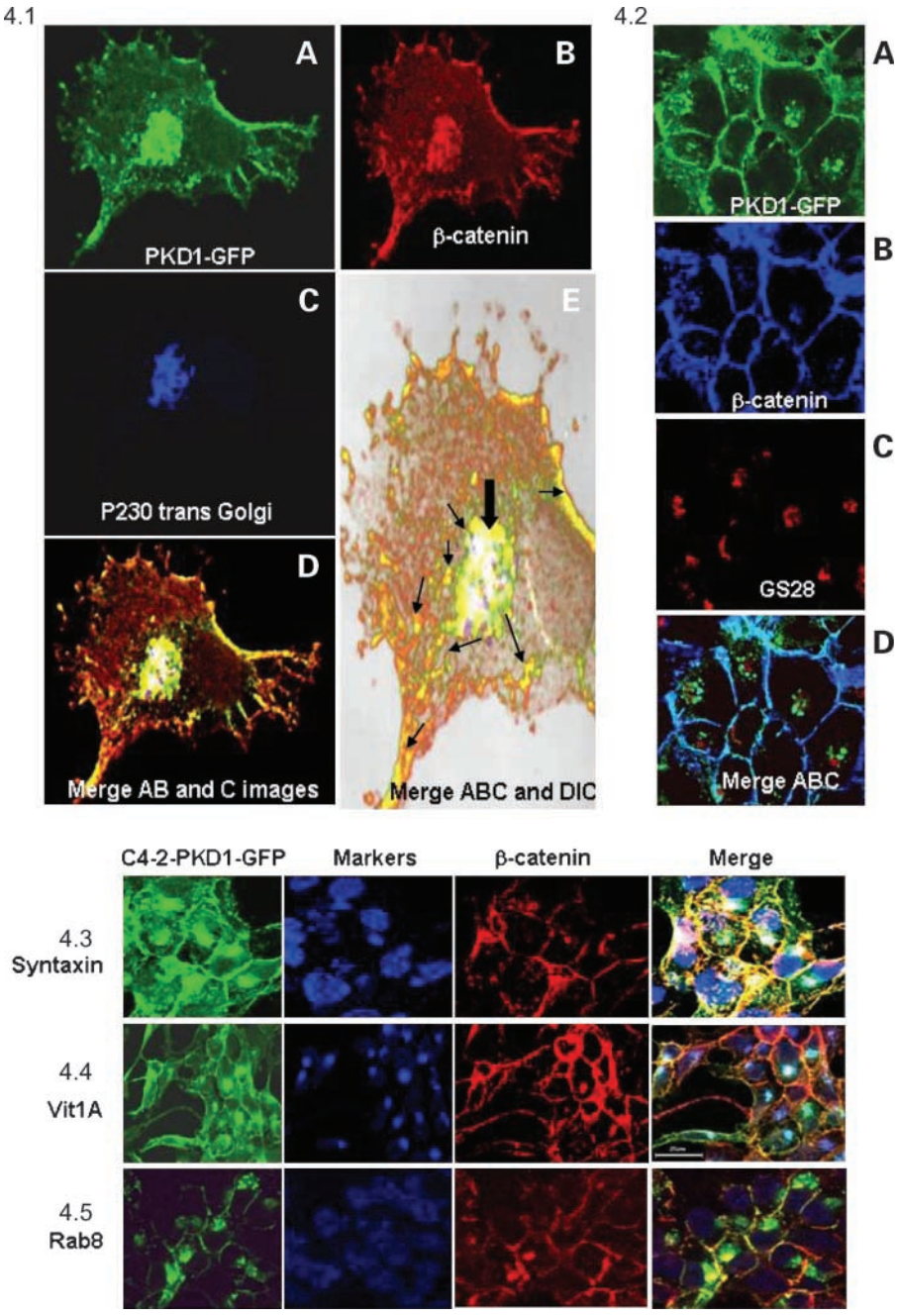

Figure 4. Immunofluorescence images of C4-2-PKD1-GFP cells at 1 h following treatment with 10 nmol/L Bryostatin showing subcellular trafficking of β-catenin: C4-2-PKD1 cells were activated by 10 nmol/L Bryostatin 1 for 1 h before fixation and antibody staining. **4.1,** using antibodies against β-catenin (*red*) and p230 TGN (*blue*). *Green,* PKD1-GFP. All three proteins colocalized at perinuclear area (**E,** *bold arrow*). PKD1 (*green*) and β-catenin (*red*) also colocalized in vesicle-like structures around TGN (*yellow dots, thin arrows*) and at cell membrane (*thin arrow*). **4.2,** using antibodies against β-catenin (*blue*) and GS28 (*red*). **4.3,** using antibodies against β-catenin (*red*) and Syntaxin 11 (*blue*). **4.4,** using antibodies against β-catenin (*red*) and Vtila (*blue*). **4.5,** using antibodies against β-catenin (*red*) and Rab8 (*blue*). β-Catenin and PKD1 are shown to colocalize with Syntaxin 11 and Vtila but not with GS28 or Rab8.

adhesion and proliferation functions. The E-cadherin-catenin complex influences cell proliferation primarily through subcellular distribution of β-catenin by altering protein stability and transport.

Molecular mechanisms involved in protein sorting and delivery are poorly understood. Localization of transmembrane proteins to plasma membrane is achieved by protein sorting in the Golgi and subsequent delivery to specific membrane domains (35). A glycosylphosphatidylinositol linkage is a known sorting signal for apical membrane proteins in MDCK cells (36–38). It has been proposed that the glycosylphosphatidylinositol anchor enables the protein to enter glycosphingolipid clusters and thereby to be sorted to the apical membrane domain (39, 40). Here, we have reported the colocalization of PKD1, β-catenin, and p230 after Bryostatin 1 activation in C4-2 cells overexpressing PKD1 in TGN, suggesting that PKD1 may be involved in the transport of E-cadherin/β-catenin from TGN to plasma membrane.

In the absence of Wnt signaling, the level of β-catenin is kept low through degradation of cytoplasmic β-catenin that is in excess of binding sites, such as cadherins at the plasma

Published OnlineFirst September 2, 2008; DOI: 10.1158/1535-7163.MCT-08-0119

membrane. β-Catenin is targeted for ubiquitination and degradation in the 26S proteasome by paired phosphorylation through the serine/threonine kinases casein kinase I and glycogen synthase kinase-3β bound to a scaffolding complex of axin and adenomatous polyposis coli protein (41). CK2 and the monomeric serine-threonine kinase glycogen synthase kinase-3 have opposing actions on β-catenin: glycogen synthase kinase-3 phosphorylation of the NH_2-terminus of β-catenin promotes degradation, whereas phosphorylation by CK2 in the armadillo repeat protein interaction domain protects it (42). There is a study showing that transcription activity of β-catenin can be inhibited by overproduction of cadherin through competitive binding with TCF (43).

In this study, we show that down-regulation of PKD1 decreases β-catenin expression and PKD1 activation with

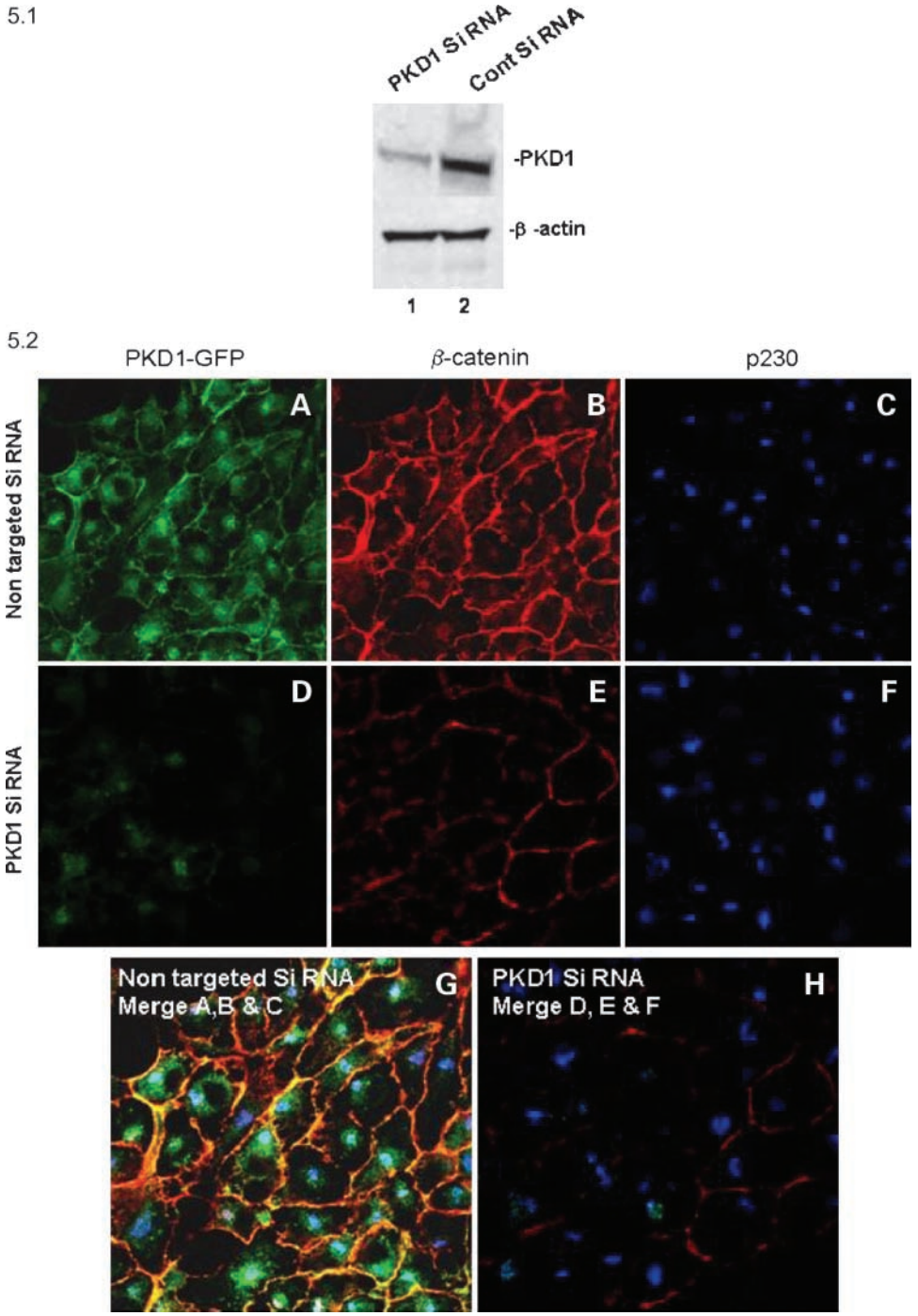

Figure 5. Inhibition of PKD1 on β-catenin subcellular localization. **5.1,** RNA interference silencing of PKD1 in C4-2-PKD1-GFP cells. Cell lysates from C4-2-PKD1-GFP cells transfected with PKD1 silencing siRNA and nontargeted siRNA were immunoblotted with PKD1 and β-actin antibodies. Western blot shows more than 90% silencing of PKD1 (*lane 1*) compared with the negative control (*lane 2*). β-Actin shows equal loading of protein in the two lanes. **5.2,** Bryostatin 1-treated C4-2-PKD1-GFP cells were transfected with a PKD1 silencing siRNA and stained for β-catenin and p230 antibodies. Confocal images taken at identical settings shows inhibition of PKD1-GFP expression (**D**) compared with control nontargeted siRNA (**A**). β-Catenin shows strong membrane localization and perinuclear localization in control siRNA-transfected cells (**B** and **D**), whereas PKD1 silenced cells showed punctuate membrane staining (**F**) and does not show perinuclear staining (**F** and **G**).

Published OnlineFirst September 2, 2008; DOI: 10.1158/1535-7163.MCT-08-0119

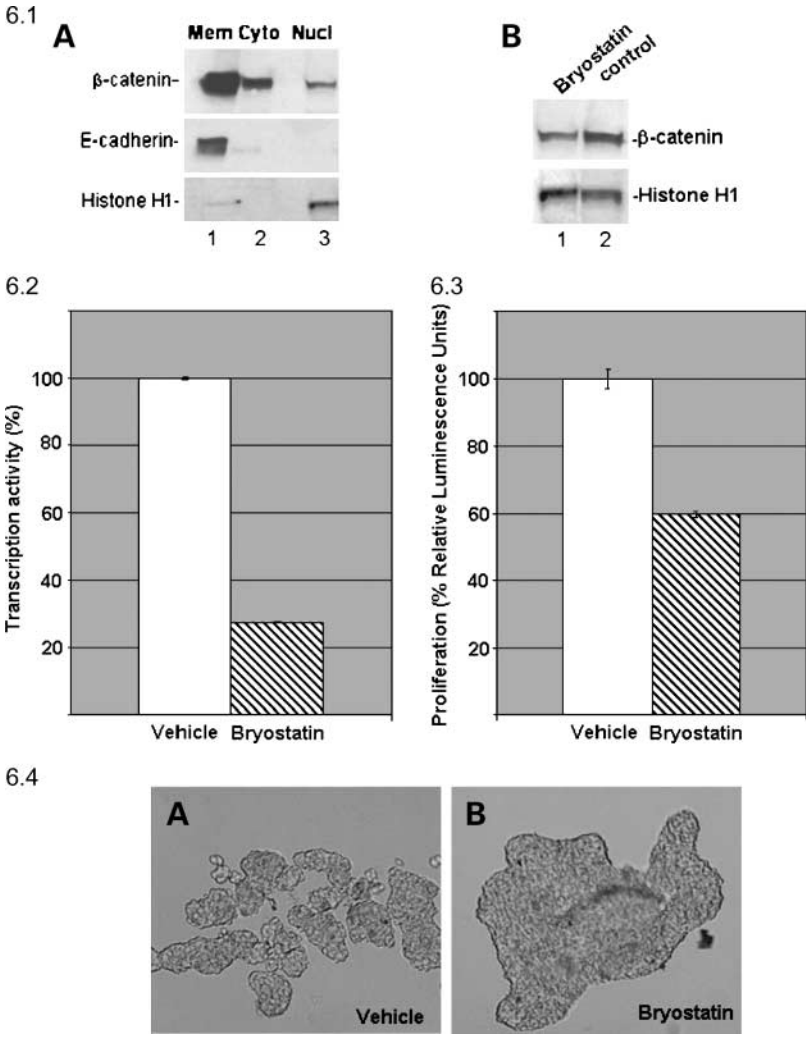

Figure 6. **6.1,** PKD1 activation decrease in nuclear β-catenin. **A,** nuclear and cytoplasmic extracts were prepared using Active Motif nuclear extraction kit. Membrane extracts were made in Tris. Proteins were resolved by SDS-PAGE followed by immunoblotting with anti-mouse β-catenin and E-cadherin antibodies. *Lane 1,* membrane extracts showing β-catenin; *lanes 2* and *3,* β-catenin protein expression in cytoplasmic and nuclear fractions, respectively. The absence of E-cadherin in cytoplasmic and nuclear extracts (*lanes 2* and *3*), presence of histone H1 in nuclear extract (*lane 3*), and absence of both E-cadherin and histone H1 in the cytoplasmic extract confirm the purity of the extracts. **B,** Western blot analysis shows that nuclear β-catenin in Bryostatin 1-treated (10 nmol/L for 1 h) C4-2 -PKD1-GFP cells decreased (*lane 1*) compared with control (*lane 2*). **6.2,** effect of PKD1 on β-catenin transcription activity. C4-2-PKD1-GFP cells were transfected with TOPFlash or FOPFlash firefly luciferase reporter constructs and treated with 10 nmol/L Bryostatin 1 or vehicle only. Data are expressed as fold induction normalized to the cotransfected *Renilla* luciferase-encoding pRL-TK plasmid. Treatment of C4-2-PKD1-GFP cells significantly decreased β-catenin-mediated transcriptional activity compared with vehicle only treated cells ($P = 0.019$). *Bars,* SE. **6.3,** proliferation assay of Bryostatin 1-treated C4-2-PKD1-GFP cells. Proliferation assay after 48 h of 10 nmol/L Bryostatin 1 treatment (see Materials and Methods) expressed as percent of control cells treated with vehicle only ($P = 0.001$). *Bars,* SE. **6.4,** increased PKD1 activity in prostate cancer cells increases cellular aggregation. Bryostatin 1-treated C4-2 -PKD1-GFP cells showed increased cellular aggregation (**B**) compared with vehicle only treated cells (**A**).

Bryostatin 1 is associated with decreased β-catenin transcriptional activity. We have published previously that β-catenin is capable of rescuing growth inhibition in prostate cancer cells caused by overexpression of PKD1 (32). Ongoing work in our laboratory show that PKD1 phosphorylates β-catenin at T112 and T120 residues and nonphosphorylated double mutants of these residues result in increased nuclear expression of β-catenin with paradoxical decrease in β-catenin/TCF transcriptional activity.[3] PKD1 seems to regulate β-catenin activity through subcellular trafficking, phosphorylation, and altered expression. Therefore, it is conceivable that antineoplastic properties of Bryostatin 1 may be mediated by altering β-catenin function by PKD1 activation, which provides for a novel target to improve efficacy of Bryostatin 1.

Although this study establishes PKD1-mediated alteration of β-catenin function as a mechanism of Bryostatin 1 action, several other effects of Bryostatin 1 can also be potentially influenced by PKD1. In several hematologic and solid tumor cell lines, Bryostatin 1 inhibits proliferation, induces differentiation, and promotes apoptosis (44), although the mechanism of Bryostatin 1 effects on cells are poorly understood. PKD1 has also been shown to play a role in several of these cellular processes raising a possibility that several effects of Bryostatin 1 on cells may involve PKD1 (1). For example, although Bryostatin 1 does not directly affect the activity of matrix metalloproteinases, it has been shown to inhibit the production of matrix metalloproteinase-1, -3, -9, -10, and -11 (45). Other studies show that PKD1 is a critical signal transducer of increased expression of matrix metalloproteinase-1, 3, and 9 by tumor necrosis factor-α (46). These data raise intriguing possibilities that PKD1 may mediate inhibitory effects of Bryostatin 1 on cell invasion by altering matrix metalloproteinase secretion and other mechanisms, which merit further investigation.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

[3] Unpublished data.

Published OnlineFirst September 2, 2008; DOI: 10.1158/1535-7163.MCT-08-0119

Acknowledgments

We thank Janice Taylor (Confocal Laser Scanning Microscope Core Facility at University of Nebraska Medical Center) and Dr. R. Moon (Washington University) for providing β-catenin mutant constructs and TCF/LIF constructs and Drs. K.R. Johnson and M.J. Wheelock (University of Nebraska) for providing E-cadherin and β-catenin antibodies and SW-480 cells. Confocal microscopy studies were also conducted at the Sanford Research/University of South Dakota Imaging Core, Cardiovascular Research Center.

References

1. Jaggi M, Du C, Zhang W, Balaji KC. Protein kinase D1: a protein of emerging translational interest. Front Biosci 2007;12:3757 – 67.

2. Johannes FJ, Prestle J, Eis S, Oberhagemann P, Pfizenmaier K. PKCμ is a novel, atypical member of the protein kinase C family. J Biol Chem 1994; 269:6140 – 8.

3. 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.

4. Hayashi A, Seki N, Hattori A, Kozuma S, Saito T. PKCν, a new member of the protein kinase C family, composes a fourth subfamily with PKCμ. Biochim Biophys Acta 1999;1450:99 – 106.

5. Waldron RT, Rozengurt E. Protein kinase C phosphorylates protein kinase D activation loop Ser^{744} and Ser^{748} and releases autoinhibition by the pleckstrin homology domain. J Biol Chem 2003;278:154 – 63.

6. Wong C, Jin ZG. Protein kinase C-dependent protein kinase D activation modulates ERK signal pathway and endothelial cell proliferation by vascular endothelial growth factor. J Biol Chem 2005;280:33262 – 9.

7. Bollag WB, Dodd ME, Shapiro BA. Protein kinase D and keratinocyte proliferation. Drug News Perspect 2004;17:117 – 26.

8. Haworth RS, Cuello F, Herron TJ, et al. Protein kinase D is a novel mediator of cardiac troponin I phosphorylation and regulates myofilament function. Circ Res 2004;95:1091 – 9.

9. Lemonnier J, Ghayor C, Guicheux J, Caverzasio J. Protein kinase C-independent activation of protein kinase D is involved in BMP-2-induced activation of stress mitogen-activated protein kinases JNK and p38 and osteoblastic cell differentiation. J Biol Chem 2004;279:259 – 64.

10. Jaggi M, Johansson SL, Baker JJ, Smith LM, Galich A, Balaji K. Aberrant expression of E-cadherin and β-catenin in human prostate cancer. Urol Oncol Semin Orig Invest 2005;23:402 – 6.

11. Hausser A, Storz P, Martens S, Link G, Toker A, Pfizenmaier K. Protein kinase D regulates vesicular transport by phosphorylating and activating phosphatidylinositol-4 kinase IIIβ at the Golgi complex. Nat Cell Biol 2005;7:880 – 6.

12. Yap AS. The morphogenetic role of cadherin cell adhesion molecules in human cancer: a thematic review. Cancer Invest 1998;16:252 – 61.

13. Nagafuchi A, Takeichi M. Cell binding function of E-cadherin is regulated by the cytoplasmic domain. EMBO J 1988;7:3679 – 84.

14. Peifer M, Polakis P. Wnt signaling in oncogenesis and embryogenesis—a look outside the nucleus. Science 2000;287:1606 – 9.

15. Nelson WJ, Nusse R. Convergence of Wnt, β-catenin, and cadherin pathways. Science 2004;303:1483 – 7.

16. Mulholland DJ, Dedhar S, Coetzee GA, Nelson CC. Interaction of nuclear receptors with the Wnt/β-catenin/Tcf signaling axis: Wnt you like to know? Endocr Rev 2005;26:898 – 915.

17. Brembeck FH, Rosario M, Birchmeier W. Balancing cell adhesion and Wnt signaling, the key role of β-catenin. Curr Opin Genet Dev 2006;16: 51 – 9.

18. Malbon CC. β-Catenin, cancer, and G proteins: not just for frizzleds anymore. Sci STKE 2005;2005:pe35.

19. Kallakury BV, Sheehan CE, Ross JS. Co-downregulation of cell adhesion proteins α- and β-catenins, p120CTN, E-cadherin, and CD44 in prostatic adenocarcinomas. Hum Pathol 2001;32:849 – 55.

20. Chesire DR, Ewing CM, Gage WR, Isaacs WB. *In vitro* evidence for complex modes of nuclear β-catenin signaling during prostate growth and tumorigenesis. Oncogene 2002;21:2679 – 94.

21. Ozawa M, Kemler R. Correct proteolytic cleavage is required for the cell adhesive function of uvomorulin. J Cell Biol 1990;111:1645 – 50.

22. Hinck L, Nathke IS, Papkoff J, Nelson WJ. Dynamics of cadherin/catenin complex formation: novel protein interactions and pathways of complex assembly. J Cell Biol 1994;125:1327 – 40.

23. 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;65:83 – 92.

24. 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.

25. Jaggi M, Rao PS, Smith DJ, Hemstreet GP, Balaji KC. Protein kinase Cμ is down-regulated in androgen-independent prostate cancer. Biochem Biophys Res Commun 2003;307:254 – 60.

26. 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.

27. Jaggi M, Rao PR, Smith DJ, Hemstreet GP, Balaji KC. Protein kinase Cμ (PKCμ) interacts with E-cadherin and β-catenin in prostate cancer *in vitro* and *in vivo*. Proc AACR 2004;45:1187.

28. Liljedahl M, Maeda Y, Colanzi A, Ayala I, Van Lint J, Malhotra V. Protein kinase D regulates the fission of cell surface destined transport carriers from the trans-Golgi network. Cell 2001;104:409 – 20.

29. Bard F, Malhotra V. The formation of TGN-to-plasma-membrane transport carriers. Annu Rev Cell Dev Biol 2006;22:439 – 55.

30. Gleeson PA, Anderson TJ, Stow JL, Griffiths G, Toh BH, Matheson F. p230 is associated with vesicles budding from the trans-Golgi network. J Cell Sci 1996;109:2811 – 21.

31. Salaun C, James DJ, Greaves J, Chamberlain LH. Plasma membrane targeting of exocytic SNARE proteins. Biochim Biophys Acta 2004;1693: 81 – 9.

32. Syed V, Mak P, Du C, Balaji KC. 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.

33. Raftopoulou M. Tumorigenesis: TCF4 feeds c-Jun. Nat Cell Biol 2005; 7:932.

34. Luu HH, Zhang R, Haydon RC, et al. Wnt/β-catenin signaling pathway as a novel cancer drug target. Curr Cancer Drug Targets 2004;4:653 – 71.

35. Chen JS, Mehta K. Tissue transglutaminase: an enzyme with a split personality. Int J Biochem Cell Biol 1999;31:817 – 36.

36. Brown DA, Crise B, Rose JK. Mechanism of membrane anchoring affects polarized expression of two proteins in MDCK cells. Science 1989; 245:1499 – 501.

37. Lisanti MP, Caras IW, Davitz MA, Rodriguez-Boulan E. A glycophospholipid membrane anchor acts as an apical targeting signal in polarized epithelial cells. J Cell Biol 1989;109:2145 – 56.

38. Nosjean O, Briolay A, Roux B. Mammalian GPI proteins: sorting, membrane residence and functions. Biochim Biophys Acta 1997;1331: 153 – 86.

39. Simons K, Wandinger-Ness A. Polarized sorting in epithelia. Cell 1990;62:207 – 10.

40. Weimbs T, Low SH, Chapin SJ, Mostov KE. Apical targeting in polarized epithelial cells: there's more afloat than rafts. Trends Cell Biol 1997;7:393 – 9.

41. Polakis P. Casein kinase 1: a Wnt'er of disconnect. Curr Biol 2002;12: R499 – 501.

42. Seldin DC, Landesman-Bollag E, Farago M, Currier N, Lou D, Dominguez I. CK2 as a positive regulator of Wnt signalling and tumourigenesis. Mol Cell Biochem 2005;274:63 – 7.

43. Fagotto F, Funayama N, Gluck U, Gumbiner BM. Binding to cadherins antagonizes the signaling activity of β-catenin during axis formation in *Xenopus*. J Cell Biol 1996;132:1105 – 14.

44. Kortmansky J, Schwartz GK. Bryostatin-1: a novel PKC inhibitor in clinical development. Cancer Invest 2003;21:924 – 36.

45. Wojtowicz-Praga SM, Dickson RB, Hawkins MJ. Matrix metalloproteinase inhibitors. Invest New Drugs 1997;15:61 – 75.

46. Alexander JP, Acott TS. Involvement of protein kinase C in TNFα regulation of trabecular matrix metalloproteinases and TIMPs. Invest Ophthalmol Vis Sci 2001;42:2831 – 8.

AACR American Association for Cancer Research

Molecular Cancer Therapeutics

Bryostatin 1 modulates β-catenin subcellular localization and transcription activity through protein kinase D1 activation

Meena Jaggi, Subhash C. Chauhan, Cheng Du, et al.

Mol Cancer Ther 2008;7:2703-2712. Published OnlineFirst September 2, 2008.