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Transformation of Mammary Epithelial Cells by 3-Phosphoinositide-dependent Protein Kinase-1 Activates β-Catenin and c-Myc, and Down-Regulates Caveolin-1[1]

Zhihui Xie, Xiao Zeng, Todd Waldman, and Robert I. Glazer[2]

Department of Oncology and Lombardi Cancer Center, Georgetown University School of Medicine, Washington, DC 20057

ABSTRACT

3-Phosphoinositide-dependent protein kinase-1 (PDK1) plays a pivotal role in coupling growth factor receptor signaling to tumor cell proliferation, survival, and invasion. Protein kinase C (PKC) α, but not Akt1, was found previously to be downstream of PDK1-mediated transformation of mammary epithelial cells. To determine the basis for its oncogenic activity, signal transduction pathways mediated by PDK1 in mammary epithelial cells were investigated. β-Catenin/T-cell factor-dependent promoter activity was markedly activated in PDK1- and PKCα-expressing cells, but not in Akt1-expressing cells, which resulted in increased levels of the β-catenin/T-cell factor target genes *c-myc* and *cyclin D1*. In contrast, caveolin-1, of which the transcription is suppressed by c-myc, was down-regulated in PDK1- and PKCα-expressing, but not in Akt1-expressing cells. Analysis of 16 breast cancer cell lines established that caveolin-1 expression was either absent or reduced compared with breast epithelial cells, and that PDK1 was elevated in all of the cell lines. Interestingly, all of the cell lines known to be invasive expressed caveolin-1 to some degree, whereas, 5 of 6 cell lines that are not invasive did not express caveolin-1. Therefore, it appears that a concomitant gain of c-myc function and a loss or reduction of caveolin-1 are major determinants of PDK1- and PKCα-mediated mammary oncogenesis.

INTRODUCTION

PDK1[3] was first identified as a protein-Ser/Thr kinase that linked phosphatidylinositol 3′-kinase to Akt activation through growth factor-mediated signaling (1, 2). PDK1 contains an NH_2-terminal catalytic domain and a COOH-terminal pleckstrin homology domain (2), and is responsible for phosphorylating AGC kinases in their activation domain, a process that is essential for their full catalytic activity (3, 4). PDK1 activates a number of AGC kinases, including Akt (5, 6), PKC (7, 8), serum- and glucocorticoid-induced kinase (9, 10), and ribosomal p70S6-kinase (5, 11), implicating PDK1 as a pivotal signaling molecule in response to growth factors and metabolic effectors.

Akt, the most extensively studied PDK1 substrate, has been implicated in human cancers by promoting proliferation and survival, and inhibiting apoptosis (12, 13). Akt1 is the predominant isoform in most tissues and is highly expressed in breast cancer cells (14), as well as in primary breast cancer (15). PKCα also plays a role in tumor proliferation and survival as demonstrated by the antitumor activity produced by an antisense cDNA and antisense oligonucleotides (16, 17), as well as its oncogenic activity in mammary epithelial cells (18).

Apart from the similarities between Akt1 and PKCα in their proliferative and antiapoptotic functions, there are also clear differences in their tumorigenic potential. Akt1 was neither transforming *in vitro* (18) nor tumorigenic when expressed in the mammary gland of transgenic mice (19–21), whereas PKCα was highly oncogenic when overexpressed in mammary epithelial cells (18). Although both Akt1 and PKCα are downstream of PDK1, only PDK1 and PKCα exhibited transformation activity (18). To define the mechanistic differences between these protein kinases, they were overexpressed in mammary epithelial cells, and downstream signaling pathways were analyzed. The results of this study define for the first time an oncogenic pathway downstream of PDK1 and PKCα that is associated with increased c-Myc and cyclin D1 expression through β-catenin activation, as well as activation of the PKCα promoter and suppression of caveolin-1 expression. These data provide a unique framework for investigating the regulatory networks associated with PDK1-mediated transformation.

MATERIALS AND METHODS

Cells and Antibodies. Mouse mammary epithelial cell line COMMA-1D (obtained from Dr. Robert Dickson, Georgetown University, Washington, DC) and COMMA-1D cells expressing Akt1, PDK1, PKCα, or vector control (18) were maintained at 37°C under 5% CO_2 in IMEM supplemented with 2.5% fetal bovine serum, 10 ng/ml epidermal growth factor, and 5 μg/ml insulin. Human breast cancer cell lines MDA-MB-157, BT20, BT549, MCF-7/ADR, MDA-MB-468, BT474, Hs578T, MCF-7/Ras, MDA-MB-231, MDA-MB-436, BT483, ZR75-1, SK-Br-3, MCF-7, MDA-MB-435, and T47-D, and human mammary epithelial cell line MCF-10A were obtained from the Lombardi Cancer Center Tissue Culture Core Facility. HCT116 cells were obtained from the American Type Culture Collection (Manassas, VA), and HAB85 cells have been described previously (22). Monoclonal antibodies to cyclin D1, c-Myc, β-actin, and rabbit anti-caveolin-1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse anti-caveolin-1 was obtained from BD Biosciences Co. (San Diego, CA). Biotinylated goat antirabbit IgG, ABC reagent, and diaminobenzidine were purchased from Vector Laboratories (Burlingame, CA).

Plasmids. Plasmids were obtained from the following sources: β-catenin/TCF luciferase (TopFlash) from Upstate Biotechnology (Lake Placid, NY), c-Myc promoter luciferase plasmid pDel-1 from Dr. Burt Volgelstein, Johns Hopkins University (Baltimore, MD), TK-*Renilla*-luciferase from Promega (Madison, WI), PKCα reporter plasmid −1571/+77 as described (23), human myc-tagged PDK1 in pCMV5 from Dr. Dario Alessi, University of Dundee (Dundee, United Kingdom), human Akt1 in pTarget as described (14), and human c-Myc in the RCAS viral vector from Dr. Yi Li, Memorial Sloan-Kettering Cancer Center (New York, NY). c-Myc was amplified by PCR and subcloned into pCR3.1-TA (Invitrogen Corp., Carlsbad, CA) using the forward and reverse primers, 5′-GCC ACC ATG CCC CTC AAC GTT and 5′-CC TTA CGC ACA AGA GTT CCG, respectively.

Western Blotting. Total cell lysates from 2×10^5 cells were prepared by lysing the washed cell pellet directly in Laemmli sample buffer and boiling for 10 min. Caveolin-1 was extracted in lysis buffer containing: 1% Triton X-100, 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 60 mM octylglucoside, and a protease inhibitor mixture (Boehringer-Mannheim Co., Indianapolis, IN) for 45 min at 4°C. Lysates were clarified by centrifugation at 13,000 × *g* for

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[2] To whom requests for reprints should be addressed, at Georgetown University School of Medicine, 3970 Reservoir Rd., NW, Research Building, Room W318, Washington, DC 20057. Phone: (202) 687-8324; Fax: (202) 687-7505; E-mail: glazerr@georgetown.edu.

[3] The abbreviations used are: PDK1, 3-phosphoinositol-dependent protein kinase-1; PKC, protein kinase C; TCF, T-cell factor; IMEM, improved minimal essential medium; GFR, growth factor receptor; IGF-1, insulin-like growth factor-1; PAGE, polyacrylamide gel electrophoresis; PH, preckstrin homologue; P13K, phosphoinositide 3-kinase; GSK, serum- and glucocorticoid-induced kinase.

[4] Z. Xie, and R. I. Glazer, unpublished observations.

15 min at 4°C, and mixed with 10× Laemmli sample buffer and boiled. Lysates were separated by SDS-PAGE in 10% polyacrylamide gels, blotted onto nitrocellulose, and analyzed by Western blotting with the antibodies described.

Immunostaining. Cells were seeded on cover slips in a six-well plate overnight, and fixed with 4% formaldehyde in PBS for 30 min after removing the medium. Fixed cells were permeabilized with 0.5% Triton X-100 in PBS for 10 min, blocked in 2% goat serum in PBS for 30 min, and washed three times in PBS. Slides were stained with rabbit anti-caveolin-1 antibody in blocking buffer for 1 h, washed three times with PBS, and incubated with secondary biotinylated goat antirabbit IgG for an additional hour. Antigen was visualized using ABC Vectastain and diaminobenzidine as substrate. Slides were counterstained with Harris-modified hematoxylin (Fisher Scientific, Pittsburgh, PA) and mounted in Permount.

Luciferase Assay. Cells were seeded in a 24-well plate at a density of 40,000 cells/well overnight. Cells were then transfected with 100 ng TopFlash, 50 ng pDel-1/c-Myc, or 50 ng p[-1571/+77]PKCα promoter, and 5 ng TK-*Renilla*-luciferase (Promega) using either Effectene (Qiagen Inc., Valencia, CA) or Lipofectamine Plus reagent (Invitrogen Corp.) according to the manufacturer's instructions. In some experiments, reporter plasmids were cotransfected with 50 ng of pTarget/Akt1, pCMV5/PDK1, pCR3.1/c-Myc, or the empty vector. Transfection efficiency was monitored by transfection with 100 ng pEGFP-C1 (BD Biosciences, Palo Alto, CA); ≥50% efficiency was observed in all of the cell lines. Luciferase activity was measured 24 h after transfection using the Luciferase Assay System (Promega), normalized to *Renilla*-luciferase activity, and is expressed as the activity relative to vector-transfected controls.

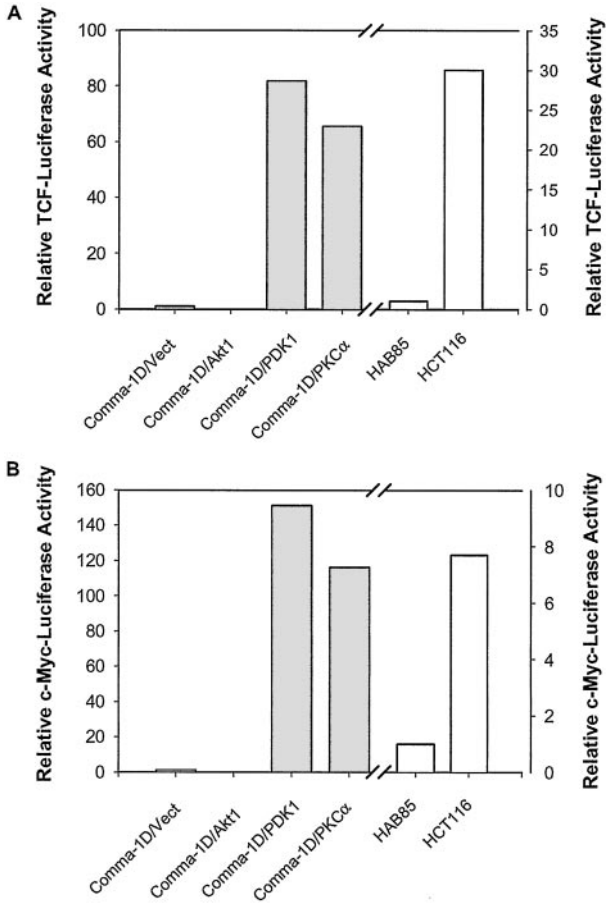

Fig. 1. β-Catenin/TCF reporter activity in PDK1- and PKCα-expressing cells. *A*, vector-, Akt1-, PDK1-, or PKCα-expressing COMMA-1D cells were transfected with a β-catenin/TCF luciferase plasmid and a *Renilla*-luciferase plasmid to correct for efficiency, and luciferase activity was measured 24 h later. Each value represents the mean of three experiments that have been normalized to *Renilla*-luciferase activity, and is expressed as activity relative to control cells; *bars,* ±SE. HAB85 cells deficient in a mutated, activated β-catenin allele and parental HCT116 cells are shown for comparison. *B*, cells were transfected with the pDel-1/c-Myc promoter luciferase plasmid, and luciferase activity was measured 24 h later. Each value represents the mean of three experiments, and is expressed as activity relative to control cells. HAB85 and HCT116 cells are shown for comparison.

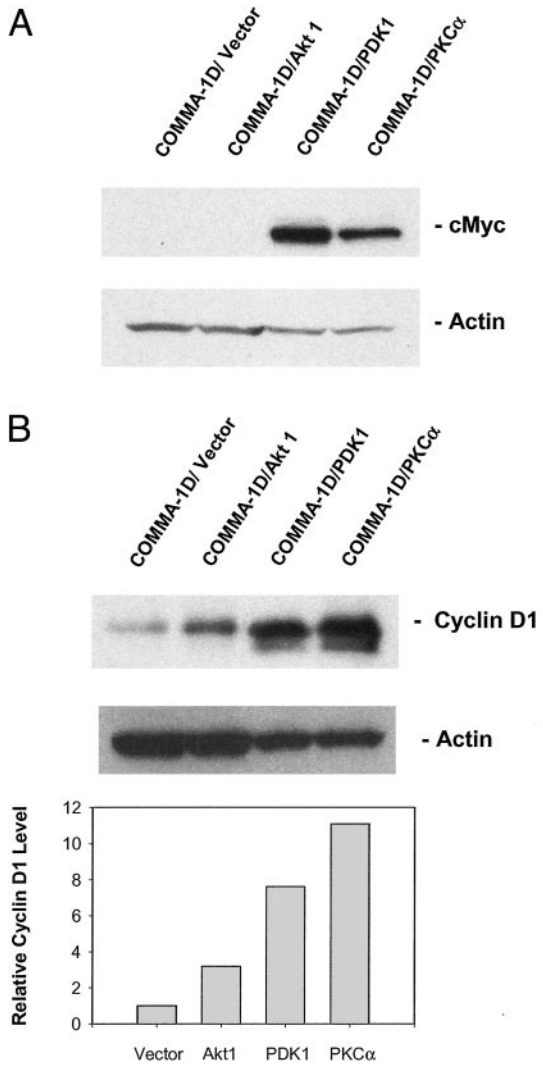

Fig. 2. c-Myc and cyclin D1 levels in PDK1- and PKCα-expressing cells. Cell lysates were separated by SDS-PAGE, transferred to nitrocellulose, and analyzed by Western blotting. *A,* c-Myc levels. Blots were probed with a c-Myc antibody, stripped, and reprobed with a β-actin antibody. *B,* cyclin D1 levels. Blots were probed with a cyclin D1 antibody, stripped, and reprobed with a β-actin antibody. *Bar graph*, relative cyclin D1 levels normalized to β-actin determined by densitometric scanning of *B*. Results are the mean of two independent experiments.

RESULTS

One oncogenic pathway suggested to be downstream of PDK1 is the β-catenin/TCF pathway. Activation of the Wnt1/β-catenin pathway in the mammary gland is highly oncogenic (24), and GSK-3β, which suppresses β-catenin function, is inhibited downstream of PDK1 (13, 25). To assess the activity of this pathway in our cell lines, cells were transfected with a TCF-dependent promoter luciferase plasmid (26). β-Catenin/TCF reporter activity was markedly increased by ~80- and 65-fold, respectively, in PDK1- and PKCα-expressing cells, but not in Akt1-expressing cells (Fig. 1*A*). HAB85 cells heterozygous for the wild-type *β-catenin* allele and deficient in the mutated, oncogenic allele (22) were included as controls for the specificity of the assay. β-Catenin/TCF-dependent activity was markedly suppressed in HAB85 cells in comparison with parental HCT116 cells containing the oncogenic allele (Fig. 1*A*).

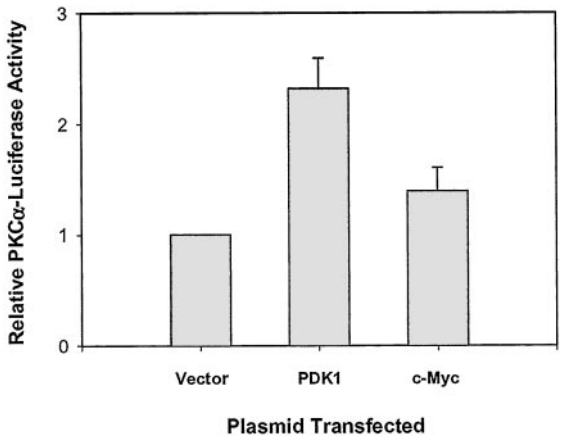

Fig. 3. PKCα promoter activity in PDK1-expressing cells. Cells were cotransfected with the p[-1571/+77]PKCα promoter luciferase plasmid, and a control, c-Myc, or PDK1 plasmid, and luciferase activity was measured 24 h later. Each value represents the mean ± SEM of three experiments and is expressed as activity relative to control cells.

The β-catenin target, c-Myc (27), which is oncogenic in the mammary gland (28), was next evaluated using a c-Myc promoter reporter gene (Fig. 1*B*). c-Myc promoter activity was markedly increased in PDK1- and PKCα-expressing cells, but not in Akt1-expressing cells; promoter dependence on β-catenin activation was again absent in HAB85 cells lacking the oncogenic β-*catenin* allele (Fig. 1*B*).

To additionally characterize the downstream effectors of the β-catenin pathway operative in PDK1- and PKCα-expressing cells, levels of the β-catenin targets, c-Myc and cyclin D1 (29), were determined. c-Myc was undetectable in control and Akt1-expressing cells, but was increased significantly in PDK1- and PKCα-expressing cells (Fig. 2*A*). Levels of cyclin D1 were also much greater in PDK1- and PKCα-expressing cells than in control or Akt1-expressing cells (Fig. 2*B*). Cyclin D2 was not detectable (4), although it has also been suggested to be the mediator of c-Myc-induced mammary tumorigenesis (30).

We found previously that PKCα was up-regulated in PDK1-expressing cells (18). To determine whether this was linked to c-Myc expression, a PKCα promoter reporter plasmid (23) that contains several c-Myc response elements was tested (Fig. 3). Although, PDK1 increased PKCα promoter activity 2.3-fold, coexpression of c-Myc did not markedly enhance promoter activity, suggesting that additional transcription factors likely contribute to regulating PKCα transcription downstream to PDK1.

To additionally delineate the role of c-Myc in PDK1-mediated oncogenesis, expression of the tumor suppressor caveolin-1 was determined. Caveolin-1 is the principal component of plasma membrane caveolae that are involved in sequestering several signal transduction effectors (31, 32). Caveolin-1 functions as a tumor suppressor gene in breast cancer (33), and its transcription is suppressed by c-Myc (34, 35). In PDK1- and PKCα-expressing cells, caveolin-1 expression was undetectable, although it remained unchanged in Akt1-expressing cells (Fig. 4*A*). Immunostaining for caveolin-1 also indicated clear differences among control, Akt1-, PKCα-, and PDK1-expressing cells, where it was undetectable in the latter two cell lines (Fig. 4*B*).

To examine the relationship between PDK1 and caveolin-1 expression, 16 breast cancer cell lines and 1 breast epithelial cell line were analyzed by Western blotting (Fig. 5*A*). Caveolin-1 was either reduced or undetectable in most cell lines, and all of the cell lines expressed 1.4–5-fold higher PDK1 levels in comparison with MCF-10A cells (Fig. 5*B*). Interestingly, 7 of 7 cell lines known to be invasive expressed caveolin-1, whereas 5 of 6 cell lines known not to be invasive did not express caveolin-1 (Fig. 5*B*). The invasive status of 3 cell lines is unknown.

DISCUSSION

PDK1 and its downstream effector, PKCα (36), were shown previously to mediate mammary epithelial cell transformation and tumorigenesis (18). Akt1, a well-characterized PDK1 substrate, did not exhibit such activity. The present study now suggests that activation

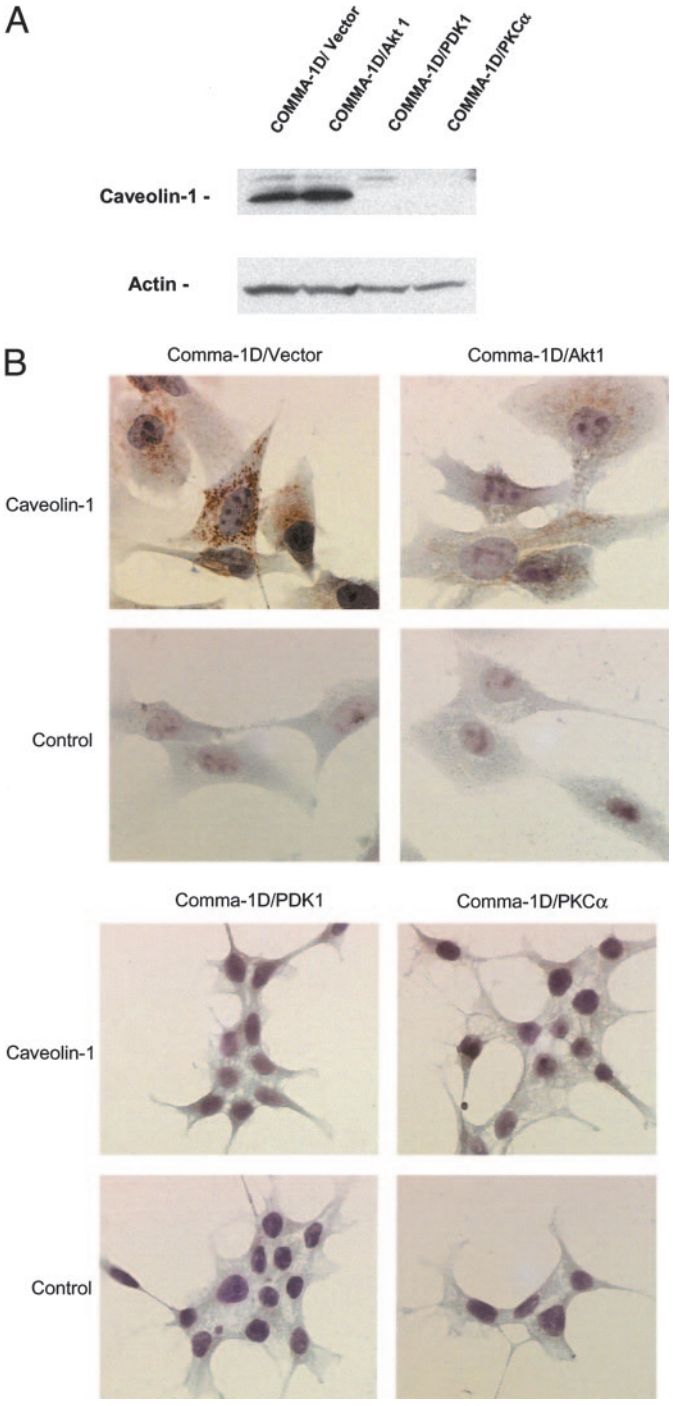

Fig. 4. Caveolin-1 expression in PDK1- and PKCα-expressing cells. *A*, cell lysates were separated by SDS-PAGE, blotted onto nitrocellulose, and probed with an anti-caveolin-1 antibody. Blots were stripped and reprobed for β-actin. *B*, cells were fixed and stained with an anti-caveolin-1 antibody, and antigen was detected by peroxidase staining. Magnification ×400.

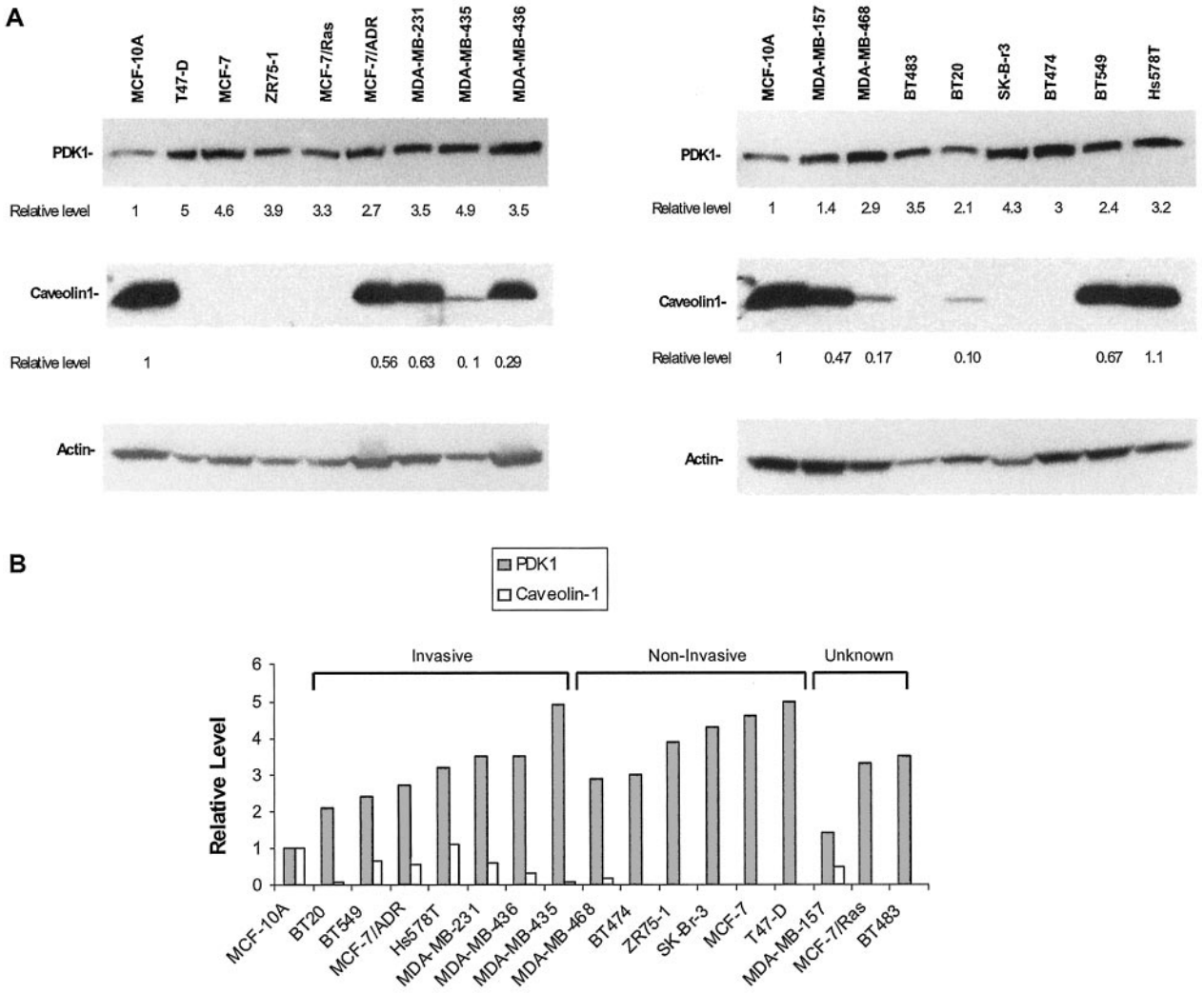

Fig. 5. Caveolin-1 and PDK1 expression in human breast cancer cell lines. *A*, cell lysates were separated by SDS-PAGE, blotted onto nitrocellulose, and probed with an anti-caveolin-1, anti-PDK1, or antiactin antibodies. The values listed below each *lane* are the level of caveolin-1 or PDK1 normalized to β-actin levels relative to MCF-10A cells. *B*, PDK1 and caveolin-1 levels relative to MCF-10A cells. Each point is based on the data in *A* where PDK1 and caveolin-1 levels in MCF-10A were set to 1.

of β-catenin/TCF and two of its target genes, c-Myc and cyclin D1, correlate with the oncogenic potential of PDK1 and PKCα in mammary epithelial cells, and that activation of this signaling axis results in the down-regulation of the tumor suppressor, caveolin-1 (Fig. 6). These changes appear to explain the lack of oncogenicity of both wild-type and constitutively active Akt1 in the mammary gland, despite its ability to block apoptosis, increase cyclin D1 levels, and cause hyperplasia (19–21). However, the lack of Akt1 oncogenicity in the mammary gland may not pertain to all of the Akt isoforms and target tissues, because expression of lymphocyte-targeted Akt2 caused lymphomas (37).

c-Myc up-regulation occurred downstream of PDK1 and PKCα, but not Akt1, and the expression of this oncogene correlated with the relative oncogenic activity of PDK1 and PKCα in mammary epithelial cells (18), and the known oncogenic activity of c-Myc in fibroblasts (38, 39) and the mammary gland (28). However, c-Myc activation did not appear to explain the up-regulation of PKCα occurring in PDK1-expressing cells, because it had only a limited effect on PKCα promoter activity (Fig. 3). This suggests that other transcription factors (23) or post-translational processes modulated by PDK1 are likely to be involved.

Activation of the TCF coactivator, β-catenin, is associated with transformation in many tissues (40, 41). This is particularly striking in the mammary gland where expression of a constitutively active β-catenin transgene resulted in rapid tumorigenesis and elevated cyclin D1 expression (42). In the present study, it is less clear how elevated cyclin D1 levels found in PDK1- and PKCα-expressing cells relate to transformation, because Akt1 also increased cyclin D1 levels, albeit to a lesser extent. Because Akt1 expression in the mammary gland was not tumorigenic (19), and the phenotype of mouse mammary tumor virus-cyclin D1 mice is not strongly tumorigenic (43), the contribution of cyclin D1 to transformation may be more quantitative than qualitative or require a secondary event such as tumor suppressor down-regulation. More than 50% of human breast cancers overexpress cyclin D1 (44, 45), and cyclin D1 is required for Neu- and Ras-mediated transformation, but not for c-Myc- and Wnt1-induced mammary tumorigenesis (30). Although, Akt1 has a key role in cell survival (13, 14, 46) and induces cyclin D1 expression in the mammary gland (19), its lack of oncogenicity (18) suggests that cyclin D1 may be necessary but insufficient for transformation through the PDK1 signaling pathway.

Although the β-catenin/c-Myc pathway activates a number of proto-oncogenes (47, 48), c-Myc also serves to down-regulate the tumor suppressor, caveolin-1, by inhibiting initiator element function in the caveolin-1 promoter (34, 35). This was readily apparent in PDK1- and PKCα-expressing cells where caveolin-1 was absent, and

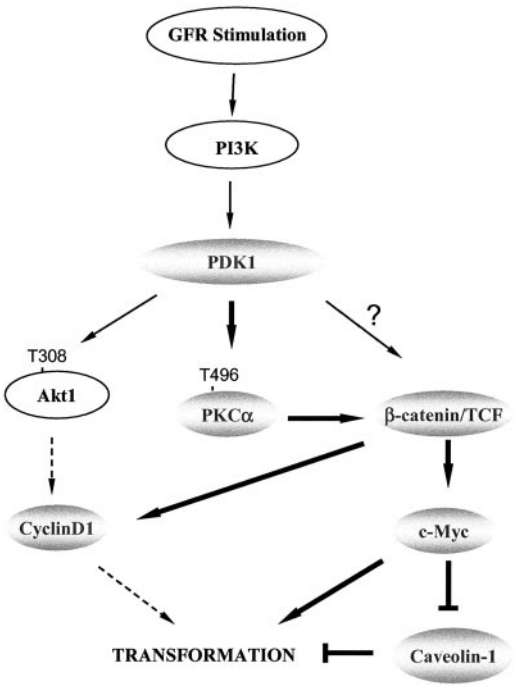

Fig. 6. PDK1 transformation pathway in mammary epithelial cells. Growth factor receptor (*GFR*) stimulation activates phosphatidylinositol 3′-kinase to generate phosphatidylinositol-3,4,5-trisphosphate, which recruits PDK1 to the plasma membrane. PDK1 phosphorylates Akt1 and PKCα at T308 and T496, respectively, to prime catalytic activity. PDK up-regulates PKCα expression and activates the β-catenin pathway; it is uncertain whether PDK1 directly activates β-catenin. β-Catenin activates cyclin D1 and c-Myc transcription, and c-Myc suppresses caveolin-1 transcription. Increased c-Myc activity and loss of caveolin-1 lead to transformation. *Solid bold arrow*, marked activation; *solid arrow*, activation; *dashed arrow*, weak activation; *blunt line*, inhibition.

correlated inversely with c-Myc and PDK1 expression. Caveolin-1 has been proposed to function as a scaffolding protein to bind and sequester signaling molecules such as PKCα, c-Src, eNOS, and G proteins in caveolar membrane rafts, where their activity is inhibited (31, 49). The suppressor effect of caveolin-1 on malignant transformation has been illustrated by its ability to reverse H-Ras- and v-Abl-induced transformation in fibroblasts (50), as well as the tumorigenicity of MCF-7 breast cancer cells (51). In the present study, cell morphology was also markedly changed in PDK1- and PKCα-expressing cells, which suggests changes in the actin cytoskeleton, such as those observed in 3T3 cells expressing an oncogenic caveolin-1 mutant (33). Thus, loss of caveolin-1 expression by PDK1 and PKCα likely plays a key role in their ability to mediate mammary epithelial cell transformation.

The low levels or absence of caveolin-1 and the high expression of PDK1 in all 16 of the breast cancer cell lines examined is of great interest, because it implies a functional link between these proteins. A tumor suppressor role for caveolin-1 is suggested by its absence in mammary tumors in mouse mammary tumor virus-c-Myc, -Neu, -Src, and -Ha-Ras transgenic mice (52), as well as the presence of a transforming P132L caveolin-1 mutation in 16% of highly aggressive scirrhous breast cancers (33). However, studies in primary prostate and pancreatic cancer indicate just the converse, *i.e.,* that positive caveolin-1 expression is associated with tumor progression and poor clinical outcome (53, 54). Our results identified a subset of breast cancer cell lines that have been characterized previously as invasive (55–57), and that express caveolin-1 and PDK1. The association of caveolin-1 mRNA expression with invasiveness in MDA-MB-435, BT549, MDA-MB-231, and Hs578T cells was also noted previously (58). The reappearance of caveolin-1 in drug-resistant MCF-7/ADR cells (59), and its association with P-glycoprotein (60), indeed suggests a role for caveolin-1 in pleiotropic drug resistance and tumor progression.

In summary, the present study demonstrates that PDK1 and PKCα, but not Akt1, results in activation of β-catenin-mediated c-Myc and cyclin D1 expression that is associated with the down-regulation of caveolin-1. The concomitant gain of c-Myc function and loss of caveolin-1 function suggest they are major determinants of PDK1- and PKCα-mediated mammary oncogenesis.

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