Transformation of Mammary Epithelial Cells by 3-Phosphoinositide-dependent Protein Kinase 1 (PDK1) Is Associated with the Induction of Protein Kinase Ca

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ABSTRACT

3-Phosphoinositide-dependent protein kinase 1 (PDK1) is a mediator of multiple signaling pathways coupled to growth factor receptor activation in human cancers. To evaluate the role of PDK1 in mammary gland oncogenesis, COMMA-1D mouse mammary epithelial cells were retrovirally transduced with PDK1, and transformation was measured by anchorage-independent growth in soft agar. PDK1-expressing cells exhibited a high degree of transformation that was associated with the activation of Akt1 and an elevation of protein kinase Ca (PKCa) expression. Cells overexpressing Akt1 did not exhibit anchorage-independent growth, whereas PKCa overexpression produced significant transformation, although to a lesser extent compared with PDK1. Coexpression of Akt1 and PKCa led to a more than additive effect on transformation activity. Isografts of either PDK1- or PKCa-expressing cells but not Akt1-expressing cells in syngeneic mice led to formation of poorly differentiated mammary carcinomas. PDK1 was highly expressed in a majority of human breast cancer cell lines. These results suggest that activation of PDK1 can lead to mammary tumorigenesis, in part through PKCa, and that PDK1 expression may be an important target in human breast cancer.

INTRODUCTION

The deregulation of protein kinases involved in modulating cell proliferation and survival is often associated with malignant transformation (1). One pathway that prominently plays a role in oncogenesis is the growth factor receptor-coupled activation of PI3-K (2) and its central effector, PDK1 (3, 4). PDK1 was originally identified on the basis of its ability to phosphorylate and activate Akt at Thr308 (5–9). Phosphatidylinositol-3,4-bisphosphate and phosphatidylinositol-3,4,5-trisphosphate, generated by PI3-K at the plasma membrane, direct membrane localization of PDK1 through its pleckstrin homology domain (10) resulting in the autophosphorylation of PDK1 at Ser241 within the activation loop (7). Activation of PDK1, in turn, effects transphosphorylation within the activation loop of several protein Ser/Thr kinase families, including AKT (5), PKC (11–15), ribosomal p90 S6 kinase, S6K (16–19), and SGK (20, 21). In addition, PDK1 activates the Rho kinase, PKN/PKC-related kinase 1, and the Rac/Cdc42 kinase, PAK1 (22–24), which are associated with increased invasion and metastasis (25). This suggests that PDK1 functions as a master control point for the activation of a number of signaling pathways involved in proliferation, survival, and invasion.

There are few studies of the functional role of PDK1 in cancer cells. Recently, it was found that antisense oligonucleotides targeting PDK1 could block the proliferation of U-87 glioblastoma cells by promoting apoptosis (26). This effect could have resulted from inhibition of one or more downstream targets of PDK1 such as Akt or PKC. The PDK1 substrates, Akt1, Akt2, and Akt3, are highly expressed in several human cancers (27), and one or more PKC isoforms are elevated in breast and colon tumors (28, 29). Akt2 and PKCα have also been shown to transform rodent fibroblasts in vitro, whereas Akt1 and Akt3 were not oncogenic (30, 31). Despite these findings, a direct functional link between PDK1 activation and processes involved in malignant transformation has not been explored.

In the present study, we demonstrate for the first time that expression of PDK1 in mouse mammary epithelial cells is sufficient to elicit transformation in vitro and tumor formation in vivo. This process was associated with activation of Akt1 and PKCa, as well as an increase in PKCa levels, and expression of PKCa but not Akt1 in mammary epithelial cells resulted in transformation in vitro and tumorigenesis in vivo. These results suggest that the PKCa signaling pathway is a major route mediating PDK1-dependent transformation.

MATERIALS AND METHODS

Cells and Antibodies. Mouse mammary epithelial cell line COMMA-1D (Ref. 32; provided by Dr. Robert Dickson, Georgetown University) was maintained at 37°C under 5% CO2 in IMEM with 1× supplement (2.5% fetal bovine serum, 10 ng/ml epidermal growth factor, and 5 μg/ml insulin). Cell pellets of human breast cancer cell lines MCF-7, ZR-75–1, T47D, MDA-MB-231, MDA-MB-436, MDA-MB-157, BT483, and SK-Br-3, and human mammary epithelial cell line MCF-10A were obtained from the Lombardi Cancer Center Tissue Culture Core Facility. Polyclonal antibodies to PDK1, Akt1, Akt1/Thr308, PKCα, pSer67/PKCα, PKCγ, SGK, S6K, and pThr412S6K, and monoclonal antibodies to the myc epitope tag (9E10) were obtained from Upstate Biotechnology, Inc (Lake Placid, NY). Monoclonal antibodies to PKβ, δ, ε, η, θ, and μ were obtained from Transduction Laboratories (Lexington, KY) and to β-actin (C-11) from Santa Cruz Biotechnology (Santa Cruz, CA).

Plasmid Construction, Virus Production, and Transduction of COMMA-1D cells. The human PDK1 cDNA with an NH2-terminal myc epitope tag was provided by Dr. Dario Alessi, University of Dundee, Dundee, United Kingdom. The mouse Akt1 cDNA was obtained from Drs. Peter Vogt and Masahiro Aoki, The Scripps Research Institute, La Jolla, CA. Rabbit PKCa cDNA was provided by Dr. Shigeo Ohno, Yokohama University, Yokohama, Japan. Akt1 and PKCa were amplified by PCR and subcloned into vectors pGAD77 and pGBK77 (Clontech, Inc.), respectively, to obtain an NH2-terminal hemaglutinin epitope tag for Akt1 and an NH2-terminal myc epitope tag for PKCa. All of the cDNAs were then cloned into the retroviral vector pSRαMSV/Neo (33). Retroviral vector pCMV/hyg was generated by replacement of the Tet on/off control element on vector pRevTRE (Clontech, Inc.) with the CMV promoter from pCMV (Invitrogen). Either pSRαMSV/Neo or pSRαMSV/hyg encoding PKCa, Akt1, or PKCα were cotransfected with the pSV-ψ-E-MLV ecotropic vector into 293T cells. After 48 h, the supernatants were collected, mixed with an equal volume of fresh IMEM medium plus 2× supplement in the presence of 4 μg/ml Polybrene, and added to COMMA-1D cells. After four rounds of infection, G418-resistant COMMA-1D cells were selected for 2 weeks. To generate cells coexpressing Akt1+PKCa, Akt1+PKD1, or PKCa+PKD1, a second round of transduction was carried out.
out using amphotrophic viruses produced in 293T cells cotransfected with pCMV/hyg-Akt1 and the pSV-ψ-A-MLV amphotropic vector. After 2 weeks of selection in 50 μg/ml hygromycin, the expression of both genes was confirmed by Western blotting.

**Western Analysis for pThr308Akt1, pSer673PKCa, and pThr412S6K.** Subconfluent cells were lysed in Buffer A containing: 50 mM Tris-HCl (pH 7.5), 0.1% (v/v) Triton X-100, 1 mM EDTA, 1 mM EGTA, 50 mM NaF, 10 mM sodium β-glycerol phosphate, 5 mM Na3VO4, 1 mM sodium orthovanadate, 0.1% (v/v) 2-mercaptoethanol, 1 μM Microcystin LR, and protease inhibitor mixture (Boehringer-Mannheim). Whole cell extracts containing 50 μg of protein were analyzed by SDS-PAGE and Western blotting. To measure IGF-I-dependent activation of Akt1 and PKCa, cells were grown to subconfluence, washed once in serum-free IMEM medium, and incubated overnight (12–14 h) in serum-free medium. Fresh serum-free medium was added with or without 100 ng/ml IGF-I, and cells were incubated for 10 min at 37°C. Stimulation was stopped by the addition of serum-free medium prechilled to 4°C. Cells were collected on ice by scraping into prechilled Buffer A. Lysates were clarified by centrifugation at 13,000 × g for 15 min. Lysates (400 μg protein) were then incubated at 4°C for 2 h with 4 μg of prewashed protein A/G-agarose (Santa Cruz Biotechnology) adsorbed with anti-Akt1, anti-PKCa, or anti-S6K antibodies. After washing four times in Buffer A, bound proteins were eluted directly into Laemmli sample buffer by boiling for 5 min. The eluted proteins were then analyzed by Western blotting using the antibodies specific for pThr308Akt1, pSer673PKCa, and pThr412S6K.

**PDK1 Kinase Assay.** Immunoprecipitation was carried out as described above with 4 μg of anti-myc IgG and 400 μg protein of whole cell lysate. PDK1 activity was measured with the PDK1 Kinase Assay kit (Upstate Biotechnologies, Inc.) according to the manufacturer’s instructions. This assay measures PDK1-dependent phosphorylation and activation of recombinant SGK and the subsequent incorporation of [γ-32P]ATP into a basic peptide substrate.

**Soft Agar Assay for Anchorage-independent Growth.** After eight passages of the retrovirally transduced cells, exponentially growing cells in 1% agarose/1% methylcellulose were suspended in 2 ml 0.33% (w/v) SeaPlaque agar (BMA, Rockland, ME) dissolved in IMEM containing 1× supplement and layered over 1% agar dissolved in IMEM containing 1× supplement in six-well plates. Cultures (in triplicate for each condition) were fed with fresh medium once a week. Colonies >50 cells were counted visually at the end of 3 weeks.

**Isograft Transplantation into Syngeneic Mice.** COMMA-1D cells were trypsinized, washed, and resuspended in serum-free IMEM medium at a concentration of 106 cells/ml. Aliquots of 10 μl were injected into the cleared inguinal mammary fat pad of 3 week-old female BALB/c mice (34). Eight weeks after transplantation, mice were sacrificed, and isografts were fixed in 10% formalin in 1× PBS, embedded in paraffin, and stained with H&E by the Histopathology Core Facility, Lombardi Cancer Center. In instances where COMMA-1D/PDK1 and COMMA-1D/PKCa cells produced adenocarcinomas, part of the tumor was removed aseptically and mechanically dispersed with a 28-gauge needle in 1× PBS. The suspension was centrifuged and re plated in IMEM containing 1× supplement, 600 μg/ml G418 (Life Technologies, Inc.), and 50 μg/ml gentamicin. After four passages, cells were collected and cell extracts prepared in Buffer A for Western blotting with the myc-tag antibody. Histopathology was assessed by Dr. Baljit Singh, Histopathology Core Facility, Lombardi Cancer Center.

### RESULTS

To evaluate the role of PDK1 in mammary epithelial cell transformation, mouse mammary epithelial cell line COMMA-1D was retrovirally transduced with PDK1 (Fig. 1). Cells expressing PDK1 exhibited a high degree of anchorage-independent growth in soft agar, a feature that is considered indicative of transformation.

To determine whether the activity of downstream substrates of PDK1 were changed, immunocomplex kinase assays were carried out (Fig. 2). Cells expressing PDK1 exhibited higher Akt1 activity as shown by enhanced phosphorylation of Thr308 (Fig. 2A). The activity of PKCa, as measured by autophosphorylation of Ser673, was increased almost 7-fold in response to IGF-I stimulation (Fig. 2B), but the phosphorylation of another PDK1 substrate, S6K, was not changed (Fig. 2C).

To determine whether PDK1 altered the levels of PKCa or other PKC isoforms, cell lysates were analyzed by Western blotting (Fig. 3). The steady state level of PKCa was increased 6-fold, whereas other PKC isoforms remained unchanged (Fig. 3). PKCδ and θ were absent in these cell lines. We also noted that the steady state levels of Akt1, S6K, and SGK were not changed in COMMA-1D/PDK1 cells (Fig. 3).

Because COMMA-1D/PDK1 cells expressed greater Akt1 and PKCa activity, transformation was measured in COMMA-1D cells transduced with either Akt1 or PKCa (Fig. 4). In contrast to Akt1-expressing cells, which did not exhibit anchorage-independent growth in soft agar, PKCa-expressing cells showed significant growth, although less than PDK1-expressing cells (Fig. 4A). These results suggested that a portion of the transforming ability of PDK1 might be caused by the up-regulation of PKCa. When cells expressing PKCa were cotransduced with Akt1, cell transformation was increased by 20% in comparison to cells expressing PKCa alone (Fig. 4B), indicating a small degree of potentiation between the Akt1 and PKCa signaling pathways. When either Akt1 or PKCa was coexpressed with PDK1, no additional change in transformation occurred (Fig. 4C).

To determine the tumorigenicity of the various COMMA-1D cell lines, isografts of each cell line were transplanted into the cleared fat pad of syngeneic BALB/c mice, and the mammary gland was examined 8 weeks later (Fig. 5). Mice receiving isografts of COMMA-1D cells retrovirally transduced with either neo (Fig. 5A, Ctl) or Akt1 (Fig. 5A, Akt1) exhibited a normal ductal morphology. In contrast, all mice receiving isografts of COMMA-1D/PDK1 (Fig. 5A, PDK1) or COMMA-1D/PKCa (Fig. 5A, PKCa) cells presented with poorly differentiated mammary adenocarcinomas. PDK1-derived tumors exhibited an acinar morphology with an absence of glandular structure and were highly invasive to the musculature, as well as highly vascular (results not shown). PKCa-derived tumors were more focal,
not as invasive, and exhibited a squamous morphology. To ascertain the origin of the tumors, a portion of the tumor was removed and cultured in vitro in the presence of G418. PKCα and PDK1 expression in the respective COMMA-1D cell lines used for the isografts were comparable with their levels expressed in the cell lines derived from these tumors (Fig. 5B, C).

PDK1 expression was also evaluated in several human breast cancer cell lines by Western blotting (Fig. 6). In all instances, PDK1 levels were greater in the cancer cells compared with untransformed human mammary epithelial cell line MCF-10A. It was also noted that all of the cell lines expressed variable levels of a NH₂-terminally truncated PDK1.

**DISCUSSION**

The purpose of this study was to determine whether activation of the PDK1 signaling pathway resulted in the transformation of mouse mammary epithelial cells. Our results indicate that wild-type PDK1 was highly transforming in mammary epithelial cells both in cell culture and as isografts in syngeneic mice. Although PDK1 expression by retroviral transduction was high by physiological standards, our findings that some human breast cancer cells also contain very high levels of PDK1 suggest that perhaps this signaling pathway is relevant to the malignant phenotype. Because PDK1 controls the activation of several protein kinase families, we wished to determine whether its known activation of Akt1 and PKCα (14, 15) was associated with its ability to produce transformation. The finding that PDK1 increased the steady state level of PKCα is consistent with the findings that PKCα is down-regulated in embryonic stem cells null for PDK1 (35). Whether PDK1 modulates PKC expression transcriptionally or enhances post-translational stability (35) remains to be established; however, we have noted that the human PKCα promoter (36) is activated in PDK1-expressing cells, which may contribute to enhanced PKCα expression in this cell line. PKCα can regulate PKCα activity directly through phosphorylation of its activation loop at Thr497 as well as its COOH terminus to allow autophosphorylation at Ser657 (11, 37, 38), and this is also consistent with our results in COMMA-1D/PDK1 cells. The present study also indicates that PKCα is oncogenic in its own right. Although, PKCα has not been found previously to transform mouse and rat fibroblasts in vitro or to be tumorigenic in xenografts of these cell lines (39, 40), ectopic expression of PKCα in either human mammary epithelial cell line MCF-10A (41) or mouse 3T3 fibroblasts (42) has been shown to increase anchorage-independent growth and motility that were reminiscent of a transformed phenotype (39, 41, 42).

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**Fig. 4.** Akt1 enhances PKCα-mediated transformation of COMMA-1D cells. A, COMMA-1D cells were retrovirally transduced with neo (Ctl), Akt1 (Akt1), PKCα (PKCα), or PDK1 (PDK1). Colony formation in soft agar was determined as the number of colonies per 10,000 cells plated. Each value is the mean of three determinations; bars, ± SD. B, a second round of retroviral transduction was carried out in COMMA-1D/PKC cells with either hyg (PKCα) or Akt1 (PKCα)-Akt1, or in COMMA-1D/PDK1 cells with hyg (PKD1), Akt1 (PKD1-Akt1), or PKCα (PKD1+PKCα). Colony formation in soft agar was determined as in A. Each value is the mean of three experiments; bars, ± SD. C, Western analysis of PKCα, PDK1, and Akt1 expression in the cell lines shown in B.
Alternative signaling pathways other than those directly associated with PDK1 may also be involved in the regulation of PKCa and Akt1. Ha-Ras and polyoma middle T antigen activate PI3-K (43–45), and induce and activate PKCa (29, 46), as well as Akt1 activity (47, 48). Because PKCa is also an upstream activator of the Ras/Raf pathway (49), PKCa could conceivably regulate its own expression through the Ras pathway by a positive feedback loop. Coexpression of activated Ras and constitutively active Akt1 have also been shown to act synergistically to induce gliomagenesis in vivo (50). Because PKCa can directly stimulate Akt1 phosphorylation at Ser473 (51, 52), this could account for the small degree of potentiation of PKCa-mediated transformation by Akt1.

One or more Akt isoforms highly expressed in human breast cancer cells are coupled to estrogen receptor and growth factor receptor activation (53–57). However, none of the Akt isoforms have shown oncogenicity in chick embryo and mouse 3T3 fibroblasts (30, 58–60), and mouse mammary epithelial cells (25), with the exception of constitutively activated forms of Akt (30, 59, 60). Moreover, mammary gland-directed expression of either constitutively active or wild-type Akt1 in transgenic animals has failed to induce a malignant phenotype (61–63), suggesting that additional factors or signaling pathways are required for transformation. The latter findings are consistent with the present study indicating that overexpression of Akt1 alone does not suffice to induce transformation or tumorigenesis.

The invasive characteristics of the tumors formed from allografts of COMMA-1D/PDK1 cells agree with our earlier finding that Akt1-expressing mammary epithelial cells exhibited increased extracellular matrix invasion through induction of MMP-2 (25). The acquisition of an invasive phenotype is also in accord with the findings that PKCa expression in MCF-7 breast cancer cells leads to increased anchorage-independent growth in soft agar (64), as well as increased motility and adhesion in human MCF-10A breast epithelial cells (41). Interestingly, isografts of COMMA-1D cells overexpressing v-Ha-Ras showed an undifferentiated morphology (65) that was very similar to the tumors arising from isografts of COMMA-1D/PDK1 cells in the present study.

In summary, PDK1 expression in mouse mammary epithelial cells caused an increase in anchorage-independent growth in soft agar and

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**Fig. 5.** Neoplastic transformation in isografts of COMMA-1D/PDK1 and COMMA-1D/PKCa cells. A, H&E-stained paraffin-embedded tissue obtained from isografts of COMMA-1D cells retrovirally transduced with neo (Ctl), Akt1 (Akt1), PKCa (PKCa), or PDK1 (PDK1). A representative section is shown for each tissue. Tumors derived from COMMA-1D/PKCa cells show an absence of glandular structure and a squamous morphology. Tumors derived from COMMA-1D/PDK1 cells show an acinar pattern with an absence of glandular structure. Magnification: ×100. B, Western analysis of PKCa expression in COMMA-1D/PKCa cells (Lane 1) and in a cell line derived from its respective tumor (Lane 2), and maintained in tissue culture. PKCa was analyzed by Western blotting for the myc tag. C, Western analysis of PDK1 expression in COMMA-1D/PDK1 cells (Lane 1), and in a cell line derived from its respective tumor (Lane 2) and maintained in tissue culture. PDK1 was analyzed by Western blotting for the myc tag.

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**Fig. 6.** PDK1 expression in human breast cancer cell lines. PDK1 levels in human breast cancer cell lines. Cell lysates were prepared, and Western blotting was carried out as described in "Materials and Methods." The relative level of PDK1 versus MCF-10A cells is denoted at the bottom of the figure.
malignant transformation in vivo. Although, PDK1 up-regulated PKCε expression and Akt1 activity, only expression of PDKCα was associated with transformation and tumor formation. These results suggest that the PDKCα signaling pathway is a major component of the PDK1 oncogenic pathway.

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