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Award Number: DAMD17-99-1-9195

TITLE: The Role of AKT1 in Mammary Tumorigenesis and Transformation

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REPORT DATE: September 2002

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

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20030519 020

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	September 2002	Final (1 Sep 99 - 31 Aug 02)

4. TITLE AND SUBTITLE
The Role of AKT1 in Mammary Tumorigenesis and Transformation

5. FUNDING NUMBERS
DAMD17-99-1-9195

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
report contains color

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 Words)*

AKT1 belongs to the AKT/PKB protein-serine kinase family and is the cellular homolog of the v-akt oncogene. Gene amplification and overexpression of AKT are associated with adenocarcinomas of the stomach, breast, ovary and pancreas. AKT1 is coupled to growth factor-dependent proliferation and resistance to apoptosis, but these processes have not been studied in either mammary epithelial cells or in breast cancer. The broad objectives of this proposal are to determine the role of the AKT1 in the proliferation, survival and transformation of mammary epithelial cells using two transgenic mouse strains in which expression of either AKT1 or a constitutively active form of AKT1 is directed to the mammary gland under the control of a mammary gland-specific promoter. The use of these two transgenic models will allow determination of the role of AKT1 in mammary gland hyperplasia, dysplasia and tumorigenesis, and will address the hypothesis that AKT1 is involved in the growth, survival and transformation of mammary epithelial cells. This hypothesis will be tested using two experimental approaches. **First**, transgenic mice will be generated with mammary gland-directed expression of wild-type AKT1 or constitutively active myristoylated AKT1 (myrAKT1) to determine their effects on mammary hyperplasia and tumorigenesis. **Second**, primary mouse mammary epithelial cells will be transduced with ecotropic retroviruses expressing either AKT1 or myrAKT1 to determine their impact on growth factor-dependent proliferation, susceptibility to apoptosis induced by serum deprivation or the PI3K inhibitor, LY294002, as well as transformation determined by anchorage-independent growth in soft agar and tumor formation in nude mice. Gene arrays or suppression subtractive hybridization will be used to identify genes modulated by AKT1 that are involved in its anti-apoptotic and proliferative activities. These studies will determine the role of the potentially oncogenic *AKT1* gene that is linked to growth factor activation and inhibition of apoptosis, on the etiology of mammary gland hyperplasia, dysplasia and transformation, and provide a rationale for the development of antiproliferative and chemopreventive therapies for breast cancer.

14. SUBJECT TERMS
breast cancer

15. NUMBER OF PAGES
33

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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3) Zeng, X., Xu, H. and Glazer, R.I. Transformation of mammary epithelial cells by 3-phosphoinositide-dependent protein kinase-1 (PDK1) is associated with the induction of protein kinase Cα. Cancer Res. 62:3538-3543, 2002.

INTRODUCTION:

AKT1 (protein kinase B, PKB) is the human cellular protein-serine/threonine kinase homolog of the v-akt oncogene (1, 2) that was rescued from the transforming AKT-8 retrovirus responsible for spontaneous lymphomas in the AKR mouse (3). AKT exists as three closely related subtypes, AKT1/PKBα , AKT2/PKBβ and AKT3/PKBγ that have 70% homology to the protein kinase C catalytic domain (4). AKT1 is 480 amino acids and encodes a 56 kDa protein that shares 98% homology with murine akt1 and 89% homology with other AKT isoforms. AKT1 maps to chromosome 14q32, the same locus that is rearranged in T-cell lymphomas and leukemias and ataxia telangiectasia (5). AKT2 maps to chromosome 19q13.1-13.2, the region amplified in ovarian carcinoma (6), and AKT3 maps to chromosome 1q43-q44 (7). Gene amplification or overexpression of one or more isoforms of AKT has been noted in human primary tumors of the breast, prostate, ovary and brain (8), where AKT activation is coupled to growth factor-dependent proliferation and resistance to apoptosis (9-12). Growth factors such as IGF-I, PDGF-B, EGF and bFGF as well as the v-Ha-Ras and v-src oncogenes activate AKT1 (13, 14); however, not all growth factor signaling pathways affect AKT1 equally since dominant-negative Ha-Ras abolishes bFGF-dependent AKT1 activation, but partially blocks the effects of PDGF and EGF (15). In addition, Ha-Ras and R-Ras, but not c-Raf, Rho, Rac and Cdc42 stimulate AKT1 activity (16). Growth factor-coupled signaling through phosphatidylinositol 3-kinase (PI3-K) results in AKT activation (17), and PI3-K inhibitors such as wortmannin and LY294002 block this effect (18, 19). Mammary gland oncogenes such as polyoma middle-T antigen, ErbB-2 and a constitutively active EGFR, activate PI3-K (20-22), and hence, maintain AKT in an activated state (23). This also occurs *in vivo* in the mammary gland of mice expressing a constitutively active AKT1 transgene (24-26), as well as in PTEN knockout transgenic mice expressing the Wnt-1 transgene (27).

Cellular stresses such as heat shock and oxygen radicals activate AKT (28) linking it to a survival pathway that is similar to the apoptotic stress response to toxins and anticancer drugs (29). v-Akt protects cells against apoptosis induced by IL-3 withdrawal, etoposide or paclitaxel (30), and overexpression of AKT1 or v-akt prevents apoptosis induced by serum deprivation or wortmannin (9, 31). AKT1 prevents apoptosis mediated by MAP kinase p38 and caspase-3 inhibition (32), and v-akt protects cells from apoptosis (anoikis) induced by detachment from the extracellular matrix (33). AKT1 activation is associated with integrin-mediated adhesion in platelets and fibroblasts (34), but not with membrane ruffling or lamellopodia formation (15).

AKT is involved in the post-translational regulation of many proteins through the ubiquitination/proteasome pathway. GSK-3 is a substrate and downstream effector of AKT (35) and is inactivated upon phosphorylation. Inhibition of GSK-3 leads to greater resistance to proteasomal degradation for β-catenin, which enhances tumor cell growth and invasion (36, 37) . GSK3 also inhibits c-jun, and therefore, GSK3 inhibition by AKT would be expected to increase transcription factor AP-1 activity (38) leading to activation of matrix metalloproteases (MMPs) (39). The seminal finding that AKT1 and v-akt activate matrix metalloproteinase-2 (MMP-2) in mammary epithelial cells by increasing its stability to proteosomal degradation provides a basis for extracellular matrix invasion (40).

The purpose of this proposal was to investigate the role of AKT in mammary tumorigenesis and transformation by the generation of two transgenic mouse models, as well as in such processes as transformation and invasion, where its role has not been well-defined.

BODY:

Statement of Work:

Year 1: [note Tasks 3-8 were incorrectly numbered Task 4-9]

1. Backcross AKT1 mice to develop pure transgenic strains. Determine histopathological changes and mammary tumor formation in nulliparous and multiparous AKT1 mice. Characterize mammary gland expression of AKT1 by Southern, PCR, northern, RT-PCR, western and *in situ* hybridization assays.

2. Begin pronucleus injections with the MMTV/Gag-akt1 plasmid and generate founder mice.

3. Evaluate Gag-akt1 founder mice for mammary gland expression of Gag-akt1 by Southern, PCR, northern, RT-PCR, western and *in situ* hybridization assays. Characterize mammary gland morphology and histopathology in progeny from Gag-akt1 mice.

4. Prepare AKT1, AKT1K179E and Gag-akt1 retroviruses.

5. Transduce non-transgenic primary mouse mammary epithelial cells with AKT1 and Gag-akt1 retroviruses and determine their proliferative response to IGF-I, PDGF-B, EGF and b-FGF.

6. Test AKT1- and Gag-akt1-transduced primary epithelial cells from non-transgenic mice for their apoptotic response to the PI3K inhibitor, LY294002, by measuring DNA fragmentation by *in situ* end-labeling.

7. Test primary mammary epithelial cells from AKT1 mice for their proliferative response to IGF-I, PDGF-B, EGF and b-FGF.

8. Test primary mammary epithelial cells from AKT1 mice for their apoptotic response to the PI3K inhibitor, LY294002, and to the dominant-negative AKT1K179E, by measuring DNA fragmentation by *in situ* end-labeling.

Results:

1. Characterization of MMTV-AKT1 transgenic mouse was completed and the results published (see Appendix for reprint) (26). These mice did not exhibit mammary tumors over their lifespan, but did show marked mammary gland hyperplasia and suppression of mammary gland involution following cessation of lactation, indicating a block in mammary epithelial cell apoptosis. This was associated with elevation of cyclin D1 and phosphorylation of the proapoptotic protein, BAD, which results in its inactivation (26).
2. Pronuclear injections to generate MMTV-Gag-akt1 mice were completed.
3. Generation of MMTV-Gag-akt1 mice was not completed since founder MMTV-Gag-akt1 mice were unable to be produced, suggesting that this transgene was embryonic lethal. As an alternative approach, the ovine β-lactoglobulin (BLG) promoter (provided by Dr. Bruce Whitelaw, Roslin Institute, Edinburgh, Scotland) was used to express a constitutively active myristoylated form of AKT1. The BLG promoter is strongly activated in alveolar secretory cells only during late pregnancy and lactation. Founder mice expressing a constitutively active AKT1 gene under the control of the BLG promoter were produced and two founder lines are being characterized for mammary gland morphology and transgene expression by western blotting and IHC. Since the BLG promoter is only activate during late pregnancy and lactation, characterization can only be done in multiparous mice, thereby necessitating a considerably longer time for analysis.
4. Preparation of Akt1, Akt1K179E and Gag-Akt1 retroviruses was completed.
5. Mouse mammary epithelial cell lineCOMMA-1D derived from mammary epithelial cells from Balb/c mice was transduced with retroviruses expressing AKT1, AKT179E and Gag-akt1 to complete this task. This cell line, rather than primary epithelial cells, was used for consistency and reproducibility since primary cells were difficult to grow and maintain in culture. Their proliferative response to IGF-1, PDGF-B, EGF and b-FGF was not tested, but no changes in growth were noted in the presence of 10% fetal calf serum.
6. Cell lines from non-transgenic mice were not prepared or tested for their apoptotic response to LY294002
7. Cell lines from MMTV-AKT1 mice were not prepared or tested for their proliferative response to growth factors.
8. Primary cells from MMTV-AKT1 mice were not prepared or tested for their apoptotic response to LY294002.

Other results: COMMA-1D cells expressing AKT1 did not exhibit transformation when grown in soft agar; cells

expressing myrAKT1 or v-akt were weakly transforming (see Appendix for reprint) (41). Cells expressing AKT1 were not tumorigenic as isografts in syngeneic Balb/c mice (41). COMMA-1D cells were also engineered to express the avian retrovirus receptor, tva, to make them permissive to infection by avian retroviruses (see Appendix for reprint) (40). Cells expressing AKT1 or v-akt exhibited invasivness in Matrigel and produced matrix metalloprotease-2 (MMP-2), suggesting that extracellular matrix degradation occurred separately from transformation. MCF-7 human breast carcinoma cells were also engineered to express tva and were subsequently transduced with an avian retrovirus expressing the AKT kinase, PDK1, Akt1K179E (kinase-dead) or both PDK1 and AKT1K179E to see if a dominant-negative AKT1 could inhibit PDK1 signaling. Neither cell growth nor apoptosis induced by LY294002 was affected by expression of either PDK1, AKT1K179E or both genes together (unpublished results).

Year 2:

1. Finish evaluating nulliparous and multiparous AKT1 mice for mammary tumor formation.

2. If no tumor formation is observed in AKT1 mice, administer DMBA and determine the incidence and latency of tumor formation between non-transgenic and AKT1 mice.

3. Backcross Gag-akt1 mice to develop pure transgenic strains. Determine histopathology and mammary tumor formation in nulliparous and multiparous Gag-akt1 mice. Characterize mammary gland expression of Gag-akt1 by Southern, PCR, northern, RT-PCR, western and *in situ* hybridization assays.

4. Assess gene expression by differential hybridization in AKT1 and Gag-akt1-transduced primary mammary epithelial cells from non-transgenic mice vs. cells transduced with an empty virus. Assess gene expression by suppression subtractive hybridization in AKT1 and Gag-akt1-transduced primary mammary epithelial cells from non-transgenic mice after treatment with LY294002 or vehicle.

5. Test primary mammary epithelial cells from Gag-akt1 mice for their proliferative response to IGF-I, PDGF-B, EGF and b-FGF.

6. Test primary mammary epithelial cells from Gag-akt1 mice for their apoptotic response to the PI3K inhibitor, LY294002, and to the dominant-negative AKT1K179E, by measuring DNA fragmentation by *in situ* end-labeling.

Results:

1. MMTV-AKT1 mice did not exhibit mammary tumor formation during their life span.
2. MMTV-AKT1 mice were not tested for their response to DMBA, but we expect to do these studies in the coming year.
3. MMTV-Gag-Akt1 mice were unable to be produced. BLG-myrAkt1 mice are in the process of being evaluated, but no mammary gland expression of myrAkt1 or a significant phenotype has been noted so far in lactating mice.
4. COMMA-1D/Akt1 cells were examined for changes in gene expression using the Mouse Angiogenesis and Mouse Pathway Finder arrays from SuperArray, Inc. Each array contained 96 genes associated with either angiogenesis or multiple signal transduction pathways. The following genes exhibited changes in AKT1-expressing cells vs control cell s transduced with an empty virus: BMP2 [bone morphogenetic protein 2] (+329%), BMP4 [bone morphogenetic protein 4 (+280%), Bcl2 (+423%), Bcl2-like [Genbank L35049] (+423%), En1 [engrailed homolog 1, Genbank U41751] (+186%). Changes in BMP4 mRNA levels could not be confirmed by northern or western blot and were not pursued further.
5. and 6. Not completed since Gag-Akt1 mice were unable to be not produced.

Year 3:

1. Finish evaluating nulliparous and multiparous Gag-akt1 mice for mammary tumor formation.

2. If no tumor formation is observed in Gag-akt1 mice, administer DMBA and determine the incidence and latency of tumor formation between non-transgenic and Gag-akt1 mice.

3. Finish evaluating AKT1 mice for tumor formation following DMBA treatment, if required.

4. Sequence and identify genes that are regulated by AKT1 and Gag-akt1 that were identified by suppression subtractive hybridization. Determine the expression of these genes in primary epithelial cells from non-transgenic and AKT1 and Gag-akt1 transgenic mice, and in response to apoptosis induced by LY294002, serum-deprivation and a dominant-negative AKT1K179E.

6. Evaluate the mammary gland and mammary tumors from AKT1 and gag-akt1 transgenic mice for their expression of genes identified by suppression subtractive hybridization.

Results:

1. and 2. not completed since MMTV-Gag-Akt1 mice could not be produced.
3. DMBA administration to MMTV-Akt1 mice was not initiated since we had to rederive our transgenic line due to a MPV infection outbreak in our animal facility. Frozen sperm from our two founder lines will be used to rederive the transgenic lines.
4. Subtractive hybridization has not been initiated. This will require more manpower and since MMTV-AKT1 transgenic mice did not exhibit a malignant phenotype, we have not commited more time to this task. The results of gene array analysis suggest that AKT1 may regulate Bcl2 mRNA expression and will be addressed in future studies if confirmed by northern analysis.
5. Affymetrix mouse gene array analysis of the mammary gland from MMTV-AKT1 transgenic mice was not initiated since the Cancer Center only recently made this available as a core resource. In addition, MMTV-AKT1 mice need to be rederived, and we expect this task to be completed as soon as this can be accomplished.

KEY RESEARCH ACCOMPLISHMENTS:

- Completed and published the characterization of MMTV-AKT1 transgenic mice (see Appendix, Ackler *et al.*).
- Produced BLG-myrAKT1 transgenic founder mice, which are being characterized (unpublished results).
- Completed and published a study showing that COMMA-1D mammary epithelial cell lines made amenable to avian retroviral infection and expressing AKT1or v-akt exhibited increased MMP-2 activity and invasion (see Appendix, Park *et al.*).
- Completed and published a study showing that mammary epithelial cells expressing AKT1 were not transformed *in vitro* or tumorigenic as isografts in syngeneic mice, but that the AKT kinase, PDK1, was highly tumorigenic (see Appendix, Zeng et al.).
- Using the yeast two-hybrid system, discovered that glutathione peroxidase and a protein of unknown function interact with AKT1 (unpublished results).
- Produced COMMA-1D mammary epithelial cells expressing AKT1 by murine retroviral transduction (see Appendix, Park et al.).
- Produced COMMA-1D mammary epithelial and breast cancer cell lines expressing the avian retrovirus receptor, tva, which are amenable to avian retroviral transduction (see Appendix, Zeng et al.).

REPORTABLE OUTCOMES:

Abstracts:

Ackfer, S., Ahmad, S., Tobias, C., Johnson, M. and Glazer, R.I. The development of mammary hyperplasia in MMTV-AKT1 transgenic mice. Proc. Amer. Assoc. Cancer Res. 41:714, 2000 (S. Ackler chosen for a Young Investigator Award)

Ackler, S., Ahmad, S., Johnson, M. and Glazer, R.I. Delayed involution in MMTV-c-AKT transgenic mice. Mouse Models of Cancer, La Jolla, CA, November 29-December 3, 2000.

Park, B-K, Zeng, X. and Glazer, R.I. AKT1 induces invasion on Matrigel and MMP-2 activity in mouse mammary epithelial cells. Proc. Amer. Assoc. Cancer Res. 42:16, 2001. (B-K Park chosen for a Young Investigator Award).

Zeng, X., Xu, H. and Glazer, R.I. 3-Phosphoinositide-dependent protein kinase1 (PDK1) transforms mouse mammary epithelial cells: association with enhanced expression of protein kinase Cα and activation of protein kinase Cα and AKT1. Proc. Amer. Assoc. Cancer Res. 43:375, 2002.

Papers:

Park, B.-K., Zeng, X. and Glazer, R.I. Induction of MMP-2 and invasion in mammary epithelial cells expressing AKT1. Cancer Res. 61:7647-7653, 2001.

Ackler, S., Ahmad, S., Tobias, C., Johnson, M. and Glazer, R.I. Delayed involution and mammary hyperplasia in MMTV-AKT1 transgenic mice. Oncogene 21:198-206, 2002.

Zeng, X., Xu, H., Park, B.-K., and Glazer, R. I. Transformation of mammary epithelial cells by 3-phosphoinositide-dependent protein kinase-1 (PDK1) is associated with the induction of protein kinase Cα. Cancer Res. 62:3538-3543, 2002.

Cell lines. COMMA-1D/tva, COM/AKT1, COMMA-1D/v-akt1, COMMA-1D/myrAKT1 and COMMA-1D/PDK1 mouse mammary epithelial cell lines; MCF-7/tva, MCF-7/PDK1 and MCF-7/dnAKT1 human breast carcinoma cell lines.

Animal models.

MMTV-AKT1 (completed) and BLG-myrAKT1transgenic mice (in progress)

CONCLUSIONS:

We have realized most of our goals for the third year, including generation of a new transgenic mouse, and have published three papers describing these results. The second transgenic mouse model, BLG-myrAKT1, is expected to be completed this year. Using the yeast two-hybrid system, we have identified two gene products not previously known to interact with AKT1, cellular glutathione peroxidase, and an EST that is highly expressed in several primary human cancers, but is as yet undefined.

PERSONNEL:

Robert I. Glazer, Ph.D., P.I., 10%
Shakeel Ahmad,, Ph.D., Res. Asst. Prof., 10%
Xiao Zeng, Ph.D., Res. Assoc., 30%

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Oncogene (2002) 21, 198–206
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Delayed mammary gland involution in MMTV-AKT1 transgenic mice

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AKT1/protein kinase Bα is a protein-serine/threonine kinase that regulates multiple targets involved in cell survival and cell cycle progression in a variety of cell types including breast cancer cells. To explore the role of Akt1 in mammary gland function and tumorigenesis, transgenic mice were generated that express human AKT1 under the control of the MMTV promoter. Virgin transgenic mice did not exhibit a dominant phenotype, but upon cessation of lactation, a notable delay in involution occurred compared to age-matched non-transgenic mice. The delay in involution coincided with increased hyperplasia as evidenced by an increased number of binucleated epithelial cells and a marked elevation in cyclin D1 expression in mammary epithelium. The delayed involution phenotype corresponded to increased phosphorylation of Thr308 in AKT1 and Ser136 in BAD, but not phosphorylation of Ser21 in GSK-3α. There was no evidence of mammary dysplasia or neoplasia during the lifespan of multiparous transgenic mice. These data suggest that AKT1 is involved in cell survival in the lactating and involuting mammary gland, but that overexpression of AKT1 alone is insufficient to induce transformation.
Oncogene (2002) **21,** 198–206. DOI: 10.1038/sj/onc/1205052

Keywords: AKT1; transgenic; mammary gland; cyclin D1

Introduction

The protein-serine/threonine kinase, Akt/protein kinase B, is the cellular homolog of the viral oncogene v-akt that was rescued from the AKT8 retrovirus infecting AKR mice (Staal, 1987). Akt is activated following receptor tyrosine kinase activation by several growth factors including epidermal growth factor (EGF) (Okano *et al.*, 2000; Wang *et al.*, 2000), heregulin (Olayioye *et al.*, 2000), hepatocyte growth factor (Bowers *et al.*, 2000), vascular endothelial growth factor (Gille *et al.*, 2000), insulin and insulin-like growth factor (IGF-1) (Alessi *et al.*, 1996), interleukins (Ahmed *et al.*, 1997; del Peso *et al.*, 1997) and nerve growth factor (Andjelkovic *et al.*, 1998). Akt activation occurs upon its recruitment to the plasma membrane via its pleckstrin homology domain (Bellacosa *et al.*, 1998) and phosphorylation of Thr308 and Ser473 by PDK1 and a postulated secondary protein kinase, respectively (Cohen *et al.*, 1997; Toker and Newton, 2000).

Activation of Akt leads to the modulation of several proteins involved in apoptosis and cell cycle regulation through either transcriptional or post-translational mechanisms. Phosphorylation of CREB by Akt leads to increased transcription of the cytoprotective protein Bcl-2 (Pugazhenthi *et al.*, 2000). The Forkhead family of transcription factors, including FKHR, FKHRL1 and AFX are phosphorylated by Akt, which results in their association with 14-3-3 proteins to prevent their translocation to the nucleus and activation of Fas ligand transcription (del Peso *et al.*, 1999). The pro-apoptotic Bcl-2 family member, BAD, is serially phosphorylated at Ser136 and Ser155 by Akt, which also leads to its association with 14-3-3 proteins, thereby preventing its association with and inactivation of the antiapoptotic proteins Bcl-2 and Bcl-X_L (Datta *et al.*, 2000). Akt phosphorylation of IκB kinase leads to its activation and phosphorylation of IκB targeting IκB for, proteasomal degradation and leading to induction of NF-κB-dependent transcription (Romashkova and Makarov, 1999). Glycogen synthase kinase-3 (GSK-3) has also been identified as a target of Akt, where phosphorylation of Ser9 inhibits its activity (Cross *et al.*, 1995) and reduces proteasomal degradation of cyclin D (Diehl *et al.*, 1998). Akt can also enhance translation of cyclin D mRNA by an unknown mechanism (Muise-Helmericks *et al.*, 1998). Increased cyclin D levels lead to increased cell division by maintaining the retinoblastoma protein (RB) in a hyperphosphorylated, inactivated state (Muller and Helin, 2000; Pavletich, 1999).

Of the three Akt isoforms, AKT1 and AKT2 overexpression have been linked to breast cancer. *AKT2* was amplified in approximately 12% of ovarian cancers and 3% of breast cancers and was associated with a poor prognosis (Bellacosa *et al.*, 1995). AKT1 levels were elevated in virtually all breast cancer cell

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Received 5 June 2001; revised 27 September 2001; accepted 9 October 2001

lines, and its activity was stimulated by IGF-1, estradiol and heregulin (Ahmad *et al*., 1999; Altiok *et al*., 1999; Gibson *et al*., 1999; Liu *et al*., 1999), resulting in resistance to apoptosis induced by TNFα, Fas ligand or wortmannin (Ahmad *et al*., 1999; Gibson *et al*., 1999; Zhou *et al*., 2000).

To examine the role of Akt1 in the etiology of mammary tumorigenesis, transgenic mice were generated that express human AKT1 under the control of the mouse mammary tumor virus (MMTV) long terminal repeat (LTR). In this study, we demonstrate for the first time that AKT1 expression during lactation results in a pronounced delay in involution, which was associated with hyperplasia and marked elevation of cyclin D1. Although MMTV-AKT1 mice did not exhibit mammary dysplasia or neoplasia in multiparous mice, our data suggest that Akt1 is an important survival factor in mammary epithelial cells during lactation and involution.

Results

Four transgenic founder lines 6, 19, 23 and 44, were produced, which express AKT1 under the control of the MMTV LTR. Mice were screened by PCR analysis with primers designed to detect a 446 bp fragment unique to the *AKT1* transgene (Figure 1a). DNA from mice testing positive for *AKT1* by PCR analysis was then assessed by Southern analysis where founder 23 expressed the highest copy number followed by founders 6 and 44 (Figure 1b).

Expression of AKT1 was also determined using Western blotting (Figure 2a). Akt1 was overexpressed in the mammary glands of virgin mice in founder lines 44 and 6, but not in founder line 23. Although commercially available AKT1 antibodies proved unsuitable for immunohistochemistry, activated AKT1 could be detected using an anti-phosphoThr308 AKT1 antibody (Figure 2b). Expression of activated Akt1 was low but detectable in the mammary epithelium in the glands of all virgin mice with expression greatest in founder lines 44 and 6.

Whole mounts of mammary glands from founder lines 6 and 44 revealed differences in ductal growth compared to their wild-type littermates (Figure 3a). Little difference in mammary gland morphology was evident in founder 6, whereas founder line 44 showed increased tubule branching. Multiparous mice from founder line 6 displayed marked tubule dilation and increased end-bud density compared to wild-type littermates (Figure 3b).

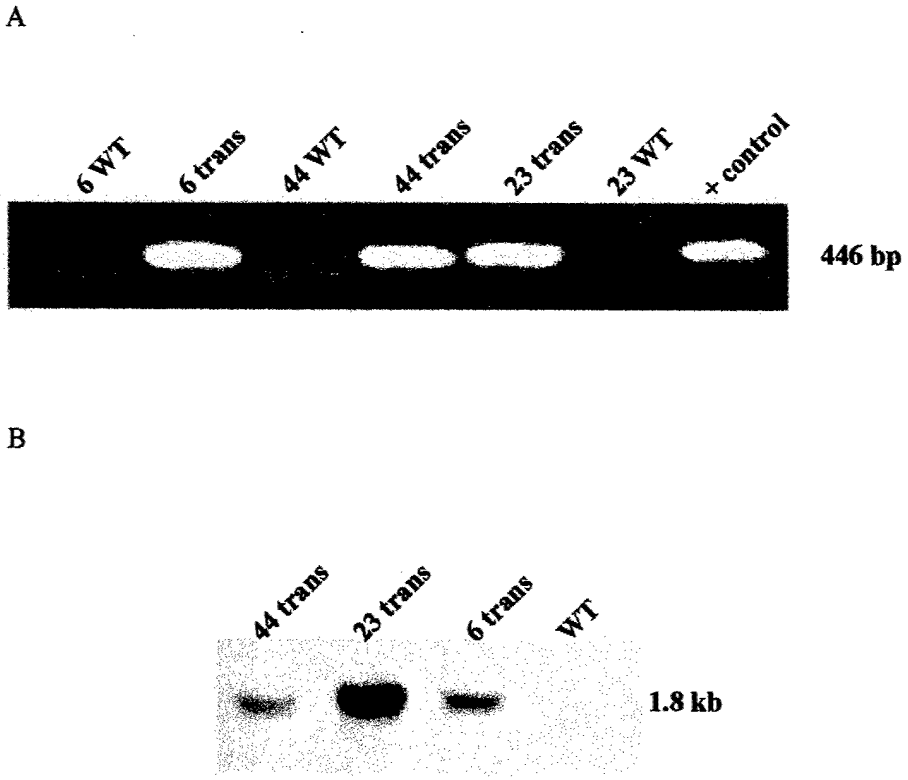

Figure 1 *AKT1* levels in transgenic mice. (**a**) PCR analysis. DNA was prepared from tail snips of littermates from each founder line, and analysed by PCR with primers specific for the *AKT1* transgene. Samples were compared to a DNA standard (+ control) prepared from a founder mouse identified previously by Southern blotting. (**b**) Southern blotting. Tail DNA from transgenic (trans) and wild-type (WT) littermates from each founder line (6, 44, or 23) was digested with *Eco*RI, separated in a 1% agarose gel and subjected to Southern analysis using an *AKT1* probe

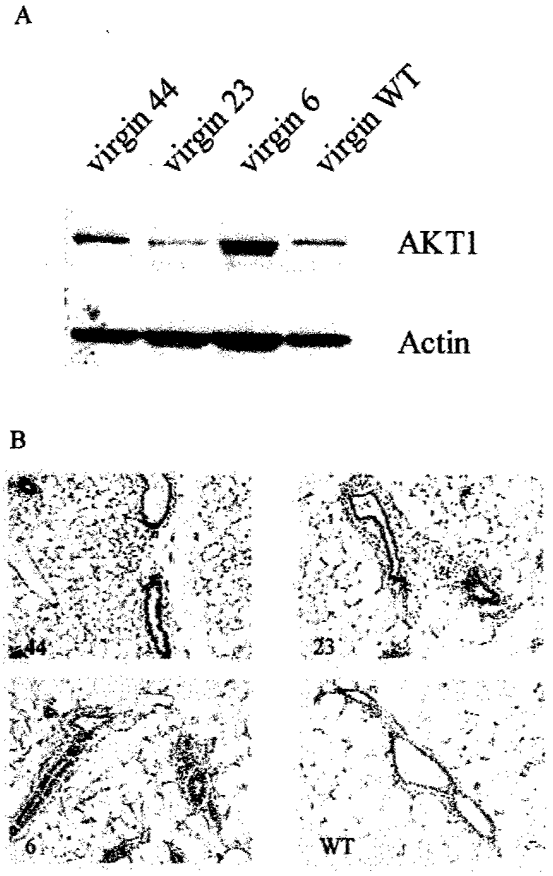

Figure 2 AKT1 expression in nulliparous mice. (**a**) Western analysis of AKT1 and actin in mammary gland extracts from nulliparous transgenic and wild-type mice. (**b**) Immunohistochemical detection of phospho-T308 AKT1 in mammary glands of nulliparous mice from transgenic founder lines 44 (**a**), 23 (**b**), 6 (**c**) and wild type (**d**). Magnification 100×

Transgene expression under the control of the MMTV LTR is strongly induced during pregnancy (Krane and Leder, 1996). Since Akt is involved in multiple cell survival pathways that are relevant to mammary epithelium (Dufourny *et al*., 1997, 2000; Jackson *et al*., 2000; Lu *et al*., 1999; Zhou *et al*., 2000), the effect of AKT1 overexpression in mammary epithelium during involution was examined. Female transgenic and non-transgenic littermates from founders 6 and 44, ranging in age from 16–22 weeks, were bred and allowed to nurse their pups. Involution was induced in individual glands by teat sealing at days 10, 3 and 1 before weaning (Li *et al*., 1997). In all instances, pups were weaned 18 days after birth and the glands harvested. Sealing had no overt effect on the ability of the pups to suckle or on their overall health, and had no effect on neighboring glands. Notable differences in gland histology between transgenic and wild-type mice during involution were observed (Figure 4). In wild-type mice (Figure 4c,f,i,l), obvious signs of involution were seen at day 3 when the gland became engorged with milk, the acinar structure collapsed and significant loss of epithelial cells occurred. By day 10, the gland had returned to a more normal, ductal structure, resembling the virgin gland. In founder line 6, the gland underwent apoptosis at approximately the same rate as the wild-type mouse (Figure 4b,e,h,k), but remained hyperplastic at day 10 of involution. Many regions within the gland had still not receded, and ducts still secreted milk as seen by the eosin-stained protein within the ductal lumina. A more severe phenotype was noted in founder line 44, where the mammary gland did not show any sign of involution at day 10 (Figure 4a,d,g,j). Accompanying the delay in involution was the appearance of many binucleated epithelial cells and an increase in granulocytic cells within the lumina (Figure 5), and was observed in both founder lines.

Expression of activated AKT1 was determined by immunohistochemistry using an antibody to phosphorylated Thr308 in AKT1. There was no evidence of staining at any stage of lactation and involution in the wild-type gland (Figure 6). However, in the glands of transgenic mice, strong expression of activated AKT1 could be seen in the glands of lactating mice and at day 1 and day 10 of involution.

Since there was histological evidence of hyperplasia in all founder lines following lactation, and AKT1 has been shown to increase cyclin D levels (Diehl *et al*., 1998; Muise-Helmericks *et al*., 1998), mammary glands were analysed immunohistochemically for cyclin D1 expression (Figure 7). In all mice, cyclin D1 staining was evident during lactation on day 0. On day 1 of involution, cyclin D1 expression was absent in the mammary gland from wild-type mice, but was strongly expressed in all transgenic founder lines. By day 10 of involution, cyclin D1 levels remained high in all transgenic lines, even when almost fully involuted, but remained below detection in wild-type mice.

Western blotting was used to examine the phosphorylation of potential downstream targets of AKT1 and markers of involution in founder line 6 following lactation and involution (Figure 8). While levels of AKT1 did not differ between transgenic and wild-type mice, phosphorylation of Thr308 in AKT1 was elevated in transgenic mice and corresponded to the onset of involution. The increase in phospho-Thr308 AKT1 also corresponded to Ser136-BAD phosphorylation in transgenic mice during lactation and day 3 of involution. Phosphorylation of Ser21 in GSK-3α was also evident during lactation and day 3 of involution in both wild-type and transgenic mice and did not correlate with delayed involution. Phosphorylation of Tyr705 in STAT3, a marker of involution, was apparent in both wild-type and transgenic mice, but parsisted lougorin transgenic mice.

Discussion

The present study was designed to determine if mammary gland-directed expression of AKT1 would

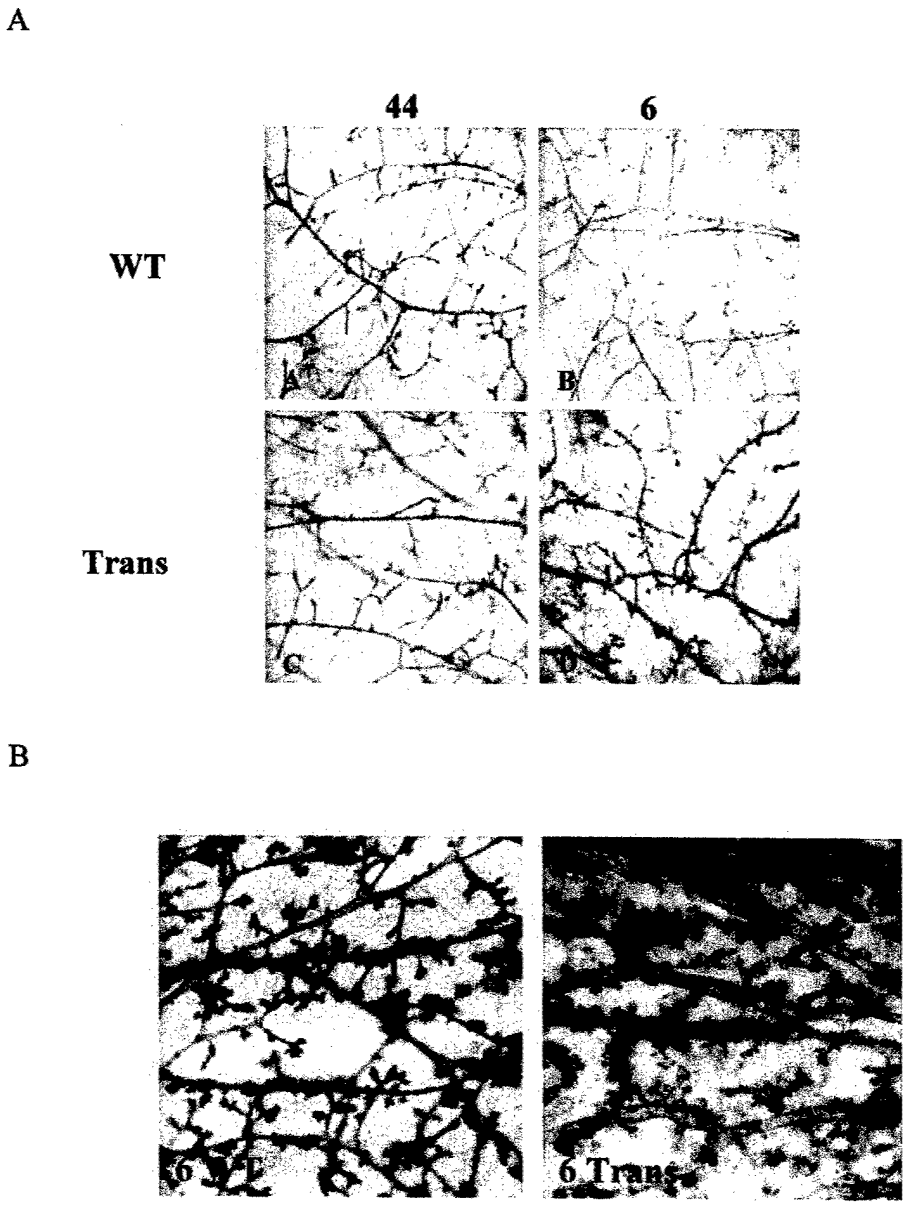

Figure 3 Whole mount preparations from nulliparous and multiparous mice. **(a)** Nulliparous mice. Whole mounts were prepared from littermates of wild-type mice (**a,b**) and transgenic mice (**c,d**) from founder lines 6 (**b,d**) and 44 (**a,c**). **(b)** Multiparous mice. Whole mounts were prepared from littermates of wild-type mice ('6 WT') and transgenic founder line 6 ('6 Trans')

result in proliferative, dysplastic or neoplastic changes or alterations in tissue remodeling during mammary gland development. MMTV-AKT1 mice universally exhibited varying degrees of delayed involution, which coincided with elevated cyclin D1 levels, increased AKT1 activation and an increased number of mitotic cells (Figures 5 and 7). The delay in involution correlated with the persistence of phospho-Tyr705-STAT3, which disappeared upon involution (Li *et al.*, 1997; Chapman *et al.*, 1999). There was a direct correlation between prolonged expression of activated AKT and cyclin D1 in the involuting mammary gland of both founder lines (Figures 6 and 7). While AKT activation was only moderately increased during lactation in wild-type mice (Figures 6 and 7), continued AKT activation in transgenic mice appeared to maintain a pool of cyclin D1 that prevented involution. These results are consistent with the elevation of cyclin D1 by AKT through both translational (Muise-Helmericks *et al.*, 1998) and post-translational mechanisms (Diehl *et al.*, 1998). Although inhibition of GSK-3 phosphorylation by AKT1 has been reported to increase cyclin D1 levels (Diehl *et al.*, 1998), both wild-type and transgenic mice exhibited a strong increase in GSK-3α phosphorylation during the early stages of involution. Since cyclin D1-dependent activa-

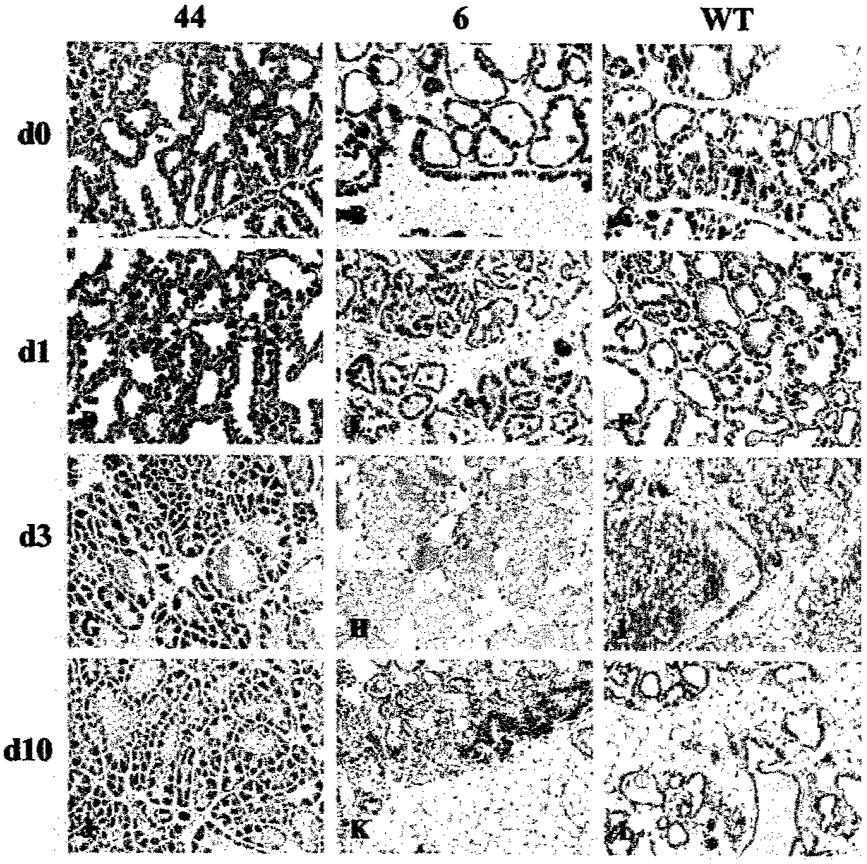

Figure 4 Mammary gland morphology following lactation and involution. Mammary glands from founder lines 44 (**a,d,g,j**) and 6 (**b,e,h,k**) and wild-type (**c,f,i,l**) mice were obtained during lactation at day 0 (**a–c**) and on day 1 (**e–f**), day 3 (**g–i**) and day 10 (**j,l**) after induced involution as described under Materials and methods. H & E staining. Magnification 100×

tion of CDK4 plays a key role in the inactivation of RB to permit G1-S transit through the cell cycle (Pavletich, 1999), increased cyclin D1 levels suggest an important mechanism for regulating mammary gland involution. Previous studies have shown that MMTV-regulated expression of cyclin D1 in transgenic mice leads to mammary oncogenesis (Wang *et al.*, 1994), and that cyclin D1 acts in a cooperative manner with the molecular chaperone CDC37 to accelerate mammary tumor formation (Stepanova *et al.*, 2000). MMTV-Cdc25B mice also exhibited elevated cyclin D1 levels, delayed involution and hyperplasia (Ma *et al.*, 1999), which was similar to the phenotype observed in MMTV-AKT1 mice. These studies all appear to provide a causal link between prolonged AKT activity, inappropriate cyclin D1 expression and delayed involution.

It is interesting that the phenotype observed in MMTV-AKT1 mice is similar in some respects to that observed in transgenic mice expressing the *TGFα* transgene. Metallothionein (MT)-TGFα mice exhibited up to a six week delay in involution and the gland contained many binucleated cells showing hyperplasia (Smith *et al.*, 1995). TGFα activates the same pathway as EGF (Beerli and Hynes, 1996), which through activation of erbB1 results in phosphoinositide 3-kinase (PI3-K) and AKT activation (Okano *et al.*, 2000; Wang *et al.*, 2000), suggesting a possible point of convergence for the ErbB1 and the AKT signaling pathways in the mammary gland. Another potential point of convergence is the regulation of cyclin D as shown by the elevated cyclin D1 levels in the mammary gland of WAP-TGFα mice (Sandgren *et al.*, 1995). However, unlike TGFα transgenic mice, AKT1 mice or mice expressing constitutively active forms of AKT1 (Hutchinson *et al.*, 2001; Schwertfeger *et al.*, 2001) did not exhibit neoplasia. This suggests that targets downstream to TGFα other than AKT1 contribute to the malignant process and that activation of at least one additional signaling pathway is required for oncogenesis to occur.

AKT1 overexpression has been shown in numerous studies to prevent apoptosis induced by serum- or cytokine-deprivation (for review see Datta *et al.* (1999)). Overexpression of AKT1 would therefore, be expected to tip the balance from apoptosis to survival. The appearance of phospho-Ser136 BAD in the mammary gland at lactation and at day 3 of

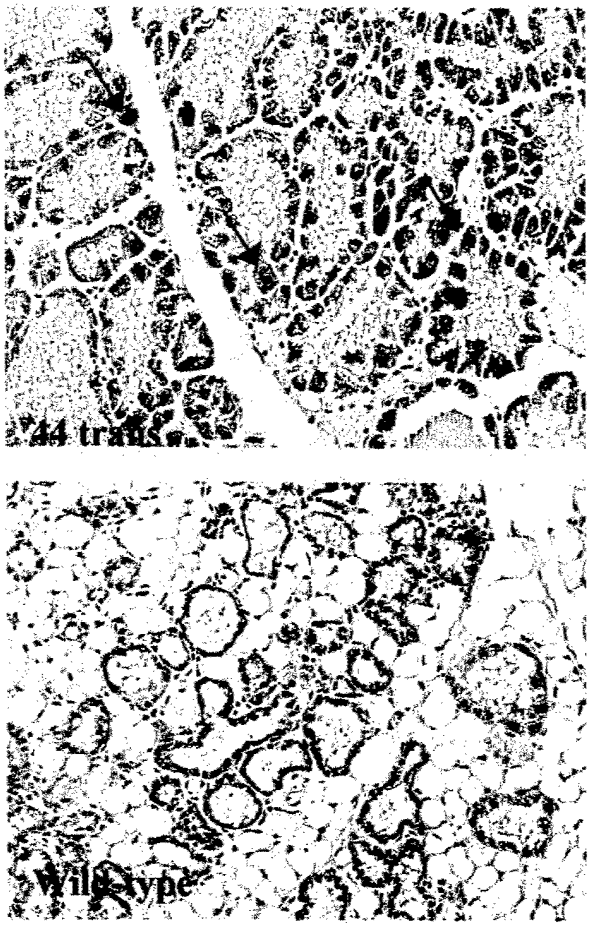

Figure 5 Binucleated cells in the mammary gland undergoing involution. Binucleated cells (*arrow*) were present in the mammary gland from founder line 44 ('44 Trans'), but not in the mammary gland from wild-type mice ('Wild-type') at day 10 after inducing involution as described under Materials and methods. Magnification 100×

involution in transgenic mice also suggests such a mechanism. Phosphorylation of BAD leads to its sequestration in the cytoplasm by 14-3-3 proteins (Datta *et al*., 2000), preventing its translocation to the mitochondria and subsequent inhibition of apoptosis (Datta *et al*., 1997). Thus, in addition to enhanced proliferation induced by elevated cyclin D1, BAD phosphorylation, at least early in involution, may contribute to the delay in involution resulting from AKT1 activation.

In conclusion, mammary gland-directed expression of AKT1 resulted in delayed involution associated with enhanced cyclin D1 expression and BAD phosphorylation. The delayed involution phenotype was neither associated with mammary tumor formation nor sustained hyperplasia. It appears therefore, that AKT1 overexpression alone is insufficient for driving the tumorigenic process in mammary epithelial cells and that one or more additional signaling pathways are required.

Materials and methods

Generation of transgenic mice

MMTV-AKT1 was prepared by insertion of a 1.8 kb *Eco*RI fragment of the human AKT1 cDNA (Ahmad *et al*., 1999) into the *Eco*RI site in the plasmid MMTV-SV40-Bssk (kindly provided by Dr Philip Leder). This construct was then digested with *Sal*I and *Spe*I, and the fragment containing the MMTV promoter, AKT1 sequences and SV40 poly-adenylation signal was isolated by electrophoresis in a 0.8% agarose gel and purified using QIAquick™ (Qiagen). The plasmid was adjusted to a concentration of 2 μg/ml in 10 mM Tris pH 7.5, 0.1 mM EDTA (TE buffer), and injected into the male pronucleus of FVB mouse embryos using standard techniques by the Transgenic Shared Resource, Lombardi Cancer Center. Four independent founders were generated and designated founder lines 6, 19, 23, and 44.

Southern analysis

DNA was extracted from 25 mg liver from transgenic and non-transgenic mice using DNeasy™ (Qiagen). Approximately 8–10 μg of DNA was digested with *Eco*RI overnight and precipitated with ethanol at −80°C. DNA was dissolved in 50 μl TE buffer and separated in a 1% agarose gel. Following Southern blot transfer (Southern, 1975) onto Immobilon™-Ny+ membranes (Millipore), DNA was hybridized with a human AKT1 cDNA probe labeled with biotinylated dCTP (Detector Random Primer DNA biotinylation kit, Kirkegaard & Perry Laboratories) and visualized with DNAdetector™ (Kirkegaard & Perry Laboratories) using CDP-Star as a substrate.

PCR analysis

DNA was extracted from mouse tail snips using DNeasy™ (Qiagen), and 200 ng DNA was used in each PCR reaction. The primers were designed to differentiate between the *AKT1* transgene and endogenous *akt1*. For AKT1, the forward primer (nt 477–500), 5′-TGAGGAGCGGGAGGAGTGGACAAC-3′, and the reverse primer (nt 900–922), 5′-CCCGGGA-CAGGTGGAAGAACAGC-3′, yielded a 446 bp fragment. DNA from a transgenic mouse, which tested positive by Southern blot analysis, was used as a positive control in all experiments. PCR was performed using a Biometra Thermocycler under the following conditions: denaturation at 95°C for 1 min, annealing at 60°C for 1 min and elongation at 72°C for 1 min for 30 cycles. Samples were separated in a 1% agarose gel.

Whole mounts

Non-pregnant female mice were euthanized using CO_2 and cervical dislocation, and mammary glands were harvested, placed on dry, silanized glass slides and fixed overnight in 1 part glacial acetic acid : 3 parts 100% ethanol. Tissues were rehydrated through successive incubations with 70% ethanol followed by distilled water, and stained with Carmine Red alum overnight. Tissues were then dehydrated through successive incubations in graded ethanol followed by mixed xylenes and mounted in Permount® (Fisher Scientific).

Involution studies

Mammary glands were harvested at days 0, 1, 3 and 10 following induced involution using the sealing procedure of Li *et al* (1997). Involution was induced by painting the #4

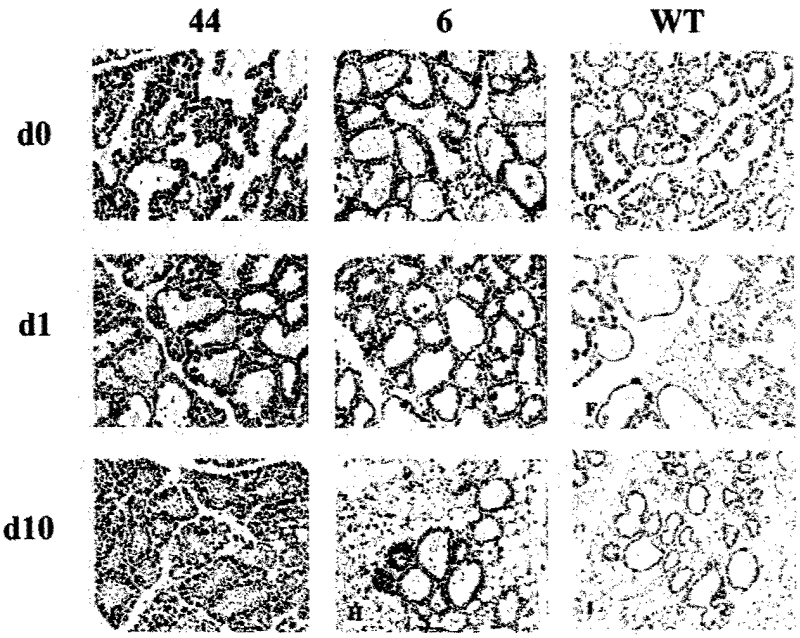

Figure 6 Phospho-Thr308-AKT1 expression in the mammary gland following lactation and involution. Mammary glands from founder lines 44 and 6 as well as wild-type mice were stained immunohistochemically for phospho-Thr308-AKT1. Tissue was obtained from the lactating mammary gland on day 0, day 1 and day 10 after inducing involution as described under Materials and methods. Magnification 100×

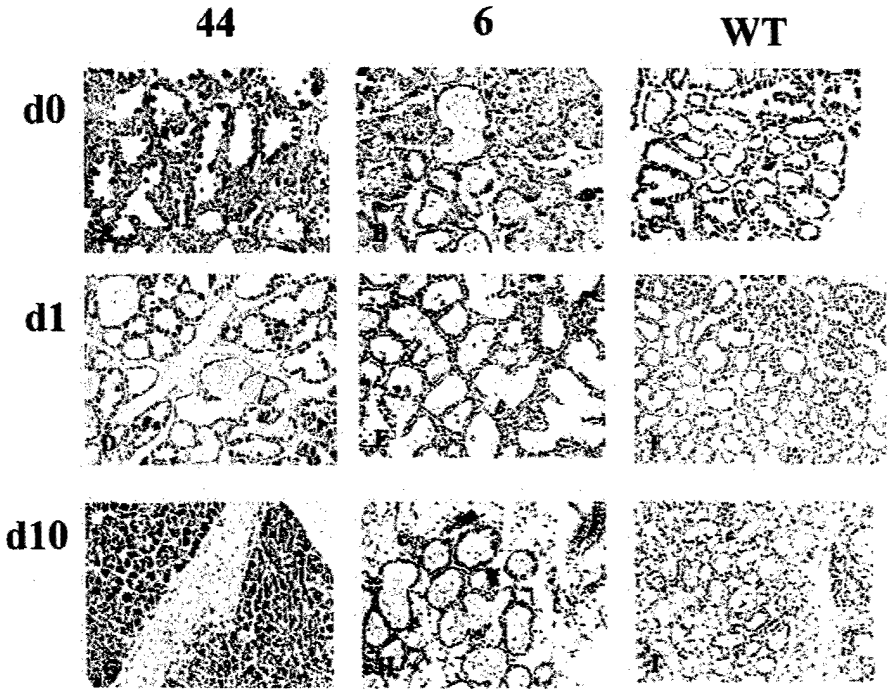

Figure 7 Cyclin D1 expression in the mammary gland following lactation and involution. Mammary glands from founder lines 44 (**a**,**d**,**g**) and 6 (**b**,**e**,**h**) as well as wild-type (**c**,**f**,**i**) mice were stained immunohistochemically for cyclin D1. Tissue was obtained from the lactating mammary gland on day 0 (**a**–**c**), day 1 (**d**–**f**) and day 10 (**g**–**i**) after inducing involution as described under Materials and methods. Magnification 100×

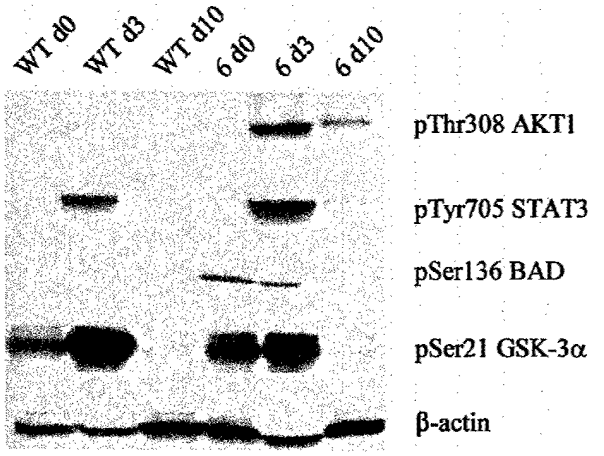

Figure 8 Expression of phospho-Thr308 AKT1, phospho-Ser136 BAD, phospho-Ser21 GSK-3α and phospho-Tyr705 STAT3 in the mammary gland from wild-type ('WT'), and transgenic mice from founder line 6 ('6'). Western analysis of mammary gland extracts was carried out in the lactating gland at day 0 (d0) and in the involuting gland at day 3 (d3) and day 10 (d10) post-involution. Transgenic and wild-type mice correspond to the respective immunohistochemical data presented in Figures 4, 6 and 7

(day 10), #9 (day 3) and #3 (day 1) teats with acrylate at days 10, 3 and 1, respectively, prior to weaning. Mammary gland #8 was used as the non-involuting control (day 0). On the day of weaning, nursing females were sacrificed as described above, and the #3, #4, #8 and #9 glands were excised. Half of each gland was snap frozen in liquid nitrogen for protein extraction and immunoblot analysis, and the other half was stored in formalin overnight for further processing for histopathology and immunohistochemistry.

Histopathology and immunohistochemistry

Formalin-fixed mammary glands from virgin and lactating mice were embedded in paraffin by the Histopathology and Tissue Core Facility, Lombardi Cancer Center. Tissue sections were stained with either hematoxylin and eosin (H & E) for histopathology or with hematoxylin alone for nuclear staining.

For immunostaining, slides were deparaffinized with xylenes, and rehydrated in graded alcohols. Slides were treated in 0.1% trypsin/phosphate-buffered saline (PBS) at 37°C for 30 min, followed by a 10 min rinse under running deionized water. Endogenous peroxidases were quenched with 0.3% H_2O_2 in methanol for 30 min. Blocking solution consisted of 2% rabbit or goat serum (depending on source of secondary antibody), 5% BSA in PBS, and was applied for 1 h. Primary and secondary antibodies were applied for 1 h each in blocking solution. ABC reagent coupled to peroxidase (#PK-6001, Vector Laboratories) was applied for 1 h. Reactivity was detected using diaminobenzidine (DAB, Dako) staining, and the slides were counterstained with Harris-modified hematoxylin (Fisher Scientific) according to the manufacturer's instructions. Slides were coverslipped in Permount® (Fisher Scientific). The following antibodies were used: rabbit anti-phosphoT308Akt1 (Upstate Biotechnology) diluted 1:100 and goat anti-cyclin D1 (Santa Cruz) diluted 1:200; biotinylated goat anti-rabbit IgG (Kierkegaard and Perry Laboratories) and biotinylated rabbit anti-goat IgG (Kirkegaard and Perry Laboratories) diluted 1:100.

Western analysis

Total protein was extracted from snap-frozen mammary glands by homogenization using a Dounce homogenizer in 2 ml extraction buffer (25 mM Tris pH 7.4, 150 mM NaCl, 0.5 mM sodium vanadate, 50 mM sodium fluoride, 10 mM sodium pyrophosphate, 1 mM PMSF, 10 μg/ml trypsin inhibitor). Homogenates were centrifuged at 10000 *g* for 3 min at 4°C, and fat was removed from the surface by vacuum aspiration. The precipitate was resuspended by vortexing, and NP-40 was added to a final concentration of 0.5%. Samples were again homogenized using a Dounce homogenizer and centrifuged. Supernatants were decanted, and protein concentrations determined using the Coomassie protein assay (Pierce). Protein solutions were stored at −80°C until use.

Approximately 75 μg of protein were diluted in Laemmli sample buffer, boiled and loaded onto a 4–20% PAGEr® gradient Tris-glycine gel (BioWhittaker). Separated proteins were transferred to Protran nitrocellulose membrane (Schleicher and Schuell). Membranes were blocked with 1% polyvinylpyrrolidone (PVP) in Tris-buffered saline-0.1% Tween 20 (TBST) as described by Haycock (1993). Primary and horseradish-peroxidase (HRP) conjugated secondary antibodies were appropriately diluted in TBST. Bound antibody was detected using SuperSignal West Pico chemiluminescent substrate (Pierce). The following antibodies were used: rabbit anti-phospho-T308-Akt1, rabbit anti-phospho-Y705-STAT3, rabbit anti-phospho-S21-GSK3α, and sheep anti-phospho-S136-BAD were from Upstate Biotechnology and diluted 1:1000; goat anti-actin was from Santa Cruz and diluted 1:200. Rabbit anti-goat IgG (Pierce) and goat anti-rabbit IgG (Biorad) were diluted 1:15000. Rabbit anti-sheep IgG (Dako) was diluted 1:5000.

Acknowledgments

S Ackler was supported by Predoctoral Fellowship DAMD17-00-1-0258 from the US Army Breast Cancer Research Program. This work was supported by NIH grant R01 CA881565 and by DAMD17-99-9195 from the US Army Breast Cancer Research Program (RI Glazer). The Lombardi Cancer Center Transgenic Shared Resource and Tissue Culture Shared Resources are supported by NIH grants P50-CA58185 and P30-CA51008. The authors would also like to acknowledge Dr Sandy McLeskey for assistance with the immunohistochemical staining techniques.

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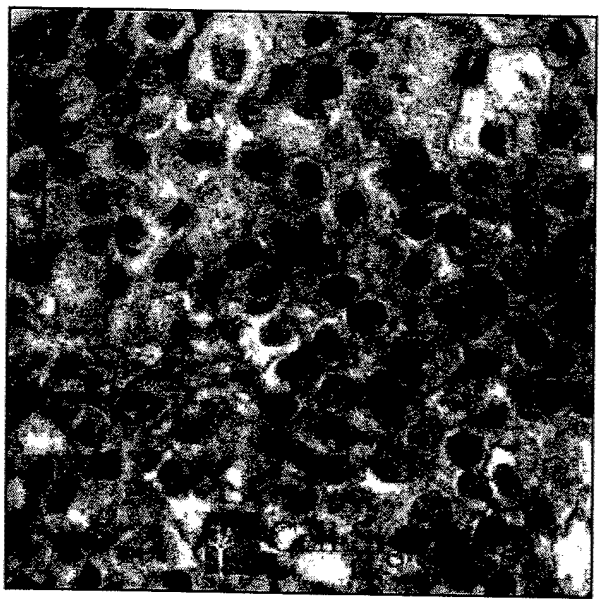

[CANCER RESEARCH 61, 7647–7653, October 15, 2001]

Akt1 Induces Extracellular Matrix Invasion and Matrix Metalloproteinase-2 Activity in Mouse Mammary Epithelial Cells[1]

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ABSTRACT

The roles of the protein-serine/threonine kinase, Akt1, in signaling pathways associated with cell motility and extracellular matrix invasion were examined in the immortalized mouse mammary epithelial cell line, COMMA-1D. COMMA-1D cells were engineered to express the avian leukosis subtype A receptor, tv-a, to permit infection by recombinant avian leukosis virus produced by the replication-competent avian splice vector, RCAS. COMMA-1D/tv-a cells transduced with RCAS/v-akt, but not RCAS/Akt1, formed anchorage-independent colonies in soft agar; however, cells overexpressing either v-akt or Akt1 became highly invasive when grown on the ECM, Matrigel. Zymography of extracellular protease activity shed into the medium by COMMA-1D/Akt1 or COMMA-1D/v-akt cells revealed elevated gelatinase activity that was confirmed to be matrix metalloproteinase-2 (MMP-2; gelatinase A) by Western blotting and immunoprecipitation-zymography. The MMP inhibitor, BB-94, blocked MMP-2 activity and invasion associated with Akt1- and v-akt-expressing cells. The proteasome inhibitor, lactacystin, markedly increased MMP-2 levels and invasion in control cells but not in Akt1- and v-akt-expressing cells. These results suggest that the invasive behavior of mammary epithelial cells induced by Akt1 is associated with increased MMP-2 expression that may result from inhibition of MMP-2 degradation by the proteasome pathway.

INTRODUCTION

The serine/threonine-protein kinase, Akt/PKB,[3] originally identified by its homology to protein kinases A and C (1, 2), is the transforming protein expressed by the murine AKT8 retrovirus (3, 4). Akt is composed of an NH_2-terminal pleckstrin homology domain that binds to membrane-associated phosphatidylinositol-3,4,5-trisphosphate and phosphatidylinositol-3,4-bisphosphate generated by phosphoinositide 3-kinase (5, 6), a kinase catalytic domain and a COOH-terminal regulatory domain. Akt1 is one of three mammalian isoforms and is activated by several growth factor pathways that elicit phosphorylation at T308 in the activation loop of the catalytic domain and at S473 in the COOH terminus (7). Akt1 is a downstream target of phosphoinositide 3-kinase (8) and translocates to the plasma membrane upon growth factor stimulation (9), where it binds to phosphatidylinositol-3,4-bisphosphate and phosphatidylinositol-3,4,5-trisphosphate. This results in its activation by transphosphorylation at T308 by phosphoinositide-dependent kinase 1 (7), and either autophosphorylation (10) or transphosphorylation at S473 by phosphoinositide-dependent kinase 1 (11, 12), integrin-linked kinase (13), and protein kinase C (14). Akt1 has been implicated in a variety of cellular functions, such as survival, transcription, and translation (8, 15). Overexpression of Akt blocks apoptosis *in vitro* and is overexpressed in ovarian, pancreatic, and breast cancer (16–19).

Cancer cell invasion through the ECM and basement membranes is mediated by degradation of collagen IV, the major structural component of the basement membrane (20). Matrix metalloproteinases play a critical role in tumor progression, growth and metastasis (21). The association between the activation of MMP-2 (type IV collagenase/gelatinase A) and ECM invasion *in vitro* constitutes a model for metastatic progression in human breast cancer (22). Similar to other secreted MMPs, the latent proenzyme of MMP-2 is activated by its association with TIMP-2 (23, 24) and cell surface-anchored MT1-MMP (25, 26), and a stoichiometric imbalance in this ternary complex may be associated with tumor progression. Overexpression of MT1-MMP induces abnormalities and tumor formation in the mammary glands of transgenic mice (27) and increases the invasiveness of prostate cancer cells (28).

In this study, we sought to examine the role of Akt1 in the transformation and invasion of mammary epithelial cells and to determine whether these processes were controlled by the same determinants. In the course of these studies, we discovered that the induction of invasion and transformation were independent events, and that the invasive activity of Akt1-expressing cells correlated with increased MMP-2 expression and activation.

MATERIALS AND METHODS

Antibodies and Reagents. Antibodies and reagents were obtained from the following sources: monoclonal (Ab-3) and polyclonal (Ab-7) anti-MMP-2 antibodies (Oncogene Science, Boston, MA); rabbit polyclonal anti-Akt1 antibody (06-558; Upstate Biotechnology Lake Placid, NY); mouse monoclonal anti-Akt1 antibody (610860; BD Transduction Laboratories, Lexington, KY); rhodamine-conjugated goat antirabbit IgG and Texas Red-conjugated goat antimouse IgG (KPL, Gaithersburg, MD); BB-94 (Batimastat; British Biotech, Oxford, United Kingdom); APMA (Aldrich-Sigma Chemical Co. Chemical, St. Louis, MO); and lactacystin (Calbiochem, San Diego, CA).

Cell Line Establishment and Culture. COMMA-1D cells (29) were engineered to express the gp800 *tv-a* cDNA (Ref. 30; kindly provided by Dr. Yi Li, Memorial-Sloan Kettering Cancer Institute, New York, NY). *tv-a* was cloned into the pSRαMSVtkneo retroviral vector (31) and cotransfected into 293T cells with the pSV-ψ^--E-MLV ecotropic *env* vector by calcium phosphate precipitation. COMMA-1D cells were infected with virus-containing medium and selected in 600 μg/ml G418 for 2 weeks. The expression and the proper function of tv-a in G418-selected COMMA-1D cells were confirmed by immunostaining with a rabbit polyclonal anti-tv-a antibody (generously provided by Dr. Andrew Leavitt, University of California, San Francisco, CA) and by transduction with a recombinant ALV encoding the *GFP* gene (see below). High-titer ALV was generated by transfecting DF-1 chicken embryo fibroblast cells (Ref. 32; kindly provided by Dr. Stephen Hughes, National Cancer Institute, Bethesda, MD) growing in DMEM containing 10% FCS, 10% tryptose phosphate broth, and 1% chicken serum, with ALV generated with RCAS/Akt1 and RCAS/v-akt (Ref. 33; generously provided by Drs. Peter Vogt and Masahiro Aoki, The Scripps Research Institute, La Jolla, CA) using calcium phosphate precipitation (34). After 6 days, the medium was centrifuged at 3000 × *g* for 5 min, and the virus-containing supernatant was added to COMMA-1D/tv-a cells and incubated overnight. Cells were transduced by three sequential rounds of infection over 2 days. The medium was then aspirated, and the COMMA-1D/tv-a cell lines were cultured as monolayers in

Received 5/4/01; accepted 8/16/01.

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[1] Supported by National Cancer Institute Grant CA81565 and Department of Defense Grant DAMD17-99-1-9195.

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[3] The abbreviations used are: Akt/PKB, Akt/protein kinase B; ALV, avian leukosis virus; ECM, extracellular matrix; FACS, fluorescence-activated cell sorting; GFP, green fluorescent protein; MMP-2, matrix metalloproteinase-2; MT1-MMP, membrane-type 1 matrix metalloproteinase; RCAS, replication-competent avian splice vector; TIMP, tissue inhibitor of metalloproteinase; tv-a, avian leukosis subtype A receptor; APMA, 4-aminophenylmercuric acetate.

IMEM containing 2.5% FCS, epidermal growth factor (Upstate Biotechnology, Lake Placid, NY; 10 ng/ml), and insulin (Aldrich-Sigma Chemical Co., St. Louis, MO; 5 μg/ml).

Anchorage-independent Growth. COMMA-1D/Akt1 or COMMA-1D/v-akt cells (1×10^5) were suspended in 2 ml of 0.5% (wt/vol) SeaPrep agar (BMA, Rockland, ME) dissolved in IMEM containing 10% FCS and gentamicin (50 μg/ml) and overlaid on 1% agar dissolved in IMEM containing 2% FCS in six-well plates. Cultures were fed once a week for 5 weeks.

Akt Kinase Assay. A rabbit polyclonal Akt1 antibody (Upstate Biotechnology) was adsorbed on 50 μl of protein A/G-agarose (Santa Cruz Biotechnology, Santa Cruz, CA), and incubated with 250 μg of cell lysate. Immunoprecipitates were incubated in 30 μl of kinase buffer containing 10 μCi of [γ-^{32}P]ATP, 5 μM ATP, 75 mM $MgCl_2$, 20 mM 4-morpholinepropanesulfonic acid (pH 7.2), 25 mM β-glycerol phosphate (pH 7.0), 1 mM Na_3VO_4, 1 mM DTT, 17 μM protein kinase A inhibitor peptide, and 30 μM histone H2B as substrate (16). The relative amounts of incorporated radioactivity were determined by autoradiography and densitometry.

Preparation of Samples from Conditioned Media. Monolayer cultures were grown in 75-cm^2 plastic flasks for 48 h, washed three times with PBS, and then incubated for 18 h in serum-free IMEM. The medium was collected and concentrated using a Centricon 10 centrifugal filter with a M_r 3000 cutoff (Amicon, Bedford, MA).

Western Blotting Analysis. Whole cell lysates were prepared by lysing cells in a buffer containing 50 mM Tris-HCl (pH 7.4), 1% NP40, 150 mM NaCl, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na_3VO_4, 1 mM NaF and Complete protease inhibitor cocktail (Boehringer Mannheim, Mannheim, Germany). Lysates (20 μg of protein) were mixed with Laemmli sample buffer, boiled for 5 min, and separated in 10% PAGEr Gold precast gels (BMA, Rockland, ME) by SDS-PAGE. Resolved proteins were electrophoretically transferred to a nitrocellulose membrane (Protran; Schleicher & Schuell, Keene, NH) and blocked for 1 h in 5% nonfat dry milk in TBST buffer [10 mM Tris-HCl (pH 7.2), 50 mM NaCl, and 0.2% Tween 20]. Blots were incubated overnight with primary antibody at 4°C and then for 1 h at room temperature with horseradish peroxidase-conjugated secondary antibody diluted 1:5000. Labeled proteins were visualized with the ECL detection system (Amersham, Arlington Heights, IL) according to the manufacturer's instructions.

Matrigel Invasion Assay. Assays were carried out with untreated cells or cells treated overnight with either 100 nM BB-94 or 1 μM lactacystin. Cells were then plated on Matrigel-coated Biocoat Cell Culture Insert chambers (Becton Dickinson Labware, Bedford, MA) containing 23-mm diameter, 8-μm pore size filters. Matrigel was diluted with cold, serum-free IMEM and 850 μg of Matrigel were applied to each filter, dried in a hood, and incubated at 37°C for 30 min. Cells (1×10^5) were suspended in serum-free IMEM and added to the Matrigel-coated chamber, and conditioned medium was placed in the lower chamber as a chemoattractant. After incubation for 72 h at 37°C in 5% CO_2, the Matrigel-coated side of the filter was removed with a cotton swab, and cells on the opposite side of the filter were fixed in 10% formaldehyde, stained with H&E, and counted. Each assay was performed in triplicate.

Gelatin Zymography. Two μg of protein from concentrated cell-conditioned medium were loaded onto 10% acrylamide-0.1% gelatin gels (Novex, San Diego, CA) and separated by electrophoresis under nonreducing conditions. After electrophoresis, gels were renatured by soaking for 30 min at room temperature in 2.5% Triton X-100 with gentle agitation. Zymogram gels were then equilibrated for 30 min at room temperature in developing buffer [50 mM Tris, 40 mM HCl (pH 7.4), 200 mM NaCl, 5 mM $CaCl_2$, and 0.02% Brij35] and incubated overnight at 37°C. Transparent bands of gelatinolytic activity were visualized by staining with 0.5% Coomassie Blue R250.

Immunocytofluorescence. Invasion assays were performed as described above, and after 60 h, attached cells were fixed for 30 min at 4°C with 2% paraformaldehyde and blocked for 30 min at room temperature in 10% normal goat serum, 1% gelatin in PBS. Cells attached to Matrigel were incubated for 2 h at room temperature with a polyclonal anti-MMP-2 antibody diluted 1:100 in blocking buffer and washed for 5 min with PBS three times. The sections were stained for 1 h with rhodamine-conjugated goat antirabbit IgG diluted 1:200, followed by washing with PBS.

For subcellular localization of Akt, chamber-mounted glass slides (Nalge Nunc International, Naperville, IL) were coated with a concentrated cell suspension and fixed for 10 min at room temperature by addition of an equal volume of 10% formaldehyde to the growth medium. The supernatant was removed, and cells were permeabilized with 0.05% Triton X-100 in PBS. Slides were washed with PBS three times and blocked for 1 h at room temperature with 20% goat serum in PBS. Cells were incubated for 1 h at room temperature with a monoclonal anti-Akt1 antibody diluted 1:100 in 20% goat serum/PBS. Slides were washed with PBS three times and then incubated for 1 h with Texas Red-conjugated goat antimouse antibody diluted 1:200, followed by washing with PBS. Slides were treated with ProLong Antifade reagent (Molecular Probes, Inc.), and stained sections were visualized by confocal microscopy.

RESULTS

Transduction of Mammary Epithelial Cells by Recombinant ALV. To examine gene expression in mammary epithelial cells, COMMA-1D cells were engineered to express the ALV subtype A receptor, tv-a, to make them permissive to infection by RCAS viruses. COMMA-1D/tv-a cells were then transduced with RCAS/GFP to allow selection by FACS (Fig. 1). A single clone sorted from the high tv-a-expressing cells was expanded and used in all subsequent studies.

Anchorage-independent Growth and Invasion. COMMA-1D cells transduced with RCAS-Akt1, and particularly RCAS–v-akt, exhibited high kinase activity compared with the parental cell line expressing GFP alone, as measured by immune-complex kinase assay (Fig. 2). To measure transformation potential, anchorage-independent growth of these cell lines in soft agar was determined (Fig. 3*A*). After 5 weeks in culture, cells transduced with v-akt, but not Akt1, formed colonies, which appeared as early as 1 week after infection.

To determine whether these cells also possessed invasive potential, Boyden chamber assays were performed with Matrigel as a substrate (Fig. 3*B*). Both COMMA-1D/Akt1 and COMMA-1D/v-akt cells exhibited 25–30% of the invasive potential of MDA-MB-231 breast carcinoma cells, in contrast to parental cells, which were not invasive.

No morphological differences were observed between control and Akt-transduced cells when grown on a plastic substratum (Fig. 4, *A*, *C*, and *E*); however, when cells were grown for 72 h on Matrigel, COMMA-1D/tv-a cells formed simple, spherical colonies (Fig. 4*B*), in

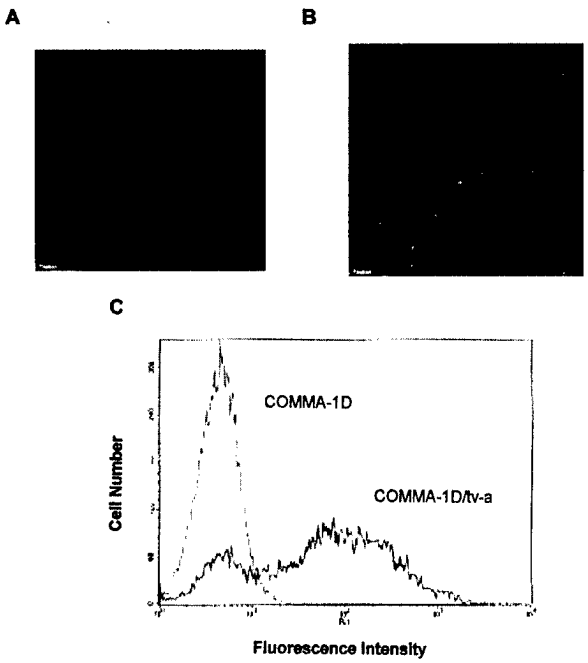

Fig. 1. Selection of COMMA-1D/tv-a cells. COMMA-1D/tv-a cells were transduced with RCAS-GFP and sorted by FACS. Fluorescence microscopy of COMMA-1D (*A*) and COMMA-1D/tv-a (*B*) cells expressing GFP is shown. ×100. *C*, FACS analysis of COMMA-1D and COMMA-1D/tv-a cells expressing GFP.

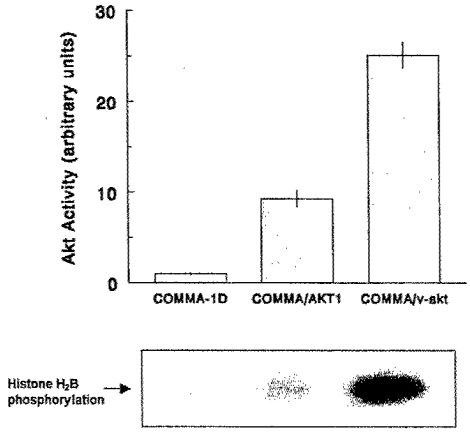

Fig. 2. Akt kinase activity in COMMA-1D/Akt1 and COMMA-1D/v-akt cells. Immunocomplex kinase assays were carried out using histone H2B as substrate, and incorporated radioactivity was determined by autoradiography and densitometry. Each value is the mean of duplicate results from two experiments; *bars,* SD.

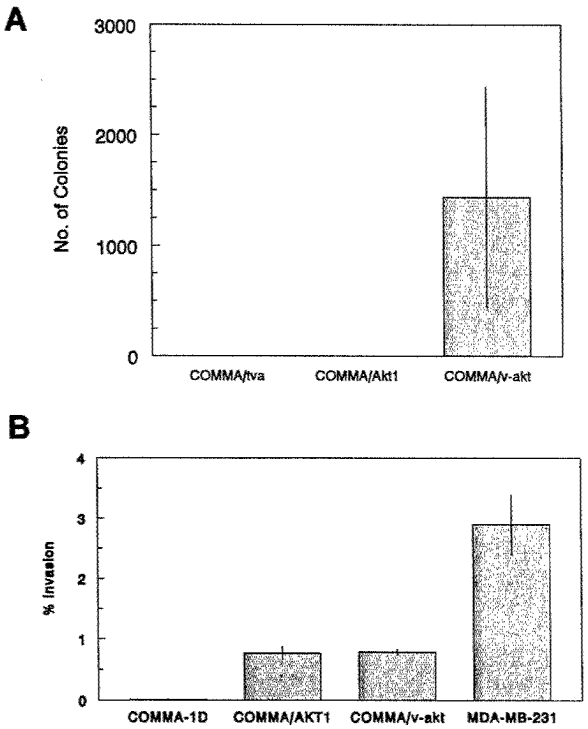

Fig. 3. Anchorage-independent growth and Matrigel invasion activity of COMMA-1D/Akt1 and COMMA-1D/v-akt cells. *A*, anchorage-independent growth in soft agar. Colony formation was measured 3 weeks after plating. Each value is the mean of three experiments; *bars,* SD. *B*, ECM invasion was measured in a modified Boyden chamber with Matrigel-coated filters as described in "Materials and Methods." The number of cells migrating to the underside of the filter was measured after 72 h and is expressed as a percentage of the number of cells applied to the filter. MDA-MB-231 breast carcinoma cells were used as a positive control. Each value is the mean of three experiments; *bars,* SD.

contrast to the more dense and branched lattices of cells formed by the Akt1- and v-akt-transduced cells (Fig. 4, *D* and *F*).

Akt1 Induces MMP-2 Activity. To determine whether secreted protease activity was associated with invasion activity, cell-conditioned medium was assayed by gelatin zymography (Fig. 5). Several bands of activity were revealed under nondenaturing, nonreducing conditions (Fig. 5*A*), and immunoblotting of the concentrated cell-conditioned medium from COMMA-1D/Akt1 and COMMA-1D/v-akt cells indicated the presence of an M_r 85,000 form of MMP-2 (Fig. 5*B*), the activity of which was confirmed by immunoprecipitation and gelatin zymography (Fig. 5*C*). To determine whether the M_r 85,000 form of MMP-2 was proMMP-2, samples were incubated with the cysteine-modifying agent, APMA, to induce autocatalysis (Fig. 6*A*). APMA induced the autocatalytic processing of M_r 85,000 MMP-2 to

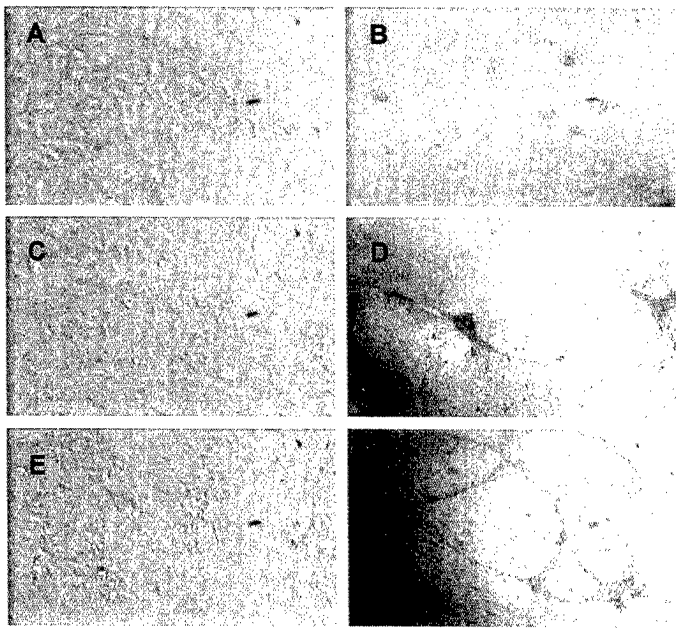

Fig. 4. Light microscopy of COMMA-1D/Akt1 and COMMA-1D/v-akt cells growing on plastic or Matrigel. *A* and *B*, COMMA-1D/tv-a cells; *C* and *D*, COMMA-1D/Akt1 cells; *E* and *F*, COMMA-1D/v-akt cells grown on plastic (*A, C,* and *E*) or Matrigel (*B, D,* and *F*). ×100.

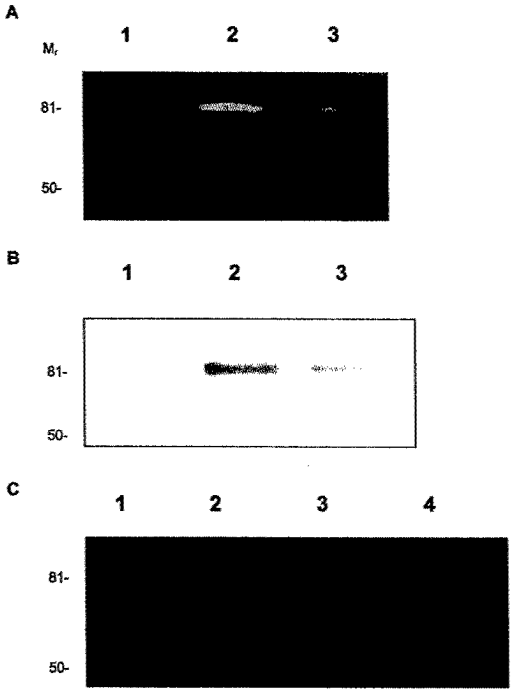

Fig. 5. MMP-2 activity associated with the conditioned medium from COMMA-1D/Akt1 and COMMA-1D/v-akt cells. *A*, gelatin zymography of proteolytic activity in the conditioned medium. *Lane 1,* COMMA-1D/tv-a; *Lane 2,* COMMA-1D/Akt1; *Lane 3,* COMMA-1D/v-akt cells. *Ordinate,* the position of prestained protein markers in thousands. *B,* immunoblot of the conditioned medium used for gelatinase assays in *A. Ordinate,* the position of prestained protein markers in thousands. *C,* MMP-2 immunoprecipitation-gelatinase assay using the conditioned medium in *A. Lane 1,* COMMA-1D/tv-a; *Lane 2,* COMMA-1D/Akt1; *Lane 3,* COMMA-1D/v-akt cells; *Lane 4,* recombinant MMP-2. *Ordinate,* the position of prestained protein markers.

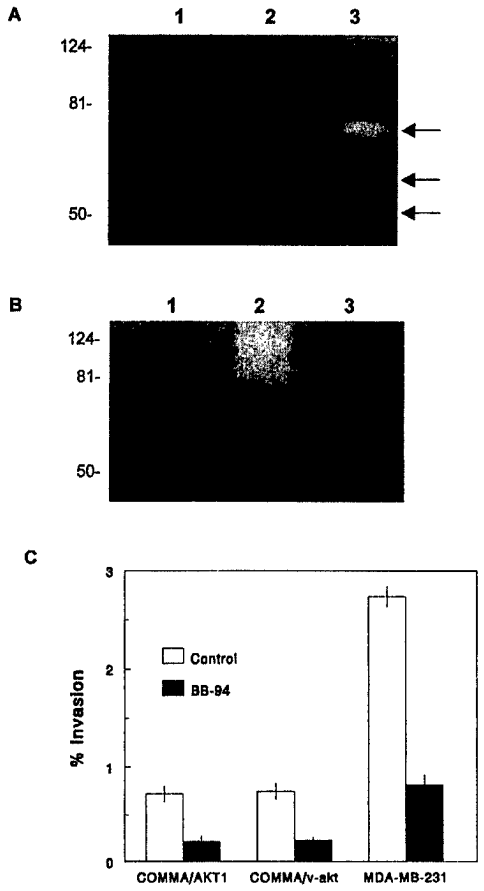

Fig. 6. Gelatinase and invasion activity of COMMA-1D/Akt1 cells treated with APMA or BB-94. *A*, conditioned medium from COMMA-1D/Akt1 cells was treated *in vitro* with 1 mM APMA prior to zymography. *Lane 1*, conditioned medium from COMMA-1D/tv-a cells; *Lane 2*, untreated COMMA-1D/Akt1 cells; *Lane 3*, APMA-treated COMMA-1D/Akt1. *Arrows*, active forms of MMP-2 resulting from APMA-induced autocatalysis of proMMP-2. *Ordinate*, the position of prestained protein markers in thousands. *B*, cells were treated for 24 h with 100 nM BB-94, and the conditioned medium was concentrated and tested for gelatinase activity as described in "Materials and Methods." *Lane 1*, conditioned medium from COMMA-1D/tv-a cells; *Lane 2*, control COMMA-1D/Akt1 cells; *Lane 3*, COMMA-1D/Akt1 cells treated with BB-94. *Ordinate*, the position of prestained protein markers in thousands. *C*, cells were treated as in *B*, and Matrigel invasion assays were carried out as described in Fig. 3*B*. Each value is the mean of triplicate determinations; *bars*, SD.

M_r 50,000–72,000 species, suggesting that the high molecular weight MMP-2 complex consisted predominantly of proMMP-2.

To further characterize MMP-2 activity, conditioned medium from Akt-transduced cells was incubated with the pan MMP inhibitor, BB-94 (Fig. 6*B*). BB-94 inhibited the gelatinolytic activity of the M_r ~85,000 form as well as lower molecular weight forms of MMP-2. Treatment of COMMA-1D/Akt1 and COMMA-1D/v-akt cells for 24 h with 100 nM BB-94, a noncytotoxic concentration, inhibited Matrigel invasion by 70% (Fig. 6*C*), suggesting a causal relationship between MMP-2 expression and invasion.

Immunolocalization of MMP-2 and Akt1. To determine whether MMP-2 was localized to the cell surface, MMP-2 expression was measured in COMMA-1D/Akt1 cells by immunohistochemical staining (Fig. 7*A*). MMP-2 was present in cell aggregates growing on Matrigel (Fig. 7A, *left panel*) but was barely visible in control cells (Fig. 7A, *left panel*). Immunohistochemical staining for Akt1 and v-akt indicated that both enzymes were concentrated in the plasma membrane and cytosol to the same degree (Fig. 7*B*).

Posttranslational Regulation of MMP-2 Expression. One mechanism by which Akt might regulate MMP-2 expression is by the modulation of proteasome activity either indirectly though GSK-3β inhibition (35, 36) or directly through MMP-2 phosphorylation. Therefore, cells were treated for 24 h with 1 μM lactacystin, a noncytotoxic concentration, and MMP-2 levels in the conditioned medium were determined (Fig. 8*A*). Lactacystin treatment dramatically increased MMP-2 levels in control cells but had only a marginal effect on MMP-2 levels in Akt1- and v-akt-transduced cells. Treatment of COMMA-1D/tv-a cells with lactacystin also resulted in a dramatic increase in their invasive activity but only a slight increase in the activity of COMMA-1D/Akt1 and COMMA-1D/v-akt cells (Fig. 8*B*).

DISCUSSION

The present study has examined the ability of Akt1 and its oncogenic homologue, v-akt, to activate ECM invasion in mouse mammary epithelial cells. To achieve this objective, we developed a cell line that is amenable to transduction by one or more recombinant avian retroviruses (37). This system is based on the introduction of the ALV subtype A receptor, tv-a, into mammalian cells making them permissive to infection by recombinant ALV (38). The mouse mammary epithelial cell line, COMMA-1D, was used for this retrovirus delivery system because these cells have been shown to differentiate *in vitro* and to give rise to normal gland structures in the cleared fat pad of the BALB/c mouse (39, 40). Although these immortalized mammary epithelial cells contain one mutated *p53* allele (41), they do not exhibit a transformed phenotype either *in vitro* or *in vivo* (39, 40).

Transduction of COMMA-1D/tv-a cells with Akt1 or v-akt led to an interesting dichotomy between their ability to induce transformation *versus* their ability to induce invasion. Although it is believed that the constitutive activity and plasma membrane localization of v-akt are responsible for its oncogenic activity (3, 33, 42), mammary epithelial cells were found to be susceptible to transformation by v-akt, but not by Akt1, despite little difference in membrane localization of these proteins (Fig. 7*B*); however, v-akt did exhibit 2.5-fold greater activity, which may account, in part, for its transforming activity. Because v-akt and Akt1 induced similar invasion activity and MMP-2 expression, differences other than absolute activity and membrane localization appear to be associated with MMP-2 expression. One clue to the mechanism for this effect may be deduced from the lactacystin experiments. Control cells treated with this proteasome inhibitor exhibited a dramatic increase in MMP-2 levels and invasion activity in comparison with its marginal effect on Akt- and v-akt-expressing cells. This suggests that the mechanism of action of Akt1 and v-akt is similar to that of lactacystin, *i.e.*, to block proteasome activity. This effect may occur indirectly though GSK-3β inhibition by Akt (35, 36) or directly through MMP-2 phosphorylation, although the latter possibility has not been reported.

Another way by which Akt may increase MMP-2 expression is by transcriptional activation. The MMP-2 promoter contains several *cis*-acting regulatory elements including cyclic AMP-responsive element binding, Sp1, Ets-1 and AP-2 (43). Akt is known to up-regulate ets-2 (44) and cyclic AMP-responsive element binding (45), and we are currently examining whether this is a potential regulatory pathway for MMP-2 expression by Akt1 in mammary epithelial cells.

ECM invasion depends on the cooperative processes of cell adhesion and membrane-associated proteolysis (46). MMP-2 has been implicated in ECM invasion in breast carcinoma cell lines (47–49) as well as in mammary epithelial cells transduced with H-Ras or stimulated by nitric oxide activators (50, 51). Invasion correlated with phosphoinositide 3-kinase and Akt activation in instances where ECM invasion was induced by epidermal growth factor or IGF-1 (52, 53). Akt activation has also been implicated in ECM adhesion and H-Ras-mediated transformation by blocking anoikis-induced cell death (54,

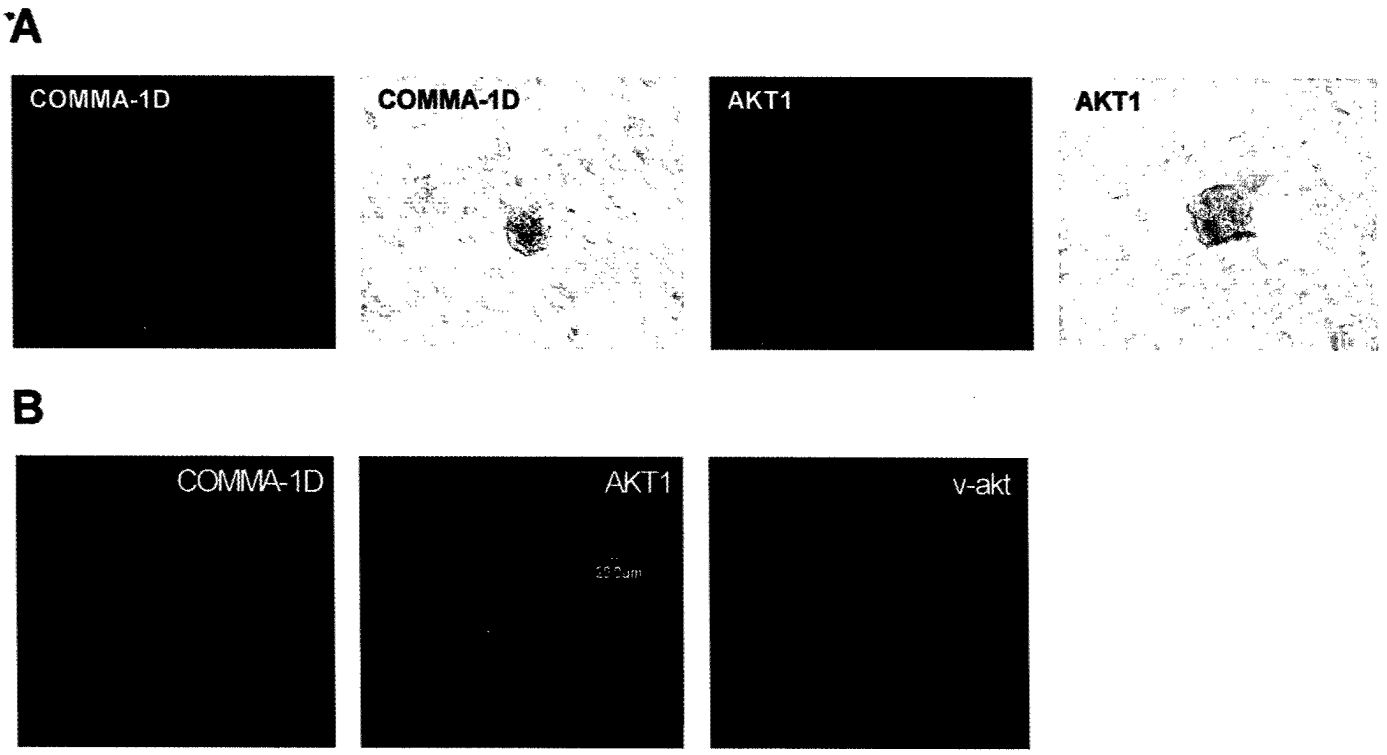

Fig. 7. Confocal microscopy of COMMA-1D/tv-a and COMMA-1D/Akt1 cells immunostained for MMP-2, Akt1 and v-akt. *A*, cells were grown for 60 h on Matrigel and stained for MMP-2 using a polyclonal MMP-2 antibody and a rhodamine-conjugated secondary antibody. Confocal immunofluorescence (*left panel*) or phase contrast microscopy (*right panel*) is shown. ×400. *B*, cells were grown as described in *A*, except that they were stained using a monoclonal Akt1 antibody and a Texas Red-conjugated secondary antibody. ×600.

55), and Akt has been shown to localize to sites of epithelial cell-matrix contact (56). MMP-2 localizes to the surface of invading tumor and endothelial cells and interacts through its COOH-terminal hemopexin-like domain with $\alpha v\beta 3$ integrin (57–59). Cleavage of the ECM by MMP-2 induces migratory activity of breast epithelial cells (60), suggesting a close relationship between cell adhesion and cell invasion in tissue remodeling. Because $\alpha v\beta 3$-mediated cell migration in tumor cells was dependent on Akt activation (61), Akt and MMP-2 appear to be critically linked to cell adhesion.

MMP-2 exists as a M_r 100,000 complex of proMMP-2 and TIMP-2, which results in multiple forms of secreted MMP-2 (23). Incubation of this complex with the cysteine-reactive compound, APMA, promoted the autocatalytic processing of proMMP-2 to active M_r 72,000 to M_r 50,000 species (Fig. 6*B*; Refs. 23, 24, 62). On the cell surface, proMMP-2 is activated by MT1-MMP after its association with TIMP-2 via its hemopexin domain (63). Formation of a complex of proMMP-2 with MT1-MMP correlated with ECM invasion where the complex localizes to invadopodia (64) and to sites of collagenolytic activity (25). Although MT1-MMP-dependent invasion was independent of proMMP-2 activation, both enzymes were required to be membrane-anchored to the cell surface to function as pericellular collagenases (25). Therefore, these studies suggest that the concentration of MMP-2 along basement membranes at sites of tissue remodeling and at the leading edge of invading cells (65, 66) is a major factor contributing to tumor progression and invasion (67–69). The present study further delineates that the Akt1 signaling pathway is involved in up-regulating this process and suggests a mechanism by which tumors with high Akt1 expression may be more invasive and metastatic.

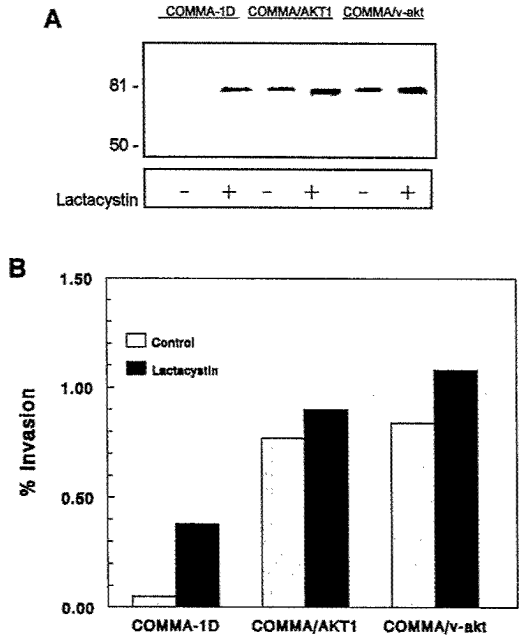

Fig. 8. Effect of lactacystin treatment on MMP-2 expression. *A*, cells were treated for 18 h with 1 μM lactacystin, and the conditioned medium was concentrated, separated by SDS-PAGE, and transferred to nitrocellulose. MMP-2 was detected by immunoblotting using a polyclonal MMP-2 antibody. Each lane contains 20 μg of protein. *B*, cells were treated for 24 h with 1 μM lactacystin, and Matrigel invasion assays were carried out as described in Fig. 3*B*. Each value is the mean of duplicate determinations; *bars*, SD.

ACKNOWLEDGMENTS

We thank Drs. Peter Vogt and Masahiro Aoki (Scripps Research Institute) for providing RCAS/Akt1 and RCAS/v-akt, Dr. Stephen Hughes (National Cancer Institute) for providing the RCAS vector and DF-1 cells, Dr. Yi Li (Memorial Sloan-Kettering Cancer Institute) for providing the *tv-a* cDNA, and Dr. Andrew Leavitt (University of California, San Francisco, CA) for providing the polyclonal anti-tv-a antibody.

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[CANCER RESEARCH 62, 3538–3543, June 15, 2002]

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

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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 Cα (PKCα) expression. Cells overexpressing Akt1 did not exhibit anchorage-independent growth, whereas PKCα overexpression produced significant transformation, although to a lesser extent compared with PDK1. Coexpression of Akt1 and PKCα led to a more than additive effect on transformation activity. Isografts of either PDK1- or PKCα-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 PKCα, 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[3] (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 PKCα, as well as an increase in PKCα levels, and expression of PKCα but not Akt1 in mammary epithelial cells resulted in transformation *in vitro* and tumorigenesis *in vivo*. These results suggest that the PKCα 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% CO_2 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/pThr308, PKCα, pSer657PKCα, PKCζ, 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 PKCβ, 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 NH_2-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 Masashiro Aoki, The Scripps Research Institute, La Jolla, CA. Rabbit PKCα cDNA was provided by Dr. Shigeo Ohno, Yokohama University, Yokohama, Japan. Akt1 and PKCα were amplified by PCR and subcloned into vectors pGADT7 and pGBKT7 (Clontech, Inc.), respectively, to obtain an NH_2-terminal hemaglutinin epitope tag for Akt1 and an NH_2-terminal myc epitope tag for PKCα. All of the cDNAs were then cloned into the retroviral vector pSRαMSVtkneo (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 pRc/CMV (Invitrogen). Either pSRαMSVtkneo or pSRαMSVtkneo encoding PDK1, 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+PKCα, Akt1+PDK1, or PKCα+PDK1, a second round of transduction was carried

Received 11/28/01; accepted 4/18/02.

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[1] Supported by National Cancer Institute Grant CA81565 and Department of Defense Grant DAMD17-99-9195.

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[3] The abbreviations used are: PI3-K, phosphoinositide 3-kinase; Akt1, protein kinase Bα; IGF-I, insulin-like growth factor-1; IMEM, Improved Minimal Essential Medium; PDK, 3-phosphoinositide-dependent protein kinase; CMV, cytomegalovirus; PKC, protein kinase C; S6K, ribosomal p70 S6 kinase-1; SGK, serum- and glucocorticoid-regulated kinase.

out using amphotropic 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, pSer657PKCα, 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 Na PP_i, 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 PKCα, 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 $\times$ *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-PKCα, 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, pSer657PKCα, 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 [^{32}P]ATP into a basic peptide substrate.

Soft Agar Assay for Anchorage-independent Growth. After eight passages of the retrovirally transduced cells, exponentially growing cells (1×10^5) were suspended in 2 ml 0.33% (w/v) SeaPlaque agar (BMA, Rockland, ME) dissolved in IMEM containing 1$\times$ supplement and layered over 1% agar dissolved in IMEM containing 1$\times$ 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 10^8 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$\times$ 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/PKCα cells produced adenocarcinomas, part of the tumor was removed aseptically and mechanically dispersed with a 28-gauge needle in 1$\times$ PBS. The suspension was centrifuged and replated in IMEM containing 1$\times$ 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 and Tissue Shared Resource, 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. 2*A*). The activity of PKCα, as measured by autophosphorylation of Ser657, was increased almost 7-fold in response to IGF-I stimulation (Fig. 2*B*), but

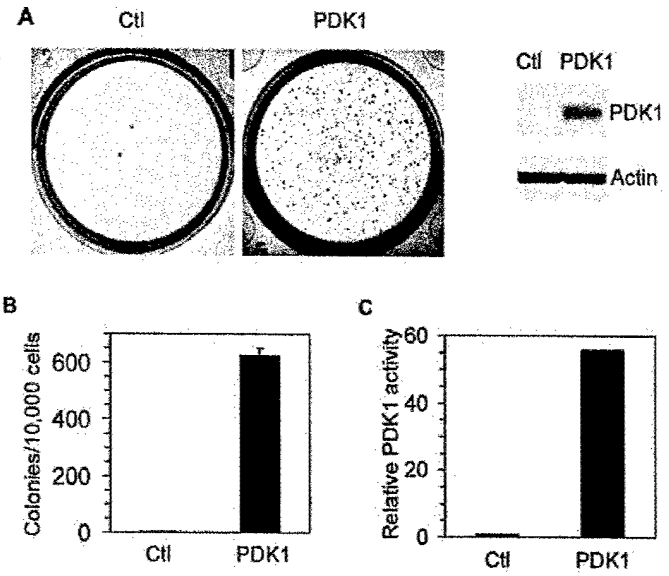

Fig. 1. Transformation of COMMA-1D cells by PDK1. *A*, COMMA-1D cells were retrovirally transduced with either *neo* (*Ctl*) or PDK1 (*PDK1*), and colony formation in soft agar was determined as the number of colonies per 10,000 cells plated. PDK1 and β-actin expression were determined in control (*Ctl*) and PDK1-expressing cells (*PDK1*) by immunoblotting with a myc-tag and β-actin antibody, respectively (*right*). *B*, quantitation of colony formation in soft agar as depicted in *A*. Each value is the mean of three experiments; *bars*, $\pm$SD. *C*, PDK1 activity was determined in control (*Ctl*) and COMMA-1D/PDK1 cells (*PDK1*) as described in "Materials and Methods," and is expressed in arbitrary units relative to control cells transduced with the empty retrovirus (*Ctl*). Each value is the mean of three experiments.

the phosphorylation of another PDK1 substrate, S6K, was not changed (Fig. 2*C*).

To determine whether PDK1 altered the levels of PKCα or other PKC isoforms, cell lysates were analyzed by Western blotting (Fig. 3). The steady state level of PKCα 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 PKCα activity, transformation was measured in COMMA-1D cells transduced with either Akt1 or PKCα (Fig. 4). In contrast to Akt1-expressing cells, which did not exhibit anchorage-independent growth in soft agar, PKCα-expressing cells showed significant growth, although less than PDK1-expressing cells (Fig. 4*A*). These results suggested that a portion of the transforming ability of PDK1 might be caused by the up-regulation of PKCα. When cells expressing PKCα were cotransduced with Akt1, cell transformation was increased by 20% in comparison to cells expressing PKCα alone (Fig. 4*B*), indicating a small degree of potentiation between the Akt1 and PKCα signaling pathways. When either Akt1 or PKCα was coexpressed with PDK1, no additional change in transformation occurred (Fig. 4*C*).

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. 5*A*, *Ctl*) or Akt1 (Fig. 5*A*, *Akt1*) exhibited a normal ductal morphology. In contrast, all mice receiving isografts of COMMA-1D/PDK1 (Fig. 5*A*, *PDK1*) or COMMA-1D/PKCα (Fig. 5*A*, *PKCα*) 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). PKCα-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. 5*B*, *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 M_r 60,000 form in addition to the M_r 63,000 form of PDK1 and may represent an NH_2-terminally truncated PDK1.

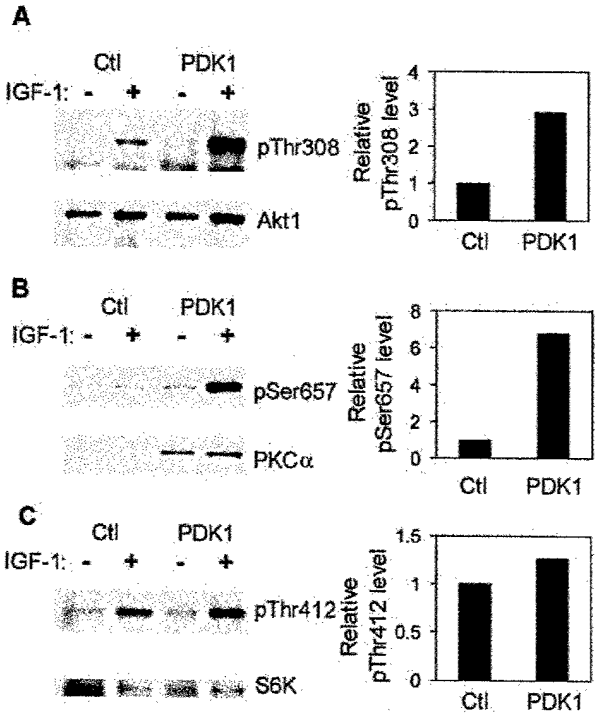

Fig. 2. Akt1 and PKCα activity, but not S6K activity are increased in COMMA-1D/PDK1 cells. *A*, COMMA-1D cells were retrovirally transduced with either *neo* (*Ctl*) or PDK1 (*PDK1*), serum-starved overnight, and then grown in the absence (−) or presence (+) of 100 ng/ml IGF-I. Akt1 was immunoprecipitated from cell lysates, and pThr308Akt1 and Akt1 were measured by immunoblotting. The *bar graph* represents the net increase in pThr308 produced by IGF-I normalized to the level of Akt1. *B*, cell lysates were prepared as described in *A*, PKCα was immunoprecipitated, and pSer657PKCα and PKCα were measured by immunoblotting. The *bar graph* represents the net increase in pSer657 normalized to the level of PKCα. *C*, cell lysates were prepared as described in *A*, S6K was immunoprecipitated, and pThr412S6K and S6K were measured by immunoblotting. The *bar graph* represents the net increase in pThr412S6K normalized to the level of S6K.

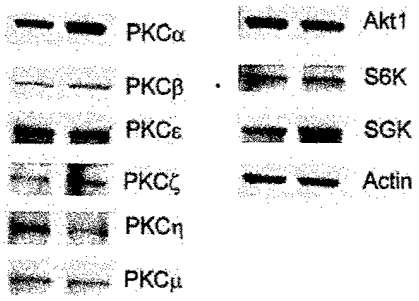

Fig. 3. PKC, Akt1, S6K, and SGK levels in COMMA-1D cells. Cell lysates were prepared from COMMA-1D/PDK1 cells, and protein kinase levels were analyzed by Western blotting as described in "Materials and Methods."

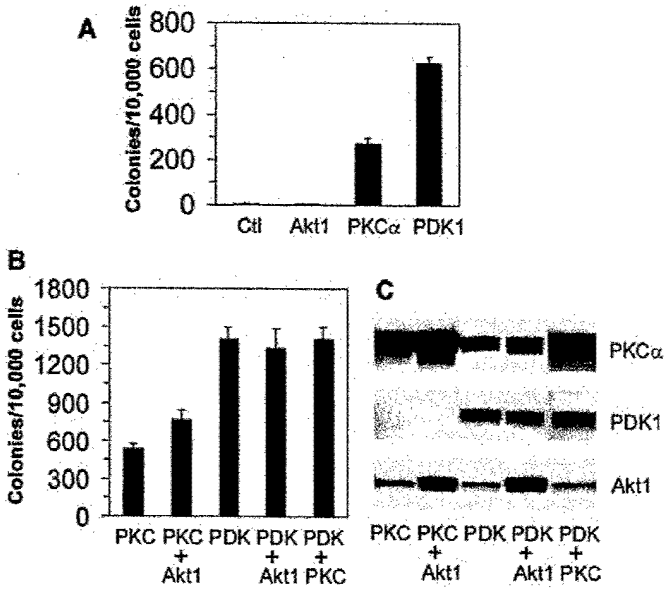

Fig. 4. Akt1 enhances PKCα-mediated transformation of COMMA-1D cells. *A*, COMMA-1D cells were retrovirally transduced with *neo* (*Ctl*), Akt1 (*Akt1*), PKCα (*PKCα*), or PDK1 (*PDK1*). Colony formation in soft agar was determined as the number of colonies per 10,000 cells plated. Each value is the mean of three determinations; *bars*, ±SD. *B*, a second round of retroviral transduction was carried out in COMMA-1D/PKC cells with either *hyg* (*PKC*) or Akt1 (*PKC+Akt*), or in COMMA-1D/PDK1 cells with *hyg* (*PDK*), Akt1 (*PDK+Akt*), or PKCα (*PDK+PKC*). 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*.

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,[4] which may contribute to enhanced PKCα expression in this cell line. PDK1 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).

[4] B-K. Park, X. Zeng, and R. I. Glazer, unpublished observations.

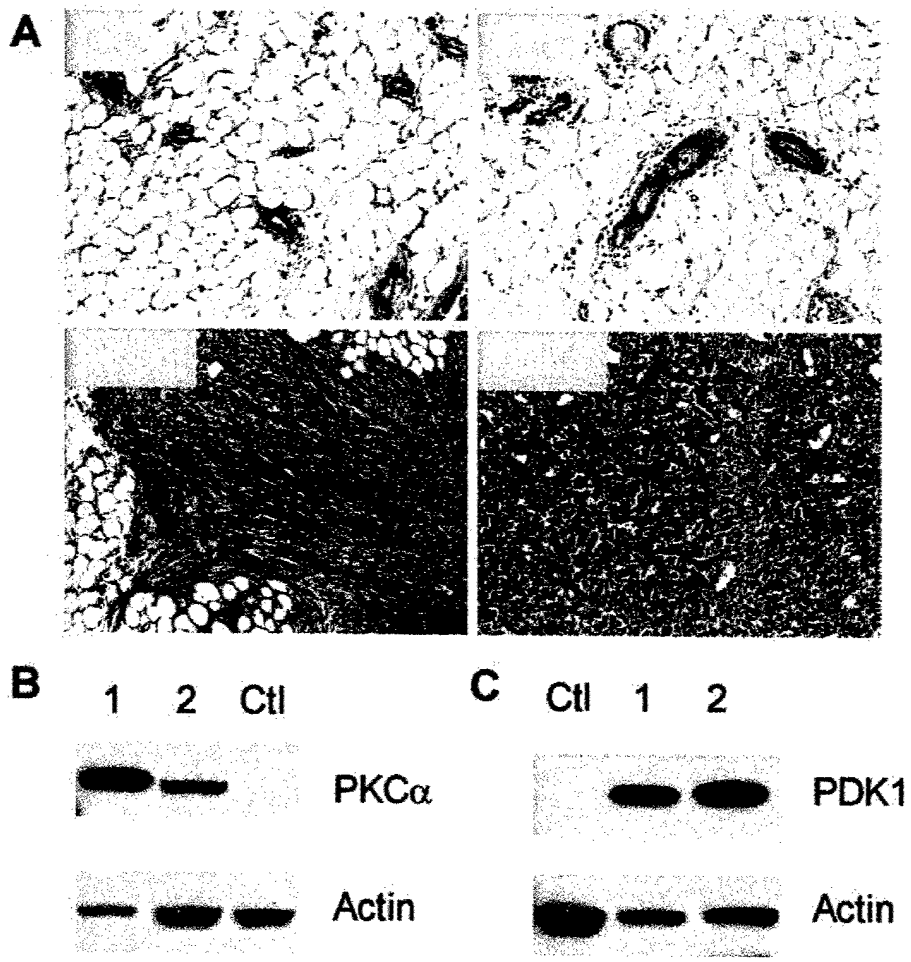

Fig. 5. Neoplastic transformation in isografts of COMMA-1D/PDK1 and COMMA-1D/PKCα cells. *A*, H&E-stained paraffin-embedded tissue obtained from isografts of COMMA-1D cells retrovirally transduced with *neo* (*Ctl*), Akt1 (*Akt1*), PKCα (*PKCα*), or PDK1 (*PDK1*). A representative section is shown for each tissue. Tumors derived from COMMA-1D/PKCα 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 PKCα expression in COMMA-1D/PKCα cells (*Lane 1*) and in a cell line derived from its respective tumor (*Lane 2*), and maintained in tissue culture. PKCα 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.

Alternative signaling pathways other than those directly associated with PDK1 may also be involved in the regulation of PKCα and Akt1. Ha-Ras and polyoma middle T antigen activate PI3-K (43–45), and induce and activate PKCα (29, 46), as well as Akt1 activity (47, 48). Because PKCα is also an upstream activator of the Ras/Raf pathway (49), PKCα 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 PKCα can directly stimulate Akt1 phosphorylation at Ser473 (51, 52), this could account for the small degree of potentiation of PKCα-mediated transformation by Akt1.

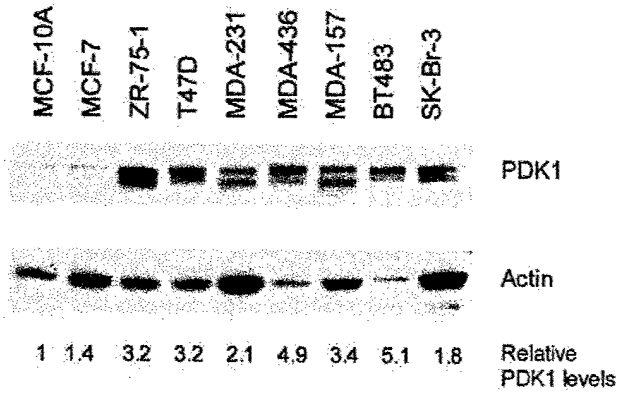

Fig. 6. PDK1 expression in human breast cancer cell lines. PDK1 levels in human breast cancer cell lines. Cell lysates were prepared, and Western blotting was carried out as described in "Materials and Methods." The relative level of PDK1 *versus* MCF-10A cells is denoted at the bottom of the figure.

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 PKCα expression in MCF-7 breast cancer cells leads to enhanced 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/PDK1cells in the present study.

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

malignant transformation *in vivo*. Although, PDK1 up-regulated PKCα expression and Akt1 activity, only expression of PKCα was associated with transformation and tumor formation. These results suggest that the PKCα signaling pathway is a major component of the PDK1 oncogenic pathway.

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