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Polyoma Middle T Antigen Activates the Ser/Thr Kinase Akt in a PI3-Kinase-Dependent Manner[1]

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Polyoma middle T antigen (PMT) was originally identified as the tumorigenic component of the polyomavirus genome. To investigate whether the serine/threonine kinase Akt/PKB, which is the proto-oncogene transduced by the transforming AKT8 retrovirus, is activated by PMT, 3T3-L1 fibroblasts were stably transfected with wild type PMT. PMT expression accelerated glucose transport and increased phosphorylation of p70 S6-kinase and MAPK. PMT expression also stimulated Akt kinase activity 7 fold as compared to untreated, mock infected cells. This stimulation rivaled that obtained following insulin treatment of both mock and PMT infected cells. Akt activation and phosphorylation were eliminated in a PMT mutant incapable of interacting with PI3-kinase, but not one which does not interact with Shc, and correlated closely to the amount of PI3-kinase activity in anti-phosphotyrosine immunoprecipitates. These results indicate that the PI3-kinase pathway is requisite, but the Shc pathway is dispensable, for Akt activation. The studies further suggest that Akt may participate in PMT and PI3-kinase's regulation of cellular transformation and tumorigenesis.

Inoculation of mice with the murine polyomavirus gives rise to a diverse range of tumors (1). A milestone in polyomavirus research was the discovery of polyoma middle T antigen (PMT), one of three proteins encoded by the polyomavirus genome (2) as the source of transformation initiation (3). Expression of PMT DNA in vivo by either direct injection (4) or retroviral infection (5-7) results in the development of endothelial tumors or hemangiomas. Similarly, transgenic animals expressing PMT develop mammary adenocarcinomas, hemangiomas, or neuroblastomas (8-10). Work in cultured cells further emphasizes the importance of PMT in cellular transformation. Expression of the 56 kDa protein in various cell lines induces a fully transformed phenotype (3) characterized by accelerated rates of glucose uptake (11), increased phosphorylation of ribosomal protein S6 (12) and MAPK (13), enhanced expression of early response genes (14), and increased levels of the 3′phospholipids PI-3,4-P2 and PI-3,4,5-P3 (15,16).

PMT is a docking protein which initiates cellular responses by complexing with other signaling molecules. This mode of action is analogous to that used by the cytoplasmic tails of activated growth factor receptors. Once phosphorylated by the cellular tyrosine kinases Src, Yes, and Fyn (17,18), PMT recruits Shc (19), PI3-kinase (12) protein phosphatase 2A (20), PLC-γ1 (21), and 14-3-3 proteins (22). Shc binding requires phosphorylation of tyrosine-250 (19) while PI3-kinase requires phosphorylation of tyrosine-315 (12). Mutations which abolish either of these interactions (315YF or 250YS) greatly reduce the protein's transforming ability (19,23,24); both are unable to induce growth in soft agar (25) and the 315YF mutant has a weakened potential to stimulate tumor formation in newborn mice (26) and glucose uptake (27) and pp70 S6-kinase (24) in fibroblast cell lines.

The ser/thr kinase Akt may link PI3-kinase to cellular transformation. While there is abundant evidence that many agonist require PI3-kinase for activation of Akt, the role of the Ras-dependent signaling pathways remains unclear. An oncogenic form of Akt was identified as the transforming component of AKT8, an acute transforming retrovirus isolated from a rodent T-cell lymphoma (28). Moreover, cellular forms of Akt1 and Akt2 are overexpressed in a number of ovarian (29), gastric (30), and pancreatic (31) carcinomas. The following experiments demonstrate that PMT expression into 3T3-L1 fibroblasts markedly stimulates Akt kinase activity as compared to mock infected cells. Furthermore, studies with various polyoma middle T antigen mutants demonstrate that PI3-kinase, but not the Shc signaling pathway, is required for Akt phosphorylation and activation.

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METHODS

Antibodies. Polyclonal anti-Akt antibodies were raised against the sequence CDQTHFPQFSYSASIRE found in Akt2. Polyclonal anti-phospho S6 antibodies were raised against the major phosphorylation site in the ribosomal S6 subunit [CRRLS-(P)S-(P)LRAS-(P)TSKS-(P)EES-(P)QK]. Monoclonal mouse anti-polyoma middle T antigen antibodies raised against a common region in small, middle, and large tumor antigen from the polyomavirus genome and were generously provided by Jean Dahl, Harvard Medical School (32). Anti phospho-Akt antibodies were from New England Biolabs (Beverly, MA) and anti-Shc and agarose bound anti-phosphotyrosine antibodies and were from Upstate Biotechnology, Inc. (Lake Placid, NY)

Generation of clonal polyoma middle T-expressing cell lines. Plasmids encoding wild type middle T antigen (PMT), 315YF-PMT (315YF), and 250YS-PMT (250YS) were cloned into the murine retrovirus PWZL-neo, which bears a neomycin resistance gene to allow for selection of infected cells. 315YF-PMT encodes a polyoma middle T antigen construct with the tyrosine at position 315 changed to a phenylalanine, thus preventing its association with the 85 kDa regulatory subunit of PI3-kinase. Similarly, 250YS-PMT encodes a construct with the tyrosine at position 250 changed to a serine, thus preventing its association with Shc. Constructs were packaged into replication-incompetent retrovirus that was used to infect 3T3-L1 fibroblasts using the method of Hudson et al. (33). Cells infected with the retrovirus were selected with 800 μg/ml G418 (active concentration) and colonies of G418 resistant cells were cloned. Of over 150 different clones selected, three were selected which demonstrated similar levels of expression for further experimental analysis.

Immunoblot analysis of total cell lysates. Confluent 100mm diameter dishes containing each of the four cell lines were serum starved for 18-24 hrs in DMEM supplemented with 0.5% BSA and 10 mM HEPES. Following stimulation at 37°C with or without 1 μM insulin, cells were washed twice with ice cold PBS and lysed in 100μl of 66 mM Tris (pH 8.0) containing 2% SDS and 100 mM vanadate. The sample was boiled for 2 minutes and the DNA sheared by passing the extract through a 27 gauge needle several times. Protein concentrations were determined using the bicinchoninic protein assay kit from Pierce and 40 μg of total protein was loaded onto a 7.5 or 10% PAGE gel. Proteins were transferred to nitrocellulose and blotted with the appropriate antibodies. Antibody detection was performed using the enhanced chemiluminescence kit from Amersham.

Immunoprecipitation of phosphotyrosine-containing proteins. Briefly, confluent 100mm diameter dishes containing each of the four cell lines were serum starved and stimulated with insulin as described above. Cells were washed twice with ice cold PBS and then solubilized in ice cold lysis buffer (20mM TRIS-Cl, ph7.5, 137 mM NaCl, 10% glycerol, 1% NP-40, 1 mM CaCl2, 1 mM MgCl2, and 100μM Na3VO4). Following centrifugation at 13,000g for 10 minutes to remove insoluble material, the supernatant was mixed with agarose bound monoclonal mouse anti-phosphotyrosine antibody. Proteins were separated by SDS-PAGE, transferred to nitrocellulose, and Western blotted with indicated antibodies.

PI3-kinase assays. PI3-kinase assays were performed using the method of Sun et al.(34).

Akt kinase assays. For Akt kinase assays, cells were solubilized as above and incubated with 5 μl anti-Akt antibodies. Following a 1-3h incubation at 4C, 20 μl of washed protein A agarose (Gibco/BRL) was added and the incubation extended for another 20-30 minutes. The protein A was then washed twice in ice cold lysis buffer without protease inhibitors, twice in ice cold high salt buffer (25 mM HEPES, ph7.2, 10% glycerol, 1% Triton X-100, 1M NaCl, and 0.1% BSA), and twice in room temperature kinase buffer (20mM HEPES, ph7.2, 10mM MgCl, 10mM MnCl, and 1mM DTT). The kinase reaction is initiated by the addition of 40 μl kinase mix (20 mM HEPES, pH 7.2, 10mM MgCl, 10mM MnCl, 1mM DTT, 5μM ATP, 0.2mM EGTA, 2μgm/rxn protein kinase inhibitor from Sigma (cat#P-0300), 25 μgm/rxn Histone H2B (Boehringer Mannheim cat#223-514), and 5μCi/rxn [gamma-32P] ATP (Amersham cat#AA-0018 redivue >5000Ci/mmol). The reaction is allowed to proceed for 20-30 minutes at room temperature and is then stopped by the addition of an equal volume of 2X Laemmli buffer, and boiling for 2min. Beads are pelleted by centrifugation, and the entire reaction was loaded on a 12.5% PAGE gel, transferred to nitrocellulose, and processed on a phosphorimager.

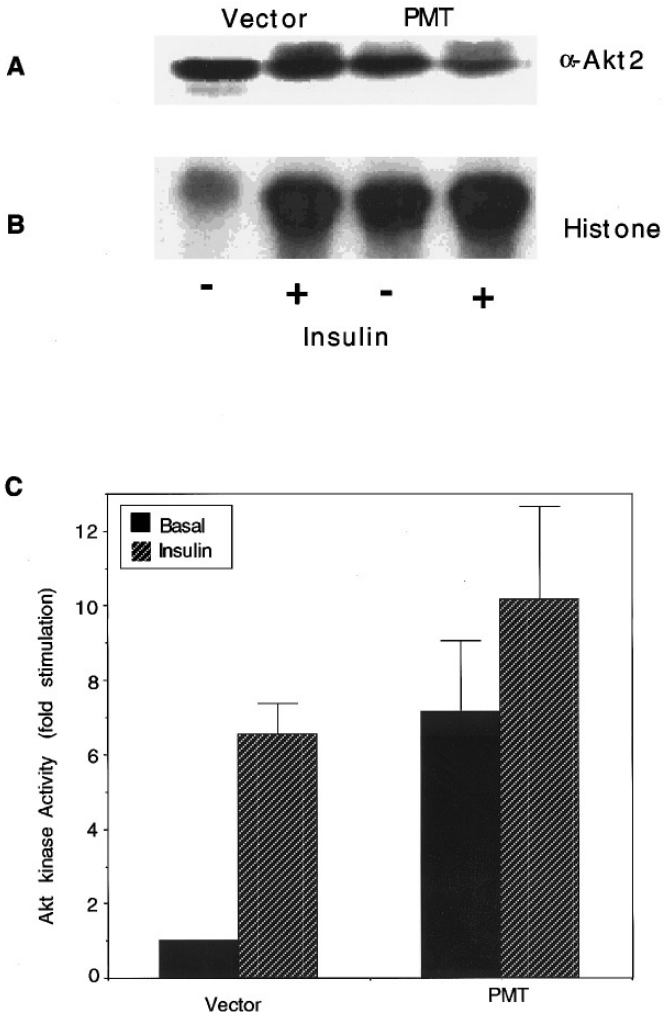

FIG. 1. PMT stimulates Akt kinase activity. 3T3-L1 fibroblasts stably expressing vector or PMT were serum deprived in L-15 medium for 2 hours prior to stimulation for 15 minutes with or without 1 μM insulin. In A, total cell lysates were separated by SDS page, transferred to nitrocellulose, and immunoblotted with anti-Akt2 antibodies. In B and C, Akt kinase activity towards histone was determined. Experiments are representative of 5 independent experiments.

RESULTS

Stable expression of PMT into 3T3-L1 fibroblasts was associated with a 7 fold stimulation in Akt kinase activity towards histone and a corresponding mobility shift on polyacrylamide gels (Fig. 1). Subsequent insulin addition had little effect. To investigate the relative contribu-

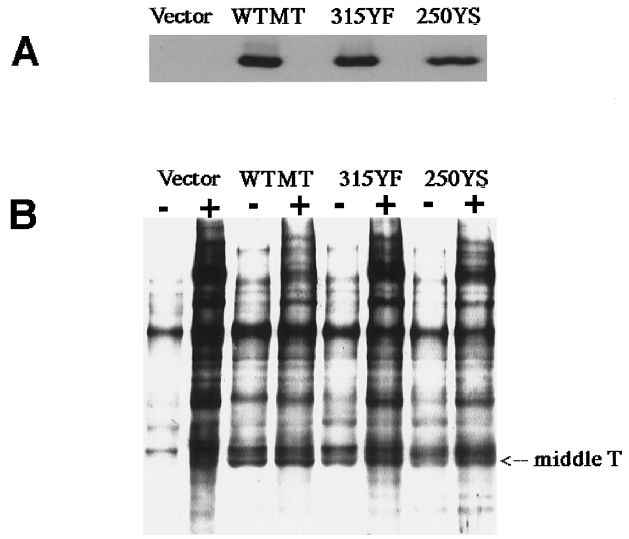

FIG. 2. Expression of PMT, 315YF, and 250YS. 3T3-L1 fibroblasts stably expressing vector, PMT, 315YF, or 250YS were serum deprived in L-15 medium for 2 hours prior to stimulation for 15 minutes with or without 1 μM insulin. Total cell lysates were separated by SDS page, transferred to nitrocellulose, and immunoblotted with anti-PMT (A) or anti-phosphotyrosine (B) antibodies.

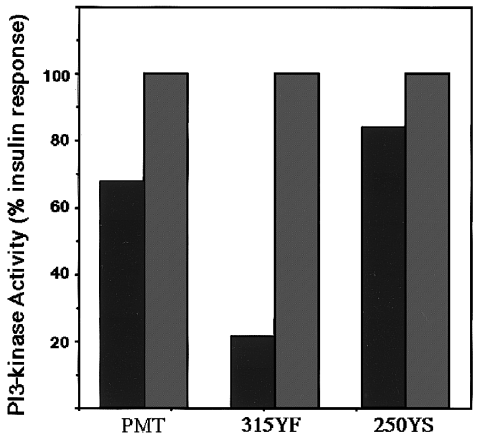

FIG. 3. PI3-kinase activity in anti-phosphotyrosine immunoprecipitates. 3T3-L1 fibroblasts stably expressing vector, PMT, 315YF, or 250YS were serum deprived in L-15 medium for 2 hours prior to stimulation for 15 minutes with or without 1 μM insulin. Following precipitation with anti-phosphotyrosine antibodies, PI-kinase activity was measured. Results are normalized to the amount of PI3-kinase activity found in anti-phosphotyrosine precipitates following insulin stimulation and are representative of two independent experiments.

tion of the PI3-kinase and Shc pathways to Akt activation, the 315YF and 250YS mutants described were also stably expressed in 3T3-L1 fibroblasts using retroviral infection. Clonal cell lines which expressed comparable levels of PMT, 315YF, or 250YS were isolated (Fig. 2A) were selected for further analysis. Anti-phosphotyrosine immunoblots indicate that PMT and its respective mutants tyrosine phosphorylate a similar array of proteins, including one which co-migrates with PMT (Fig. 2B). The relative PI3-kinase activity in anti-phosphotyrosine immunoprecipitates was determined by incubating the immune complexes with phosphatidylinositol and [32P]-ATP and measuring the incorporation of [32P] into phosphatidylinositol phosphate. Significantly less PI3-kinase activity was precipitated from cell lines expressing the 315YF mutant (Fig. 3). In contrast, when the anti-phosphotyrosine immunocomplexes were probed for Shc protein, significantly less Shc protein was associated with the 250YS mutant (Fig. 4).

The pattern of Akt activation recapitulated the stimulation in PI3-kinase activity. Whereas PMT and 250YS alone stimulated Akt ~70% as well as insulin, the 315YF mutant stimulated Akt kinase activity only ~20% as well (Fig. 5). These results indicate that stimulation of PI3-kinase is a prerequisite, but the Shc signaling cascade is dispensable, for subsequent Akt activation.

Although PI-3,4-P2 can activate Akt directly in vitro (35-37), the protein is regulated primarily by phosphorylation (38,39). To investigate whether PMT promotes phosphorylation of Akt, and whether phosphorylation of this site is dependent upon PI3-kinase, total cell lysates were probed with antibodies raised against the phosphorylated form of the Akt carboxyl-terminus. These antibodies recognize the phosphorylated S473, a site previously reported as critical for complete Akt activation (38,39). The upstream kinase responsible for phosphorylating this residue has not been identified. The degree of phosphorylation on this residue corresponded to the degree of Akt activation and the amount of PI3-kinase in anti-phosphotyrosine immunoprecipitates. Whereas PMT and 250YS promoted phosphorylation of Akt nearly as well as insulin, 315YF did not stimulate Akt phosphorylation appreciably in the absence of insulin (Fig. 6).

DISCUSSION

PI3-kinase activity was first discovered in a complex with a viral src protein and polyoma middle T antigen

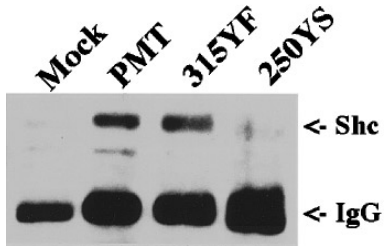

FIG. 4. p66 Shc in anti-phosphotyrosine immunoprecipitates. Phosphotyrosine containing proteins from 3T3-L1 fibroblasts expressing vector, PMT, 315YF, or 250YS were immunoprecipitated, separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with anti-Shc antibodies. Data are representative of two independent experiments.

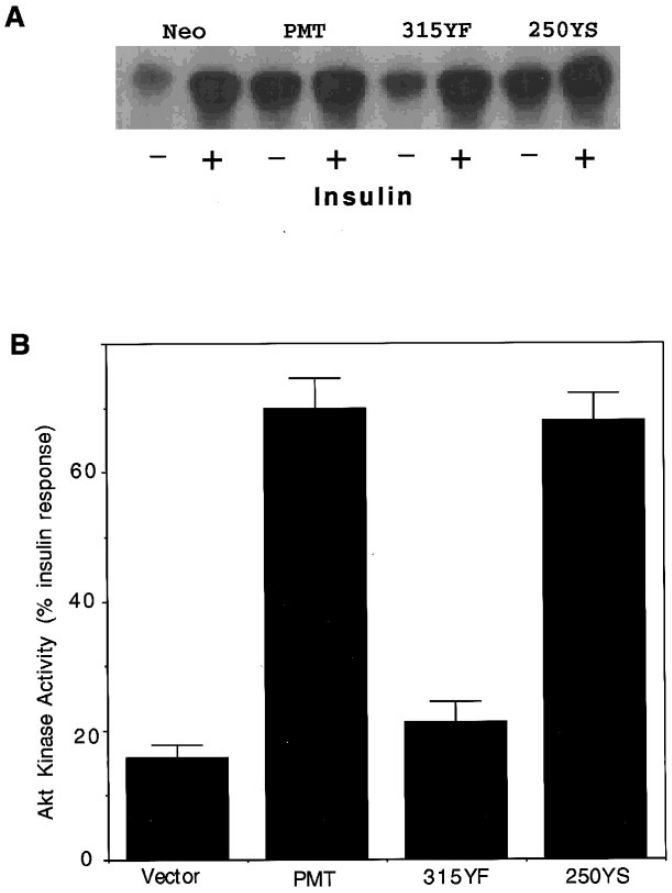

FIG. 5. PMT and 250YS, but not 315YF, stimulate Akt kinase activity. 3T3-L1 fibroblasts stably expressing vector, PMT, 315YF, or 250YS were serum deprived in DMEM containing 0.2 % BSA and 10 mM HEPES for 24 hours prior to stimulation for 15 minutes with or without 1 μM insulin. Akt kinase activity towards histone was observed by autoradiography (A) or quantitated on a phosphorimager (B). Data are means +/− S.E.M of 5 independent experiments.

(40). Studies describing the weakened transforming potential of PMT mutants incapable of activating PI3-kinase suggest a role for the enzyme in neoplastic transformation. Moreover, a retrovirus-encoded PI3-kinase can cause haemangiosarcomas in chickens and can transform chicken embryo fibroblasts in culture (41). Additionally, transformed cells have increased basal PI3-kinase activity (41,42). The anionic phospholipids produced by PI3-kinase are not degraded by phosphoinositide specific phospholipase C (PI-PLC) (43) and their mechanism of action in cellular transformation is not fully understood.

Several recent studies indicate that PI3-kinase is necessary for Akt activation by peptide growth factors. Stimulation of Akt is blocked by the following: (1) expression of growth factor receptor mutants incapable of activating PI3-kinase, (2) expression of dominant negative forms of PI3-kinase; or, (3) treatment with PI3-kinase inhibitors (44-46). Whether PI3-kinase is sufficient to stimulate Akt kinase activity is less clear. Although overexpression of activated forms of PI3-kinase stimulates Akt kinase activity in various cell systems (35,36,41,46), whether the same activation can be accomplished by maximal stimulation of endogenous PI3-kinase is uncertain. For example, although overexpression of PI3-kinase is sufficient to stimulate glucose transporter GLUT4 translocation (47,48), activation of endogenous PI3-kinase by an activating peptide, PDGF, or IL-4 is not sufficient (49-51). This study demonstrates that PMT mediated activation of endogenous PI3-kinase, without activation of the Shc signaling cascade, promotes Akt activation and phosphorylation. Future studies will address whether some additional signaling event in addition to PI3-kinase, but exclusive of Shc signaling pathway, is important for PMT's activation and phosphorylation of Akt.

The role of Akt in cellular transformation and/or proliferation is unclear. To date, Akt is implicated in regulation of various metabolic events [i.e., stimulation of glu-

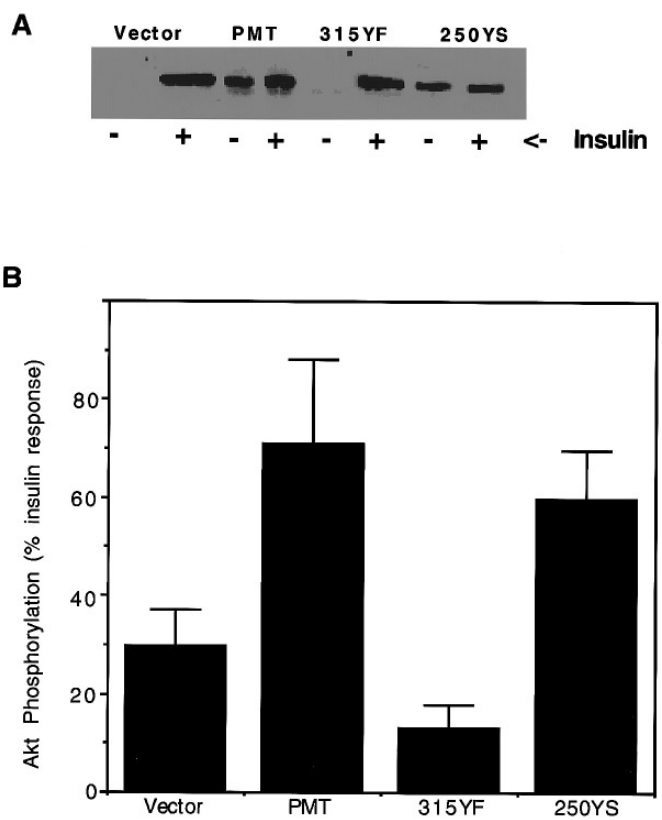

FIG. 6. PMT and 250YS, but not 315YF, stimulate Akt phosphorylation. 3T3-L1 fibroblasts stably expressing vector, PMT, 315YF, or 250YS were serum deprived in L-15 medium containing 0.2 % BSA 2 hours prior to stimulation for 15 minutes with or without 1 μM insulin. Total cell lysates were separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with anti-phospho-Akt antibodies. Akt phosphorylation was visualized by chemiluminescence (A) or quantitated on a densitometer (B). Data are means +/− S.E.M of 5 independent experiments.

cose transporter GLUT4 translocation, lipogenesis, glycogen synthesis, and others (39,52-54)] activation of pp70 S6-kinase through an unknown intermediate kinase (44) protection from apoptosis through Bcl2 induction and BAD phosphorylation (55-57), and induction of cell cycle progression through c-myc induction (57). 3T3-L1 fibroblasts expressing PMT do not express GLUT4 (data not shown), however, they do demonstrate accelerated glucose transport attributable in part to a post-translation modification of the glucose transporter GLUT1 (27). Moreover, cells expressing PMT had increased phosphorylation of p70 S6 kinase and the ribosomal S6 subunit (data not shown). Whether Akt contributes to these events, or has some other role in transformation, will require further investigation.

Polyomavirus is a powerful carcinogen which causes tumors in vivo with very short latency periods (3-4 weeks) (58). Moreover, PMT apparently interacts with a relatively small number of cellular proteins, simplifying the search for altered molecules which participate in tumorigenesis. The studies above indicate that PMT activates Akt via a PI3-kinase dependent mechanism, and suggest that Akt could participate in some of the multiple events leading to cellular transformation.

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