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Phosphatidylinositol 4,5-bisphosphate (PIP₂)-specific AKT1 is oncogenic

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Abstract

The protein kinase AKT1 (v-akt murine thymoma viral oncogene homologue 1), also referred to as PKB (protein kinase B), is an essential mediator of the phosphatidylinositol 3-kinase signaling pathway. Elevated activity of AKT1 is common in human cancer. Localization at the plasma membrane, leading to enhanced phosphorylation and activation of AKT1, is an important factor determining the oncogenicity of this kinase. Although the phosphatidylinositol 3-kinase signaling pathway is frequently upregulated in cancer, cancer-specific mutations in AKT1 are not common. Recently, such a mutation has been identified in breast, colon and ovarian cancers. The mutation is located in the pleckstrin homology (PH) domain of AKT1 and results in a glutamic acid to lysine substitution at residue 17. The resultant change in the conformation of the PH domain facilitates membrane binding of the mutant protein. Here we show that exchange of the PH domain leading to preferential binding of phosphatidylinositol 4,5-bisphosphate (PIP₂) over phosphatidylinositol 3,4,5-trisphosphate (PIP₃) constitutively activates AKT1. AKT1 with this altered PIP affinity induces oncogenic transformation in cultures of chicken embryo fibroblasts and causes neoplastic growth and angiogenesis in the chorioallantoic membrane of the chicken embryo. Gain-of-function mutants of AKT1 may not be affected by PI3K inhibitors that are currently in development. Therefore, AKT1 remains a distinct and important cancer target.

The serine/threonine kinase AKT1 (v-akt murine thymoma viral oncogene homologue 1), also referred to as PKB (protein kinase B), is an important target of PI3K (phosphatidylinositol 3-kinase) and a central mediator of the PI3K signaling pathway. Upon activation of receptor tyrosine kinases or G-protein coupled receptors by growth factors or other extracellular stimuli, PI3K Class I enzymes catalyze the production of phosphatidylinositol 3,4,5-trisphosphate (PIP₃) from phosphatidylinositol 4,5-bisphosphate (PIP₂). The PH domain of AKT1 binds to PIP₃, facilitating the recruitment of the enzyme to the cell membrane. AKT1 then becomes phosphorylated at threonine 308 (T308) and serine 473 (S473) ^{1–5}. The binding of AKT1 to PIP₃ induces a conformational change which makes T308 accessible for phosphorylation by PDK1 (3-phosphoinositide-dependent kinase) and is the crucial step in AKT1 activation ^{6–11}. Both phosphorylations at T308 and S473 are essential for a complete activation of the kinase function of AKT1 ¹².

Enhanced membrane recruitment and elevated AKT1 kinase activity are frequently observed in human cancer. Constitutive activation of AKT1 is commonly induced by recurring mutations

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in enzymes that directly or indirectly regulate levels of PIP₃. Among these are gain-of-function mutations in receptor tyrosine kinases and in the catalytic subunit p110 α of PI3K and loss-of-function mutations in PTEN (phosphatase and tensin homologue lipid phosphatase), the antagonist of PI3K^{13–20}. These genetic changes lead to elevated levels of PIP₃ that facilitate increased membrane recruitment and phosphorylation of AKT1. In the absence of enhanced membrane association, overexpressed wild-type AKT1 is not oncogenic *in vitro* or *in vivo*. Fusion to a membrane-targeting myristylation signal leads to constitutive activity and oncogenicity²¹. All isoforms of Akt have been observed to be amplified or overexpressed in human cancer, but this change alone is insufficient to cause transformation^{21–23}.

As an intermediary between mutated upstream regulatory proteins and downstream signaling molecules, AKT1 seemed to have a passive role in human cancer, until Carpten and coworkers discovered a cancer-specific mutation that results in a glutamic acid to lysine substitution at amino acid 17 in the PH domain of AKT1²⁴. The E17K mutation alters the affinity of AKT1 for phosphoinositides²⁵. The mutation leads to elevated membrane association of AKT1 and to constitutive activation of the kinase^{24–27}.

In this study, we determined the properties of the E17K mutation in greater detail and explored the role of the PH domain-PIP interaction in oncogenesis and signaling. The PH domain of wild-type AKT1 was substituted with either the PH domain of general receptor for phosphoinositides-1 (GRP1) that is specific for PIP₃ or the PH domain of phospholipase C-delta1 (PLC δ 1), specific for PIP₂^{28, 29}. E17K-AKT1, GRP-AKT1 and PLC-AKT1 were studied in tests for oncogenic potential and signaling. Wild-type AKT1 served as negative control, and the constitutively active and oncogenic myristylated AKT1 (myrAKT1) served as positive control²¹ (Figure 1a). To facilitate identification, the AKT1 proteins used in this study were fused to a C-terminal HA-tag. We expressed the different proteins from the retroviral vector RCAS in chicken embryonic fibroblasts (CEF). Cells transfected with this vector release infectious avian retrovirus that expresses the insert and infects surrounding cells. Tests for oncogenic potential were carried out on monolayers of primary CEF. In this system, transformed cells, under an overlay of nutrient agar, grow into multilayered microtumors, termed foci³⁰. E17K-AKT1 induced such foci of transformation in CEF, confirming the oncogenic activity seen in previous experiments with Rat1 cells²⁴. E17K-AKT1 was comparable in oncogenic activity to the positive control, myrAKT1. The PLC-AKT1 chimera was also potently transforming. In contrast, wild-type AKT1 did not induce focus formation within 17 days of incubation, and the oncogenic activity of GRP-AKT1 was marginal, only detectable at high concentrations of transfected DNA (Figure 1b). The efficiencies of transformation for the various constructs were as follows: E17K-AKT1: 400 foci/ μ g plasmid DNA, PLC-AKT1: 750 foci/ μ g plasmid DNA, myrAKT1: 650 foci/ μ g plasmid DNA and GRP-AKT1: 20 foci/ μ g plasmid DNA.

Tests for *in vivo* oncogenicity were carried out on the chorioallantoic membrane (CAM) of the chicken embryo. The CAM is a vascularized membrane, located underneath the shell and engulfing the embryo. It is a readily accessible and sensitive model for the evaluation of angiogenesis and tumor formation *in vivo*^{31–33}. The CAMs of 9-day-old chicken embryos were inoculated with the various AKT1 constructs. Inoculation with wild-type AKT1 or empty RCAS vectors served as negative controls, and the oncogenic myrAKT1 served as a positive control. All CAMs inoculated with myrAKT1, E17K-AKT1 or PLC-AKT1 displayed increased vascularization and formation of neoplastic nodules (Figure 2). Areas with abnormal cell growth were marked by elevated angiogenesis. Histological analysis of the tumor confirmed the malignant nature of the growths on E17K-AKT1, PLC-AKT1- and myrAKT1-inoculated CAMs (data not shown). One CAM out of five inoculated with CEF expressing the non-transforming GRP-AKT1 variant showed a small area of hyperplasia that did not reveal

malignant features in hematoxylin- and eosin-stained sections, confirming the observations in cell culture.

Activated AKT1 is phosphorylated at T308 and S473 and catalyzes the phosphorylation of numerous downstream targets. Among them glycogen synthase kinase-3 β (GSK3 β), a substrate of AKT1, and ribosomal protein S6, a substrate of p70S6K which is activated downstream of AKT1. In CEF expressing E17K-AKT1, PLC-AKT1 or myrAKT1, AKT1 was highly phosphorylated at T308 and S473 in the presence of serum and under serum-starved conditions. Wild-type AKT1- or GRP-AKT1-expressing cells showed only low levels of AKT1 phosphorylation in the presence of serum, and under serum-starved conditions, phosphorylation of AKT1 was barely detectable despite efficient expression of the proteins (Fig. 3). The downstream targets GSK3 β and S6 were preferentially phosphorylated in E17K-AKT1-, PLC-AKT1- or myrAKT1-expressing cells and were not phosphorylated in CEF expressing wild-type AKT1 or GRP-AKT1. Wild-type AKT1 and GRP-AKT1 were, however, functionally intact, because they were capable of being phosphorylated in the presence of an upstream activating signal such as serum stimulation as shown in figure 3 and can be transformed by overexpression of p110 δ (data not shown). GRP-AKT1 also does not function as a dominant negative in cells transformed by gain-of-function mutants of AKT1 (data not shown).

PLC-AKT1 expression leads to constitutive signaling and cellular transformation, possibly by constitutive membrane localization. The PH domain of PLC δ 1 is a high affinity and high specificity binder of PIP₂ in the plasma membrane. Furthermore, the PH domain of PLC δ 1 is especially attracted to particular arrangements of PIP₂ found in caveolae³⁴. In order to document the preferential localization of PLC-AKT1 to specific domains of the plasma membranes, PLC-AKT1- and GRP-AKT1-infected cells were seeded onto glass coverslips, serum starved, fixed with formaldehyde, probed using the Anti-HA antibody and visualized with FITC labeled anti-mouse antibody (Sigma, St Louis, MO). PLC-AKT1 localized primarily to apical sites of the fibroblasts which may be enriched in the PIP₂-concentrating structures described recently³⁴. In contrast, GRP-AKT1 did not localize strongly to these areas or the plasma membrane in general (Fig. 4). There is increased association of GRP1-Akt with the nucleus or nuclear membrane. This localization pattern has been previously noted, but the origin of this distribution pattern is unknown³⁵.

E17K-AKT1, PLC-AKT1 and myrAKT1 induce oncogenic transformation of CEF as well as tumor formation and angiogenesis in CAMs of chicken embryos. These constructs also initiate constitutive downstream signaling under serum-starved conditions. In contrast, the mere overexpression of wild-type AKT1 is insufficient to induce oncogenesis and fails to generate a significant downstream signal in the absence of growth factors. Constitutive localization at the plasma membrane and the resultant elevated phosphorylation and kinase activity are essential requirements for the oncogenicity of AKT1. This study shows that changes in the PH domain that enhance membrane recruitment can trigger the latent oncogenic potential of AKT1. It is not known to what extent the gain of function observed with the AKT1 constructs studied here is caused by an enhanced interaction with all phosphoinositides or with the more abundant PIP₂ specifically. In wild-type AKT1, the E17 occupies the phosphoinositide-binding pocket. Upon binding to PIP₃ or PIP₂, E17 swings away from the pocket. The E17K mutation changes the conformation and surface charge of the PH domain; K17 does not occupy the binding pocket, but forms a hydrogen bond with Y18²⁴. The mutation leads to constitutive membrane localization of AKT1, possibly because of an increased affinity in the AKT-phosphoinositide interaction or a reduced off rate^{24, 25}. The oncogenic activity of PLC-AKT1 is mediated by its ability to bind to PIP₂. The cellular concentrations of PIP₂ are higher than those of PIP₃, and binding to PIP₂ may effectively cause constitutive membrane localization but may also involve changes in affinity^{29, 36}. GRP-AKT1 shows marginal oncogenic activity and is

inactive in signaling, probably because it binds exclusively to the much less abundant PIP₃³⁷. In this construct, the PH domain may not occlude T308 as effectively as the native PH domain of wild-type AKT1. A low level of PDK1-mediated phosphorylation of T308 may thus occur constitutively and may account for the marginal oncogenicity of GRP-AKT1. AKT1 from which the PH domain is deleted is also constitutively active^{38, 39}. PDK1 is largely but not entirely membrane-associated. Its PH domain interacts with high affinity with PIP₃ and with lower affinity with PIP₂, and it can activate AKT1 under serum-starved conditions²⁹.

Our study confirms the potent oncogenic activity of the E17K mutation described by Carpten and coworkers²⁴ and suggests that other changes in the PH domain of AKT1 could lead to a gain of function and to oncogenicity. In human cancer, E17K would likely function as a driver mutation, determining the neoplastic phenotype of the cancer cell^{24, 27}. Current research efforts to control PI3K-AKT signaling focus heavily on inhibitors of the catalytic subunit of PI3K^{40–44}. Such inhibitors would not affect gain-of-function mutations in AKT1. AKT1 therefore remains an important therapeutic cancer target.

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Figure 1. PIP₂-specific AKT1 induces oncogenic transformation in CEF

(a) Schematic structure of AKT1 constructs.

Human full-length AKT1 (NM_005163.2) was fused to an HA epitope tag on the carboxy terminus. E17K-AKT1 and myrAKT1 were generated using human wild-type AKT1 as template for PCR. The chimeric constructs GRP-AKT1 and PLC-AKT1 were obtained by substituting the PH domain of AKT1 with either the PH domain of GRP1 (NM_004227.3) or PLCδ1 (NM_006225). All constructs were subsequently cloned into the avian retroviral expression vector RCAS(A)²¹.

(b) Focus assay with AKT1 constructs.

CEF were transfected with RCAS(A) encoding wild-type AKT1, E17K-AKT1, myrAKT1, GRP-AKT1 or PLC-AKT1 using the dimethyl sulfoxide/polybrene method⁴⁵. Cells were overlaid with agar medium every 2–3 days and stained with 2% (w/v) crystal violet in 10% (v/v) methanol after 17 days. A representative experiment out of three independent ones is shown.

Figure 2. Tumor induction and angiogenesis induced by PIP₂ affine AKT1 constructs in the CAM of the chicken embryo

CAM assays were performed with fertilized chicken eggs (White Leghorn) obtained from Charles Rivers Breeding Laboratories (Wilmington, MA). Eggs were incubated at 37.5 °C and 70% humidity for 9 days. Eggs were candled to confirm embryo growth and to locate vascularized areas of the CAM. Two holes were drilled, one into the air sac at the bottom of the eggs, another one at the lateral end above the blood vessels, penetrating through the outer shell membrane without injuring the CAM. Applying suction to the air sac exposed the CAM. CAMs were inoculated with AKT1, E17K-AKT1, myrAKT1, GRP-AKT1, PLC-AKT1 or empty vector. Eggs were sealed and incubated for another 9 days. A representative experiment out of three independent ones is shown.

Figure 3. Phosphorylation events associated with expression of Akt constructs

CEF transfected with RCAS constructs encoding wtAKT, E17K-AKT, myrAKT, GRP-AKT, PLC-AKT or empty vector were incubated under normal and serum starved condition. Whole cell lysates were electrophoresed and analysed by immunoblotting with anti-phospho-AKT (S473), anti-phospho-AKT (T308), anti-phospho-S6 (S235/S236), anti-phospho-GSK-3b (S9), anti-HA and anti-β-Actin, all obtained from Cell Signaling Technology® (Danvers, MA).

PLC-AKT1

GRP-AKT1

Figure 4. Intracellular localization of Akt constructs

CEF transfected with PLC-AKT1 (A–C) or GRP-AKT1 (D–F) constructs were incubated under serum starved conditions. Cells were fixed with formaldehyde and probed using an Anti-HA antibody. Images were taken at 100× magnification on an Axiovert 100TV fluorescence microscope.