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AKT upregulates B-Raf Ser445 phosphorylation and ERK1/2 activation in prostate cancer cells in response to androgen depletion

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Abstract

Upregulated ERK1/2 activity is often correlated with AKT activation during prostate cancer (PCa) progression, yet their functional relation needs elucidation. Using androgen-deprived LNCaP cells, in which ERK1/2 activation occurs in strong correlation with AKT activation, we found that AKT-mediated B-Raf regulation is necessary for ERK1/2 activation.

Specifically, in response to androgen deprivation, AKT upregulated B-Raf phosphorylation at Ser445 without affecting A-Raf or C-Raf-1. This effect of AKT was abolished by Arg25 to Ala mutation or truncating ($\Delta 4-129$) the pleckstrin homology domain of AKT, indicating that the canonical AKT regulation is important for this signaling. Intriguingly, although a constitutively active AKT containing N-terminal myristoylation signal could sufficiently upregulate B-Raf phosphorylation at Ser445 in LNCaP cells, subsequent MEK/ERK activation still required hormone deprivation. In contrast, AKT activity was sufficient to induce not only B-Raf phosphorylation but also MEK/ERK activation in the hormone refractory LNCaP variant, C4-2. These data indicate that androgen depletion may induce MEK/ERK activation through a synergy between AKT-dependent and -independent mechanisms and that the latter may become deregulated in association with castration resistance. In support, consistent AKT-mediated BRAF regulation was also detected in a panel of PCa lines derived from the *cPten*^{-/-} *L* mice before and after castration. Our results also demonstrate that AKT regulates androgen receptor levels partly *via* the Raf/MEK/ERK pathway. This study reveals a novel crosstalk between ERK1/2 and AKT in PCa cells.

Keywords

Prostate cancer; androgen withdrawal; AKT; B-Raf; ERK1/2

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Conflict of interest

The authors declare no conflict of interest for this article.

Introduction

Prostate cancer (PCa) is the most commonly diagnosed cancer among men and the second leading cause of male cancer death. PCa often reprogram their signal transduction pathways to develop higher malignancy and therapy resistance [1-5]. For example, increased activity of extracellular signal-regulated kinase (ERK) 1 and its homologue, ERK2, (collectively referred to as ERK1/2) is implicated in the progression and poor prognosis of PCa [6-11]. Because activation mutations of Ras or Raf are relatively rare in human PCa [12-18], ERK1/2 activation should also be attributed to other mechanisms. Notably, ERK1/2 activity is upregulated in strong correlation with AKT (also known as protein kinase B) activation in PCa that arises in prostate-specific phosphatase and tensin homolog (*Pten*) knockout mice, especially during PCa conversion into a hormone-refractory state [19]. Moreover, in a large cohort study (n = 535), ERK1/2 activation was correlated with deregulated activation of all components of the AKT pathway in 21% of human PCa cases and with deregulation of at least one component of the pathway in 42% of the cases, respectively [11]. These studies strongly implicate AKT in ERK1/2 activation in PCa, although the underlying mechanism is yet unclear.

The Raf/MEK/ERK and PTEN/phosphatidylinositol 3-kinase (PI3K)/AKT signaling pathways have pivotal roles in cell survival, cell cycle progression, metabolism, and differentiation, and their deregulation is a central signature of many epithelial cancers [20, 21]. In response to Ras signals, the Ser/Thr kinase Raf (A-Raf, B-Raf or C-Raf-1) activates the dual-specificity kinases MEK1 and MEK2 (collectively referred to as MEK1/2) which, in turn, sequentially phosphorylate Tyr and Thr in the activation loop of the ubiquitously expressed Ser/Thr kinase, ERK1/2. ERK1/2, currently the only known substrate of MEK1/2, serves as the focal point of Raf/MEK/ERK signaling by regulating a wide variety of proteins [22]. The PI3K/AKT pathway can also mediate Ras signaling [23]. Upon activation, PI3K produces phosphatidylinositol-3,4,5-trisphosphate, which is then dephosphorylated by PTEN. Phosphatidylinositol-3,4,5-trisphosphate interacts with the N-terminal pleckstrin homology (PH) domain of the Ser/Thr kinase AKT to facilitate AKT recruitment to the plasma membrane, where AKT is activated through Thr308 and Ser473 phosphorylation in its activation segment [21].

It has been known that AKT can negatively regulate MEK/ERK signaling by directly inhibiting C-Raf *via* Ser259 phosphorylation, which promotes Raf interaction with the inhibitory scaffold 14-3-3 ζ [24, 25]. Similarly, AKT can also negatively regulate B-Raf [26]. In contrast, recent studies have demonstrated that AKT can positively regulate MEK/ERK signaling by promoting Raf activation through the Rac/p21-activated kinase (PAK) pathway, wherein c-Raf Ser338 phosphorylation is a key regulatory mechanism [27, 28]. Therefore, AKT can differentially regulate Raf/MEK/ERK signaling depending upon biological contexts. Because Ser338 of c-Raf is conserved in B-Raf and A-Raf, these Raf proteins may also be subject to AKT-mediated positive regulation. Further, it has not been tested whether positive regulation of Raf by AKT is involved in the upregulation of ERK1/2 activity in PCa.

Previously, we and others reported that hormone depletion upregulates AKT and ERK1/2 activity in the androgen-dependent human PCa line, LNCaP, to mediate androgen receptor (AR) downregulation and subsequent neuroendocrine differentiation [29-31]. AR is a nuclear transcription factor pivotal to prostate carcinogenesis [4, 5], and its downregulation is implicated in neuroendocrine differentiation of PCa, a process associated with the development of castration resistance [2, 32]. Using this model, this study investigates a crosstalk between AKT and ERK1/2 pathways. Our results suggest that MEK/ERK activation is a downstream event of AKT activation, which is mediated by B-Raf and is

partly required for AKT-mediated AR downregulation. Specifically, AKT could sufficiently upregulate Ser445 phosphorylation of B-Raf, but subsequent MEK/ERK activation required additional signals from androgen depletion. These findings were also validated using an androgen refractory variant of LNCaP, C4-2, and the PCa cell lines derived from the prostate specific *cPten*^{-/-} *L* mice.

Materials and Methods

Cell culture and reagents

LNCaP (ATCC) and CWR22Rv1 (ATCC) were maintained in phenol red-deficient RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS), 100 U of penicillin and 100 µg of streptomycin per ml. LAPC4 (ATCC) was grown in Iscove's medium with 10% FBS. LNCaP C4-2 cells were maintained in phenol red-deficient RPMI 1640 supplemented with 10% charcoal/dextran-stripped FBS (c.s.FBS). The mouse PCa lines, E8, E2, E4, CE1 and CE2, derived from the *cPten*^{-/-} *L* mouse [33], were previously described [34, 35]. The androgen-dependent mouse PCa line, E8, was maintained in phenol red-deficient DMEM (Invitrogen) supplemented with 10% FBS. The castration-resistant CE1 and CE2 lines, derived from a 13 month old male *cPten*^{-/-} *L* mouse that was castrated at the age of 8 months, were maintained in phenol red-deficient DMEM supplemented with 10% c.s.FBS. The castration-resistant E2 and E4 cells, derived after long-term culture of E8 cells under androgen-depleted conditions, were maintained in phenol red-deficient DMEM supplemented with 10% c.s.FBS. IPA-3, AKTi, MK-2206, and AZD6244 were purchased from Sigma (St. Louis, MO), EMD Chemicals (Gibbstown, NJ), Active Biochem (Maplewood, NJ), and Selleck Chemicals (Houston, TX), respectively.

Recombinant lentiviral constructs

pHAGE-GFP-AKT1wt, pHAGE-GFP-AKT1-E40K, and pHAGE-GFP-AKT1-K179M were generated by ligating mouse c-AKT1 (wild type), AKT1-E40K and AKT1-K179M into the BamHI site of the pHAGE vector, respectively. To generate pHAGE-GFP-AKT1-R25A, wild type c-AKT1 in pHAGE was mutagenized using Quickchange II site directed mutagenesis kit (Stratagene, Cedar Creek, TX) and the primers TTAACCTGGCGGCCAGCCTACTTCCTCTCAAGAAC and GTTCTTGAGGAGGAAGTAGGCTGGCCGCCAGGTTTAA. The E40K mutation is known to increase affinity of the PH domain to phosphatidylinositol-3,4,5-trisphosphate and subsequent plasma membrane localization, whereas the R25A mutation disables the PH domain function [36]. The K179M mutation disables the catalytic site of AKT. pHAGE-GFP-AKT-myr and pHAGE-GFP-AKT-null were generated by ligating human N-terminal myristoylated AKT1 and the PH domain truncated AKT1 (AKT-null) into the NotI and BamHI sites of the pHAGE vector, respectively. AKT-myr lacks the PH domain (Δ4-129) and contains a Src myristoylation signal sequence, which results in constitutive activation upon expression in mammalian cells. AKT-null lacks the PH domain and does not contain the Src myristoylation signal sequence [37]. pHAGE-GFP-AKT1-dn was generated by ligating a dominant-negative bovine AKT1 into the PmeI and XhoI sites of the pHAGE vector. The dominant-negative variant of AKT has three substitutions (K179A, T308A and S473A) and is phosphorylation-deficient and kinase-inactive [38].

RNA interference

The human B-Raf-specific small hairpin RNA (shRNA) expressing lentiviral pGIPZ constructs (Open Biosystems) target three different regions of mRNA, CCUGUCAAUAUUGAUGACUUG, GCAGAUGAAGAUCAUCGAAAU, and GGUGUGGAAUAUCAAACAAAU, respectively. The mouse AKT1-specific small interfering RNA (siRNA) targets CUGAGGUGCUGGAGGACAAUU.

Viral infection

Virus was produced as previously described [39]. Briefly, lentiviral constructs were co-transfected with packaging vectors into 293T cells and the resulting supernatant was collected after 48 h. Viral titers were determined by infecting the recipient cell lines with serially diluted viral supernatants mixed with polybrene (Sigma) at 8 µg/ml and scoring cells expressing GFP at 48 h post-infection. Cells were infected overnight and were switched into fresh culture media.

Quantitative real time polymerase chain reaction (qPCR)

qPCR was performed by reverse transcription of 0.25 µg total RNA and subsequent polymerase chain reaction using the Mx3005P™ instrument (Stratagene) and Brilliant SYBR® Green QPCR Core Reagent Kit (Stratagene) according to the manufacturer's protocol. Thermocycling conditions were 10 min at 95°C as first denaturation step, followed by 40 cycles at 95°C for 30 seconds, 55°C for 60 seconds and 72°C for 30 seconds. For normalization, expression of β-actin (ACTB) was measured. Primers were CTACATCAAGGAAGCTCGATC and CTGGGTGTGGAAATAGATT (AR 2,502–2,760), CCGACCAGCAGATGAAGATCAT and TCAACATTTTCACTGCCACATCAC (B-Raf 1,098–1,516), and GTCCTCTCCCAAGTCCACAC and GGGAGACCAAAAAGCCCTTCAT (ACTB 1,543–1,731). Calculation of the gene copy number was carried out using delta-delta-Ct method [40], as previously described [29].

Immunoblot analysis

Cells harvested at various times were lysed in 62.5 mM Tris (pH 6.8)-2% SDS mixed with the protease inhibitor cocktail (Sigma) that contains 4-(2-aminoethyl) benzenesulfonyl fluoride, pepstatin A, E-64, bestatin, leupeptin, and aprotinin, and briefly sonicated before determining the protein concentration using the BCA reagent (Pierce, Rockford, IL). 50 µg of protein was resolved by SDS-PAGE, transferred to a polyvinylidene difluoride membrane filter (Bio-Rad, Hercules, CA), and stained with Fast Green reagent (Thermo Fisher Scientific, Waltham, MA). Membrane filters were then blocked in 0.1 M Tris (pH 7.5)-0.9% NaCl-0.05% Tween 20 with 5% nonfat dry milk, and incubated with appropriate antibodies. Antibodies were diluted as follows: ERK1/2, 1:2,500; phospho-ERK1/2 (Thr202/Tyr204), 1:2,500; phospho-MEK1/2 (Ser217/221), 1:2,500; AKT, 1:2,500; phospho-AKT (Ser473), 1:5,000; phospho-GSK-3β (Ser9), 1:2,500; AR, 1:2,500; phospho-A-Raf (Ser299), 1:2,500; phospho-B-Raf (Ser445), 1:2,500; phospho-C-Raf (Ser338), 1:2,500; phospho-C-Raf (Ser259), 1:2,500; phospho-PAK1/2 (Thr423), 1:2,500; Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), 1:5,000 (Cell Signaling Technology, Danvers, MA); Poly (ADP-ribose) polymerase, 1:1000; E2F1, 1:1,000; PSA, 1:1,000; β-tubulin, 1:1,000; β-actin, 1:1,000 (Thermo Fisher Scientific); p21^{CIP1}, 1:2,500 (Santa Cruz Biotech, Santa Cruz, CA). The Supersignal West Pico and Femto chemiluminescence kits (Pierce) were used for visualization of the signal. For densitometry, immunoblots were scanned and analyzed using LabWorks™ (UVP BioImaging Systems, Upland, CA).

Results

Correlative activation of AKT and ERK1/2 in LNCaP cells deprived of androgen

To determine whether AKT activation occurs in correlation with ERK1/2 activation in LNCaP cells deprived of androgen, we monitored these kinases over 12 days in LNCaP cells maintained in the RPMI medium containing c.s.FBS (c.s.FBS culture). During this period, AKT phosphorylation at Ser473, an indication of AKT activation [21], gradually increased and was accompanied by increasing ERK1/2 phosphorylation in its activation loop (Thr202/Tyr204 of ERK1; Thr183/Tyr185 of ERK2), indicating a strong correlation between AKT

and ERK1/2 activation (Fig. 1A). Along with these changes, AR downregulation (Fig. 1A) and growth inhibition (Supplemental Fig. 1) were also detected. In conjunction with previous reports that these c.s.FBS-induced changes can be reversed by androgen analogues, R1881 or DHT [29, 31], and that activation of AKT or ERK1/2 pathway is sufficient to induce AR downregulation in LNCaP cells [29, 41], these data led us to hypothesize that androgen deprivation elicits a crosstalk between AKT and Raf/MEK/ERK in PCa cells to mediate AR downregulation.

Inhibition of B-Raf/MEK cannot block AKT activation in LNCaP cells deprived of androgen

To test whether AKT and Raf/MEK/ERK crosstalk, we determined whether inhibition of Raf/MEK/ERK could affect AKT activation in the LNCaP c.s.FBS culture. Raf proteins are selectively used to phosphorylate MEK1/2 in different biological contexts [20, 22]. Therefore, we first investigated which Raf mediates MEK/ERK activation in the LNCaP c.s.FBS culture. A key modification required for Raf activation is its phosphorylation at a conserved Ser residue, i.e., Ser445 of B-Raf, Ser338 of C-Raf-1 and Ser299 of A-Raf, which primes Raf for subsequent activation [42, 43]. In the LNCaP c.s.FBS culture maintained for 6 days, B-Raf phosphorylation at Ser445 was significantly increased, whereas phosphorylation of C-Raf or A-Raf at the corresponding residue did not change (Fig. 1B; Supplemental Fig. 2). In a subsequent analysis using three different B-Raf-specific lentiviral shRNA constructs, B-Raf knockdown consistently abrogated c.s.FBS-induced MEK1/2 and ERK1/2 phosphorylation (Fig. 1B), indicating that, among the Raf family members, B-Raf mainly mediates c.s.FBS-induced MEK/ERK activation in LNCaP cells. However, B-Raf knockdown did not affect AKT phosphorylation (Fig. 1B). Moreover, the highly specific MEK1/2 inhibitor, AZD6244, also did not affect AKT phosphorylation although it effectively blocked ERK1/2 phosphorylation (Fig. 1C). Therefore, B-Raf/MEK/ERK activation is not required for AKT activation in LNCaP cells deprived of hormone.

AKT activation is required for B-Raf phosphorylation at Ser445 in LNCaP cells deprived of androgen

We next determined the effects of AKT inhibition on B-Raf/MEK/ERK activity in the LNCaP c.s.FBS culture. A dominant-negative c-AKT1 mutant (AKT-dn), defective of the catalytic site (Lys179 to Ala mutated) and the activation segment (Thr308 to Ala; Ser473 to Ala mutated), can competitively inhibit endogenous AKT [38]. We observed that overexpression of this AKT mutant inhibited c.s.FBS-induced ERK1/2 phosphorylation (Fig. 2A). However, because AKT-dn inhibited B-Raf phosphorylation only mildly, we also used the AKT-specific inhibitors, MK-2206 and AKTi, to more effectively deplete AKT activity in cells. These inhibitors substantially depleted cellular AKT activity, as determined by phosphorylation of AKT and GSK3 β , a *bona fide* AKT substrate (Fig. 2B and 2C). These inhibitors indeed significantly decreased the levels of phosphorylated B-Raf at Ser445 in the LNCaP c.s.FBS culture (Fig. 2B and 2C), suggesting that AKT has a role in regulating B-Raf phosphorylation at Ser445 in LNCaP cells. However, unexpectedly, these AKT inhibitors did not downregulate but upregulated ERK1/2 phosphorylation in LNCaP cells (Fig. 2B and 2C). Of note, MEK1/2 phosphorylation was not increased under these conditions (Fig. 2B and 2C), indicating that the upregulation of ERK1/2 phosphorylation was independent of Raf/MEK. These effects were limited to LNCaP cells in our study; in mouse PCa cell lines, AKT inhibition decreased phosphorylation of both B-Raf and ERK1/2 (shown below), suggesting that AKT may have biphasic effects on ERK1/2 specifically in LNCaP cells.

AKT overexpression promotes B-Raf phosphorylation at Ser445 and MEK/ERK activation in response to hormone depletion

We further evaluated the role of AKT in c.s.FBS-induced B-Raf/MEK/ERK activation using wild type AKT and a mutant containing disabled active site but intact activation segment (AKT-K179M). Virally-mediated overexpression of wild type AKT, but not AKT-K179M, increased basal as well as c.s.FBS-induced AKT activity in LNCaP cells, as determined by phosphorylation of GSK3 β (Fig. 3A; infection efficiency shown in Supplemental Fig. 3A). AKT overexpression did not significantly increase ERK1/2 phosphorylation in the FBS culture although it mildly increased phosphorylation of MEK1/2 and B-Raf at Ser445 (Fig. 3A). In contrast, AKT overexpression robustly increased phosphorylation of ERK1/2, MEK1/2, and B-Raf in the c.s.FBS culture (Fig. 3A), suggesting the requirement of androgen depletion for the AKT effects. Under these conditions, phosphorylation of C-Raf or A-Raf were not significantly affected (Fig. 3A), further supporting the specific relationship between AKT and B-Raf. Because AKT-K179M could not mediate any similar effects, catalytic activity of AKT is necessary for this signaling. Further, AKT-induced ERK1/2 phosphorylation in the c.s.FBS culture was reversed by addition of low concentration of R1881 (Supplemental Fig. 4). These data suggest that AKT can facilitate B-Raf/MEK/ERK activation in LNCaP cells in response to hormone deprivation.

Of note, compared to the control and cells expressing AKT-K97M, relatively high total B-Raf protein levels were detected in cells expressing wild type AKT (Fig. 3A; also shown in Fig. 4B) without any significant changes in B-Raf mRNA levels (Fig. 3B), indicating that the increases in phosphorylated B-Raf levels was partly due to increased B-Raf proteins. Nevertheless, the degrees of increase were similar in both FBS and c.s.FBS cultures, indicating that B-Raf phosphorylation at Ser445 was specifically upregulated in the c.s.FBS culture.

MEK/ERK activity is partly required for AKT-mediated AR regulation

AR downregulation is a common effect of AKT or MEK/ERK activation [29, 41]. Because Raf/MEK/ERK activation could downregulate AR at mRNA levels in LNCaP cells [29], we determined whether AKT can also regulate AR at mRNA levels. Our qPCR analysis revealed that AKT overexpression could downregulate AR at mRNA levels regardless of culture conditions (Fig. 3B). We also tested whether AKT overexpression could downregulate the activity of a luciferase reporter containing -1,238 to +307 base pairs of AR promoter DNA. The activity of this reporter did not decrease in response to AKT overexpression (Supplemental Fig. 5), suggesting that AKT may not downregulate AR transcription.

Consistent with its effect on MEK/ERK, AKT overexpression augmented AR downregulation in the LNCaP c.s.FBS culture (Fig. 3A). We therefore determined whether MEK/ERK activation is required for AKT-induced AR downregulation. Although effective, the MEK1/2 specific inhibitor AZD6244 could not completely inhibit AKT-mediated AR downregulation in the c.s.FBS culture (Fig. 3D), indicating that the mechanisms underlying AKT-mediated ERK1/2 and AR regulation may not entirely overlap. In support of this notion, despite its insignificant effect on basal ERK1/2 activity, AKT overexpression induced significant AR downregulation in the FBS culture (Fig. 3A).

We also examined AKT effects on LNCaP cell growth and survival. Overexpression of wild type AKT, but not AKT-K179M, mildly decreased S-phase cell population although it did not significantly affect cell growth rates in the FBS culture (Supplemental Table 1; Supplemental Fig. 3B). Consistent with these effects, overexpression of wild type AKT, but not AKT-K179M, upregulated p21^{CIP1} in the FBS culture (Fig. 3A). However, both AKT

constructs commonly increased p21^{CIP1} in the c.s.FBS culture (Fig. 3A) and augmented c.s.FBS-induced S-phase depletion (Supplemental table 1). Of note, AKT-mediated p21^{CIP1} upregulation was mildly inhibited by AZD6244 (Fig. 3D), suggesting a role of MEK/ERK for AKT-regulated LNCaP cell growth. In contrast, AKT overexpression or AZD6244 did not affect PARP and E2F1, a marker for apoptosis and a key transcription factor mediating cell cycle progression [5], suggesting that the AKT-ERK axis mediates specific cellular responses, i.e., AR downregulation and p21^{CIP1} induction (Fig. 3A and 3D). Together, these data identify B-Raf/MEK/ERK and AR as major targets of AKT regulation in LNCaP cells.

The N-terminal pleckstrin homology (PH) domain is necessary for AKT to regulate B-Raf/MEK/ERK, but not AR, under hormone-depleted conditions

AKT consists of a central kinase domain, a C-terminal regulatory tail containing a hydrophobic motif, and a specific N-terminal PH domain, whose interaction with phosphatidylinositol-3,4,5-trisphosphate is pivotal to AKT recruitment to the plasma membrane and subsequent activation [21]. To determine whether AKT requires its PH domain to regulate B-Raf/MEK/ERK and AR, we examined the effect of Arg25-to-Ala (R25A) mutation, which disables the PH domain function [36]. Unlike the wild type AKT expressed at a similar level, AKT-R25A was not efficiently phosphorylated, nor could it mediate GSK3 β phosphorylation in the LNCaP c.s.FBS culture (Fig. 4A). AKT-R25A overexpression could not also increase phosphorylation of ERK1/2, MEK1/2, or B-Raf, indicating that AKT requires its PH domain to regulate Raf/MEK/ERK. Nevertheless, AKT-R25A could mediate AR downregulation, although not as efficiently as wild type AKT (Fig. 4A), indicating that the canonical PH domain control is important but not necessary for AKT-mediated AR regulation. These contrasting effects further distinguish AKT-mediated regulation of B-Raf/MEK/ERK and AR.

AKT activity is sufficient to upregulate B-Raf Ser445 phosphorylation, but subsequent MEK/ERK activation requires hormone deprivation

AKT-myr, which contains an N-terminal myristoylation signal in replacement of the PH domain, constantly translocates to the plasma membrane to be activated [37]. Using this mutant, we determined whether AKT activity is sufficient for Raf/MEK/ERK activation in LNCaP cells. As expected, contrary to the control AKT-null (Δ 4-129) mutant, AKT-myr phosphorylation occurred at equivalently high levels in both FBS and c.s.FBS cultures of LNCaP, which was correlated with equivalently increased GSK3 β phosphorylation (Fig. 4B). This indicated persistent AKT activity regardless of the culture conditions. Indeed, in both cultures, AKT-myr expression increased B-Raf expression and Ser445 phosphorylation to similar degrees (Fig. 4B), indicating that AKT activity is sufficient for B-Raf upregulation in LNCaP cells. Nevertheless, very intriguingly, subsequent MEK/ERK activation in the FBS culture was mild and much lower than the levels detected in the c.s.FBS culture (Fig. 4B), indicating that AKT activity alone is insufficient to fully activate the Raf/MEK/ERK pathway. Therefore, MEK/ERK activation in the c.s.FBS culture requires not only AKT but also an AKT-independent mechanism.

Of note, when assessed in the LNCaP c.s.FBS culture, wild type AKT was as effective as AKT-myr or AKT-E40K, another constitutively active AKT mutant [36], in mediating phosphorylation of GSK3 β , B-Raf, and ERK1/2 as well as AR downregulation (Fig. 4C), suggesting that hormone depletion may induce a strong signal for AKT activation. All these AKT constructs consistently increased B-Raf Ser445 phosphorylation without affecting other Raf proteins. However, although AKT can catalyze the inhibitory C-Raf Ser259 phosphorylation [24], none of these constructs upregulated that phosphorylation (Fig. 4C), suggesting that AKT controls Raf mainly in a positive regulatory context in LNCaP cells deprived of androgen.

Involvement of PAK in AKT-mediated B-Raf/MEK/ERK regulation

PAK1 overexpression can upregulate phosphorylation of the conserved amino acid residues in Raf, C-Raf Ser338 and B-Raf Ser445 [44]. Given the requirement of Rac/PAK signaling for AKT-mediated C-Raf Ser338 phosphorylation [27, 28], we investigated whether PAK is also involved in AKT-mediated B-Raf regulation in LNCaP cells. PAK activation in the c.s.FBS culture of LNCaP was indicated by increased phosphorylation of PAK1/2 (Fig. 5A). c.s.FBS- induced PAK phosphorylation was inhibited by overexpression of AKT-dn (Fig. 5A), whereas it was augmented by overexpression of wild type AKT (Fig. 5B). Furthermore, whereas the PH domain defective AKT-R25A could not mediate PAK phosphorylation (Fig. 5C), AKT-myr sufficiently induced PAK phosphorylation (Fig. 5D). Because these effects were consistent with AKT-mediated B-Raf regulation, we subsequently examined the effects of IPA-3, a PAK1 specific inhibitor, on AKT-mediated B-Raf/MEK/ERK regulation. In the LNCaP cells overexpressing AKT, increasing doses of IPA-3 induced commensurate inhibitory effects on c.s.FBS-induced ERK1/2 and B-Raf phosphorylation without significantly affecting the levels of B-Raf protein and phosphorylation of AKT and GSK3 β (Fig. 5E). These data suggest that PAK1 may be involved in AKT-mediated B-Raf/MEK/ERK regulation in LNCaP cells.

AKT-mediated MEK/ERK activation can occur without hormone deprivation in the androgen-refractory LNCaP variant, C4-2

Because hormone-refractory PCa cells often present altered signal transduction caused by androgen depletion [1-5], we investigated whether AKT differently regulates B-Raf/MEK/ERK signaling in the androgen refractory progeny of LNCaP, C4-2. Similarly as in the parental LNCaP cells, AKT-myr expression was sufficient to upregulate phosphorylation of B-Raf and PAK in C4-2 cells (Fig. 6A). However, in stark contrast to its effects in LNCaP, in which AKT-myr required hormone depletion to increase ERK1/2 phosphorylation (shown in Fig. 4B), AKT-myr expression could increase ERK1/2 phosphorylation in the FBS culture of C4-2 to the similar levels as detected in the c.s.FBS culture (Fig. 6A). LNCaP and C4-2 exhibited similar basal levels of total and phosphorylated B-Raf, MEK1/2, and ERK1/2 regardless of the culture conditions (Supplemental Fig. 6), suggesting that the difference was not attributed to different cellular composition of these proteins. Moreover, similar effects of AKT-myr were observed in CWR22R *v*1, an androgen-refractory progeny of CWR22 (Fig. 6B). Therefore, these hormone-refractory PCa variants do not require androgen deprivation to fully activate MEK/ERK in response to AKT activity, possibly because of an alteration associated with castration resistance.

Consistent with its effects in LNCaP, C4-2, and CWR22R *v*1 cells, AKT-myr also mediated phosphorylation of B-Raf and PAK, and AR downregulation in LAPC4 cells, another androgen-dependent human PCa cell line (Fig. 6C). However, unlike in LNCaP, AKT-myr could not upregulate ERK1/2 phosphorylation in the c.s.FBS culture of LAPC4 (Fig. 6C), indicating that not all AKT effects are conserved in different PCa cell types.

AKT regulates B-Raf/MEK/ERK activation in the PCa cell lines derived from prostate-specific conditional Pten knockout mice

To substantiate our findings on AKT-mediated B-Raf regulation, we examined the mouse PCa cell lines derived from the prostate tumors that arise before castration and relapse after castration in the *cPten*^{-/-} L mice [33-35]. Fig. 7A shows relatively high levels of Ser445 phosphorylated B-Raf, but not A-Raf or C-Raf, and phosphorylation of AKT, ERK1/2, and PAK in CE1 and CE2 cell lines (derived from the castration resistant PCa) compared to the control E8 cells (derived before castration and androgen-dependent). Moreover, E2 and E4 cells, the androgen refractory progenies of E8 generated after prolonged *in vitro* maintenance using c.s.FBS [35], also exhibited relatively high B-Raf/ERK and AKT activity

(Fig. 7B). These expression patterns are correlated with castration resistance and consistent with the c.s.FBS-induced effects in LNCaP cells.

When CE2 cells were treated with the AKT specific inhibitor AKTi for 48 hours, B-Raf levels (both total and phosphorylated) and phosphorylation of ERK1/2 and PAK were significantly decreased (Fig. 7C). AKTi also blocked B-Raf phosphorylation in E2 cells although its effect on ERK1/2 was insignificant (Fig. 7D). Moreover, siRNA-mediated partial knockdown of AKT1 induced mild but consistent B-Raf downregulation in CE2 cells (Fig. 7E). These data indicate that AKT is also required for B-Raf regulation in these murine PCa cells. Furthermore, consistent with its effect in LNCaP cells, the PAK1 inhibitor IPA-3 decreased B-Raf Ser445 phosphorylation in CE2 cells without significantly affecting total B-Raf levels (Fig. 7C), and B-Raf mRNA levels were not affected by AKT inhibition (Fig. 7F). Together, our results strongly suggest that AKT has a specific role in regulating the B-Raf/MEK/ERK pathway in PCa.

Discussion

Based upon the known correlation between AKT and ERK activation during PCa conversion into hormone-refractory state [11, 19] and recently reported AKT-mediated positive regulation of Raf [27, 28], we postulated a role for AKT in Raf/MEK/ERK regulation in PCa cells. Our results demonstrate that AKT can positively regulate the Raf/MEK/ERK pathway at the level of B-Raf in a subset of PCa cells, particularly in response to androgen depletion. Specifically, AKT catalytic activity was sufficient to upregulate the levels of Ser445 phosphorylated B-Raf, but it could not induce strong MEK/ERK activation in LNCaP cells without androgen withdrawal. Therefore, it appears that androgen deprivation induces MEK/ERK activation through a synergy between AKT and as yet unidentified AKT-independent mechanisms (depicted in Fig. 8).

B-Raf phosphorylation at Ser445 is a key regulatory mechanism, which is conserved among Raf family members [42, 43]. Phosphorylation of the Ser residue decreases autoinhibitory interaction between N-terminal regulatory and C-terminal catalytic domains of Raf, and the resulting conformational change promotes the interaction of Raf with Ras [24, 44]. Although necessary, this phosphorylation is not sufficient for Raf activation [24, 44]. Therefore, AKT can increase the levels of B-Raf proteins primed for subsequent activation in PCa cells. This mechanism may contribute to the reprogrammed ERK1/2 signaling detected during PCa progression. Additionally, although it has been reported that B-Raf is constitutively phosphorylated at Ser445 whereas phosphorylation of other Raf proteins at the corresponding residue is inducible [42, 44], our results argue that B-Raf phosphorylation at Ser445 is also subject to a specific regulation in certain cell types.

Intriguingly, the androgen refractory C4-2 cells did not require androgen withdrawal to exhibit strong MEK/ERK activation in response to AKT activity. We speculate that this may be due to an alteration in these castration-resistant cells. A possible scenario is as follows. It was previously reported that Ras activity is upregulated in LNCaP cells deprived of androgen, and its basal level is constitutively high in C4-2 cells [45]. Nonetheless, basal ERK1/2 phosphorylation levels are not significantly different between LNCaP and C4-2, which was also shown by others [46]. Accordingly, it is conceivable that Ras may have to synergize with AKT to fully activate Raf/MEK/ERK in C4-2 cells. Nonetheless, because B-Raf can also be activated by another small G protein, Rap1B [47], other scenarios are also available. Regardless of these possibilities, our current study identifies AKT as a major determinant of ERK1/2 activity in PCa cells, particularly in the context of hormone deprivation responses.

How does androgen deprivation upregulate AKT activity? PTEN antagonizes AKT activation by depleting phosphatidylinositol-3,4,5-trisphosphate [21]. Because PTEN activity is absent in LNCaP and the *cPten*^{-/-} L mouse PCa cells, a PTEN-independent mechanisms might be involved in the upregulation of AKT activity. Given that the PH domain of AKT was critical for AKT activation, phosphatidylinositol-3,4,5-trisphosphate might be important in the upregulation of AKT activity. It is therefore conceivable that androgen depletion increases PI3K activity to produce more phosphatidylinositol-3,4,5-trisphosphate. Indeed, a recent study demonstrated that inhibition of AR activity upregulates AKT activity by promoting PI3K activity and by downregulating the AKT phosphatase, PHLPP, *via* FKBP5, a chaperone for PHLPP [48]. Moreover, AKT activity can also be upregulated through increased activity of mTOR, a kinase that activates AKT [21]. In support, mTOR activity is upregulated during the conversion of PCa into a hormone-refractory state [11, 49]. While these studies reveal the mechanisms that underlie AKT activation in PCa upon androgen depletion, our present study connects this AKT activation to the activation of B-Raf/MEK/ERK in PCa deprived of androgen.

Our observation of PAK is consistent with the previous reports implicating PAK in AKT-mediated Raf regulation [27, 28], although the underlying mechanisms need to be elucidated. While PAK1 can directly phosphorylate Ser338 of c-Raf [50, 51], it has been recently proposed that PAK1 indirectly mediates c-Raf Ser338 phosphorylation by stimulating Raf autophosphorylation [52]. Moreover, recent studies have shown that PAK1 can also phosphorylate MEK1 at Ser298 to facilitate subsequent Raf-mediated phosphorylation or autophosphorylation of MEK1 [53-55]. These mechanisms need to be evaluated in our future study to better understand the role of PAK in Raf/MEK/ERK regulation in PCa cells.

We and others previously showed that activation of AKT or ERK1/2 is sufficient for AR downregulation in a subset of human PCa cells [29-31]. Our current study further elaborates this signaling by demonstrating that AKT can regulate AR levels partly *via* ERK1/2 and partly *via* an ERK1/2-independent mechanism. Other data in this study also support these dual-mechanisms. Although the PH domain-disabled AKT-null or AKT-R25A could not effectively activate MEK/ERK, these mutants could still downregulate AR, suggesting that the PH domain of AKT is necessary for ERK1/2 regulation but not for AR regulation. Furthermore, AKT-myr downregulated AR in LAPC4 cells without affecting MEK/ERK activity. Exploration of the mechanism underlying AR downregulation is important because many recurrent PCa cells still rely on AR, and thus AR downregulation is recognized as a therapeutic strategy for PCa regardless of hormone sensitivity [56].

The significance of AKT-mediated regulation of Raf/MEK/ERK has been noted in different tumors [57-59]. Intriguingly, it appears that AKT-mediated B-Raf regulation contrasts in two opposing contexts depending upon tumor types. For example, it was previously reported that AKT3 can mediate Ser364/428 phosphorylation of B-Raf^{V600E} to decrease MEK/ERK activity below the threshold that triggers innate tumor suppressive responses, yet high enough to stimulate melanoma cell proliferation [60]. In contrast, our study demonstrates that AKT can upregulate MEK/ERK activity in PCa by increasing Ser445 phosphorylation of B-Raf. This is a novel example of positive regulation of the Raf/MEK/ERK pathway by AKT.

In conclusion, our study provides an insight into the mechanism underlying AKT and ERK1/2 signaling in PCa cells by demonstrating that (i) AKT regulates B-Raf Ser445 phosphorylation to control ERK1/2 activity in PCa cells deprived of androgen and (ii) subsequent MEK/ERK activation additionally requires, as yet unidentified, AKT-independent signals.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviation

AR	androgen receptor
c.s.FBS	charcoal/dextran-stripped fetal bovine serum
ERK	extracellular signal-regulated kinase
MEK	mitogen activated protein kinase kinase
PAK	p21-activated kinase
PCa	prostate cancer
PH	pleckstrin homology
PI3K	phosphatidylinositol 3-kinase
PTEN	phosphatase and tensin homolog

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Figure 1. Androgen deprivation induces AKT activation and B-Raf-mediated ERK1/2 activation in LNCaP cells

(A) LNCaP cells were maintained in the medium containing c.s.FBS over 12 days. Total cell lysates harvested at indicated time points were analyzed by Western blotting for expression of phosphorylated AKT (pAKT), phosphorylated ERK1/2 (pERK1/2), ERK1/2, and AR. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was detected to validate equal protein loading. Western blot results were then analyzed by densitometry (right panel). Data (mean $\pm$ standard error) are from three independent experiments and show optical density normalized for GAPDH levels for pERK1 and pERK2, and % changes for pAKT and AR. $P < 0.05$ for pAKT, pERK1 and pERK2 at days 9 and 12 relative to day 0 (Student's t test). (B) LNCaP Cells, infected with the B-Raf specific lentiviral shRNA constructs (shBraf #1, #2, and #3) or the control pGIPZ, were maintained in FBS or c.s.FBS medium for 6 days. Total cell lysates were analyzed by Western blotting for expression of the indicated proteins. pB-Raf, pA-Raf, pC-Raf, and pMEK1/2 denote phosphorylated forms of B-Raf, C-Raf, A-Raf, and MEK1/2, respectively. β -tubulin was detected to validate equal protein loading. 74 kDa indicates the expected region for the detection of C-Raf phosphorylated at Ser338. The efficacy of Raf antibodies is validated in Supplemental Fig. 2. (C) LNCaP cells were treated with the MEK1/2 inhibitor AZD6244 in c.s.FBS medium for the indicated time periods. Total cell lysates were analyzed by Western blotting for expression of the indicated proteins.

Figure 2. AKT inhibition attenuates c.s.FBS-induced phosphorylation of B-Raf
(A) LNCaP cells, infected with the lentivirus expressing the dominant-negative AKT (AKT-dn) or the control pHAGE virus, were maintained in FBS or c.s.FBS medium for 6 days. **(B and C)** LNCaP cells were treated with the AKT inhibitors, MK-2206 and AKTi, in c.s.FBS medium. Total cell lysates were analyzed by Western blotting for expression of the indicated proteins. pGSK3β denotes GSK3β phosphorylated at Ser9.

Figure 3. AKT promotes B-Raf/MEK/ERK activation and AR downregulation

(A) LNCaP cells, infected with the pHAGE lentivirus expressing wild type AKT (AKT-wt) or the kinase-deficient AKT mutant (AKT-K179M), were maintained in FBS or c.s.FBS medium for 48 hours. pHAGE is the control empty virus. (B) LNCaP cells, infected with lentiviral AKT-wt, were maintained in FBS or c.s.FBS medium for 48 hours. mRNA levels of AR and B-Raf were examined by qPCR. Data (mean $\pm$ standard error) are from a representative experiment performed in triplicate and are expressed as fold changes relative to the levels in pHAGE-infected cells in FBS medium. P value is < 0.005 for AKT relative to pHAGE (Student's t test). (C) LNCaP cells, infected with the pHAGE lentivirus expressing wild type AKT, were treated with increasing dose of AZD6244 in c.s.FBS medium for 48 hours. Total cell lysates from each experiment were analyzed by Western blotting for expression of the indicated proteins.

Figure 4. Roles of the PH domain for AKT-mediated B-Raf/MEK/ERK activation and AR downregulation

(A) LNCaP cells, infected with the pHAGE lentivirus expressing wild type AKT or the PH domain-defective AKT mutant (AKT-R25A), were maintained in c.s.FBS medium for 48 hours. (B) LNCaP cells, infected with the pHAGE lentivirus expressing the PH domain-truncated AKT mutant (AKT-null) or the PH domain-truncated and N-terminal-myristoylation-tagged AKT mutant (AKT-myr), were maintained in FBS or c.s.FBS medium for 48 hours. (C) LNCaP cells, infected with the pHAGE lentivirus expressing wild type AKT or the constitutively active AKT mutant (AKT-myr or AKT-E40K), were maintained in c.s.FBS medium for 48 hours. Total cell lysates from each experiment were examined by Western blotting for expression of the indicated proteins. AKT-myr migrates faster than other AKT constructs because of the N-terminal truncation.

Figure 5. Involvement of PAK in AKT-mediated B-Raf/MEK/ERK1/2 regulation

(A) LNCaP cells, infected with the pHAGE lentivirus expressing AKT-dn, were maintained in c.s.FBS medium for 6 days. Cell lysates are identical as those in Fig. 1B. The GAPDH image was also used in Fig. 1B. (B to D) LNCaP cells, infected with the pHAGE lentivirus expressing wild type AKT, AKT-K79M, AKT-R25A, AKT-null or AKT-myr, were maintained in FBS or c.s.FBS medium for 48 hours. Cell lysates are identical as those in Figures 2A, 3A and 3B. The GAPDH images were also used in those figures. (E) LNCaP cells, infected with the pHAGE lentivirus expressing wild type AKT, were maintained in c.s.FBS medium containing different amount of the PAK1 inhibitor IPA-3 for 48 hours. Total cell lysates were analyzed by Western blotting for expression of the indicated proteins. pPAK1/2 denotes PAK1 and PAK2 phosphorylated at Thr423 and Thr402, respectively.

Figure 6. Effects of constitutively active AKT expression in different PCa cells
Cells of LNCaP C4-2 (A) CWR22Rv1 (B), and LAPC4 (C), infected with the pHAGE lentivirus expressing AKT-null or AKT-myr, were maintained in c.s.FBS medium for 48 hours. Total cell lysates from each experiment were analyzed by Western blotting for expression of the indicated proteins.

Figure 7. AKT regulates B-Raf/ERK1/2 in PCa cells derived from the prostate-specific Pten knockout mice

(**A and B**) E8 cell line (derived before castration and androgen-dependent), CE1 and CE2 cell lines (derived from the castration resistant PCa), and E2 and E4 lines (androgen refractory progenies of E8 generated *in vitro*) were maintained in FBS medium for 48 hours before harvest. (**C and D**) CE2 and E2 cells were treated with increasing dose of the AKT inhibitor AKTi or the PAK1 inhibitor IPA-3 in c.s.FBS medium for 48 hours. (**E**) CE2 cells were treated with increasing dose of AKT-specific siRNA or the control scrambled siRNA oligomers in c.s.FBS medium for 72 hours. Total cell lysates from each experiment were analyzed by Western blotting for expression of the indicated proteins. (**F**) CE2 cells were treated with increasing dose of AKTi in c.s.FBS medium for 48 hours. mRNA levels of AR were examined by qPCR. Data (mean $\pm$ standard error) are from a representative experiment performed in triplicate and are expressed as fold changes relative to the levels in untreated cells.

Figure 8. A proposed model for AKT-mediated regulation of Raf/MEK/ERK and AR in PCa
 In a subset of PCa cells, androgen deprivation activates AKT to increase the levels of Ser445 phosphorylated B-Raf partly *via* PAK1 (i). For B-Raf/MEK/ERK to be fully activated, additional AKT-independent signals are required (ii). As a physiological effect, AKT mediates AR downregulation partly *via* Raf/MEK/ERK (iii) and partly *via* a Raf/MEK/ERK-independent pathway (iv).