Regulation of Mammalian Target of Rapamycin Activity in PTEN-Inactive Prostate Cancer Cells by IκB Kinase α

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Abstract

The mammalian target of rapamycin (mTOR) is a mediator of cell growth, survival, and energy metabolism at least partly through its ability to regulate mRNA translation. mTOR is activated downstream of growth factors, insulin, and Akt-dependent signaling associated with oncoprotein expression or loss of the tumor-suppressor PTEN. In this regard, mTOR activity is associated with cancer cell growth and survival. Here, we have explored an involvement of the IκB kinase (IKK) pathway, associated with nuclear factor-κB activation, in controlling mTOR activity. The experiments show that IKKα controls mTOR kinase activity in Akt-active, PTEN-null prostate cancer cells, with less involvement by IKKβ. In these cells, IKKα associates with mTOR, as part of the TORC1 complex, in an Akt-dependent manner. Additionally, IKKα is required for efficient induction of mTOR activity downstream of constitutively active Akt expression. The results indicate a novel role for IKKα in controlling mTOR function in cancer cells with constitutive Akt activity. [Cancer Res 2007;67(13):6263–9]

Introduction

The mammalian target of rapamycin (mTOR) is a Ser/Thr kinase that is activated downstream of increases in amino acid levels, by exposure of cells to growth factors or insulin, in response to hypoxia, by expression/activation of oncoproteins and by the loss of the PTEN tumor suppressor (1–4). mTOR controls cell growth, at least partly, through its ability to phosphorylate S6K and 4EBP1, key regulators of mRNA translation. The control of mTOR downstream of insulin-dependent signaling involves the Ser/Thr kinase Akt, which regulates mTOR through the ability of this kinase to phosphorylate tuberous sclerosis complex 2 (TSC2; refs. 5, 6). TSC1 and 2 form a complex that exhibits GTPase activity, blocking the activity of Rheb, a GTPase that controls mTOR activation (7). Mutations in the TSC genes lead to tuberous sclerosis, a disease characterized by the appearance of benign tumors.

mTOR interacts with the regulatory protein Raptor to comprise a rapamycin-sensitive complex (TORC1) that controls mTOR downstream functions (8, 9). A second mTOR complex (TORC2) contains the protein Rictor and this complex has been shown to function as the Pdk2 activity to phosphorylate Akt on Ser^{473} (10). Importantly, although Raptor has been shown to be required for mTOR function in the TORC1 complex, a strong association between Raptor and mTOR has been proposed to negatively regulate mTOR activity (8). An additional protein termed GβL associates with mTOR to control mTOR kinase activity via stabilization of mTOR-Raptor interaction (11).

The serine-threonine kinase Akt is widely activated in human cancers, where it controls suppression of apoptosis, cell growth and proliferation, and energy metabolism (4). In cancers, Akt is constitutively activated downstream of growth factor receptor signaling, through activating mutations in phosphatidylinositol 3-kinase (PI3K), or after PTEN loss of expression or mutation (4, 12–15). The initiating step in Akt activation is the binding of PIP3 to the PH domain of Akt, leading to translocation of Akt to the cell membrane where it is activated by phosphorylation through PDK1 (16, 17) and by a second activity termed PDK2 (10). Two important downstream effectors of Akt are the FOXO family of transcription factors and mTOR (4). Thus, mTOR activation is associated with a number of cancers, including those that are Akt active (15, 18).

The nuclear factor-κB (NF-κB) pathway is activated downstream of a variety of inflammatory mediators and growth factor pathways (19, 20). NF-κB activation in most signal transduction cascades uses the IKK complex, containing IKKα, IKKβ, and IKKγ. IKKα and IKKβ drive the catalytic activity of IKK, with IKKβ appearing to be dominant in inflammatory-mediated pathways and IKKα functioning in the so-called alternative pathway (19, 21). The IKK complex phosphorylates the inhibitory IκB proteins leading to their ubiquitination and subsequent proteasome-dependent degradation. This allows efficient NF-κB nuclear accumulation and binding to target sequences in the promoters and regulatory sequences of genes encoding cytokines, chemokines, and regulators of apoptosis. The dysregulation of NF-κB activity is associated with numerous cancers and with the inflammatory process that promotes cancer (see refs. 20, 22, 23). Interestingly, it has been reported that Akt can activate NF-κB transcriptional activity (24, 25) and nuclear accumulation (26) through a pathway involving IKK.

Here, we have explored a relationship between the IKK family of proteins and the regulation of mTOR activation downstream of cancer cell–associated, constitutively active Akt. Expression of IKKα promotes mTOR activity and knockdown of IKKα inhibits mTOR in PTEN-inactive prostate cancer cells. The ability of Akt to effectively activate mTOR requires IKKα as shown using IKKα−/− mouse embryonic fibroblast (MEF). Our data indicate that the IKKα subunit interacts with mTOR strongly in PTEN null prostate cancer cells in a manner that is dependent on Akt activity. In this setting, IKKα is required for mTOR activity, controlling its kinase activity. IKKα also regulates protein synthesis rates, paralleling its effects on mTOR function. The results show a new and unexpected role for IKKα in controlling the phenotype of cancer cells with constitutively active Akt.

Materials and Methods

Antibodies and reagents. Antibodies were obtained from the following sources. Antibodies against IKKα, IKKβ, and mTOR were obtained from Upstate Biotechnology. Raptor and Rictor antibodies were obtained from

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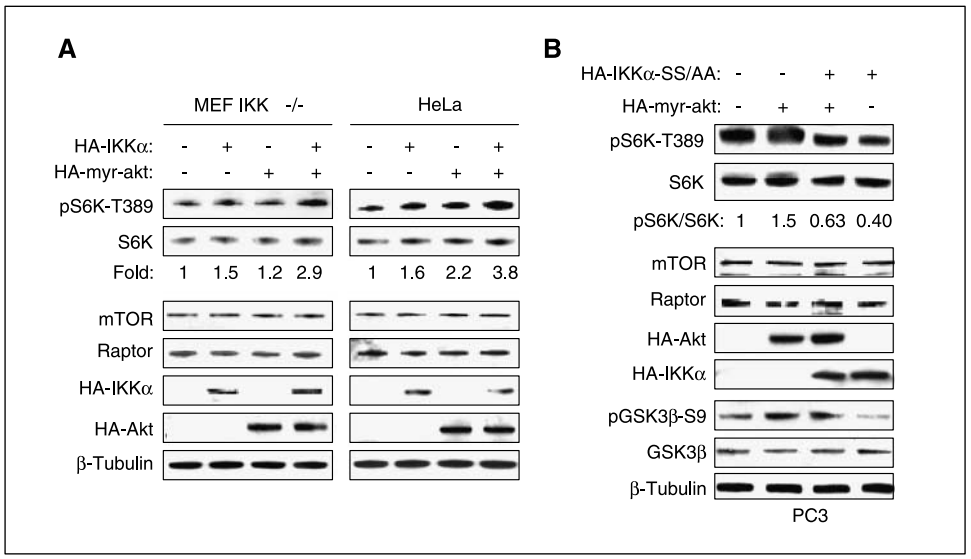

Figure 1. IKKα is involved in Akt-mediated mTOR activation. *A,* IKKα−/− MEFs and HeLa cells were transfected with HA-myr-Akt and/or HA-IKKα-WT and kept in full serum. Cell lysates were generated and blotted with mTOR, Raptor, HA, and tubulin antibodies as indicated. The bands phospho-S6K and S6K were quantified and the ratio of pS6K/S6K was measured as described in Fig. 3 legend. *B,* PC3 cells were transfected with HA-myr-Akt and/or HA-IKKα-SS-AA as indicated. Lysates were blotted with phospho-S6K, S6K, mTOR, Raptor, HA, and tubulin antibodies as in (*A*).

Bethyl Laboratories. Anti-HA and anti-Flag antibodies were obtained from Roche and Sigma, respectively. Anti-actin was obtained from Calbiochem. The anti-myc (9E-10), anti-tubulin, anti-S6K, and control rabbit IgG, as well as horseradish peroxidase–labeled anti-mouse and anti-rabbit secondary antibodies were from Santa Cruz Biotechnology. All other antibodies were from Cell Signaling. Other reagents were obtained from the following sources: Protease and phosphatase inhibitor cocktails were from Roche. CHAPS was from Pierce. Protein A and protein G agarose beads were from Invitrogen Life Technologies. All radiochemicals used were obtained from New England Nuclear.

Cell lines, cell culture, and transfection. IKKα wild-type and IKKα$^{-/-}$ MEFs were provided by I. Verma and M. Karin. HEK293T, HeLa, as well as prostate cancer cell lines PC3, LNCaP, and DU145 cell lines were from American Type Culture Collection. All cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS), 2 mmol/L glutamine, and 100 units/mL penicillin and streptomycin (Life Technologies). Transfections were done using Polyfect Transfection Reagent (Qiagen) or LipofectAMINE Plus (Invitrogen) following the manufacturer's instructions. Three to four hours after transfection, cells were recovered in full serum for 36 h or in full serum for 24 h and then serum-starved for 16 to 24 h as indicated.

RNA interference. Small interfering RNA (siRNA) SMARTpool IKKα, PTEN, IKKβ, Akt1, and Akt2 were from Dharmacon. Each of these represents four pooled SMART selected siRNA duplexes that target the indicated gene. HeLa, PC3, LNCaP, and DU145 cell lines cells were transfected with indicated SMARTpool siRNA or nonspecific control pool using DharmaFECT 1 reagent (Dharmacon) according to the manufacturer's instructions. In brief, 20 nmol/L final concentration of siRNA was used to transfect cells at 60% to 70% confluency. Twenty-four hours after transfection, cells were recovered in full serum or were serum starved 16 h before harvest. Cells were harvested 48 to 72 h after siRNA transfection.

Cell lysis, immunoblotting, and coimmunoprecipitations. Cells growing in 100 mm dishes were rinsed twice with cold PBS and then lysed on ice for 20 min in 1 mL lysis buffer [40 mmol/L HEPES (pH 7.5), 120 mmol/L NaCl, 1 mmol/L EDTA, 10 mmol/L pyrophosphate, 10 mmol/L glycerophosphate, 50 mmol/L NaF, 0.5 mmol/L orthovanadate, and EDTA-free protease inhibitors; Roche] containing 1% Triton X-100. After centrifugation at 13,000 × *g* for 10 min, samples containing 20 to 50 μg of protein were resolved by SDS-PAGE and proteins were transferred to Pure Nitrocellulose Membrane (Bio-Rad Lab.), blocked in 5% nonfat milk, and blotted with the indicated antibodies. For immunoprecipitation experiments, the lysis buffer contained 0.3% CHAPS instead of 1% Triton. Four micrograms of the indicated antibodies were added to the cleared cellular lysates and incubated with rotation for 6 to 16 h. Then, 25 μL of protein G agarose were added and the incubation continued for 1 h. Immunoprecipitates captured with protein G-agarose were washed three times with the CHAPS lysis buffer, twice by wash buffer A [50 mmol/L HEPES, (pH 7.5), 150 mmol/L NaCl], and boiled in 4× SDS sample buffer for Western blot.

***In vitro* mTOR kinase assay.** Transfected HEK293T cells were grown in 100 mm dishes for 48 h in DMEM containing 10% FBS, and lysed in 1 mL lysis buffer with 0.3% CHAPS. Half of total cell lysate was incubated with

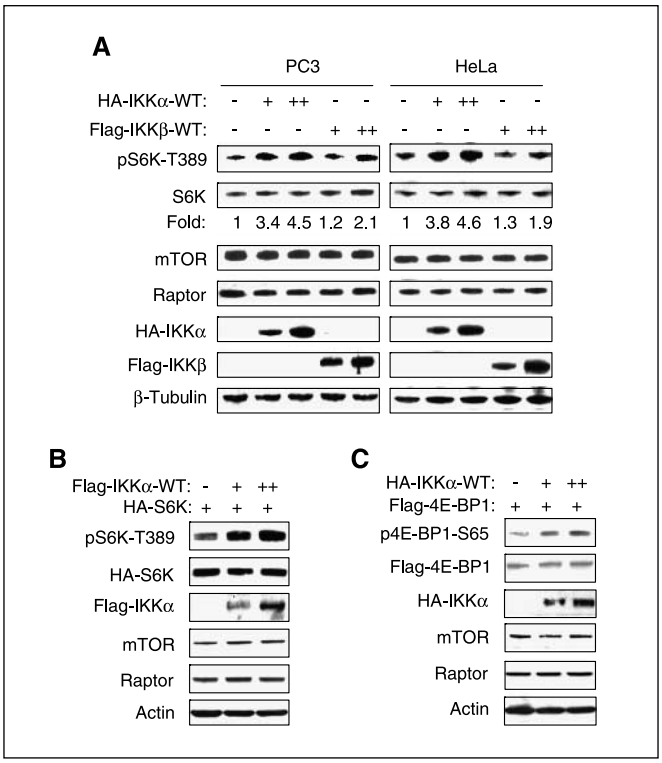

Figure 2. Overexpression of IKKα increases mTOR activity. *A,* PC3 and HeLa cells were transfected with HA-IKKα-WT and Flag-IKKβ as indicated, and the levels of endogenous phosphorylation of S6K and β-tubulin were detected and the ratio of pS6K/S6K was measured. Antibodies to HA and Flag showed the expression of the exogenous proteins. Results are representative of at least three experimental repetitions. *B* and *C,* transfection of IKKα into PC3 cells promotes phosphorylation of HA-tagged S6K (*B*) or 4E-BP1 (*C*).

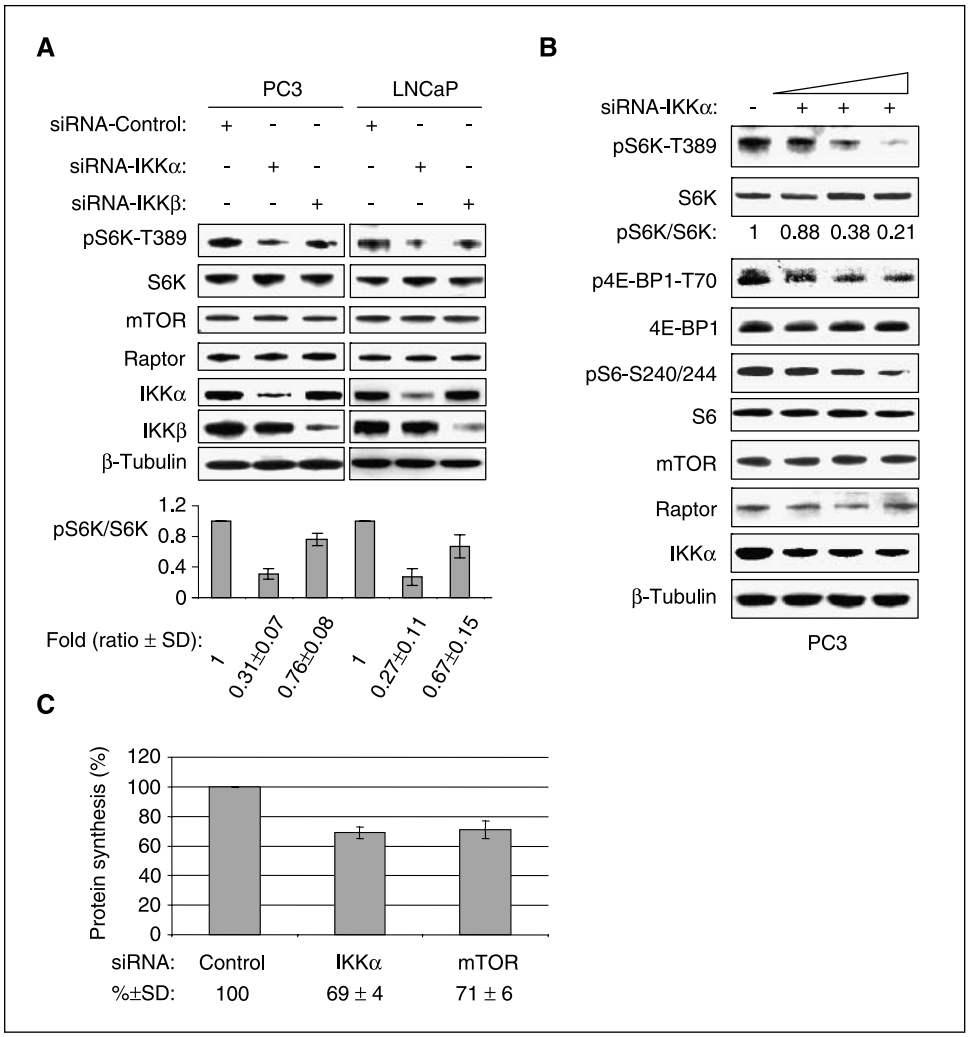

Figure 3. IKKα is a positive regulator of the mTOR pathway in PTEN-inactive cancer cells. *A,* PC3 and LNCaP cells were transfected with control siRNA, and siRNAs to IKKα and IKKβ as indicated. The cells were lysed 48 h after transfection and the levels of IKKα, IKKβ, and β-tubulin and of endogenous phosphorylation of S6K were determined by immunoblotting with the indicated antibodies. The experiments were carried out on three separate occasions. The bands were quantified by scanning densitometry using the IMAGE QUANT 5.0 software. The relative mean densitometry of different transfected siRNAs was calculated. *Columns,* mean ratios. *B,* PC3 cells were transfected with different amounts of siRNA IKKα. The levels of endogenous phosphorylation of S6K and 4E-BP1, as well as of IKKα, mTOR, and β-tubulin, were determined by immunoblotting with the indicated antibodies as in (*A*). The ratio of pS6K/S6K was measured by quantitation. Results are representative of at least three experimental repetitions. *C,* siRNA to IKKα inhibits protein synthesis in PC3 cells. PC3 cells were transfected with control siRNA and siRNAs to IKKα and mTOR as indicated. The cells were incubated with 12.5 μCi/mL [^{35}S]methionine for 20 min before harvesting in lysis buffer after 48 h transfection. The incorporation of radiolabel into cell protein was measured as described in Materials and Methods. All samples were equilibrated for protein levels and standarized against the control siRNA (100%).

anti-mTOR antibody for 3 h, followed by another hour of incubation with 25 μL protein G agarose beads. Immunoprecipitates were washed twice by lysis buffer, twice by wash buffer B [20 mmol/L Tris (pH 7.5), 500 mmol/L NaCl, 1 mmol/L EDTA, 20 mmol/L β-glycerophosphate, 5 mmol/L EGTA, 1 mmol/L DTT, 1 mmol/L orthovanadate, 40 mg/mL phenylmethylsulfonyl fluoride (PMSF), 10 μg/mL leupeptin, 5 μg/mL pepstatin], once with wash buffer C [10 mmol/L HEPES (pH 7.4), 50 mmol/L glycerophosphate, 50 mmol/L NaCl, 1 mmol/L DTT, 1 mmol/L orthovanadate, 40 mg/mL PMSF, 10 μg/mL leupeptin, 5 μg/mL pepstatin], and once with mTOR kinase assay buffer without ATP [10 mmol/L HEPES (pH 7.4), 50 mmol/L NaCl, 50 mmol/L glycerophosphate, 1 mmol/L DTT, 10 mmol/L $MgCl_2$, 4 mmol/L $MnCl_2$]. Kinase assay toward recombinant GST-S6K1 (amino acids 308–400) in washed immunoprecipitates was done for 30 min at 30°C in 30 μL mTOR kinase buffer with 100 μmol/L ATP unlabeled and 10 μCi [γ-^{32}P]ATP (New England Nuclear). To stop the reaction, 6 μL of 4× SDS sample buffer was added to each reaction, which was boiled for 10 min. The reaction was then separated by 4% to 12% SDS-PAGE, and transferred to polyvinylidene difluoride membrane. ^{32}P incorporated into GST-S6K was assessed by autoradiography.

Assay for protein synthesis. The rates of protein synthesis were assayed in 70 to 80% confluent PC3 or HeLa cells, grown in 60 mm plates, by measuring the incorporation of [^{35}S]methionine into acid-insoluble protein. In brief, the cells were labeled with 12.5 μCi/mL [^{35}S]methionine for 20 min before harvesting in lysis buffer. The protein concentrations in the extracts were measured using the Bio-Rad reagent and protocol. Aliquots (20 μL) of cell extract were applied to 1 × 1 cm squares of 3MM filter paper (Whatman), which were then washed thrice for 1 min in boiling 5% (w/v) trichloracetic acid containing a trace of cold L-methionine. Filters were rinsed once in ethanol and dried before radioactivity was determined by scintillation counting. Data were normalized to the protein content of each extract.

Results

Effective Akt activation of mTOR requires IKKα. To determine a potential role of IKKα as an intermediate in mTOR activation downstream of Akt, two approaches were chosen. First, it was determined if IKKα is required for Akt to activate mTOR. Constitutively activated (myristoylated) Akt (myr-Akt) was transfected into IKKα null MEFs and S6K phosphorylation was analyzed. As shown in Fig. 1*A*, myr-Akt poorly activated S6K phosphorylation in IKKα null cells. However, reexpression of IKKα in IKKα null cells in parallel with myr-Akt expression, led to a significant activation of S6K phosphorylation. Similarly, expression of IKKα in HeLa cells potentiated the S6K activation response to myr-Akt expression (Fig. 1*A*). S6K, mTOR, and Raptor levels did not change under the different experimental conditions. Second, a mutant of IKKα (SS-AA, which is inhibited in its ability to be activated by upstream signals) was tested for its activity in PC3 cells via transfection and measurement of S6K phosphorylation. Expression of IKKα SS-AA

reduced Akt-induced as well as basal levels of S6K phosphorylation in PC3 cells (Fig. 1*B*). These experiments provide evidence for a requirement for IKKα in mediating the ability of constitutively expressed Akt to induce mTOR activity.

IKKα expression induces mTOR activity. To provide an additional link between IKKα and mTOR activation, it was determined if exogenous IKKα expression could induce mTOR activity as measured through phosphorylation of S6K. PC3 cells and HeLa cells were transfected with an expression vector for IKKα or for IKKβ. As shown in Fig. 2*A*, IKKα expression leads to S6K phosphorylation as measured through the S6K phosphospecific antibody. Additionally, IKKβ expression led to a weaker, but clearly positive, effect on S6K phosphorylation (Fig. 2*A*). These transfection conditions did not affect the levels of endogenous mTOR, S6K, or Raptor. We then determined whether IKKα expression could induce phosphorylation of transfected/expressed S6K and 4E-BP1. As shown in Fig. 2*B* and *C*, IKKα expression induced the phosphorylation of both of these well-established downstream targets of mTOR.

Knockdown of IKKα suppresses mTOR activity in PTEN-null, Akt-active prostate cancer cells. To address a potential involvement of IKK in controlling mTOR activity in a manner associated with Akt activity, we analyzed two prostate cancer cell lines (PC3 and LNCaP). PC3 cells do not express PTEN whereas LNCaP cells express a mutated form of PTEN. To block expression of IKKα or IKKβ, siRNA to these kinases was used. A scrambled siRNA was used as a control. As shown in Fig. 3*A*, siRNA specific for IKKα or IKKβ was effective at reducing the expression levels of these two kinases. Subsequently, mTOR activity was assayed through measurement of phosphorylation of downstream kinase targets of mTOR. The results of these experiments revealed that knockdown of expression of IKKα, and to a lesser degree IKKβ, suppressed the phosphorylation of S6K at Thr^{389}, one of the well-established phosphorylation targets of mTOR kinase activity, in the two cancer cells tested (Fig. 3*A*). Additionally, knockdown of IKKα in PC3 cells blocked phosphorylation of a second known mTOR target, 4E-BP1, as well as one of the downstream targets of S6K, namely S6 (Fig. 3*B*). Importantly, there was no effect on the levels of endogenous mTOR, Raptor, or S6K under these experimental conditions (Fig. 3*A* and *B*). To further address an involvement of IKKα in controlling mTOR, we compared knockdown of IKKα to knockdown of mTOR relative to effects on rates of protein synthesis in PC3 cells (Fig. 3*C*). Knockdown of IKKα or mTOR led to an approximate 30% decrease in protein synthesis cells. These results suggest that IKKα controls mTOR activity in PTEN-inactive cancer cells.

IKKα controls mTOR kinase activity in PTEN null cancer cells. To determine if IKKα regulates mTOR kinase activity, two different experiments were done. First, an IKKα expression vector was transfected into 293T cells. Subsequently, mTOR was immunoprecipitated and assayed in an *in vitro* kinase assay using GST-S6K as a substrate. As shown in Fig. 4*A*, expression of IKKα enhanced the levels of endogenous mTOR kinase activity as quantified in the *in vitro* kinase assay. Second, siRNA to IKKα was used to knockdown IKKα in PC3 cells, which exhibit elevated mTOR activity. mTOR was immunoprecipitated from these cells and assayed for *in vitro* kinase activity against GST-S6K (Fig. 4*B*). The results indicated a significantly reduced level of mTOR kinase activity when IKKα levels are reduced. These data indicate that IKKα regulates mTOR kinase activity. To address whether IKKα is involved with mTORC1 and/or mTORC2 activity, Raptor or Rictor was immunoprecipitated from PC3 cells under conditions associated with IKKα siRNA or control siRNA exposure. *In vitro* kinase activity was measured in the immunoprecipitate with recombinant S6K as a substrate. The results revealed (Fig. 4*C*) that knockdown of IKKα has a significantly stronger effect on the activity of the Raptor-mTOR complex (mTORC1) compared with the Rictor-mTOR (mTORC2) complex. Under these experimental conditions, there were no changes in the endogenous levels of mTOR, Raptor or Rictor (Fig. 4*C*).

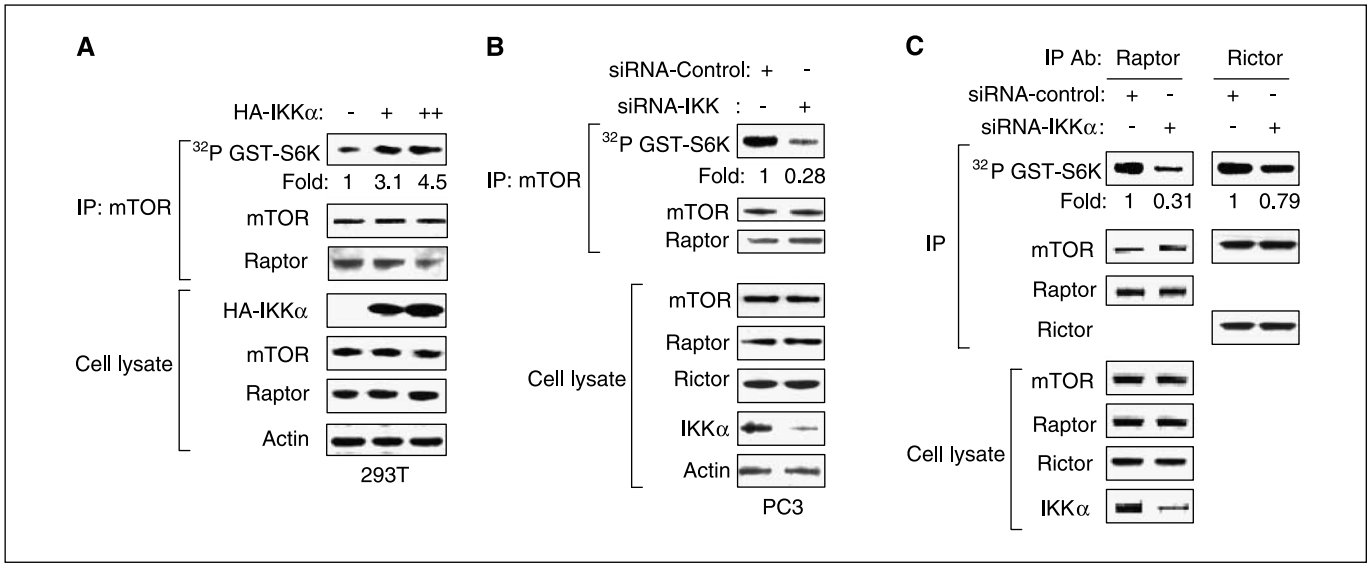

Figure 4. IKKα controls mTOR kinase activity. *A,* expression of IKKα enhances *in vitro* mTOR kinase activity. HEK293T cells were transfected with different amounts of HA-IKKα. Endogenous mTOR was immunoprecipitated (*IP*) with antibody and mTOR kinase activity toward GST-S6K was determined in the immunoprecipitates. *B,* depletion of IKKα with siRNA decreases the mTOR kinase activity in PC3 cells, as measured by *in vitro* mTOR kinase assay using GST-S6K1 as substrate. *C,* PC3 cells were transfected with control siRNA and siRNAs to IKKα as indicated. Endogenous Raptor and Rictor were immunoprecipitated, respectively, with the indicated antibodies. mTOR kinase activity toward GST-S6K was determined in the immunoprecipitates.

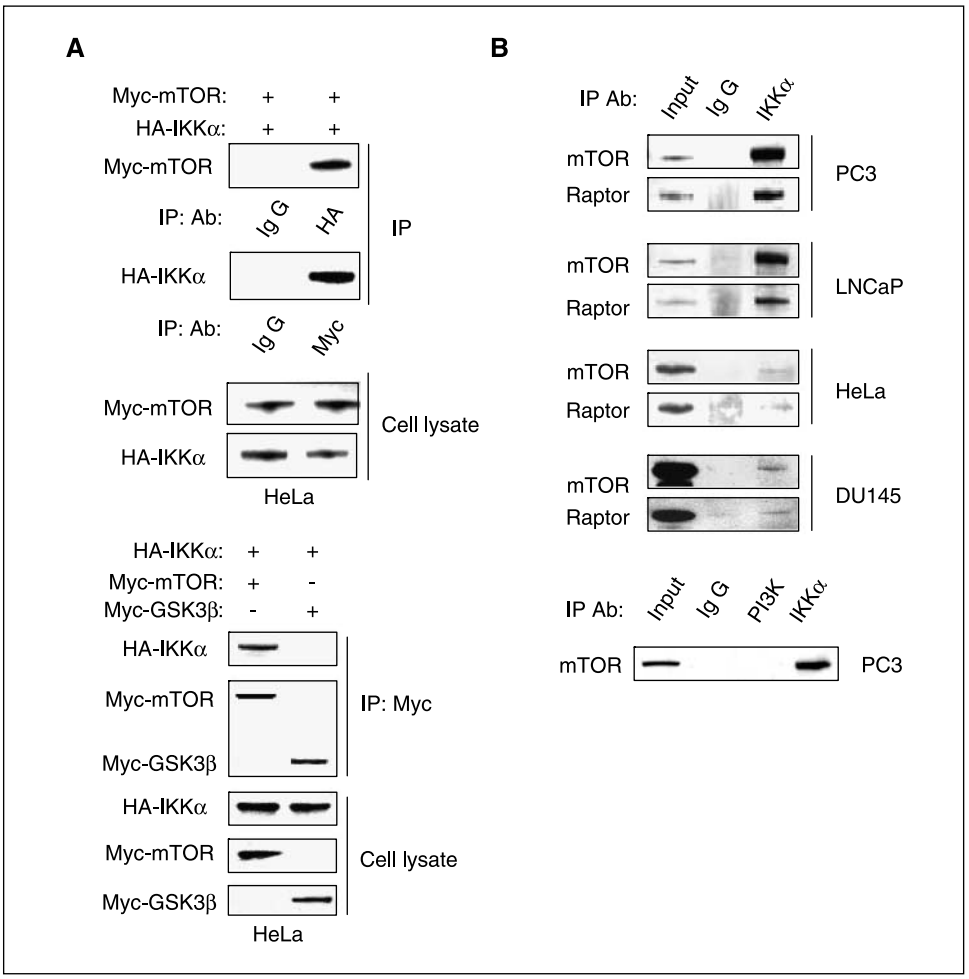

Figure 5. IKKα associates with the mTOR/Raptor complex. *A,* HeLa cells were cotransfected with myc-mTOR and HA-IKKα or myc-GSK3β as indicated. Western blot analyses of immunoprecipitates prepared from the cells using indicated antibodies (*Ab*) are shown after exposure to HA or Myc or antibodies. *B,* immunoprecipitations of endogenous IKKα prepared from indicated cells were analyzed for endogenous mTOR or Raptor levels using the indicated antibodies. Mouse IgG was used as negative control, and total cell lysate (1% input) indicates expression of mTOR.

IKKα interacts with the mTOR-Raptor complex in cells exhibiting constitutively active Akt. To determine if IKKα interacts with the mTOR complex, transfection experiments were carried out in HeLa cells. Myc-tagged mTOR and HA-tagged IKKα were expressed and cell lysates were generated. As shown in Fig. 5*A*, immunoprecipitation with the HA antibody revealed robust association with the myc-tagged mTOR protein. Similarly, immunoprecipitation with the myc antibody showed association with the HA-tagged IKKα protein. Control experiments reveal that Myc-tagged GSK3β does not interact with HA-tagged IKKα (Fig. 5*A, bottom*). To measure a potential interaction between the endogenous mTOR complex and IKKα, immunoprecipitation experiments were carried out from extracts of PC3 and LNCaP cells. As described above, these cells are PTEN-null/inactive and, therefore, exhibit constitutively activated Akt. As examples of PTEN-positive cells, HeLa and DU145 cells were analyzed. Immunoprecipitation of IKKα from these cells, followed by gel electrophoresis and immunoblotting, revealed robust associations between IKKα and mTOR (or an mTOR-associated protein) in PC3 and LNCaP cells but only weak interactions in HeLa cells and DU145 prostate cancer cells, both of which are PTEN positive (Fig. 5*B*). Additionally, Raptor was coimmunoprecipitated with IKKα and this paralleled mTOR levels in the immunoprecipitate (Fig. 5*B*). As a control, it was shown that endogenous PI3K does not interact with mTOR (Fig. 5*B, bottom*). These experiments reveal that IKKα interacts with mTOR and Raptor constitutively in PC3 cells and LNCaP cells.

IKKα-TORC1 interaction in PC3 prostate cancer cells is Akt dependent. Based on the coimmunoprecipitation results described above, we asked whether the interactions between TORC1 and IKKα are dependent on Akt-induced signaling. First, myr-Akt expression in HeLa cells and in DU145 cells resulted in an enhanced mTOR and IKKα interaction as measured by coimmunoprecipitation experiments. Myr-Akt also further enhanced the association between IKKα and TORC1 in PC3 and LNCaP cells (Fig. 6*A*). Myr-Akt promoted the interaction between Myc-tagged mTOR and Flag-tagged IKKα and this paralleled the induction of phosphorylation of a known target of Akt, namely GSK3β (Fig. 6*B*). Second, siRNA against Akt in PC3 cells was used and interaction between TORC1 and IKKα was measured through coimmunoprecipitation experiments. These data revealed (Fig. 6*C*) a significant loss of interaction between mTOR and IKKα after knockdown of Akt2 expression. Knockdown of Akt1, although quantitatively effective, was less efficient in reducing mTOR-IKKα interaction (data not shown), indicating a more dominant role for Akt2 in controlling mTOR and IKKα interaction in PC3 cells. Consistent with this, Akt2 is expressed at significantly higher levels than Akt1 in PC3 cells (data not shown). We then used siRNA to PTEN to reduce levels of this protein in HeLa and DU145 cells, and then measured the interaction between mTOR and IKKα. These experiments revealed that loss of PTEN promoted Akt phosphorylation and enhanced interaction between IKKα and mTOR (Fig. 6*D*). Correspondingly, expression of PTEN in 293T cells with

mTOR and IKKα reduced the interaction between these two proteins while inhibiting the phosphorylation of Akt (Fig. 6*E*). Similarly, expression of PTEN in the PTEN-null/inactive PC3 and LNCaP cells reduced interaction between endogenous mTOR and IKKα (Fig. 6*F*). These experiments reveal that active Akt in PTEN-null cancer cells promotes a functional association between IKKα and the mTOR-Raptor complex.

Discussion

mTOR is a key effector of Akt downstream signaling mechanisms through its ability to control mRNA translation via direct phosphorylation of two key regulatory proteins S6K and 4E-BP1. This mechanism is likely to be important in the progression of certain cancers, as mTOR can regulate the translation of mRNAs encoding proteins important for cell proliferation and survival (2, 4, 14). Previously reported studies indicate that Akt stimulates mTOR activity through its ability to phosphorylate TSC2, ultimately leading to the release of TSC inhibitory activity on the GTPase Rheb (5). Subsequently, Raptor was identified as a key regulator of mTOR functional activity (8, 9). Furthermore, it was proposed that Raptor and mTOR exist in two states, one negative and one positive, with strong/stable interaction being associated with inhibition of mTOR activity (8). Additionally, it was shown that GβL interaction with mTOR is required for nutrient-sensitive interaction between Raptor and mTOR (11). Although stimuli such as insulin are known to regulate mTOR through Akt and downstream regulation on the TSC complex, less is known regarding the ability of constitutively active, cancer-associated Akt to control mTOR function.

Based on evidence that IKK functions downstream of Akt to control its ability to stimulate NF-κB activity (24–26), we explored a potential link between IKK and mTOR regulation. Our data show that IKKα is required for efficient induction of mTOR activity and that IKKα associates with the mTOR-Raptor TORC1 complex in a manner dependent on Akt expression levels in two PTEN-inactive cell lines. Restoration of PTEN expression in these cells reduces the association between IKKα and the mTOR-Raptor complex as measured through coimmunoprecipitation assays. Consistent with these findings, knockdown of IKKα significantly reduces mTOR kinase activity in PC3 and LNCaP cells.

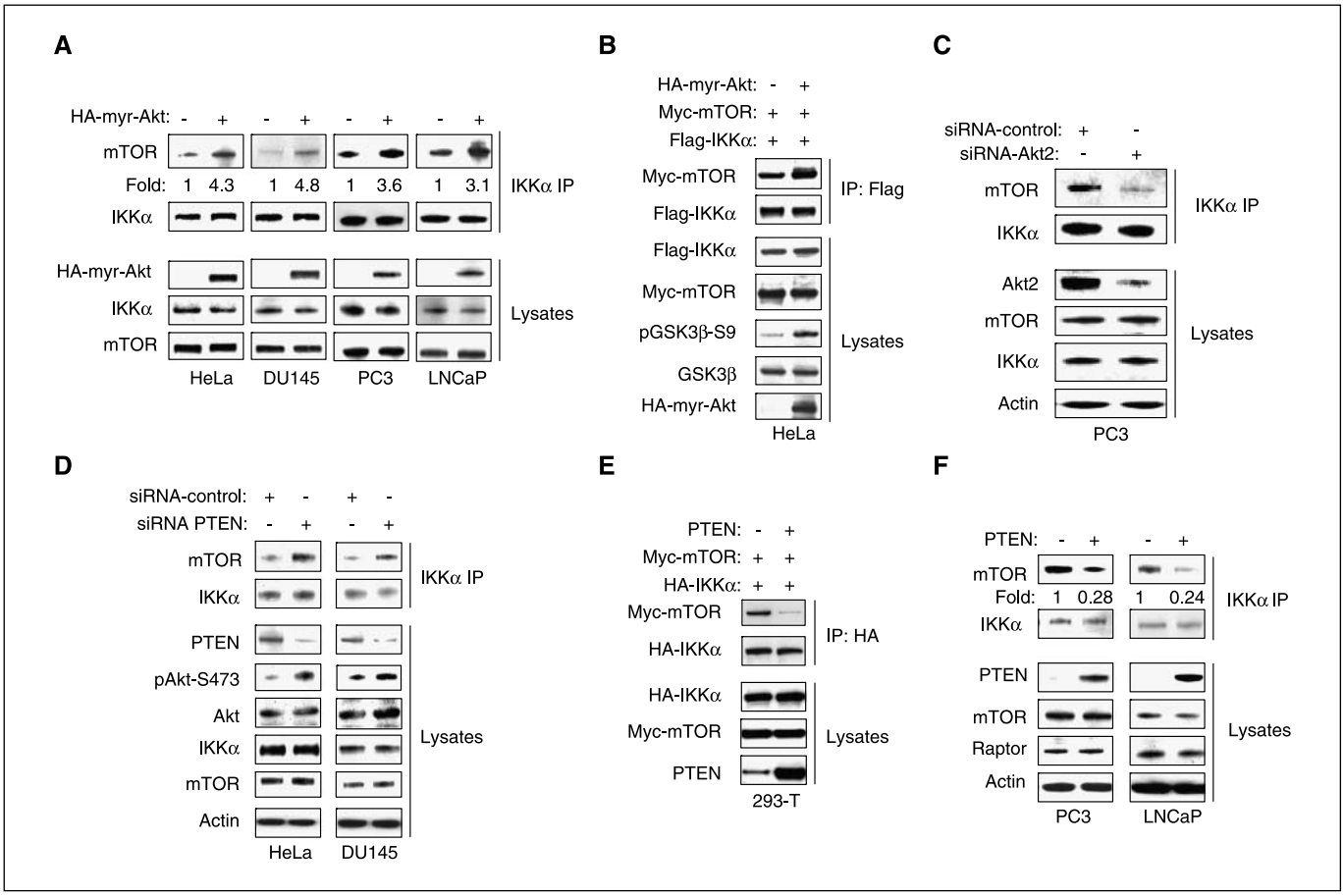

Figure 6. Akt drives IKKα association with the mTOR/Raptor complex. *A,* overexpression of myr-Akt induces the interaction between IKKα and mTOR. HeLa, DU145, PC3, and LNCaP cells were transfected with pcDNA3 or HA-myr-Akt. Lysates were immunoprecipitated with anti-IKKα and blotted with mTOR and IKKα antibodies, respectively. *B,* HeLa cells were transfected with myr-Akt, myc-tagged mTOR, and flag-tagged IKKα, as indicated, followed by immunoprecipitation with the Flag antibody. Lysates were blotted with the myc or flag antibody, as well as antibodies recognizing GSK3β or phosphorylated GSK3β. *C,* PC3 cells were transfected with siRNA control or Akt siRNA as indicated, and the interaction between IKKα and mTOR was measured by coimmunoprecipitation as described for (*A*). *D,* either HeLa or DU145 were treated with siRNA control or siRNA to PTEN and immunoprecipitation carried as described in (*A*). Reconstitution of PTEN suppresses the interaction between IKKα and mTOR, and stabilized mTOR/Raptor complex formation. PC3 and LNCaP cells were transfected with PTEN. Lysates were immunoprecipitated with anti-IKKα or anti-mTOR antibodies, and blotted with indicated antibodies. *E,* PTEN was either coexpressed with myc-tagged mTOR and HA-tagged IKKα and immunoprecipitations were carried out with the HA antibody followed by blotting with myc- or HA-specific antibodies or (*F*) PTEN was expressed in PC3 or LNCaP cells followed by analysis from immunoprecipitation with IKKα antibody and blotting for endogenous mTOR and IKKα.

Our results in PTEN-defective cancer cells suggest that constitutive Akt signaling promotes a robust association between IKKα and TORC1. Presumably, this response reflects a natural role for IKKα in controlling mTOR function, possibly associated with a role in growth factor/nutrient signaling, that has been constitutively co-opted for cancer cell growth and survival under constitutively active Akt conditions. We are presently analyzing the involvement of IKKα and/or IKKβ in regulating mTOR activation downstream of insulin-dependent and nutrient-dependent signaling. However, we cannot rule out a unique, gain-of-function role for IKKα in cancer cells that exhibit loss of PTEN and/or constitutively active Akt. Overall, our results suggest an important role for IKKα in prostate cancer-associated mTOR activity, which is important in the progression of the disease (13).

Although the data show a key role for IKKα in controlling mTOR activity in PC3 and LNCaP prostate cancer cells, we cannot rule a role for IKKβ in this process. As shown in Fig. 1, knockdown of IKKβ has a moderate effect on basal mTOR activity under our experimental conditions. Additionally, overexpression of IKKβ activates mTOR activity (Fig. 2) and coimmunoprecipitation reveals that IKKβ is associated with mTOR (data not shown). Thus, it is possible that a complex containing both IKKα and IKKβ, which is important for classic NF-κB–dependent activation, controls mTOR activity with IKKα serving as the dominant functional subunit. It is also possible that residual mTOR activity found when IKKα is either deleted or knocked down is due to the remaining IKK activity derived from IKKβ.

A potential mechanism to explain the role of IKKα in mediating mTOR function in Akt constitutive cells is unclear at present. It is possible that IKK directly phosphorylates mTOR or an associated protein such as Raptor and that this modulates mTOR activity. Consistent with the idea that IKK catalytic activity is important for its ability to control mTOR, expression of a mutant of IKKα in PC3 cells blocks mTOR activation, both basally and induced by myr-Akt (Fig. 1*B*). It is also possible that a physical association between IKKα and TORC1, potentially not involving IKK catalytic activity, affects the mTOR activity through modulation of a regulatory factor such as Raptor. Future experiments are being designed to address these issues. In conclusion, the results reveal a novel role for IKK, and particularly the IKKα subunit, in mediating mTOR function in cancer cells that exhibit constitutively active Akt.

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