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Protein Phosphatase 2A and DNA-dependent Protein Kinase Are Involved in Mediating Rapamycin-induced Akt Phosphorylation*

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Inhibition of mammalian target of rapamycin complex 1 (mTORC1), for example with rapamycin, increases Akt phosphorylation while inhibiting mTORC1 signaling. However, the underlying mechanisms have not been fully elucidated. The current study has uncovered a previously unknown mechanism underlying rapamycin-induced Akt phosphorylation involving protein phosphatase 2A (PP2A)-dependent DNA protein kinase (DNA-PK) activation. In several cancer cell lines, inhibition of PP2A with okadaic acid, fostriecin, small T antigen, or PP2A knockdown abrogated rapamycin-induced Akt phosphorylation, and rapamycin increased PP2A activity. Chemical inhibition of DNA-PK, knockdown or deficiency of DNA-PK catalytic subunit (DNA-PKcs), or knock-out of the DNA-PK component Ku86 inhibited rapamycin-induced Akt phosphorylation. Exposure of cancer cells to rapamycin increased DNA-PK activity, and gene silencing-mediated PP2A inhibition attenuated rapamycin-induced DNA-PK activity. Collectively these results suggest that rapamycin induces PP2A-dependent and DNA-PK-mediated Akt phosphorylation. Accordingly, simultaneous inhibition of mTOR and DNA-PK did not stimulate Akt activity and synergistically inhibited the growth of cancer cells both in vitro and in vivo. Thus, our findings also suggest a novel strategy to enhance mTOR-targeted cancer therapy by co-targeting DNA-PK.

The mammalian target of rapamycin (mTOR), a phosphatidylinositol 3-kinase (PI3K)-related serine/theronine kinase, plays a central role in regulating multiple cell functions including cell growth, proliferation, and survival primarily through interactions with other proteins such as raptor (forming mTOR complex 1, mTORC1) and rictor (forming mTOR complex 2, mTORC2) (1–3). The best characterized downstream effectors of mTORC1 are the 70-kDa ribosomal S6 kinase (p70S6K) and the eukaryotic translation initiation factor 4E-binding protein 1 (4EBP1) (1). In response to mitogenic stimuli or nutrient availability, mTORC1 is activated (4), leading to phosphorylation of p70S6K and 4EBP1, and the subsequent enhanced translation of mRNAs that are critical for cell cycle progression and proliferation (1). Compared with mTORC1, the upstream and downstream regulation of mTORC2 signaling has not been fully elucidated. However, mTORC2 targets or phosphorylates a number of the AGC family members, including Akt (Ser473), protein kinase Ca (PKCa; Ser657), and serum and glucocorticoid-induced protein kinase (SGK1; Ser622) (5, 6).

PI3K and Akt signaling represents a major cell survival pathway. It is generally thought that mTOR (i.e. mTORC1) functions downstream of the PI3K and Akt pathway and is phosphorylated (or activated) in response to stimuli that activate the PI3K and Akt pathway (1, 7). However, the discovery of mTORC2 as an Akt Ser473 kinase also places mTOR upstream of Akt. Although mTORC2 is thought to be insensitive to rapamycin, it has been shown that prolonged rapamycin exposure inhibits mTORC2 assembly and Akt activity in certain types of cancer cells (8). We and others have shown that the conventional mTOR inhibitor rapamycin and its analogs (rapalogs) activate Akt while suppressing mTORC1 signaling in different types of cancer cell lines and clinical human tumor samples (9–11). Our previous study suggested that sustained Akt phosphorylation during rapamycin treatment seems associated with...
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the development of rapamycin resistance (11). A recent study suggests that early increase in Akt phosphorylation (particularly at Thr\(^{308}\)) predicts a better response of cancer cells to rapalogs (12). Currently, it is unclear how mTOR inhibitors activate Akt survival signaling.

Insulin receptor substrate-1 (IRS-1) is an important mediator of insulin receptor-dependent activation of PI3K. Chronic insulin stimulation causes the phosphorylation and degradation of IRS-1 protein in a rapamycin-sensitive manner (13, 14). Thus, studies on insulin signaling in mammalian skeletal muscle cells, adipocytes, and fibroblasts have demonstrated that mTOR activation by insulin initiates a feedback inhibition of PI3K and Akt, likely through p70S6K and its subsequent phosphorylation of IRS-1. The phosphorylation of IRS-1 promotes IRS-1 degradation and reduces its abundance, leading to decreased activity of PI3K and Akt. Thus, it has been proposed that rapamycin suppresses p70S6K and thus relieves this negative feedback inhibition of Akt, leading to activation of the PI3K and Akt survival pathway (13, 14). Recent studies have suggested that mTORC1 exerts feedback inhibition of PI3K/Akt signaling by activating and destabilizing Grb10 through subsequent inhibition and destabilization of IRS proteins, particularly in cells lacking tuberous sclerosis complex 2. Accordingly, chronic mTORC1 inhibition (e.g. with long term rapamycin exposure) inactivates and destabilizes Grb10, relieving this feedback inhibition followed with Akt activation (15, 16).

Protein phosphatase 2A (PP2A) is the major protein serine/threonine phosphatase that modulates, particularly down-regulates, activated protein kinases in eukaryotic cells. The core enzyme of PP2A comprises a 36-kDa catalytic subunit (PP2Ac or C) that is always associated with a 65-kDa scaffolding subunit (PR65 or A), which modulates its enzymatic properties, substrate specificity, and subcellular localization (17). PP2A has long been suggested as an mTOR effector in both yeast and mammals (18). Through suppression of PP2A activity, mTOR (or TOR) regulates p70S6K activity (19), ERK signaling (20), and other biological processes (21–25).

DNA-dependent protein kinase (DNA-PK) is a complex of the DNA-PK catalytic subunit (DNA-PKcs) and the DNA end-binding Ku70-Ku80 (or Ku86) heterodimer. Like mTOR, it belongs to the PI3K-like kinase family of proteins. DNA-PK is required for the repair of DNA double-strand breaks (DSBs) through the process of nonhomologous end joining (26, 27). DNA-PK undergoes phosphorylation and dephosphorylation, which regulate its activity. Phosphorylation of DNA-PK (including both Ku and DNA-PKcs components) correlates with loss of protein kinase activity and disruption of DNA-PKcs from the Ku-DNA complex (28). In contrast, PP2A-mediated dephosphorylation of DNA-PK enhances the formation of the Ku-DNA-PKcs complex and increases DNA-PK activity (29, 30). In addition to its role in DNA repair, DNA-PK has been shown to function as an Akt Ser\(^{473}\) kinase, particularly under the condition of DNA damage (31–37).

The current study aimed to explore other possible mechanisms by which rapamycin increases Akt phosphorylation, particularly after an acute exposure. Consequently we identified a previously unknown mechanism underlying rapamycin-induced Akt phosphorylation involving PP2A and DNA-PK.

EXPERIMENTAL PROCEDURES

Reagents—Rapamycin and okadaic acid (OA) were purchased from LC Laboratories (Woburn, MA). RAD001 was provided by Novartis Pharmaceuticals Corporation (East Hanover, NJ). Nu7441 was provided by KuDOS Pharmaceuticals Inc. (Cambridge, UK). Nu7026 was purchased from Cayman (Ann Arbor, MI). These agents were dissolved in dimethyl sulfoxide at a concentration of 20 mM, and aliquots were stored at –80 °C. Stock solutions were diluted to the desired final concentrations with growth medium just before use. Antibodies against Akt, mTOR, raptor, p70S6K, S6, p-Akt (Ser\(^{473}\)), p-p70S6K (Thr\(^{389}\)), p-S6 (Ser\(^{235/236}\)), p-4EBP1 (Ser\(^{65}\)), lamin A/C, c-Myc, and cyclin D1 were purchased from Cell Signaling Technology. Mouse monoclonal anti-PP2A antibody (clone 1D6) was purchased from Upstate Biotechnology. Mouse monoclonal anti-DNA-PKcs (G-4) and rabbit polyclonal anti-Mcl-1 (S-19) antibodies and fostriecin were purchased from Santa Cruz Biotechnology. Rabbit polyclonal anti-actin and anti-tubulin antibodies were purchased from Sigma. Rabbit polyclonal anti-GAPDH antibody was purchased from Trevigen (Gaithersburg, MD).

Cell Lines and Cell Culture—Human cancer cell lines used in this study were purchased from the American Type Culture Collection (ATCC, Manassas, VA) and were grown as described previously (9). Wild-type (WT), Ku86-KO and DNA-PKcs-KO mouse embryonic fibroblasts (MEFs) were provided by Dr. David J. Chan (University of Texas Southwestern Medical Center, Dallas, TX). The human embryonic kidney (HEK) cell lines, HEK-TERST, HEK-TERST110, and HEK-TERV, which stably express Small T (ST) antigen, mutant ST antigen (ST100), and empty vector, respectively (38), were provided by Dr. David Pallas (Emory University, Atlanta, GA).

Western Blot Analysis—The procedures for preparation of whole cell protein lysates from cells and tumor xenografts and for Western blotting were described previously (9, 11, 39). Protein band intensities were quantitated with National Institutes of Health ImageJ software (Bethesda, MA).

Gene Knockdown by Small Interfering RNA (siRNA)—Control (nonsilencing) (1022076) siRNA, raptor siRNA that targets 5′-AAAGGCTAGTCTGTTTCGAAAT-3′ and DNA-PKcs siRNA that targets 5′-AAAGGGCCAAGCTGTCACTCT-3′ (40) were synthesized by Qiagen. Human PP2Ac siRNA (sc-43509) was purchased from Santa Cruz Biotechnology. The transfection of siRNA was conducted in a 12-well or 24-well plate using Lipofectamine 2000 (Invitrogen) or HiPerFect (Qiagen) following the manufacturer’s instructions. Forty-eight hours after transfection, cells were treated with dimethyl sulfoxide or rapamycin for the given times and then subjected to detection of the given proteins by Western blot analysis.

PP2A Activity Assay—PP2A activity was determined using a PP2A Immunoprecipitation Phosphatase Assay kit from Upstate Biotechnology following the manufacturer’s instructions.

DNA-PK Activity Assay—The given cell lines were treated with dimethyl sulfoxide or rapamycin for 1 h and then harvested for preparation of whole cell protein lysates. These lysates (800 μg) were incubated with 2 μg of DNA-PKcs antibody (G-4; Santa Cruz Biotechnology) and 60 μl of protein A/G

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beads with shaking at 4 °C overnight. The beads were washed thoroughly with lysis buffer, cold 1× PBS, and DNA–PK reaction buffer, sequentially. Each immunoprecipitation sample was split equally into three tubes for the subsequent DNA–PK ex vivo activity assay using the SignalTECT DNA–PK assay system (Promega) according to the manufacturer’s instructions.

Preparation of Cytoplasmic and Nuclear Fractions—Nuclear and cytoplasmic extracts were prepared with NE-PER® Nuclear and Cytoplasmic Extraction Reagents (Pierce) according to the manufacturer’s protocol. Tubulin and lamin A/C were used as cytoplasmic- and nucleus-specific markers for the purity of the fractions.

Growth Inhibition Assay—Cell numbers were estimated by the sulforhodamine B assay, and growth inhibition was calculated as described previously (42). Moreover, we used the colony formation assay as described previously (11) to determine the long term growth inhibitory effect. The combination index for drug interaction (e.g., synergy) was calculated using CompuSyn software (ComboSyn, Paramus, NJ).

Cancer Xenografts and Treatments—Animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of Emory University and were conducted as described previously (11, 43). In brief, H460 cells at 5 × 10^6 in serum-free medium were injected subcutaneous into the flank region of nude mice. H460 xenografts were treated (once a day) with vehicle control, RAD001 (4 mg/kg, oral gavage), Nu7026 (20 mg/kg, intraperitoneally) and their combination (RAD + Nu) starting on the same day after grouping for 14 days.

Immunohistochemistry—Immunohistochemical analysis on formalin-fixed, paraffin-embedded nude mouse xenograft tissues was done as described previously (11). The primary antibodies against p-Akt (Ser^473^) (Epitomics; Burlingame, CA) and p-S6 (Ser^235/236^) (Cell Signaling Technology) were used at 1:25 and 1:50 dilutions, respectively.

Statistical Analysis—The statistical significance of differences between two groups or among multiple groups was analyzed with two-sided unpaired Student’s t tests (for equal variances) or with Welch’s corrected t test (unequal variances) or one-way analysis of variance by use of GraphPad InStat 3 software. Results were considered to be statistically significant at p < 0.05.

RESULTS

Inhibition of PP2A Abrogates mTORC1 Inhibition-induced Akt Phosphorylation—Through an effort to demonstrate the potential mechanisms underlying rapamycin-induced Akt phosphorylation, we found that OA, a PP2A inhibitor, attenuated the ability of rapamycin to increase p-Akt amounts in a dose-dependent manner, although it weakly increased p-Akt amounts itself at high concentration ranges (e.g., 25–50 nM; Fig. 1A). With another PP2A inhibitor, fostriecin, we generated similar results (Fig. 1B). These findings led us to ask whether PP2A is involved in rapamycin-induced Akt phosphorylation. We then compared the effects of rapamycin on Akt phosphorylation among HEK cells transfected with vector (TERV), ST antigen (TERST) that is known to inactivate PP2A, or mutant ST antigen (TERST110). Treatment with rapamycin at both 1 nM and 10 nM for 1, 3, or 24 h increased the amount of p-Akt in both TERV and TERST110 cell lines, but not in TERST cells (Fig. 1C), indicating that the presence of ST antigen abrogated rapamycin-induced Akt phosphorylation. These findings suggest that rapamycin may increase Akt phosphorylation through a PP2A-dependent mechanism.

To demonstrate robustly the involvement of PP2A in rapamycin–or mTORC1 inhibition-induced Akt phosphorylation, we directly knocked down PP2Ac to inhibit PP2A activity and then examined its impact on rapamycin-induced Akt phosphorylation. Depending on the silencing effects, PP2Ac siRNA either attenuated or abolished rapamycin-induced Akt phosphorylation. Specifically, PP2Ac siRNA at 5 nM, which partially reduced the amount of PP2Ac, attenuated rapamycin-induced Akt phosphorylation; however, at 20 nM, which exerted a more substantial effect on decreasing the amount of PP2Ac, it abolished rapamycin-induced Akt phosphorylation in both H157 and A549 cells (Fig. 1D). Similar results were also generated when we extended our study to a few more cancer cell lines (i.e., H358, Calu-1, and Du145) (Fig. 1E). Because experiments in Fig. 1, D and E, used a 1-h treatment with rapamycin, we further analyzed the effects of silencing PP2Ac on p-Akt in cells exposed to rapamycin for 24 h. Again, we observed that knock-down of PP2Ac attenuated or abolished rapamycin-induced Akt phosphorylation (data not shown).

We further investigated whether inhibition of PP2A suppresses Akt phosphorylation induced by raptor knockdown-mediated mTORC1 inhibition. Increased p-Akt was detected in cells transfected with raptor siRNA alone (lanes 3 and 7), but not in cells transfected with both PP2Ac and raptor siRNAs (lanes 4 and 8) in both H157 and A549 cells (Fig. 1F). These data clearly indicate that PP2A also mediates raptor inhibition-induced Akt phosphorylation.

Rapamycin Increases PP2A Activity—It has been suggested that mTOR negatively regulates PP2A activity (19, 20). Thus, we next determined whether rapamycin increases PP2A activity in our cell systems. Indeed, treatment of both H157 cell and Du145 cells with 10 nM rapamycin exhibited a time-dependent effect in increasing PP2A activity (Fig. 1G). Therefore, rapamycin activates PP2A in our cell systems.

Inhibition of DNA–PK Prevents Akt Phosphorylation Induced by Rapamycin or Raptor Silencing—Given that DNA–PK functions as an Akt Ser^473^ kinase (44) and its activity is enhanced by PP2A-mediated protein dephosphorylation (29, 30), we asked whether DNA–PK activation is involved in mTORC1 inhibition-induced Akt phosphorylation. To this end, we first treated cells with rapamycin in the absence and presence of the DNA–PK inhibitor, Nu7441 (e.g., 1 and 5 μM) and then compared alterations in the amount of p-Akt. Rapamycin increased the amount of p-Akt in the absence of Nu7441 and failed to do so in the presence of Nu7441 in both H157 and A549 cell lines (Fig. 2A). To avoid possible off-target effects caused by high concentrations of small molecules, we further reduced the concentrations of Nu7441 to 100 nM and found that Nu7441 at low concentration ranges (e.g., 100–1000 nM) still abrogated rapamycin-induced Akt phosphorylation (Fig. 2B). In agreement, the presence of another DNA–PK inhibitor, Nu7026, also abrogated rapamycin-induced Akt phosphorylation in both A549 and H157 cells (data not shown). Thus,
these data suggest that rapamycin induces a DNA-PK-dependent Akt phosphorylation.

We next inhibited DNA-PK through knockdown of DNA-PKcs and then examined its impact on rapamycin-induced Akt phosphorylation. We detected reduced amounts of DNA-PKcs in all three cancer cell lines transfected with DNA-PKcs siRNA, indicating the successful knockdown of DNA-PKcs. Rapamycin increased p-Akt amounts in control siRNA-transfected cells, but not in cells transfected with DNA-PKcs siRNA (Fig. 2C–D). Finally, we analyzed the effects of rapamycin on Akt phosphorylation in cells deficient in either DNA-PKcs (M059K or DNA-PKcs-KO MEFs) or Ku80 (Ku80-KO). In agreement with the data from the above knockdown experiments, rapamycin at the tested concentrations (1–100 nM) increased p-Akt amounts both in M059K cells (with DNA-PKcs) and wild-type (WT) MEFs, but not in M059 cells, DNA-PKsc-KO MEFs, or Ku80-KO MEFs (Fig. 2, C–D). These data hence provide strong evidence supporting the critical role of DNA-PK in mediating rapamycin-induced Akt phosphorylation.

We also examined the impact of DNA-PK inhibition on raptor knockdown-induced Akt phosphorylation. Consistent with the aforementioned experiments, siRNA-mediated raptor knockdown increased p-Akt amounts in M059K cells, but not in M059 cells (Fig. 2G). These data again support the notion that DNA-PK mediates mTORC1 inhibition-induced Akt phosphorylation.

Rapamycin Increases PP2A-dependent DNA-PK Activity—We further determined whether rapamycin is able to increase DNA-PK activity. To this end, we treated two cancer cell lines (A549 and H157) for 1 h with rapamycin (Rap) and OA or fostriecin for an additional 1 h. SE, short exposure. C, the given cell lines were treated with the indicated concentrations of OA or fostriecin for 30 min and then co-treated with 10 nM rapamycin (Rap) and OA or fostriecin for an additional 1 h. NT, no transfection. E, the given cell lines were transfected with the indicated siRNAs and 48 h later were treated with 10 nM rapamycin for an additional 1 h. NT, no transfection. F, the given cell lines were transfected with control (Ctrl) or PP2Ac siRNA as indicated and 48 h later were treated with 10 nM rapamycin for an additional 1 h. NT, no transfection. F, the given cell lines were transfected with the indicated siRNAs and 48 h later were treated with 10 nM rapamycin for an additional 1 h. NT, no transfection. F, the given cell lines were transfected with the indicated siRNAs and 48 h later were treated with 10 nM rapamycin for an additional 1 h. NT, no transfection. F, the given cell lines were transfected with the indicated siRNAs and 48 h later were treated with 10 nM rapamycin for an additional 1 h. NT, no transfection.
sured its activity. We detected significantly increased DNA-PK activity either with radioactive counting or with dot blotting in both A549 and H157 cell lines exposed to rapamycin or VP16 (Fig. 3A), indicating that rapamycin activates DNA-PK. To determine whether rapamycin-induced DNA-PK activation is secondary to PP2A activation, we knocked down PP2Ac to inhibit PP2A and then examined its impact on rapamycin-induced DNA-PK activation. Knockdown of PP2Ac not only reduced basal levels of DNA-PK activity, but also significantly attenuated rapamycin-induced DNA-PK activity compared with DNA-PK activity in control siRNA-transfected cells (Fig. 3B). Thus, we assume that rapamycin-induced PP2A and DNA-PK-dependent Akt phosphorylation can occur in both the cytoplasm and nucleus.

**Rapamycin-induced Akt Phosphorylation Occurs in Both the Cytoplasm and Nucleus**—To determine the locations in which rapamycin-induced Akt phosphorylation occurs, we treated A549 cells with 10 nM rapamycin for 1 h and then harvested cells for fractioning cytoplasmic and nuclear proteins. In this study, we used tubulin (a cytoplasmic protein) and lamin A/C (a nuclear protein) to control the purity of the fractions. We could easily detect increased p-Akt in the cytoplasmic fraction treated with rapamycin. After a prolonged exposure, we also detected increased p-Akt in the nuclear fraction treated with rapamycin. Although DNA-PK was detected mainly in the nuclear fraction, we did detect basal amounts of cytoplasmic DNA-PKcs and an increased amount of cytoplasmic DNA-PKcs in cells treated with rapamycin after a long exposure of the film. PP2A could be easily detected in both fractions (Fig. 3C). Thus, we assume that rapamycin-induced PP2A and DNA-PK-dependent Akt phosphorylation can occur in both the cytoplasm and nucleus.

**Co-inhibition of mTOR and DNA-PK Synergistically Inhibits the Growth of Cancer Cells in Vitro and in Vivo**—Given that inhibition of DNA-PK abrogates rapamycin-induced Akt activation, we wondered whether co-inhibition of mTOR and DNA-PK would exert enhanced anticancer activity. In six lung cancer cell lines, the combination of rapamycin and Nu7026 exhibited much more potent growth-inhibitory effects than either agent alone. The combination index for the combination in each cell line was <1, indicating synergistic growth-inhibitory effects (Fig. 4A). In a 12-day colony formation assay with repeating treatments, the combination of rapamycin and Nu7026 was also more effective than either agent alone in inhibiting the growth of cell colonies in both A549 and H157 cells (Fig. 4, B and C). In agreement, the combination of RAD001 and Nu7026 was more potent than either single agent.
in inhibiting the growth of lung cancer xenografts. In this study, both RAD001 and Nu7026 alone at the tested doses weakly inhibited the growth of H460 xenografts \((p > 0.05)\); however, their combination significantly inhibited the growth of H460 tumor growth \((p < 0.05\) to 0.001) (Fig. 5A). Importantly, the body weights of mice treated with RAD001 and Nu7026 were not significantly reduced and were comparable with those of other treatment groups (Fig. 5B). Collectively, the combination of RAD001 and Nu7026 exerts enhanced \(i.e.\) synergistic anticancer activity both \textit{in vitro} and \textit{in vivo}, without enhanced toxicity.

By analyzing protein markers involved in mTOR signaling in these xenograft tissues, we found that the amount of \(p\)-Akt was significantly increased in RAD001-treated tissues \((p < 0.001)\) but not in tissues exposed to both RAD001 and Nu7026 (Fig. 5, C–E). These data indicate that Nu7026 abrogates Akt phosphorylation induced by RAD001 \textit{in vivo}. We noted that the amounts of both \(p\)-S6 and \(p\)-p70S6K were abolished in tumors exposed to either RAD001 alone or the combination of RAD001 and Nu7026 (Fig. 5, C–E). Amounts of \(p\)-4EBP1 \((\text{Ser}^{65})\) were weakly reduced in RAD001-treated tissues \((p < 0.05)\) but greatly reduced in tissues treated with both RAD001 and Nu7026 \((p < 0.01)\). Consistently, we detected the lowest amounts of cyclin D1, c-Myc, and Mcl-1 in tissues treated with the RAD001 and Nu7026 combination (Fig. 5, C and D). These results together suggest that the combination of RAD001 and Nu7026 exerts enhanced effects on the inhibition of mTORC1 signaling.

**DISCUSSION**

It is known that mTOR negatively regulates PP2A activity and accordingly rapamycin activates PP2A activity \((19, 20, 45)\). Indeed, we detected increased PP2A activity in cancer cells exposed to rapamycin (Fig. 1G). Intriguingly, we have shown that mTORC1 inhibition by both rapamycin and raptor siRNA induces Akt phosphorylation through a PP2A-dependent mechanism based on the following evidence: (i) the known small molecule PP2A inhibitors, OA and fostriecin, attenuated rapamycin-induced Akt phosphorylation; (ii) overexpression of ST antigen, a well known viral protein that inactivates PP2A, abolished rapamycin-induced Akt phosphorylation; (iii) silencing of the PP2Ac subunit that is responsible for PP2A catalytic activity in several cancer cell lines attenuated or abolished Akt phosphorylation induced by both rapamycin and raptor siRNA (Fig. 1).

How PP2A activation mediates rapamycin-induced Akt phosphorylation is unknown. Given the fact that rapamycin rapidly increases Akt phosphorylation \((within 1 h)\) (9), it is logical to speculate that the rapamycin-activated PP2A dephosphorylates and activates an Akt kinase that directly phosphorylates Akt or an unknown protein that indirectly activates an Akt kinase, leading to Akt activation. DNA-PK has been documented as an Akt \(\text{Ser}^{373}\) kinase, and its activity is regulated by protein phosphorylation \((44)\). It has been shown that PP2A activates DNA-PK activity through dephosphorylation \((29, 30)\). In our study, we found that rapamycin-induced Akt phosphorylation could be abolished by the presence of the DNA-PK inhibitor Nu7441 or Nu7026, by knockdown of DNA-PKcs, and by deficiency of DNA-PKcs or Ku80. In agreement, deficiency of DNA-PKcs also abrogated raptor knockdown-induced Akt phosphorylation (Fig. 2). Furthermore, rapamycin could significantly increase DNA-PK activity (Fig. 3A). Collectively, these findings robustly demonstrated that DNA-PK indeed mediates Akt phosphorylation induced by mTORC1 inhibition \(e.g.\) with rapamycin or raptor knockdown.

Importantly, we have shown that rapamycin induces PP2A-dependent DNA-PK activation because inhibition of PP2A by knockdown of PP2Ac significantly impaired the ability of rapa-
mycin to increase DNA-PK activity (Fig. 3B). Thus, we propose that rapamycin or mTORC1 inhibition (e.g. with raptor knockdown) activates PP2A, which dephosphorylates and activates DNA-PK, leading to increased Akt phosphorylation. This process is likely to be parallel with p70S6K and 4EBP1 inhibition (Fig. 6).

It is well known that DNA-PK is activated by DSBs (44). Two recent studies have shown that rapamycin inhibits yeast nucleotide excision repair (46) and suppresses homologous recombination and nonhomologous end joining, two major mechanisms required for repairing DNA DSBs, in cancer cells (47), suggesting that rapamycin inhibits DNA repair. Accordingly, rapamycin treatment could result in persistent radiation-induced DNA DSBs (47). Although rapamycin itself does not directly damage DNA as reported (48), it is possible that persistent inhibition of DNA repair by sustained treatment with rapamycin may cause accumulation of spontaneously damaged DNA, leading to increased DNA-PK activity. In our previous study, rapamycin induced a rapid (within 1 h) and sustained (up to 96 h) Akt phosphorylation (9, 11). Our current study focused on demonstrating how rapamycin induces rapid Akt phosphorylation through primarily using a short treatment time (e.g. 1 h).

Therefore, we propose that acute rapamycin treatment rapidly increases Akt phosphorylation through a PP2A-dependent DNA-PK activation, and sustained rapamycin treatment further enhances DNA-PK activity, resulting in a persistent increase in Akt phosphorylation, through increasing DNA damage via sustained inhibition of DNA repair (Fig. 6). In this way, we may explain how rapamycin induces sustained AKT phosphorylation.

Cytoplasmic and even membrane-bound DNA-PKcs, including the DNA-PK complex, has been reported although it is primarily a nuclear protein or kinase (49–51). In our study, we could clearly detect cytoplasmic DNA-PK in our cell system, which was even increased upon treatment with rapamycin. Moreover, we detected increased p-Akt in both cytoplasmic and nuclear fractions from cells exposed to rapamycin (Fig. 3C). Thus, we suggest that rapamycin-induced Akt phosphorylation may occur in both the cytoplasm and nucleus.

Given that PP2A is generally thought to be an Akt phosphatase that inhibits Akt phosphorylation (17), our finding of PP2A mediation of mTORC1 inhibition-induced Akt phosphorylation seems surprising. In our study, we noted that OA alone weakly increased p-Akt amounts in our cell systems (Fig. 1A),

FIGURE 4. The combination of a rapalog with Nu7026 synergistically inhibits the growth of human lung cancer cells in vitro. A, the indicated cell lines seeded in 96-well plates were treated with the given concentrations of rapamycin (Rap) alone, Nu7026 (Nu) alone, or their combination. After 3 days, cell numbers were estimated with the sulforhodamine B assay, and combination indexes were calculated with CompuSyn software as labeled inside the graphs. The data are means ± S.D. (error bars) (n = 4). B and C, the given cell lines were seeded in 12-well plates and the next day treated with the indicated concentrations of Rap alone, Nu7026 alone, or their combination. The same treatments were repeated every 3 days. After 12 days, the cell colonies were fixed and stained for photography (B) and counting (C). The data are means ± S.D. (n = 3).
although co-treatment with OA and rapamycin attenuated rapamycin-induced Akt phosphorylation. However, both overexpression of ST antigen and silencing of PP2Ac (Fig. 1) did not increase p-Akt amounts; in contrast, we observed decreased p-Akt in almost every tested cell line transfected with PP2Ac siRNA (Fig. 1). Thus, the specific genetic suppression of PP2A with the approaches discussed above in our systems failed to demonstrate a suppressive role of PP2A in inhibiting Akt phosphorylation.

In this study, we have demonstrated that co-inhibition of mTOR and DNA-PK (e.g. by the combination of RAD001 and Nu7026) synergistically inhibits the growth of lung cancer cells both in vitro and in vivo without detectable toxicity (Figs. 4 and 5). The presence of Nu7026 not only prevented Akt activation induced by RAD001, but also enhanced the effect of RAD001 on suppressing 4EBP1 phosphorylation and down-regulating cyclin D1, c-Myc, and Mcl-1, which are known to be regulated by mTOR signaling. Thus, our findings suggest a novel and effective strategy to enhance mTOR-targeted cancer therapy by inhibition of DNA-PK activity. Further clinical investigation in this regard is warranted.

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