Rapamycin Induces Bad Phosphorylation in Association with Its Resistance to Human Lung Cancer Cells

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Abstract

Inhibition of mTOR signaling by rapamycin has been shown to activate extracellular signal-regulated kinase 1 or 2 (ERK1/2) and Akt in various types of cancer cells, which contributes to rapamycin resistance. However, the downstream effect of rapamycin-activated ERKs and Akt on survival or death substrate(s) remains unclear. We discovered that treatment of human lung cancer cells with rapamycin results in enhanced phosphorylation of Bad at serine (S) 112 and S 136 but not S 155 in association with activation of ERK1/2 and Akt. A higher level of Bad phosphorylation was observed in rapamycin-resistant cells compared with parental rapamycin-sensitive cells. Thus, Bad phosphorylation may contribute to rapamycin resistance. Mechanistically, rapamycin promotes Bad accumulation in the cytosol, enhances Bad/14-3-3 interaction, and reduces Bad/Bcl-XL binding. Rapamycin-induced Bad phosphorylation promotes its ubiquitination and degradation, with a significant reduction of its half-life (i.e., from 53.3–37.5 hours).

Inhibition of MEK/ERK by PD98059 or depletion of Akt by RNA interference blocks rapamycin-induced Bad phosphorylation at S112 or S136, respectively. Simultaneous blockage of S112 and S136 phosphorylation of Bad by PD98059 and silencing of Akt significantly enhances rapamycin-induced growth inhibition in vitro and synergistically increases the antitumor efficacy of rapamycin in lung cancer xenografts. Intriguingly, either suppression of Bad phosphorylation at S112 and S136 sites or expression of the nonphosphorylatable Bad mutant (S112A/S136A) can reverse rapamycin resistance. These findings uncover a novel mechanism of rapamycin resistance, which may promote the development of new strategies for overcoming rapamycin resistance by manipulating Bad phosphorylation at S112 and S136 in human lung cancer.

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Introduction

The mTOR is a serine/threonine kinase and functions as a central regulator of cell growth, cell proliferation, and survival (1, 2). mTOR has been identified as a downstream target of the PI3K/Akt survival pathway. The discovery and clinical development of the highly specific and potent mTOR inhibitor rapamycin and its derivatives (i.e., RAD001, CCI-779, and AP23573) as anticancer agents has further enhanced our ability to elucidate mTOR biological function (3–5). Inhibition of the mTOR pathway has been proposed to represent a promising therapeutic approach for lung cancer (5–7). However, it is increasingly recognized that the clinical activity of rapalog(s) as a single agent is insufficient for achieving a broad and robust anticancer effect (1, 8). The molecular mechanisms underlying resistance of some cancer cells to mTOR inhibition are not fully understood. We and others have previously discovered that, in addition to inhibiting the kinase activity of mTOR, rapamycin and RAD001 can also activate Akt and extracellular signal-regulated kinase 1 or 2 (ERK1/2) survival signaling pathways. This effect contributes to the resistance of lung and breast cancer cells to mTOR inhibition (6, 9–11). However, the downstream survival or death substrates of rapamycin-activated Akt and ERK signaling pathways remain unclear.

Bcl2 family members are key regulators of apoptosis at the decision phase and share homology clustered within 4 conserved Bcl2 homology (BH) domains: BH1, BH2, BH3, and BH4. Only the antiapoptotic proteins, such as Bcl2, Bcl-XL, Bcl-w, and A1 bear the NH2-terminal BH4 domain (12). The proapoptotic family members are divided into 2 subgroups based on the presence of BH domains, including the BH123 multidomain proteins (i.e., Bax and Bak)
and the BH3-only molecules (i.e., Bad, Bid, Bim, Bik, Nix, Noxa, PUMA, etc.; refs. 13, 14). The BH3-only proapoptotic proteins are upstream sensors of cellular damage that selectively respond to specific, proximal death, and survival signals (15). BH3-only proteins exert their proapoptotic activity by hierarchical and tightly choreographed interactions with other Bcl-2 family members. The BH3-only proapoptotic proteins share homology within a single amphithetic BH segment, the BH3 domain, which is also known as the minimum death domain. BH3-only proteins are cell death initiators and their posttranslational modifications (i.e., phosphorylation), proteolytic processing and lipid modification, are potential mechanisms that integrate extracellular survival and death signals with the core apoptotic machinery (15). These molecules are also being explored as possible tools for cancer therapy, based on the expectation that molecules mimicking the BH3 domain of these proteins could selectively and efficiently cooperate with chemotherapeutic drugs in cell killing (16). Bad is one of the BH3-only proapoptotic members and can couple death signals to mitochondria and promote apoptosis by quelling the protective action of Bcl-XL (17). Phosphorylation of Bad at serine (S) 112, S136, and S155 has been shown to inactivate its proapoptotic function (18, 19) through a mechanism involving binding to 14-3-3 scaffold proteins that results in sequestering Bad from mitochondria and dissociation of Bad from mitochondrial Bcl2 and/or Bcl-XL (20–22). The active Bad exists in a dephosphorylated form that localizes to the mitochondria and interacts with Bcl-XL to neutralize its antiapoptotic function. Akt and the mitogen-activated protein kinases (MAPK) ERK1/2 are reported to function as physiologic Bad kinases (23–25). Here, we report that inhibition of mTOR by rapamycin stimulates Bad phosphorylation at S112 and S136 through activation of ERK1/2 and Akt, which results in inactivation of the proapoptotic function of Bad and decreased sensitivity of lung cancer cells to mTOR inhibition. Blockage of rapamycin-induced Bad phosphorylation significantly sensitizes lung cancer cell lines and lung tumors to mTOR inhibition.

Materials and Methods

Materials

Rapamycin was purchased from LC Laboratories. PD98059 was purchased from EMD Chemicals, Inc. Phospho-Akt (S473), Akt, ERK1/2, mTOR, p-mTOR, phospho-Bad (S136), phospho-Bad (S112), phospho-Bad (S155), phospho-ERK1/2, p-ERK1/2, phospho-Bad (S155), and ERK1/2 antibodies were purchased from Cell Signaling Technology. 14-3-3, pERK1/2, proliferating cell nuclear antigen (PCNA), prohibitin and β-actin antibodies were purchased from Santa Cruz Biotechnology. Bad and Bcl-XL were purchased from Epitomics, Inc. NanoJuice Transfection Kit was obtained from EMD Chemical, Inc. TumorTACS In Situ Apoptosis Detection Kit was purchased from Trevigen, Inc. Murine WT-Bad and mutant Bad S112A/S136A (AA) cDNAs in pcDNA3 plasmids were obtained from Addgene. All other reagents used were obtained from commercial sources unless otherwise stated.

Cell lines and cell culture

H460, H157, and A549 were purchased from the American Type Culture Collection, and no authentication for these cell lines was done by the authors. H460 and H157 cells were maintained in RPMI 1640 with 10% fetal bovine serum. A549 cells were maintained in F-12K medium with 10% fetal bovine serum. The rapamycin-resistant A549 cell line (A549-RR) was established as described previously (6). Briefly, A549-RR was established by exposing the rapamycin-sensitive A549 parental cells (A549-P) to gradually increasing concentrations of rapamycin from the initial 1 nmol/L to the final 20 μmol/L over a 6-month period as described (6).

Preparation of cell lysate and Western blot

Cells were washed with cold PBS and resuspended in ice-cold EBC buffer (0.5% Nonidet P-40, 50 mmol/L Tris, pH 7.6, 120 mmol/L NaCl, 1 mmol/L EDTA, and 1 mmol/L β-mercaptoethanol) containing protease inhibitor mixture set I. Following cell lysis by sonication and centrifugation at 14,000 × g for 15 minutes at 4°C, the resulting supernatant was collected as the total cell lysate. As previously described, Western blot was conducted by loading 50 μg of protein per lane on an 8% to 12% SDS-PAGE, followed by protein transfer to nitrocellulose membrane for analysis of specific protein(s) (26).

RNA interference, plasmids, and transfection

Human Akt short hairpin RNA (shRNA) plasmid is a target-specific lentiviral vector plasmid encoding a 19 to 25 nt (plus hairpin) shRNA designed to knock down gene expression. The control shRNA plasmid-A encodes a scrambled shRNA sequence that will not lead to the specific degradation of any cellular message. Both Akt shRNA and control shRNA plasmids were purchased from Santa Cruz Biotechnology. Hairpin sequence: GAT CCT GCC CTT CTA CAA CCA GGA TTC AAG AGA UUG UAG AAG GGC Att-3. Transfection of shRNA, Bad cDNA, or HA-tagged ubiquitin plasmid (27, 28) was carried out using NanoJuice Transfection Kit according to the manufacturer’s instructions (EMD Chemical, Inc.).

Subcellular fractionation. Subcellular fractionation was carried out as previously described (29). Briefly, H460 cells (2–3 × 10⁷) were washed with cold 1 × PBS and resuspended in isotonic mitochondrial buffer (210 mmol/L mannitol, 70 mmol/L sucrose, 1 mmol/L EGTA, 10 mmol/L Hepes, pH 7.5) containing protease inhibitor mixture set I, homogenized with a Polytron homogenizer operating for 4 bursts of 10 seconds each at a setting of 5 and then centrifuged at 2000 × g for 5 minutes to pellet the nuclei and unbroken cells. The supernatant was...
centrifuged at 13,000 × g for another 10 minutes to pellet mitochondria as described (30). The second supernatant was further centrifuged at 150,000 × g to pellet light membranes. The resulting supernatant containing cytosolic fraction was collected. The mitochondrial pellet was washed with mitochondrial buffer twice and resuspended in 1% NP-40 lysis buffer and rocked for 60 minutes, then centrifuged at 17,530 × g for 10 minutes at 4°C. The resulting supernatant consists of mitochondrial proteins. For nuclear fractionation, the nuclear pellet collected in 2 mL of Buffer A (10 mmol/L Tris-HCl, pH 7.4, 10 mmol/L NaCl, 3 mmol/L MgCl2, 0.03% Nonidet P-40) was removed and cell colonies were stained with crystal violet (0.1% in 20% methanol). Pictures were taken using a BX41 microscope (Olympus America). The semiquantitative evaluation of IHC staining of pBad was carried out using immunoscore based on both percentage of stained cells and staining intensity as described (36, 37). The intensity score for p-Bad detection was defined as follows: 0, no appreciable staining; 1, weak intensity; 2, moderate intensity; 3, strong intensity; 4, very strong intensity. The fraction score was based on the proportion of positively stained cells (0–100%). The proportion of cells staining at each intensity was multiplied by the corresponding intensity value and these products were added to obtain an immunoscore ranging from 0 to 400. The mean of the immunoscores was obtained from ten microscopic high power fields.

**Statistical analysis**

The statistical significance of differences between 2 groups was analyzed with 2-sided unpaired Student t test. Results were considered to be statistically significant at P < 0.05. Statistical analysis was carried out with Graphpad InStat 3 software (10).

**Results**

**Rapamycin induces Bad phosphorylation at S136 and S112 but not S155 via activation of Akt and ERK**

Previous findings reveal that inhibition of mTOR by rapamycin can activate Akt and ERK (6, 9–11). Because
ERK1 and ERK2 are physiological S112 Bad kinases while Akt is an S136 Bad kinase (38–40), it is possible that rapamycin may stimulate Bad phosphorylation via activation of Akt and ERKs. To test this, human lung cancer H460 cells were treated with increasing concentrations of rapamycin for 45 minutes. Phosphorylation of Bad was analyzed by Western blot using phospho-specific Bad antibodies. Results reveal that inhibition of mTOR by rapamycin results in increased Bad phosphorylation at S112 and S136 but not S155 in association with activation of ERK1/2 and Akt (Fig. 1). As expected, decreased phosphorylation of mTOR, p70S6K (the 70-kDa ribosomal S6 kinase) or 4EBP1 (the eukaryotic translation initiation factor 4E–binding protein 1) was observed following rapamycin treatment (Fig. 1C), indicating that mTOR kinase activity was inhibited (41). It has already been established that phosphorylation of Bad at S112 or S136 inactivates its proapoptotic function (38). Our findings thus suggest that rapamycin-induced Bad phosphorylation may lead to the loss of death-promoting activity of Bad and thereby contribute to the resistance of human lung cancer cells to rapamycin. Similar results were also obtained in H157 cells (Supplementary Fig. S1). This confirms that rapamycin-induced Bad phosphorylation is not limited to a specific cell type.

**Rapamycin resistance is associated with increased Bad phosphorylation**

To further show whether rapamycin resistance involves Bad phosphorylation, rapamycin-sensitive (i.e., A549 parental: A549-P) and rapamycin-resistant lung cancer cells (i.e., A549-RR) were generated as described previously (6). Phosphorylation levels of Bad at S112, S136, and S155 were compared in A549-P and A549-RR cells. Intriguingly, higher levels of Bad phosphorylation at S112 and S136 but not S155 in association with increased activities of ERK1/2 and Akt were observed in A549-RR cells as compared with A549-P cells (Fig. 2A). Importantly, SRB colorimetric and colony formation assays reveal that A549-P is sensitive but A549-RR is insensitive to rapamycin (Fig. 2B and C). These results provide strong evidence that rapamycin-induced Bad phosphorylation at S112 and S136 contributes to rapamycin resistance in human lung cancer cells. To test whether the nonphosphorylatable mutations of Bad at S112 and S136 sites (i.e., S → A) are sufficient to reverse rapamycin resistance, the nonphosphorylatable S112A/S136A (AA) mutant murine...
Bad and the WT murine Bad were transfected into A549 parental (A549-P) and rapamycin-resistant A549 cells (A549-RR). After transfection, cells were treated with rapamycin (100 nmol/L) for 48 hours. Results reveal that expression of the AA mutant Bad but not WT-Bad reverses rapamycin resistance of A549-RR cells (Supplementary Fig. S2A and S2B). Previous reports have shown that murine Bad and human Bad have conserved structural homology and function (39, 42). However, the number of amino acids (aa) in murine Bad and human Bad are different (210 aa vs. 168 aa; refs. 43, 44). Human Bad lacks a stretch of 42 amino acids as previously reported (44). This is consistent with our findings that the molecular weight of exogenous murine Bad is larger than the endogenous human Bad (Supplementary Fig. S2A).

Treatment of lung cancer cells with rapamycin results in Bad accumulation in the cytosol, increased Bad/14-3-3 association, and decreased Bad/Bcl-XL binding

Our results indicate that rapamycin induces phosphorylation of Bad at S112 and S136 (Fig. 1A). Phosphorylation has been reported to promote Bad translocation from mitochondria into cytosol, interaction with the scaffold protein 14-3-3 and dissociation from Bcl-XL (18, 21). To test whether rapamycin-stimulated Bad phosphorylation affects its subcellular localization and interactions with 14-3-3 or Bcl-XL, human lung cancer H460 cells were treated with rapamycin (10 nmol/L) for 45 minutes, and subcellular distributions of total Bad, pBad, 14-3-3, and Bcl-XL were examined by subcellular fractionation analysis as previously described (45). After treatment with rapamycin, Bad was translocated from mitochondria into cytosol, increased Bad/14-3-3 association, and decreased Bad/Bcl-XL binding.
the cytosol (Fig. 3A). Because rapamycin increases the phosphorylated forms of Bad in the cytosol and only the phosphorylated Bad could be observed in the cytosolic fraction (Fig. 3A), this indicates that rapamycin-mediated sequestration of Bad from mitochondria may occur through its phosphorylation. By contrast, rapamycin has no significant effect on the subcellular localization of 14-3-3 or Bcl-XL (Fig. 3A). To determine the purity of the subcellular fractions obtained, fraction-specific proteins were assessed by probing the same filters. Prohibitin, an exclusively mitochondrial protein (46), was detected only in the mitochondrial fraction (Mt) whereas PCNA, a nuclear marker (47), was detected exclusively in the nuclear fraction (Nuc), and glyceraldehydes-3-phosphate dehydrogenase (GAPDH), the cytosol marker (48), was only observed in the cytosolic fraction (Fig. 3A). This determination further confirmed the purity of these subcellular fractions without artifactual cross-contamination.

In addition to accumulation of Bad in the cytosol, rapamycin also enhances Bad/14-3-3 interaction in association with decreased Bad/Bcl-XL binding (Fig. 3B and C). These findings indicate that rapamycin-induced Bad phosphorylation results in sequestering Bad from the mitochondria and functionally blocking its proapoptotic function.

**Rapamycin promotes Bad ubiquitination and degradation**

Phosphorylation has been shown to regulate ubiquitination and degradation of the Bcl2 family proteins (49–51). To test whether rapamycin-induced Bad phosphorylation affects its stability in human lung cancer cells, the half-life of Bad was measured using cycloheximide block method as described (52). H460 cells were treated with 100 μg/mL cycloheximide in the absence or presence of rapamycin (100 nmol/L) for various times as indicated. Levels of Bad were analyzed by Western blot and further quantified by the ImageJ software for calculating the half-life as described (53). Results reveal that rapamycin significantly reduces the half-life of Bad from 53.3 to 37.5 hours (Fig. 4A and B), indicating that rapamycin-induced Bad phosphorylation may promote Bad degradation. To further uncover the mechanism by which rapamycin reduces Bad stability, ubiquitination was measured following rapamycin treatment as described (27, 28). First, the HA-tagged ubiquitin expression...
plasmid was transfected into H460 cells. After 24 hours, cells were treated with increasing concentrations of rapamycin from 1 nmol/L to 1 μmol/L for 48 hours. A co-immunoprecipitation (co-IP) was carried out using a Bad antibody. Bad ubiquitination was analyzed by Western blot using anti-HA antibody. Results reveal that rapamycin induces a dose-dependent ubiquitination of Bad, which is characterized as the typical higher molecular weight smear of the polyubiquitin chains on Bad protein (Fig. 4C, left panel). Total cell lysate was used as input control before co-IP (Fig. 4C, right panel). These findings suggest that rapamycin-induced reduction in the half-life of Bad may occur through its ubiquitination and degradation.

Inhibition of rapamycin-induced Bad phosphorylation by PD98059 or depletion of AKT sensitizes lung cancer cells to rapamycin

Our findings suggest that rapamycin-induced Bad phosphorylation may inactivate its proapoptotic function (Figs. 1, 3, and 4). Inhibition of rapamycin-induced Bad phosphorylation may restore the proapoptotic activity of Bad and sensitize lung cancer cells to rapamycin. To test this hypothesis, H460 parental cells, H460 cells expressing Akt shRNA or control shRNA were treated with rapamycin (Supplementary Fig. S3) in the absence or presence of PD98059 (Supplementary Fig. S3). Results reveal that inhibition of MAPK ERK1/2 by PD98059 specifically blocks rapamycin-induced S112 site phosphorylation of
Bad but has no significant effect on Bad phosphorylation at S136 or S155 (Fig. 5A). By contrast, depletion of AKT by RNA interference using Akt shRNA specifically blocks rapamycin-induced S136 site phosphorylation and has no effect on Bad phosphorylation at S112 or S155 (Fig. 5B). Intriguingly, simultaneous shutdown of MAPK/ERK1/2 and Akt by PD98059 and Akt shRNA blocks rapamycin-stimulated phosphorylation of Bad at both S112 and S136 sites (Fig. 5B), which additively enhances rapamycin-induced growth inhibition of human lung cancer cells (Fig. 5C). To test whether ERK and Akt inhibition is effective to reverse rapamycin resistance, A549-P and A549-RR cells were treated with PD98059 and/or transfected with Akt shRNA in the presence or absence of rapamycin for 48 hours. Results show that simultaneous inhibition of ERK and Akt not only significantly sensitizes A549-P cells to rapamycin but also reverses rapamycin resistance of A549-RR cells, suggesting that inhibition of Bad phosphorylation at S112 and S136 by blocking ERK and Akt signal pathways can reverse rapamycin resistance (Supplementary Fig. S2C).

Suppression of rapamycin-induced Bad phosphorylation by PD98059 or depletion of Akt enhances antitumor efficacy of rapamycin in lung cancer xenografts

To further test whether blockage of rapamycin-enhanced Bad phosphorylation increases rapamycin’s antitumor efficacy in vivo, we generated lung cancer xenografts using H460 cells or H460 cells expressing Akt shRNA. Xenograft mice were randomly grouped and treated with rapamycin or PD98059 or the combination for 2 weeks as described in “Materials and Methods.” Results indicate that either treatment with PD98059 or...
silencing of Akt using shRNA in lung cancer xenografts significantly enhances the antitumor efficacy of rapamycin in vivo. Importantly, PD98059 plus Akt shRNA block rapamycin-stimulated Bad phosphorylation at both S112 and S136 sites in tumors (Fig. 6B and C), and more efficiently represses lung tumor growth than either PD98059 or Akt shRNA alone (Fig. 6A). Consistent with in vitro results, PD98059 and Akt shRNA have no significant effect on S155 site phosphorylation of Bad in vivo (Fig. 6B and C). These findings suggest that blockage of rapamycin-induced Bad phosphorylation at both S112 and S136 sites may not only sensitize cancer cells to rapamycin but also can overcome rapamycin resistance leading to increased antitumor activity in vivo.

To evaluate the role of apoptosis in tumor growth, a terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay was used for measuring apoptosis in tumor tissues using a Tumor TACS In Situ Apoptosis Detection Kit ( Trevigen, Inc.). Results reveal that inhibition of Bad phosphorylation by PD98059 and Akt shRNA significantly enhances apoptosis in tumor tissues (Supplementary Fig. S4).

Discussion

Lung cancer, a major cigarette smoke-related cancer, is the primary cause of cancer-related mortality in the United States, accounting for more deaths than breast, prostate, and pancreatic cancer combined (54). mTOR

Figure 6. Inhibition of rapamycin-induced Bad phosphorylation by PD98059 or depletion of Akt enhances antitumor efficacy of rapamycin in vivo. A, seven groups of Nu/Nu nude mice with H460 or Akt shRNA H460 xenografts were treated as indicated. Each group included 8 mice. After 14 days, the mice were sacrificed and the tumors were removed. Tumor sizes were measured once every 2 days. B and C, phosphorylation of Bad at S112, S136, or S155 in tumor tissues from various groups was detected with IHC using phospho-specific Bad antibodies (B) and quantified by analyzing immunoscore (C) as described in Materials and Methods. Error bars represent ± SD.
inhibitors, such as rapamycin (Supplementary Fig. S3) and everolimus, have been evaluated as lung cancer therapeutics but with limited success (1). Previous reports indicate that rapalog-activated Akt and MAPK ERK1/2 may contribute to the development of resistance to these agents (6, 9, 10). However, the downstream mediators of the survival consequence of rapamycin-activated Akt and ERK1/2 remain unclear. Here, we discovered that rapamycin, in addition to mTOR inhibition, potently stimulates Bad phosphorylation at S112 and S136 sites via activation of Akt and MAPK ERK1/2 (Figs. 1 and 5), which can lead to rapamycin resistance because increased levels of Bad phosphorylation were observed in rapamycin-resistant cells (Fig. 2). Intriguingly, either blockage of Bad phosphorylation at S112 and S136 sites or expression of the nonphosphorylatable Bad mutant (S112A/S136A) can reverse rapamycin resistance (Supplementary Fig. S2), suggesting that manipulation of Bad phosphorylation at these 2 sites should be an effective approach for overcoming rapamycin resistance. Because PKA is the physiologic S155 Bad kinase (55) and a previous study has shown that rapamycin does not influence the activity of PKA (56), this helps explain why rapamycin has no effect on S155 Bad phosphorylation in vitro and in vivo (Figs. 1, 5, and 6).

It is known that phosphorylation of Bad at 1 or more sites (S112, S136, or S155) can inactivate the proapoptotic function of Bad (18, 19, 39). Therefore, we expect that rapamycin-induced Bad phosphorylation at S112 or S136 will abolish the death-promoting activity of Bad. In support of this, rapamycin facilitates Bad translocation from mitochondria into the cytosol, and promotes Bad interaction with 14-3-3 and its dissociation from Bcl-XL (Fig. 3). The overall outcome of this series of effects results in the inability of Bad to overcome the antiapoptotic function of Bcl-XL in the mitochondria. In addition, treatment of lung cancer cells with rapamycin promotes Bad ubiquitination and degradation, leading to a reduced half-life (Fig. 4) and ultimately a loss of function.

Because Bad is a potent BH3-only proapoptotic protein that is ubiquitously expressed in both SCLC and NSCLC cells (38), blocking rapamycin-induced Bad phosphorylation may represent a novel therapeutic strategy for improving the antitumor efficacy of rapamycin. Although rapamycin can induce Bad phosphorylation at 2 sites (i.e., S112 and S136; Fig. 1A), PD98059 blocks Bad phosphorylation only at the S112 site while depletion of Akt blocks Bad phosphorylation only at the S136 site in either lung cancer cells or in lung tumor tissues (Figs. 5 and 6). These findings provide strong evidence that rapamycin-induced S112 site phosphorylation occurs through the MEK/ERK1/2 signaling pathway while rapamycin-induced S136 site phosphorylation occurs through the Akt pathway (Figs. 1, 5, and 6). Abrogation of rapamycin-stimulated phosphorylation of Bad at S112 and S136 led to increased growth inhibition of lung cancer cells in vitro and synergistic enhancement of rapamycin activity against lung cancer tumor xenografts in vivo (Figs. 5 and 6).

In summary, our studies identify a novel rapamycin survival signal transduction pathway that depends on phosphorylation of Bad at S112 and S136 but not S155 through activation of MAPKs ERK1/2 and Akt. Rapamycin-induced double-site phosphorylation results in translocation of Bad from the mitochondria, sequestration in the cytosol where it interacts with 14-3-3, dissociation from Bcl-XL in mitochondria and reduced stability via ubiquitination, which leads to loss of the apoptotic function of Bad and rapamycin resistance. Our findings have established Bad as a new signaling target of rapamycin in human lung cancer cells. Therefore, activation of Bad by reducing or blocking its phosphorylation may represent a new therapeutic strategy to overcome resistance to mTOR inhibition in patients with lung cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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