Rapamycin Resistance Is Linked to Defective Regulation of Skp2

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Abstract

The mammalian target of rapamycin (mTOR) plays a role in controlling malignant cellular growth. mTOR inhibitors, including rapamycin (sirolimus), are currently being evaluated in cancer trials. However, a significant number of tumors are rapamycin resistant. In this study, we report that the ability of rapamycin to downregulate Skp2, a subunit of the ubiquitin protein ligase complex, identifies tumors that are sensitive to rapamycin. RNA interference (RNAi)–mediated silencing of Skp2 in human tumor cells increased their sensitivity to rapamycin in vitro and inhibited the growth of tumor xenografts in vivo. Our findings suggest that Skp2 levels are a key determinant of antitumor responses to mTOR inhibitors, highlighting a potentially important pharmacogenomic marker to predict sensitivity to rapamycin as well as Skp2 silencing strategies for therapeutic purposes. Cancer Res; 72(7); 1836–43. ©2012 AACR.

Introduction

Dysregulated cell-cycle progression is a hallmark of cancer. Cell-cycle progression is normally regulated by the ubiquitin-mediated proteolysis of key regulatory proteins including the cyclins and cyclin-dependent kinase (CDK) inhibitors (1). The anaphase-promoting complex (APC) and the Skp1-Cul1-F-box (SCF) complex are the 2 main classes of E3 ubiquitin ligases. Skp2 is an F-box protein that constitutes the substrate recognition subunit of the SCFSkp2 ubiquitin ligase complex and is the rate-limiting component required for the turnover of proteins involved in cell-cycle control such as the CDK inhibitor p27 (2). Skp2 is considered to be a proto-oncogene as Skp2 overexpression causes increased proliferation, invasion, and inhibits apoptosis (3–5). Many clinical studies have indicated that upregulation of Skp2 and loss of p27 are common in malignant tumors and are associated with poor prognosis (6).

The mechanisms that regulate Skp2 protein levels remain controversial. Rodier and colleagues showed that phosphorylation of Skp2 on Ser-64 and Ser-72 increases its stability (7). Moreover, Lin and colleagues and Gao and colleagues observed that Akt-mediated phosphorylation of Skp2 at Ser-72 impairs its degradation and relocalizes it to the cytosol contributing to its oncogenic function (8, 9). More recently, conflicting reports from Boutonnet and colleagues and Bashir and colleagues showed that phosphorylation of Skp2 on Ser-72 does not control Skp2 binding to Skp1 and Cul1, has no influence on SCFSkp2 ubiquitin ligase activity, and does not affect its subcellular localization (10, 11) but do not dispute its phosphorylation by Akt at Ser-72. Interestingly, it has been shown that Akt phosphorylates CDK2 regulating its cellular localization, which is required for cell-cycle progression (12). At the transcriptional level, the phosphoinositide 3-kinase (PI3K)/Akt pathway regulates Skp2 through E2F1 binding to its promoter (13, 14). This finding implies a positive feedback loop composed of Skp2, p27, cyclinE-CDK2, and Rb-E2F termed the Skp2 autoinduction loop that promotes progression through the restriction point (15, 16).

The serine/threonine kinase mammalian target of rapamycin (mTOR) plays a central role in controlling cell growth (17). mTOR exists in 2 complexes. mTOR complex 1 (mTORC1) regulates protein translation via modulation of S6K1 and 4EBP1, and mTOR complex 2 (mTORC2) regulates cell survival via phosphorylation of Akt on Ser-473, which is necessary for full activation of Akt, along with PDK1 phosphorylation of Thr308 (18). The immunosuppressant drug rapamycin, when bound to the FK506-binding protein 12 (FKBP12), inhibits the activity of the CDK4/cyclin D and CDK2/cyclin E complexes by increasing the CDK inhibitor p27 leading to cell-cycle arrest at G1 (19, 20). The FKBP12–rapamycin complex does not bind to mTORC2, however, prolonged rapamycin treatment suppresses the assembly of mTORC2 and its ability to activate Akt in a cell-type–specific manner (21). In many cell lines, active mTORC1 suppresses PI3K activity by a negative feedback loop mediated by S6K1. Inhibition of mTORC1 release this inhibitory feedback loop resulting in increased Akt activity (22).

Because mTOR activation is common in cancers, we investigated the role of Skp2 in the response of tumor cells to the...
mTOR inhibitor, rapamycin. Here, we show that Skp2 is downregulated upon rapamycin treatment in rapamycin-sensitive human tumor cells, whereas Skp2 expression was not affected in rapamycin-resistant tumor cells. Moreover, when tumor xenografts derived from rapamycin-resistant cells were treated with rapamycin, the tumor volume was significantly increased. Silencing Skp2 in rapamycin-resistant tumor cells using short hairpin RNA (shRNA) was sufficient to increase the sensitivity to rapamycin and to inhibit the growth of tumor xenografts.

Materials and Methods

Materials

Reagents were obtained from the following sources: Dulbecco’s Modified Eagle’s Medium (DMEM), RPMI, and McCoy’s from Invitrogen; HAT from American Type Culture Collection (ATCC); FBS from Atlanta Biologicals; rapamycin from Biovision; cDNA synthesis kit from NE Biolabs; qRT-PCR Master Mix from Roche; and VWR. All animal studies were approved by the Institutional Animal Care and Use Committee at Columbia University Medical Center. PC3 cells (3 × 10⁶) were injected subcutaneously in the upper region of mice that had been anaesthetized with isoflurane. Mice were treated with rapamycin (10 mg/kg) or with NVP-BEZ235 (30 mg/kg in 10% NMP:90% PEG, given per os in 100 μL of volume) as described (23). At completion of all xenograft studies mice were sacrificed, the tumors were excised and tumor volumes estimated with the following formula: volume = (a² × b)/2, where a = short and b = long tumor lengths, respectively, in millimeters.

Results and Discussion

Rapamycin resistance correlates with increased Skp2 and decrease in p27

The potential role of Skp2 in the response of tumor cells to the mTOR inhibitor, rapamycin, was investigated by comparing Skp2 protein levels in a rapamycin-sensitive murine cell line, BC3H1, derived from a mouse brain tumor, and 2 rapamycin-resistant BC3H1 clonal cell lines (RR1 and RR3) developed in our laboratory (24). Rapamycin inhibited cell growth in the BC3H1 cells but not in RR1 or RR3 cells (Fig. 1A). Skp2 protein and mRNA levels were low in rapamycin-sensitive BC3H1 cells, and rapamycin treatment or serum starvation
resulted in a further reduction (Fig. 1B and C). In contrast, in RR1 and RR3 cells, Skp2 protein and mRNA levels were elevated more than 2-fold than the parental BC3H1 cells and were unchanged by rapamycin treatment or serum starvation (Fig. 1B and C). Moreover, Skp2 protein levels were inversely correlated with p27 protein levels (Fig. 1B and C). These data are consistent with previous reports of decreased p27 levels in RR cells (24) and may suggest that low levels of p27 protein might be due to increased Skp2 expression. The increase in Skp2 at the transcriptional level is consistent with previous data showing persistent retinoblastoma (Rb) hyperphosphorylation in RR cells (24), releasing the transcription factor E2F (13). In addition, downregulation of p27, which activates CDK2 in RR cells, may increase the stability of Skp2 at the protein level by phosphorylating it at the conserved S64 site (7).

Rapamycin resistance is associated with defective regulation of Skp2 in human tumor cell lines

We next tested whether Skp2 and p27 protein levels correlated with the response of 6 different human tumor cell lines to rapamycin. Cells that were resistant to rapamycin (HeLa, MDA-MB-231, LNCaP; Fig. 2A) exhibited high levels of Skp2 (both protein and mRNA) that was not affected by rapamycin treatment (Fig. 2B and C). Conversely, rapamycin-sensitive cells (U937, T47D, PC3; Fig. 2A) exhibited approximately 2-fold decrease in Skp2 protein levels and approximately 1.5-fold decrease in Skp2 mRNA levels upon rapamycin treatment (Fig. 2B and C). Notably, p27 and Skp2 protein levels were inversely correlated (Fig. 2B). These data agree with previous reports that low p27 and high Skp2 levels are associated with more aggressive tumors (25) and indicate that impaired downregulation of Skp2 by rapamycin correlates with rapamycin resistance.

Dephosphorylation of Skp2 and Akt correlates with human tumor cell line sensitivity to rapamycin

In addition to the transcriptional dysregulation of Skp2 in rapamycin-resistant cells, aberrant regulation of Skp2 protein stability may also play a critical role in determining rapamycin sensitivity. Phosphorylation of Skp2 at Ser-64 and Ser-72 by CDK2 and Akt, respectively, were shown to increase Skp2 stability by interfering with its association with Cdh1 (7–9). Because prolonged rapamycin treatment (24 hours) inhibited the phosphorylation and activation of Akt in certain tumor cells (21), we hypothesized that cell lines with rapamycin-sensitive Akt phosphorylation would have low amounts of Skp2 after rapamycin treatment. Indeed, cell lines with rapamycin-sensitive Akt phosphorylation (U937, PC3, T47D) exhibited decreased Skp2 protein levels upon rapamycin treatment (Fig. 3A). In contrast, cell lines with rapamycin-insensitive Akt phosphorylation (LNCaP, HeLa, and MDA-MB-231) showed no change in Skp2 levels upon rapamycin treatment (Fig. 3A).

To test the effect of Akt on Skp2 expression, we treated the same tumor cell lines with the PI3K inhibitor LY294002, or the dual inhibitor of PI3K and mTOR, NVP-BEZ235 (26). LNCaP, HeLa, and MDA-MB-231 cells treated with LY294002 or NVP-BEZ235 exhibited inhibition of Ser-473-Akt phosphorylation and approximately 2-fold decrease in Skp2 expression (Fig. 3B and C). Moreover, the decrease in Skp2 expression correlated with the increased sensitivity of cells to LY294002 or NVP-BEZ235 (Fig. 3D). In addition, treatment of these cells with the
representative experiment carried out in triplicate. Followed by cell counts. Results presented as mean ± SD from a representative experiment carried out in triplicate, compared with DMSO-treated cells (P < 0.01). B, representative immunoblot for Skp2, p27, and GAPDH from lysates from the indicated cells treated with DMSO or with 100 nmol/L rapamycin (Rap) or serum starved (Ser) for 24 hours. Results presented as mean ± SD (n = 3). C, RT-PCR using total RNA extracted from the indicated cells treated with DMSO or with 100 nmol/L rapamycin (Rap) for 24 hours. Data shown are mean ± SD (n = 3), compared with DMSO-treated cells (P < 0.01). AU, arbitrary units.

Downregulation of Skp2 by rapamycin is phosphorylation dependent

Recent reports have suggested that phosphorylation of Skp2 at Ser-64 and/or Ser-72 by CDK2 and/or Akt increases its stability (7–9). Because both CDK and Akt are downstream of mTOR, we tested whether the downregulation of Skp2 in the rapamycin-sensitive cells is phosphorylation dependent.

We therefore replaced both Ser-64 and Ser-72 with the phosphomimetic amino acid aspartic acid using a flag-tagged Skp2 expressing plasmid (DD-Skp2). Upon rapamycin treatment, the rapamycin-sensitive cells (PC3, T47D) downregulated the endogenous Skp2 and the WT-Flag-Skp2 but not the DD-Flag-Skp2 (Fig. 4A and B). The downregulation of Skp2 correlated with the rapamycin sensitive decrease in Akt phosphorylation. Interestingly, rapamycin-resistant cells (HeLa), showed no change in the expression levels of the endogenous Skp2, the WT-Flag-Skp2, or the DD-Flag-Skp2 upon rapamycin treatment and correlated with the rapamycin-insensitive Akt phosphorylation (Fig. 4C). These data show that dephosphorylation of Skp2 at Ser-64 and/or Ser-72 is critical for Skp2 downregulation by rapamycin.

Skp2 determines tumor xenograft sensitivity to rapamycin

We further investigated the biologic significance of Skp2 expression on the cellular response to rapamycin, by ectopically overexpressing WT-Flag-Skp2 in rapamycin-sensitive cells (PC3). Stably transfected PC3 cells with WT-Flag-Skp2 exhibited reduced sensitivity to rapamycin (Fig. 5A and B). To determine the role of Skp2 in the response of tumors to rapamycin in vivo, control or WT-Flag-Skp2 stably transfected PC3 cells were xenografted onto nude mice. Rapamycin inhibited the growth of tumors derived from control PC3 cells by 85%, whereas tumors derived from WT-Flag-Skp2 overexpressing PC3 cells were relatively resistant to rapamycin exhibiting less than 50% reduction in tumor growth (Fig. 5C and D).

Silencing the endogenous Skp2 using Skp2-specific shRNA in rapamycin-resistant cells (HeLa) increased the expression of p27 compared with control cells by more than 2-fold (Fig. 6A). When treated with rapamycin, sh-SKp2–transfected cells showed a further increase in the expression of p27 whereas control cells showed a slight decrease (Fig. 6A). Moreover, HeLa cells transfected with sh-Skp2 had a reduced proliferation rate and exhibited increased sensitivity to rapamycin (Fig. 6B and C). These data are in agreement with previous reports showing that mouse embryonic fibroblasts (MEF) derived from Skp2−/− embryos and other organs from Skp2−/− mice exhibit increased levels of p27 protein, indicating that Skp2 regulates p27 protein degradation. Moreover, Skp2−/− MEFs proliferate slowly in culture (27). Notably, tumor xenografts of Hela cells stably transfected with sh-control vector exhibited a 2-fold increase in tumor size upon rapamycin treatment (Fig. 6D). These data are in agreement with reports of acceleration in tumor growth after the use of mTOR inhibitors, which may be due to the release of the negative feedback loop by rapamycin (28).

dual PI3K and mTOR inhibitor increased the expression of p27 (Fig. 3C). These data show a direct correlation between the degree of Akt phosphorylation, Skp2 protein levels, and the cellular response to rapamycin, LY294002, and to the dual inhibitor NVP-BEZ235. These data are supported by Sarbassov and colleagues who showed that tumor xenografts of PC3 cells stably transfected with S473D-Akt but not wild-type Akt were resistant to rapamycin-induced decrease in tumor size (21).
Remarkably, tumor xenografts of HeLa cells stably transfected with Skp2-shRNA (designed to mimic the low Skp2 expression and low phospho-Ser-473-Akt response of sensitive cells to rapamycin) exhibited more than 90% inhibition of tumor growth (Fig. 6D). However, when tumor xenografts of HeLa cells transfected with Skp2-shRNA were treated with rapamycin, which increased phospho-Ser-473-Akt, tumor growth was increased by approximately 4-fold compared with nontreated HeLa cells transfected with Skp2-shRNA (Fig. 6D). We used the dual PI3K and mTOR inhibitor to test whether the detrimental effect of rapamycin observed in these tumor models was due to rapamycin insensitive Akt. HeLa cells transfected with Skp2-shRNA or with sh-control exhibited approximately 80% inhibition of tumor growth compared with nontreated HeLa cells transfected with sh-control upon treatment with the dual PI3K and mTOR inhibitor NVP-BEZ235.

The existence of the negative feedback loop downstream of mTORC1 may contribute to rapamycin resistance observed in HeLa cells. Therefore, when the dual PI3K and mTOR inhibitor was used, it exhibited greater antiproliferative effect and reduction in tumor volume compared with that of rapamycin (Figs. 3D and 6D). Moreover, these data show that targeting Skp2 alone is sufficient to inhibit the growth of a tumor derived from rapamycin-insensitive Akt in mouse xenografts. However, the combination of rapamycin with Skp2 inhibitors would not have a beneficial effect in tumors that have rapamycin-insensitive Akt phosphorylation.

Figure 3. Rapamycin sensitivity correlates with downregulation of Skp2 and Akt phosphorylation. A to C, representative immunoblots and quantification bar graph for the indicated proteins and phosphorylation states from the indicated cell lines treated with DMSO, 100 nmol/L rapamycin (Rap), 20 μmol/L LY 294002 (LY), or 100 nmol/L NBP-EZ235 for 24 hours. D, indicated cells were plated (10⁴ cells per well) in 24-well plates, treated as in A to C for 72 hours followed by cell counts. Results presented as mean ± SD from a representative experiment carried out in triplicate. *, compared with DMSO-treated cells (P < 0.01). AU, arbitrary units.
Our data are in agreement with recent reports showing that the combined knockout of Skp2 and Pten in mice inhibits the tumor formation which was observed in Pten-knockout mice (29), although tumors with PTEN loss are particularly susceptible to mTOR inhibitors (30). However, PTEN loss alone is neither necessary nor sufficient to result in rapamycin-sensitive regulation of Akt phosphorylation (21). In fact, in the present study we chose 2 prostate cancer cell lines that do not express PTEN, the PC3 cells are sensitive to rapamycin whereas LNCaP are resistant, and of the 2 breast cancer cell lines, both of which express PTEN, the T47D cells were sensitive whereas MDA-MB-231 cells were resistant.

Figure 4. Downregulation of Skp2 by rapamycin is phosphorylation dependent. A to C, indicated cells stably expressing empty vector (EV), wild-type Flag-Skp2 (WT), or DD Flag-Skp2 (DD) were treated with DMSO or 100 nmol/L rapamycin (Rap) for 24 hours, and cell lysates were analyzed by immunoblotting for the indicated proteins and phosphorylation states. Bar graphs indicate the quantification of the levels of endogenous Skp2 (Endo) and the Flag-Skp2. Data are presented as means ± SD (n = 3). *, compared with DMSO-treated cells (P < 0.01).

Figure 5. Overexpression of Skp2 increases cellular resistance to rapamycin. A, representative immunoblots for indicated proteins from PC3 cells stably expressing empty vector (EV) or wild-type Flag-Skp2 (WT) treated with DMSO or with 100 nmol/L rapamycin for 24 hours. B, PC3 cells described in A were plated (10^4 cells per well) in 24-well plates and treated with DMSO or rapamycin as indicated for 72 hours followed by cell counts. Results presented as mean ± SD (n = 3). *, compared with DMSO-treated cells or rapamycin-treated cells, respectively (P < 0.01). C and D, mice were injected with 3 x 10^6 PC3 cells described in A and were allowed to grow to at least 50 mm³ in size and then treated with rapamycin or vehicle for 4 executive days. After 14 days, the volumes of tumor xenografts were determined. Results presented as mean ± SD for tumor volume, *, compared with the percentage of vehicle-treated mice (n = 6 per condition, P < 0.01).
propose that rapamycin treatment is effective primarily in tumors that exhibit rapamycin-sensitive dephosphorylation of Akt at Ser-473 and consequently decreased Skp2 expression. Upon validation in the clinical setting, Skp2 expression levels could serve as a marker for predicting the response of tumors to mTOR inhibitors. For tumors resistant to mTOR inhibitors in which rapamycin fails to inhibit or increases Ser-473 phosphorylation of Akt, targeting Skp2 might be a therapeutic alternative.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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