High-dose rapamycin induces apoptosis in human cancer cells by dissociating mTOR complex 1 and suppressing phosphorylation of 4E-BP1

Paige Yellen,[1] Mahesh Saqcena,[1] Darin Salloum,[1] Jiangnan Feng,[1] Angela Preda,[1] Limei Xu,[1] Vanessa Rodrik-Outmezguine[2] and David A. Foster[1,*]

[1]Department of Biological Sciences; Hunter College of the City University of New York; New York, NY USA; [2]Program in Molecular Pharmacology and Chemistry; Memorial Sloan-Kettering Cancer Center; New York, NY USA

mTOR, the mammalian target of rapamycin, has been widely implicated in signals that promote cell cycle progression and survival in cancer cells. Rapamycin, which inhibits mTOR with high specificity, has consequently attracted much attention as an anticancer therapeutic. Rapamycin suppresses phosphorylation of S6 kinase at nanomolar concentrations; however, at higher micro-molar doses, rapamycin induces apoptosis in several human cancer cell lines. While much is known about the effect of low-dose rapamycin treatment, the mechanistic basis for the apoptotic effects of high-dose rapamycin treatment is not understood. We report here that the apoptotic effects of high-dose rapamycin treatment correlate with suppressing phosphorylation of the mTOR complex 1 substrate, eukaryotic initiation factor 4E (eIF4E) binding protein-1 (4E-BP1). Consistent with this observation, ablation of eIF4E also resulted in apoptorsis in MDA-MB 231 breast cancer cells. We also provide evidence that the differential dose effects of rapamycin are correlated with partial and complete dissociation of Raptor from mTORC1 at low and high doses, respectively. In contrast with MDA-MB-231 cells, MCF-7 breast cancer cells survived rapamycin-induced suppression of 4E-BP1 phosphorylation. We show that survival correlated with a hyperphosphorylation of Akt at S473 at high rapamycin doses, the suppression of which conferred rapamycin sensitivity. This study reveals that the apoptotic effect of rapamycin requires doses that completely dissociate Raptor from mTORC1 and suppress that phosphorylation of 4E-BP1 and inhibit eIF4E.

Introduction

The mammalian target of rapamycin (mTOR) is an important integrator of signals that sense nutrients and energy.[1] mTOR is also critical for controlling cell cycle progression and survival and is commonly activated by oncogenic alterations in human cancer.[2] Consequently, there has been strong interest in targeting mTOR as an anticancer therapeutic strategy.[3,4] mTOR is inhibited with high specificity by rapamycin; however, a confounding aspect concerning the effect of rapamycin on mTOR is that the concentrations of rapamycin required to suppress different actions of mTOR can vary dramatically.[5-8] mTOR exists in two complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2), most commonly distinguished by their association with the companion proteins Raptor and Rictor, but also by a differential sensitivity to rapamycin. mTORC1 is generally sensitive to rapamycin, but mTORC2 is relatively resistant.[9] While rapamycin suppresses phosphorylation of the mTORC1 substrate S6 kinase in the low nano-molar range,[10] rapamycin induces

apoptosis in several human cancer cell lines but at micro-molar concentrations.[7,11,12] Virtually nothing is known about what the high-dose rapamycin treatment is doing to cause apoptosis.

Rapamycin retards cell cycle progression,[10] which is considered the basis for its immune suppressive and anticancer properties. Thus, rapamycin has been referred to as a "cytostatic" drug.[13] The cytostatic effects of rapamycin and rapamycin analogs (rapalogs) are likely due to the suppression of S6 kinase, because these studies have used nano-molar concentrations that suppress S6 kinase phosphorylation. Although mTORC2 is considered to be rapamycin-insensitive, conditions where rapamycin suppresses mTORC2 have been reported in references 6 and 8. Thus, it is possible that the apoptotic effects of high-dose rapamycin treatment are due to an effect on mTORC2, which has also been implicated in cancer cell survival.[5,14] Another plausible target is eukaryotic initiation factor 4E (eIF4E)-binding protein 1 (4E-BP1). Although 4E-BP1 is thought to be an mTORC1 substrate,[10] 4E-BP1 phosphorylation is insensitive to rapamycin at doses up to 500 nM.[10,15,16] Sonenberg and colleagues recently
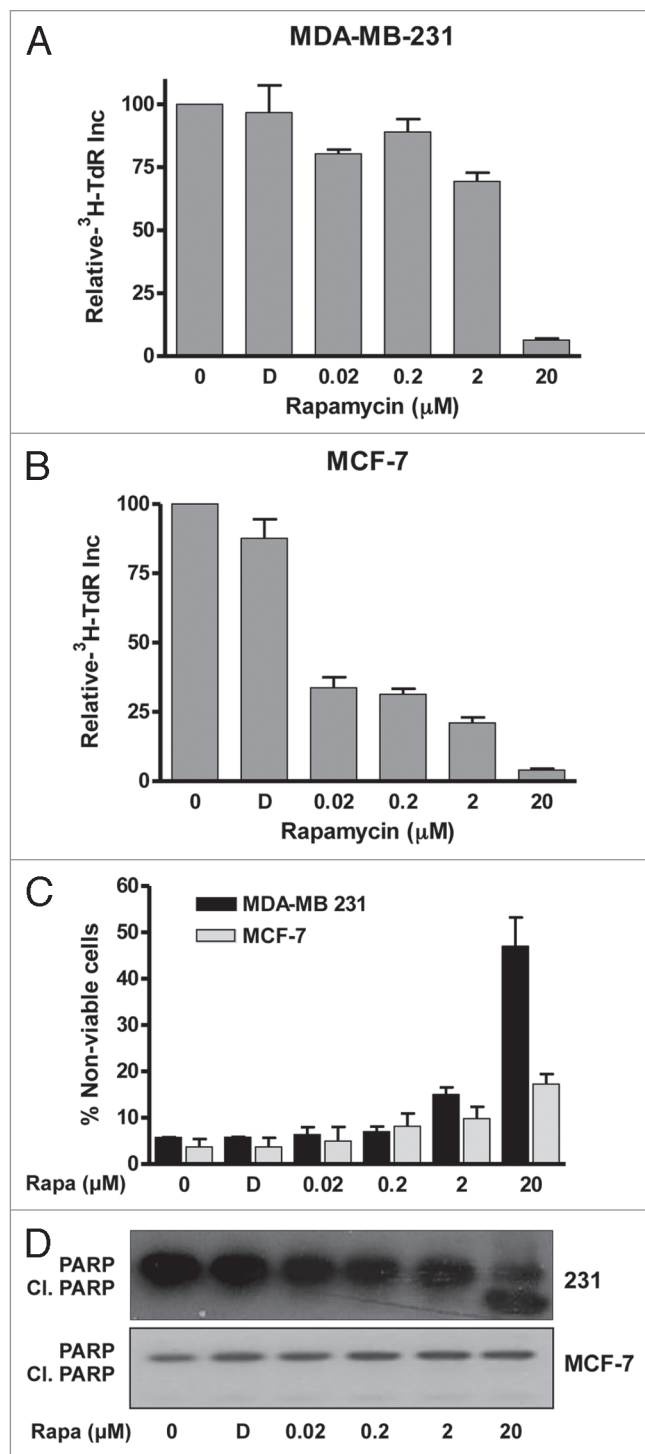
Figure 1. Differential suppression of G_1 cell cycle progression and cell viability by low- and high-dose rapamycin treatment. MDA-MB-231 (A) and MCF-7 (B) cells were plated at 50% confluence in a 10 cm dish in complete medium containing 10% serum. After 24 h, the cells were treated with nocodazole at 200 ng/ml for 16 h to block cells at mitosis. At this point, mitotic cells were collected and plated in complete media containing 10% serum. Four hr later, after the cells reattached, the indicated concentrations of rapamycin or DMSO vehicle (D) were added along with [^{3}H]-thymidine (TdR) (1 μCi/ml). After 24 h, lysates were collected, and the incorporated label was determined as described in Material and Methods. Error bars represent the standard deviation from three independent experiments. (C) MDA-MB-231 and MCF-7 cells were plated in regular (10% serum) medium. Twenty-four hours later, at 90% confluence, cells were exposed to 0% serum and treated with DMSO or rapamycin (Rapa) at indicated doses and harvested after 24 h. Cell viability was determined as described in Materials and Methods. (D) MDA-MB-231 cells were plated as in (A) and treated with DMSO (D) or rapamycin at indicated doses and harvested after 4 h (the 4-h time point established from a time course at 20 μM rapamycin at which strong induction of cleaved PARP was observed). Cell lysates were collected and immunoblotted with PARP antibody. Data shown are representative of experiments repeated at least two times.

implications for the use of rapamycin, and other compounds that suppress mTOR, as an anticancer therapeutic agents.

Results

Differential effects of low and high-dose rapamycin treatment on G_1 cell cycle progression and cell viability. We reported previously that while high-dose rapamycin treatment induced apoptosis in MDA-MB-231 cells in the absence of serum, in the presence of serum, high-dose rapamycin treatment induced G_1 cell cycle arrest.[11] Cell cycle arrest has been reported with low-dose nano-molar concentrations of rapamycin;[18-21] however, it has been noted that rapamycin may only slow cell cycle progression through G_1 at the nano-molar concentrations used.[10,16] We therefore examined the rapamycin doses required to block G_1 cell cycle progression of MDA-MB-231 cells and the less malignant MCF-7 breast cancer cell line. Since MDA-MB-231 cells do not arrest in G_1/G_0 in response to serum withdrawal, we synchronized the MDA-MB-231 and MCF-7 cells with nocodazole, which reversibly arrests cells at mitosis.[22] Mitotic cells were collected and then monitored for progression into S phase by uptake of [^{3}H]-thymidine. Concentrations of rapamycin up to 200 nM slightly reduced the incorporation of [^{3}H]-thymidine in the MDA-MB-231 cells, but 20 μM rapamycin dramatically suppressed uptake of [^{3}H]-thymidine (**Fig. 1A**). Progression of the MCF-7 cells into S phase was more sensitive to nano-molar levels of rapamycin, but, like the MDA-MB-231 cells, complete G_1 arrest required 20 μM rapamycin (**Fig. 1B**). Thus, while low nano-molar rapamycin concentrations retard G_1 cell cycle progression, micro-molar concentrations caused a complete G_1 arrest. These data reveal a specific effect of rapamycin that occurs between 2 and 20 μM that results in complete G_1 arrest in the presence of serum.

In contrast to the rapamycin-induced G_1 cell cycle arrest observed in the presence of serum, we have previously noted that in the absence of serum, high-dose rapamycin treatment causes

reported that cell proliferation is controlled by 4E-BPs.[17] Thus, suppression of 4E-BP1 phosphorylation could be responsible for the effects of high-dose rapamycin treatment.

We report here that the apoptotic effect of high-dose rapamycin is due to the suppression of 4E-BP1 phosphorylation and the subsequent sequestration and inhibition of eIF4E. Interestingly, high-dose rapamycin treatment dramatically elevated Akt phosphorylation at S473 in MCF-7 breast cancer cells, which suppresses the drug's apoptotic effects. These data have significant

apoptosis.[11,12] To establish the precise concentrations needed to induce apoptosis, dose curves were performed for the effect of rapamycin on the viability of MDA-MB-231 and MCF-7 cells in the absence of serum. As shown in **Figure 1C**, loss of cell viability in the MDA-MB-231 cells occurred between 2 and 20 μM, notably, the same concentration range that induced complete G_1 cell cycle arrest in the presence of serum. A corresponding increase in cleavage of the caspase 3 substrate poly-ADP-ribose polymerase (PARP) was observed at 20 μM (**Fig. 1D**, indicating apoptotic cell death. Surprisingly, the MCF-7 cells survived the rapamycin treatment (**Fig. 1C**) and no PARP cleavage was observed (**Fig. 1D**). Thus, compared with the MCF-7 cells, MDA-MB-231 cells are less sensitive to low-dose rapamycin treatment with regard to G_1 cell cycle progression but more sensitive to the apoptotic effect of high-dose rapamycin treatment. These data further establish a critical effect of rapamycin between 2 and 20 μM.

Differential rapamycin sensitivity of mTOR substrates in MDA-MB-231 cells. We next examined the sensitivity of mTOR substrates to rapamycin in MDA-MB-231 cells. As shown in **Figure 2A**, S6 kinase phosphorylation at the mTORC1 site at T389 was sensitive to 20 nM rapamycin, significantly lower than the concentration needed to induce apoptosis. Rapamycin at 20 μM had no effect on the phosphorylation of Akt at the mTORC2 site at S473, indicating that the apoptotic effect of rapamycin was not likely due to suppression of mTORC2. There are four sites on 4E-BP1 that are phosphorylated in response to the activation of mTORC1: T37/T46, S65 and T70.[23] Phosphorylation at all of these sites was sensitive to rapamycin; however, there were profound differences in the doses needed to suppress the different sites. Phosphorylation at T70 was sensitive to doses of rapamycin that suppressed S6 kinase phosphorylation; however, phosphorylation of T37/46 and S65 were sensitive to the doses that induced apoptosis. Thus, rapamycin induces apoptosis at the same concentration that suppress 4E-BP1 phosphorylation, most significantly at T37/46 and S65.

It has been reported that nano-molar rapamycin concentrations disrupt the association of mTOR with Raptor, a critical component of the mTORC1 complex.[24] However, co-treatment with a cross-linking reagent in lysis buffer maintained the mTOR-Raptor association, suggesting that low-dose rapamycin treatment weakens but does not abolish the association between mTOR and Raptor. This finding suggests that low-dose rapamycin treatment disrupts the structure of mTORC1 sufficiently to inhibit phosphorylation of S6 kinase, but not 4E-BP1. We therefore examined the association of mTOR with Raptor in the presence of low (200 nM) and high (20 μM) rapamycin doses with and without cross-linking. As shown in **Figure 2B**, low-dose rapamycin treatment partially dissociated mTOR from Raptor, and as reported previously in references 6 and 24, the cross-linking reagent reversed this effect and rescued the association between mTOR and Raptor. In contrast, high-dose rapamycin proved to irreversibly dissociate mTOR from Raptor, as cross-linking failed to rescue the complex association. These data suggest that the differential effects of high- and low-dose rapamycin treatment on mTORC1 is due to differential disruption of the mTORC1 complex where low doses loosely disrupt the structure

of mTORC1, such that S6 kinase is no longer recognized as a substrate, and high doses completely dissociate the complex such that 4E-BP1 is no longer recognized. This is shown schematically in **Figure 2C**.

Hyperphosphorylation of Akt at S473 suppresses rapamycin-induced apoptosis. The sensitivity of mTOR substrate phosphorylation to rapamycin was also examined in the MCF-7 cells, which did not undergo apoptosis in response to high-dose rapamycin but did arrest in G_1. As shown in **Figure 3A**, rapamycin suppressed the phosphorylation of S6 kinase at nano-molar doses and 4E-BP1 at micro-molar doses in MCF-7 cells as was observed in the MDA-MB-231 cells. Thus, while high-dose rapamycin treatment also suppressed the 4E-BP1 phosphorylation in the MCF-7 cells, this treatment did not induce apoptosis. Interestingly, there was a substantial increase in the phosphorylation of Akt at S473 observed between 2 and 20 μM rapamycin (**Fig. 3A**). A modest increase in Akt phosphorylation was observed with nano-molar doses of rapamycin as has been reported previously in references 25–27. However, at 20 μM rapamycin, there was dramatic hyperphosphorylation of Akt at S473 in the MCF-7 cells. Since Akt phosphorylation at S473 has been implicated in survival signaling,[28] we examined whether suppression of Akt phosphorylation sensitized the MCF-7 cells to high-dose rapamycin. Phosphorylation of Akt at S473 is dependent on phosphatidylinositol-3-kinase.[28] Treatment with the phosphatidylinositol-3-kinase inhibitor wortmannin suppressed Akt phosphorylation at S473 and sensitized the MCF-7 cells to high-dose rapamycin, which now induced PARP cleavage (**Fig. 3B**). These data indicate that the hyperphosphorylation of Akt in MCF-7 cells protects against the apoptotic effects of high-dose rapamycin.

If MCF-7 cells are surviving the high-dose rapamycin by increasing Akt phosphorylation at S473, then catalytic mTOR inhibitors,[4,29] which inhibit both mTORC1 and mTORC2, the kinase that phosphorylates Akt at S473, should induce apoptosis in the MCF-7 cells. As shown in **Figure 3C**, the catalytic mTOR inhibitor AZD8055 induced PARP cleavage in the MCF-7 cells,[30] providing further evidence that the hyperphosphorylation of Akt at S473 was responsible for the resistance to the high-dose rapamycin treatment.

Next, we examined whether inducing Akt phosphorylation could desensitize MDA-MB-231 cells to high-dose rapamycin treatment. We previously reported that insulin stimulates Akt phosphorylation at S473 in these cells.[6] As shown in **Figure 3D**, insulin treatment caused transient stimulation of Akt phosphorylation, which completely abolished PARP cleavage induced by high-dose rapamycin treatment. Collectively, these data reveal that stimulating Akt phosphorylation at S473 prevents rapamycin-induced apoptosis.

Suppression of eIF4E expression leads to apoptosis. Upon phosphorylation of 4E-BP1 by mTORC1, eIF4E dissociates and facilitates translation of RNAs encoding proteins involved in cell cycle progression.[31,32] Therefore, if suppressed 4E-BP1 phosphorylation induces apoptosis, then ablation of eIF4E expression should mimic the effect of high-dose rapamycin treatment. siRNAs targeted against S6 kinase, 4E-BP1 and eIF4E were
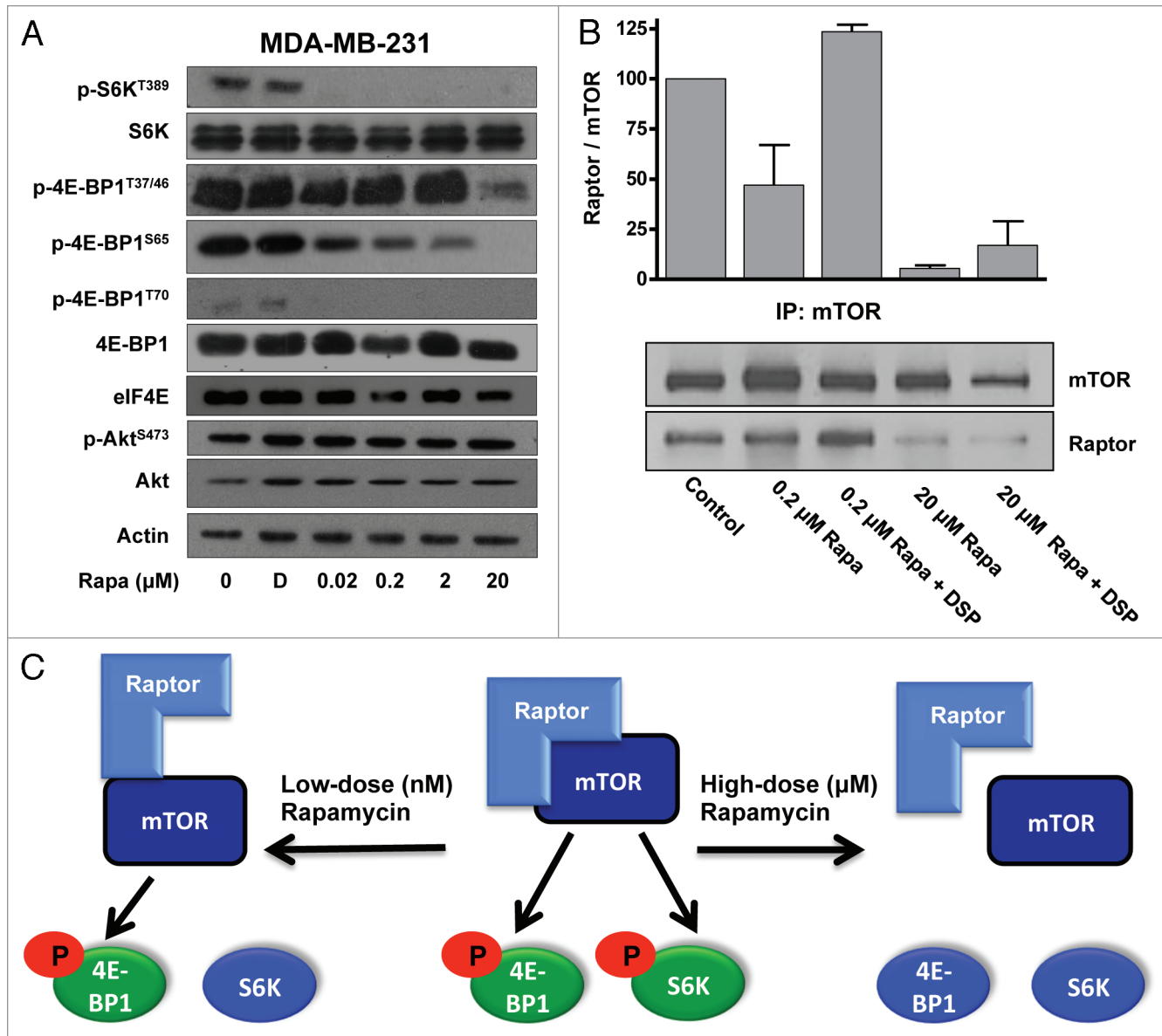
Figure 2. Differential rapamycin sensitivity of mTOR substrates in MDA-MB-231 cells. (A) MDA-MB-231 cells were plated in regular medium. Twenty-four hours later, at 90% confluence, cells were exposed to 0% serum and treated with DMSO (D) or rapamycin (Rapa) at the indicated concentrations for 4 h. Cells were harvested and lysates were immunoblotted with the indicated antibodies. (B) Cells were plated as in (A) exposed to 0% serum and treated with indicated rapamycin concentrations for 4 h. Cells were harvested and then lysed in the absence or presence of DSP cross-linking reagent and immunoprecipitated with mTOR. The immunoprecipitates were then subjected to protein gel blot analysis using either mTOR or Raptor antibody. The data shown are representative of experiments repeated at least two times. (C) A model for the differential sensitivity of mTORC1 substrate phosphorylation by rapamycin.

introduced into the MDA-MB-231 cells, which were then examined for PARP cleavage. As shown in **Figure 4A**, a substantial increase in PARP cleavage and loss of cell viability was observed with eIF4E ablation but not with S6 kinase or 4E-BP1 ablation. These data further support a model whereby the apoptotic effect of high-dose rapamycin is due to suppression 4E-BP1 phosphorylation and subsequent inhibition of eIF4E.

We also examined the effect of suppressing eIF4E in the MCF-7 cells. In contrast with MDA-MB-231 cells, suppression of eIF4E did not significantly increase PARP cleavage or reduce

cell viability (**Fig. 4B**). However, if MCF-7 cells were treated with wortmannin to suppress phosphorylation of Akt at S473, then a substantial increase in PARP cleavage and loss of cell viability was observed in the eIF4E-ablated cells (**Fig. 4B**). These data reinforce the observation that the MCF-7 cells are resistant to the suppression of 4E-BP1 phosphorylation by rapamycin and that this resistance is due at least in part to a feedback activation of Akt.[25-27]

Since, it appeared that the apoptotic effect of high-dose rapamycin treatment is due to sequestration eIF4E by unphosphorylated
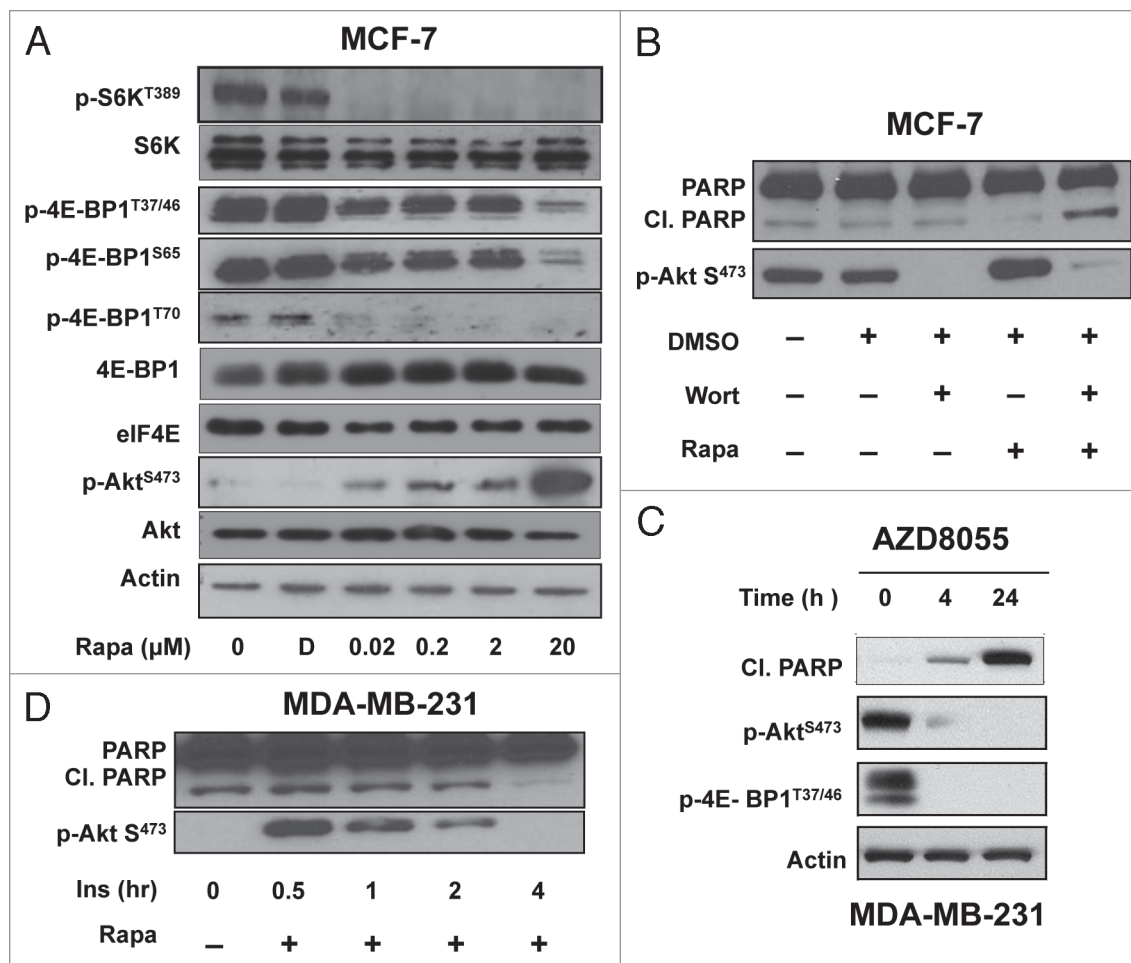
Figure 3. Phosphorylation of Akt at suppresses rapamycin-induced apoptosis. (A) MCF-7 cells were plated and treated as in Figure 2A. Cells were harvested, and lysates were immunoblotted with the indicated antibodies. (B) MCF-7 cells plated as in (A) and treated in 0% serum with DMSO (D), wortmannin (Wort) (1 μM) or rapamycin (Rapa) (20 μM) for 4 h. Cell lysates were then immunoblotted with the indicated antibodies. (C) MCF-7 were prepared as in (A) and then treated with 500 nM μM AZD8055 for the indicated times. The cells were harvested at the indicated times, lysates were then prepared and immunoblotted with the indicated antibodies. (D) MDA-MB 231 were plated as in (A) and treated in 0% serum with insulin or rapamcyin (20 μM) for indicated times. Cell lysates were immunoblotted with the indicated antibodies. All data are representative of at least two independent experiments.

4E-BP1, we reasoned that suppressing 4E-BP1 expression would desensitize the cells to high-dose rapamycin because there would be no 4E-BP1 to bind eIF4E. We therefore examined the effect of 20 μM rapamycin on cell viability on MDA-MB-231 cells treated with siRNA targeted for 4E-BP1. As shown in **Figure 4C**, MDA-MB-231 cells treated with a control siRNA were sensitive to 20 μM rapamycin. However, cells treated with siRNA for 4E-BP1 were resistant to 20 μM rapamycin. These data further support the hypothesis that the effects of high-dose rapamycin are due to the suppression of 4E-BP1 phosphorylation and the suppression of eIF4E. Importantly, this result also establishes that the apoptotic effect of high-dose rapamycin treatment is not a nonspecific effect of high-dose rapamycin on another cellular protein.

Discussion

In this study, we investigated the effect of cytotoxic doses of rapamycin on mTOR substrates. A clear correlation emerged between the induction of apoptosis and the suppression of 4E-BP1 phosphorylation at T37/46 and S65. Suppression of 4E-BP1 phosphorylation results in the sequestration of eIF4E by unphosphorylated 4E-BP1,[23] and consistent with these findings, ablation of eIF4E expression resulted in apoptotic cell death. Thus, rapamycin-induced apoptosis appears to be a direct result of suppressing 4E-BP1 phosphorylation by mTORC1 and the indirect result of inactivating eIF4E. The finding that suppression of 4E-BP1 phosphorylation by high-dose rapamycin treatment is responsible for the observed apoptosis is consistent with reports implicating eIF4E in cell proliferation and oncogenic transformation.[17,33-38] Significantly, phosphorylated 4E-BP1 is also associated with poor patient survival in melanoma.[39] Thus, data provided here are consistent with an emerging paradigm that mTOR survival signals are mediated by the phosphorylation of 4E-BP1 and the subsequent release of eIF4E. These data have important implications for the many ongoing clinical trials involving rapamycin and the targeting of mTOR.
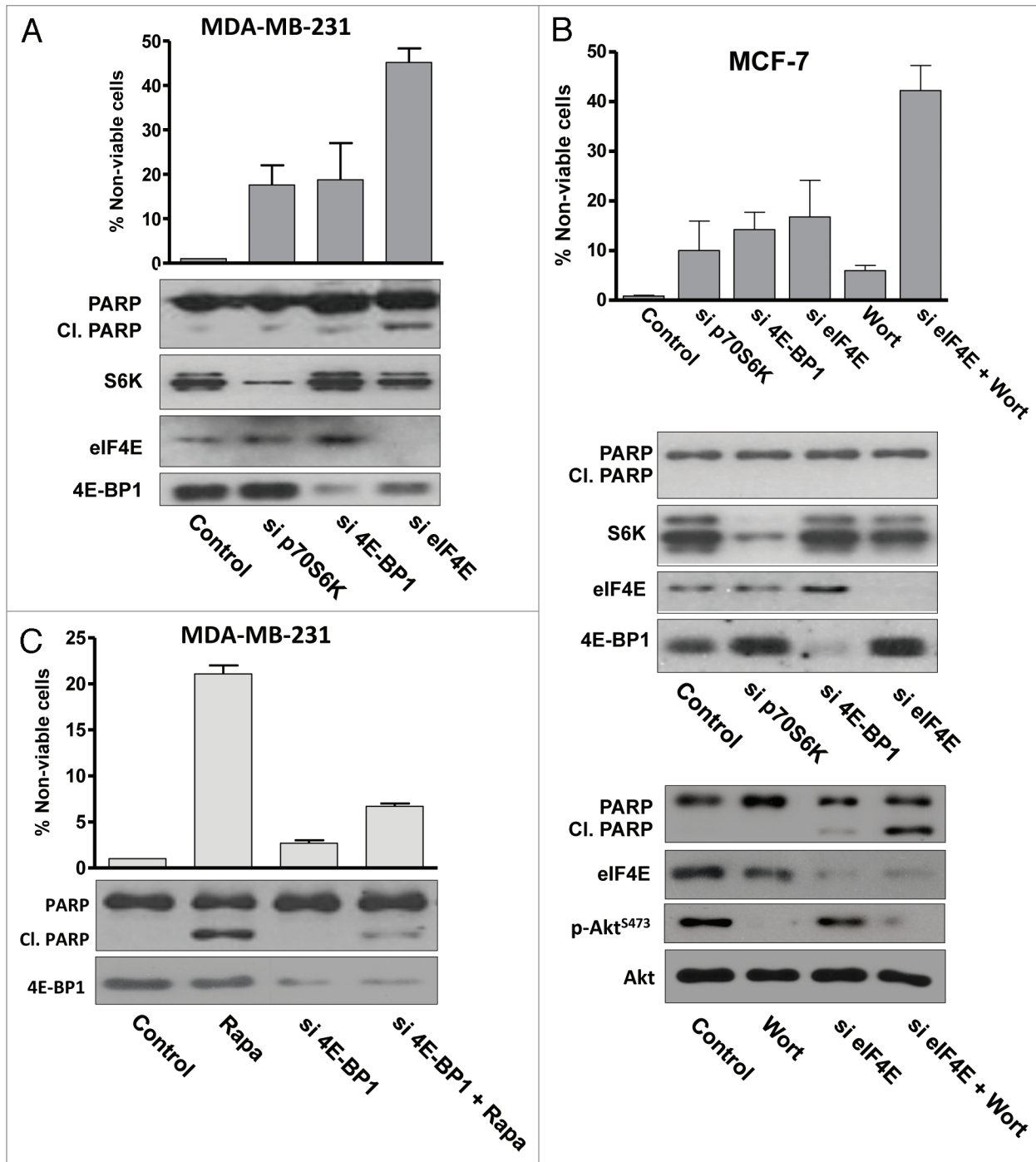
Figure 4. Suppression of eIF4E expression leads to apoptosis. (A) MDA-MB-231 cells were transfected at 50% confluence with negative control siRNA or siRNA targeted for S6 kinase, 4E-BP1 or eIF4. 48 h later, immunoblotting was used to analyze the indicated proteins. The percentage of non-viable cells was determined at 48 h as described in Materials and Methods. (B) MCF-7 cells were transfected with indicated siRNAs as in (A). Cells were evaluated for the indicated proteins and phosphoproteins and cell viability at 48 h. Wortmannin (1 uM) was added where indicated 24 h prior to evaluating cell viability and 4 h prior to evaluating protein levels and PARP cleavage. (C) MDA-MB-231 cells were either transfected at 50% confluence with negative control siRNA or siRNA targeted for 4E-BP1. Cells were treated with rapamycin (Rapa) (20 μM) for 4 h for PARP cleavage and 24 h for cell viability. All results are representative of experiments repeated at least two times.

Clinical trials with rapamycin or rapalogs have been largely disappointing.[3,40] In general, rapamycin has been considered a cytostatic, rather than cytotoxic compound,[13] which may explain the limited response in clinical trials. However, doses of rapamycin that suppress 4E-BP1 phosphorylation are, in fact, cytotoxic and induce apoptosis, at least in the absence of serum.[11] In the presence of serum, low-dose rapamycin treatment only retarded cell cycle progression, whereas high-dose rapamycin

treatment was required to cause complete G_1 cell cycle arrest. The efficacy of rapamycin-based strategies in clinical trials is commonly evaluated by effective suppression of S6 kinase.[41,42] The study presented here indicates that 4E-BP1 phosphorylation would be a better indicator of drug efficacy. It is widely believed that phosphorylation of 4E-BP1 is not inhibited by rapamycin; however, as shown here, 4E-BP1 phosphorylation is suppressed by rapamycin, but requires higher doses than are required to suppress phosphorylation of S6 kinase. The question as to whether levels of rapamycin or rapalogs that suppress 4E-BP1 phosphorylation at T37/46 can be achieved with tolerated toxicity has not been evaluated.

The differential effects of low- and high-dose rapamycin treatment were apparently due to the partial vs. complete dissociation of mTOR and Raptor. Low-dose rapamycin treatment reduced association between mTOR and Raptor, but if a cross-linking reagent was added during cell lysis, Raptor remained associated with mTOR. The conclusion was that mTOR was able to phosphorylate 4E-BP1, but not S6 kinase, at the low dosage where Raptor is still weakly associated with mTOR. However, at high dosage, the lack of any Raptor associated with mTOR prevents phosphorylation of 4E-BP1 (**Fig. 2C**). This model is conceptually similar to one recently proposed based on a structural analysis of mTORC1.[43] In this study, prolonged treatment (2 h) with 100 nM rapamycin suppressed the phosphorylation of 4E-BP1 by purified mTORC1 at T37/46 in vitro, whereas S6 kinase phosphorylation was suppressed after a 5 min treatment. Their conclusion was similar to ours, whereby the short-term treatment resulted in partial disruption of the mTORC1 complex and prevented phosphorylation of S6 kinase, and long-term treatment completely disrupted the complex such that 4E-BP1 did not get phosphorylated. Longer-term treatment with intact cells, at least over the 2 h time course used for the in vitro study, is not sufficient to break down the mTOR complex in vivo. This could be due to different effective concentrations of rapamycin when it is administered to intact cells. However, the principle is the same in both studies, that mTORC1 can be partially disrupted by rapamycin such that S6 kinase is no longer a substrate, yet 4E-BP1 still gets phosphorylated.

MCF-7 cells have a more benign phenotype than the MDA-MB-231 cells and retain differentiated epithelial cell characteristics. These cells were not killed by high-dose rapamycin treatment even though 4E-BP1 phosphorylation was suppressed. Significantly, the high-dose rapamycin treatment induced hyperphosphorylation of Akt at S473, substantially higher than that observed at the nano-molar concentrations as reported previously in references 25–27. MCF-7 cells were sensitized to rapamycin treatment upon suppression of Akt phosphorylation. Similarly, stimulation of Akt phosphorylation in MDA-MB-231 cells resulted in resistance to high-dose rapamycin treatment. It is possible that normal and less malignant cells like MCF-7 cells could be protected from high-dose rapamycin treatments by virtue of elevated Akt phosphorylation. A clinical trial for everolimus revealed an increase in the phosphorylation of Akt at S473 observed in 50% of the tumors.[44] It was not apparent how this impacted on the effect of everolimus; however, there could be interesting differences in the effect of the rapalogs in cells where Akt phosphorylation is elevated.

Rapamycin-based therapeutic strategies are problematic because of the relative resistance to rapamycin of mTORC2 and the phosphorylation of 4E-BP1 by mTORC1.[45] Consequently, there has been strong interest in catalytic inhibitors of mTOR that target both mTORC1 and mTORC2 [4,29] and suppress phosphorylation of S6 kinase, 4E-BP1 and Akt at S473 with equal efficiency.[15,45] Elevated Akt phosphorylation protected the more benign MCF-7 cells from the apoptotic effect of high-dose rapamycin. As shown here, a catalytic mTOR inhibitor was able to induce apoptosis in the MCF-7 cells. Thus, while the catalytic inhibitors kill cancer cells with elevated Akt phosphorylation, this class of inhibitor may also be more toxic to normal cells. In this regard, rapamycin may be advantageous because of its selective inhibition of mTORC1.

The high doses of rapamycin needed to suppress 4E-BP1 phosphorylation in cultured cells reported here are not likely to be achieved in clinical trials. This is because maximum tolerated doses or rapamycin result in nano-molar levels of the drug in plasma.[37] Thus, delivering an effective dosage capable of inducing the apoptotic effects observed here would require a vehicle that targets tumors with high specificity. One possible strategy would be to tag rapamycin with glucose, which is taken up preferentially by most cancer cells due to an altered metabolism known as the Warburg effect.[46] This strategy has been used previously to enhance the uptake of a photodynamic therapeutic agent into cancer cells.[47] With such a strategy, tolerable levels of rapamycin could result in the delivery of higher toxic levels to tumor. This strategy could also result in additional benefits conferred by the low-dose treatments that have been implicated prolonging lifespan.[48-50] Although the low-dose rapamycin treatments are not likely to kill the cancer cells or significantly block cell cycle progression, the low doses have been able to sensitize cancer cells to other treatments.[51-53] Thus, the current low-dose strategies may still be of value in combination with other chemotherapeutic agents.

In summary, the effects of rapamycin on cancer cells have been have been difficult to evaluate because of differential effects observed in different cancer cells and the varying concentrations needed to suppress different downstream targets of mTOR. In this study, we have revealed that the cytotoxic effect of high-dose rapamycin treatment is due to the complete dissociation of mTOR from Raptor, which results in the inhibition of 4E-BP1 phosphorylation. We also show that rapamycin-resistance can be achieved by hyperphosphorylation of Akt at the mTORC2 site at S473. These complex responses to rapamycin reveal both problems and opportunities for targeting mTOR, which has been implicated in the signals that promote cell cycle progression and survival in human cancers.

Materials and Methods

Cells, cell culture conditions and cell viability. The human cancer cell lines MDA-MB-231 and MCF-7 cells were obtained from the American Tissue Type Culture Collection (ATCC) and cultured in Dulbecco's modified Eagle medium (DMEM) (Sigma)

supplemented with 10% Fetal Bovine Serum (Sigma). Cell viability was determined by trypan blue exclusion or by counting viable attached cells. For trypan blue exclusion, cells were harvested, washed and treated with trypan blue at a concentration of 0.4% v/v. After 5 min, trypan blue uptake (dead cells) was scored using a hemocytometer. Cell viability was also determined by counting adherent cells 24 h after treatment using a hemacytometer or Coulter counter.

Antibodies and reagents. The following antibodies were used: PARP, cleaved PARP, p-S6 kinase T389, S6 kinase, p-4E-BP1 T37/46, p-4E-BP1 S65, p-4E-BP1 T70, 4E-BP1, p-Akt S473, Akt (Cell Signaling); α-actin (Sigma); eIF4E (Santa Cruz Biotechnology). Negative control siRNA (Dharmacon), siRNAs targeted against S6 kinase, 4E-BP1 and eIF4E (Santa Cruz Biotechnology) were purchased. Lipofectamine RNAiMax (Invitrogen) was used for transient transfections. Rapamycin and wortmannin were obtained from Calbiochem, and insulin was purchased from Sigma. [3H]-thymidine was from Perkin Elmer and the catalytic mTOR inhibitor AZD8055 was from Axon Medchem.

Protein gel blot analysis. Extraction of proteins from cultured cells and protein gel blot analysis of extracted proteins was performed using the ECL system (Amersham) as described previously in reference 6.

Transient transfections. Cells were plated in 6-well plates in medium containing 10% FBS. The next day (50% confluence), transfections with siRNAs (75 nM) in Lipofectamine RNAiMAX were performed. After 6 h, reagents were replaced with fresh 10% FBS, and cells were allowed to incubate for an additional 48 h.

Cross-linking assay and immunoprecipitation. The cross-linking of mTOR with Raptor dithiobis(succinimidyl) propionate (DSP) (Sigma) and co-immunoprecipitation was performed as described previously in reference 6. Quantitative changes in protein levels were analyzed by densitometry using Image-J software.

Cell cycle synchronization and progression. Cell cycle progression under different concentrations of rapamycin was studied using thymidine incorporation assay. Cells were plated, synchronized in M phase using nocodazole (Sigma) and then treated with the indicated concentration of rapamycin along with [3H]-thymidine (1 mCi/ml, 20 Ci/mMole) (Perkin Elmer) label as described in the figure legend. Twenty-four hours later, the cells were washed twice with PBS and then precipitated twice with 10% trichloroacetic acid. The precipitates were solubilized in 0.5 ml of 0.5% SDS/0.5 M NaOH solution. The extent of thymidine incorporation was quantified using 75 µl of sample and 3 ml of scintillation fluid.

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