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Serum and glucocorticoid-regulated kinase 1 (SGK1) activation in breast cancer: Requirement for mTORC1 activity associates with ER-alpha expression

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Abstract

Mammalian target of rapamycin (mTOR) is an attractive target for cancer treatment. While rapamycin and its derivatives (e.g. everolimus) have been shown to inhibit mTOR signaling and cell proliferation in preclinical models of breast cancer, mTOR inhibition has demonstrated variable clinical efficacy with a trend toward better responses in estrogen receptor alpha positive (ERα+) compared to ERα negative (ERα-) tumors. Recently, serum and glucocorticoid-regulated kinase 1 (SGK1) was identified as a substrate of mTOR kinase activity. Previous studies have alternatively suggested that either mTORC1 or mTORC2 is exclusively required for SGK1's Ser422 phosphorylation and activation in breast cancer cells. We investigated the effect of rapamycin on the growth of several ERα+ and ERα- breast cancer cell lines and examined differences in the phosphorylation of mTOR substrates (SGK1, p70S6K and Akt) that might account for the differing sensitivity of these cell lines to rapamycin. We also examined which mTOR complex contributes to SGK1-Ser422 phosphorylation in ERα+ versus ERα- breast cell lines. We then assessed whether inhibiting SGK1 activity added to rapamycin-mediated cell growth inhibition by either using the SGK1 inhibitor GSK650394A or expressing an *SGK1* shRNA.

We observed sensitivity to rapamycin-mediated growth inhibition and inactivation of insulin-mediated SGK1-Ser422 phosphorylation in ERα+ MCF-7 and T47D cells, but not in ERα- MDA-MB-231 or MCF10A-Myc cells. In addition, either depleting SGK1 with shRNA or inhibiting SGK1 with GSK650394A preferentially sensitized MDA-MB-231 cells to rapamycin. Finally, we found that rapamycin-sensitive SGK1-Ser422 phosphorylation required ERα expression in MCF-7 derived cell lines. Therefore, targeting SGK1 activity may improve the efficacy of rapamycin and its analogues in the treatment of ERα- breast cancer.

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Authors' contributions

BAH and SDC conceived, designed the study, and wrote the manuscript. BAH carried out the majority of the experiments. TYK carried out SRB assays and assisted with the generation of *RPTOR* and *RICTOR* shRNA clones and Westerns blots of protein lysates from MCF-7 cells. MNS performed the SRB assays. All authors proofread and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Keywords

Breast cancer; Serum and glucocorticoid-regulated kinase 1 (SGK1); Mammalian target of rapamycin (mTOR); Phosphoinositide 3-kinase (PI3K); Rapamycin; Estrogen receptor alpha (ER α)

Introduction

The mammalian target of rapamycin complexes 1 and 2 (mTORC1 and mTORC2) mediate both extracellular and intracellular signals, functioning as central regulators of cell metabolism, growth and survival. Aberrant mTOR signaling has been implicated in the etiology of cancer [1]. mTOR is a highly conserved serine/threonine kinase, expressed in a range of cell types, including normal breast epithelial [2] and breast cancer cells [3]. At least two complexes exist: mTORC1 and mTORC2; however, only mTORC1 is inhibited by short-term rapamycin treatment [4], allowing rapamycin to be used to dissect the requirement for mTORC1 (versus mTORC2) activity.

Serum and glucocorticoid-regulated protein kinase 1 (SGK1) plays a critical role in promoting epithelial cell survival under conditions of cellular stress such as serum-deprivation and chemotherapy treatment [5,6]. Similar to Akt, SGK1 mediates anti-apoptotic signals following its phosphorylation and activation downstream of Phosphoinositide-3-kinase (PI3K) signaling [7,8]. However, the precise upstream mechanisms regulating SGK1's phosphorylation at Ser422 and subsequent activation have been the subject of debate. For example, while recent evidence shows a requirement for the mTOR pathway in the phosphorylation of SGK1's Ser422, independent studies have alternatively identified either mTORC1 [9] or mTORC2 [10] as the upstream complex required for SGK1 activation.

Previous reports also suggest that rapamycin more effectively inhibits the proliferation of estrogen receptor alpha positive (ER α +) compared to ER α -negative (ER α -) breast cancer cell lines [11-13]. We hypothesized that the difference in rapamycin sensitivity between ER α and ER α - cell types could be due to differential molecular circuitry downstream of mTOR signaling. We therefore compared the phosphorylation of key downstream mTOR substrates following PI3K/mTOR activation in ER α MCF-7 and T47D cell lines versus ER α - MDA-MB-231 and MCF10A-MYC cell lines. We found that in ER α cell lines, rapamycin-mediated growth inhibition correlated with a loss of SGK1-Ser422 phosphorylation. Furthermore, in ER α - MDA-MB-231 cells, knockdown of SGK1 expression or treatment with an SGK1 inhibitor GSK650394A, combined with rapamycin, significantly increased the effectiveness of rapamycin-mediated growth inhibition. These findings suggest that breast cancer sensitivity to rapamycin correlates with downstream inhibition of SGK1 kinase activity and with ER α expression.

Materials and methods

Reagents

Dexamethasone, insulin and thapsigargin (Sigma-Aldrich), LY294002 (Calbiochem), Rapamycin (LC-laboratories) and PI-103 (Alexis), GSK650394A (Tocris). Torin1 [14,15] was a gift from Drs. N. Gray and D. Sabatini (Harvard University).

Antibodies

α -P-Akt Ser473, α -Akt, α -P-p70S6K Thr389, α -p70S6K, α -mTOR, α -P-NDRG1 (Thr346, Thr356 and Thr366), α -NDRG1, α -RAPTOR, and α -RICTOR (Cell Signaling Technology),

α -P-Ser422-SGK1 (Santa Cruz Biotechnology), α -SGK1 (Assay Designs), α - β -actin (Sigma-Aldrich). The ER α antibody (H222.2) was a gift from Dr. G. Greene (University of Chicago).

Cell culture

MDA-MB-231 and MCF-7 cells (ATCC) were cultured in DMEM (BioWhittaker) with 10% fetal calf serum. MCF10A-MYC cells were cultured as described previously [16]. C4-12 and FLAG-ER α -C4-12 cells (from Dr. G. Greene, University of Chicago) were cultured in phenol red-free DMEM with 10% charcoalstripped FCS.

Immunoprecipitations

Cells were transfected with plasmids, SGK-1-FLAG-pLPCX, HA-SGK1-pLPCX or pLPCX control, using Fugene HD. Following 24h, cells were serum-deprived for 24h and treated with insulin $\pm$ rapamycin. Cells were lysed as described previously [17]. Equal protein lysates were immunoprecipitated using α -FLAG- or α -HA-agarose or magnetic beads (Sigma-Aldrich). SGK1-FLAG and HA-SGK1 were eluted in Laemmli buffer.

Western blot analysis

Parental cells or cells stably transfected with SGK1-FLAG or HA-SGK1 were serum-deprived for the indicated time and treated with dexamethasone (1 μ M), insulin (300nM), or thapsigargin (1 μ M). Cells were lysed as in [17] and equal protein lysates (measured by BCA assay) were loaded into 7.5-10% SDS/polyacrylamide gels and transferred to 0.45 μ m nitrocellulose. Membranes were blocked in 5% milk in 0.1% Tween20 in TBS and incubated with the primary and secondary HRP-conjugated, AlexFluor680 (Invitrogen) or Dylight800 (Thermoscientific) antibodies. Membranes were exposed to autoradiography film or scanned using the Odyssey infrared imager (LI-COR).

Generation of stable *RPTOR*, *RICTOR* and *SGK1* shRNA-expressing cell lines

MCF-7 and MDA-MB-231 cell lines stably expressing either *RPTOR* or *RICTOR* shRNA were generated by transfecting with *RPTOR* or *RICTOR* pLKO.1 shRNA plasmids (Addgene, [18]). Two shRNAs were used to validate knockdown of both RAPTOR and RICTOR proteins. Cells were then selected in puromycin (400-800ng/ml) and clones of *RPTOR* or *RICTOR* shRNA-expressing cells were screened. Generation of stable *SGK1* or scrambled sequence shRNA-expressing MDA-MB-231 and MCF-7 cell lines was performed as described previously [16].

Sulforhodamine B assay

The sulforhodamine B (SRB) assay was used to measure total cellular protein as described previously [19,20]. MCF-7 and MDA-MB-231 cells were treated with 10nM or 100nM rapamycin or DMSO vehicle for four days. In some experiments, the SGK1 inhibitor GSK650394A or DMSO vehicle was also added.

Results

mTORC1 activity contributes to insulin-induced SGK1-Ser422 phosphorylation in MCF-7 and T47D cells

Consistent with previous reports [11,12] we found that ER α +, MCF-7 and T47D breast cancer cell lines were more sensitive to the growth inhibitory effects of rapamycin compared to ER α -, MDA-MB-231 and MCF10A-MYC breast cell lines (Supplementary Fig. S1). Using these ER α and ER α - cell lines, we examined potential differences in the phosphorylation and activation of the mTOR substrates, p70S6K, Akt and SGK1 that might

account for differing cell sensitivity to rapamycin. Previous studies had shown that the mTORC1 target, p70S6K, loses phosphorylation following rapamycin treatment [21], while the mTORC2 target, Akt Ser473 phosphorylation is generally rapamycin-insensitive [4,18]. Interestingly, SGK1 activation has been reported to require either mTORC1 [9] or mTORC2 [10] activity. We therefore investigated whether endogenous SGK1-Ser422 phosphorylation is lost following mTORC1 inhibition with short-term (1-hour) rapamycin treatment, while attempting to confirm p70S6K sensitivity and Akt insensitivity to rapamycin.

MCF-7 cells were serum-starved and endogenous SGK1 expression was induced with dexamethasone treatment. We found that 8h of dexamethasone treatment also increased p70S6K phosphorylation (supplementary Fig. S2), suggesting that dexamethasone treatment promotes mTORC1 activity in these cells. Cells were then stimulated for the last 1-hour with insulin alone or in combination with an mTORC inhibitor. Figure 1A demonstrates that endogenous SGK1 expression and Ser422 phosphorylation were induced, as expected, following concomitant dexamethasone and insulin treatment. The α -P-SGK1-Ser422 antibody detected low levels of endogenous P-SGK1-Ser422 (~52kDa) following insulin (Fig. 1A), and as described previously, cross-reactive P-p70S6K (~70kDa) was also detected [9,10]. We also confirmed the observed increase in insulin-mediated p70S6K phosphorylation using the α -P-p70S6K-Thr389 antibody. Inhibition of mTORC1 by rapamycin inhibited both insulin-induced SGK1-Ser422 and p70S6K-Thr389 phosphorylation, while Akt-Ser473 phosphorylation remained unchanged. This suggested that mTORC1 is required for insulin-mediated SGK1-Ser422 phosphorylation in MCF-7 cells. Treatment with the dual mTORC1/mTORC2 inhibitor PI-103 [22] or the PI3K inhibitor LY294002 resulted in a similar reduction in P-SGK1-Ser422 and a loss of both P-p70S6K-Thr389 and P-Akt-Ser473. Treatment of cells with a lower concentration of LY294002 also reduced P-p70S6K-Thr389 and P-SGK1-Ser422 (Supplementary Fig. S3). The specificity of the observed P-SGK1-Ser422 and total SGK1 immunoreactive bands was confirmed by *SGK1* shRNA expression, which significantly diminished SGK1 immunoreactive bands by Western analysis (Supplementary Fig. S4).

We next examined SGK1-Ser422 phosphorylation of immunoprecipitated SGK1 from MCF-7 cells ectopically expressing SGK1-FLAG to ensure that the immunoreactive P-SGK1-Ser422 bands observed were indeed SGK1 species. Figure 1B shows that insulin and concurrent rapamycin treatment of MCF-7 cells inhibited Ser422 phosphorylation in SGK1-FLAG immunoprecipitates (Fig. 1B); these data support a requirement, at least in part, for mTORC1 activity in insulin-stimulated SGK1 activation. As expected, rapamycin treatment also inhibited insulin-stimulated p70S6K-Thr389 phosphorylation, under conditions in which Akt-Ser473 phosphorylation did not change significantly (Fig. 1B). The dual mTORC1/mTORC2 inhibitor, Torin1, inhibited both P-p70S6K-Thr389 and Akt-Ser473 phosphorylation. Torin1 treatment also resulted in inhibition of SGK1-Ser422 phosphorylation to a greater degree than rapamycin alone, suggesting that while mTORC1 activity is required for SGK1-Ser422 phosphorylation, mTORC2 might also play a role.

To further examine the requirement for mTORC1 in mediating SGK1-Ser422 phosphorylation following insulin stimulation in MCF-7 cells, we examined SGK1 activation in cells lacking either mTORC1 or mTORC2 activity resulting from shRNA knockdown of their respective regulatory domain proteins. Specifically, the regulatory proteins, RAPTOR or RICTOR, required for functional mTORC1 or mTORC2 activity respectively, were depleted by the stable expression of *RPTOR*, or *RICTOR* shRNAs. MCF-7 cells expressing control, *RPTOR*, or *RICTOR* shRNAs were transfected with a plasmid encoding SGK1-FLAG, serum-deprived for 24h and then treated with insulin (Fig. 1C). Depletion of RAPTOR abrogated SGK1-Ser422 phosphorylation following insulin stimulation of MCF-7 cells while depletion of RICTOR had no significant effect on SGK1-

Ser422 phosphorylation. As expected, depletion of RAPTOR also enhanced Akt-Ser473 phosphorylation [likely via inhibition of the negative feedback loop whereby mTORC1 phosphorylates insulin receptor substrate 1, IRS-1 (reviewed in [1])], while depletion of RICTOR inhibited Akt-Ser473 phosphorylation. These results are consistent with a requirement for mTORC1 in SGK1-Ser422 phosphorylation following insulin.

To assess the requirement for mTORC1 in insulin-stimulated SGK1-Ser422 phosphorylation in T47D cells, another ER α + cell line, we transfected SGK1-FLAG, and immunoprecipitated SGK1 following insulin treatment with or without rapamycin. We also examined p70S6K and Akt phosphorylation in T47D cells. Consistent with our findings in MCF-7 cells, rapamycin inhibited insulin-induced SGK1-Ser422 and p70S6K-Thr389 phosphorylation (Fig. 1D), while P-Akt-Ser473 was unchanged. These data suggest that insulin-induced SGK1-Ser422 phosphorylation requires mTORC1 activity in both ER α + MCF-7 and T47D cells.

mTORC2 activity is required for insulin-induced SGK1 phosphorylation in MDA-MB-231 and MCF10A-MYC cells

To determine whether or not rapamycin could also inhibit SGK1-Ser422 phosphorylation in ER α -breast cell lines, we performed parallel experiments using MDA-MB-231 and MCF10A-MYC cells. Figure 2A shows that both p70S6K and SGK1-Ser422 phosphorylation were increased following dexamethasone and insulin treatment of MDA-MB-231 cells. In these cells, as in MCF-7 cells, p70S6K-Thr389 phosphorylation was inhibited with concomitant rapamycin treatment. However, unlike ER α + MCF-7 and T47D cells, MDA-MB-231 SGK1-Ser422 phosphorylation was not inhibited by rapamycin (but enhanced), suggesting that mTORC1 activity is not required for SGK1-Ser422 phosphorylation in these ER α - cells. As expected, PI-103 and LY294002 treatment inhibited SGK1, p70S6K and Akt phosphorylation.

We next examined SGK1-Ser422 phosphorylation in MDA-MB-231 cells transfected with SGK1-FLAG. Figure 2B shows that insulin-induced SGK1-FLAG Ser422 phosphorylation was not inhibited following rapamycin treatment, under conditions in which P-p70S6K-Thr389 was inhibited. As expected, treatment with Torin1 blocked insulin-induced SGK1-Ser422, p70S6K-Thr389 and Akt-Ser473 phosphorylation. These data support a requirement for mTORC2 activity, but not mTORC1 activity, in insulin-stimulated SGK1-Ser422 phosphorylation in MDA-MB-231 cells.

To further investigate the mTORC2 requirement for SGK1-Ser422 phosphorylation in MDA-MB-231 cells, we performed experiments in which RAPTOR or RICTOR were depleted. With stable RAPTOR knockdown, phosphorylation of SGK1-FLAG-Ser422 was enhanced in MDA-MB-231 cells compared to phosphorylation of SGK1-FLAG-Ser422 in control shRNA-expressing cells (Fig. 2C). However, in RICTOR-depleted cells, SGK1-Ser422 phosphorylation was significantly inhibited, suggesting that SGK1-Ser422 phosphorylation requires mTORC2 (and not mTORC1) in MDA-MB-231 cells. Similarly, mTORC2-dependent Akt-Ser473 phosphorylation was enhanced in cells with depleted RAPTOR following insulin, while insulin-induced Akt-Ser473 phosphorylation was inhibited in RICTOR-depleted cells. In addition, Torin1 treatment inhibited SGK1-Ser422 and Akt-Ser473 phosphorylation in all cases.

We also assessed ER α - MCF10A-MYC cells ectopically expressing SGK1-FLAG. Consistent with the results from MDA-MB-231 cells, insulin-induced SGK1-Ser422 phosphorylation of immunoprecipitated SGK1-FLAG from MCF10A-MYC cells was enhanced with contaminant rapamycin treatment (Fig. 2D), while P-p70S6K-Thr389 was

inhibited and P-Akt-Ser473 was enhanced. Furthermore, Torin1 treatment inhibited SGK1-Ser422, p70S6K-Thr389 and Akt-Ser473 phosphorylation.

Thapsigargin treatment induces mTORC2-dependent SGK1 phosphorylation in MDA-MB-231 cells and mTORC1-dependent SGK1 phosphorylation in MCF-7 cells

Thapsigargin is a sarco/endoplasmic reticulum Ca^{2+} -ATPase (SERCA) pump inhibitor [23] that also stimulates PI3K activity in fibroblasts and MCF-7 cells [24]. We found that thapsigargin induces both *SGK1* expression and subsequent SGK1-Ser422 phosphorylation in MDA-MB-231 cells (Hall and Conzen, unpublished observations). We therefore investigated whether thapsigargin-induced endogenous SGK1-Ser422 phosphorylation would have the same cell context-dependent mTORC requirement as insulin stimulation. Figure 3A demonstrates that MDA-MB-231 cells treated with $1\mu\text{M}$ thapsigargin for 2-hours resulted in both increased SGK1 expression and SGK1-Ser422 phosphorylation. Although, p70S6K phosphorylation was noticeably decreased, the addition of rapamycin for the last 1-hour of treatment did not alter SGK1-Ser422 phosphorylation. In contrast, the use of PI-103 or Torin1 markedly inhibited SGK1-Ser422 phosphorylation, which is consistent with an mTORC2 requirement and not an mTORC1 requirement.

In MCF-7 cells, thapsigargin did not induce a significant increase in total SGK1 expression (data not shown), although it has been reported to increase Akt-Ser473 phosphorylation through PI3K activation [24]. Consistent with this finding, we found that thapsigargin alone increased both p70S6K-Thr389 and Akt-Ser473 phosphorylation in MCF-7 cells (Supplementary Fig. S5), demonstrating that thapsigargin increased mTORC1 and mTORC2 activity in this cell line. We therefore used dexamethasone to induce endogenous SGK1 and examined SGK1-Ser422 phosphorylation following thapsigargin treatment (Fig. 3B). As with insulin-mediated activation of PI3K/mTOR, rapamycin inhibited thapsigargin-induced SGK1-Ser422 phosphorylation in MCF-7 cells, comparable to the inhibitory effect of Torin1. Collectively, these results suggest that PI3K-mediated stimulation of SGK1-Ser422 phosphorylation requires mTORC1 signaling in MCF-7 cells and mTORC2 activity in MDA-MB-231 cells.

Inhibition of SGK1 kinase activity is required for sensitivity to rapamycin-mediated growth inhibition

To examine a possible functional role for the loss of SGK1 kinase activity following rapamycin treatment, we assessed the effect of rapamycin and concomitant treatment with an SGK1 inhibitor GSK650394A on MCF-7 and MDA-MB-231 cell growth. GSK650394A has been shown to inhibit the growth of both prostate and cervical cancer cells [25]. MCF-7 and MDA-MB-231 cells were treated with either vehicle, $0.1\mu\text{M}$ or $1\mu\text{M}$ of this SGK1 inhibitor in the absence or presence of rapamycin for four days and total cellular protein (which correlates with cell number) was measured with the SRB assay (Fig. 4A and 5B). In addition, inhibition of SGK1 activity was assessed by measuring phosphorylation of the SGK1 substrate, N-myc downstream-regulated 1 (NDRG1) [26,27] (Fig. 4C and 5D). In the absence of rapamycin, treatment with $1\mu\text{M}$ GSK650394A inhibited the growth of both MCF-7 and MDA-MB-231 cells (Fig. 4A and 4B, $P < 0.05$). However, in the presence of rapamycin, treatment with $1\mu\text{M}$ of GSK650394A had no measurable effect on MCF-7 cell growth (Fig. 4A) while significantly inhibiting MDA-MB-231 cell growth (Fig. 4B, $P < 0.05$). These data suggest that MDA-MB-231 cells are more sensitive than MCF-7 cells to a combination of GSK650394A and rapamycin; therefore SGK1 activity may contribute to the relative rapamycin-resistance of MDA-MB-231 cells.

To measure SGK1 kinase activity, NDRG1 was assessed for phosphorylation of its known SGK1-dependent residues (Thr346/Thr356/Thr366). In both MCF-7 (Fig. 4C) and MDA-

MB-231 cells (Fig. 4D), NDRG1 phosphorylation was significantly inhibited with GSK650394A (1 μ M) treatment alone (Fig. 4C and 4D). While 0.1 μ M GSK650394A reduced NDRG1 phosphorylation in MDA-MB-231 cells (but not in MCF-7 cells) it was not until a concentration of 1 μ M SGK1 inhibitor was used that an effect on cell growth and a significant reduction in NDRG1 phosphorylation was observed (Figures 4B and 4D). Following rapamycin treatment, NDRG1 phosphorylation was inhibited in MCF-7 cells but slightly enhanced in MDA-MB-231 cells (Fig 4C and 4D). This is consistent with a requirement for mTORC1 activity for SGK1 activation in MCF-7 but not in MDA-MB-231 cells. Rapamycin led to increased Akt-Ser473 phosphorylation in both cell lines, presumably as a result of inhibition of the negative feedback of mTORC1 activity on the mTORC2 pathway [28]. Concomitant treatment with GSK650394A and rapamycin minimally affected P-NDRG1 in MCF-7 cells (Fig. 4C). In contrast, MDA-MB-231 cells treated with rapamycin and 1 μ M GSK650394A resulted in a significant reduction in NDRG1 phosphorylation (Fig 4D) and corresponded to a significant decrease in MDA-MB-231 total cellular protein (Fig. 4B). In addition, the use of a lower concentration of rapamycin (10nM) combined with GSK650394A also sensitized MDA-MB-231 cells to rapamycin-mediated growth inhibition (Supplementary Fig. S6).

To further examine the role of SGK1 kinase activity on the sensitivity of breast cancer cells to rapamycin-mediated growth inhibition, MCF-7 and MDA-MB-231 cell lines stably expressing either a control shRNA or *SGK1* shRNA (Fig. 4E and 4F) were examined for total cellular protein following rapamycin treatment. Western blot analysis of the SGK1 shRNA-expressing cells (Fig. 4E and 4F) showed effective knockdown of SGK1 and was accompanied by a loss of NDRG1 phosphorylation in both cell lines. Figure 4E also shows that SGK1 depletion in MCF-7 cells had no significant effect on rapamycin-mediated growth inhibition following treatment with either 10nM or 100nM rapamycin, while MDA-MB-231 cells expressing *SGK1* shRNA (Fig. 4F) exhibited significantly less total cellular protein following rapamycin treatment compared to cells expressing a control shRNA ($P < 0.001$). In both cell lines, *SGK1* shRNA-expressing cells had equivalent total cellular protein to control shRNA-expressing cells following vehicle treatment. In summary, either SGK1 inhibition or SGK1 depletion enhanced rapamycin's ability to block cell growth in MDA-MB-231 but not MCF-7 cells, consistent with the notion that SGK1 was effectively inhibited by rapamycin treatment alone in MCF-7 cells.

Estrogen receptor alpha expression is required for rapamycin-mediated inhibition of SGK1-Ser422 phosphorylation

In view of the disparate effect of rapamycin in ER α + versus ER α - cells, we assessed whether the sensitivity of SGK1-Ser422 phosphorylation to rapamycin was affected by varying ER α expression. We measured P-SGK1-Ser422 of ectopically expressed HA-SGK1 in an MCF-7 cell sub-line, C4-12, previously selected for low ER α expression using long-term estrogen deprivation [29]. We compared SGK1-Ser422 phosphorylation in isogenic C4-12 cells ectopically expressing FLAG-ER α [30]. Both cell lines were cultured in the absence of estrogenic factors and treated with insulin or a combination of insulin and estradiol (E2) $\pm$ rapamycin. As shown in Figure 5A, insulin-induced SGK1-Ser422 phosphorylation was detectable in both C4-12 and FLAG-ER α C4-12 cells. Interestingly, SGK1-Ser422 phosphorylation was insensitive to rapamycin in C4-12 cells but rapamycin-sensitive in FLAG-ER α C4-12 cells. While concomitant insulin and E2 treatment increased SGK1-Ser422 phosphorylation in the FLAG-ER α -C4-12 cells, E2 was not required to observe an inhibitory effect of rapamycin on SGK1-Ser422 phosphorylation. This implies that ER α expression *per se* (and not ligand-bound ER α) is associated with rapamycin-sensitive SGK1-Ser422 phosphorylation. Independently of ER α expression, p70S6K-Thr389 phosphorylation was rapamycin-sensitive and Akt-Ser473 phosphorylation was rapamycin-

insensitive, suggesting that the differential rapamycin sensitivity is unique to SGK1. Taken together, these data suggest that ER α expression is associated with a requirement for mTORC1 activity in SGK1-Ser422 phosphorylation (Fig. 5B).

Discussion

mTORC1 and mTORC2 are central mediators of cell proliferation and survival, yet rapamycin and its analogues have had limited clinical efficacy in breast cancer. Here we report that in ER α +, but not ER α -, breast cancer cell lines, SGK1-Ser422 phosphorylation requires mTORC1 activity. This is in contrast to p70S6K and Akt phosphorylation, which are consistently either rapamycin-sensitive or -insensitive, respectively. Recently, angiotensin II-induced SGK1-Ser422 phosphorylation was also found to be blocked by rapamycin in a fibrosarcoma cell line, γ 2A [31], further supporting a requirement for mTORC1 activity in SGK1 activation under certain physiological conditions.

Our conclusions concerning SGK1-Ser422 phosphorylation differ from a recent publication that examined MCF-7 cells under non-insulin stimulating conditions [10]. This study found that in MCF-7 cells maintained in 10% FCS, Ser422 phosphorylation of ectopically expressed GST-SGK1 was not significantly changed by rapamycin treatment. Our use of serum-deprivation and insulin stimulation, rather than continuous culture of cells in FCS [10], may contribute to a differential requirement for mTORC1 activity. In addition, the length of rapamycin treatment may reflect a differential requirement. To test this, we compared 0.5h, 1h, and 24h of rapamycin treatment of MCF-7 cells maintained in FCS and found that rapamycin did not completely inhibit SGK1-Ser422 phosphorylation at the indicated times (Supplementary Fig.S7). This suggests a potential partial requirement for mTORC1 in SGK1-Ser422 phosphorylation under conditions of 10% FCS.

Interestingly, we also found that SGK1 sensitivity to rapamycin treatment correlated with ER α expression. ER α can regulate target gene expression as well take part in rapid 'estrogen independent' signaling. Figure 5B shows a model suggesting that in the absence of ER α , SGK1 phosphorylation is downstream of mTORC2 (left panel). However, in ER α + cells (right panel), SGK1-Ser422 phosphorylation requires mTORC1 activity, possibly because ER α is involved in promoting the interaction and subsequent phosphorylation of SGK1 by mTORC1. In this case, we propose a direct requirement for mTORC1 activity, whereby ER α alters SGK1's conformation and/or localization to allow for SGK1's preferential interaction with mTORC1. It is also plausible, that in ER α + cells, mTORC2 (and not mTORC1) remains the immediate upstream kinase complex responsible for directly phosphorylating SGK1 at Ser422 and there is an essential, but indirect requirement for mTORC1 in ER α + cells. For example, mTORC1-dependent p70S6K phosphorylation of ER α [32] could be required for the interaction of mTORC2 with its substrate SGK1 in ER α + cells. This possibility will be tested in future work.

Understanding the molecular mechanisms contributing to rapamycin sensitivity (or resistance) is critical for the successful use of mTOR inhibitors in breast cancer treatment. To date, single agent rapamycin analogues have shown limited success in clinical trials of advanced breast cancer [33,34]. Recently, however, two clinical trials reported a benefit in combining the rapamycin analog, everolimus, with either an aromatase inhibitor, exemestane [35], or with a selective ER modulator, tamoxifen [36]. Based on our findings that SGK1-Ser422 phosphorylation and subsequent *in vivo* kinase activity (as measured by NDRG1 phosphorylation) is rapamycin-sensitive exclusively in ER α + cells, SGK1 may be a key mTORC1 target in ER α + breast cancers. Our data suggest that rapamycin's effectiveness in inhibiting both mTORC1 and SGK1 activity correlates with growth

inhibition of ER α + breast cancer cells. Furthermore, the addition of SGK1 kinase inhibitors may improve the efficacy of rapamycin analogues in the treatment of ER α - breast cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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List of abbreviations

The abbreviations used are:

Dex	dexamethasone
DMEM	Dulbecco's modified essential medium
ERα	estrogen receptor alpha
HRP	horseradish peroxidase
mTOR	mammalian target of rapamycin
shRNA	short hairpin RNA
SGK1	serum and glucocorticoid-regulated protein kinase 1
PI3K	phosphoinositide 3-kinase

Figure 1. mTORC1 activity contributes to insulin-induced SGK1 Ser422 phosphorylation in MCF-7 and T47D breast cancer cells

A, MCF-7 cells were serum-starved for 48h followed by treatment with dexamethasone (1 μ M) for 8h and insulin (300nM) for the last 1h. Rapamycin (100nM), PI-103 (1 μ M) or LY294002 (50 μ M) was added with insulin in lanes 3, 4 and 5, respectively. B, MCF-7 cells were transiently transfected with a plasmid encoding SGK1-FLAG, serum-starved for 24h and subsequently treated with insulin (300nM) for 1h alone or in combination with rapamycin (100nM) or Torin1 (1 μ M). SGK1-FLAG was immunoprecipitated using α -FLAG M2 magnetic beads. Cell lysates and immunoprecipitates were analyzed by Western blotting for the indicated proteins. Lane 6 in the total SGK1 panel and lanes 6-10 in the P-SGK1 Ser422 panel shows cross-reactivity with heavy chain IgG of the secondary anti-mouse antibody, however total phosphorylated species of SGK1 are detectable above the 50kDa IgG band. C, MCF-7 cells stably expressing a scrambled sequence shRNA (control shRNA), *RPTOR* shRNA, or *RICTOR* shRNA were transiently transfected with a plasmid encoding SGK1-FLAG, serum-starved for 24 h and subsequently treated with insulin (300nM) for 1h. D, T47D cells were transiently transfected with a plasmid encoding SGK1-FLAG, serum-starved for 24 h and subsequently treated with insulin (300nM) for 1h alone or in combination with rapamycin (100nM). SGK1-FLAG was immunoprecipitated using α -FLAG M2 magnetic beads. Cell lysates and immunoprecipitates were analyzed by Western blotting for the indicated proteins. N.S. indicates a non-specific band.

Figure 2. mTORC2 activity is required for insulin-induced SGK1 Ser422 phosphorylation in MDA-MB-231 and MCF10A-Myc cells

A, MDA-MB-231 cells were serum-starved for 48h followed by treatment with dexamethasone (1μM) for 8h and insulin (300nM) for the last 1h. Rapamycin (100nM), PI-103 (1μM) or LY294002 (50μM) was added with insulin in lanes 3, 4 and 5, respectively. B, MDA-MB-231 cells were transiently transfected with a plasmid encoding SGK1-FLAG, serum-starved for 24h and subsequently treated with insulin (300nM) for 1h alone or in combination with rapamycin (100nM) or Torin1 (250nM). SGK1-FLAG was immunoprecipitated with α-FLAG M2 magnetic beads. Cell lysates and immunoprecipitated proteins were analyzed by Western blotting as indicated. C, MDA-MB-231 cells stably expressing scrambled sequence shRNA (control shRNA), *RPTOR* shRNA or *RICTOR* shRNA were transfected with a plasmid encoding SGK1-FLAG. Following 24h, cells were then serum-starved for an additional 24h and subsequently treated with insulin (300nM) alone or in combination with Torin1 (250nM) for 1h. D, MCF10A-Myc cells were transiently transfected with a plasmid encoding SGK1-FLAG, serum-starved for 24h and subsequently treated with insulin (300nM) for 1h alone or in combination with either rapamycin (100nM) or Torin 1 (250nM). SGK1-FLAG was immunoprecipitated using α-FLAG M2 magnetic beads. Cell lysates and immunoprecipitated proteins were analyzed by Western blotting.

Figure 3. Thapsigargin treatment results in mTORC2-dependent SGK1 Ser422 phosphorylation in MDAMB- 231 cells and mTORC1-dependent phosphorylation in MCF-7 cells

A, MDA-MB-231 cells were treated with thapsigargin (1 μ M) or vehicle (DMSO) for 2h. For the last 1h, cells were treated with rapamycin (100nM), PI-103 (1 μ M), Torin1 (250nM), or vehicle (DMSO). B, MCF-7 cells were serum-starved for 48h, treated with dexamethasone for 8h $\pm$ thapsigargin (1 μ M) for the last 1h. Rapamycin (100nM) or Torin1 (250nM) was added for the last 1h, respectively (lanes 4 and 5). Cell lysates were analyzed by Western blotting for the indicated proteins and β -actin was used as a loading control. N.S. indicates a non-specific band.

Figure 4. SGK1 kinase activity contributes to the resistance of ER α - MDA-MB-231 cells to rapamycin treatment

A, MCF-7 or B, MDA-MB-231 cells were treated with vehicle, 0.1 μ M or 1 μ M of the SGK1 inhibitor GSK650394A in combination with vehicle (-rapamycin) or with rapamycin (+rapamycin, 100nM) for 4 days (inhibitors were replaced every 2 days) and total cellular protein was measured using an SRB assay (n=3). Error bars represent S.E.M., * P <0.05. C, MCF-7 or D, MDA-MB-231 cells were treated with vehicle or the SGK1 inhibitor GSK650394A (0.1 μ M or 1 μ M) $\pm$ rapamycin (100nM) for 24h. Cell lysates were analyzed by Western blotting for the indicated phosphorylated and total NDRG1 (as a measure of SGK1 kinase activity) and P-Akt Ser473/ total Akt. E, MCF-7 or F, MDA-MB-231 cells stably expressing *SGK1* shRNA or scrambled sequence (control) shRNA were treated with either 10nM or 100nM rapamycin or DMSO (vehicle) for four days. On day 4, total cellular protein was determined using an SRB assay (three independent experiments with three replicate wells per condition). Error bars represent S.E.M. of three independent experiments. Differences in total cellular protein were tested using paired two-tailed unequal variance Student's t -tests, ** P <0.001. Western blots of SGK1, P-NDRG1 (3xThr) and total NDRG1 from cell lysates expressing control shRNA or *SGK1* shRNA, $\pm$ dexamethasone (Dex) treatment.

Figure 5. Dependence of SGK1 Ser422 phosphorylation on mTORC1 activity requires ER α

A, C4-12 or C4-12-FLAG-ER α cells were transiently transfected with a plasmid encoding HA-SGK1, and subsequently serum and estradiol-deprived for 24h. Cells were then treated with insulin (300nM), estradiol (E2), or a combination for 1h, in the presence or absence of rapamycin (100nM). HA-SGK1 was immunoprecipitated using anti-HA ab-conjugated agarose beads. Immunoprecipitated proteins and input cell lysates were analyzed by Western blotting for the indicated proteins. Densitometric analysis of the P-SGK1 Ser422/total SGK1 ratio from two independent experiments is shown. Error bars represent S.E.M. of the densitometry experiments. B, A model illustrating mTORC2 exclusively mediates SGK1 Ser422 phosphorylation in the absence of ER α , while in ER α -expressing cells, SGK1 Ser422 phosphorylation requires mTORC1 activity.