mTORC1 Signaling Can Regulate Growth Factor Activation of p44/42 Mitogen-activated Protein Kinases through Protein Phosphatase 2A*

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The mTORC1 complex (mammalian target of rapamycin (mTOR)-raptor) is modulated by mitogen-activated protein (p44/42 MAP) kinases (p44/42) through phosphorylation and inactivation of the tuberous sclerosis complex. However, a role for mTORC1 signaling in modulating activation of p44/42 has not been reported. We show that in two cancer cell lines regulation of the p44/42 MAPks is mTORC1-dependent. In Rh1 cells rapamycin inhibited insulin-like growth factor-I (IGF-I)-stimulated phosphorylation of Thr202 but not Tyr204 and suppressed activation of p44/42 kinase activity. Down-regulation of raptor, which inhibits mTORC1 signaling, had a similar effect to rapamycin in blocking IGF-I-stimulated Tyr204 phosphorylation. Rapamycin did not block maximal phosphorylation of Tyr204 but retarded the rate of dephosphorylation of Tyr204 following IGF-I stimulation. IGF-I stimulation of MEK1 phosphorylation (Ser217/221) was not inhibited by rapamycin. Higher concentrations of rapamycin (≥100 ng/ml) were required to inhibit epidermal growth factor (EGF)-induced phosphorylation of p44/42 (Thr202). Rapamycin-induced inhibition of p44/42 (Thr202) phosphorylation by IGF-I was reversed by low concentrations of okadaic acid, suggesting involvement of protein phosphatase 2A (PP2A). Both IGF-I and EGF caused dissociation of PP2A catalytic subunit (PP2Ac) from p42. Whereas low concentrations of rapamycin (1 ng/ml) inhibited dissociation of PP2Ac after IGF-I stimulation, it required higher concentrations (≥100 ng/ml) to block EGF-induced dissociation, consistent with the ability for rapamycin to attenuate growth factor-induced activation of p44/42. The effect of rapamycin on IGF-I or insulin activation of p44/42 was recapitulated by amino acid deprivation. Rapamycin effects altering the kinetics of p44/42 phosphorylation were completely abrogated in Rh1mTORrr cells that express a rapamycin-resistant mTOR, whereas the effects of amino acid deprivation were similar in Rh1 and Rh1mTORrr cells. These results indicate complex regulation of p44/42 by phosphorytases downstream of mTORC1. This suggests a model in which mTORC1 modulates the phosphorylation of Thr202 on p44/42 MAPks through direct or indirect regulation of PP2Ac.

Evidence increasingly implicates the Ser/Thr kinase mammalian target of rapamycin (mTOR)2 as a central controller of cell growth, proliferation, and survival. mTOR exists in two complexes that have different cellular functions in yeast and mammalian cells (reviewed in Ref. 1). The mTORC1 complex comprises mTOR, raptor, mLST8, PRAS40, and controls initiation of translation of ribosomal proteins and several proteins that regulate cell cycle. Activation of ribosomal S6K1 after mitogen stimulation is dependent on mTORC1 (2). Cap-dependent translation is facilitated by mTORC1’s phosphorylation and inactivation of 4E-BP1, the suppressor of eukaryotic initiation factor 4E (3, 4). The emerging picture places mTORC1 in a central role in which it senses mitogenic stimuli and amino acid (5) nutrient (6) conditions or AMP levels (7, 8) and coordinates many cellular processes related to growth and proliferation. Rapamycin, a macrocyclic lactone antibiotic, is a potent and highly selective inhibitor of mTORC1 (9).

The mTORC2 complex (mTOR, rictor, mSin1, and mLST8) is thought to control actin cytoskeleton organization and protein kinase C (reviewed in Refs. 9–12). The mTORC2 complex also phosphorylates Akt(Ser173), required for full activation (13) and negative regulation of FOXO1A (14). Activation of mTORC1 by insulin and insulin-like growth factors occurs through activation of phosphatidylinositol 3-kinase and Akt. Akt negatively regulates the tuberous sclerosis complex (TSC) composed of hamartin and tuberin that acts as a GTPase-activating protein to maintain Rheb in the GDP (inactive) form (15). Exactly how Rheb activates mTOR is unknown. The activity of TSC is regulated by hypoxia, increases in AMP that activate AMP-PK, a positive regulator of TSC activity, and by MAPks p44/42 that phosphorylate and inactivate TSC, thus promoting mTORC1 activation (16–20). Akt also phosphorylates and inactivates PRAS40, another negative regulator of mTOR (21).

To date, there is no clear evidence of the exact mechanism by which mTORC1 controls growth-related cellular processes. In vitro, mTORC1 phosphorylates at least two residues (Thr37 and Thr46) of 4E-BP1 (3, 22, 23). Direct phosphorylation of S6K1 has also been reported (24). However, an alternative mechanism has been proposed in which mTOR kinase activity

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The abbreviations used are: mTOR, mammalian target of rapamycin; mTOR, rapamycin-resistant mTOR; TSC, tuberous sclerosis complex; PK, protein kinase; MAPk, mitogen-activated protein kinase; PP2A, protein phosphatase 2A; PP2Ac, PP2A catalytic subunit; MEK, MAPK/ERK kinase; BSA, bovine serum albumin; shRNA, short hairpin RNA; IGF-I, insulin-like growth factor I; EGF, epidermal growth factor.
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represses the activity of a Ser/Thr phosphatase (protein phosphatase 2A; PP2A) physically associated with S6K1 (25). This proposal is based on the prevention of rapamycin-induced dephosphorylation of 4E-BP1 by calyculin A and its slight reversal of inhibition of S6K1 activity. Nutrient starvation or rapamycin treatment of Saccharomyces cerevisiae causes PP2A homologues Pph21p and Pph22p and another phosphatase, Sic4p, to dissociate from Tap42p, resulting in G1 arrest (26). However, Tap42 can both inhibit and activate protein phosphatases demonstrating the complex events underlying TOR regulation of transcription (27). In mammalian cells, α4, the homolog of Tap42p, has been shown to bind the catalytic subunit of PP2A, resulting in enhanced phosphatase activity and an altered substrate specificity (28). Some studies using mammalian cells have also shown rapamycin-induced dissociation of PP2A from α4 (28, 29). Although the relationship between G₁ arrest and rapamycin-induced release of PP2A from α4 is unknown, such a relationship could imply that mTORC1 exerts pleiotropic effects on many cellular processes involving PP2A (or other protein phosphatases). For example, Type-2A phosphatase activity has been implicated in regulation of transcription, translation, replication, cell growth, metabolism, and survival (30–32). An alternative interpretation is that α4 differentially regulates Type-2A protein phosphatases (33). Mitogens, such as insulin, rapidly inactivate PP2A (34), activate MAPKs, and also activate mTORC1, thus inducing both protein synthesis and transcriptional activation. mTORC1 signaling also appears to positively regulate PP5 activity (35).

Although previously it had been shown that in NIH3T3 cells rapamycin does not inhibit activation of MAPKs (2), there are anecdotal reports that rapamycin can influence activation of MAPK pathways (36). We were interested in the possible role of mTORC1 in coordinating both translational initiation and MAPK activation after stimulation by growth factors reported to be mitogenic in sarcoma cells (37–40).

MATERIALS AND METHODS

Reagents—Rapamycin was obtained from the NCI, National Institutes of Health drug repository (Frederick, MD). PD 98059 and okadaic acid were purchased from Calbiochem (Cambridge, MA). Each compound was dissolved in Me₂SO before incubation with rabbit polyclonal anti-p44/42 MAPK (Thr202/Tyr204), and pS6K, and pS6 followed by incubation with goat anti-rabbit or anti-mouse IgG conjugated to horseradish peroxidase. Immunoreactive bands were visualized by using Pierce SuperSignal™ chemiluminescence substrate (Pierce) on Kodak Biomax™ MR film (Eastman Kodak, Rochester, NY).

Quantitation of Immunoreactive Bands—Quantitation of band intensities was achieved following scanning of films using a CanoScan LiDE 35 scanner coupled to a Macintosh G5 computer using NIH Image Software. Signal Intensity (optical density) was calculated by converting gray scale values by comparison with a calibrated photographic gray scale card (Kodak).

Reactivity of Antibodies for p44/42 Phospho-peptides—To test the specificity of the anti-p44/42 MAPK (Thr202/Tyr204, Cell Signaling Technology), rabbit polyclonal anti-p42, or mouse monoclonal anti-p44/42 MAPK (Tyr204), antibodies, we...
prepared the following BSA-conjugated peptides with no sites phosphorylated, single phosphorylation (Tyr\textsuperscript{204}), or dual phosphorylation (Thr\textsuperscript{202}/Tyr\textsuperscript{204}): BSA-Gly-Gly-ADPHEHDHTG-FLTEYVATRWRYAPEIM, ADPHEHDHTGFLTEYVATRWYRAPEIM, and ADPHEHDHTGFLTEYVATRWRYAPEIM.

**Down-regulation of Raptor**—To detect whether down-regulation of raptor has the same effects as rapamycin, we used lentivirus encoding raptor shRNA PT972 (American Pharma Source, LLC, Gaithersburg, MD). Rh1 cells (0.3 × 10\textsuperscript{6}) were seeded in 35-mm dishes. On the second day, cells were infected with lentivirus (0.6 × 10\textsuperscript{6} viral particles) encoding mismatched control shRNA, raptor shRNA, or left untreated. After 24 h, cells were washed twice and placed in serum-free medium. After 48 h, cells were stimulated with IGF-I for 10 min and cell lysates were obtained. Human raptor shRNA (accession number: KIAA1303) were as follows: sense, 5′-GATCCAGGCTAGTCT-GTTCGAAAATTTCTCTCCTGCAAATTTTGAACTAGCCTTTTTG; antisense, 5′-AATTCCAAAA GGCTAG-TCTGTTTCGAAATTCTTCCTGTCAAAATTTCGAAACAGACTAGCCTGG; Control shRNA: sense, 5′-GATCCAGTCC-TAAGTTAAAGTGCACCCTTGTTATGGACAGGGCGACTT-AAAATCGAGGTTTTTG; antisense, 5′-AATTCCAAAA-ACCTCAGTTTAAAGTCGCCCTCGTGAACAGGCGGACTTACCTTAGACTG.

**RESULTS**

**Kinetics for p44/42 Phosphorylation Is Growth Factor-dependent**—Although it is well established that mTORC1 regulates cap-dependent translation and transcription of specific genes, whether it integrates these activities with growth factor-induced activation of the p44/42 pathway is unclear. To investigate if mTOR regulated growth factor activation of these MAPKs, we initially used Rh1 human Ewing sarcoma cells (43). In serum-free culture these cells are sensitive to rapamycin (44). In serum-free culture these cells are sensitive to rapamycin (44). A highly specific inhibitor of mTOR in the mTORC1 complex. Full activation of p44/42 (insulin-stimulated MAP2 kinase (ERK1) and MAPK 2 (ERK2)) requires phosphorylation of both Thr\textsuperscript{202} and Tyr\textsuperscript{204} (45) mediated by a single kinase, MEK1. As shown in Fig. 1A when serum-starved Rh1 cells were stimulated with growth factors, the p44/42 kinases were rapidly activated, as shown by assays with an antibody that recognizes simultaneously phosphorylated Thr\textsuperscript{202} and Tyr\textsuperscript{204}. Phosphorylation was maximal after 5 min of stimulation with IGF-I and after 15 min of stimulation with platelet-derived growth factor, after which phosphorylation declined. Maximal phosphorylation occurred after 5 min of stimulation with epidermal growth factor (EGF) and was maintained for at least 60 min. Phosphorylation of p44/42 in Rh1 Cells Is mTOR-dependent—We next determined whether phosphorylation of p44/42 was dependent on mTORC1 signaling (Fig. 1B). The cells were grown overnight in serum-free conditions, exposed to various concentrations of rapamycin for 2 h, then stimulated for 5 min with growth factors. Although each growth factor stimulated p44/42 phosphorylation in cells that had not been treated with rapamycin, exposure to 10 ng/ml rapamycin significantly decreased activation by platelet-derived growth factor and essentially abrogated activation by IGF-I. Activation by EGF was less sensitive to rapamycin inhibition but was markedly attenuated at higher concentrations (0.1–1 μg/ml).

To determine whether this inhibitory effect resulted specifically from rapamycin inhibition of mTORC1 signaling, we used Rh1 cells that express an mTOR mutant with an amino acid substitution (Ser\textsuperscript{2033}→Ile). This mutant has reduced binding affinity for the rapamycin-FKBP12 (FK506-binding protein) complex (3). The mutant mTOR is thus rapamycin-resistant (designated mTORrr), hence retains mTORC1 signaling in the presence of rapamycin that inhibits endogenous mTOR in the mTORC1 complex. Rh1 and Rh1mTORrr cells were grown in serum-free medium and stimulated with IGF-I for 5 min. Rapamycin suppressed phosphorylation of p44/42 (Thr\textsuperscript{202}/Tyr\textsuperscript{204}) in Rh1 cells but had no inhibitory effect in Rh1mTORrr cells (Fig. 1C). Thus, the effect of rapamycin on p44/42 phosphorylation appears to be mediated specifically through inhibition of mTORC1. IGF-I-induced phosphorylation of Akt (Thr\textsuperscript{208} and Ser\textsuperscript{473}), a protein kinase proximal to mTORC1 in the IGF-I signaling pathway (47), was similar in both lines; hence, the signaling from the IGF-I receptor to mTORC2, the putative Akt(Ser\textsuperscript{473}) kinase (48), in these clones appears to be intact. Thus, the initial mitogen induced phosphorylation of p44/42 appears to be dependent on mTORC1.

We next determined whether the effect of rapamycin was sustained. Serum-starved Rh1 and Rh1mTORrr cells were stimulated with IGF-I, and p44/42 phosphorylation was measured over 60 min. As shown in Fig. 1D, IGF-I caused a rapid, but transient increase in p44/42 phosphorylation that was maximal at 5 min, but decreased thereafter. Rapamycin inhibited phosphorylation of p44/42 at both 5 and 15 min during the peak period of stimulation, but at subsequent time points there was no difference between control and rapamycin-treated Rh1 cells. In contrast, rapamycin had no effect on IGF-I-induced phosphorylation of p44/42 in Rh1mTORrr cells. Thus, inhibition of mTOR by rapamycin abrogates the initial robust stimulation of phosphorylation of p44/42 by IGF-I. In contrast, rapamycin did not inhibit IGF-I-stimulated phosphorylation of Tyr\textsuperscript{204} but did retard the rate of dephosphorylation in Rh1 cells. Rapamycin had no effect in Rh1mTORrr cells (Fig. 1E), again supporting the contention that effects on p44/42 phosphorylation are mTORC1-dependent. Thus, signaling through mTORC1 has contrasting effects on the kinetics of phosphorylation and dephosphorylation of p44/42 at Thr\textsuperscript{202} and Tyr\textsuperscript{204}. To further test the role of mTORC1 signaling in regulating p44/42 phosphorylation, we used lentivirus delivery of shRNA to down-regulate raptor. Down-regulation of raptor was confirmed by Western blot analysis and inhibited IGF-I-stimulated phosphorylation of S6 (data not shown). As shown in Fig. 1F, down-regulation of raptor had an essentially identical effect to treatment with rapamycin, inhibiting IGF-I-stimulated dual phosphorylation of p44/42 at Thr\textsuperscript{202}/Tyr\textsuperscript{204}. However, down-regulation of raptor also inhibited IGF-I-stimulated phosphorylation of Tyr\textsuperscript{204}. In contrast, the control shRNA had no effect on IGF-I-stimulated phosphorylation of p44/42 at either residue. Rapamycin Inhibits IGF-I Stimulation of Phosphorylation of Thr\textsuperscript{202}, but Not Tyr\textsuperscript{204} Phosphorylation of p44/42—We decided to focus on how signaling through mTORC1 inhibited the ini-
tial IGF-I-induced phosphorylation of Thr^{202}. As discussed above, the mechanism by which mTORC1 regulates downstream targets remains controversial. mTORC1 demonstrates in vitro kinase activity, but it has been proposed that activation of S6K1 in vivo is through suppression of protein phosphatase 2A (25). Although there is no evidence to associate mTOR
physically with p44/42, a role for protein phosphatase 2A in regulating p44/42 activation has been proposed. It thus seemed possible that the effect of rapamycin on p44/42(Thr202) phosphorylation was a consequence of protein phosphatase activation that either directly affected p44/42 or affected an upstream kinase. As little as 15 min of exposure to rapamycin almost completely eliminated IGF-I-induced dual Thr202 and Tyr204 phosphorylation of p44/42. In contrast, rapamycin treatment did not inhibit Tyr204 phosphorylation after 5-min stimulation with IGF-I (Fig. 2, A and B).

Phosphorylation of Thr202 and Tyr204 is mediated by a single kinase, MEK1 (49). Because Tyr204 phosphorylation was stimulated by IGF-I in the presence of rapamycin it suggested that the effect of rapamycin is directed at p44/42 rather than against MEK1 or other upstream kinases. To test this, we examined the effect of rapamycin on IGF-I-induced phosphorylation of MEK1 (Ser217/221) in the activation loop of subdomain VIII. As shown in Fig. 2A, MEK1 was equally phosphorylated in the absence or presence of rapamycin. We also used the MEK1 inhibitor PD098059. As shown in Fig. 2C, IGF-I-stimulated phosphorylation of p44/42 was inhibited in a concentration-dependent manner by the MEK1 inhibitor. Importantly, inhibition was detected by both the antibody that recognizes dual Thr202/Tyr204 phosphorylation and the antibody that recognizes only Tyr204 phosphorylation. Thus, inhibition of MEK1 equally prevented Thr202 and Tyr204 phosphorylation (quantitated in Fig. 2C, right panels). In contrast, in rapamycin-treated cells IGF-I still stimulated phosphorylation of Tyr204, indicating that MEK1 activity is maintained. The result with rapamycin is consistent with a Ser/Thr phosphatase activity, which would maintain hypophosphorylation of Thr202, rather than with MAPK phosphatase activity that would dephosphorylate both phosphotyrosine and phosphothreonine residues of p44/42 (50).

**Rapamycin Inhibits Activation of p44/42**—Consistent with the finding that rapamycin treatment inhibited Thr202 phosphorylation induced by IGF-I stimulation, rapamycin blocked IGF-I stimulation of p44/42 kinase activity (Fig. 2D). Rh1 and Rh1mTORrr cells were serum-starved and stimulated with IGF-I with or without rapamycin treatment, and p44/42 activity was measured in immunoprecipitates using Elk-1 peptide as a substrate. IGF-I-mediated p44/42 kinase activity was inhibited by rapamycin in Rh1 cells but not in cells that expressed the rapamycin-resistant mTOR. Further, concentrations of rapamycin that inhibited Thr202 phosphorylation inhibited p44/42 activity.

**Specificity of the Anti-phospho-p44/42 Antibodies**—To ensure that the antibodies truly identified dual (pThr202/pTyr204) or single (pTyr204) phosphorylated sites, we tested their specificity against BSA-conjugated p44/42 phosphopeptides that incorporated these sites. As shown in Fig. 2E, the Tyr204-specific antibody recognized peptide phosphorylated at both sites (pThr202/pTyr204), or just at pTyr204. The “dual” phospho-antibody recognized only the peptide with both Thr202/Tyr204 phosphorylated. Neither antibody detected the unphosphorylated peptide conjugated to BSA-Gly-Gly. Thus, the antibodies accurately identify phosphorylation of p44/42 at these residues.

**Okadaic Acid Reverses Rapamycin Inhibition of IGF-I-stimulated Phosphorylation of p44/42**—In one model of growth factor-stimulated activation of ribosomal S6K1, mTOR kinase activity suppresses protein phosphatase 2A associated with S6K1 (25). To determine whether a similar mechanism could explain rapamycin inhibition of IGF-I activation of p44/42, we used okadaic acid. Okadaic acid at concentrations ~100 nm or less is a relatively specific inhibitor of PP2A (30, 51) but not PP1α (52, 53). Rh1 cells were serum-starved and incubated with okadaic acid for 2 h, then stimulated with IGF-I for 5 min. At higher concentrations (>100 nm), okadaic acid slightly increased IGF-I-stimulated phosphorylation of p44/42 (Fig. 3A). To determine whether okadaic acid could prevent rapamycin inhibition of IGF-I-induced p44/42 phosphorylation, we treated cells for 2 h with rapamycin (10 ng/ml) in the presence of increasing concentrations of okadaic acid (0–1000 nm). As shown in Fig. 3B, IGF-I-stimulated p44/42 phosphorylation, and this effect was inhibited by rapamycin. At concentrations between 30 and 300 nm, okadaic acid reversed rapamycin inhibition of IGF-I-stimulated p44/42 phosphorylation. Thus, okadaic acid at concentrations where it would selectively inhibit PP2A (30–100 nm) reversed the rapamycin block of IGF-I-induced p44/42 phosphorylation. At these concentrations of okadaic acid p44/42 were not phosphorylated in the absence of growth factor stimulation (i.e. this is not a consequence of MEK1 activation by inhibiting PP2A under these conditions). Similar results were obtained with calyculin A, another PP2A inhibitor (data not shown). The induction of p44/42 phosphorylation in unstimulated cells by okadaic acid at high concentrations (>300 nm) suggests that the hypophosphorylation is...
dependent on a phosphatase activity (54). These results support the premise that IGF-I stimulation inactivates a protein phosphatase, probably PP2A, and that this inactivation is prevented by rapamycin, hence mTORC1-dependent. Rapid inactivation of PP2A by insulin, and the inhibition of this effect by rapamycin has been reported (34). Similarly, treatment of active preparations of p44/42 kinases \textit{in vitro} with the PP2A catalytic subunit (PP2Ac) causes dephosphorylation of phospho-threonine and inhibition of kinase activity (45). Our results are also consistent with the proposal that PP2A is involved in the rapid deactivation of the MAPK pathway (49, 55–57), and implicate mTORC1 as a regulator of this pathway in Rh1 cells. To test this
directly, we examined the ability of rapamycin to inhibit IGF-I-induced phosphorylation of p44/42 in four additional cancer cell lines. Rapamycin inhibited p44/42 phosphorylation in Rh30 cells, but not in Rh18, Rh36 and Rh41 rhabdomyosarcoma cell lines (Fig. 3C). Thus, the effect of rapamycin on inhibiting IGF-I-mediated p44/42 phosphorylation appears to be cell line specific.

Rapamycin Inhibits IGF-I-stimulated Dissociation of PP2Ac from p42—To see if the effect of rapamycin may be due to differences in association of PP2A with p44/42, we were serum-starved overnight, p42 was immunoprecipitated, and associated PP2Ac was determined by immunoblotting. As shown in Fig. 4A, levels of p42 were similar in each cell line. PP2Ac associated with p42 was readily detected in Rh1, Rh30, and Rh18 cells, with only trace amounts associated with p42 immunoprecipitated from either Rh36 or Rh41 cells. We next asked whether IGF-I-stimulated dissociation of PP2Ac from p42, and whether rapamycin could inhibit this. In serum-starved Rh1 cells PP2Ac associated with p42 but dissociated rapidly after stimulation of cells with IGF-I, Fig. 4B. Rapamycin retarded the dissociation of PP2Ac from p42 following IGF-I stimulation. PP2Ac re-associated with p42 by 60 min to a greater degree in rapamycin-treated cells, consistent with the transient effect of IGF-I on phosphorylation of p42. As in Rh1 cells, IGF-I induced a robust but transient phosphorylation of p44/42 in Rh30 cells (Fig. 4C), phosphorylation being maximal at 5 min. Consistent with maximal activation of p44/42, IGF-I stimulation decreased the PP2Ac that co-precipitated with p42. Rapamycin blocked this dissociation (Fig. 4D). These results support the idea that mTORC1 may regulate the activity of PP2Ac associated with p44/42 at least in some cell lines and thus present the first mechanistic evidence for cross-talk between mTORC1 and the MAPK pathway. These data provide, also, a partial explanation as to why in some cell lines (Rh36 and Rh41) rapamycin does not block IGF-I-induced p44/42 phosphorylation. Virtually no PP2Ac was associated with p44/42 in Rh36 and Rh41 cells, hence an inhibitory effect of rapamycin would not be anticipated. These results do not explain why rapamycin blocks the ability of IGF-I, but not EGF, to activate p44/42. However, activation of p44/42 by EGF was attenuated at higher concentrations of rapamycin (Fig. 1B). We therefore compared the effect of rapamycin over a wide range of concentration (1–1000 ng/ml) on association of PP2Ac with p42. Rapamycin at 1 ng/ml, inhibited IGF-I-stimulated dissociation of PP2Ac following IGF-I stimulation. In contrast, 100 ng/ml rapamycin was required to prevent EGF-induced dissociation of PP2Ac from p42 (Fig. 4E). Irrespective of the growth factor stimulation, rapamycin (10 ng/ml) completely suppressed phosphorylation of S6K1 (Thr249), confirming inhibition of mTORC1 signaling.

Amino Acid Depletion Mimics the Effect of Rapamycin and Inhibits p44/42 Phosphorylation (Thr202) Induced by IGF-I or Insulin—Inhibition of mTORC1 signaling by rapamycin recapitulates the effect of nutritional deprivation as depletion of amino acids also leads to decreased mTORC1 signaling. We were interested also in whether rapamycin influenced the activation of p44/42 by insulin compared with its effects on IGF-I-induced p44/42 activation. To determine whether amino acid deprivation altered the ability of growth factors to activate p44/42, serum-starved Rh1 cells were incubated for 2 h in medium where levels of leucine, lysine, methionine, and glutamine were reduced by 20–100% relative to regular growth medium. Cells were stimulated with either IGF-I or insulin (10 ng/ml), and phosphorylation of p44/42 was determined at 5 min. Rapamycin (10 ng/ml) blocked IGF-I (Fig. 5A), and insulin (Fig. 5B) induced activation of p44/42, and this effect was similar to that caused by amino acid deprivation. In both conditions Tyr204 was phosphorylated, but Thr202 remained hypophosphorylated. We next determined how quickly amino acid

**FIGURE 3. Okadaic acid reverses rapamycin inhibition of IGF-I-induced p44/42 phosphorylation.** A, effect of okadaic acid on IGF-I stimulation of p44/42 phosphorylation. Rh1 cells were serum-starved overnight, incubated for 2 h with or without okadaic acid (0–1 μM), and stimulated for 5 min with IGF-I. Left panel, Western blot of cell lysates after incubation with antibody that recognizes dual (Thr202 and Tyr204) phosphorylation. Right panel, duplicate blot after incubation with antibody to total p42. B, Rh1 cells were serum-starved overnight, incubated for 2 h with or without 100 ng/ml rapamycin in the presence of okadaic acid (0–1 μM), and stimulated for 5 min with IGF-I. Upper panel, Western blot of cell lysates incubated with antibody that recognizes dual (Thr202 and Tyr204) phosphorylation. Okadaic acid reversed the effect of rapamycin at concentrations of 30 nM and 100 nM but did not induce phosphorylation of p44/42 in unstimulated cells at these concentrations. Lower panel, duplicate blot after incubation with anti-total-p42 antibody. C, the ability of rapamycin to inhibit IGF-I-stimulated p44/42 phosphorylation was examined in Rh30, Rh18, Rh36, and Rh41 rhabdomyosarcoma cell lines. Serum-starved cells were incubated for 2 h with or without rapamycin (100 ng/ml) then stimulated for 5 min with IGF-I. Western blot of cell lysates incubated with antibody that recognizes dual (Thr202 and Tyr204) phosphorylation. Lower panel, duplicate blot after incubation with anti-total-p42 antibody.
deprivation affected the ability of IGF-I to activate p44/42. Cells were serum-starved as before, then exposed to medium without the four amino acids for 5–120 min. At each time point IGF-I was added, and phosphorylation of p44/42 determined after 5 min. A period as short as 5 min amino acid deprivation completely blocked the ability of IGF-I to activate p44/42 (Fig. 5C).

DISCUSSION

The extracellular signal-regulating protein kinases p44/42 (ERK1/2) are unique among the Ser/Thr protein kinases requiring both Thr and Tyr phosphorylation in the Thr–Glu–Tyr motif of the activation loop (58) for full activation. These kinases play a critical role in mediating growth factor stimulation of multiple cellular processes, including transcription, DNA replication, and protein translation. Regulation of protein translation is in part, regulated through p44/42-mediated phosphorylation and negative regulation of TSC leading to increased signaling by the mTORC1 (mTOR-raptor) complex. Activation of mTORC1 leads to phosphorylation and inactivation of 4E-BP1, the suppressor of eukaryotic initiation factor 4E, activation of S6K1, and increased formation of the eIF4F pre-initiation complex. Thus, cross-talk between the MAPK pathway and the mTOR pathway potentially integrates transcription and translation. However, the converse regulation, by which mTORC1 signaling feeds back to regulate p44/42 signaling, has not been explored extensively in many cell systems. In NIH3T3 cells, inhibition of mTOR by rapamycin did not alter activation of MAPKs or growth-related protein kinases following EGF, phorbol 12-myristate 13-acetate, or heat shock stimulation (2). In contrast, rapamycin partially suppressed serum stimulation of p44/42 in VMM18 melanoma cells (36). Previously, we and others have shown that IGF-I receptor-mediated signaling is implicated in the autocrine or paracrine growth of childhood Ewing sarcoma (59, 60) and rhabdomyosarcoma (38, 61).

Consequently, we were interested in determining whether inhibition of mTORC1 signaling impacted on the ability of IGF-I to activate the p44/42 kinases. IGF-I induced a robust but relatively transient increase in p44/42 phosphorylation, being maximum at 5 min in Rh1 (Ewing sarcoma) cells. Pretreatment with rapamycin (100 ng/ml) for 5 min blocked the ability of IGF-I to activate p44/42 (Fig. 5C).

FIGURE 4. Association of PP2A catalytic subunit (PP2Ac) with p42. A, cells (Rh1, Rh30, Rh18, Rh36, and Rh41) were serum-starved overnight, and p42 was immunoprecipitated from lysates. Upper panel, Western blot after incubation with antibody to PP2Ac. Lower panel, duplicate blot after incubation with antibody to total p42. B, time course for PP2Ac association with p42 in IGF-I-stimulated Rh1 cells. Rh1 cells were serum-starved overnight, incubated for 2 h with or without 100 ng/ml rapamycin, and stimulated for up to 60 min with IGF-I. p42 was immunoprecipitated as described above, and associated PP2Ac was determined by Western blot (upper panel). Total p42 is shown in the lower panel. Densitometric quantification of PP2Ac immunoprecipitated with p42 in the presence or absence of rapamycin is shown for Rh1 cells in the lower graph. C, IGF-I stimulation of p44/42 phosphorylation in Rh30 cells. Cells were serum-starved overnight and stimulated with 10 ng/ml IGF-I for up to 60 min. IGF-I-stimulated p44/42 phosphorylation was determined by using an antibody that recognizes dual phosphorylation of Thr202 and Tyr204. Lower panel, duplicate blot after incubation with antibody to total p42. D, IGF-I stimulation induces mTOR-dependent dissociation of PP2Ac from p42. Rh30 cells were serum-starved overnight then incubated with or without rapamycin (100 ng/ml) for 2 h. Cells were stimulated with IGF-I (10 ng/ml) for 5 or 15 min. p42 was immunoprecipitated from cell lysates. Upper panel, Western blot after incubation with antibody to PP2Ac. Lower panel, duplicate blot after incubation with antibody to total p42. E, Rh1 cells were serum-starved overnight then incubated with or without rapamycin at different concentrations (0–1000 ng/ml) for 2 h. Cells were stimulated with IGF-I (10 ng/ml) or EGF (10 ng/ml) for 5 min. p42 was immunoprecipitated from cell lysates. Upper panel, Western blot after incubation with antibody to PP2Ac. IgG and anti-p38 were used as negative controls. Center panel, Western blot of cell lysates for Rh1 cells showing inhibition of p70S6 kinase phosphorylation (Thr389), demonstrating inhibition of mTORC1 signaling. Lower panel, duplicate blot after incubation with antibody to phospho-p44/42(Tyr106).

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ng/ml) completely abrogated phosphorylation of p44/42, as determined using an antibody that recognizes dual phosphorylation of Thr202 and Tyr204. In contrast, the effect of rapamycin on EGF-stimulated p44/42 was seen only at much higher concentrations of drug. The effect of rapamycin appears to be mediated through mTORC1, as in Rh1mTORrr, a clone expressing a rapamycin-resistant mTOR(Ser2035→Ile), the effect of rapamycin on inhibiting IGF-I stimulation of p44/42 phosphorylation was abrogated. Further, down-regulation of raptor, which inhibited mTORC1 signaling, had essentially identical effects as rapamycin in blocking the dual phosphorylation of p44/42, although it also blocked phosphorylation at Tyr204. This may be because down-regulation of raptor more effectively inhibits mTORC1 signaling than does rapamycin. Alternatively, inhibition of mTORC1, by rapamycin or down-regulation of raptor, appears to decrease the rate of de-phosphorylation, or increase the basal level of phosphorylation under serum-free conditions, respectively, of p44/42 at Tyr204 (Fig. 1E and lane 4 of Fig. 1F). Rapamycin exerted no apparent effect on the initial burst of p44/42 phosphorylation, if an antibody recognizing only phospho-Tyr204 was used. Rather, rapamycin treatment retarded the loss of pTyr204 signal, maintaining phosphorylation of this residue. Rapamycin had no effect on the kinetics of Tyr204 phosphorylation in Rh1mTORrr cells, demonstrating that the kinetics of phosphorylation of Tyr204 is also mTORC1-dependent. Amino acid deprivation recapitulated the effect of rapamycin, but had equal effects in both Rh1 and Rh1mTORrr cell lines (data not shown). Thus, the role of mTORC1 on phosphorylation of p44/42 appears complex. mTORC1 appears to negatively regulate the phosphatase involved in maintaining hypophosphorylation of Thr202, but positively regulates the phosphatase that dephosphorylates Tyr204 following initial activation by IGF-I stimulation. Because the primary event controlled by mTORC1 appeared to be regulation of IGF-I-induced phosphorylation of Thr202, we chose to investigate this phenomenon further.

Rapamycin did not inhibit IGF-I-induced phosphorylation of MEK1 (Ser217/221), thus does not act upstream of p44/42. To test the specificity of the antibodies used, reactivity against p44/42 peptides that were unphosphorylated, or phosphorylated at a single site (equivalent to Tyr204) or dual phosphorylation (Thr202/Tyr204) was examined. Antibody against pTyr204 recognized both peptides containing the phosphorylated residue, whereas the “dual specific” antibody recognized only peptide phosphorylated at the equivalent sites of pThr202/pTyr204 of the p44/42 peptides. Neither phospho-specific antibody reacted with unphosphorylated peptide (indicating also that there was also no reactivity with conjugated BSA). Thus, we consider that the antibodies used accurately report the phosphorylation status at these residues in p44/42. Consistent with hypophosphorylation of Thr202, the kinase activity of p44/42 activity was inhibited in Rh1 cells as rapamycin decreased the activity of immunoprecipitated p42 in in vitro kinase assays using Elk-1 peptide as substrate. Rapamycin had no effect in Rh1mTORrr cells, again pointing to mTORC1 as the target of rapamycin activity. In contrast to the effect of rapamycin, the MEK1 inhibitor, PD98059 reduced phosphorylation of p44/42 to a similar extent as determined by either antibody recognizing Tyr204 or the antibody recognizing dual phosphorylation. Because p44/42...
are phosphorylated on both sites by MEK1, the effect of rapamycin seemed consistent with maintenance of Thr202 in a hypophosphorylated state by a protein phosphatase. Protein phosphatases may positively regulate the MAPK pathway, as for Raf-1 (53, 62) or negatively regulate activity, as in the case of p44/42 (49, 57). In yeast TOR regulates the activity of several protein phosphatases (26, 27). Further support for the possible involvement of a protein phosphatase was the reversal of the rapamycin effect on blocking IGF-I-stimulated p44/42 phosphorylation by low concentrations of okadaic acid. Similar results were obtained using calyculin A. The sensitivity to reversal by okadaic acid suggested involvement of a Type-2 protein phosphatase, probably PP2A. To determine if inhibition of p44/42 activation by rapamycin was a more general phenomenon, we examined four additional cell lines derived from childhood rhabdomyosarcoma. Previously, we have shown that the proliferation of Rh30 cells is IGF-IR-dependent, and proliferation is inhibited by an antibody directed against the IGF-1 receptor (44). Rapamycin inhibited IGF-I-stimulated phosphorylation of p44/42 in Rh30 cells, but not in three other cell lines (Rh18, Rh36, and Rh41). Thus, the effect of rapamycin in modulating p44/42 activation appears to be cell context-specific. Immunoprecipitation studies indicated that PP2Ac was associated with p42 in each cell line, but with only trace amounts co-precipitating with p42 in Rh36 and Rh41 cell lysates, suggesting that the level of PP2Ac associated with p42 may relate to the ability of rapamycin to block p44/42 activation by growth factors. However, this does not explain why rapamycin did not inhibit p44/42 activation in Rh18 cells. We focused on the two lines, Rh1 and Rh30, where rapamycin did inhibit p44/42 activation by IGF-I. In Rh1 cells, stimulation with IGF-I caused dissociation of PP2Ac from p42 that was partially inhibited by rapamycin. Similar results were obtained in Rh30 cells, suggesting that mTORC1 signaling functions to regulate association of PP2Ac with p42. Interestingly, although low concentrations of rapamycin blocked IGF-I-induced dissociation of PP2Ac from p42, it required far higher concentrations of drug to block EGF-induced dissociation. Why rapamycin selectively inhibits the effect of IGF-I compared with EGF requires further investigation. Finally, we examined whether amino acid deprivation, which inhibits mTORC1 signaling, had similar effect to rapamycin on IGF-I or insulin stimulation of p44/42 phosphorylation. As before, rapamycin ablated p44/42 phosphorylation induced by either IGF-I or insulin. Depletion of four amino acids (Leu, Lys, Met, and Glu) abrogated activation of p44/42 induced by these growth factors. The effect of amino acid deprivation appears to be rapid, because within 5-min exposure to medium completely devoid of the four amino acids, the ability of IGF-I to induce phosphorylation of p44/42 was inhibited. Thus, in Rh1 cells amino acid deprivation mimics the effect of rapamycin. These data suggest that, in some malignant cell lines, signaling through the mTORC1 complex impacts on IGF-I activation of p44/42 MAPK. The exact mechanism by which mTORC1 regulates the association of PP2Ac with p42 is unknown, but the subject of ongoing experimentation. The biological consequences of the rapamycin-induced attenuation of p44/42 activation are also unclear. One possibility is that inhibition of mTORC1 signaling induces feedback to enhance protein translation. For example, inhibition of global translation by inhibitors such as cycloheximide or emetine activates mTORC1 signaling (63–65). Conversely, overexpression of eukaryotic initiation factor 4E, the protein considered rate-limiting in the formation of elf4F pre-initiation translation complex, suppresses mTORC1 signaling (66). Thus, mTORC1 signaling appears tightly regulated by the process of translation. The p44/42 kinases phosphorylate MAPK signal-integrating kinases (Mnk1 and Mnk2) (42), and their phosphorylation diminishes cap-dependent translation (46). The best characterized substrate for Mnk1 and Mnk2 is eukaryotic initiation factor 4E (Ser209). Consequently, inhibition of p44/42 activation by rapamycin may lead to decreased phosphorylation of Mnk1 and the potential for increased cap-dependent translation. This possibility is being explored.

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