

Mammalian target of rapamycin regulates IRS-1 serine 307 phosphorylation

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Abstract

Insulin signaling can be negatively regulated by phosphorylation of serine 307 of the insulin receptor substrate (IRS)-1. Rapamycin, an inhibitor of the kinase mTOR, can prevent serine 307 phosphorylation and the development of insulin resistance. We further investigated the role of mTOR in regulating serine 307 phosphorylation, demonstrating that serine 307 phosphorylation in response to insulin, anisomycin, or tumor necrosis factor was quantitatively and temporally associated with activation of mTOR and could be inhibited by rapamycin. Amino acid stimulation activated mTOR and resulted in IRS-1 serine 307 phosphorylation without activating PKB or JNK. Okadaic acid, an inhibitor of the phosphatase PP2A, activated mTOR and stimulated the phosphorylation of serine 307 in a rapamycin-sensitive manner, indicating serine 307 phosphorylation requires mTOR activity but not PP2A, suggesting that mTOR itself may be responsible for phosphorylating serine 307. Finally, we demonstrated that serine 307 phosphorylated IRS-1 is detected primarily in the cytosolic fraction.

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Insulin is important for the control of many metabolic processes including carbohydrate, protein, and fat metabolism, as well as reproduction, and cellular growth and survival. The cellular effects of insulin are mediated by the activation of the insulin receptor's intrinsic tyrosine kinase activity. Subsequently, insulin receptor substrate (IRS) proteins are recruited to the insulin receptor, where they are also tyrosine phosphorylated. These tyrosine phosphorylation events result in the generation of binding sites for SH2 domain containing proteins such as the regulatory subunit of phosphatidylinositol-3 kinase (PI-3 kinase), Grb2, and SHP-2 ([1] and references therein). The insulin signal is then propagated primarily along PI-3 kinase and mitogen activated protein kinase (MAPK) pathways, leading to the regulation of target molecules ([1] and references therein).

Insulin receptor substrate proteins are docking proteins that are crucial to the insulin-mediated activation

of PI3 kinase [1]. In addition to tyrosine phosphorylation sites, IRS proteins contain many potential serine phosphorylation sites [2]. Serine phosphorylation of IRS-1 is capable of regulating insulin signal transduction both positively [3,4] and negatively [4–6]. Current research has focused upon serine phosphorylation events that are increased in insulin resistance, and thus may contribute to the diminished insulin signaling associated with diseases such as Type 2 diabetes and the metabolic syndrome.

Recently, phosphorylation of IRS-1 upon serine 307 has been shown to negatively regulate insulin signal transduction [7]. Phosphorylation of serine 307 inhibits insulin-stimulated tyrosine phosphorylation and IRS-1-associated PI-3 kinase activity, potentially through disruption of the protein–protein interaction between IRS-1 and the insulin receptor [8]. Furthermore, mutation of serine 307 can prevent the insulin-stimulated degradation of IRS-1 [9]. Serine 307 phosphorylation can be induced by factors such as cellular stressors [7,8,10], insulin [7,11], TNF- α [11], and lipids [12], all factors that cause insulin resistance. Numerous kinases

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have been suggested to contribute to phosphorylation of serine 307 [11,13,14]. For instance, insulin stimulates serine 307 phosphorylation by a PI-3 kinase dependent pathway, whereas TNF- α utilizes a PD98059-sensitive pathway that may not involve Erk 1/2 [11], as well as salicylic acid-sensitive pathway [13,14]. Finally, serine 307 phosphorylation can be stimulated by anisomycin, potentially by Jun N-terminal kinase (JNK) [7].

Several lines of evidence support a central role for mTOR in the development of insulin resistance and regulating the phosphorylation of serine 307. Rapamycin, an inhibitor of the kinase mammalian target of rapamycin (mTOR), has been shown to prevent the development of insulin resistance and the degradation of IRS-1, likely through the inhibition of serine phosphorylation of IRS-1 [15–17]. In addition, inhibition of mTOR with rapamycin can prevent serine 307 phosphorylation in response to insulin [18] or osmotic stress [10]. We expand upon these previous findings to demonstrate that serine 307 phosphorylation in response to multiple stimuli is related to mTOR activation and likely does not require activity of PP2A, a phosphatase regulated by mTOR. Furthermore, we demonstrate that serine 307 phosphorylated IRS-1 is localized primarily to the cytosolic fraction.

Materials and Methods

Cell culture. 3T3-L1 cells were grown and maintained as fibroblasts in Dulbecco's modified Eagle's medium (DMEM, Life Technologies, Gaithersburg, MD) with high glucose containing 10% fetal bovine serum (FBS, HyClone Logan, UT) in a humidified atmosphere composed of 95% air and 5% CO₂. Cells were differentiated into adipocytes by exposure to DMEM high glucose with 10% FBS, 0.4 μ g/ml dexamethasone, 0.5 mmol/L isobutylmethylxanthine, and 5 μ g/ml insulin. Following 3 days, the media were changed and the cells were maintained with DMEM containing 10% FBS. Day 10–12 adipocytes were starved for 2 h in DMEM/0.1% FBS prior to incubation in DMEM/0.1% FBS without or with insulin (100 nM), anisomycin (Boehringer-Mannheim), PDGF (Gibco), TNF- α (Gibco), or okadaic acid (Calbiochem) for the time specified. In some cases the cells were pretreated with rapamycin (Alexis Biochemicals) for 30 min prior to exposure to insulin or other reagents. At the appropriate time, media were aspirated from the cells and the cells were washed with ice-cold PBS. Subsequently, cells were lysed in lysis buffer [25 mM Tris-HCl (pH 7.4), 0.5 mM EGTA, 25 mM NaCl, 1% Nonidet P-40, 1 mM Na₃VO₄, 10 mM NaF, 0.2 mM leupeptin, 1 mM benzamidine, and 0.1 mM of 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride] (Calbiochem, La Jolla, CA) and rocked for 30 min at 4°C. Detergent insoluble material was sedimented by centrifugation at 12,000g for 10 min at 4°C. Protein concentration of the cell lysates was determined by bicinchoninic acid assay (Pierce Chemical, Rockford, IL).

Amino acid starvation and stimulation. The effects of amino acids upon mTOR signaling in 3T3-L1 adipocytes were investigated using a protocol modified from Pham et al. [19]. Briefly, 3T3-L1 adipocytes were starved of amino acids for 2 h in Krebs-Ringer Hepes (118 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO₄, 2.5 mM CaCl₂, 1.2 mM KH₂PO₄, 25 mM Hepes, pH 7.4, 1 mM EDTA, 2 mM glucose, and 200 nM adenosine). Following the 2 h starvation, the cells were rinsed and Krebs-Ringer Hepes media containing 0, 1 \times , 2 \times , or 4 \times the concen-

tration of amino acids (Gibco) normally found in DMEM (Gibco) were added to the cells. At the appropriate time the cells were rinsed in ice-cold PBS and lysed as described above.

Immunoprecipitation. IRS-1 was immunoprecipitated from 150 μ g of total protein. Lysates were diluted to 0.5 μ g/ μ l in lysis buffer. The lysates were pre-cleared with the addition of protein-A-agarose beads and rocked at 4°C for 30 min. Beads were sedimented by brief centrifugation at no more than 2500g at 4°C. Supernatants were transferred to new tubes and 4 μ g of rabbit anti-IRS-1 (Upstate Biotechnology) was added to each immunoprecipitation. Samples were rocked for 90 min at 4°C. Subsequently, beads were washed three times with lysis buffer and denatured by the addition SDS-PAGE sample buffer. Samples were then subjected to immunoblot analysis.

Immunoblot analysis. Twenty micrograms of protein was separated on SDS-PAGE gels (Bio-Rad, Hercules, CA) and transferred to nitrocellulose. Following transfer, proteins were stained with Ponceau S (Sigma) to confirm transfer and equal gel loading. Membranes were blocked in Tris-buffered saline 0.05% Tween 20 (TBS-T, Sigma) supplemented with 4% non-fat dried milk (Bio-Rad). Phospho-specific antibodies were used to assess phosphorylation status of threonine 389 p70 S6K (Cell Signaling Technologies; 1:1000), serine 473 PKB (Cell Signaling Technologies; 1:1000), dual phosphorylated threonine 183 and tyrosine 185 JNK (Promega; 1:5000), serine 63 c-Jun or phosphotyrosine (Upstate Biotechnology; 1:1000), and serine 307 of IRS-1 [11] (1:7500). Antibodies used to detect protein levels independent of phosphorylation status were IRS-1 (Upstate Biotechnology; 0.5 μ g/ml), JNK (Santa Cruz Biotechnology; 1:5000), p70 S6K (Santa Cruz Biotechnology; 1:5000), and PKB (Cell Signaling Technology; 1:1000). Horseradish peroxidase (HRP) conjugated goat anti-rabbit antibodies (Amersham) or HRP-coupled protein-A (Amersham) were used as secondary antibodies. Bound antibody was detected using enhanced chemiluminescence (ECL-plus, Amersham) and autoradiography (Hyperfilm, Amersham).

Subcellular fractionation. Isolation of cytosolic and intracellular membrane fractions of 3T3L1 adipocytes was performed as a modification of [20]. Briefly, 3T3L1 cells were cultured and differentiated as described above in 75 cm² tissue culture flasks. Cells were treated as described above. Cells were rinsed (140 mM NaCl, 20 mM Hepes, pH 7.4, 5 mM KCl, 2.5 mM MgSO₄, and 1 mM CaCl₂), lifted with a rubber policeman, and homogenized in homogenization buffer (225 mM sucrose, 4 mM Na₂EDTA, 20 mM Hepes, pH 7.4, 1 μ M leupeptin, 200 μ M AEBSF, and 1 μ M pepstatin) using a glass-Teflon homogenizer chilled to 4°C. The homogenate was transferred to centrifuge tubes and centrifuged at 19,000g for 20 min at 4°C. The resulting supernatant was subsequently centrifuged at 41,000g for 90 min at 4°C in an ultracentrifuge. The resulting pellet containing the high density microsomes (HDM) was resuspended in homogenization buffer, whereas the supernatant containing the cytosolic fraction, low density microsome, and plasma membrane were concentrated in Ultra-Free concentrators (Millipore). Subcellular fractions were subjected to IRS-1 immunoprecipitation and immunodetection for analysis of serine 307 phosphorylation.

Results

Insulin-, anisomycin-, and TNF- α -stimulated serine 307 phosphorylation is rapamycin-sensitive

Inhibition of mTOR with rapamycin is known to inhibit serine phosphorylation of IRS-1 in response to insulin [16], thus we tested whether insulin-induced serine 307 phosphorylation was rapamycin-sensitive. Insulin-stimulated serine 307 phosphorylation at 5 min, but more dramatically at 1 h (Fig. 1A). Phosphorylation

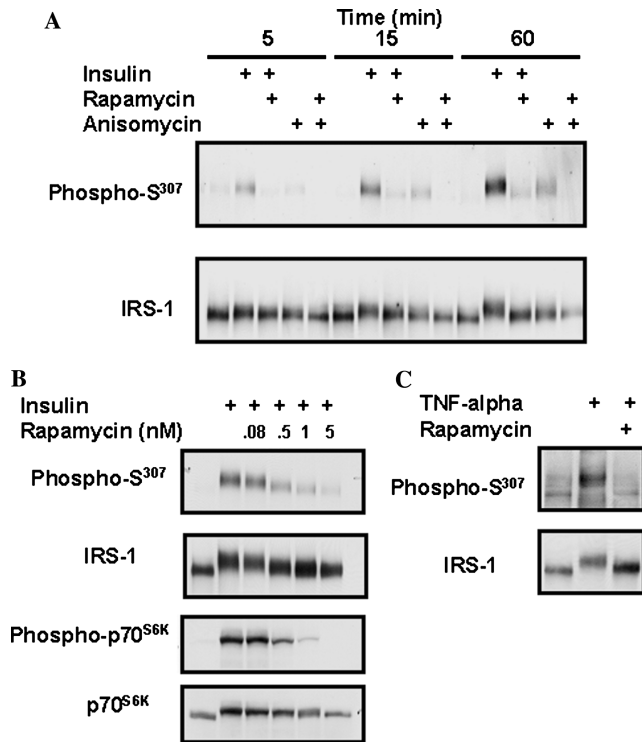


Fig. 1. Time course and rapamycin sensitivity of serine 307 phosphorylation. Data shown are representative blots from at least three independent observations. (A) 3T3-L1 adipocytes were serum-starved for 2 h prior to incubation with 200 nM rapamycin or vehicle alone (DMSO) for 30 min. Cells were then stimulated with insulin (100 nM) or anisomycin (5 μ g/ml) for the specified time. (B) 3T3-L1 adipocytes were serum-starved for 2 h prior to incubation with 0.08–5 nM rapamycin or vehicle alone (DMSO) for 30 min. Cells were then stimulated with insulin (100 nM) for 60 min. (C) 3T3-L1 adipocytes were treated as described in A, except cells were stimulated with 20 ng/ml TNF- α for 1 h. Cell lysates were prepared and IRS-1 immunoprecipitated prior to SDS-PAGE and Western blotting. Serine 307 phosphorylation was immunodetected with phospho-specific antibodies. Phosphorylation of p70 S6K was determined by immunodetection of whole cell lysates using anti-phospho-threonine 389 specific polyclonal antibodies.

of ser307 was accompanied by decreased electrophoretic mobility of IRS-1, consistent with increased serine phosphorylation. Pretreatment of the cells with rapamycin prevented the insulin-stimulated phosphorylation of ser307, as well as the shift in IRS-1 electrophoretic mobility. Rapamycin prevented both IRS-1 serine 307 phosphorylation and p70 S6K threonine 389 phosphorylation with an IC_{50} of approximately 0.5 nM (Fig. 1B). p70 S6K can be phosphorylated on threonine 389 [21] by mTOR in response to insulin and other factors, and was thus used as a marker of mTOR activation in this experiment. Activated recombinant p70 S6K was not able to phosphorylate serine 307 in vitro (data not shown), indicating that mTOR itself or another rapamycin-sensitive kinase other than p70 S6K was responsible for serine 307 phosphorylation.

Several other signaling pathways might mediate serine 307 phosphorylation, including an anisomycin–JNK

pathway and a PD98059-sensitive-TNF- α -mediated pathway [11]. To test whether these different pathways were also sensitive to rapamycin, we treated 3T3-L1 adipocytes with anisomycin and TNF- α without or with rapamycin (Figs. 1A and C). Anisomycin resulted in a delayed increase in serine 307 phosphorylation, similar to the effects of insulin. Surprisingly, anisomycin-stimulated phosphorylation of serine 307 was also rapamycin-sensitive. Furthermore, serine 307 phosphorylation in response to TNF- α was also rapamycin-sensitive (Fig. 1C). Taken together these experiments indicate that mTOR plays a central role in the phosphorylation of IRS-1 upon serine 307 in response to diverse stimuli, including insulin, anisomycin, and TNF- α .

Activation of the mTOR signaling pathway

To characterize the signaling pathways potentially used by insulin and anisomycin in the phosphorylation of IRS-1 upon serine 307, we assayed the phosphorylation status of members of the JNK and mTOR signaling pathways (Fig. 2). Insulin transiently increased JNK phosphorylation at 5 min and was returned to baseline by 60 min. Interestingly, insulin-stimulation resulted in the phosphorylation of specific JNK isoforms that migrated at approximately 44 kDa. In contrast, anisomycin stimulated phosphorylation of JNK isoforms migrating at approximately 46 and 54 kDa. JNK phosphorylation was detectable at 15 min and was further increased at 60 min. Pretreatment of the cells with rapamycin did not inhibit JNK phosphorylation in response to insulin or anisomycin.

As a marker of mTOR activity, we determined the phosphorylation status of p70 S6K. Phosphorylation of p70 S6K was increased by insulin, anisomycin (Fig. 2) in a rapamycin-sensitive manner, indicating that these pathways were capable of activating mTOR. Additionally, peak p70 S6K phosphorylation was observed to occur at the same time point as peak serine 307 phosphorylation in response to both insulin and anisomycin.

To determine if insulin and anisomycin share similar pathways upstream of mTOR, we determined the phosphorylation status of PKB. PKB is downstream of PI3 kinase and is capable of phosphorylating and activating mTOR [22,23]. PKB phosphorylation was detected only in response to insulin, whereas anisomycin did not increase PKB phosphorylation at the timepoints examined. Pretreatment of the cells with rapamycin did not prevent the insulin-induced increase in PKB phosphorylation, consistent with PKB acting upstream of mTOR. These data are consistent with previous reports that insulin may increase serine 307 phosphorylation through a PI3 kinase–PKB–mTOR pathway [11], however, the observation that anisomycin activated mTOR, but not PKB, suggests that the pathways used by insulin and anisomycin are divergent upstream of mTOR.

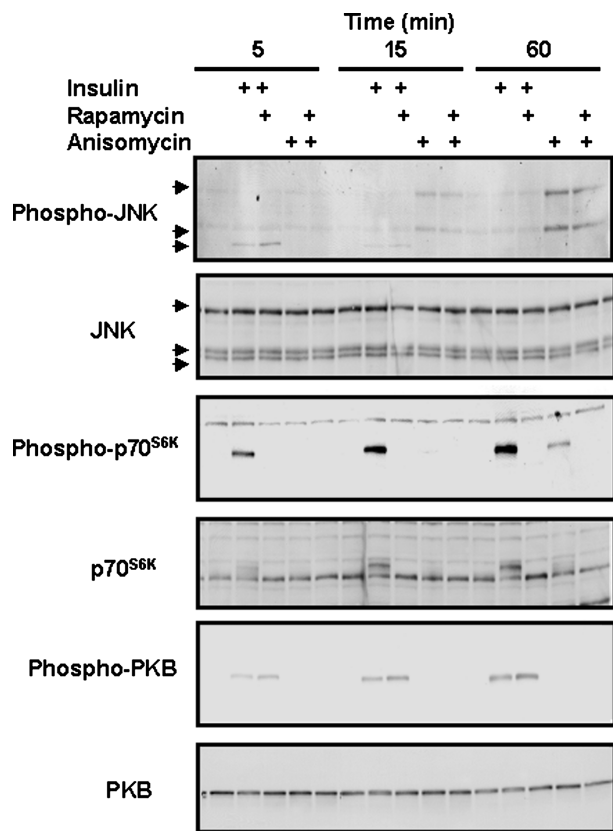


Fig. 2. Timecourse and rapamycin sensitivity of JNK, p70 S6K, and PKB phosphorylation. Data shown are representative blots from at least three independent observations. 3T3-L1 adipocytes were serum-starved for 2 h prior to incubation with 200 nM rapamycin or vehicle alone (DMSO) for 30 min. Cells were then stimulated with insulin (100 nM) or anisomycin (5 μ g/ml) for the specified time. Cell lysates were prepared and analyzed by immunodetection. Dual phosphorylated JNK was detected with anti-phospho-threonine 183/phosphotyrosine 185 polyclonal antibodies. Phosphorylation of p70 S6K was detected with anti-phospho-threonine 389 specific polyclonal antibodies. PKB phosphorylated upon serine 473 was detected with polyclonal phosphospecific antibodies.

Amino acids induce serine 307 phosphorylation and are necessary for optimal ser307 phosphorylation in response to insulin and anisomycin

Amino acids are capable of modulating the activity of mTOR [19]. Thus, we further tested the role of mTOR in phosphorylating serine 307 by incubating cells for 2 h in serum-free media without amino acids. Amino acids were then added back to levels equivalent to the normal amino acid content of DMEM (1 \times), or 2- and 4-fold the amount in DMEM (2 \times and 4 \times , respectively). Amino acids by themselves transiently increased serine 307 phosphorylation as well as p70 S6K phosphorylation (Fig. 3A). Amino acids did not increase phosphorylation of PKB or JNK (data not shown). Amino acid treatment did not have any effect upon IRS-1, p70 S6K, PKB, or JNK protein levels. When the cells were stim-

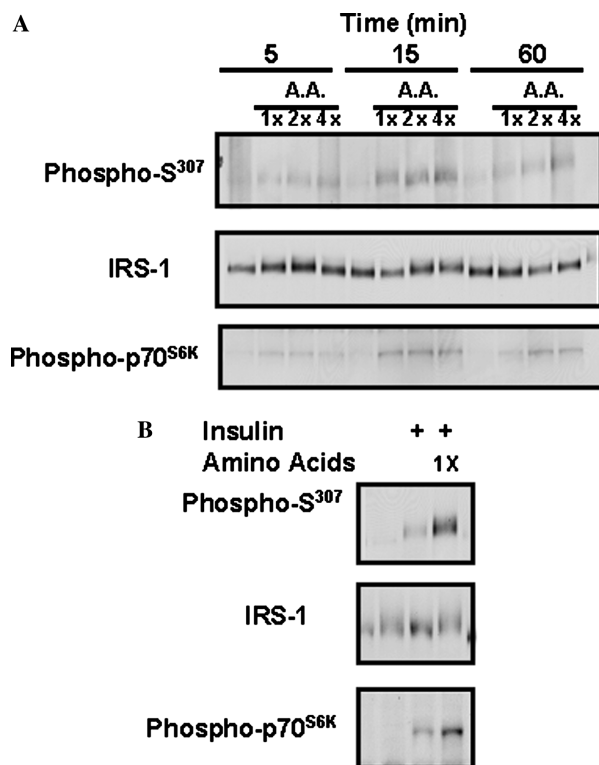


Fig. 3. Amino acids transiently activate mTOR and increase serine 307 phosphorylation. Data shown are representative blots from at least three independent observations (A) 3T3-L1 adipocytes were serum starved for 2 h in Krebs–Ringer buffer without amino acids. Subsequently, cells were stimulated with Krebs–Ringer buffer supplemented with 1 \times , 2 \times or 4 \times the amount of amino acids found in DMEM for the indicated time. (B) 3T3-L1 adipocytes were serum starved for 2 h in Krebs–Ringer buffer without or with amino acid concentrations similar to DMEM (1 \times). Subsequently, cells were stimulated with insulin (100 nM) for 60 min. Cell lysates were prepared and IRS-1 immunoprecipitated prior to SDS–PAGE and Western blotting. Serine 307 phosphorylation was immunodetected with phospho-specific antibodies. Phosphorylation of p70 S6K was determined by immunodetection of whole cell lysates using anti-phospho-threonine 389 specific polyclonal antibodies.

ulated with insulin, phosphorylation of serine 307 and p70 S6K was greater in the presence of 1 \times amino acids compared to insulin-stimulation without amino acids (Fig. 3B). The presence of amino acids was also important for optimal anisomycin-induced phosphorylation of p70 S6K and serine 307 (data not shown). These experiments further demonstrate a quantitative relationship between the degree of activation of mTOR (as assessed by p70 S6K phosphorylation) and phosphorylation of IRS-1 upon serine 307.

Okadaic acid-induced serine 307 phosphorylation is rapamycin-sensitive

Phosphorylation of serine 307 is likely the result of a balance between specific kinases and phosphatases. mTOR is capable of deactivating the phosphatase PP2A

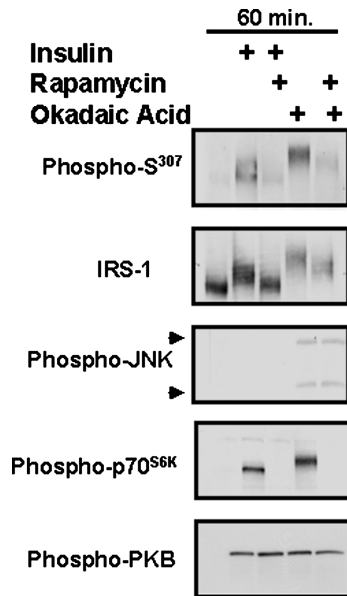


Fig. 4. Okadaic acid increases serine 307 phosphorylation in a rapamycin-sensitive manner. Data shown are representative blots from at least three independent observations. 3T3-L1 adipocytes were serum-starved for 2 h prior to incubation with 2.5 μ M okadaic acid for 60 min. Cell lysates were prepared and IRS-1 immunoprecipitated prior to SDS-PAGE and Western blotting. Serine 307 phosphorylation was immunodetected with phospho-specific antibodies. The remaining immunodetections were performed on whole cell lysates. Dual phosphorylated JNK was detected with anti-phospho-threonine 183/ phospho-tyrosine 185 polyclonal antibodies. Phosphorylation of p70 S6K was detected with anti-phospho-threonine 389 specific polyclonal antibodies. PKB phosphorylated upon serine 473 was detected with polyclonal phosphospecific antibodies.

[24], thus mTOR might increase serine 307 phosphorylation through the deactivation of PP2A. Similar to the effects of insulin, inhibition of PP2A with okadaic acid increased the phosphorylation of serine 307, as well as PKB and p70 S6K (Fig. 4). JNK phosphorylation was increased by exposure to okadaic acid for 1 h, whereas insulin did not activate JNK at this time point. Inhibition of mTOR by rapamycin prevented the phosphorylation of serine 307 and p70 S6K, but not PKB or JNK. Exposure to okadaic acid had no effect upon p70 S6K, JNK, or PKB protein abundance. These results are consistent with okadaic acid acting upstream of mTOR, potentially by activating PKB and thus stimulating mTOR activity. The data suggest that inhibition of PP2A is not directly responsible for regulating phosphorylation of serine 307, and that mTOR activity is necessary for serine 307 phosphorylation, independent of PP2A activity.

Subcellular localization of serine 307 phosphorylated IRS-1

Cellular insulin resistance is associated with an accumulation of IRS-1 in the cytosolic fraction [25]. In-

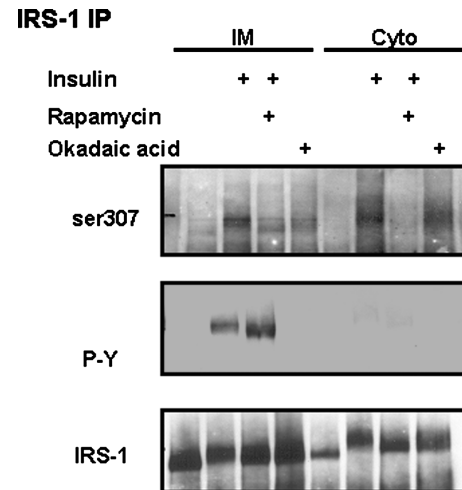


Fig. 5. Serine 307 phosphorylation is detected primarily in cytosolic IRS-1. Data shown are representative blots from at least three independent observations. 3T3-L1 adipocytes were serum-starved for 2 h prior to incubation with 100 nM insulin with or without rapamycin (200 nM) or 2.5 μ M okadaic acid for 60 min. Cell lysates were subjected to isolation of subcellular compartments including the high speed pellet (HSP) and cytosolic fractions. IRS-1 was immunoprecipitated prior to SDS-PAGE and Western blotting. Serine 307 phosphorylation or tyrosine phosphorylation was detected with phospho-specific antibodies.

sulin and okadaic acid are capable of inducing the movement of IRS-1 from high density microsomes (HDM) to the cytosolic fraction, an event that may be regulated by mTOR [26]. The rapamycin sensitivity of serine 307 suggested that phosphorylation of this residue may be associated with subcellular localization of IRS-1. Thus, we analyzed the phosphorylation state of IRS-1 in HDM and cytosolic fractions from 3T3L1 adipocytes treated with insulin or okadaic acid (Fig. 5). Insulin or okadaic acid stimulation for 1 h increased serine 307 phosphorylation which was detectable in the HDM, but was proportionately greater in the cytosolic fraction. Tyrosine phosphorylation of IRS-1 was induced only by insulin treatment and was restricted to the HDM fraction. Treatment with rapamycin inhibited serine 307 phosphorylation in both the HDM and cytosolic fractions and potentiated tyrosine phosphorylation of IRS-1.

Discussion

The results of the current experiments indicate that mTOR may be an important factor in the regulation of serine 307 phosphorylation. Serine 307 phosphorylation induced by insulin, anisomycin, or TNF- α was found to be quantitatively and temporally associated with mTOR activity (determined by p70 S6K phosphorylation). Because insulin and anisomycin can activate many signaling pathways, the observation that amino acids alone

were able to stimulate serine 307 phosphorylation further demonstrates that mTOR is involved in the phosphorylation of serine 307. Our observation that okadaic acid-induced serine 307 phosphorylation is rapamycin-sensitive indicates that mTOR activity but not PP2A activity is necessary for serine 307 phosphorylation. Taken together these data suggest that mTOR itself may be a serine 307 kinase. mTOR has a substrate specificity conforming to either (S/T)P as in PHAS-I, or h(S/T)h (where h = a hydrophobic residue) as in p70 S6K [27]. Serine 307 conforms to the (S/T)P motifs that can be recognized by both mTOR and MAP kinases ([28], for review). Thus, it is possible that mTOR is responsible for phosphorylation of serine 307.

Other kinases such as JNK and IKK are also potential kinases for serine 307 [7,29], although our data indicate that mTOR may be an important kinase in 3T3-L1 adipocytes. It is improbable that JNK is downstream of mTOR, as JNK phosphorylation was not temporally associated with mTOR activation, nor was it rapamycin-sensitive. Furthermore, the observation that amino acids were capable of activating mTOR and serine 307 phosphorylation without activating JNK indicates that JNK activation is not necessary for serine 307 phosphorylation. However, this does not rule out the possibility that mTOR may be downstream of JNK in some situations, although there are no published data describing a link between JNK and the regulation of mTOR. The observation that mutation of the JBD in IRS-1 prevents the phosphorylation of serine 307 in response to anisomycin [8] and that inhibition of JNK prevents insulin-stimulated serine 307 phosphorylation [30] raises the possibility that mTOR and JNK may interact in some way to achieve phosphorylation of serine 307. In addition to the possibility that JNK is upstream of mTOR, an alternative model might be that phosphorylation of serine 307 by JNK may require phosphorylation of other sites within IRS-1 by mTOR. In this situation, mTOR activity may modify IRS-1 in such a way to make it accessible to subsequent phosphorylations. In support of this, numerous serine sites within IRS-1 have been shown to be rapamycin-sensitive, and rapamycin can prevent the decreased electrophoretic mobility of IRS-1 in response to many stimuli [31,32]. Future work is necessary to determine the nature and existence of any potential relationship between JNK and mTOR.

Salicylic acid can improve insulin sensitivity in insulin-resistant animals and cells [13,14]. High doses of salicylic acid can inhibit the inhibitor of κ B kinase (IKK) and has been shown to inhibit serine 307 phosphorylation in response to TNF- α , consistent with IKK as a serine 307 kinase. Furthermore, IKK has been shown to phosphorylate serine 307 in vitro raising the possibility that IKK may be downstream of mTOR. However, treatment with salicylic acid can prevent the

activation of mTOR in response to TNF- α , suggesting that IKK may be upstream of mTOR [13].

In addition to p70 S6K, mTOR is capable of phosphorylating and inactivating the protein phosphatase 2A (PP2A) [24]. PP2A has been suggested to contribute to insulin resistance and IRS-1 protein degradation through the regulation of IRS-1 serine phosphorylation [32]. Because mTOR is capable of inhibiting PP2A activity, it was possible that mTOR may regulate serine 307 phosphorylation indirectly through regulation of PP2A. Our observation that okadaic acid-stimulated serine 307 phosphorylation was sensitive to rapamycin indicated that serine 307 phosphorylation requires mTOR activity, but not PP2A activity. PKB phosphorylation was increased by okadaic acid, and was not rapamycin-sensitive, consistent with PKB acting upstream of mTOR. Thus, inhibition of PP2A by okadaic acid activated PKB likely leading to activation of mTOR. This is consistent with the results of Resjo et al. [33] who have demonstrated that PKB is a primary target of PP2A.

Phosphorylation of serine 307 has been shown to inhibit insulin-stimulated tyrosine phosphorylation, potentially through disrupting the interaction between IRS-1 and the activated insulin receptor [8]. We observed that IRS-1 containing serine 307 phosphorylation is primarily localized to the cytosolic fraction and is devoid of tyrosine phosphorylation. Inhibition of mTOR prevented serine 307 phosphorylation and promoted tyrosine phosphorylation. These results are consistent with the role of serine 307 in regulating the association of IRS-1 with the insulin receptor.

The current experiment demonstrates that the mTOR pathway is a primary component of several signaling pathways responsible for the phosphorylation of IRS-1 upon serine 307. Considering that rapamycin prevents the onset of insulin resistance in 3T3-L1 adipocytes [17], the mTOR pathway may play a critical role in the development of insulin resistance, potentially through the phosphorylation of IRS-1 upon serine 307.

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