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Role of mTOR in the Degradation of IRS-1: Regulation of PP2A Activity

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Abstract We have investigated the role of PI 3-kinase and mTOR in the degradation of IRS-1 induced by insulin. Inhibition of mTOR with rapamycin resulted in approximately 50% inhibition of the insulin-induced degradation of IRS-1. In contrast, inhibition of PI-3 kinase, an upstream activator of mTOR, leads to a complete block of the insulin-induced degradation. Inhibition of either PI-3 kinase or mTOR prevented the mobility shift in IRS-1 in response to insulin, a shift that is caused by Ser/Thr phosphorylation. These results indicate that insulin stimulates PI 3-kinase-mediated degradation of IRS-1 via both mTOR-dependent and -independent pathways. Platelet-derived growth factor (PDGF) stimulation leads to a lower level of degradation, but significant phosphorylation of IRS-1. Both the degradation and phosphorylation of IRS-1 in response to PDGF are completely inhibited by rapamycin, suggesting that PDGF stimulates IRS-1 degradation principally via the mTOR-dependent pathway. Inhibition of the serine/threonine phosphatase PP2A with okadaic acid also induced the phosphorylation and degradation of IRS-1. IRS-1 phosphorylation and degradation in response to okadaic acid were not inhibited by rapamycin, suggesting that the action of mTOR in the degradation of IRS-1 results from inhibition of PP2A. Consistent with this, treatment of cells with rapamycin stimulated PP2A activity. While the role of mTOR in the phosphorylation of IRS-1 appears to proceed primarily through the regulation of PP2A, we also provide evidence that the regulation of p70S6 kinase phosphorylation requires the direct activity of mTOR. J. Cell. Biochem. 85: 304–314, 2002.

Key words: rapamycin; phosphatase; insulin; p70S6 kinase; PI-3 kinase

The primary substrates of the insulin receptor are a family of large molecular weight docking proteins, the insulin receptor substrates (IRS proteins) [White, 1997]. There are currently four members of the IRS family identified in mammalian cells with a high degree of sequence identity between each member. Each IRS protein has an amino-terminal pleckstrin homology (PH) domain with an adjacent phosphotyrosine-binding domain (PTB). The PTB binds an autophosphorylated site in the juxtamembrane region of the insulin receptor. The remainder of the protein contains numerous tyrosine phosphorylation sites that are involved in the recruitment of SH2 domain-containing proteins such as the p85 subunit of phosphatidylinositol-3 kinase (PI-3 kinase). The liver appears to rely on IRS-2 for insulin responsiveness more than IRS-1, while adipose and muscle tissues rely more on IRS-1 [Kerouz et al., 1997; Kido et al., 2000].

Insulin resistance is coincident with and sometimes precedes overt diabetes. It is a state, whereby, insulin-sensitive tissues have dramatically reduced, or no ability to respond to insulin. Mouse models have demonstrated that reductions in the level of individual IRS proteins alone can lead to insulin resistance and the development of diabetes [Kerouz et al., 1997; Kido et al., 2000].

Studies have suggested that insulin signaling leads to the degradation of IRS-1 by a proteasome dependent pathway [Haruta et al., 2000]. This insulin-induced degradation of IRS-1 can be prevented by the addition of inhibitors of PI-3 kinase [Egawa et al., 2000; Haruta et al., 2000].

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Conversely, it has been shown in 3T3-L1 adipocytes that stable expression of a constitutively activated PI-3 kinase catalytic subunit leads to a 50% reduction in IRS-1 protein levels, as well as the induction of insulin resistance [Egawa et al., 1999, 2000]. These data implicate PI-3 kinase in the onset of insulin resistance, likely as part of negative feedback signaling.

PI-3 kinase is an important mediator of insulin signaling and leads to the activation of the Ser/Thr kinase Akt/PKB. Akt is believed to be the critical protein involved in the stimulation of glucose transport by insulin [Kohn et al., 1996; Hill et al., 1999], and also promotes the initiation of protein translation in part by phosphorylating and activating another Ser/Thr kinase, the mammalian target of rapamycin (mTOR) [Scott et al., 1998]. Interestingly, the IRS-1 degradation induced by insulin can also be inhibited by rapamycin, a specific inhibitor of mTOR [Haruta et al., 2000]. Therefore, it appears that the signals resulting in the degradation of IRS-1 proceed from PI-3 kinase through Akt and mTOR, though it has not been determined what the exact contribution of any of these molecules is.

In mammalian cells, at least two proteins are regulated by the activity of mTOR. 4E-BP1 and p70S6 kinase are both involved in the initiation of protein translation and are regulated by Ser/Thr phosphorylation. Some data suggest that mTOR directly phosphorylates p70S6 kinase and 4E-BP1, while other data suggest that the mTOR-dependent phosphorylation of p70S6 kinase and 4E-BP1 is an indirect result of restraining the Ser/Thr phosphatase PP2A [Gingras et al., 2001]. TOR regulation of 4E-BP1 and p70S6 kinase has best been characterized in yeast. In yeast, TOR phosphorylates a docking protein, TAP42 [Jiang and Broach, 1999]. Phosphorylation of TAP42 provides a docking site for the catalytic subunits of several Ser/Thr phosphatases including the yeast homologs of PP2A. Treatment of yeast with rapamycin blocks the phosphorylation of TAP42 and induces the dissociation of TAP42 from the phosphatases [Di Como and Arndt, 1996]. Therefore, the model suggests that TOR is able to regulate protein translation indirectly by restraining Ser/Thr phosphatases. There appear to be some differences in mammalian mTOR signaling. The alpha4 protein is the mammalian homolog of yeast, TAP42p. While alpha4 has been shown to bind the catalytic subunit of PP2A [Murata et al., 1997; Chen et al., 1998], the rapamycin sensitivity of this association remains to be clearly demonstrated. It is unclear whether the mTOR regulation of p70S6 kinase in mammalian cells involves the phosphorylation of alpha4 or the catalytic subunit of PP2A [Murata et al., 1997; Peterson et al., 1999; Liu et al., 2001]. Phosphorylation of PP2A will likely inhibit its activity [Janssens and Goris, 2001].

The sensitivity of insulin-induced IRS-1 degradation to rapamycin may result from the direct inhibition of mTOR or the release of PP2A from mTOR regulation. We commenced these studies to better understand mTOR signaling, and the role of mTOR in IRS-1 degradation. We have used 3T3-L1 adipocytes, which resemble normal brown adipose tissue, for our studies. As mentioned above, IRS-1 protein levels appear to be more important in adipose tissue for determining the onset of insulin resistance than do other IRS family members. Our results demonstrate that mTOR is only part of the overall signal emanating from PI-3 kinase activation that leads to IRS-1 degradation in response to insulin. The data further suggest that the role of mTOR in the degradation of IRS-1 is by the way of restraining the activity of PP2A. In addition, we provide evidence that the kinase activity of mTOR is necessary for efficient phosphorylation of p70S6 kinase apart from the ability of mTOR to inhibit PP2A.

MATERIALS AND METHODS

Cells, Differentiation, and Inhibitors

Sixth passage 3T3-L1 cells were a generous gift from Dr. Silvia Corvera. Cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum (FCS), 50 U/ml penicillin, 50 μg/ml streptomycin, and grown in 10% CO_2. For fibroblast experiments, cells were plated into 60-mm tissue culture dishes, and allowed to just become confluent. The cells were then starved in 0.5% FCS for 16 h prior to stimulation with 100 ng/ml platelet-derived growth factor (PDGF)-BB (Sigma) for the indicated times. For adipocyte experiments, cells were plated into 75-cm^2 flasks, and again allowed to become confluent. At confluence, the growth medium was replaced by differentiation medium (DMEM containing 0.25 μM dexamethasone (Sigma), 0.5 mM isobutylmethylxanthine

(Sigma), 10% FCS, and 5 μg/ml porcine insulin (Sigma)). Cells were kept in differentiation medium for 48–72 h at 37°C in 10% CO_2. After this incubation, the differentiation medium was replaced by normal growth medium. On day 6 or 7 from the start of differentiation, the cells were trypsinized and seeded into 60-mm dishes. Cells were stimulated with 100 ng/ml porcine insulin or 200 nM okadaic acid (Sigma) after starving in 0.5% FCS for 16 h. In each case, inhibitors other than okadaic acid were added 15 min prior to stimulation. Okadaic acid was resuspended in DMSO, wortmannin in sterile water, and both were purchased from Sigma. LY294002 was purchased from Biomol and resuspended in ethanol. Rapamycin was resuspended in DMSO and also purchased from Biomol.

Antibodies

Rabbit antisera to IRS-1 and PP2A was purchased from Upstate Biotechnology Incorporated. Phosphorylation-specific rabbit antisera to Akt (Ser 473), p70S6 kinase (Thr 389), and p70S6 kinase (Thr 421/Ser 424) were purchased from Cell Signaling Technologies. The PY72 antisera specific for phosphorylated tyrosine residues was obtained from Dr. Bartholomew M. Sefton.

Lysis, Immunoprecipitation, and Immunoblotting

After stimulation, the cells were washed once in cold PBS on ice. The cells were then scraped into a standard lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% NP-40, 2 mM EDTA, 4 μM PMSF, 1 mM NaF, 2 mM sodium vanadate). These crude lysates were incubated at 4°C for 30 min before centrifugation in a microfuge at 14,000*g* for 15 min, also at 4°C. Clarified lysates were tested for protein concentration by a standard Bradford assay (Bio-Rad). Lysates were separated by 5% SDS–PAGE to accentuate the mobility shift induced in IRS-1 by stimulation. Polyacrylamide gels were then transferred to nitrocellulose. Blots were blocked for 30 min at room temperature or at 4°C overnight (0.5% BSA, 3% dried milk, 0.1% Tween-20, 50 mM NaCl). Blots were incubated in primary antibody (in blocking solution) overnight at 4°C. The primary antibody was detected using horseradish peroxidase (HRP)-conjugated goat anti-rabbit antisera (Bio-Rad), and enhanced chemiluminescence (NEN). Images were scanned, and the relative band intensities were analyzed by ImageQuant software (Molecular Dynamics). For immunoprecipitations, the clarified lysates were incubated with antibody for 30 min at 4°C with rotation. Protein A-sepharose (Amersham Pharmacia Biotech) was added and the lysates were incubated for an additional 60 min. The precipitates were then washed twice in wash buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% NP-40, and 0.1% SDS), once in PBS, and then boiled in SDS-sample buffer.

Phosphatase Assays

Cells were stimulated and lysed as above with the following exception. A modified lysis buffer (50 mM Tris-HCl, pH 7.0, 1% NP-40, 2 mM EDTA, and 4 μM PMSF) was used that lacks phosphatase inhibitors and more closely resembles the assay buffer. Lysates (100 μg) were immunoprecipitated with antisera to PP2A (4 μg). These precipitates were washed three times in lysis buffer and twice in assay buffer (50 mM Tris-HCl, pH 7.0, and 0.1 mM $CaCl_2$). The phosphatase activity was measured in each precipitate using the Ser/Thr phosphatase assay kit 1 from Upstate Biotechnology. This kit utilizes the malachite green dye to measure free phosphate. Briefly, the precipitates were resuspended in 40 μl of assay buffer, and kept on ice until assayed. The reactions were started by adding 10 μl of the substrate stock to give a 50 μl reaction volume containing 200 μM of phosphopeptide (K-R-pT-I-R-R). Reactions were incubated for 12 min in a room temperature water bath. The reactions were stopped by pelleting the precipitates and removing the supernatant to a tube containing the malachite green reagent. Free phosphate was then quantified by measuring the absorbance at 640 nm.

RESULTS

Sensitivity of Insulin-Induced IRS-1 Degradation to Kinase Inhibitors

Rapamycin pre-treatment was previously shown to inhibit the insulin-induced degradation of IRS-1 [Haruta et al., 2000]. Initial studies were performed to better characterize the role of rapamycin-sensitive signaling in this process. Insulin treatment, in the absence of any protein synthesis inhibitors, leads to the rapid loss of IRS-1 protein from differentiated 3T3-L1 adipocytes (Fig. 1A). Interestingly, while rapamycin treatment reduces the amount of IRS-1

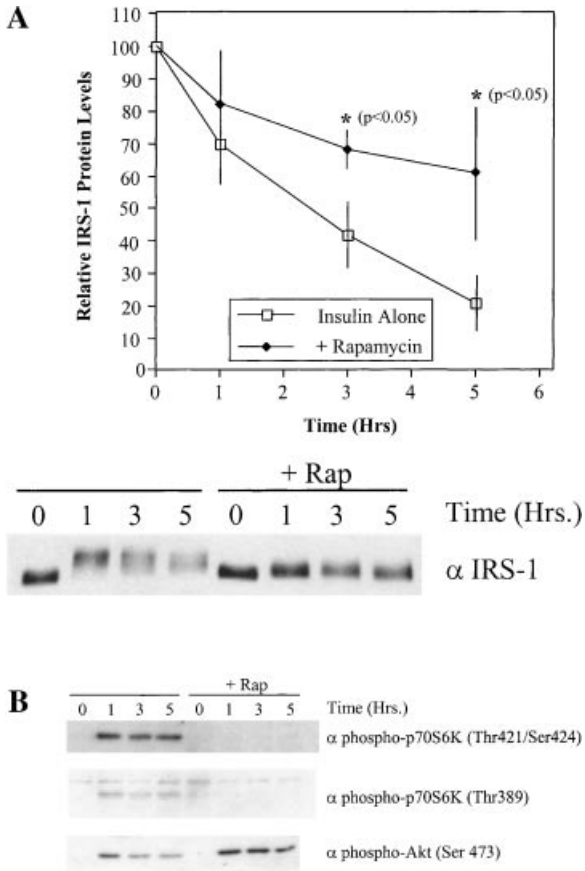

Fig. 1. Partial inhibition of insulin-induced IRS-1 degradation by rapamycin. 3T3-L1 adipocytes were starved in media containing 0.5% FCS for 16 h prior to stimulation with 100 ng/ml porcine insulin for the indicated times. Some cells were pre-treated with rapamycin (22 nM) for 15 min prior to insulin stimulation. Cells were then lysed and the lysates clarified by centrifugation. **A**: Samples (10 μg each) were separated on 5% SDS–PAGE and transferred to nitrocellulose. The blots were probed for the presence of IRS-1 and detected using chemiluminescence. The bands representing IRS-1 were scanned and analyzed by ImageQuant software. Given the abundance of IRS-1 and the high affinity of the IRS-1 antisera, we determined that 10 μg of lysate was sufficient to maintain immunoblots within the linear range of the chemiluminescence used for western detection. Data are presented as the percent of IRS-1 in the sample when compared to unstimulated cells (with or without rapamycin treatment). The blot is a single representative experiment, while the graph was obtained from four independent experiments. The asterisk (*) denotes where rapamycin treatment is significantly different from insulin alone ($P < 0.05$). **B**: Lysates (20 μg) from the experiment shown in (A) were immunoblotted for the presence of p70S6 kinase phosphorylated at Thr421/Ser424 or Thr389, and Akt phosphorylated at Ser473 using phosphorylation specific antisera.

degraded by insulin, it only inhibits the insulin-induced degradation by about 50%. The amounts of rapamycin used in these experiments are sufficient to completely inhibit known mTOR-dependent processes as evidenced by the complete inhibition of p70S6 kinase phosphorylation (Fig. 1B). The phosphorylation at Thr389 and Thr421/Ser424 in p70S6 kinase is induced by insulin and completely blocked by rapamycin treatment as assayed by blotting with phosphorylation specific antisera. Insulin treatment leads to a large mobility shift in IRS-1 that is caused by increases in serine and threonine phosphorylation (Fig. 1A) [Zick, 1989]. Rapamycin prevents most of this shift in IRS-1 mobility induced by insulin, suggesting that mTOR plays a critical role in regulating the Ser/Thr phosphorylation of IRS-1.

Typical signaling leading to the activation of mTOR proceeds from PI-3 kinase to Akt/PKB, which can activate mTOR [Scott et al., 1998]. Given that mTOR inhibition does not provide a complete block to IRS-1 degradation, we next determined the effect of PI-3 kinase inhibition on the insulin-induced degradation of IRS-1. As expected, PI-3 kinase inhibitors block the phosphorylation of both Akt/PKB (Ser473) and p70S6 kinase by insulin (Fig. 2B), whereas rapamycin treatment only inhibited the phosphorylation of p70S6 kinase (Fig. 1B). In addition, inhibition of PI-3 kinase blocks most of the mobility shift in IRS-1 induced by insulin stimulation similar to rapamycin treatment (Fig. 2A). Unlike inhibition of mTOR with rapamycin, PI-3 kinase inhibition consistently provides a complete block to the insulin-induced degradation of IRS-1 in 3T3-L1 adipocytes (Fig. 2A). Similar inhibition of IRS-1 degradation was observed whether PI-3 kinase was inhibited with wortmannin or LY294002. Therefore, PI-3 kinase generates signals leading to IRS-1 degradation, only part of which is mediated by mTOR.

IRS-1 Degradation is Induced by PDGF-BB

PDGF treatment of cells has been shown to lead to a mobility shift in IRS-1 on SDS–PAGE [Li et al., 1999]. This mobility shift, as with insulin treatment, is primarily due to increases in the Ser/Thr phosphorylation of IRS-1. We treated cells with PDGF-BB to see if increases in Ser/Thr phosphorylation would lead to the degradation of IRS-1. PDGF-BB failed to have any effect on IRS-1 mobility or degradation in fully differentiated 3T3-L1 adipocytes due to the downregulation of the receptor during differentiation [Shigematsu et al., 2001] (data not shown). Therefore, we stimulated undifferentiated 3T3-L1 fibroblasts with PDGF-BB and

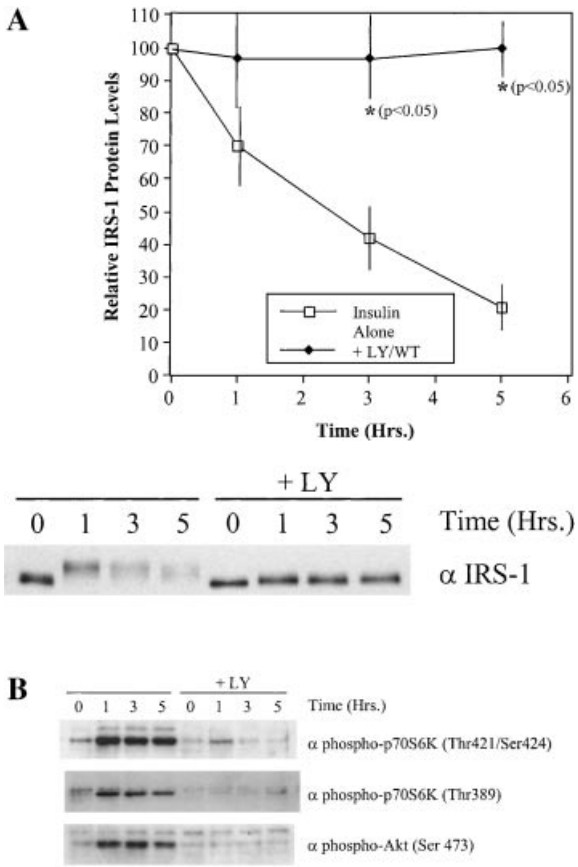

Fig. 2. Complete inhibition of insulin-induced IRS-1 degradation by inhibition of PI-3 kinase. 3T3-L1 adipocytes were starved for 16 h prior to stimulation. In some experiments, the cells were treated with 50 μM LY294002 for 15 min prior to insulin stimulation. Other experiments used 100 nM wortmannin in place of LY294002. Given the rapid degradation of wortmannin in culture, experiments using wortmannin had cells treated with additional wortmannin every 2 h. **A**: The graph represents data from six independent experiments, three with wortmannin and three with LY294002. No difference was observed in the effects of these different PI-3 kinase inhibitors. Immunoblots shown are from a single experiment performed with and without LY294002. IRS-1 immunoblots were scanned and the relative amount of IRS-1 protein quantified as in Figure 1. The asterisk (*) denotes where rapamycin treatment is significantly different from insulin alone ($P < 0.05$). **B**: Lysates (20 μg) from the experiment shown in (A) were immunoblotted for the presence of p70S6 kinase phosphorylated at Thr421/Ser424 or Thr389, and Akt phosphorylated at Ser473 using phosphorylation specific antisera.

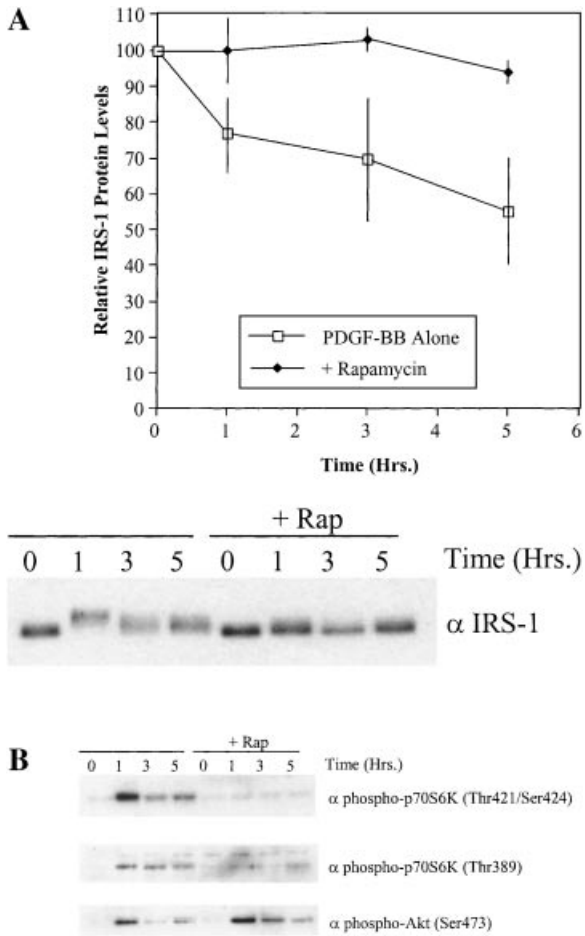

Fig. 3. Rapamycin completely inhibits PDGF-induced degradation of IRS-1. Confluent cultures of undifferentiated 3T3-L1 cells were starved for 16 h in media containing 0.5% FCS. Some samples were incubated with 22 nM rapamycin for 15 min prior to stimulation. Cells were stimulated with 100 ng/ml PDGF-BB for the indicated times prior to lysis. **A**: The graph represents data from four separate experiments, while the immunblot is derived from one representative experiment. **B**: Lysates (20 μg) from the experiment shown in (A) were immunoblotted for the presence of p70S6 kinase phosphorylated at Thr421/Ser424 or Thr389, and Akt phosphorylated at Ser473 using phosphorylation specific antisera.

observed a large mobility shift in IRS-1 (Fig. 3A), though only about 50% of the degradation induced by insulin in adipocytes. It is important to note that while the level of IRS-1 degradation induced by PDGF-BB stimulation is small, it is completely inhibited by pre-treatment with rapamycin. Similar to the insulin-induced mobility shift in IRS-1 being inhibited by rapamycin, the PDGF-induced mobility shift is also prevented by the inhibition of mTOR (Fig. 3A). Therefore, PDGF stimulation is able to activate mTOR-dependent signaling that promotes IRS-1 degradation (Fig. 3A), but does not lead to significant IRS-1 degradation via the mTOR-independent pathway that is stimulated by insulin (Figs. 1 and 2).

PDGF treatment of 3T3-L1 fibroblasts will lead to increases in the activities of PI-3 kinase, Akt/PKB, mTOR, and p70S6 kinase similar to insulin stimulation (Fig. 3B) [Staubs et al.,

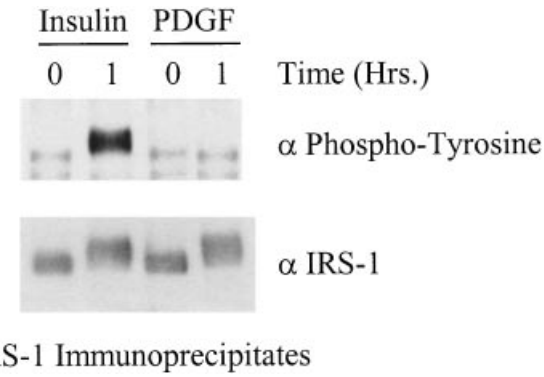

Fig. 4. PDGF stimulation fails to induce the tyrosine phosphorylation of IRS-1. Differentiated or undifferentiated 3T3-L1 cells were starved in 0.5% serum for 16 h. Fibroblasts were left unstimulated or stimulated for 1 h with 100 ng/ml PDGF-BB, while adipocytes were stimulated with 100 ng/ml insulin. Cells were lysed and the lysates were precipitated using antisera to IRS-1. The precipitates were then resolved on SDS–PAGE followed by immunoblotting for the presence of IRS-1 or phosphotyrosine.

1998]. However, PDGF will not lead to the tyrosine phosphorylation of IRS-1 (Fig. 4), and thus will not induce the direct association of PI-3 kinase or the clustering of other signaling molecules with IRS-1. It is, therefore, possible that the reduced amount of IRS-1 degradation induced by PDGF stimulation is due to the lack of colocalization of IRS-1 and PI-3 kinase in PDGF stimulated cells. Alternatively, this might be due to the differentiation state of the cells or to other differences between insulin and PDGF signaling.

Inhibition of PP2A is Sufficient to Induce IRS-1 Degradation

The preceding data demonstrate that mTOR can regulate the Ser/Thr phosphorylation and degradation of IRS-1. Given the connection between mTOR and PP2A in regulating the initiation of protein translation, we next tested whether the regulation of IRS-1 phosphorylation and degradation by mTOR also involved PP2A. Okadaic acid is a cell permeable inhibitor of type-2A Ser/Thr phosphatases, with some ability to also inhibit the Ser/Thr phosphatase PP1 at higher concentrations [Cohen, 1997]. We treated resting cells with okadaic acid to determine if inhibition of type-2A Ser/Thr phosphatases was sufficient for inducing the degradation of IRS-1. It was shown previously that okadaic acid treatment leads to large increases in the Ser/Thr phosphorylation of IRS-1 [Clark et al., 2000]. Similarly, we find that okadaic acid induces a large mobility shift in IRS-1, as well as a significant level of degradation (Fig. 5). This demonstrates that both Ser/Thr phosphorylation and the degradation of IRS-1 are negatively regulated by PP2A. The degradation of IRS-1 induced by phosphatase inhibition occurred in either differentiated or undifferentiated 3T3-L1 cells with similar kinetics indicating that there is no significant difference in the PP2A-regulated signaling pathway in these cell types.

If mTOR regulates IRS-1 Ser/Thr phosphorylation by controlling the activity of phosphatases, then okadaic acid-induced phosphorylation of IRS-1 should not be inhibited by rapamycin. Indeed, we find that rapamycin does not prevent either the mobility shift, or the degradation of IRS-1 induced by okadaic acid treatment (Fig. 6A). In contrast to the lack of effect that rapamycin has on okadaic acid-induced phosphorylation of IRS-1, at short time-points, PI-3 kinase inhibition is able to block the mobility shift induced by okadaic acid. It is possible that PI-3 kinase inhibition also blocks the degradation of IRS-1, but this is not

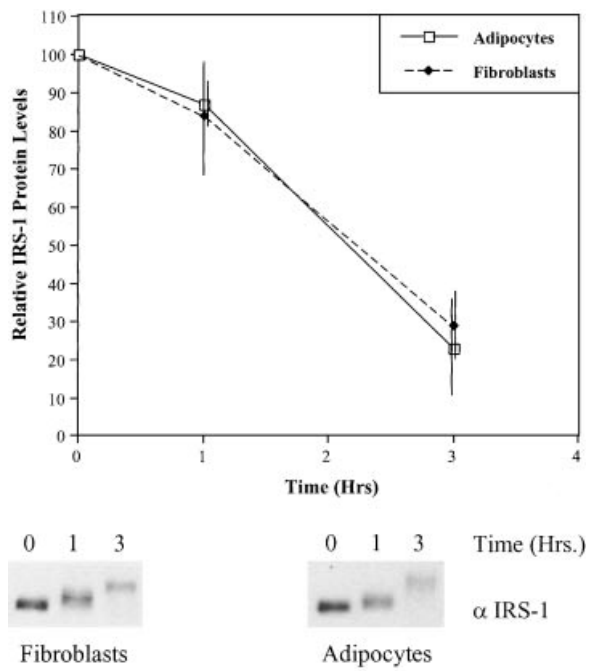

Fig. 5. Okadaic acid induces the degradation of IRS-1 in both adipocytes and fibroblasts. Starved 3T3-L1 cells, adipocytes and fibroblasts, were treated with 200 nM okadaic acid for the indicated times. Cells were lysed, and 10 μg of cell lysate was probed for the presence of IRS-1. The graph represents data from four experiments for each cell state.

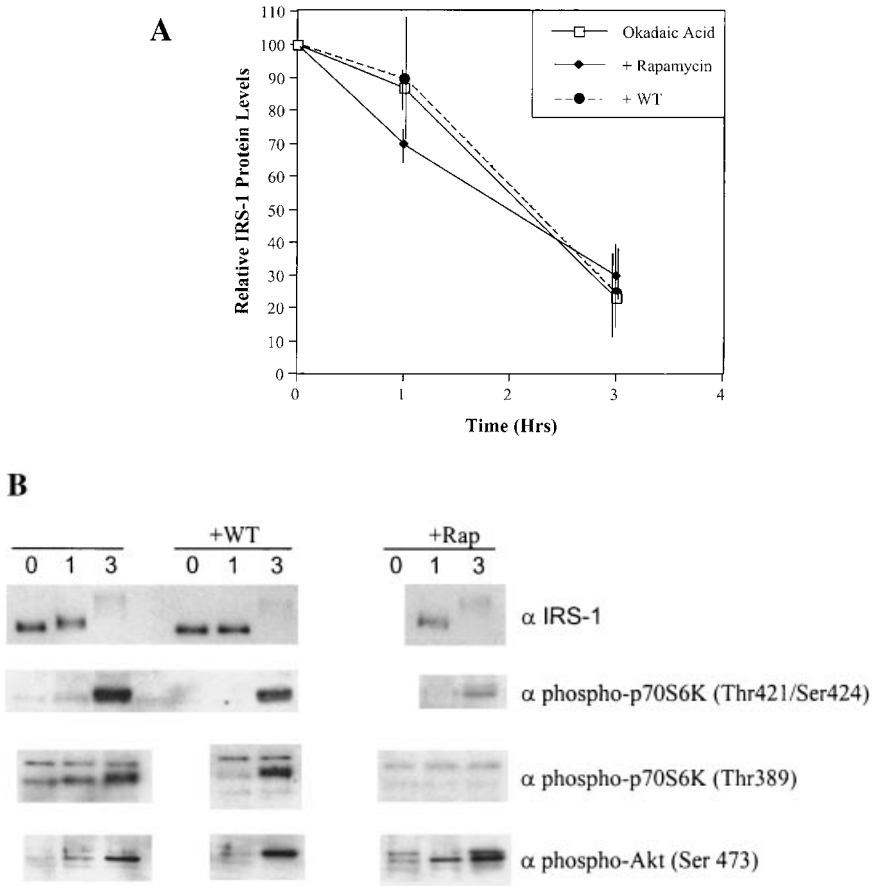

Fig. 6. Sensitivity of okadaic acid-induced phosphorylation and degradation to kinase inhibitors. 3T3-L1 adipocytes were left untreated or incubated with either 22 nM rapamycin or 100 nM wortmannin for 15 min prior to treatment with 200 nM okadaic acid for the indicated times. Clarified lysates (10 μg) were separated on 5% SDS–PAGE and immunoblotted for the presence of IRS-1. **A**: The graph is derived from three independent experiments. **B**: Lysates (20 μg) from a representative experiment were immunoblotted for the presence of p70S6 kinase phosphorylated at Thr421/Ser424 or Thr389, and Akt phosphorylated at Ser473 using phosphorylation specific antisera.

detectable due to the limited degradation following short incubations with okadaic acid.

The activity of many protein kinases is regulated by an equilibrium of activating phosphorylation and deactivating phosphatase action even under non-stimulated conditions [Millward et al., 1999]. Consistent with this, we find that after 3 h of okadaic acid treatment, Akt, p70S6 kinase, and IRS-1 all become phosphorylated even in the presence of PI-3 kinase inhibitors (Fig. 6B). Interestingly, the okadaic acid-induced phosphorylation of p70S6 kinase is significantly inhibited by rapamycin (Fig. 6B). In contrast, rapamycin did not alter the okadaic acid-induced phosphorylation of Akt or IRS-1. Therefore, the phosphorylation of p70S6 kinase requires the activity of mTOR, while the phosphorylation of IRS-1 is only regulated by mTOR in a manner involving the inhibition of PP2A.

mTOR Regulates PP2A Activity

The preceding data support the hypothesis that mTOR regulates IRS-1 phosphorylation by controlling the activity of PP2A. We, therefore, examined the effect of inhibiting mTOR on PP2A activity. Fully differentiated 3T3-L1 adipocytes were starved overnight in 0.5% serum and then treated for various times with rapamycin. Cells were then lysed, and PP2A was immunoprecipitated and assayed for activity using a synthetic phosphopeptide (K-R-pT-I-R-R) as a substrate. Phosphate released was linear with increasing amounts of immunoprecipitate added to the reactions (Fig. 7A). In addition, phosphatase activity was inhibited

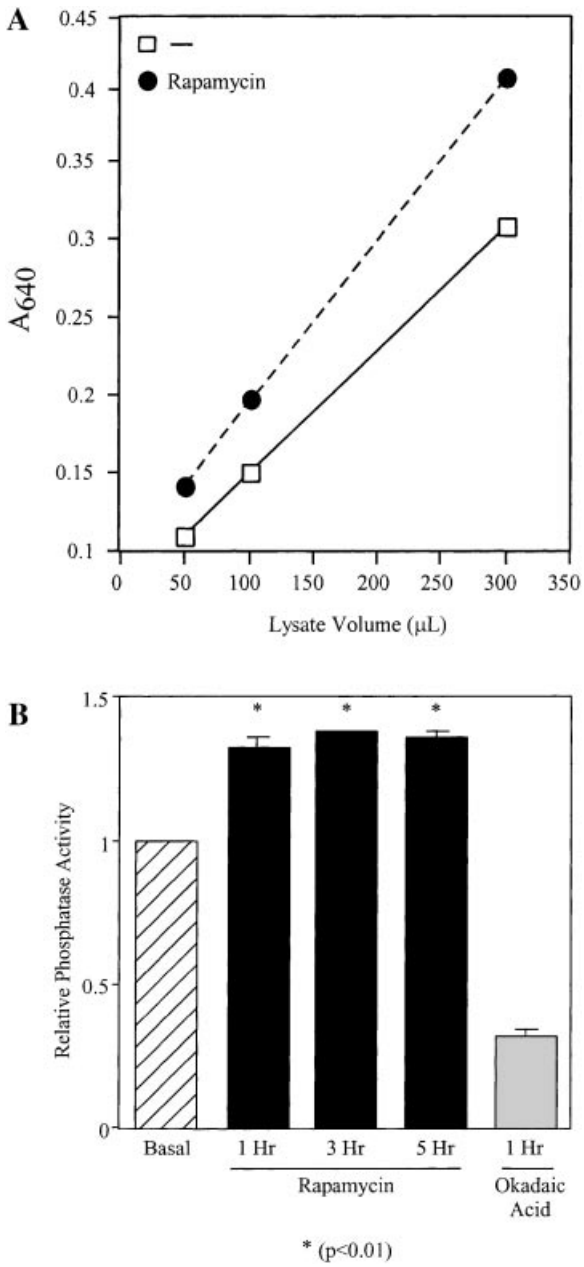

Fig. 7. mTOR inhibits PP2A activity. **A**: Fully differentiated 3T3-L1 adipocytes were treated with 22 nM rapamycin for 1 h or left untreated. The cells were then lysed, and the indicated amounts of clarified lysate were precipitated with 4 μg of antisera to PP2A. The precipitates were washed and assayed for phosphatase activity using a synthetic phospho-peptide as a substrate (K-R-pT-I-R-R). **B**: Depicted is PP2A activity from five separate experiments performed on precipitates derived from 100 μl of lysate. Cells were treated with 22 nM rapamycin for 1, 3, or 5 h, or with 200 nM okadaic acid for 1 h. Samples where okadaic acid was added into the assay mixture had 14% of basal phosphatase activity (not shown). All values are normalized to samples from untreated cells (basal activity) within each experiment.

over 90% by addition of okadaic acid to the reactions (data not shown), indicating that the assay was specific for PP2A.

Treatment of cells with rapamycin increased PP2A activity by approximately 30% (Fig. 7B). The increase is highly reproducible ($P < 0.01$) and similar in magnitude to the results of others [Begum and Ragolia, 1996]. The increase in PP2A activity did not change with longer incubations times of 1–5 h in the presence of rapamycin. As expected, treatment of intact cells with okadaic acid substantially inhibited PP2A activity.

DISCUSSION

We commenced these studies to better understand mTOR signaling and the role of mTOR in IRS-1 degradation. Previous studies [Egawa et al., 1999, 2000; Haruta et al., 2000] have demonstrated the dependence of IRS-1 degradation on the PI 3-kinase signaling pathway and on mTOR, but have not defined the relative contribution of these pathways to IRS-1 degradation. Consistent with previous results [Haruta et al., 2000], we found that the insulin-induced degradation of IRS-1 was sensitive to inhibition of mTOR by rapamycin. However, rapamycin treatment of 3T3-L1 adipocytes consistently resulted in only a 50% inhibition of the degradation of IRS-1 in response to insulin. In contrast, inhibition of PI-3 kinase with wortmannin or LY294002 resulted in a complete block to the insulin-induced degradation of IRS-1. It, therefore, appears that the insulin-induced degradation of IRS-1 is mediated by PI-3 kinase signaling, which stimulates the degradation of IRS-1 by both mTOR-dependent and -independent pathways.

Degradation of IRS-1 is also induced in response to stimulation of fibroblasts with PDGF, although PDGF stimulation leads to only about 50% of the IRS-1 degradation induced by insulin treatment of adipocytes. Interestingly, however, the degradation of IRS-1 induced by PDGF is completely inhibited by rapamycin. It thus appears that PDGF stimulation activates the mTOR-dependent pathway leading to IRS-1 degradation, but does not lead to significant IRS-1 degradation via the mTOR-independent signal that contributes to IRS-1 degradation in insulin-stimulated cells. This difference may result from colocalization of IRS-1 and PI 3-kinase with the insulin receptor in cells

treated with insulin, but not with PDGF. PDGF stimulation leads to increases in PI-3 kinase activity primarily associated with the PDGF receptor, while insulin leads to increases in PI-3 kinase activity associated with the insulin receptor in distinct membrane fractions (microsomal or LDM fractions) within which the majority of IRS-1 is localized [Nave et al., 1996; Takano et al., 2001]. This localization of PI-3 kinase activity with IRS-1 may be necessary for a PI-3 kinase-dependent, mTOR-independent signal contributing to IRS-1 degradation.

Both insulin and PDGF stimulated a large mobility shift of IRS-1 indicative of increased Ser/Thr phosphorylation. Rapamycin effectively inhibited the mobility shift induced by either insulin or PDGF, indicating that phosphorylation of IRS-1 is mediated by mTOR. This is consistent with previous studies indicating that treatment of cells with PDGF or TNFα inhibits insulin signaling as a result of Ser/Thr phosphorylation of IRS-1 [Nave et al., 1996; Paz et al., 1997; Staubs et al., 1998], and that this inhibition of insulin signaling can be prevented by rapamycin treatment [Li et al., 1999; Ozes et al., 2001]. It has been shown that Ser/Thr phosphorylation of IRS-1 prior to insulin stimulation inhibits the subsequent tyrosine phosphorylation of IRS-1 [Paz et al., 1997], thereby blocking insulin signaling in addition to promoting IRS-1 degradation. Therefore, irrespective of IRS-1 degradation, these results demonstrate that the mTOR regulation of IRS-1 Ser/Thr phosphorylation is critical to the onset of insulin resistance.

It is likely that mTOR contributes to IRS-1 degradation by promoting the translocation of IRS-1 to the cytosol, where it is degraded by the proteasome [Takano et al., 2001]. Insulin stimulation normally leads to a release of IRS-1 from the microsomal compartment to the cytosol [Clark et al., 2000; Takano et al., 2001]. It has been shown that rapamycin treatment will inhibit the insulin-induced movement of IRS-1 to the cytosol [Takano et al., 2001], apparently to a similar extent as we have found that rapamycin inhibits IRS-1 degradation. PDGF can also induce the translocation of IRS-1 to the cytosol [Clark et al., 2000], with PDGF inducing a slower shift consistent with its decreased ability to induce IRS-1 degradation. This suggests that mTOR-dependent phosphorylation of IRS-1, or a protein located in the microsomal fraction [Inoue et al., 1998], is involved in the translocation of IRS-1 to the cytosol where it is then degraded by the proteasome.

A possible candidate for the mTOR-independent signal that contributes to the insulin-induced degradation of IRS-1 is phosphorylation of IRS-1 by Akt. Akt has been shown to directly phosphorylate IRS-1 within and near the PTB domain [Paz et al., 1999], which can inhibit the function of this domain and block the ability of IRS-1 to be recruited to the insulin receptor [Paz et al., 1997]. It has further been shown that prior activation of Akt is sufficient to inhibit insulin signaling [Li et al., 1999]. Phosphorylation of IRS-1 by Akt may, therefore, inhibit insulin signaling and contribute to the insulin-induced degradation of IRS-1 by promoting the release of IRS-1 from the insulin receptor and into the cytosol.

Although mTOR-dependent phosphorylation is known to regulate the activities of p70S6 kinase and 4E-BP1 in mammalian cells, it is unclear whether this results from direct phosphorylation of these proteins by mTOR or as a secondary result of mTOR inhibition of the protein phosphatase PP2A [Jiang and Broach, 1999]. We, therefore, examined the possible role of PP2A in the phosphorylation and degradation of IRS-1. Inhibition of PP2A with okadaic acid induced both the phosphorylation mobility shift and degradation of IRS-1. These effects of okadaic acid were not blocked by treatment of cells with rapamycin, indicating that inhibition of PP2A was sufficient to induce IRS-1 phosphorylation and degradation in the absence of mTOR activity. It, therefore, appears that the regulation of IRS-1 Ser/Thr phosphorylation by mTOR results from inhibition of PP2A. In contrast, the induction of p70S6 kinase phosphorylation by okadaic acid is inhibited by rapamycin (Fig. 6B). This suggests that the activity of mTOR is directly required for the phosphorylation of p70S6 kinase, possibly in addition to suppression of PP2A activity.

Consistent with the involvement of PP2A in IRS-1 degradation, we found that inhibition of mTOR resulted in a consistent 30% increase in PP2A activity. The majority of the PP2A catalytic subunit exists as part of a trimeric complex within cells (A:B:C trimer) [Janssens and Goris, 2001]. The A and B subunits serve as docking and regulatory factors for the catalytic subunit of PP2A. In yeast, TOR regulates PP2A by phosphorylating TAP42 (alpha4 in

mammalian cells), causing a redistribution of the PP2A catalytic subunit into a complex with TAP42 and away from the normal A and B subunits [Jiang and Broach, 1999]. The binding of PP2A by TAP42 leads to the inhibition of its activity or a dramatic change in its substrate specificity, preventing PP2A from then acting on p70S6 kinase and 4E-BP1 [Murata et al., 1997]. In mammalian cells, it is unclear how mTOR may regulate PP2A, but it is thought that this involves the pool of PP2A associated with alpha 4. There is conflicting data on whether mTOR regulates the association of PP2A with alpha4 as has been shown in yeast. Only a small portion of the cellular pool of PP2A is present in a complex with alpha4 [Janssens and Goris, 2001], and this perhaps accounts for the relatively small change we find in total PP2A activity following treatment with rapamycin.

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