Insulin activates a PD 098059-sensitive kinase that is involved in the regulation of p70^{S6K} and PHAS-I

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Abstract Incubating either Chinese hamster ovary (CHO) cells or 3T3-L1 adipocytes with insulin increased the phosphorylation of the eIF-4E-binding protein, PHAS-I. Insulin also activated p70^{S6K} and the Erk-1 and Erk-2 isoforms of mitogen-activated protein kinase (MAP kinase). However, the concentrations of the hormone needed to activate MAP kinase were 10–100 times higher than those needed to increase PHAS-I phosphorylation and p70^{S6K} activity. Incubating cells with the inhibitor of MAP kinase kinase (MEK) activation, PD 098059, blocked the effects of low concentrations of insulin on PHAS-I and p70^{S6K}. The effects of the inhibitor were overcome by increasing concentrations of insulin. The results indicate that insulin activates a PD 098059-sensitive kinase that is involved in the regulation of both p70^{S6K} and PHAS-I.

Key words: Insulin; p70^{S6K}; PD 098059; MAP kinase kinase; PHAS-I

1. Introduction

Insulin rapidly stimulates protein synthesis by increasing the rate of mRNA translation [1,2]. This hormonal effect involves phosphorylation of ribosomal proteins and translation factors, including the eIF-4E-binding protein, PHAS-I (also known as 4E-BP1) [3–5]. Non-phosphorylated PHAS-I binds tightly to eIF-4E, the mRNA cap-binding protein, and inhibits its translation of capped mRNA both in vitro and in vivo [6,7]. When PHAS-I is phosphorylated in response to insulin, the PHAS-I/eIF-4E complex dissociates, thereby allowing eIF-4E to participate in translation initiation [7–10].

PHAS-I is an excellent substrate for MAP kinase in vitro, and the kinase phosphorylates the protein in the same five sites that are phosphorylated when adipocytes are incubated with insulin [11,12]. However, neither the Erk-1 nor Erk-2 isoforms of MAP kinase appears to be the major insulin-stimulated PHAS-I kinase in cells. This conclusion is based in part on the finding that inhibiting MAP kinase activation with PD 098059 did not block PHAS-I phosphorylation in response to insulin in 3T3-L1 adipocytes or CHO cells [10,13]. PD 098059 is a specific inhibitor of the enzymes that activate MAP kinase [14], although 10 times higher concentrations of PD 098059 are required to inhibit MEK2 than to inhibit MEK1 [14]. Further evidence of MAP kinase-independent regulation of PHAS-I is provided by the findings that rapamycin promoted dephosphorylation of PHAS-I and attenuated the effect of insulin on phosphorylating the protein [10,15]. Rapamycin is a selective inhibitor of the function of mTOR, the mammalian homologue of the TOR1p and TOR2p proteins that have been implicated in translational control in *Saccharomyces cerevisiae* [16]. Rapamycin does not interfere with activation of MAP kinase, but it potently inhibits the insulin-stimulated phosphorylation and activation of p70^{S6K}, a downstream element of the mTOR pathway [17–21]. p70^{S6K} itself does not phosphorylate PHAS-I [11]. Indeed, there is reason to suspect that PHAS-I and p70^{S6K} proteins might be phosphorylated by the same proline-directed protein kinase, as all five phosphorylation sites in PHAS-I and the four sites in the 'SKAIPS' domain of p70^{S6K} have a Ser/Thr-Pro motif [22].

The recent finding that PD 098059 promoted dephosphorylation of PHAS-I in non-stimulated CHO cells suggests that under some conditions the MAP kinase signaling pathway contributes to the control of PHAS-I [13]. In view of the similarities in the phosphorylation sites in PHAS-I and p70^{S6K}, we decided to investigate the effects of PD 098059 on the regulation of these two important proteins by insulin.

2. Materials and methods

2.1. Materials

Porcine insulin (27 U/mg) was purchased from Eli Lilly. Antibodies to PHAS-I and p70^{S6k} were generated as described previously [10,23]. PD 098059 was a gift from Alan Saltiel at Parke-Davis. [γ-^{32}P]ATP was from DuPont NEN and rapamycin was obtained from Calbiochem. Most commonly used chemicals were from Sigma Chemical Co. Tissue culture reagents were purchased from Gibco BRL.

2.2. Cell culture and treatment of cells

3T3-L1 fibroblasts were cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum (FCS) on plastic culture dishes (10 cm diameter), and converted to adipocytes by using differentiation medium as described previously [10]. Experiments were performed 10–14 days after withdrawal from differentiation medium. Prior to treatment, the growth medium was replaced with buffer containing 0.5% bovine serum albumin (BSA) [10], and the cells were incubated for a total time of 3 h. Additions of PD 098059 and insulin were made at the appropriate times prior to the end of this incubation. To terminate the incubation, cells were washed twice in ice-cold phosphate-buffered saline and homogenized in 1 mM EDTA, 5 mM EGTA, 10 mM $MgCl_2$, 2 mM dithiothreitol (DTT), 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM benzamidine, 0.1 μM microcystin-LR, 10 mM KP_i, and 50 mM β-glycerophosphate (pH 7.4).

Chinese hamster ovary cells expressing human insulin receptors (CHO-IR) were grown to confluency in alpha-minimal essential medium containing nucleotides and 10% FCS as described previously [24]. Cells were incubated in serum-free medium containing 0.5% BSA for 16 h, then incubated with hormone or drug for appropriate times. Incubations were terminated by removing the medium and freezing the cells with liquid nitrogen. Whole-cell lysates were prepared by thawing cells in lysis buffer containing 20 mM HEPES (pH 7.4), 10 mM $MgCl_2$, 2 mM EDTA, 10 mM sodium pyrophos-

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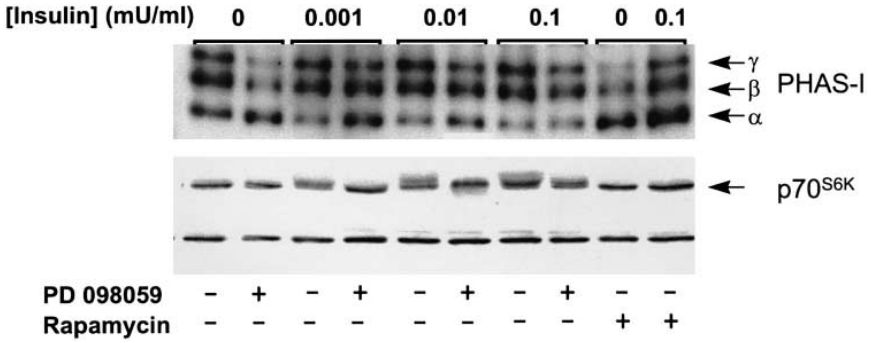

Fig. 1. Effect of rapamycin and PD 098059 on PHAS-I and $p70^{S6K}$ phosphorylation. Serum-starved CHO-IR cells were incubated at 37°C without additions or with 25 nM rapamycin or 50 μM PD 098059 for 60 min, prior to adding increasing concentrations of insulin. Incubations were continued for 30 min. Samples were subjected to SDS-PAGE, before PHAS-I or $p70^{S6K}$ were detected by immunoblotting as described in Section 2.

phate, 2 mM sodium orthovanadate, 1% Triton X-100, and the same protease and phosphatase inhibitors that were used to homogenize the adipocytes.

2.3. Immunoprecipitations and electrophoretic analyses

Extracts were prepared by centrifuging homogenates at $12000 \times g$ for 20 min at 4°C. PHAS-I was analyzed as described previously [10]. $p70^{S6K}$ was immunoprecipitated by incubating samples (100 μl of extract) with antibody (4 μg) for 16 h at 4°C with constant mixing. Protein A-agarose beads (10 μl/sample), equilibrated in cell lysis buffer, were then added. After incubating for 2 h at 4°C, the beads were washed 4 times with lysis buffer without Triton X-100.

Immunoprecipitated proteins were eluted using SDS sample buffer and subjected to SDS-PAGE [25] before proteins were electrophoretically transferred to polyvinylidene difluoride membrane (Millipore). For immunoblotting, membranes were incubated with antibodies (1 μg/ml) and washed as described previously [10]. Antibody binding was detected by enhanced chemiluminescence using alkaline phosphatase conjugated to goat anti-rabbit IgG. The intensities of the bands corresponding to PHAS-I or $p70^{S6K}$ were determined by two-dimensional scanning using a laser densitometer (Molecular Dynamics Inc.).

2.4. Protein kinase activities

The activities of the Erk-1 and Erk-2 isoforms of MAP kinase were measured using [γ-^{32}P]ATP and myelin basic protein as substrates in the gel renaturation method described by Wang and Erikson [26], or after immunoprecipitation as described previously [27]. The activity of $p70^{S6K}$ was measured in an immune complex assay by using intact 40S ribosomes as substrate essentially as described previously [23]. Briefly, protein A-agarose beads bound to immune complexes were incubated in a reaction mixture composed of 20 mM HEPES (pH 7.4), 100 mM β-glycerophosphate, 3 mM EGTA, 0.2 mM sodium orthovanadate, 2 mM DTT, 9 μM inhibitory peptide of cAMP-dependent protein kinase, 20 mM $MgCl_2$, 10 μM calmidazolium, and 200 μM [γ-^{32}P]ATP (~5000000 cpm) containing 35 μg/sample of 40S ribosomes. The reaction was terminated by adding SDS sample buffer.

3. Results

3.1. Effect of PD 098059 and rapamycin on phosphorylation of PHAS-I and $p70^{S6K}$ in CHO-IR cells

To investigate the effects of insulin on the phosphorylation state of PHAS-I, CHO-IR cells were incubated with increasing concentrations of insulin before extracts were subjected to SDS-PAGE, followed by immunoblotting to detect the PHAS-I protein. As observed with extracts of other cells and tissues, PHAS-I from non-stimulated CHO-IR cells was resolved into three bands, designated α, β and γ in order of decreasing electrophoretic mobility. These forms arise from differences in the phosphorylation state of the protein. In general phosphorylation decreases the mobility. Thus, β and γ represent hyperphosphorylated forms of PHAS-I. Approximately 25% of PHAS-I in non-stimulated CHO-IR cells was found in the non-phosphorylated α form. Insulin at concen-

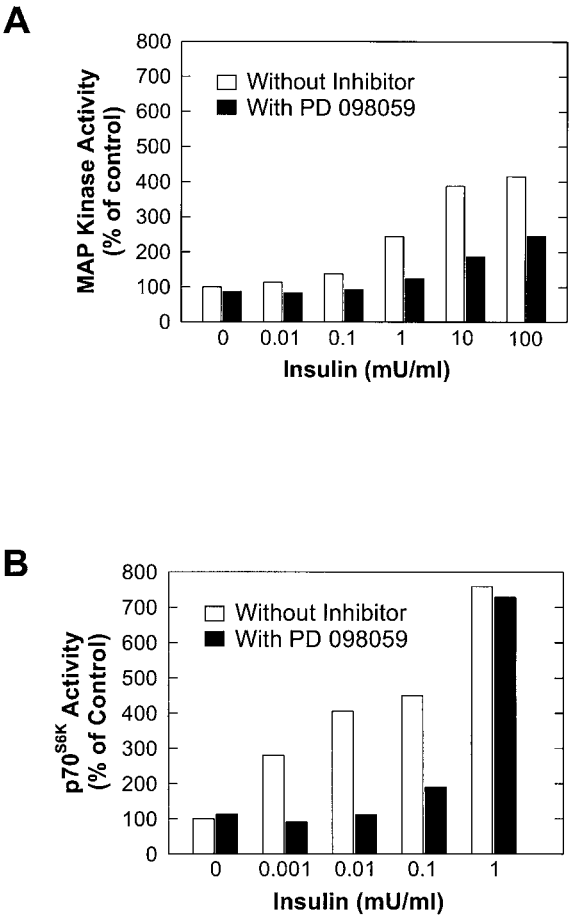

Fig. 2. Effect of PD 098059 on MAP kinase and $p70^{S6K}$ activities. CHO-IR cells were incubated as in the legend to Fig. 1, except that for MAP kinase, cells were stimulated with insulin for only 10 min. This was done because MAP kinase activity peaks at 10 min, and then declines to a level near the control with prolonged incubation. A: MAP kinase activities in extracts were assessed by using an in-gel assay with myelin basic protein as substrate. B: $p70^{S6K}$ activity was measured in an immune complex assay with 40S ribosomes as the substrate. Relative amounts of ^{32}P incorporated into myelin basic protein and ribosomal protein S6 were determined by optical density scanning of autoradiograms.

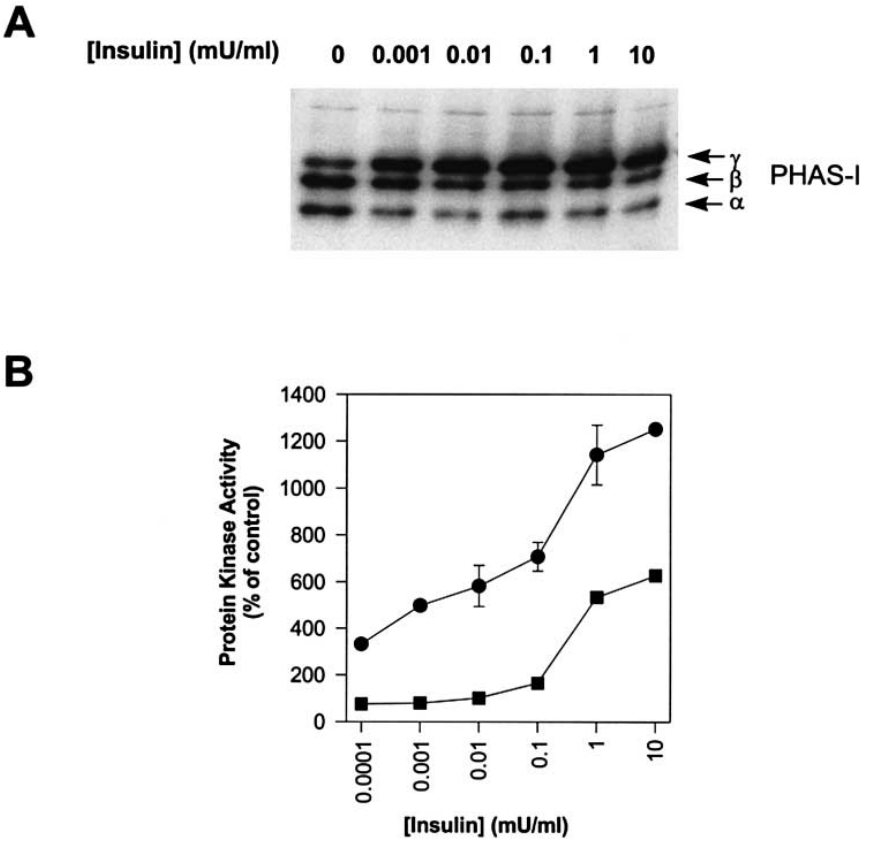

Fig. 3. Effect of increasing concentrations of insulin in 3T3L-1 adipocytes. Cells were incubated with increasing concentrations of insulin for either 10 min for MAP kinase or 30 min for PHAS-1 and $p70^{S6K}$. A: Phosphorylation of PHAS-I was analysed in cell extracts by Western blotting. B: The activities of MAP kinase (■) and $p70^{S6K}$ (●) were determined as described in the legend to Fig. 2.

trations as low as 1 μU/ml decreased the amount of the α form, and increased the relative proportion of the more highly phosphorylated γ form (Fig. 1, top). As reported by Flynn and Proud [13] PD 098059 promoted the dephosphorylation of PHAS-I in non-stimulated CHO cells. The present results extend these earlier findings by demonstrating that PD 098059 attenuated the effects of insulin on increasing PHAS-I phosphorylation. Indeed, at low concentrations of insulin, the magnitude of the effect of PD 098059 was comparable to that of rapamycin.

As with PHAS-I, the insulin-induced phosphorylation of $p70^{S6K}$ can be monitored by the decreases in electrophoretic mobility. $p70^{S6K}$ from extracts of non-stimulated CHO-IR cells appeared as a single band of $M_r = 70\,000$. Multiple forms of $p70^{S6K}$ having reduced electrophoretic mobility were observed after incubating cells with insulin at concentrations as low as 0.01 mU/ml (Fig. 1, bottom), indicative of the phosphorylation and activation of the kinase by insulin. As expected, the effect of insulin was abolished by rapamycin (Fig. 1). Incubating the cells with PD 098059 before adding insulin also markedly decreased the hormonal effect on phosphorylation of $p70^{S6K}$.

3.2. *Inhibition of MAP kinase and $p70^{S6K}$ activities by the MEK inhibitor, PD 098059, in CHO-IR cells*

To investigate further the effects of PD 098059, CHO-IR cells were incubated with and without the MEK inhibitor in the presence of increasing concentrations of insulin before the activities of MAP kinase and $p70^{S6K}$ were measured. Little, if any, increase in MAP kinase activity was observed with 10 μU/ml insulin, even though $p70^{S6K}$ activity was increased by nearly 4-fold by this concentration of the hormone (Fig. 2). PD 098059 abolished the effects of the lower concentrations of insulin on increasing both MAP kinase (Fig. 2A) and $p70^{S6K}$ (Fig. 2B) activities. However, the effects of the inhibitor were overcome by increasing concentrations of insulin. The concentration of PD 098059 (50 μM) required to inhibit the activation of $p70^{S6K}$ at low concentrations of insulin is within the concentration range that is known to abolish MEK [14].

3.3. *Regulation of PHAS-I, MAP kinase and $p70^{S6K}$ in 3T3-L1 adipocytes*

The effects of insulin and PD 098059 in 3T3-L1 adipocytes were similar to those observed in CHO-IR cells. In the adipocytes concentrations of insulin as low as 1 μU/ml increased the phosphorylation of PHAS-I (Fig. 3A). At this concentration of insulin, $p70^{S6K}$ activity was increased by over 3-fold. In contrast, MAP kinase activity was not significantly increased until the insulin concentration reached 0.1 mU/ml (Fig. 3B). Thus, in 3T3-L1 adipocytes insulin was at least 100-times more potent in increasing PHAS-I phosphorylation and $p70^{S6K}$ activity than in activating MAP kinase.

3.4. *Effect of PD 098059 on insulin-stimulated 3T3-L1 adipocytes*

As in CHO-IR cells, PD 098059 promoted the dephosphorylation of PHAS-I, and attenuated the effects of low concentrations of insulin on increasing the phosphorylation of the

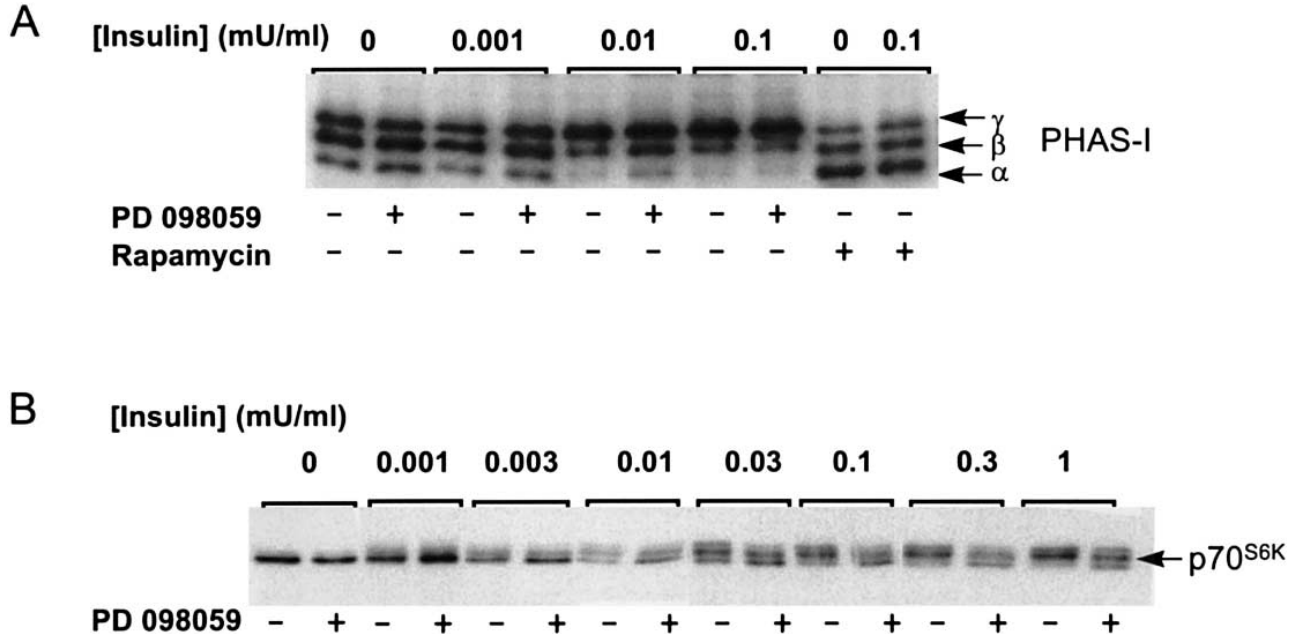

Fig. 4. Effect of PD 098059 on PHAS-I and $p70^{S6K}$ phosphorylation in 3T3-L1 adipocytes. Cells were incubated with rapamycin, PD 098059, and increasing concentrations of insulin as described in the legend to Fig. 1. Cell extracts were analyzed by Western blotting using antibodies to (A) PHAS-I or (B) $p70^{S6K}$.

protein (Fig. 4A). However, the effects of the MEK inhibitor were less pronounced in the adipocytes than in CHO-IR cells, and they were completely overcome when the insulin concentration exceeded 0.01 mU/ml (Fig. 4A). Cells were incubated with PD 098059 for 1 h prior to the addition of insulin in the present study, but for only 20 min in previous experiments in 3T3-L1 adipocytes. This difference, and the fact that high concentrations of insulin were used previously, explain why no effect of the inhibitor on PHAS-I was detected in our earlier study [10].

When assessed by gel-shift analysis, PD 098059 decreased the phosphorylation of $p70^{S6K}$ at even the highest concentration of insulin investigated in the 3T3-L1 adipocytes (Fig. 4B). However, increasing the hormone concentration overcame the inhibitory effect of PD 098059 on $p70^{S6K}$ activity. The most likely explanation for this apparent discrepancy is that with high insulin concentrations, the MEK inhibitor prevented phosphorylation of sites that affect the electrophoretic mobility but did not inhibit phosphorylation of sites most important for activating the kinase. PD 098059 was more effective in preventing activation of MAP kinase than in inhibiting $p70^{S6K}$ (Fig. 5). In the presence of the inhibitor, the highest concentration of insulin tested elicited only a slight increase in the activities of Erk-1 and Erk-2.

4. Discussion

The finding that the MEK inhibitor, PD 098059, attenuates the effect of insulin on increasing the phosphorylation of PHAS-I would seem to indicate that the MAP kinase signaling pathway is involved in the control of PHAS-I. However, this conclusion may be premature. The finding that with increasing concentrations of insulin, the effect of the hormone on PHAS-I was maximal before Erk-1 or Erk-2 activities were even increased supports the view that neither of these isoforms of MAP kinase is the major mediator of insulin action on PHAS-I. Relatively low concentrations of insulin were needed to activate $p70^{S6K}$, a downstream element of the mTOR-signaling pathway. Moreover, PD 098059 decreased $p70^{S6K}$ phosphorylation and inhibited activation of the kinase by insulin. Thus, the effects of PD 098059 are consistent with the increasing evidence that implicates the mTOR/$p70^{S6K}$ signaling pathway in the control of PHAS-I phosphorylation.

$p70^{S6K}$ is thought to be regulated independently of the MAP kinase signaling pathway, which is believed to be specifically inhibited by PD 098059 [14,28]. Regardless of the specificity of the inhibitor, the present findings indicate that inhibition of the $p70^{S6K}$ pathway must be considered as a potential explanation for effects observed with PD 098059 in the increasing number of studies in which the MEK inhibitor is being used to investigate the MAP kinase signaling pathway. Previously, Alessi et al showed that PD 098059 does not directly inhibit $p70^{S6K}$ [14]. Unfortunately, the kinases responsible for activating $p70^{S6K}$ are not known, so that it is not feasible to investigate the specificity of the inhibitor using these enzymes. Nevertheless, based on the present findings, we propose that in order to ascribe an effect of PD 098059 to the MAP kinase pathway the effect should be shown to be resistant to rapamycin, which blocks $p70^{S6K}$ activation.

PD 098059 appears to bind to a region of MEK-1 outside of the ATP-binding site, thereby blocking access to enzymes that activate the kinase [29]. It has been argued that because the compound does not bind to the highly conserved kinase domain, it is likely to be a more selective inhibitor than the class of kinase inhibitors that act competitively with ATP. Based on the defined specificity of the inhibitor, our findings suggest that MEK-1 and/or MEK-2 is involved in the activation of $p70^{S6K}$ by insulin. In view of their extremely restricted substrate specificity, it is unlikely that MEK-1 or MEK-2 phosphorylate $p70^{S6K}$. Erk-2 does not directly phosphorylate $p70^{S6K}$ in vitro [28]. Moreover, it is unlikely that inhibition of the Erk-1 and Erk-2 account for the effects on $p70^{S6K}$, as the

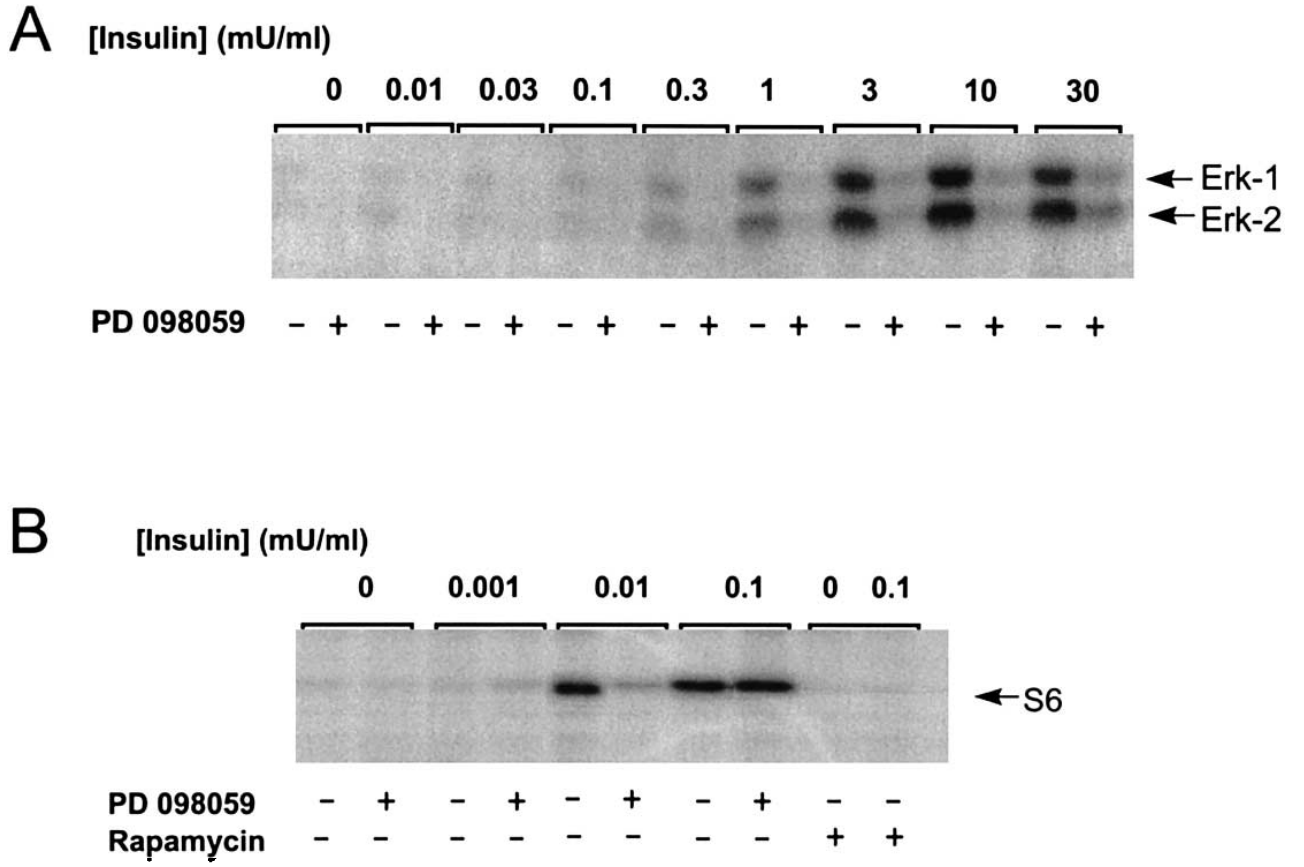

Fig. 5. Effect of PD 098059 on MAP kinase and $p70^{S6K}$ activities in rat adipocytes. Cells were treated as in Fig. 4 except that for MAP kinase cells were stimulated with insulin for only 10 min. MAP kinase activity using an in-gel assay with myelin basic protein as substrate (A) or $p70^{S6K}$ activity was measured in an immune complex assay (B).

effect of PD 098059 on $p70^{S6K}$ was observed under conditions in which the activities of these MAP kinase isoforms were not changed. Our findings are consistent with those of Lenormand et al. [30] who have shown that constitutively active MEK leads to activation of $p70^{S6K}$, independently of MAP kinase, in a cell line which stably expresses ΔRaf 1:ER, an estradiol-regulated form of oncogenic Raf. Interestingly, Holt et al. [31] have presented evidence that the phosphorylation of mSOS is also regulated by insulin through a MEK-dependent and MAP kinase-independent pathway.

$p70^{S6K}$ is regulated by the phosphorylation of two classes of Ser/Thr sites [reviewed in [32]]. In one class the phosphorylated residue is flanked by hydrophobic amino acids residues. The other class of sites has a Ser/Thr-Pro motif. As selectivity of protein kinases is often determined by the amino acids surrounding a Ser/Thr residue, it seems likely that $p70^{S6K}$ is controlled by multiple protein kinases. There is evidence that the enzyme is controlled by both phosphatidylinositol 3-kinase-dependent and -independent pathways [33–35]. Our findings suggest that a MEK-dependent, but Erk-independent, pathway participates in the control of $p70^{S6K}$. It is interesting to speculate that this pathway leads to activation of a proline-directed protein kinase that phosphorylates both $p70^{S6K}$ and PHAS-I.

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