Identification of Phosphorylation Sites in the Translational Regulator, PHAS-I, That Are Controlled by Insulin and Rapamycin in Rat Adipocytes*

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Phosphorylation of PHAS-I by mitogen-activated protein (MAP) kinase in vitro decreased PHAS-I binding to eukaryotic initiation factor (eIF)-4E. The decrease in binding lagged behind the phosphorylation of PHAS-I in Ser64, the preferred site of MAP kinase. Binding of the Ala64 mutant of PHAS-I to eIF-4E was abolished by MAP kinase, indicating that phosphorylation of sites other than Ser64 control binding. To identify such sites, PHAS-I was phosphorylated with MAP kinase and [γ-32P]ATP and then cleaved proteolytically before the resulting phosphopeptides were isolated by reverse phase chromatography and directly identified by amino acid sequencing. Phosphorylated residues were located by determining the cycles in which 32P was released when phosphopeptides were subjected to sequential Edman degradation. With an extended incubation in vitro, MAP kinase phosphorylated Thr36, Thr45, Ser64, Thr69, and Ser82. In rat adipocytes, the phosphorylation of all five sites was increased by insulin and decreased by rapamycin although there were differences in the magnitude of the effects. A form of PHAS-I phosphorylated exclusively in Thr36 remained bound to eIF-4E, indicating that phosphorylation of Thr36 is insufficient for dissociation of the PHAS-I-eIF-4E complex. In summary, our results indicate that multiple phosphorylation sites are involved in the control of PHAS-I. All five sites identified fit a (Ser/Thr)-Pro motif, suggesting that the phosphorylation of PHAS-I in cells is mediated by a proline-directed protein kinase.

Insulin and growth factors act within minutes to stimulate protein synthesis (1, 2). This rapid response is due to activation of mRNA translation and involves phosphorylation of multiple translation factors. One of these factors is PHAS-I (also known as 4EBP-1) (3, 4), a protein of Mr ~12,500 that was first identified in [32P]-labeled adipocytes as a heat- and acid-stable species that was markedly phosphorylated in response to insulin (5). PHAS-I is now known to be expressed in a wide variety of cell types and to act as a regulator of eIF-4E, the mRNA cap-binding protein (6, 7).

eIF-4E is one of the least abundant of the known translation factors (8, 9), and the amount of eIF-4E is believed to be limiting for initiation, which is generally the rate-limiting phase of protein synthesis (1, 2). Thus, increasing eIF-4E in cells increases mRNA translation, particularly of those messages which possess a high degree of secondary structure in their 5′-untranslated regions (10). eIF-4E is a key component of the eIF-4F complex, which catalyzes melting of secondary structure in the 5′-untranslated region of the mRNA and allows efficient binding and/or scanning by the 40 S ribosomal subunit (1, 2, 8, 9). eIF-4F contains two other subunits, eIF-4A, an ATP-dependent helicase, and eIF-4G, a relatively large subunit that binds to both eIF-4A and eIF-4E (1, 2, 8, 9).

Nonphosphorylated PHAS-I binds tightly to eIF-4E (4, 11) and blocks eIF-4E binding to eIF-4G (12, 13). Consequently, increasing PHAS-I inhibits cap-dependent mRNA translation both in vitro and in intact cells (4). However, when PHAS-I is phosphorylated in response to insulin or certain growth factors, the PHAS-I-eIF-4E complex dissociates (6, 7), thereby decreasing eIF-4E available to bind to eIF-4G. Increasing the eIF-4F complex by this mechanism provides an explanation of the preferential stimulation by insulin of the translation of messages, such as ornithine decarboxylase, which have a high degree of secondary structure in their 5′-untranslated region (14).

The immunosuppressive drug, rapamycin, promotes dephosphorylation of PHAS-I in adipocytes (15–17) and a variety of other cell types (18–21). Rapamycin is a potent inhibitor of the activation of p70S6K (22, 23), but this enzyme is almost certainly not a PHAS-I kinase in cells since it does not phosphorylate PHAS-I in vitro (6, 16, 24). PHAS-I can be phosphorylated in vitro by protein kinase C and casein kinase II (6, 24, 25), but whether either of these kinases phosphorylate PHAS-I in cells is not known. PHAS-I is an excellent substrate for MAP kinase1 in vitro (11, 24); and, Ser64, the site in PHAS-I preferred by MAP kinase, is phosphorylated in response to insulin in adipocytes (24). However, several lines of evidence indicate that MAP kinase is not the major mediator of insulin action on PHAS-I (6). For example, when bound to eIF-4E, the phosphorylation of PHAS-I by MAP kinase in vitro is relatively slow (6, 15, 16), and inhibiting activation of MAP kinase with the inhibitor of MEK activation, PD 098059, did not block the effects of insulin on PHAS-I in 3T3-L1 adipocytes (15).

The amino acid sequence surrounding a particular Ser or Thr residue contributes to the specificity of many protein kinases (26). Thus, identification of phosphorylation sites can provide...
clues as to which kinases phosphorylate a protein. PHAS-I isolated from adipocytes contains both phosphothreonine and phosphoserine (27–29), and results from peptide mapping studies (24) and two-dimensional electrophoretic analyses (15, 30) indicate that PHAS-I is phosphorylated in multiple sites in cells. Only one phosphorylation site (Ser64) has been identified directly (24), and the possibility exists that the sites most important in regulating the association of PHAS-I and eIF-4E have not been determined. The objectives of our experiments were to identify the sites of phosphorylation in PHAS-I in adipocytes, to investigate the role of these sites in modulating binding of PHAS-I to eIF-4E, and to determine the changes in phosphorylation produced by insulin and rapamycin.

**EXPERIMENTAL PROCEDURES**

**Incubation of Adipocytes and Immunoprecipitation of PHAS-I**—Fat cells were isolated by collagenase digestion of adipose tissue from male rats (Wistar, 120–140 g, 20 per preparation) (31) and then washed four times and suspended in low P medium (0.2 mM sodium phosphate, 125 mM NaCl, 1.2 mM MgCl2, 4 mM KCl, 2.6 mM CaCl2, 0.5 mM glucose, 10 mg/ml bovine serum albumin, and 24 mM sodium HEPES, pH 7.4). Na32P-O4 (5 μCi/10 ml aliquot of cells) was added, and the cells were incubated at 37 °C for 90 min, a time sufficient to achieve steady-state labeling of intracellular ATP (32). After treatment with insulin and rapamycin, the incubations were terminated by homogenizing the cells as described previously (24) in Homogenization Buffer (2 ml/ml packed cells), which contained 100 mM NaF, 10 mM EDTA, 2 mM EGTA, 1 mM benzamidine, 0.2 mM phenylmethylsulfonyl fluoride, and 50 mM Tris, pH 7.8. The homogenates were centrifuged at 28,000 × g for 30 min. PHAS-I was immunoprecipitated from the supernatants by using an affinity-purified antibody (3) coupled to protein A-Sepharose (Pharmacia Biotech Inc.) as described previously (11). To elute PHAS-I from the immune complexes, the beads were suspended in 300 μl of 1% β-mercaptoethanol, 1 mM EDTA, and 10 mM Tris-Cl, pH 7.5, and incubated at 95 °C for 15 min. PHAS-I, which is relatively stable to heat (3), was recovered in the supernatant after centrifuging samples at 10,000 × g for 10 min.

**Phosphorylation of PHAS-I by MAP Kinase in Vitro—Wild-type PHAS-I (17), PHAS-I tagged at the NH2 terminus with a hexahistidine sequence ([H6]PHAS-I) (24), [H6]PHAS-I having a Ser to Ala mutation (8), the ERK2 isoform of MAP kinase (34), and a constitutively active form of MEK1 (34) were expressed in bacteria and purified as described in the references indicated. MAP kinase was activated by incubation with MEK1 as described by Scott et al. (34). Samples (160 μg/ml) of PHAS-I, [H6]PHAS-I, or [H6]PHAS-IAla64 were incubated at 23 °C in a solution containing activated MAP kinase (34) (62 μg/ml), 1 mM dithiothreitol, 10 mM MgCl2, and 50 mM Tris-Cl, pH 7.5. The reactions were stopped by heating at 95 °C for 5 min. Samples of [H6]PHAS-I and [H6]PHAS-IAla64 used in far-Western analyses of FLAG-4E binding (described later) were phosphorylated in parallel incubations with unlabeled ATP, and the reactions were stopped by adding SDS sample buffer.

**Digestion of [32P]PHAS-I with Lysyl Endopeptidase and Chymotrypsin and Resolution of [32P]-Labeled Peptides by HPLC—[32P]PHAS-I samples (300 μl) from either in vitro phosphorylation reactions or immunoprecipitated from [32P]labeled adipocytes were incubated at 37 °C for 15 h with lysyl endopeptidase (17).** Phosphoamino acid analyses were performed by subjecting samples of phosphopeptides that had been purified by reverse phase HPLC to high voltage electrophoresis at pH 1.9, which provides superior separation of phosphoserine and phosphothreonine. Phosphorylation of an Ala64 mutant PHAS-I was observed (11), PHAS-I phosphorylation exceeded 1 mol/mol (24), and some phosphorylation of an Ala64 mutant PHAS-I was observed (11), phosphoamino acid analyses were performed by subjecting samples of phosphopeptides that had been purified by reverse phase HPLC to high voltage electrophoresis at pH 1.9, which provides superior separation of phosphoserine and phosphothreonine. The fact that phosphothreonine and phosphoserine are not resolved at this pH was not a problem as PHAS-I from adipocytes is not phosphorylated on tyrosyl residues (27–29).

**RESULTS**

**Phosphorylation of Sites Other Than Ser64 Controls PHAS-I Binding to eIF-4E—PHAS-I contains seven Ser/Thr-Pro motifs that represent potential sites of phosphorylation by MAP kinase (Fig. 1), the most effective of the protein kinases that have been found to phosphorylate PHAS-I in vitro (6, 16, 24). In previous experiments Ser64 was identified as the preferred site of phosphorylation by MAP kinase (24). However, with extended incubation with MAP kinase, the stoichiometry of PHAS-I phosphorylation exceeded 1 mol/mol (24), and some phosphorylation of an Ala64 mutant PHAS-I was observed (11), phosphorylation of an Ala64 mutant PHAS-I was observed (11), and peptides identified by amino acid sequence analysis after peptide mapping studies (17). Phosphoamino acid analyses were performed by subjecting samples of phosphopeptides that had been purified by reverse phase HPLC to high voltage electrophoresis at pH 1.9, which provides superior separation of phosphoserine and phosphothreonine. The fact that phosphothreonine and phosphoserine are not resolved at this pH was not a problem as PHAS-I from adipocytes is not phosphorylated on tyrosyl residues (27–29).

**Phosphorylation Sites in PHAS-I**

![FIG. 1. Amino acid sequence of rat adipocyte PHAS-I.](http://www.jbc.org/)

The amino acid sequence was deduced from the nucleotide sequence of PHAS-I cloned from a rat adipocyte cDNA library (3). The locations of the histidine-tag region present in [H6]PHAS-I, the Ser/Thr residues phosphorylated in cells (P), the eIF-4E binding motif (bold lettering) (12, 13), and peptides identified by amino acid sequence analysis after digesting PHAS-I with lysyl endopeptidase (LE-P1, LE-P2, and LE-P3) and chymotrypsin (CP-1, CP-2, and CP-3) are shown.
Fig. 2. Resolution of [32P]phosphopeptides generated by lysyl endopeptidase digestions of PHAS-I proteins phosphorylated in vitro by MAP kinase. [H6]PHAS-I (○) and [H6]PHAS-IAla64 (●) were incubated at 23 °C with [γ-32P]ATP and MAP kinase for either 10 min (inset) or 2 h. A, the [32P]-labeled proteins were then digested with lysyl endopeptidase before the resulting [32P]phosphopeptides were applied to a reverse phase column and eluted with an increasing gradient (dotted line) of acetonitrile. The results represent the amount of [32P] recovered in fractions (1 ml), which were collected each min. B, LE-P3 and LE-P4 fractions derived from [H6]PHAS-I that had been incubated for 2 h with MAP kinase in a separate experiment were pooled and incubated with chymotrypsin. The resulting [32P]phosphopeptides were isolated by reverse phase HPLC. The results represent the amount of [32P] recovered in each fraction.

indicating that MAP kinase phosphorylated more than one site in PHAS-I. Results from peptide mapping experiments provide further evidence of multisite phosphorylation (Fig. 2). Three peaks of [32P]-labeled peptides, designated LE-P2, LE-P3, and LE-P4 in order of elution, were resolved when a sample of recombinant [H6]PHAS-I that had been phosphorylated in a 10-min incubation with [γ-32P]ATP and MAP kinase was digested with lysine endopeptidase and subjected to reverse phase HPLC (Fig. 2A, inset). LE-P2 was completely absent in digests of [H6]PHAS-IAla64 that had been phosphorylated by MAP kinase, indicating that LE-P2 represents the [32P]-labeled Ser64 phosphopeptide. After 2 h with MAP kinase, relatively more [32P] was found in sites in LE-P3 plus LE-P4 than in Ser64 (Fig. 2A).3 In addition, a relatively small peak, LE-P1, eluting very early in the acetonitrile gradient was observed. The phosphorylation of other sites by MAP kinase was more pronounced in Fig. 2 than in our previous studies (11, 24) because approximately 10-fold higher concentrations of MAP kinase were used in the present experiments. However, consistent with previous findings (11, 24), the initial rate of phosphorylation of Ser64 (LE-P2) occurred more rapidly than the initial rate of phosphorylation of the other sites (Fig. 3A), which were phosphorylated at least as rapidly in the Ala64 mutant as in the wild-type protein (Fig. 3B).

The influence of Ser64 and other sites on eIF-4E binding was assessed by far-Western blotting using a [32P]-labeled FLAG-4E. MAP kinase markedly decreased binding of [H6]PHAS-I to FLAG-4E (Fig. 3C, inset). However, the initial rate of decline in eIF-4E binding activity (Fig. 3C) produced by MAP kinase did not correlate well with the initial rate of Ser64 phosphorylation (Fig. 3A). For instance, almost no decrease in eIF-4E binding was observed after 5 min of incubation with MAP kinase, even though over half of the Ser64 in PHAS-I had been phosphorylated. The decrease in binding activity correlated much better with the phosphorylation of sites recovered in LE-P2 and LE-P3, indicating that phosphorylation of sites other than Ser64 control binding of PHAS-I to eIF-4E. Findings with [H6]PHAS-IAla64 provide additional support for this interpretation. The initial decrease in eIF-4E binding activity produced by phosphorylation of the mutant protein occurred with a time course that was similar to that obtained with [H6]PHAS-I (Fig. 3C), and extended incubation with MAP kinase abolished binding of the mutant PHAS-I to FLAG-4E (Fig. 3C, inset).

Identification of Sites in PHAS-I Phosphorylated by MAP Kinase in Vitro—The first approach to identify the sites other than Ser64 was to attempt to identify the phosphopeptides derived from lysyl endopeptidase digests of PHAS-I. Except for the 4-amino acid peptide that would result from cleavage at Lys68 and Lys72, all of the predicted peptides contain at least 12 amino acids (Fig. 1) and would be expected to elute relatively late in the acetonitrile gradient. For this reason, the 4-amino acid peptide containing Thr69 was considered the most likely candidate for LE-P1. Phosphoamino acid analysis supported this assignment, as LE-P1 was found to contain phosphothreonine but little if any phosphoserine.2 Amino acid sequencing indicated that the peptide contained Pro residues in the second and third positions (Fig. 4). Other than the Thr69 peptide, the only two other occurrences of adjacent prolines in PHAS-I are Pro29-Pro30 and Pro88-Pro89 (Fig. 1). Determining the position of the phosphorylated residue within the LE-P1 phosphopeptide solidified the assignment of Thr69 as the phosphorylation site. This was accomplished in a separate sequencing run, where the [32P] in LE-P1 was found to be released in cycle 1 (Fig. 4). Even without the sequence data, finding release in the first cycle with a peptide generated by lysyl endopeptidase would be indicative of Thr69 as this residue is the only Ser or Thr adjacent to a Lys in PHAS-I (Fig. 1).

The absence of LE-P2 in peptides derived from [H6]PHAS-IAla64 provided strong evidence that Ser64 was the site of phosphorylation in the LE-P2 phosphopeptide (Fig. 2A, inset). Proof that Ser64 was the phosphorylated residue was provided by amino acid sequencing which yielded a single sequence corresponding to the predicted Ser64 peptide (Fig. 4). As Ser64 is the only Ser/Thr in this peptide, this residue had to be the phosphorylated residue. As expected, release of [32P] from the phosphopeptide in LE-P2 occurred in cycle 8 (Fig. 4).

Amino acid sequencing indicated that LE-P3 contained the peptide generated by cleaving [H6]PHAS-I at Lys72 and Lys104. As this peptide contained multiple Ser and Thr, it was necessary to measure [32P] release during sequential Edman degradation to determine the location of the phosphorylated residue. The surge in [32P] release from LE-P3 occurred in cycle 10, identifying Ser83 as a phosphorylation site. The percentage of [32P] released from LE-P3 samples was relatively low. This was probably due in part to the NH2-terminal Asp residue, as Asp may form a cyclic imide structure after reaction with the 1-ethyl-3-dimethylaminopropyl carbodiimide used to couple the peptides to the solid support (Sequacon-AA), thereby blocking Ed-
Phosphorylation Sites in PHAS-I

Fig. 3. Phosphorylation of Ser<sup>64</sup> does not correlate with the loss of PHAS-I binding to eIF-4E. [H<sup>32</sup>P]PHAS-I and [H<sup>32</sup>P]PHAS-I<sub>Ala64</sub> were incubated for increasing times with MAP kinase before incubations were terminated. A, incubations were conducted with [γ<sup>32</sup>P]ATP before reactions were terminated, and samples were subjected to SDS-PAGE. eIF-4E binding activities were determined by far-Western blotting with [γ<sup>32</sup>P]labeled FLAG-4E. The relative amounts of [γ<sup>32</sup>P]labeled FLAG-4E bound to [H<sup>32</sup>P]PHAS-I (●) or [H<sup>32</sup>P]PHAS-I<sub>Ala64</sub> (△) were determined by phosphoimaging. The results are expressed as percentages of the [γ<sup>32</sup>P]labeled FLAG-4E bound to PHAS proteins that had not been incubated with MAP kinase. The inset shows binding to [H<sup>32</sup>P]PHAS-I and [H<sup>32</sup>P]PHAS-I<sub>Ala64</sub> prior to incubation with MAP kinase and after a 120-min incubation with the kinase.

Fig. 4. Identification of sites in [H<sup>32</sup>P]PHAS-I phosphorylated in vitro by MAP kinase. [H<sup>32</sup>P]PHAS-I was incubated for 2 h with [γ<sup>32</sup>P]ATP and MAP kinase and then digested with lysyl endopeptidase. [γ<sup>32</sup>P]Phosphopeptides in LE-P1, LE-P2, LE-P3, and LE-P4 were isolated by reverse phase HPLC as shown in Fig. 2A. [γ<sup>32</sup>P]Phosphopeptides in LE-P3 and LE-P4 were digested with chymotrypsin and CT-P1, CT-P2, and CT-P3 were isolated as shown in Fig. 2B. A sample of each peak fraction was used for amino acid sequencing as described under “Experimental Procedures.” The sequences obtained are indicated on the figure. Another sample of each peak was used to determine the position of the phosphorylated residues in the peptides. The amounts of [32P] (in cpm) applied to the sequencer were as follows: LE-P1, 1052; LE-P2, 13627; LE-P3, 7046; CT-P1, 6540; and CT-P2, 8137. The results represent the amount of [32P] released in each cycle when the peptides were subjected to sequential Edman degradation.

Phosphorylation Sites in PHAS-I

To investigate the distribution of [32P] among different sites, the immunoprecipitated PHAS-I was cleaved with lysyl endopeptidase, and samples of the digest were analyzed by HPLC under conditions used to resolve the MAP kinase phosphorylation sites (Fig. 5A). The pattern of [32P]-labeled peptides from cellular PHAS-I was similar to that obtained with the recombinant protein, although there were differences. Notably, the relative size of LE-P1 was much larger with the adipocyte PHAS-I, indicating that the site in this peak was highly phosphorylated. Incubating cells with rapamycin prior to insulin attenuated the effects of the hormone on increasing phosphorylation of PHAS-I.

Incubating cells with rapamycin caused a net decrease in the [32P]-content of PHAS-I and increased the proportion of [32P] found in the more slowly migrating form, a finding that is consistent with the previous demonstration that phosphorylation of the appropriate sites in PHAS-I decreases its electrophoretic mobility (11). Rapamycin treatment caused a net decrease in the [32P]-content of PHAS-I and increased the proportion of [32P] bound in the forms of higher electrophoretic mobility (Fig. 5A, inset). The elution positions of LE-P1 and LE-P2 from cellular PHAS-I (Fig. 5A) were identical to those of the corresponding peaks from recombinant PHAS-I (Fig. 2A). To verify that the peaks contained the same sites of phosphorylation, [32P] release was measured following Edman deg-
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Radiation of the peptides (Fig. 6). Almost all of the $^{32}$P in the LE-P1 peptide was released in cycle 1, and phosphothreonine was the only phosphoamino acid detected in this peak (Fig. 7). These findings indicate that LE-P1 contains the Thr$^{69}$ peptide. With LE-P2 a single surge of $^{32}$P occurred in cycle 8 (Fig. 6), and phosphoserine was the only phosphoamino acid detected in this peak (Fig. 7). Thus, LE-P2 appears to contain the Ser$^{64}$ peptide.

LE-P3 and LE-P4 were not as well resolved using PHAS-I from cells as when the phosphorylated recombinant protein was used. Likewise, LE-P3 and LE-P4 from non-tagged recombinant PHAS-I lacking the His-tag were not resolved. The better resolution obtained with [H$^6$]PHAS-I is presumably because the additional amino acids in the His-tag region cause the large peptide in LE-P4 to elute at higher concentrations of acetonitrile. Subjecting peptides in LE-P3 to sequential Edman degradation resulted in release of $^{32}$P in cycle 10, indicative of Ser$^{62}$ phosphorylation. LE-P3 also contains other sites found in the large NH$_2$-terminal peptide, but residues from this peptide are not released during Edman degradation because the NH$_2$ terminus of PHAS-I from adipocytes is blocked (3).

FIG. 5. Peptide mapping of PHAS-I immunoprecipitated from $^{32}$P-labeled rat adipocytes. Fat cells were incubated in medium containing $^{32}$P, for 90 min. The cells were then incubated as follows: 35 min without additions (○, lane 1); 35 min without additions followed by 15 min with 2.5 milliunits/ml insulin (●, lane 2); 20 nM rapamycin for 35 min (△, lane 3); and 20 nM rapamycin for 20 min followed by 20 nM rapamycin plus 2.5 milliunits/ml insulin for 15 min (▲, lane 4). The incubations were terminated by homogenizing the cells, and PHAS-I was immunoprecipitated from extracts. A, samples of the immunoprecipitated protein were digested with lysyl endopeptidase, and peptides were resolved by reverse phase HPLC. Samples of the immunoprecipitated protein were also subjected to SDS-PAGE, and an autoradiogram of the dried gel is shown in the inset. B, $^{32}$P phosphopeptides resulting from digesting the pooled LE-P3 fractions with chymotrypsin were resolved by reverse phase HPLC. The results represent the amount of $^{32}$P detected in each fraction.

FIG. 6. Identification of the position of phosphorylated residues in phosphopeptides derived from PHAS-I isolated from rat adipocytes. $^{[32P]}$PHAS-I was immunoprecipitated from extracts of insulin-treated $^{32}$P-labeled adipocytes, and $^{[32P]}$phosphopeptides were generated and resolved as described in the legend to Fig. 5. Samples were subjected to sequential Edman degradation, and $^{32}$P released in each cycle was measured as described in the legend to Fig. 4. The amounts of $^{32}$P (in cpm) applied to the sequencer were as follows: LE-P1, 1808; LE-P2, 4830; CT-P1, 2136; CT-P2, 3343; and CT-P3, 3585. Shown in italics are the presumed amino acid sequences of $^{[32P]}$phosphopeptides, which had the same retention times as standard $^{32}$P phosphopeptides generated by proteolytic treatment of [H$^6$]PHAS-I that had been phosphorylated in vitro with $[γ^{32}P]$ATP and MAP kinase.

FIG. 7. Phosphoamino acid analysis of $^{32}$P-labeled phosphopeptides. Samples (approximately 1,000 cpm each) of $[32P]$ phosphopeptides from $[32P]$PHAS-I that had been immunoprecipitated from insulin-treated $^{32}$P-labeled adipocytes were subjected to acid hydrolysis (6 N HCl at 110 °C for 2 h). Phosphoserine (PSer) and phosphothreonine (PThr) were resolved by high voltage electrophoresis performed at pH 1.9. An autoradiogram of the chromatogram is shown. The origin and the positions of phosphoamino acid standards that were visualized by ninhydrin-staining are indicated on the right.
the five sites are summarized in Fig. 8. Insulin increased the amount of $^{32}\text{P}$ in all five sites, but the effects of insulin on the different sites differed both in the extent of $^{32}\text{P}$ introduced into the sites and in the percentage change in $^{32}\text{P}$. Relatively little $^{32}\text{P}$ was recovered in the Ser$^{62}$ peptide, and Ser$^{62}$ was least affected by insulin, which produced only a 2-fold increase in $^{32}\text{P}$. The effects of insulin on the other four sites ranged from approximately 2.5-fold with Thr$^{69}$ to more than 4-fold with Thr$^{45}$. However, the different fold increases in $^{32}\text{P}$ in Thr$^{36}$, Thr$^{45}$, Ser$^{64}$, and Thr$^{69}$ produced by the hormone were a function of the basal state of phosphorylation, as the four sites contained approximately the same amount of $^{32}\text{P}$ after insulin treatment.

Incubating cells with rapamycin alone decreased the amount of $^{32}\text{P}$ in all of the sites except Ser$^{62}$ (Fig. 8). Differences among the sites in sensitivity to rapamycin emerged in the presence of insulin. Rapamycin markedly inhibited the insulin-stimulated phosphorylation of Thr$^{69}$ and Thr$^{45}$ (Fig. 8). In contrast, the increases in the $^{32}\text{P}$ content of Ser$^{64}$ produced by insulin in the presence and absence of rapamycin were approximately equal. Similarly, rapamycin had little if any effect on the increment in the $^{32}\text{P}$ content of Thr$^{69}$ produced by insulin.

Identification of a Phosphorylated Form of PHAS-I That Binds eIF-4E—A partially phosphorylated form of PHAS-I has been previously shown to bind labeled eIF-4E in far-Western analyses (17). Likewise, some phosphorylated PHAS-I was recovered with eIF-4E when PHAS-I-eIF-4E complexes were purified from adipocyte extracts by using m$^7\text{GTP}$-Sepharose (15, 16). To identify the phosphorylated sites in the eIF-4E-bound form of PHAS-I, PHAS-I-eIF-4E complexes were purified from extracts of PHAS-I-labeled adipocytes that had been incubated with rapamycin plus insulin. After digesting the PHAS-I with lysyl endopeptidase, essentially all of the $^{32}\text{P}$ was recovered in LE-P3.2 When the peptides in this peak were digested with chymotrypsin and subjected to HPLC, the $^{32}\text{P}$ was found in CT-P2 (Fig. 9, lower panel), indicating that the eIF-4E-bound form was phosphorylated exclusively in Thr$^{36}$. For comparison, note that the PHAS-I protein that did not bind to the cap affinity resin contained $^{32}\text{P}$ in both Thr$^{69}$ and Thr$^{45}$ (Fig. 9, upper panel).

**DISCUSSION**

Our results provide definitive evidence that in rat adipocytes insulin stimulates the phosphorylation of PHAS-I in five sites, all of which fit a Ser/Thr-Pro motif (Fig. 1). The existence of multiple sites raises the possibility that more than one site is involved in the control of PHAS-I binding to eIF-4E. Moreover, identification of the sequence of amino acids surrounding the different phosphorylated residues has provided information needed to identify the protein kinases responsible for phosphorylating PHAS-I in cells. Thus, the results have important implications with respect to not only the control of PHAS-I binding to eIF-4E but also to the mechanisms of action of insulin and rapamycin.

**Multisite Phosphorylation and the Control of PHAS-I Binding to eIF-4E—**All five of the phosphorylation sites identified in rat PHAS-I are conserved in not only the mouse (15) and human proteins (4), but also in PHAS-II, another eIF-4E binding protein that is approximately 60% identical to PHAS-I (4, 17). The conservation of sites suggests that all may be important for the function of the protein. However, our results indicate that phosphorylation of certain sites can have a much greater impact on the binding of PHAS-I to eIF-4E than phosphorylation of others. For example, phosphorylation of Ser$^{64}$, the only site that had been previously identified in PHAS-I...
unlikely that Ser 82 phosphorylation could explain the near dissociation of the PHAS-I insulin (4, 11) since Ser82 appeared to contain much less 32P complete inhibition of PHAS-I binding to eIF-4E produced by that is activated by insulin and a variety of mitogenic stimuli indicates that phosphorylation of Thr36 is not sufficient for sites such as Thr36 and Ser 64 could increase or decrease the action, complex interactions are possible, and phosphorylation of sites such as Thr36 and Ser62 may have been due to poor recovery of CT-P3. Therefore, a role for Ser62 phosphorylation in regulating PHAS-I is still possible. Moreover, it should be stressed that with five sites of phosphorylation, complex interactions are possible, and phosphorylation of sites such as Thr36 and Ser62 could increase or decrease the rate of phosphorylation and/or the influence of other sites on eIF-4E binding. Nevertheless, through the process of elimination, Thr45 and Thr69 have emerged as candidates for important regulatory sites.

Implications of Multisite Phosphorylation With Respect to Signaling Pathways—The protein kinases responsible for phosphorylating PHAS-I in adipocytes have not been determined. However, it is now possible to exclude certain protein kinases that are capable of phosphorylating PHAS-I in vitro, Diggle et al. (25) first demonstrated that casein kinase II phosphorylated PHAS-I purified from adipocyte extracts. Based on evidence that insulin activated casein kinase II in adipocytes, it was proposed that casein kinase II mediated the phosphorylation of PHAS-I by insulin (25). None of the five sites phosphorylated in rat adipocytes meet the minimum consensus requirement for phosphorylation by casein kinase II (26). We have recently found that casein kinase II preferentially phosphorylates Ser111, which is not phosphorylated in cells. Thus, it is clear that casein kinase II cannot be the major mediator of the action of insulin on PHAS-I. Similarly, none of the sites phosphorylated in vitro meet the requirements for phosphorylation by protein kinase C (26), which phosphorylates recombinant PHAS-I (6, 24).

MAP kinase is by far the most effective kinase that has been described for phosphorylating PHAS-I in vitro (6, 16, 24). MAP kinase is activated by insulin in adipocytes (39), and all five sites phosphorylated by MAP kinase in vitro were phosphorylated in response to the hormone (Fig. 6). Despite this finding, neither the ERK1 nor ERK2 isoform is the sole mediator of the phosphorylation of PHAS-I in response to insulin in adipocytes as blocking MAP kinase activation with the MEK inhibitor, PD 098059, did not attenuate the effect of insulin in these cells (15). As discussed previously (15), this result does not eliminate the possibility that MAP kinase contributes to the control of PHAS-I phosphorylation as more than one pathway may be involved. Interestingly, PD 098059 was recently found to promote dephosphorylation of PHAS-I in CHO cells (40). Thus, there is reason to suspect that MAP kinase, or another kinase regulated by MEK, is involved in the control of PHAS-I in certain cell types.

PHAS-I phosphorylation is controlled by a rapamycin-sensitive pathway that is distinct from the MAP kinase signaling pathway (15, 16, 19, 21). The most extensively characterized enzyme regulated by rapamycin is p70S6K, a protein kinase that is activated by insulin and a variety of mitogenic stimuli (23). Full activation of the kinase appears to require phosphorylation of two classes of sites. One class consists of three Ser/Thr residues (Thr229, Thr233, and Ser404) that are flanked by aromatic residues (41). The other consists of four Ser/Thr-Pro residues found in a 14-amino acid stretch located near the COOH terminus of the kinase in a region referred to as the autoinhibitory domain (42, 43). Rapamycin is a potent inhibitor of the activation of p70S6K (44, 45), and an obvious possibility was that p70S6K was responsible for phosphorylating PHAS-I in cells. However, this hypothesis was eliminated by the finding that PHAS-I was not a substrate for p70S6K (6, 16, 24).

The five phosphorylation sites in PHAS-I resemble the four sites in the autoinhibitory domain of p70S6K (42). Both sets of sites are markedly increased in response to insulin or mitogenic stimulation (6, 23), and it is an intriguing possibility that the Ser/Thr-Pro sites in the two proteins are phosphorylated by the same protein kinase. Phosphorylation of PHAS-I and p70S6K by a common kinase would provide a mechanism linking capped mRNA translation, which is dependent on eIF-4E (9), and polypyrimidine tract mRNA translation, which appears to be regulated by phosphorylation of ribosomal protein S6 (46). However, circumstances might exist in which it would not be advantageous for a cell to up-regulate translation of both classes of mRNA, and the potential exists for selective regulation of the phosphorylation of PHAS-I and p70S6K. PHAS-I lacks the hydrophobic class of sites found in p70S6K as none of the Ser/Thr residues in PHAS-I are flanked by aromatic residues. The closest resemblance to the hydrophobic sites are two Ser that are adjacent to Phe and Tyr in a tandem repeat of the sequence, (Tyr/Phe)-Ser-Thr-Thr-Pro-Gly-Gly (Fig. 1). Neither of these Ser residues were phosphorylated to a significant extent in adipocytes as relatively little 32P released in cycle 1 when the CT-P1 and CT-P2 peptides were subjected to sequential Edman degradation (Fig. 6). In view of the differences in sequence surrounding the two classes of sites, it seems clear that a single protein kinase is not responsible for phosphorylating PHAS-I and the hydrophobic sites in p70S6K. This is an important point in considering the action of rapamycin, which decreases four of the Ser/Thr-Pro sites in PHAS-I (Fig. 8) and all three hydrophobic sites in p70S6K (41). However, it should be noted that rapamycin does promote dephosphorylation of p70S6K in Ser411, a site having Pro in the +1 position (41).

It is well established that rapamycin inhibits the mTOR signaling pathway, but how mTOR signals is still a mystery (22). A region of mTOR has homology with the catalytic domain of phosphatidyl inositol 3’-OH kinase, but it is not clear that mTOR functions as a lipid kinase. Indeed, there is good reason to suspect that mTOR signals as a protein kinase as it is homologous to the new class of protein kinases, which include the catalytic subunit of DNA-dependent protein kinase and the ATM gene product that is mutated in ataxia telangiectasia (47). In addition, the protein undergoes autophosphorylation in a reaction that is subject to inhibition by rapamycin (48). The effect of rapamycin on autophosphorylation, as well as the inhibitory effect of rapamycin on mTOR in cells, requires an additional receptor protein, FKBP12, as it is the rapamycin-FKBP12 complex that actually binds to mTOR (22). Rapamycin binding to FKBP12 is competitively inhibited by FK506, and because the FK506-rapamycin complex does not bind mTOR, FK506 competitively inhibits the effects of rapamycin on mTOR-dependent pathways (22).

The findings that FK506 blocks the effects of rapamycin on PHAS-I (15, 19) and p70S6K (44, 45) provide strong evidence that the mTOR signaling pathway regulates both proteins. Results of recent experiments in T lymphoma cells (YAC-1) provide additional evidence implicating mTOR in the control of
PHAS-I (20). In wild-type lymphoma cells rapamycin potently decreased PHAS-I phosphorylation, but rapamycin had little, if any, effect on PHAS-I in mutant lymphoma cells that had been selected on the basis of resistance to the antiproliferative effects of rapamycin (20). There is strong evidence that the rapamycin-resistant phenotype is due to a mutation which decreases the affinity of mTOR for rapamycin (49). Thus, there is both pharmacologic and genetic evidence supporting the conclusion that the mTOR signaling pathway is involved in the control of PHAS-I. The challenge now is to identify the PHAS-I kinases and/or phosphatases involved in the mTOR-dependent control of PHAS-I.

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