Ser-64 and Ser-111 in PHAS-I Are Dispensable for Insulin-stimulated Dissociation from eIF4E*

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Insulin stimulates phosphorylation of multiple sites in the eIF4E-binding protein, PHAS-I, leading to dissociation of the PHAS-I/eIF4E complex and to an increase in cap-dependent translation. The Ser-64 and Ser-111 sites have been proposed to have key roles in controlling the association of PHAS-I and eIF4E. To determine whether the effects of insulin require these sites, we assessed the control of PHAS-I proteins having Ala-64 or Ala-111 mutations. The results indicate that phosphorylation of neither site is required for insulin to promote release of PHAS-I from eIF4E. Also, the mutation of Ser-111, which has been proposed to serve as a necessary priming site for the phosphorylation of other sites in PHAS-I, did not affect the phosphorylation of Thr-36/45 in Ser-64, or Thr-69. Insulin promoted the release of eIF4E from PHAS-II, a PHAS-II isoform that lacks the Ser-111 site, but it was without effect on the amount of eIF4E bound to the third isoform, PHAS-III. The results demonstrate that contrary to widely accepted models, Ser-64 and Ser-111 are not required for the control of PHAS-I binding to eIF4E in cells, implicating phosphorylation of the Thr sites in dissociation of the PHAS-I/eIF4E complex. The findings also indicate that PHAS-II, but not PHAS-III, contributes to the control of protein synthesis by insulin.

Insulin and certain amino acids stimulate cap-dependent translation by promoting the phosphorylation of PHAS-I, the best characterized member of a family of three translational repressor proteins expressed in a wide variety of cell types (1, 2). Hypophosphorylated PHAS-I binds tightly to eIF4E (3, 4), the mRNA cap-binding protein, and inhibits cap-dependent translation by blocking the association between eIF4E and eIF4G (5, 6). When phosphorylated in the appropriate sites, PHAS-I dissociates from eIF4E (3, 4), allowing the formation of the 5′ complex needed for efficient binding and/or scanning by the 40 S ribosomal subunit. The signaling pathways utilized by insulin and amino acids converge at the level of mTOR (1, 2). Thus, rapamycin and other inhibitors of mTOR attenuate the phosphorylation of PHAS-I.

The following six phosphorylation sites in PHAS-I have been identified: Thr-36; Thr-45; Ser-64; Thr-69; Ser-82; and Ser-111 (7, 8). Which sites are most important in the control of the association of PHAS-I and eIF4E in cells has not been established. The sites conform to a (Ser/Thr)-Pro motif (7) with the exception of Ser-111, which is followed by Gln. Thr-36, Thr-45, Ser-64, and Thr-69 are phosphorylated in response to insulin (7, 9, 10), and these sites are conserved in PHAS-I proteins from different species as well as in the two other PHAS isoforms (2). Ser-82 is less conserved than the other (Ser/Thr)-Pro sites, and phosphorylation of Ser-82 in vitro does not inhibit binding to eIF4E (11). Moreover, in cells, this site appears to contain relatively little phosphate and it is insensitive to insulin (7, 9). Therefore, Ser-82 does not appear to contribute significantly to the control of PHAS-I. There is a great deal of interest in the phosphorylation of the other two Ser phosphorylation sites, Ser-64 and Ser-111.

Ser-64 undergoes the most dramatic increase in phosphorylation in response to insulin (12), and it is phosphorylated only after phosphorylation of the three Thr-Pro sites (10, 13). Ser-64 has been considered to be a prime candidate for promoting dissociation of the PHAS-I/eIF4E complex, because its phosphorylation in vitro markedly decreases the affinity of PHAS-I for eIF4E (3, 9, 14). Ser-111 was first identified as the major site for in vitro phosphorylation by protein kinase CK2 (15). More recently, this site has been shown to be phosphorylated in vitro by the phosphatidylinositol 3-OH kinase-related protein kinases, ATM, ATR, and Smg1 (15–18). Phosphorylation of Ser-111 has been suggested to serve as a priming event, which is necessary for both the phosphorylation of other sites in PHAS-I and the dissociation of the PHAS-I/eIF4E complex (8, 18). This study was conducted to investigate the role of Ser-64 and Ser-111 in the control of PHAS-I binding to eIF4E.

EXPERIMENTAL PROCEDURES

Antibodies—Antibodies to the COOH-terminal regions of PHAS-I and eIF4E (19) and the phosphospecific antibodies, P-Thr-36/45, P-Ser-64, and P-Thr-69 (10), were generated in rabbits and affinity-purified as described previously (10, 19). The sequences of amino acids immediately surrounding Thr-36 and Thr-45 are identical. Consequently, the P-Thr-36/45 antibodies bind to PHAS-I phosphorylated in either Thr-36 or Thr-45 (10). Monoclonal antibody to the FLAG epitope tag was from Sigma. Monoclonal antibodies 9E10 and 12CA5, which recognize the Myc and HA epitope tags, respectively, were purified from hybridoma culture medium.

Expression Vectors—cDNA encoding rat PHAS-I (12), mouse PHAS-II (19), and PHAS-I proteins having Ser-64 to Ala (3) and Ser-111 to Ala (15) mutations were generated and subcloned into pET-14b (Novagen) as described previously (3, 12, 15, 19). To create vectors for expressing Myc epitope-tagged proteins, PHAS-I (pCMV-TagAHA3 PHAS-I), pCMV-TagAHA111 PHAS-I, and PHAS-II (pCMV-TagAHA3 PHAS-II) and PHAS-II (pCMV-TagAHA111) proteins, cDNAs were excised from pET-14b by using BamHI and inserted into the BamHI site of pCMV-Tag 3A (Stratagene).
were sequenced and found to be free of errors.2 Inserts encoding the proteins (Stratagene). PHAS and eIF4E clones having the proper orientation were selected by restriction mapping. Inserts encoding the proteins were digested with EcoRI and inserted into the EcoRI site of pCMV-Tag 2B plus either pCMV-Tag 3A PHAS-I or pCMV-Tag 3A PHAS-I. After 48 h, the cells were rinsed in low phosphate buffer and incubated as described under “Experimental Procedures” with no additions, 20 mM rapamycin (RAP), 100 milliunits/ml insulin (INS), 2.5× minimum essential medium amino acids (AA), or the combinations indicated. A, extract samples were subjected to SDS-PAGE, and immunoblots denoted the three mobility forms of Myc-tagged PHAS-I resolved. B, the relative intensities of the α, β, and γ bands were determined by optical density scanning of films. To allow comparison among experiments, the intensity of each band was expressed as a percentage of the total. Results presented are mean values ± S.E. from five experiments.

A construct (pKH2PHAS-III) for expressing HA-tagged PHAS-III in cells was generated by excising PHAS-III cDNA from pGEX-2T with BamHI and EcoRI and inserting it between these sites in pKH3 (20). Human PHAS-III in pGEX-2T was supplied by Dr. Tai-An Lin. To prepare an expression vector for FLAG-tagged eIF4E (pCMV-Tag 2B), EcoRI sites were added to the ends of human eIF4E cDNA by using the polymerase chain reaction with 5′-GGAATTCATGGCCGACTGTCGAAACCGG-3′ and 5′-GGAATTCCTAAACACAAACACCTATTATTAG-3′ as forward and reverse primers, respectively. The product was digested with EcoRI and inserted into the EcoRI site of pCMV-Tag 2B (Stratagene). PHAS and eIF4E clones having the proper orientation were selected by restriction mapping. Inserts encoding the proteins were sequenced to be free of errors.2

Cell Culture and Transfections—HEK293 cells (2.5 × 10⁶) were seeded into 150-mm dishes and cultured for 24 h at 37 °C in Dulbecco’s modified Eagle’s medium supplemented with 2 mM l-glutamine, 10% fetal bovine serum, 10 units/ml penicillin G, and 10 µg/ml streptomycin. A modified CaPO₄ procedure was used to introduce DNA (34 µg each of the PHAS-I and eIF4E expression constructs or the pCMV-Tag 2B or pCMV-Tag 3A vectors) into the cells (21). Cells were incubated with the precipitated DNA for 4 h. The medium was then replaced, and the cells were cultured in growth medium. After 15 h, cells from each 150-mm dish were removed using 0.02% trypsin, seeded into six 60-mm-diameter culture dishes, and cultured for 26 h. This procedure provided equal expression of proteins among treatment groups. The cells were serum-starved prior to incubations with insulin and/or rapamycin. For serum starvation, the growth medium was replaced with Dulbecco’s modified Eagle’s medium containing 0.2% bovine serum albumin (Intergen) and the cells were incubated at 37 °C for 15 h. The Dulbecco’s modified Eagle’s medium was replaced with buffer (145 mM NaCl, 5.4 mM KCl, 1.4 mM CaCl₂, 1.4 mM MgSO₄, 5 mM glucose, 0.5% bovine serum albumin, 0.1 mM sodium phosphate, 25 mM NaHCO₃, and 10 mM HEPES, pH 7.4), and the cells were incubated for 1.5 h. Cells were incubated with or without rapamycin (20 nM) for 1 h and then incubated for an additional 30 min after adding insulin (100 milliunits/ml) and/or amino acids (2.5× minimum essential medium amino acid mixture). To terminate the incubations, the cells were placed on ice, rinsed with chilled lysis buffer minus Nonidet P-40, and then immediately scraped into 1 ml of lysis buffer (100 mM NaCl, 50 mM NaF, 10 mM EDTA, 1% Nonidet P-40, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 500 µM microcin, 10 mM sodium pyrophosphate, and 50 mM HEPES, pH 7.4). The samples were mixed at 4 °C for 1 h and centrifuged at 13,000 × g for 20 min before the supernatants were retained for analyses. Extract protein concentrations were adjusted to 0.5 mg/ml by adding lysis buffer. Protein was measured by using bicinchoninic acid (22).

Immunoprecipitation of PHAS-I/eIF4E Complexes—eIF4E proteins bound to epitope-tagged PHAS proteins were isolated by incubating extracts (0.5 ml) for 18 h at 4 °C with protein G-agarose (Invitrogen) (10 µl of packed beads), 0.2% IgG-free bovine serum albumin, and 5 µg of the 9E10 monoclonal antibody for Myc-tagged PHAS-I and PHAS-II proteins or with 5 µg of 12CA5 for HA-tagged PHAS-III. Immunoprecipitation complexes bound to the beads were recovered by centrifugation. The beads were washed twice (1 ml/wash) with lysis buffer and twice with lysis buffer minus Nonidet P-40. Proteins were eluted from the beads by using SDS sample buffer (23).

Affinity Purification of eIF4E Complexes—eIF4E was partially purified by using m’GTP-Sepharose 4B (Amersham Biosciences). Extract samples (250–300 µg protein) were mixed with 20 µl of m’GTP-Sepharose 4B before the beads were washed essentially as described previously (29). Proteins were eluted from the beads with SDS sample buffer (23).

Electrophoretic Analyses—Samples were subjected to electrophoresis using the method of Laemmli (23). Proteins were transferred to membranes (Immobilon) and immunoblotted with the appropriate antibodies as described previously (19). Bound antibodies were detected by using alkaline phosphatase-conjugated secondary antibodies and CDP-Star (Tropix). Signal intensities were determined by scanning laser densitometry of films (Kodak X-Omat AR-5).

RESULTS

Influence of Ser-64 on the Control of PHAS-I by Insulin—To determine whether Ser-64 phosphorylation was required for the control of PHAS-I by insulin, a Ser to Ala mutation was introduced in the site to prevent its phosphorylation in cells.

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tagged PHAS-I proteins were determined by densitometric scanning (FLAG-tagged plus endogenous) coimmunoprecipitating with Myc-FLAG-tagged eIF4E, which migrates more slowly than the endogenous /H18528 arrows with eIF4E antibody. The legend to Fig. 1. Myc-tagged PHAS-I proteins were immunoprecipitated cells were transfected with vectors and incubated as described in the analyses, we attempted to maintain eIF4E levels by coexpressing the translational repressor, PHAS-I, may dramatically decrease cap-dependent translation by decreasing eIF4E availability (4). Because inhibition of protein synthesis may alter PHAS-I phosphorylation (24), which would complicate the phosphorylation of Ser-64 (2), which in the hierarchy of PHAS-I occurs with the well established action of the hormone to promote dissociation of the PHAS-I-eIF4E complex (1, 2). Mutating Ser-64 to Ala did not attenuate the response to insulin, indicating that phosphorylation of Ser-64 is not required for dissociation of the complex. Indeed, the amount of eIF4E complexed with Ala-64 PHAS-I was indistinguishable from that bound to wild type PHAS-I after all of the treatments tested (Fig. 2B). Rapamycin attenuated the effects of insulin on increasing the dissociation of eIF4E from both the wild type and mutant PHAS-I proteins. Without insulin, amino acids slightly decreased the amount of eIF4E that coimmunoprecipitated with Ala-64 PHAS-I but did not affect the amount of eIF4E that coimmunoprecipitated with Ala-64 PHAS-I. Amino acids did not significantly enhance the effect of insulin on promoting dissociation of eIF4E from either PHAS-I protein (Fig. 2B). Thus, as we have demonstrated previously (9, 10), the effect of insulin on PHAS-I does not require the addition of amino acids to the medium.

Influence of Ser-111 on Control of PHAS-I—An approach similar to that described above for investigating the role of Ser-64 was used to determine whether the phosphorylation of Ser-111 was needed for the control of PHAS-I by insulin. Mutating Ser-111 to Ala was without effect on the electrophoretic mobility of PHAS-I in either the absence or presence of insulin, rapamycin, or amino acids (Fig. 3, A and B). Thus, the mobility shift assay provided no indication that Ser-111 phosphorylation was required for the phosphorylation of other sites in PHAS-I. However, because phosphorylation of some sites (Thr-36 and Thr-45, for example) has relatively little effect on the electrophoretic mobility of PHAS-I in SDS-PAGE (2), we investigated phosphorylation of different sites by immunoblotting with phosphospecific antibodies.

In the experiments presented in Fig. 4, wild type PHAS-I and Ala-111 PHAS-I were immunoprecipitated and probed with phosphospecific antibodies to the Thr-36/45, Thr-69, and Ser-64 sites (10). Insulin increased the reactivity of PHAS-I with all three phosphospecific antibodies (Fig. 4, A–D). As noted previously (9, 10), the effect of insulin on Thr-36/45 phosphorylation (Fig. 4B) was less than the effects of the hormone on the phosphorylation of either Ser-64 (Fig. 4C) or Thr-69 (Fig. 4D). The phosphorylation of Ser-64 and Thr-69 was more sensitive to inhibition by rapamycin than the phosphorylation of Thr-36/45.

When corrected for protein expression, the reactivities of the three phosphospecific antibodies for wild type and Ala-111 PHAS-I were indistinguishable (Fig. 4, B–D), confirming that Ser-111 phosphorylation is not required for the phosphorylation of Thr-36/45, Ser-64, and Thr-69. Consistent with the lack of effect of the Ala-111 mutation on phosphorylation, eIF4E binding to Ala-111 PHAS-I assessed by coimmunoprecipitation was almost identical to binding of the initiation factor to wild type PHAS-I (Fig. 5, A and B).

Effect of Insulin and Rapamycin on PHAS-II and PHAS-III Binding to eIF4E—The five (Ser/Thr)-Pro sites found in PHAS-II are also present in PHAS-II; however, PHAS-II lacks the equivalent of the Ser-111 site (25). Therefore, we assessed the effects of insulin and rapamycin on the association of

Myc epitope-tagged forms of this mutant protein and wild type PHAS-I were transiently overexpressed in HEK293 cells. Overexpressing the translational repressor, PHAS-I, may dramatically decrease cap-dependent translation by decreasing eIF4E availability (4). Because inhibition of protein synthesis may alter PHAS-I phosphorylation (24), which would complicate the analyses, we attempted to maintain eIF4E levels by coexpressing FLAG epitope-tagged eIF4E. The transfected cells were treated with insulin, amino acids, and rapamycin before the PHAS-I proteins were immunoprecipitated with Myc antibodies. Samples were then subjected to SDS-PAGE, and immunoblots were prepared with antibodies to either PHAS-I (Fig. 1A) or eIF4E (Fig. 2A).

As described previously for endogenous PHAS-I (3), Myc-tagged wild type PHAS-I appeared in immunoblots as three bands designated α, β, and γ (Fig. 1A). No PHAS-I was detected in immunoprecipitates from the vector control cells, indicating that the signals detected were because of the overexpressed protein. Insulin decreased the α form and increased the γ form (Fig. 1B). Rapamycin opposed these actions of insulin and promoted an increase in the α form. Supplementing the medium with amino acids had little effect on the electrophoretic mobility of PHAS-I in either the absence or presence of insulin.

The mobility shifts promoted by insulin and rapamycin result from increases and decreases, respectively, in the phosphorylation of PHAS-I. The shift from β to γ occurs with the phosphorylation of Ser-64 (2), which in the hierarchy of PHAS-I phosphorylation is the last site phosphorylated (10, 13). As expected, mutating Ser-64 to Ala blocked formation of γ, even in insulin-treated cells (Fig. 1A). Although no γ form was present, insulin and rapamycin clearly affected the distribution of PHAS-I between the α and β forms. Indeed, when the α form was expressed as a percentage of the respective PHAS-I protein immunoprecipitated, the mutant protein was indistinguishable from wild type (Fig. 1B).

Insulin decreased by ~50% the amounts of both FLAG-eIF4E and endogenous eIF4E that coimmunoprecipitated with wild type PHAS-I (Fig. 2, A and B). This result is consistent with the well established action of the hormone to promote dissociation of the PHAS-I-eIF4E complex (1, 2). Mutating Ser-64 to Ala did not attenuate the response to insulin, indicating that phosphorylation of Ser-64 is not required for dissociation of the complex. Indeed, the amount of eIF4E complexed with Ala-64 PHAS-I was indistinguishable from that bound to wild type PHAS-I after all of the treatments tested (Fig. 2B). Rapamycin attenuated the effects of insulin on increasing the dissociation of eIF4E from both the wild type and mutant PHAS-I proteins. Without insulin, amino acids slightly decreased the amount of eIF4E that coimmunoprecipitated with wild type PHAS-I protein but did not affect the amount of eIF4E that coimmunoprecipitated with Ala-64 PHAS-I. Amino acids did not significantly enhance the effect of insulin on promoting dissociation of eIF4E from either PHAS-I protein (Fig. 2B). Thus, as we have demonstrated previously (9, 10), the effect of insulin on PHAS-I does not require the addition of amino acids to the medium.

In Fig. 2, Failure of Ser-64 to Ala mutation to reduce insulin-stimulated dissociation of PHAS-I-eIF4E complexes. HEK293 cells were transfected with vectors and incubated as described in the legend to Fig. 1. Myc-tagged PHAS-I proteins were immunoprecipitated using the 9E10 monoclonal antibody. A, immunoblots were prepared with eIF4E antibody. The arrows point to endogenous eIF4E and FLAG-tagged eIF4E, which migrates more slowly than the endogenous protein when subjected to SDS-PAGE. B, the total amounts of eIF4E (FLAG-tagged plus endogenous) coimmunoprecipitating with Myc-tagged PHAS-I proteins were determined by densitometric scanning and expressed as a percentage of the untreated controls. Mean values ± S.E. from three experiments are presented.
PHAS-II and eIF4E. Neither insulin nor rapamycin changed the electrophoretic mobility of PHAS-II (Fig. 6A). This was not unexpected because PHAS-II does not undergo the same shifts in electrophoretic mobility that PHAS-I does when it is phosphorylated (19). Treating cells with insulin decreased by 40% the amount of both FLAG-eIF4E and endogenous eIF4E that coimmunoprecipitated with PHAS-II (Fig. 6B), indicative of dissociation of the PHAS-II/eIF4E complex. To confirm that insulin stimulated the dissociation of PHAS-II and eIF4E, we partially purified eIF4E by using m7GTP-Sepharose to estimate the amount of PHAS-II present in a cap-binding complex with eIF4E (Fig. 6C). Insulin decreased by 40% the amount of PHAS-II that copurified with eIF4E. Amino acids did not affect the amount of eIF4E that coimmunoprecipitated with PHAS-II (Fig. 6B) or the amount of PHAS-II that copurified with eIF4E (Fig. 6C) in either the absence or presence of insulin. Rapamycin attenuated but did not abolish the effects of insulin on promoting dissociation of the PHAS-II/eIF4E complex (Fig. 6B and C). Thus, the association of PHAS-II and eIF4E appears to be controlled by insulin in a manner similar to PHAS-I and eIF4E.

We next investigated the effects of insulin, amino acids, and rapamycin on PHAS-III. Similar to PHAS-II, this isoform binds eIF4E, lacks the Ser-111 site (2), and does not undergo a shift in electrophoretic mobility in response to insulin or rapamycin (Fig. 7A). In contrast to both PHAS-I and PHAS-II, PHAS-III did not undergo appreciable dissociation from eIF4E when cells were incubated with insulin (Fig. 7B).

**DISCUSSION**

Insulin-stimulated dissociation of the PHAS-I/eIF4E complex in cells has been extensively documented, but relatively little information exists on the control of the other two PHAS isoforms. Results of this study provide the first demonstration that insulin promotes the dissociation of the PHAS-II-eIF4E complex in cells (Fig. 6). The decrease in PHAS-II bound to eIF4E produced by insulin was comparable to the decrease in PHAS-I bound to eIF4E. In contrast, insulin did not decrease the amount of PHAS-III bound to the initiation factor (Fig. 7) (26). Thus, it seems that PHAS-I and PHAS-II, but not PHAS-III, are mediators of insulin action on protein synthesis.

Phosphospecific antibodies are not available for the sites in PHAS-II. For this reason, we did not investigate the effects of insulin on the phosphorylation of individual sites in this PHAS isoform. However, previous results in 32P-labeled 3T3-L1 adipocytes indicate that insulin stimulates the phosphorylation of PHAS-II in a rapamycin-sensitive manner (19). This implies that the control of PHAS-II phosphorylation by insulin is similar to that of PHAS-I, although there must be some difference in the phosphorylation of the two proteins since PHAS-II contains Ala in the position equivalent to Ser-111 in PHAS-I. In view of the well established effect of phosphorylation on binding of PHAS-I to eIF4E, it seems reasonable to conclude that dissociation of the PHAS-II/eIF4E complex resulted from phosphorylation of PHAS-II.

Two motifs required for the efficient phosphorylation of PHAS-I in cells have been described previously (27, 28). The RAIP motif (named for the sequence Arg-Ala-Ile-Pro) is found in the NH2-terminal regions of PHAS-I and PHAS-II, but it is absent in PHAS-III (28). The TOS (for mTOR signaling) motif is formed by the last five amino acids (Phe-Glu-Met-Asp-Ile) in all three PHAS isoforms (27). Mutations in either domain also abolished tight binding of PHAS-I to eIF4E, but not PHAS-III. Therefore, it seems that the association of PHAS-II and eIF4E is controlled by insulin in a manner similar to PHAS-I and eIF4E.

**Fig. 3.** Effects of insulin, rapamycin, and amino acids on the electrophoretic mobilities of Myc-tagged wild type and Ala-111 PHAS-I. HEK293 cells were transfected with vectors alone (pCMV-Tag 2B and pCMV-Tag 3A) or with pCMV-Tag 2B*eIF4E plus either pCMV-Tag 3A*PHAS-I or pCMV-Tag 3A*PHAS-I. The cells were then incubated as described in the legend to Fig. 1. A, a PHAS-I blot is presented. B, the relative intensities of the α, β, and γ bands were determined by optical density scanning of films and expressed as percentages of the total. Mean values ± S.E. from seven experiments are presented.
to raptor and markedly decreased phosphorylation of PHAS-I by mTOR in vitro (29, 31–33). The fact that PHAS-I and PHAS-II each contain both motifs may account for similarities in their regulation by insulin. As previously proposed by Wang et al. (26), a lack of the RAIP motif may explain the relative insensitivity of PHAS-III to insulin.

The present finding that Ser-64 phosphorylation is dispensable for insulin-dependent control of the PHAS-I/eIF4E complex was surprising in view of previous observations. Ser-64 was identified as the site undergoing the largest increase in phosphorylation in response to insulin even before PHAS-I was identified as an eIF4E-binding protein (12). Initial studies demonstrated that selectively phosphorylating Ser-64 abolished high affinity binding of PHAS-I to eIF4E (3, 10). More recent measurements of binding affinity of purified proteins by surface plasmon resonance indicate that phosphorylation of this site decreases the affinity for eIF4E by 100-fold (14). Changes in affinity due to phosphorylation of other sites individually were relatively small (11, 14). Finally, there is the phosphorylation hierarchy in which the three Thr-Pro sites in PHAS-I must be phosphorylated before Ser-64 can be phosphorylated (9, 13). These observations contributed to an attractive model in which progressive phosphorylation of the three Thr-Pro sites culminates in the phosphorylation of Ser-64 and dissociation of the PHAS-I/eIF4E complex (1). The present findings indicate that this model is incorrect in the sense that Ser-64 is dispensable for insulin-stimulated dissociation of the complex (Fig. 2).

Although phosphorylating the Thr-Pro sites in PHAS-I indi-
individually had small effects on the affinity of PHAS-I for eIF4E relative to that of phosphorylating Ser-64 (11, 14), it is possible that phosphorylating a combination of the Thr-Pro sites in PHAS-I would produce more pronounced effects. Consequently, the present conclusion that phosphorylation of the Thr-Pro sites is sufficient for insulin-stimulated dissociation of the PHAS-I/eIF4E complex is not inconsistent with previous studies (11, 14), which did not address the effect of phosphorylating multiple Thr-Pro sites. We cannot exclude the possibility that Ser-64 phosphorylation contributes to the control of the association state of PHAS-I and eIF4E in response to stimuli other than insulin or in manner too subtle to allow detection with the current assays. However, Ser-64 phosphorylation might control other functions of PHAS-I such as PHAS-I binding to raptor. Mutating the five (Ser/Thr)-Pro sites markedly increased the binding of PHAS-I to raptor, suggesting that phosphorylation of PHAS-I promotes dissociation of the PHAS-I/raptor complex (33).

Diggle et al. (34) were the first to show that PHAS-I was phosphorylated by protein kinase CK2 (34). Fadden et al. (15) identified Ser-111 as the major site of phosphorylation by this kinase in vitro but questioned the role of this site because its phosphorylation was not detected when PHAS-I was immunoprecipitated from 32P-labeled rat adipocytes. A possible explanation for the failure to detect Ser-111 phosphorylation in cells was provided by Denton's group, who observed significant phosphorylation of the site only in the PHAS-I that was bound to eIF4E (8). This finding led to the proposal that Ser-111 serves as a priming site whose phosphorylation is necessary for the phosphorylation of other sites in PHAS-I and for dissociation of the PHAS-I/eIF4E complex. Such a role gained acceptance as a result of a report that mutation of Ser-111 to Ala decreased the phosphorylation of the other sites in PHAS-I overexpressed 293T cells (18). The present findings clearly do not support this model. We observe no difference in the electrophoretic mobilities of wild type PHAS-I and Ala-111 PHAS-I, indicating that mutation of Ser-111 does not affect the phosphorylation of sites involved in the mobility shift (Fig. 3). Results of multiple experiments with phosphospecific antibodies demonstrate that phosphorylation of Thr-36/45, Thr-69, and Ser-64 were not influenced by mutating Ser-111 to Ala (Fig. 4).

FIG. 6. Effects of insulin, rapamycin, and amino acids on the association of PHAS-II and eIF4E. HEK293 cells were transfected with vectors alone (pCMV-Tag 2B and pCMV-Tag 3A) or with pCMV-Tag 2B-eIF4E plus pCMV-Tag 3A-PHAS-II. The cells were incubated as described in the legend to Fig. 1 before extracts were prepared and Myc-tagged PHAS-II was immunoprecipitated with the 9E10 antibody. A, immunoblots were prepared with antibodies to PHAS-II and eIF4E. B, the amounts of total eIF4E (FLAG-tagged plus endogenous) coimmunoprecipitating with Myc-PHAS-II were estimated from optical density scans of eIF4E immunoblots. Results are expressed relative to control and are mean values ± S.E. from five experiments. C, eIF4E was partially purified by using m′GTP-Sepharose. Relative amounts of PHAS-II copurifying with eIF4E are expressed relative to control and are mean values ± S.E. from four experiments.

FIG. 7. Failure of insulin and rapamycin to change the association of PHAS-III and eIF4E. HEK293 cells were transfected with vectors alone (pCMV-Tag 2B and pH3) or with pCMV-Tag 2B-eIF4E plus pH3-PHAS-III. After 48 h, the cells were rinsed in low phosphate buffer and incubated with no additions, 20 nM rapamycin (RAP), 100 milliunits/ml insulin (INS), or the combination of rapamycin plus insulin. HA-tagged PHAS-III was immunoprecipitated with the 12CA5 monoclonal antibody before samples were subjected to SDS-PAGE. A, representative immunoblots showing HA-PHAS-III and eIF4E proteins. B, the amounts of total eIF4E (FLAG-tagged plus endogenous) coimmunoprecipitating with Myc-PHAS-III were estimated and expressed relative to control. Mean values ± S.E. from four experiments are presented.
Similar results were very recently reported by Wang et al. (26). These findings support the conclusion that Ser-111 phosphorylation is not required for the phosphorylation of other sites in PHAS-I.

Yang and Kastan (18) proposed that phosphorylation of Ser-111 by ATM mediates insulin-stimulated dissociation of the PHAS-1 eIF4E complex. This hypothesis was both provocative and exciting, not only because it linked ATM-mediated check-point control, insulin action, and cap-dependent protein synthesis, but also because it appeared to identify ATM as an important kinase in insulin action. Although our studies have not directly addressed the role of ATM, it is clear that phosphorylation of Ser-111 is not required in the control of PHAS-I and PHAS-II (Fig. 6), which lacks the Ser-111 site (2), provides confirmation that phosphorylation of the Ser-111 site is not essential for dissociation of PHAS-I eIF4E from PHAS-II (Fig. 6).

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