The role of PRAS40 in insulin action : at the intersection of protein kinase B (PKB/Akt) and mamalian target of rapamyein (mTOR)

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Chapter 4

Phosphorylation of PRAS40 on Thr246 by PKB/Akt facilitates efficient phosphorylation of Ser183 by mTORC1

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Abstract

Type 2 diabetes is associated with alterations in protein kinase B (PKB/Akt) and mammalian target of rapamycin complex 1 (mTORC1) signalling. The proline-rich Akt substrate of 40 kDa (PRAS40) is a component of mTORC1, which has a regulatory function at the intersection of the PKB/Akt- and mTORC1 signalling pathway. Phosphorylation of PRAS40-Thr246 by PKB/Akt, and PRAS40-Ser183 and PRAS40-Ser221 by mTORC1 results in dissociation from mTORC1, and its binding to 14-3-3 proteins. Although all phosphorylation sites within PRAS40 have been implicated in 14-3-3 binding, substitution of Thr246 by Ala alone is sufficient to abolish 14-3-3 binding under conditions of intact mTORC1 signalling. This suggests that phosphorylation of PRAS40-Thr246 may facilitate efficient phosphorylation of PRAS40 on its mTORC1-dependent sites. In the present study, we investigated the mechanism of PRAS40-Ser183 phosphorylation in response to insulin. Insulin promoted PRAS40-Ser183 phosphorylation after a hyperinsulinaemic-euglycaemic clamp in human skeletal muscle. The insulin-induced PRAS40-Ser183 phosphorylation was further evidenced *in vivo* in rat skeletal and cardiac muscle, and *in vitro* in A14 fibroblasts, 3T3L1 adipocytes and L6 myotubes. Inhibition of mTORC1 by rapamycin or amino acid deprivation partially abrogated insulin-mediated PRAS40-Ser183 phosphorylation in cultured cell lines. However, lowering insulin-induced PRAS40-Thr246 phosphorylation using wortmannin or palmitate in cell lines, or by feeding rats a high-fat diet, completely abolished insulin-mediated PRAS40-Ser183 phosphorylation. In addition, replacement of Thr246 by Ala reduced insulin-mediated PRAS40-Ser183 phosphorylation. We conclude that PRAS40-Ser183 is a component of insulin action, and that efficient phosphorylation of PRAS40-Ser183 by mTORC1 requires phosphorylation of PRAS40-Thr246 by PKB/Akt.

Introduction

The mammalian target of rapamycin complex 1 (mTORC1) regulates many cellular responses including protein synthesis, autophagy, mitochondrial function, and cell cycle progression (1-3). Activation of mTORC1 occurs through the amino acid-dependent binding of the GTP-bound form of the small GTP-binding protein Ras homolog-enriched in brain (Rheb) to its catalytic subunit, the mammalian target of rapamycin (mTOR) (4). The levels of GTP-bound Rheb are regulated by tuberous sclerosis complex (TSC), which acts as a GTPase activating protein on Rheb (5). Insulin inhibits the activity of TSC through phosphorylation of TSC2 by protein kinase B (PKB/Akt) (6;7). This relieves Rheb from the inhibitory GTPase activity and allows Rheb-GTP to activate mTORC1 (8).

PKB/Akt also phosphorylates a component of mTORC1, the proline-rich Akt substrate of 40-kDa (PRAS40) (9). Binding of PRAS40 to mTORC1 requires the TOR signalling motif located between Phe129 and Ile133, and may in addition involve the region between Lys182 and Pro185 (10-12). Incubation of cells with insulin, platelet-derived growth factor, nerve growth factor, or nutrients like glucose and amino acids induces the phosphorylation of PRAS40 on multiple residues, including Ser183, Ser221, and Thr246 (13-17). Whereas PKB/Akt has been identified as the main regulator of Thr246 phosphorylation (18), mTORC1 itself has been implicated in the phosphorylation of PRAS40 on Ser183, and Ser221 (19;20). Phosphorylation of PRAS40 disrupts the interaction between mTORC1 and PRAS40, which may relieve an inhibitory constraint on mTORC1 activity (21;22). Phosphorylated PRAS40 also binds to 14-3-3 chaperone proteins (23-25). The interaction between 14-3-3 proteins and PRAS40 requires both the PKB/Akt- and mTORC1-mediated phosphorylation of PRAS40 since inhibition of phosphatidylinositol 3'-kinase (PI3K) activity or amino acid deprivation almost completely abolishes insulin-induced binding of PRAS40 to 14-3-3 proteins (26-28).

Insulin resistance is characterized by an impaired insulin-mediated activation of PI3K and PKB/Akt (29;30). Accordingly, alterations in insulin sensitivity were found to affect the PKB/Akt-regulated phosphorylation of PRAS40-Thr246 *in vivo* (31;32). Specifically, insulin-mediated PRAS40-Thr246 phosphorylation is reduced in tissues from insulin-resistant high-fat diet fed rats and ob/ob-mice (33;34). Reciprocally, weight loss, which improves insulin sensitivity in obese insulin resistant patients, restores the induction of PRAS40-Thr246 phosphorylation in response to hyperinsulinaemia in skeletal muscle (35).

It is unclear whether alterations in PRAS40-Thr246 phosphorylation impact on the activity of mTORC1. However, substitution of Thr246 by Ala is sufficient to abolish its binding to 14-3-3 proteins under conditions of intact mTORC1 signalling (36;37). This raises the possibility that phosphorylation of PRAS40-Thr246 may facilitate efficient phosphorylation of PRAS40 on its mTORC1-dependent sites. In the present study, we therefore examined the regulation of PRAS40-Ser183 phosphorylation by insulin in both the physiological

situation, and under conditions of reduced Thr246-phosphorylation, by either pharmacological inhibition of PI3K, high-fat diet-induced insulin resistance, or substitution of Thr246 by Ala.

Materials and methods

Materials. Tissue culture media were purchased from Invitrogen (Carlsbad, CA, USA). Rapamycin and wortmannin were obtained from Calbiochem (Darmstadt, Germany). Insulin and palmitic acid were obtained from Sigma Aldrich (St. Louis, MO, USA). Fatty-acid free bovine serum albumin (BSA; fraction V) was purchased from Roche Biochemicals (Indianapolis, IN, USA). Palmitate was complexed to BSA by sonification (5 min) and FBS heating (10 min; 55°C), and dissolved in DMEM (final concentration of 2% fatty-acid free BSA).

Human biopsies. Muscle biopsies were obtained from 6 obese subjects with type 2 diabetes (4 male and 2 female subjects, duration of type 2 diabetes 8.7±1.9 years, age 59.0±1.6 years, with fasting plasma glucose 13.1±1.3 mmol/l, HbA1c 8.1±0.6%, body weight 100.9±5.7 kg, and BMI 35.2±1.9 kg/m^2) participating in a clinical study that was approved by the medical ethical committee of the Leiden University Medical Center. Patients used at least 20 units of exogenous insulin. Four patients also used metformin and 1 patient used sulfonylurea derivatives. Written informed consent was obtained from the patients following explanation of the experimental procedures. Muscle biopsies were collected from the vastus lateralis muscle in the basal situation (overnight fast) and 30 min after initiation of a hyperinsulinaemic-euglycaemic clamp (insulin infusion rate: 40 mU/m^2 per min) using a modified Bergström needle under localized anaesthesia with 1% lidocaine as described previously (38). Muscle samples were snap-frozen in liquid nitrogen-cooled isopentane and stored at −80°C until further analysis.

Animals. Rat tissues were obtained from a previously published study, in which adult male Wistar WU rats were fed a high- or low-fat diet for 7 weeks (39;40). This investigation conformed to the Guide for the Care and use of Laboratory Animals, as published by the National Institute of Health (NIH publ. no 85-23, revised 1996) and the regulations of the institutional animal care and use committee. Following a 6 h fast, rats received an intraperitoneal injection with saline or insulin (10 U/kg body weight; Actrapid 100 U/ml; Novo Nordisk, Alphen aan den Rijn, The Netherlands) 30 min before being sacrificed by decapitation. Tissues were rapidly removed, snap frozen in liquid nitrogen-cooled isopentane and stored at −80°C until further analysis.

Plasmid generation. cDNA encoding human PRAS40 (hPRAS40) was kindly provided by Dr. R.A. Roth (Stanford University, Stanford, CA, USA) and cloned behind a CMV promoter in a plasmid which also contains green fluorescent protein (GFP) behind an IRES (41). Via mutagenesis PCR, a stop codon was introduced at the end of the coding sequence

of hPRAS40, and Phe at position 129, Ser at position 183, and Thr at position 246, were stepwise mutated into Ala. This resulted in constructs encoding wild type hPRAS40 and mutant Phe129Ala-hPRAS40, Ser183Ala-hPRAS40, Thr246Ala-hPRAS40, and Ser183Ala/Thr246Ala-hPRAS40. All constructs were verified by sequence analysis.

Cell culture. A14 cells are NIH3T3 fibroblasts overexpressing the human insulin receptor (42). L6 myoblasts were obtained from ATCC (CRL-1458), grown to confluence on α-MEM supplemented with 10% FBS, penicillin, and streptomycin. Cells were differentiated into myotubes by culturing them for 7 days on α-MEM supplemented with 2% horse serum and antibiotics. Medium was replaced every other day. 3T3L1 adipocytes were cultured and differentiated into adipocytes as described previously (43).

Transient transfections. A14 cells grown in 6-well plates were transiently transfected using 500 ng of expression vector and Fugene 6 reagent (Roche Biochemicals, Indianapolis, IN, USA) as described previously (44).

Cell incubations. Prior to insulin stimulation, 3T3L1 adipocytes and A14 fibroblasts were starved for 6 h on DMEM supplemented with 0.5% FBS, penicillin and streptomycin. L6 myotubes were incubated for 4 h on αMEM containing penicillin and streptomycin. Where indicated, cells were pretreated with rapamycin (15 min, 100 nM), wortmannin (30 min, 100 nM) or amino acid deprived for 30 min. For amino acid deprivation cells were incubated in D-PBS supplemented with MEM vitamins, glucose and pyruvate (45). Insulin resistance was induced by overnight incubation in DMEM containing 2% BSA and 0.38 mM or 0.75 mM palmitate. Cells were subsequently stimulated with insulin (5 min, 100 nM).

Analysis of insulin signalling. Lysates from tissue biopsies or cultured cells were prepared as described (46), and cleared by centrifugation. Protein content was determined using a bicinchoninic acid protein assay kit (Pierce, Rockford, IL). Expression and phosphorylation of proteins was determined by SDS-PAGE Western blotting. Antibodies against total PRAS40 (#2691) and phospho-specific mTOR-Ser2481 (#2974), PKB/Akt-Thr308 (#9275), PKB/Akt-Ser473 (#4051), S6K-Thr389 (#9205) were from Cell Signalling Technology (Danvers, MA, USA). Antibodies against hPRAS40 (AHO1031) and phospho-specific PRAS40-Thr246 (44-1100G) were from Invitrogen (Carlsbad, CA, USA). Phospho-specific antibodies against PRAS40-Ser183 were from IBL Hamburg GmBH (Hamburg, Germany). Antibodies against PKBβ/Akt2 (07-372) were from Upstate (Billerica, MA, USA). Total S6K antibody was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Total mTOR antibody has been described (47). Bound antibodies were visualized by enhanced chemiluminescence and quantified by densitometry analysis of scanned films (Image J 1.34S; National Institutes of Health, Bethesda, MD, USA).

Statistical analysis. Data are expressed mean ± standard error of the mean. Statistical analysis was performed using SPSS version 16.0. Differences between the basal and the

hyperinsulinaemic conditions were determined with a paired students two-tailed *t*-test. The effects of insulin and palmitate in rat tissues and cultured cell lines were determined using an unpaired students two-tailed *t*-test. A $p<0.05$ was considered statistically significant.

Results

Insulin increases PRAS40-Ser183 phosphorylation both in vitro and in vivo. Incubation of cultured A14 fibroblasts, 3T3L1 adipocytes, and L6 myotubes for 5 min with 100 nM insulin increased both PRAS40-Ser183 and PRAS40-Thr246 phosphorylation (Fig. 1A). In skeletal muscle biopsies, a 1.5±0.1-fold increase (n=6, p<0.05) in PRAS40-Ser183 phosphorylation was found 30 min after initiation of a hyperinsulinaemic-euglycaemic clamp at an infusion rate of 40 mU/m^2. In line with previous findings (48;49), hyperinsulinaemia caused a 2.4±0.4-fold increase (n=6, p<0.05) in PRAS40-Thr246 phosphorylation in these biopsies (Fig. 1B and 1C). Thus, insulin promotes phosphorylation of PRAS40 on Ser183 *in vitro* and *in vivo*.

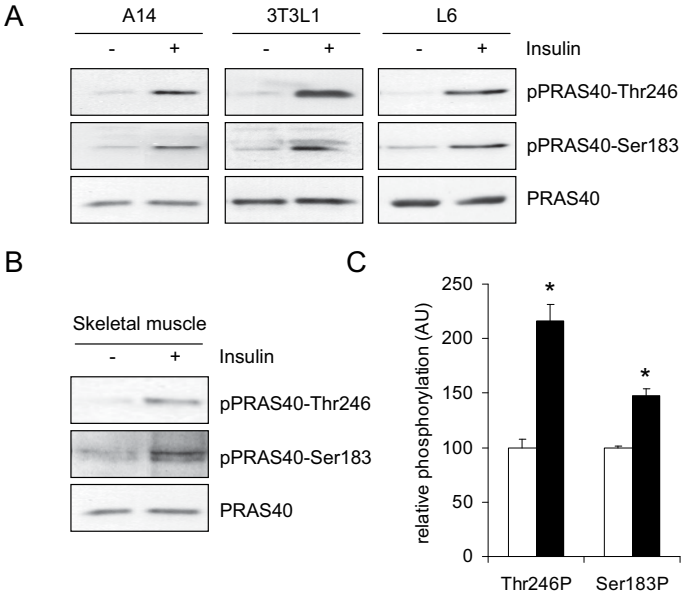

Figure 1. *Regulation of PRAS40-Ser183 phosphorylation by insulin. A. Phosphorylation and protein expression of PRAS40 in A14 fibroblasts, L6 myotubes, and 3T3L1 adipocytes. Cells were kept serum-starved (-) or stimulated with 100 nM insulin (+) for 5 minutes. Lysates were analyzed by Western blotting with antibodies recognizing PRAS40 phosphorylated on Ser183 (pPRAS40-Ser183) or Thr246 (pPRAS40-Thr246), respectively. Equal loading was verified by reprobing the stripped filters with antibody recognizing total PRAS40. B. Phosphorylation and protein expression of PRAS40 in human skeletal muscle biopsies under basal conditions and 30 min after initiation of a hyperinsulinaemic-euglycaemic clamp. Homogenates were analyzed by Western blotting with antibodies recognizing PRAS40 phosphorylated on Ser183 (pPRAS40-Ser183) or Thr246 (pPRAS40-Thr246), respectively. Equal loading was verified by reprobing the stripped filters with antibody recognizing total PRAS40. C. Quantification of PRAS40 phosphorylation on Ser183 and Thr246 under basal (open bars) and hyperinsulinaemic-euglycaemic clamp conditions (black bars). Data are corrected for total PRAS40 protein expression, and are expressed as mean ± standard error of the mean (n=6). The * indicates p<0.05 as compared to the basal condition (paired t-test).*

Effects of amino acid starvation, rapamycin and wortmannin on insulin-mediated phosphorylation of PRAS40 on Ser183 and Thr246. Studies in HEK293 cells identified mTORC1 as the main kinase regulating PRAS40-Ser183 phosphorylation (50;51). Here, we assessed the importance of mTORC1 and its upstream regulators for insulin-mediated PRAS40 phosphorylation in insulin-sensitive A14 fibroblasts and L6 myotubes.

In A14 fibroblasts, inhibition of PI3K using wortmannin completely abrogated the insulin-mediated phosphorylation of PKB/Akt-Thr308, PKB/Akt-Ser473, and of the mTORC1 substrate S6K-Thr389 (Fig. 2). The induction of PRAS40-Thr246 phosphorylation by insulin was almost completely prevented by wortmannin, whereas full inhibition was achieved for insulin-mediated PRAS40-Ser183 phosphorylation (Fig. 2). Similar results were obtained for L6 myotubes, although wortmannin completely prevented the insulin-induced increases in both PRAS40-Thr246 and PRAS40-Ser183 phosphorylation in this cell type (Fig. 3).

The mTORC1 inhibitor rapamycin, amino acid deprivation, or a combination of both, had no effect on the phosphorylation of PKB/Akt-Thr308 and PKB/Akt-Ser473, and of PRAS40-Thr246 in response to insulin in A14 fibroblasts (Fig. 2), but markedly inhibited these phosphorylations in L6 myotubes (Fig. 3). In both cell types, the insulin-mediated phosphorylationof mTOR-Ser2481 and of S6K-Thr389 were almost completely abolished by amino acid starvation and/or rapamycin treatment, indicating effective mTORC1 inhibition (Fig. 2 and 3). Remarkably, the induction of PRAS40-Ser183 phosphorylation by insulin was only partially abrogated by amino acid starvation or rapamycin, and complete inhibition of insulin-induced PRAS40-Ser183 phosphorylation in both A14 fibroblasts and L6 myotubes was only observed when amino acid deprivation was combined with rapamycin treatment (Fig. 2 and 3). Thus, the induction of PRAS40-Ser183 by insulin is sensitive to inhibition of PI3K and mTORC1. However, the molecular mechanism regulating insulin-induced PRAS40-Ser183 phosphorylation seems more complex than previously assumed.

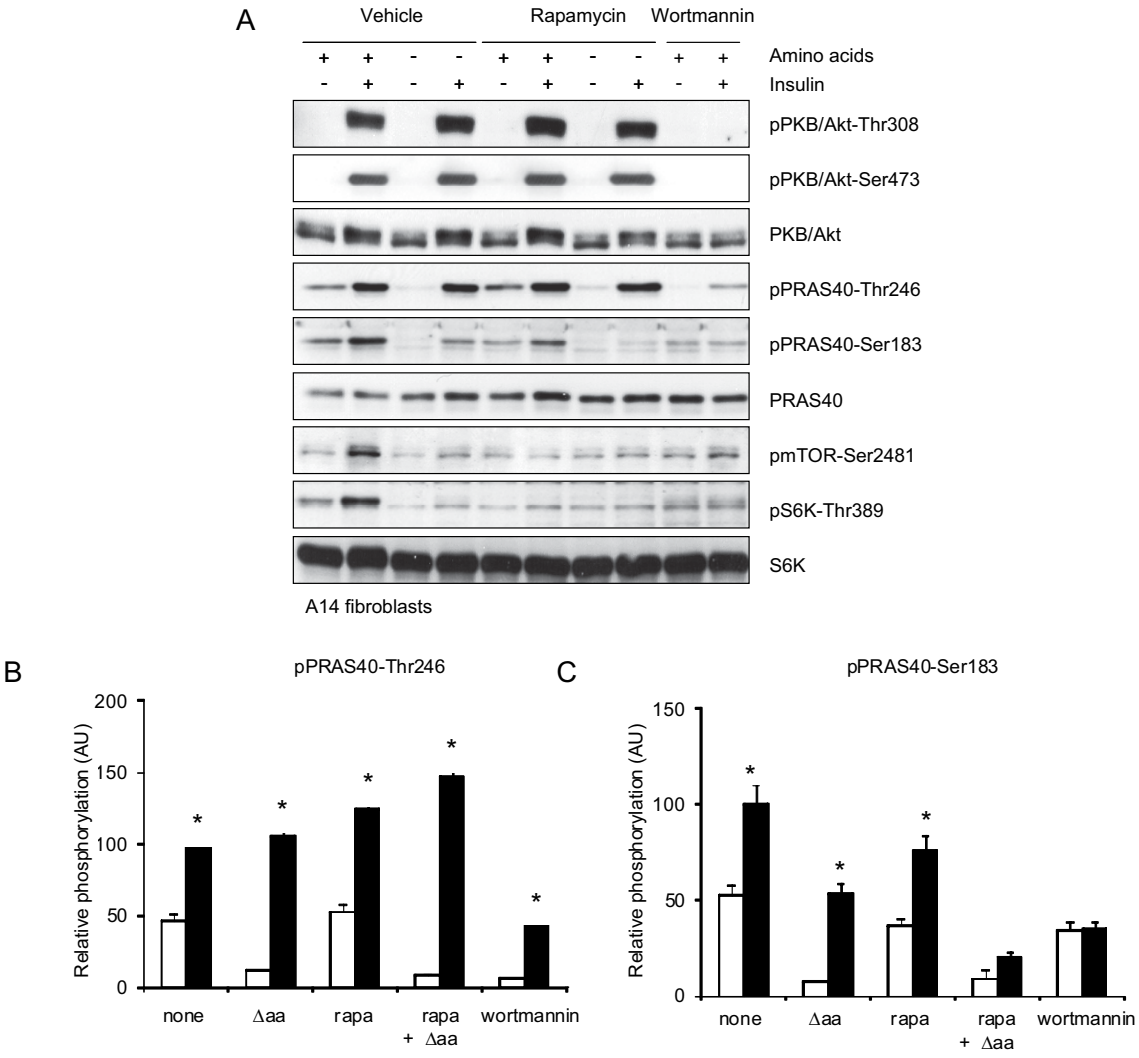

Figure 2. *Effects of amino acid starvation, rapamycin, and wortmannin on insulin-mediated phosphorylation of PRAS40 on Ser183 and Thr246 in A14 fibroblasts. A. Serum-starved A14 fibroblasts were deprived from amino acids, incubated with rapamycin (15 min, 100 nM) or wortmannin (30 min, 100 nM), and stimulated with insulin (+) (5 min, 100 nM) or left untreated (-). Lysates were analyzed by Western blotting with antibodies recognizing PKB/Akt phosphorylated on Thr308 (pPKB/Akt-Thr308) or Ser473 (pPKB/Akt-erS473), PRAS40 phosphorylated on Ser183 (pPRAS40-Ser183) or Thr246 (pPRAS40-Thr246), mTOR phosphorylated on Ser2481 (pmTORSer2481), and S6K phosphorylated on Thr389 (pS6K-Thr389). Equal loading was verified by reprobing the stripped filters with antibodies recognizing total PKB/Akt, PRAS40 and S6K, respectively. B-C. Quantification of PRAS40-Thr246 (B) and PRAS40-Ser183 (C) phosphorylation in A14 fibroblasts under basal (open bars) and insulin stimulated conditions (black bars). Data are expressed as mean + standard error of the mean (n=3). The * indicates $p<0.05$ compared to basal conditions (independent t-test). Δaa indicates absence of amino acids.*

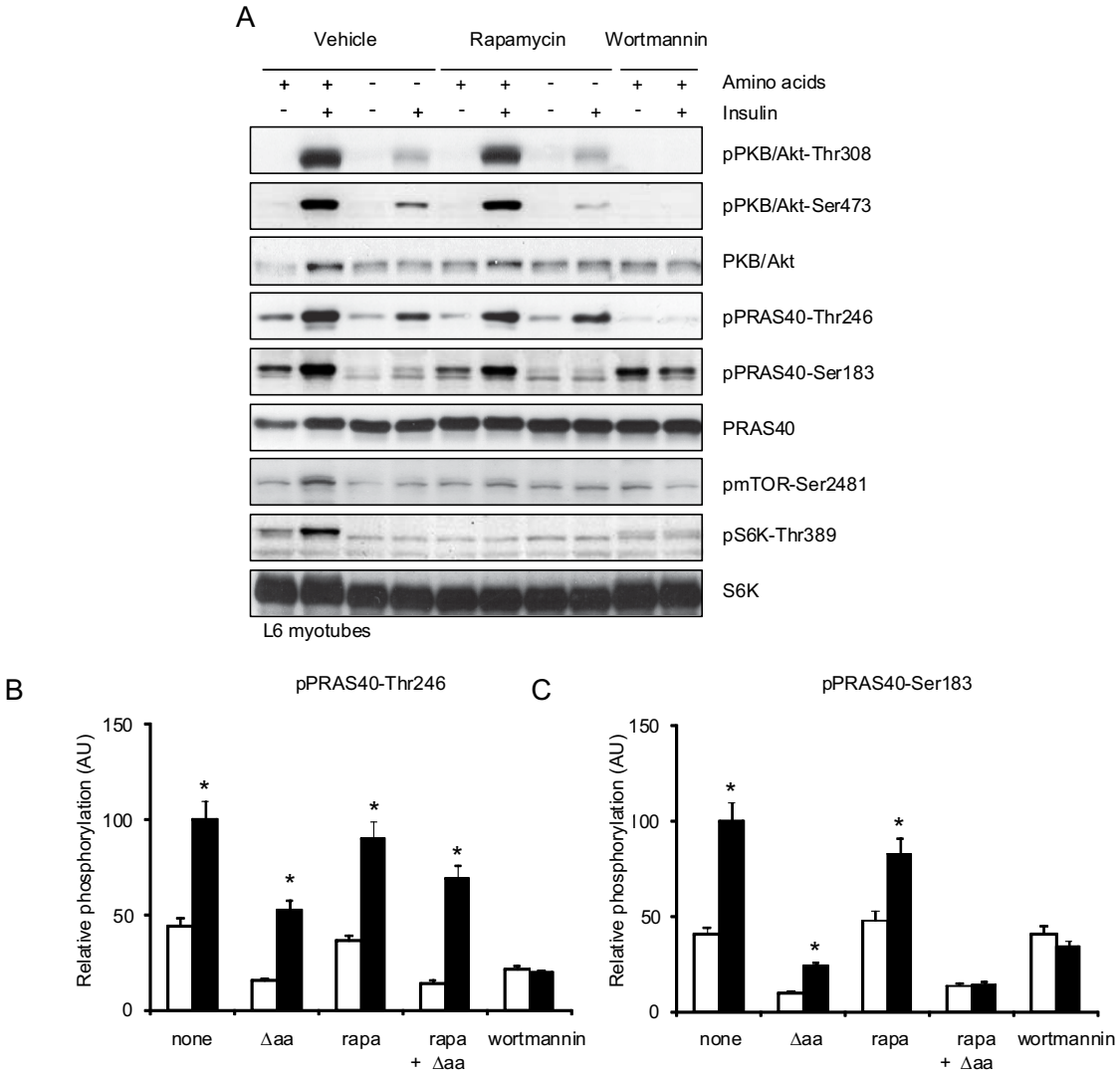

Figure 3. *Effects of amino acid starvation, rapamycin, and wortmannin on insulin-mediated phosphorylation of PRAS40 on Ser183 and Thr246 in L6 myotubes. Serum-starved L6 myotubes (B) were deprived from amino acids, incubated with rapamycin (15 min, 100 nM) or wortmannin (30 min, 100 nM), and stimulated with insulin (+) (5 min, 100 nM) or left untreated (-). Lysates were analyzed by Western blotting with antibodies recognizing PKB/Akt phosphorylated on Thr308 (pPKB/Akt-Thr308) or Ser473 (pPKB/Akt-Ser473), PRAS40 phosphorylated on Ser183 (pPRAS40-Ser183) or Thr246 (pPRAS40-Thr246), mTOR phosphorylated on Ser2481 (pmTORSer2481), and S6K phosphorylated on Thr389 (pS6K-Thr389). Equal loading was verified by reprobing the stripped filters with antibodies recognizing total PKB/Akt, PRAS40 and S6K, respectively. B-C. Quantification of PRAS40-Thr246 (B) and PRAS40-Ser183 (C) phosphorylation in A14 fibroblasts under basal (open bars) and insulin stimulated conditions (black bars). Data are expressed as mean + standard error of the mean (n=3). The * indicates $p<0.05$ compared to basal conditions (independent t-test). Δaa indicates absence of amino acids.*

Insulin resistance impairs PRAS40-Ser183 phosphorylation in response to insulin. We next determined the induction of PRAS40-Ser183 phosphorylation by insulin in tissues from control (low-fat diet; LFD) and high-fat diet (HFD) fed insulin resistant rats. Intraperitoneal injection of insulin increased the phosphorylation of PRAS40-Ser183 (n=3, $p<0.05$) in skeletal and cardiac muscle in LFD-fed rats (Fig. 4A-C). As previously shown (52), a reduced insulin-stimulated phosphorylation of PRAS40-Thr246 was evidenced in insulin-resistant HFD-fed rats (Fig 4A, 4D and 4E). Figure 4 shows that also the induction of PRAS40-Ser183 phosphorylation by insulin was markedly lower in tissues from HFD-fed rats versus LFD-fed rats.

In vitro, overnight incubation of A14 fibroblasts with increasing concentrations of palmitate induced a dose-dependent inhibition of insulin-induced phosphorylation of PKB/Akt-Thr308 and PKB/Akt-Ser473, as well as of PRAS40-Thr246 and of the mTORC1-substrate S6K-Thr389 (Fig. 5A and 5B). Remarkably, whereas activation of S6K was hardly affected at the lowest concentration of palmitate tested, the induction of PRAS40-Ser183 phosphorylation by insulin was completely absent (Fig. 5A and 5C). This indicates that activation of the mTORC1-pathway alone may not be sufficient to elicit PRAS40-Ser183 phosphorylation in response to insulin.

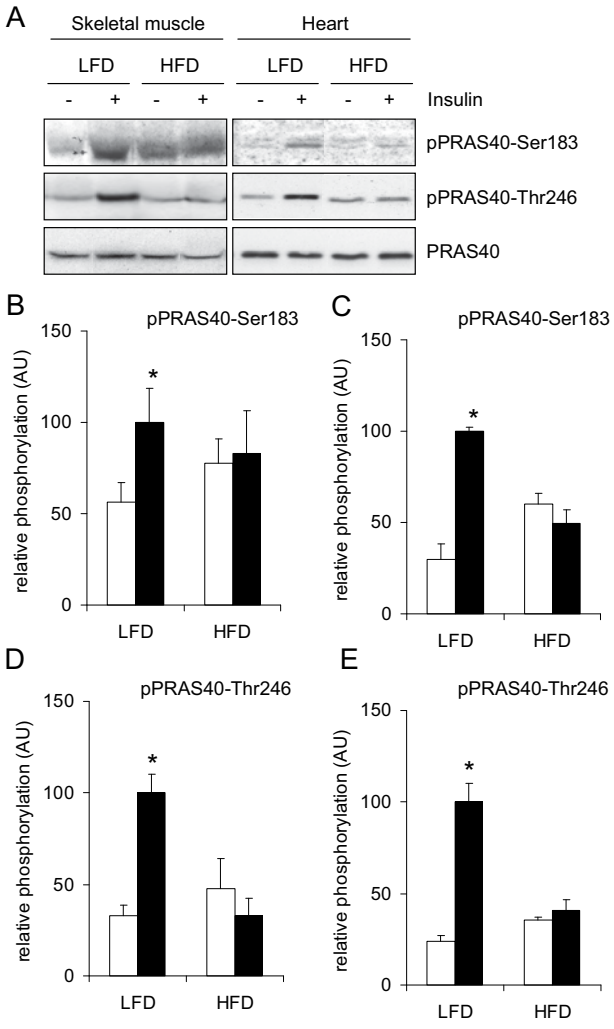

Figure 4. *Phosphorylation and protein expression of PRAS40 in tissues from low- and high-fat diet fed rats. A. Thirty minutes before sacrifice, rats fed a low-fat diet (LFD) or high-fat diet (HFD) received an intraperitoneal saline (-) or insulin (+) injection. Tissue homogenates were analyzed by Western blotting with antibodies recognizing either PRAS40 phosphorylated on Ser183 (pPRAS40-Ser183) or Thr246 (pPRAS40-Thr246), respectively. Equal loading was verified by reprobing the stripped filters with antibody recognizing total PRAS40. B-E. Quantification of PRAS40-Ser183 phosphorylation in skeletal muscle (B) and heart (C) and PRAS40-Thr246 phosphorylation in skeletal muscle (D) and heart (E) under basal (open bars) and insulin-stimulated conditions (black bars). Data are expressed as mean + standard error of the mean (n=3). The * indicates $p<0.05$ compared to saline-injected rats (independent t-test).*

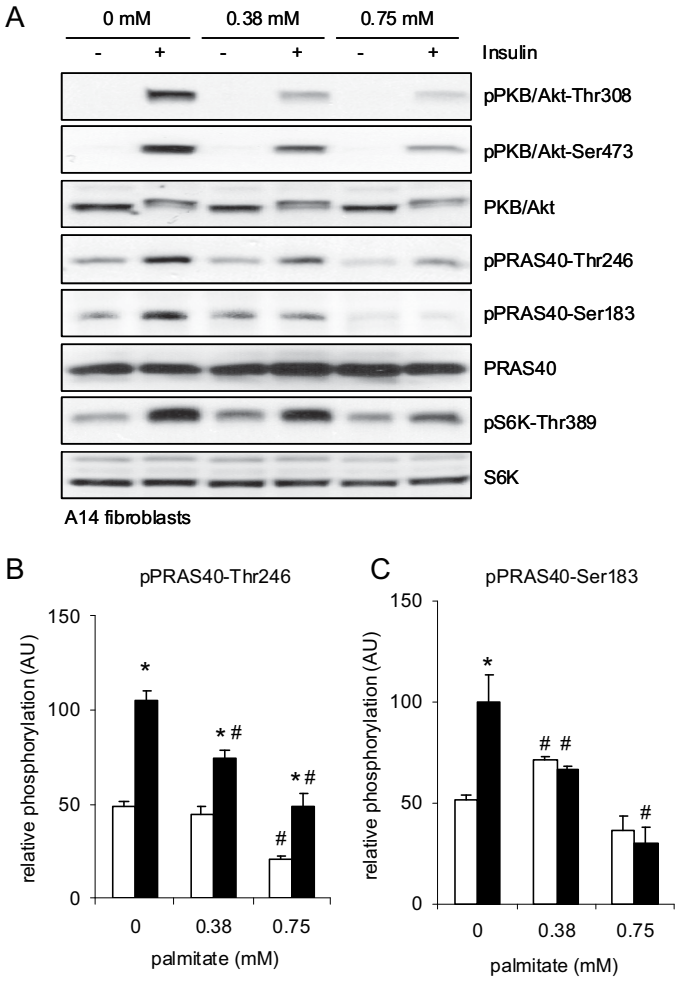

Figure 5. *Effects of palmitate on insulin-induced PRAS40 phosphorylation. A. A14 fibroblasts were incubated overnight with palmitate. Then, cells were left unstimulated (-) or stimulated with 100 nM insulin for 5 minutes (+). Lysates were analyzed by Western blotting with antibodies recognizing PKB/Akt phosphorylated on Thr308 (pPKB/Akt-Thr308) or Ser473 (pPKB/Akt-Ser473), PRAS40 phosphorylated on Ser183 (pPRAS40-Ser183) or Thr246 (pPRAS40-Thr246), and S6K phosphorylated on Thr389 (pS6K-Thr389). Equal loading was verified by reprobing the stripped filters with antibodies recognizing total PKB/Akt, PRAS40 and S6K, respectively. B-C. Quantification of PRAS40-Thr246 (B) and PRAS40-Ser183 (C) phosphorylation in A14 fibroblasts under basal (open bars) and insulin stimulated conditions (black bars). Data are expressed as mean + standard error of the mean (n=3). The * indicates $p<0.05$ compared to basal conditions (independent t-test). The # indicates $p<0.05$ compared 0 mM palmitate.*

Efficient phosphorylation of PRAS40 on Ser183 requires mTORC1 binding and functional Thr246. The absence of insulin stimulation of PRAS40-Ser183 phosphorylation under conditions where insulin-mediated phosphorylation of PRAS40-Thr246 and S6K-Thr389 are only partially inhibited, raises the possibility that phosphorylation of PRAS40-Thr246 is required for PRAS40-Ser183 phosphorylation. To test this hypothesis, we compared the insulin-induced phosphorylation of overexpressed wild type human PRAS40 (hPRAS40) with that of hPRAS40 mutants in which Phe129, Ser183 or Thr246 were substituted by Ala. In A14 fibroblasts expressing wild type or mutant hPRAS40, insulin increased the phosphorylation of PKB/Akt-Thr308 and PKB/Akt-Ser473, and of S6K-Thr389 (Fig. 6). Insulin induced similar phosphorylation of PRAS40-Thr246 in cells expressing wild type, mutant Phe129Ala- or mutant Ser183Ala-hPRAS40 (Fig. 6). As expected, no increase in ectopic PRAS40-Thr246 phosphorylation in response to insulin was observed in cells transfected with mutant hPRAS40-Thr246Ala or empty vector. Interestingly, replacement of Thr246 by Ala lowered insulin-mediated PRAS40-Ser183 phosphorylation to almost the same extent of mutating Phe129 to Ala, which results in a loss of mTORC1 binding (Fig. 6). In cells expressing mutant Ser183Ala-hPRAS40, phosphorylation of PRAS40-Ser183, but not of Thr246, was impaired (Fig. 6). Similar results for insulin-mediated PRAS40-Ser183 and PRAS40-Thr246 phosphorylation were obtained in L6 myotubes, and 3T3L1 adipocytes expressing the various hPRAS40 constructs. We conclude from these data that insulin can only induce efficient phosphorylation of PRAS40-Ser183 when PRAS40 is bound to mTORC1 and when Thr246 is present.

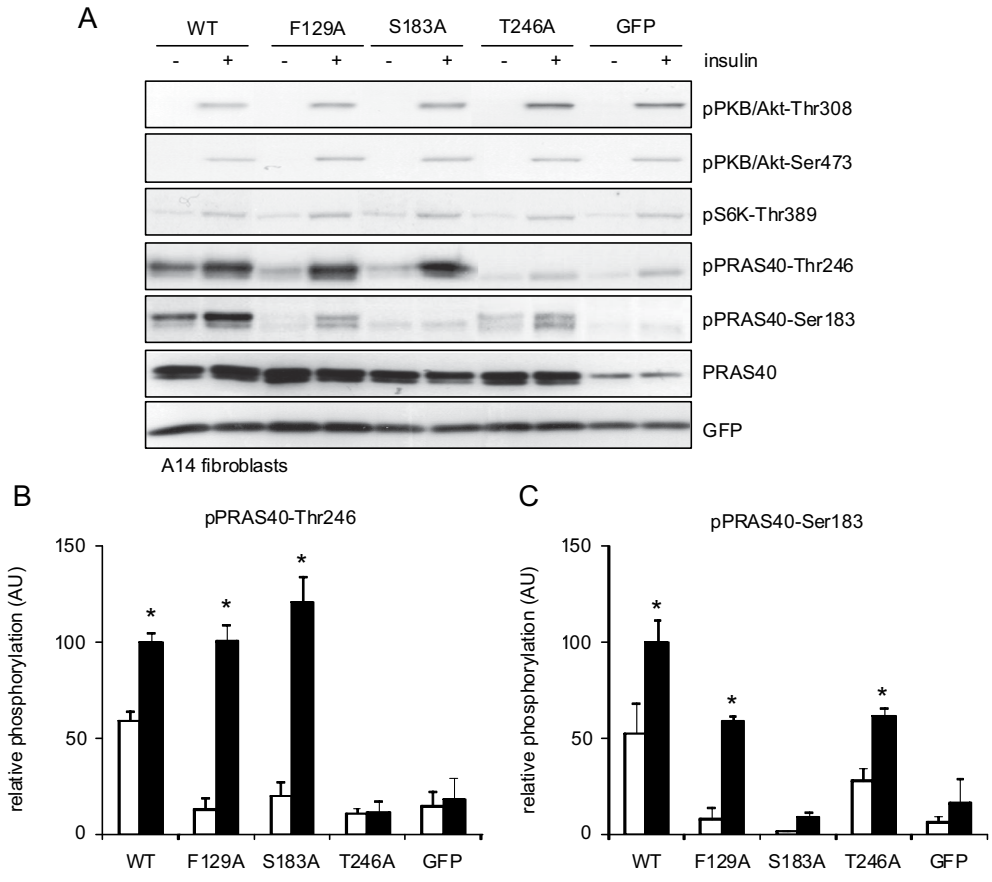

Figure 6. *Phosphorylation of PRAS40 mutants by insulin. A. A14 fibroblasts were transfected with plasmids encoding wild type hPRAS40 (WT), or hPRAS40 mutants in which Phe129, Ser183, or Thr246 were replaced by Ala (F129A, S183A, and T246A, respectively). Control cells were transfected with empty plasmid, only expressing green fluorescent protein (GFP). Following transfection, cells were serum-starved, and left untreated (-) or incubated for 5 minutes with 100 nM insulin (+). Lysates were analyzed by Western blotting with antibodies recognizing PKB/Akt phosphorylated on Thr308 (pPKB/Akt-Thr308) or Ser473 (pPKB/Akt-Ser473), S6K phosphorylated on Thr389 (pS6K-Thr389), and PRAS40 phosphorylated on Ser183 (pPRAS40-Ser183) or Thr246 (pPRAS40-Thr246). Protein loading was verified by reprobing the stripped filters with antibodies recognizing total PRAS40 and GFP, respectively. B-C. Quantification of PRAS40-Thr246 (B) and PRAS40-Ser183 (C) phosphorylation in A14 fibroblasts under basal (open bars) and insulin stimulated conditions (black bars). Data are expressed as mean + standard error of the mean (n=3). The * indicates $p<0.05$ compared to basal conditions (independent t-test).*

Discussion

PRAS40-Thr246 is among the most prominent substrates for PKB/Akt in target tissues for insulin action (53). The present study extends these observations by the identification of PRAS40-Ser183 as component of insulin action. In HEK293 cells, insulin and amino acids have been found to promote the phosphorylation of PRAS40-Ser183 (54;55). Here, we show that phosphorylation of PRAS40-Ser183 is also induced by insulin in other cell types, including A14 fibroblasts, L6 myotubes, and 3T3L1 adipocytes. The induction of PRAS40-Ser183 in response to hyperinsulinaemia *in vivo* has not been described before. Previously, we demonstrated that insulin induced the phosphorylation of PRAS40-Thr246 in human skeletal muscle, and in various rat tissues, including the liver, the heart, skeletal muscle, and adipose tissue (56). In the current study, we show that the induction of PRAS40-Thr246 phosphorylation is paralleled by the induction of PRAS40-Ser183 phosphorylation in skeletal and cardiac muscle. We also examined insulin-mediated PRAS40-Ser183 phosphorylation in the liver and white adipose tissue, but non-specific bands cross-reacting with the phospho-specific antibody for PRAS40-Ser183 prevented a reliable interpretation of the immunoblots performed on lysates of these tissues.

The signalling pathway(s) regulating insulin-induced PRAS40-Ser183 phosphorylation seems, depending on the cell type studied, more complex than previously assumed (57;58). Consistent with the insulin-mediated activation of mTORC1 via the PI3K-PKB/Akt-TSC2 axis (59;60), the PI3K-inhibitor wortmannin prevented insulin-mediated PRAS40-Ser183 phosphorylation. However, insulin-mediated PRAS40-Ser183 phosphorylation was only partially abrogated in cells expressing mutant hPRAS40-Phe129Ala, which has lost the ability to bind mTORC1. Furthermore, inhibition of mTORC1 in A14 fibroblasts and L6 myotubes using either rapamycin or amino acid deprivation also only partially affected insulin-mediated PRAS40-Ser183 phosphorylation, whereas insulin-mediated phosphorylation of S6K-Thr389, another substrate for mTORC1, was completely absent. By contrast, in E2 cardiomyocytes, rapamycin or amino acid deprivation led to full inhibition of insulin-mediated PRAS40-Ser183 phosphorylation (data not shown). The reason for these differences in efficiency of PRAS40-Ser183 inhibition between the various cell types is unknown, but these data do not exclude the possibility that protein kinases other than mTORC1 may contribute to insulin-mediated PRAS40-Ser183 phosphorylation.

Adding to the complexity is that insulin-mediated phosphorylation of PRAS40 on Ser183 and Thr246 are differentially affected by palmitate. The presence of low concentrations of palmitate completely abrogated the insulin-mediated phosphorylation of PRAS40-Ser183, while only partially blunting insulin-mediated phosphorylation of PKB/Akt, and PRAS40-Thr246. Previous studies in cell culture models for insulin resistance have shown a discordance in the efficiency of PKB/Akt substrate phosphorylation such that certain substrates are more efficiently phosphorylated than others (61). If this was the case here,

palmitate treatment should inhibit insulin-mediated activation of mTORC1 via the PKB/Akt substrate TSC2 more efficiently than insulin-mediated phosphorylation of PRAS40-Thr246 by PKB/Akt. Rather, low concentrations of palmitate that were sufficient to block insulin-mediated PRAS40-Ser183 phosphorylation hardly affected insulin-induced phosphorylation of the mTORC1-substrate S6K on Thr389. Alternatively, phosphorylation of PRAS40-Thr246 may be required to facilitate efficient PRAS40-Ser183 phosphorylation. In line with this, insulin-mediated PRAS40-Ser183 phosphorylation of mutant hPRAS40-Thr246Ala was markedly impaired. Interestingly, neither mutant PRAS40-Thr246Ala nor mutant PRAS40-Ser183Ala can bind 14-3-3 proteins. It is however unclear whether the binding of 14-3-3 proteins to PRAS40 phosphorylated on Thr246, might facilitate mTORC1-binding to phosphorylate PRAS40 on Ser183. A comparable situation has been described for activating transcription factor 2 (ATF2), where efficient phosphorylation on Thr69 by low levels of c-jun N-terminal kinase (JNK) or p38 could only be induced when ATF2 was already phosphorylated on Thr71 by extracellular-signal-regulated kinase (ERK1/2) (61-65).

Under conditions of insulin resistance, the insulin-mediated phosphorylation of PRAS40 on Thr246 and Ser183 is blunted. Phosphorylation of PRAS40 leads to dissociation from mTORC1 (66). Consequently, the amount of PRAS40 dissociated from mTORC1 in response to insulin may be lower under conditions of insulin resistance. It is not completely clear, however, whether this affects mTORC1 activity. Overexpression of PRAS40 has been found to reduce the basal phosphorylation of S6K and 4EBP1 (67-72), indicating that PRAS40 can function as substrate inhibitor on mTORC1. Accordingly, silencing of PRAS40 increases both basal and amino acid induced phosphorylation of S6K (73;74). However, other reports showed a lower insulin-stimulated phosphorylation of S6K and 4EBP1 in the absence of PRAS40, suggesting that PRAS40 is also required for proper signalling by mTORC1 (75-77). Analysis of tissues from HFD-fed rats showed a reduced induction of S6K-T389 phosphorylation in response to insulin (data not shown). However, insulin-mediated PKB/Akt phosphorylation is also lower in tissues from HFD-fed rats (78;79). Therefore, it is not clear whether the reduced mTORC1 activation in response to insulin results from a lower phosphorylation of the PKB/Akt-TSC2 axis, a reduced phosphorylation and dissociation of PRAS40 from mTORC1, or both.

In summary, this study identifies PRAS40-Ser183 as a component of insulin action. We showed that insulin promotes the phosphorylation of PRAS40-Ser183 both *in vitro* and *in vivo*. Furthermore, it was found that the induction of PRAS40-Ser183 phosphorylation was blunted under conditions of insulin resistance. Finally, our data indicate that phosphorylation of PRAS40 on Thr246 facilitates efficient phosphorylation of PRAS40-Ser183 by mTORC1.

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