Mutational analysis of sites in the translational regulator, PHAS-I, that are selectively phosphorylated by mTOR

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Received 19 April 1999; received in revised form 26 May 1999

Abstract Results obtained with PHAS-I proteins having Ser to Ala mutations in the five known phosphorylation sites indicate that mTOR preferentially phosphorylates Thr36 and Thr45. The effects of phosphorylating these sites on eIF4E binding were assessed in a far-Western analysis with a labeled eIF4E probe. Phosphorylation of Thr36 only slightly attenuated binding of PHAS-I to eIF4E, while phosphorylation of Thr45 markedly inhibited binding. Phosphorylation of neither site affected the electrophoretic mobility of the protein, indicating that results of studies that rely solely on a gel-shift assay to assess changes in PHAS-I phosphorylation must be interpreted with caution.

Key words: mTOR; Mitogen-associated protein kinase; 4E-BP1; mRNA translation initiation

1. Introduction

A key step in translation initiation is recognition of the mRNA cap (m^7GpppN, where N is any nucleotide), which is located at the 5′-end of almost all eukaryotic mRNAs [1,2]. The availability of the cap-binding protein, eIF4E, appears to control the rate of initiation, particularly of mRNAs having structured 5′ untranslated regions (UTRs) [3,4]. To facilitate translation, eIF4E must interact with eIF4G, a large scaffolding protein that also binds eIF3 and eIF4A. eIF4A is an ATP-dependent helicase, which melts secondary structure allowing more efficient translation of mRNAs with structured 5′ UTRs. eIF3 links eIF4F to the 40S ribosomal subunit. Access to eIF4E is controlled by a family of binding proteins [5,6]. PHAS-I (also known as 4E-BP1) is the best characterized of these proteins. Non-phosphorylated PHAS-I binds tightly to eIF4E and prevents eIF4E from binding to eIF4G [7]. When phosphorylated in the appropriate site(s), PHAS-I dissociates, allowing eIF4E to bind eIF4G to form eIF4F, the complex of eIF4A, eIF4E, and eIF4G. eIF4F increases translation by increasing the efficiency of binding and/or scanning by the 40S ribosomal subunit.

Insulin and growth factors increase mRNA translation by promoting the phosphorylation of PHAS-I [5,6]. In rat adipocytes insulin promotes the phosphorylation of PHAS-I in five S/T-P sites [8]. Several lines of evidence indicate that the effects of insulin are mediated in part by mTOR, a member of the family of protein kinases having sequence homology to phosphatidylinositol 3-kinase [9]. The effects of insulin on increasing the phosphorylation of PHAS-I are attenuated by rapamycin [10,11], which is known to inhibit the function of TOR proteins [9]. Expressing mTOR in cells mimics the effect of insulin by increasing phosphorylation of PHAS-I [12,13], and mTOR is able to phosphorylate PHAS-I in vitro [12,14]. Recent findings suggests that Thr36 and Thr45 in PHAS-I are preferentially phosphorylated by mTOR [14]. In the present study, we have confirmed this site selectivity of mTOR and have investigated the effects of phosphorylating Thr36 and Thr45 on the binding of PHAS-I to eIF4E.

2. Materials and methods

2.1. Preparation of recombinant PHAS-I proteins

cDNA encoding PHAS-I proteins having Ser/Thr to Ala mutations in single sites were generated by oligonucleotide-directed mutagenesis (Transformer Site-Directed Mutagenesis Kit, Clontech) using wild-type PHAS-I cDNA in pBluescript SK$^-$ (Stratagene) as template. Sequential rounds of mutagenesis were used to generate a cDNA encoding a PHAS-I having Ser/Thr to Ala mutations in all five known Ser/Thr-Pro phosphorylation sites (Table 1). This cDNA was then used as template to introduce mutations that restored Thr36, Thr45, Ser64, Thr69, and Ser82, respectively. For each mutant plasmid, the nucleotide sequence of the coding region was determined and found to be free of undesired mutations. To express PHAS-I in bacteria, the coding region was excised from pBluescript SK$^-$ by using *Afl*III and *Bam*HI, and inserted between *Nco*I and *Bam*HI sites of pET-14b (Novagen). Proper orientation and reading frame were verified by nucleotide sequencing. PHAS-I proteins were purified by taking advantage of their binding to eIF4E by using essentially the same procedure as described previously for the purification of complexes of histidine-tagged PHAS-I and eIF4E [11]. Samples of the purified complexes were incubated at 100°C for 5 min. This treatment is sufficient to denature eIF4E, which was pelleted by centrifugation at $10\,000\times g$ for 30 min. PHAS-I proteins, which are heat-stable [15], were recovered in the supernatants. The method allowed PHAS-I to be purified without resorting to an epitope tag, which could change the properties of this relatively small protein ($M_r \approx 12\,400$). When subjected to SDS-polyacrylamide gel electrophoresis, each mutant protein migrated predominantly as a single band of the same electrophoretic mobility as wild-type PHAS-I. The PHAS protein concentration of each preparation was determined from the intensity of Coomassie blue staining following SDS-PAGE. The preparations were devoid of eIF4E, as assessed by Coomassie blue staining (a picture of a stained gel of the protein preparations was provided during the review of the manuscript).

All mutants reacted with an antibody [11] directed against the COOH-terminal region of PHAS-I, indicating that the proteins were translated in the correct reading frame. Moreover, all of the proteins bound to eIF4E, both during purification and in far-Western analyses. That the proteins retained high affinity binding for eIF4E indicates that the mutations did not perturb the intrinsic properties of the PHAS-I proteins.

2.2. Phosphorylation of PHAS-I by mTOR

mTOR was immunoprecipitated from extracts of rat brain by using the antibody, mTAb2, as described previously [16]. Immune complexes were incubated at 30°C for 30 min with 1 μg of recombinant PHAS-I protein in 50 μl of reaction mixture containing 200 nM microcystin LR, 10 mM $MnCl_2$, 50 mM NaCl, 0.1 mM EGTA, 1 mM

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PII: S0014-5793(99)00762-0

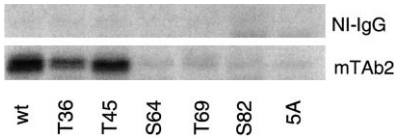

Fig. 1. Phosphorylation of wild-type and mutant PHAS-I proteins by mTOR. mTOR was immunoprecipitated from rat brain extracts by using the antibody, mTAb2. Immune complexes generated with non-immune IgG (NI-IgG) or with mTAb2 were incubated in 50 μl of reaction mixtures containing 0.5 μg of the different PHAS-I proteins. After 30 min the reactions were terminated, and samples were subjected to SDS-PAGE. Pictures of autoradiograms of the dried gels in the regions containing the PHAS-I proteins are presented.

dithiothreitol, 100 μM [γ-^{32}P]ATP, and 10 mM NaHEPES, 50 mM β-glycerophosphate, pH 7.4. The reactions were terminated by adding 5 μl 0.2 M EDTA, before samples were subjected to SDS-PAGE.

2.3. Phosphorylation of PHAS-I by mitogen-activated protein (MAP) kinase

Recombinant ERK2 isoform of MAP kinase was activated as described previously [8]. When FLAG-4E binding was to be assessed, PHAS-I proteins (20 μg/ml) were incubated at 30°C in a solution containing activated MAP kinase (7 μg/ml), 1 mM dithiothreitol, 0.5 mM ATP, 7.5 mM $MgCl_2$ and 40 mM NaHEPES, pH 7.4 for 15 h. When phosphorylation stoichiometry was to be measured, PHAS-I proteins were incubated under identical conditions except in a reaction mixture containing 0.5 mM [γ-^{32}P]ATP. The phosphorylation reactions were terminated by adding EDTA (20 mM final).

2.4. Electrophoretic analyses

Protein samples were subjected to electrophoresis in 15% polyacrylamide gels in the presence of SDS [17]. Dried gels were exposed to film to enable detection of ^{32}P-labeled PHAS-I, and bands containing the protein were excised. The amounts of ^{32}P in the gel slices were determined by measuring Cerenkov emissions. Binding of PHAS-I proteins to eIF4E was assessed by far-Western blotting performed using a ^{32}P-labeled recombinant eIF4E fusion protein (FLAG-4E) as described previously [18].

2.5. Peptide mapping

[^{32}P]Phosphopeptides resulting from the sequential digestion of PHAS-I proteins with lysyl endopeptidase C and chymotrypsin were resolved by reverse phase HPLC as described previously [8].

3. Results

3.1. Phosphorylation of PHAS-I mutants by mTOR

Wild-type PHAS-I was phosphorylated by immune complexes containing mTOR (Fig. 1). Almost no phosphorylation was detected with immune complexes generated using non-immune IgG instead of the mTOR antibody. mTOR introduced very little phosphate into a 5A PHAS-I (Table 1), a protein lacking the five known S/T-P sites that are phosphorylated in vivo [8]. To determine whether mTOR exhibited a preference for any of the five sites, reactions were conducted

Table 1
Designations of PHAS-I proteins used in this study

Designation	Ser/Thr mutated to Ala
Wild-type PHAS-I	None
T36 PHAS-I	Thr45, Ser64, Thr69, Ser82
T45 PHAS-I	Thr36, Ser64, Thr69, Ser82
S64 PHAS-I	Thr36, Thr45, Thr69, Ser82
T69 PHAS-I	Thr36, Thr45, Ser64, Ser82
S82 PHAS-I	Thr36, Thr45, Ser64, Thr69
5A PHAS-I	Thr36, Thr45, Ser64, Thr69, Ser82

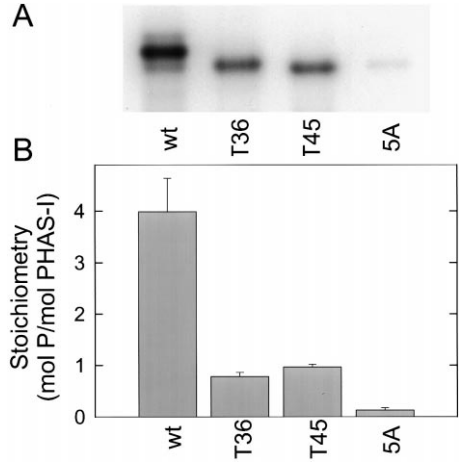

Fig. 2. Phosphorylation of wild-type, T36, T45, and 5A PHAS-I by MAP kinase. PHAS-I proteins were incubated with [γ-^{32}P]ATP and MAP kinase for 15 h before the reactions were terminated. Samples were subjected to SDS-PAGE. An autoradiogram of the dried gel is presented (A). To determine stoichiometry of phosphorylation, PHAS-I proteins were incubated without and with MAP kinase as described above, except that the reaction mixtures contained [γ-^{32}P]ATP. Samples were subjected to SDS-PAGE, and the bands containing ^{32}P-labeled PHAS-I proteins were excised. Phosphorylation stoichiometries were estimated from the amounts of ^{32}P incorporated, which were determined by scintillation counting of gel slices containing the phosphorylated proteins (B). The results presented are mean values ± S.E.M. from three experiments.

with mutant PHAS-I proteins that were generated by restoring individual sites of phosphorylation into the 5A mutant. mTOR readily phosphorylated T36 PHAS-I and T45 PHAS-I under conditions in which S64 PHAS-I, T69 PHAS-I, and S82 PHAS-I were not significantly phosphorylated. These findings

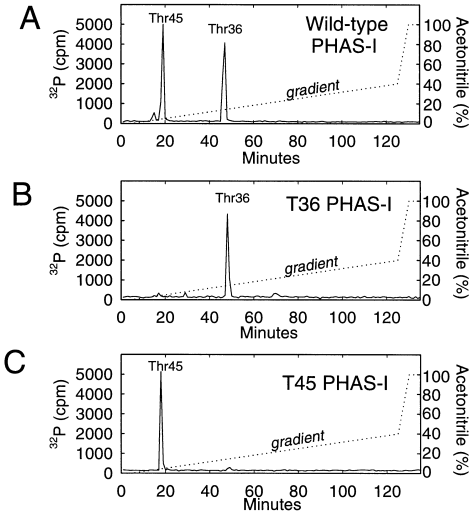

Fig. 3. Peptide mapping of PHAS-I proteins. Samples of ^{32}P-labeled wild-type PHAS-I (A), T36 PHAS-I (B) and T45 PHAS-I (C) that had been phosphorylated by incubation with [γ-^{32}P]ATP and MAP kinase for 15 h were digested with lysyl endopeptidase before samples were subjected to reverse phase HPLC as described previously [8]. More than 80% of the ^{32}P from T36 PHAS-I and T45 PHAS-I appeared in a single peak (LE-P3) (not shown), previously found to contain Thr36 and Thr45 [8]. Fractions in LE-P3 were pooled and the phosphopeptides were digested with chymotrypsin before samples were subjected to HPLC to resolve the peptides containing Thr36 and Thr45 [8]. The results represent ^{32}P (cpm) measured in the eluate by using a flow-through radiation monitor.

support the conclusion that T36 and T45 are preferentially phosphorylated by mTOR.

3.2. Effect of phosphorylating Thr36 and Thr45 on binding of PHAS-I to eIF4E

To investigate the influence of Thr36 and Thr45 on eIF4E binding, T36 PHAS-I and T45 PHAS-I were phosphorylated in vitro with MAP kinase. We chose to use MAP kinase instead of mTOR to phosphorylate PHAS-I in these experiments because with the mTOR immune complexes we were unable to confine phosphorylation to Thr36 and Thr45 in the relatively long incubations needed to approach stoichiometric phosphorylation of these sites (data not shown). MAP kinase phosphorylates PHAS-I in vitro in all five S/T-P sites that are phosphorylated in response to insulin in adipocytes [8]. Thus, MAP kinase is a useful tool for investigating the phosphorylation of PHAS-I in vitro, even if it does not phosphorylate PHAS-I in cells [19] (discussed later). Moreover, recombinant MAP kinase was available in a highly purified form, which allowed us to conduct the phosphorylation reactions under more precisely defined conditions than were possible with mTOR immunoprecipitated from cell extracts. Consequently, the extent of phosphorylation from experiment to experiment could be better controlled with recombinant MAP kinase.

T36 PHAS-I and T45 PHAS-I were incubated with MAP kinase for 15 h to allow phosphorylation of Thr36 and Thr45 to approach completion. Under these conditions 5A PHAS-I was not phosphorylated to a significant level (Fig. 2A), consistent with the interpretation that all of the major MAP kinase sites in PHAS-I have been identified. In contrast, T36 PHAS-I and T45 PHAS-I were phosphorylated to nearly 1 mol/mol (Fig. 2B). Peptide mapping experiments were conducted to confirm that Thr36 and Thr45 were phosphorylated in the mutant proteins. The elution positions of phosphopeptides containing Thr36 and Thr45 derived from wild-type PHAS-I are shown in Fig. 3A. The identities of the peptides in the two peaks were determined previously by amino acid sequencing [8]. T36 PHAS-I and T45 PHAS-I were phosphorylated almost exclusively in the peptides containing Thr36 and Thr45, respectively (Fig. 3B,C).

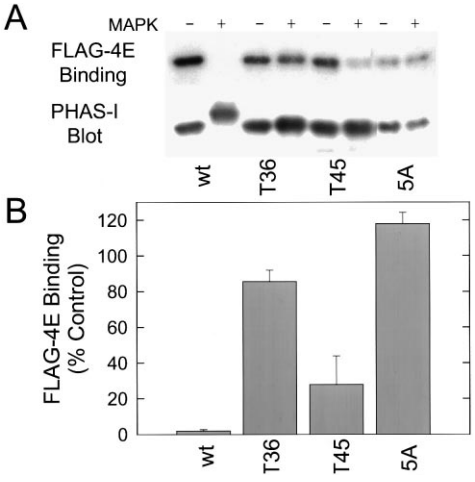

Fig. 4. Effect on eIF4E binding of phosphorylating Thr36 or Thr45. PHAS-I proteins were incubated in the absence (−) or presence (+) of MAP kinase (MAPK) for 15 h before the reactions were terminated. Samples were subjected to electrophoresis before proteins were transferred to a membrane, which was incubated with a ^{32}P-labeled FLAG-eIF4E probe (A). After washing the membrane, an autoradiogram was prepared (FLAG-4E Binding). The membrane was then stripped and reprobed with PHAS-I antibody to allow estimation of the relative amounts of PHAS-I (PHAS-I Blot) (A). FLAG-4E binding is expressed as a percentage of the control binding, which is defined as that observed with the respective samples incubated in the absence of MAP kinase (B). The results represent mean values ± S.E.M. from three experiments.

To investigate the effect of phosphorylation of Thr36 and Thr45 on eIF4E binding, samples of wild-type and mutant PHAS-I proteins that had been phosphorylated with unlabeled ATP were subjected to far-Western analysis (Fig. 4A,B). As observed previously, phosphorylation of wild-type PHAS-I abolished binding to eIF4E. Incubating 5A PHAS-I with MAP kinase for 15 h did not decrease binding of the mutant protein to ^{32}P-labeled FLAG-4E (Fig. 4A,B). Phosphorylation of T36 PHAS-I caused a relatively small decrease in binding to eIF4E, whereas phosphorylation decreased binding of T45 PHAS-I by 70%. Phosphorylation did not retard the electrophoretic mobility of either mutant protein (Figs. 2A and 4A).

4. Discussion

PHAS-I is phosphorylated in rat adipocytes in five sites, all of which conform to a Ser/Thr-Pro motif [8]. Two of these sites, Thr36 and Thr45, were preferentially phosphorylated in vitro by mTOR that had been immunoprecipitated with the non-activating antibody, mTAb2 (Fig. 1). These results are consistent with the previous findings that Thr to Ala mutations in these two sites essentially abolished phosphorylation by an epitope-tagged mTOR that had been immunoprecipitated with anti-myc antibody [14]. An extended incubation with mTOR immunoprecipitated with the activating antibody, mTAb1, resulted in the phosphorylation of all five S/T-P sites in PHAS-I [16]. Although the Thr sites in PHAS-I were more highly phosphorylated than the Ser sites in this study [16], the preference of mTOR for Thr36 and Thr45 was less apparent than in Fig. 1. This is partly explained by the high stoichiometry of phosphorylation of PHAS-I previously achieved as a result of using the activating antibody.

Results obtained with T45 PHAS-I indicate that phosphorylation of Thr45 is able to prevent PHAS-I from binding to eIF4E (Fig. 4). As Thr45 is phosphorylated in a rapamycin-sensitive manner in response to insulin [8], it seems reasonable to conclude that this site is important in regulating binding to eIF4E in cells. This conclusion is also supported by results of previous studies that provided indirect evidence that Thr45 phosphorylation regulates eIF4E binding [8,14]. However, it is important to remember that the conditions used to assess binding in vitro differ from those in intact cells. Moreover, while phosphorylation of Thr45 may be sufficient to block the association of PHAS-I and eIF4E, it may not be sufficient to promote dissociation of eIF4E, once the PHAS-I/eIF4E complex has formed. Thus, phosphorylation of other sites in PHAS-I as well as interactions with additional proteins may be needed to promote the dissociation of PHAS-I and eIF4E in cells.

The present results indicate that phosphorylation of Thr36 alone is not sufficient to block binding to eIF4E (Fig. 4). Supporting this conclusion are previous findings that PHAS-I phosphorylated in Thr36 remained bound to eIF4E in extracts of rat adipocytes [8]. Thr45 is located closer to the

eIF4E-binding domain [21] than Thr36, which may explain the greater influence of Thr45 on eIF4E binding relative to that of Thr36. Other issues remain to be resolved concerning the role of Thr36 phosphorylation in the control of eIF4E binding. The finding that mutating Thr36 to Ala increased the amount of eIF4E bound to PHAS-I expressed in HEK293 cells led Burnette et al. [14] to conclude that phosphorylation of Thr36 modulates eIF4E binding. However, these experiments did not assess directly the effect of Thr36 phosphorylation on eIF4E binding. It is possible that phosphorylation of Thr36 facilitates phosphorylation of other sites or acts in combination with the phosphorylation of other sites to promote dissociation of PHAS-I and eIF4E. Indeed, we stress that sites other than the two that are preferentially phosphorylated by mTOR may contribute to the control of eIF4E binding.

Ser64, which is the preferred site for phosphorylation by MAP kinase in vitro, was the first in vivo phosphorylation site in PHAS-I to be identified [23]. As phosphorylation of Ser64 was markedly increased in response to insulin, which promoted the dissociation of the PHAS-I/eIF4E complex, it was suggested that phosphorylation of Ser64 was responsible for the loss in eIF4E binding [18,20]. However, it was later shown that Ser64 could be phosphorylated in vitro without loss of eIF4E binding [8]. The possibility that Ser64 cooperates with other sites to influence eIF4E binding has not been eliminated. There is also reason to suspect that Thr69 has a role in controlling eIF4E binding [8]. Unfortunately, we have been unable to investigate directly the influence of Thr69 phosphorylation on eIF4E binding, as we have been unable to phosphorylate Thr69 efficiently in vitro.

The present findings have important implications with respect to the relationship between electrophoretic mobility and PHAS-I phosphorylation. The results indicate that phosphorylation of PHAS-I may occur without a change in electrophoretic mobility, provided phosphorylation is confined to Thr36 or Thr45 (Figs. 2A and 4A). Thus, the actual change in phosphorylation of PHAS-I could be significantly underestimated from the change in mobility. In any event, it cannot be concluded that the phosphorylation of PHAS-I does not change because the electrophoretic mobility of the protein is unaffected by a treatment. In other experiments we have found that phosphorylation of S82 PHAS-I, which was generated by restoring Ser82 in the 5A mutant, decreases its electrophoretic mobility but does not significantly inhibit its binding to FLAG-4E (data not shown). The phosphorylation of Ser82 provides a potential explanation for observations in cell extracts of a form of PHAS-I of reduced electrophoretic mobility that still binds to eIF4E (see [22], for example). The lack of effect of Ser82 phosphorylation on binding indicates that a mobility shift does not necessarily reflect phosphorylation of sites that modulate eIF4E binding. It seems clear from these considerations that studies of PHAS-I that rely solely on changes in electrophoretic mobility to assess phosphorylation of PHAS-I must be interpreted with caution.

PHAS-I is a substrate for MAP kinase in vitro [23]. However, it is not clear that PHAS-I serves as a substrate for MAP kinase in vivo. Perhaps the strongest argument against a role of MAP kinase was made by von Manteuffel et al. [19], who reported that MAP kinase could be activated in endothelial cells without affecting PHAS-I phosphorylation. This conclusion may have been premature since PHAS-I phosphorylation was assessed using a gel-shift assay. However, there is other evidence that MAP kinase is not a PHAS-I kinase in cells and the prevailing view is that PHAS-I is phosphorylated in cells by an insulin-activated protein kinase other than MAP kinase [24].

Identifying the protein kinases that phosphorylate PHAS-I in cells is proving to be a difficult task. Insulin appears to utilize both rapamycin-sensitive and -insensitive pathways in promoting the phosphorylation of PHAS-I [11,25]. mTOR is a candidate for the kinase that mediates the rapamycin-sensitive phosphorylation of Thr36 and Thr45 in PHAS-I. Additional research is needed to determine the kinases involved in the rapamycin-insensitive pathway, and the kinases that mediate the phosphorylation of Ser64, Thr69, and Ser82.

References

[1] Hershey, J.W.B. (1991) Annu. Rev. Biochem. 60, 717–755.
[2] Merrick, W.C. (1992) Microbiol. Rev. 56, 291–315.
[3] Rhoads, R.E., Joshi, B. and Minich, W.B. (1994) Biochimie 76, 831–838.
[4] Sonenberg, N. (1994) Biochimie 76, 839–846.
[5] Lawrence Jr., J.C. and Abraham, R.T. (1997) Trends Biochem. Sci. 22, 345–349.
[6] Sonenberg, N. and Gingras, A.-C. (1998) Curr. Opin. Cell Biol. 10, 268–275.
[7] Haghighat, A., Mader, S., Pause, A. and Sonenberg, N. (1995) EMBO J. 14, 5701–5709.
[8] Fadden, P., Haystead, T.A.J. and Lawrence Jr., J.C. (1997) J. Biol. Chem. 272, 10240–10247.
[9] Abraham, R.T. and Wiederrecht, G. (1996) Annu. Rev. Immunol. 14, 483–510.
[10] Beretta, L., Gingras, A.-C., Svitkin, Y.V., Hall, M.N. and Sonenberg, N. (1996) EMBO J. 15, 658–664.
[11] Lin, T.-A., Kong, X., Saltiel, A.R., Blackshear, P.J. and Lawrence Jr., J.C. (1995) J. Biol. Chem. 270, 18531–18538.
[12] Brunn, G.J., Hudson, C.C., Sekulic, A., Williams, J.M., Hosoi, H., Houghton, P.J., Lawrence Jr., J.C. and Abraham, R.T. (1997) Science 277, 99–101.
[13] Hara, K., Yonezawa, K., Kozlowski, M.T., Sugimoto, T., Andrabi, K., Weng, Q.-P., Kasuga, M., Nishimoto, I. and Avruch, J. (1997) J. Biol. Chem. 272, 26457–26463.
[14] Burnett, P.E., Barrow, R.K., Cohen, N.A., Snyder, S.H. and Sabatini, D.M. (1998) Proc. Natl. Acad. Sci. USA 95, 1432–1437.
[15] Hu, C., Pang, S., Kong, X., Velleca, M. and Lawrence Jr., J.C. (1994) Proc. Natl. Acad. Sci. USA 91, 3730–3734.
[16] Brunn, G.J., Fadden, P., Haystead, T.A.J. and Lawrence Jr., J.C. (1997) J. Biol. Chem. 272, 32547–32550.
[17] Laemmli, U.K. (1970) Nature 227, 680–685.
[18] Pause, A., Belsham, G.J., Gingras, A.-C., Donze, O., Lin, T.-A., Lawrence Jr., J.C. and Sonenberg, N. (1994) Nature 371, 762–767.
[19] von Manteuffel, S.R., Gingras, A.-C., Ming, X.-F., Sonenberg, N. and Thomas, G. (1996) Proc. Natl. Acad. Sci. USA 93, 4076–4080.
[20] Lin, T.-A., Kong, X., Pause, A., Haystead, T.A.J., Belsham, G., Sonenberg, N. and Lawrence Jr., J.C. (1994) Science 266, 653–656.
[21] Mader, S., Lee, H., Pause, A. and Sonenberg, N. (1995) Mol. Cell. Biol. 15, 4990–4997.
[22] Lawrence, J.C., Jr., Lin, T.-A., Kong, X., Haystead, T.A.J. and Hu, C. (1995) in: Diabetes 1994 (Baba, S. and Kaneko, T., Eds.), pp. 622–629, Elsevier, Amsterdam.
[23] Haystead, T.A.J., Haystead, C.M.M., Hu, C., Lin, T.-A. and Lawrence Jr., J.C. (1994) J. Biol. Chem. 269, 23185–23191.
[24] Sonenberg, N. (1996) in: Translational Control (Hershey, J.W.B., Mathews, M.B. and Sonenberg, N., Eds.), pp. 245–249, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[25] Diggle, T.A., Moule, S.K., Avison, M.B., Flynn, A., Foulstone, E.J., Proud, C.G. and Denton, R.M. (1996) Biochem. J. 316, 447–453.