Phosphorylation of the eIF4E-binding protein PHAS-I after exposure of PC12 cells to EGF and NGF

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Abstract PHAS-I or the eIF4E-binding protein 1 regulates the cap-binding activity of eIF4E by sequestering eIF4E. Binding of eIF4E to PHAS-I is regulated by phosphorylation of PHAS-I. PC12 cells were used to study the signal transduction pathway leading to phosphorylation of PHAS-I. Both EGF and NGF induced phosphorylation of PHAS-I. Wortmannin, a PI-3 kinase inhibitor, staurosporine, a PKC inhibitor, and rapamycin, a FRAP inhibitor all blocked the phosphorylation of PHAS-I. Of the three inhibitors, only wortmannin was able to inhibit MAPK phosphorylation. This excludes a role for MAPK in NGF- and EGF-induced PHAS-I phosphorylation in PC12 cells. Apparently, PHAS-I was phosphorylated in a PI-3 kinase-, PKC-, and FRAP-dependent manner after EGF or NGF stimulation. Only PI-3 kinase and FRAP are involved in the regulation of the basal level of PHAS-I phosphorylation.

Key words: PHAS-I; Phosphorylation; $p70^{S6K}$; FRAP; PI-3 kinase; PKC

1. Introduction

Growth and differentiation of cells are accompanied by changes in protein synthesis. These changes are induced by phosphorylation of several proteins involved in translation initiation. The cap-binding eukaryotic initiation factor (eIF) 4E is one of the proteins whose activity is regulated by changes in its phosphorylation state. Phosphorylation of eIF4E coincided with an increase in protein synthesis. Furthermore, phosphorylated eIF4E bound m^7GTP, m^7GpppG, and globin mRNA 3–4 times more efficiently than non-phosphorylated eIF4E [1]. Also, 80–85% of eIF4E present on the 48S initiation complex was phosphorylated; in contrast, only 50% of the free eIF4E was phosphorylated [2,3].

Recently, a different way to regulate eIF4E activity has been found. PHAS-I (or 4E-BP1), a heat- and acid-stable protein, is capable of binding eIF4E, and thereby is able to prevent the assembly of the eIF4F complex [4,5]. As PHAS-I and eIF4G bind to the same amino acid sequence in eIF4E [6,7], they are mutually exclusive in their binding to eIF4E [8].

PHAS-I is also regulated by phosphorylation. In vivo, PHAS-I is present in multiple forms, which are separated by one-dimensional electrophoresis into three forms (α, β, and γ), representing different forms of phosphorylation [9]. The α and β forms of PHAS-I are able to complex with eIF4E [4,5], thereby impairing the association of eIF4E with eIF4G, necessary for initiation. Phosphorylation of PHAS-I is increased after treatment of cells with insulin, epidermal growth factor (EGF), insulin-like growth factor I (IGF-I) or platelet-derived growth factor (PDGF) [10–12]. The signal transduction pathway leading to PHAS-I phosphorylation and the in vivo kinase(s) of PHAS-I remain unclear. In vitro experiments showed that PHAS-I is a substrate of mitogen-activated protein kinase (MAPK) [9,13], protein kinase C (PKC), and casein kinase II [13]. However, MAPK was not able to phosphorylate the histidine-tagged PHAS-I-eIF4E complex, and it was suggested that phosphorylation of PHAS-I by MAPK prevents reassociation with eIF4E [12]. Rapamycin, an immunosuppressive agent that blocks activation of $p70^{S6K}$ [14–16], abolished the insulin-induced phosphorylation of PHAS-I, indicating that PHAS-I was phosphorylated in a $p70^{S6K}$-dependent manner. However, $p70^{S6K}$ is not able to phosphorylate PHAS-I in vitro [9]. PD 098059, a MAP kinase kinase inhibitor blocked MAPK activation, but did not affect PHAS-I phosphorylation [12]. Apparently, PHAS-I can be phosphorylated in a MAPK- or $p70^{S6K}$-dependent manner [11,13,17].

The increase in protein synthesis in PC12 cells after nerve growth factor (NGF) treatment is accompanied by eIF4E phosphorylation [18]. However, addition of epidermal growth factor (EGF) to PC12 cells did not lead to eIF4E phosphorylation [19]. The role of PHAS-I phosphorylation in PC12 cells was studied after NGF and EGF treatment to determine whether PHAS-I phosphorylation is responsible for an increase in the availability of eIF4E for translation initiation. Furthermore, the signal transduction pathway involved in PHAS-I phosphorylation was investigated. Four important components of cellular signaling have been studied. MAPK activity was determined with a MAPK-shift assay, and the role of phosphatidylinositol-3′-OH kinase (PI-3 kinase), PKC, and FRAP were determined by using inhibitors of these kinases.

2. Materials and methods

2.1. Cell culture

PC12 cells were cultured in 1:1 DMEM and Ham's F12 (DF) containing 7.5% fetal calf serum. Cells were seeded in 60-mm diameter dishes and grown to a confluency of 60%. The cells were serum-starved for 2 h before the addition of EGF (50 ng/ml) or NGF (30 ng/ml). The inhibitors wortmannin (100 nM), staurosporine (100 nM), and rapamycin (25 ng/ml) were added 10 min prior to the addition of growth factors.

2.2. MAPK shift

After treatment with growth factors, the cells were harvested in 100

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Abbreviations: eIF, eukaryotic initiation factor; EGF, epidermal growth factor; FRAP, FKBP-rapamycin associated protein; IGF-I, insulin-like growth factor I; MAPK, mitogen-activated protein kinase; NGF, nerve growth factor; $p70^{S6K}$, 70-kDa S6 protein kinase; PDGF, platelet-derived growth factor; PHAS-I, phosphorylated heat- and acid-stable protein induced by insulin; PI-3 kinase, phosphatidylinositol-3′-OH kinase; PKC, protein kinase C.

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μl of Laemmli sample buffer [20]. Samples were heated for 10 min at 95°C, sheared, and centrifuged for 10 min at 4°C. Supernatants were analyzed by SDS-PAGE (12.5% acrylamide, 0.075% bisacrylamide) [21]. The samples were run for 2 h at 200 V on a mini-gel system (LKB) to separate the non-phosphorylated and phosphorylated forms of MAPK. Proteins were blotted onto PVDF, the blot was incubated with polyclonal ERK2 antibody (a kind gift from J.L. Bos), and MAPK was detected using the alkaline phosphatase method. The amounts of non-phosphorylated and phosphorylated forms of MAPK were quantified with a densitometer (Molecular Dynamics).

2.3. Phosphorylation of PHAS-I

The samples used for the MAPK-shift assay were also analyzed by SDS-PAGE (13.5%) [20] to separate the α-, β-, and γ-forms of PHAS-I. Proteins were blotted onto PVDF, and PHAS-I was detected using a polyclonal rabbit antibody raised against bacterially expressed His-tagged PHAS-I [13]. The phosphorylation state of PHAS-I was quantified with a densitometer (Molecular Dynamics).

2.4. Dephosphorylation of PHAS-I

PC12 cells were serum-starved for 2 h, and treated with NGF for 0 or 60 min. The cells were harvested in PBS containing 10 mM EDTA, rinsed once with PBS, and once with CIP buffer (10 mM $ZnCl_2$, 10 mM $MgCl_2$, 100 mM Tris-HCl pH 8.3). Cells were lysed in CIP buffer by freeze-thawing three times. About 50 μg of cell lysate was incubated for 16 h at 37°C with 2.5 units of calf intestine alkaline phosphatase (CIP), and after addition of an extra 2.5 units for 4 h at 56°C. The samples were analyzed by SDS-PAGE (13.5%) and immunoblotting as described above.

2.5. Association of PHAS-I and eIF4E

Cells were serum-starved for 2 h and treated with NGF for 0 or 60 min. After washing with PBS, the cells were lysed with 400 μl 10 mM HEPES, pH 7.4, adjusted with KOH, 50 mM β-glycerophosphate, 0.2 mM EDTA, 0.5% Nonidet-P40, 100 mM KCl, 7 mM β-mercaptoethanol, 0.2 mM Na_3VO_4, 0.2 mM benzamidine, and 4 μg leupeptin per ml. The lysates were centrifuged at $10\,000 \times g$ to remove cell debris and the supernatant was used to purify eIF4E. The supernatant was incubated with 5 μl packed m^7GTP Sepharose, supplemented with 15 μl packed Sepharose 4B to increase the pellet volume. After 1 h the beads were washed 4 times with lysis buffer and eIF4E was eluted with Laemmli sample buffer. Eluted eIF4E and PHAS-I were analyzed by SDS-PAGE and immunoblotting.

2.6. S6 phosphorylation assay

Cells were treated with EGF or NGF in the absence or presence of inhibitors for the times indicated in the figures. The cells were harvested in a buffer containing 20 mM Tris-HCl pH 7.6, 1% Triton X-100, 50 mM β-glycerophosphate, 0.2 mM EDTA, 0.2 mM EGTA, 1 mM sodium molybdate, 10% glycerol, 100 mM KCl, 4 μg leupeptin/ml, 0.2 mM benzamidine, 0.2 mM Na_3VO_4, and 7 mM β-mercaptoethanol. Approx. 30 ng of cell extract was incubated at 30°C for 15 min in the presence of 4 μg of 80S ribosomes derived from reticulocytes, 10 mM HEPES adjusted with KOH to pH 7.5, 100 mM KAc, 0.2 mM EDTA, 100 μM [γ-^{32}P]ATP (spec. act. 2200 dpm/pmol), 10 mM $MgAc_2$, and 7 mM β-mercaptoethanol in a total volume of 10 μl. The reaction was stopped by adding Laemmli sample buffer. The samples were heated for 5 min at 95°C, and analyzed by SDS-PAGE (12.5%) and autoradiography. S6 phosphorylation was quantified with a phospho-imager (Molecular Dynamics). This assay is specific for $p70^{S6K}$ activity [22].

3. Results

3.1. PHAS-I phosphorylation in PC12 cells

PC12 cells were treated with NGF or EGF for different time-periods (Fig. 1A). Addition of NGF as well as EGF led to a mobility shift caused by phosphorylation of PHAS-I, as will be addressed in Fig. 2. The phosphorylated γ-form changed from 15 to 44% for NGF, and from 16 to 57% for EGF. The EGF response returned to basal level after 30 min, in contrast to the NGF response which lasted for 60 min (Fig. 1B). Treatment of the cells with either NGF or EGF in the presence of wortmannin, a potent inhibitor of PI-3 kinase [23], did not induce PHAS-I phosphorylation (Fig. 1A,B), suggesting phosphorylation of PHAS-I via activation of PI-3 kinase.

Activation of the MAPK signal transduction pathway by NGF and EGF was measured by the shift from unphosphorylated to phosphorylated MAPK by SDS-PAGE [24]. Both growth factors induced a rapid phosphorylation of MAPK (Fig. 1C) as described earlier for these cells [25]. After NGF addition the amount of phosphorylated MAPK changed from 15% to 51% at 15 min, and remained at 40% up to 60 min. EGF addition led to an increase in phosphorylated MAPK from 10% to 47% at 5 min, which declined to basal level at 60 min. The amount of phosphorylated MAPK only increased from 18% to a maximum of 30% at 15 min when cells were treated with NGF in the presence of wortmannin, and from 11% to 30% at 5 min after EGF treatment in the presence of wortmannin (Fig. 1C). MAPK phosphorylation (11%) was not detectably changed in the presence of wortmannin.

3.2. Dephosphorylation of PHAS-I and association with eIF4E

Insulin-induced phosphorylation of PHAS-I in rat adipocytes [12,13], and in rat adipose tissue [5] was accompanied by dissociation of PHAS-I from eIF4E [5,13]. We wanted to know whether growth factors also regulated the association of PHAS-I and eIF4E in PC12 cells, depending upon the phosphorylation state of PHAS-I. To this end, PC12 cells were treated for 0 or 60 min with NGF. NGF induced a shift in PHAS-I to the slower migrating, phosphorylated forms (Fig. 2A, upper panel). Even though NGF induced only a small increase in PHAS-I phosphorylation in PC12 cells, this increase led to dissociation of PHAS-I from eIF4E (Fig. 2A, lower panel). The amount of eIF4E recovered was comparable. Similar results were shown for insulin-stimulated skeletal muscle cells [26]. The results suggest that eIF4E activity can be regulated by PHAS-I-eIF4E complex formation after NGF treatment in PC12 cells.

The 3 forms of PHAS-I, as revealed by one-dimensional electrophoresis, are generally assumed to arise from phosphorylation of the α-form to the β- and γ-forms. Studies with ^{32}P labeling and two-dimensional separation resulted in identification of 2 labeled forms of PHAS-I, in contrast to the 14 spots identified by immunoblotting [10,12]. To establish the relationship between the α-, β-, and γ-forms, extracts of PC12 cells were treated with alkaline phosphatase. Alkaline phosphatase treatment led to conversion from γ to β (Fig. 2B), but not from the β- to α-form, showing that at least the γ-form was a phosphorylated form of PHAS-I. The lack of conversion to the α-form will be discussed later.

3.3. The role of PKC in PHAS-I phosphorylation

PKC is able to phosphorylate PHAS-I in vitro [9]. The role of PKC in PHAS-I phosphorylation in vivo was determined by treating PC12 cells with NGF or EGF in the presence of staurosporine, a PKC inhibitor [27,28] (Fig. 3). Phosphorylation of PHAS-I was induced by NGF or EGF addition, similar to the results in Fig. 1. Treatment of cells in the presence of staurosporine blocked the effect of NGF and EGF on PHAS-I phosphorylation completely (Fig. 3A,B), suggesting a major role for PKC in the phosphorylation of PHAS-I in PC12 cells.

MAPK phosphorylation induced by EGF was not blocked by staurosporine as expected, because MAPK is activated by

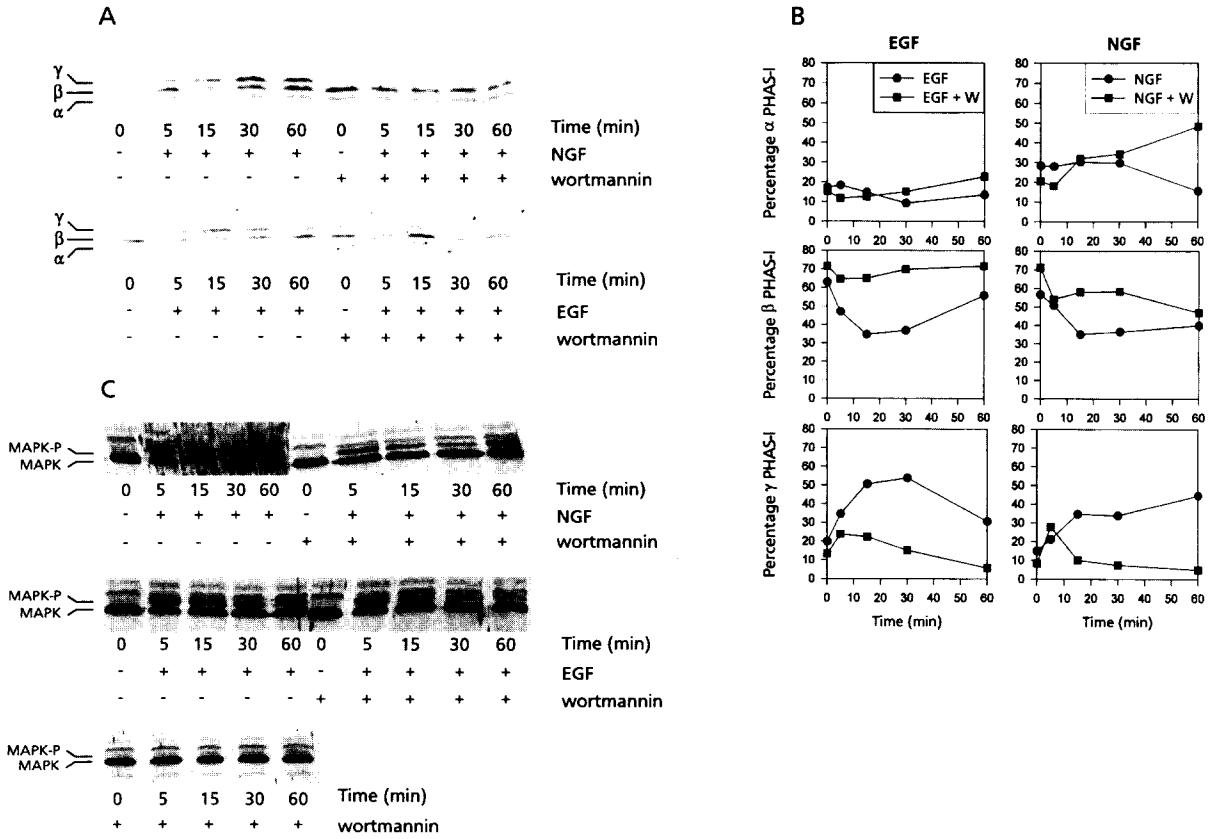

Fig. 1. Effect of wortmannin on NGF- and EGF-induced PHAS-I and MAPK phosphorylation. PC12 cells were treated without (−) or with (+) NGF or EGF in the absence (−) or presence (+) of wortmannin for the times indicated. (A) Phosphorylation state of PHAS-I analyzed by SDS-PAGE and Western blotting. (B) Quantification of the phosphorylation state of PHAS-I. The different forms of PHAS-I are given as a percentage of the total amount of PHAS-I. The quantification of PHAS-I phosphorylation in the absence of wortmannin is a mean from three independent experiments. (C) MAPK phosphorylation, shown as a shift from the unphosphorylated to the phosphorylated form of MAPK, was analyzed by SDS-PAGE, and Western blotting.

EGF in a PKC-independent way [25]. Also, staurosporine alone did not influence MAPK phosphorylation (Fig. 3C).

3.4. PHAS-I phosphorylation via FRAP

PHAS-I is phosphorylated after PDGF, IGF-I, or insulin treatment in a rapamycin-sensitive way [11,12]. Furthermore, the experiments with wortmannin (Fig. 1) and staurosporine (Fig. 3) showed no correlation between phosphorylation of PHAS-I and MAPK activation after NGF or EGF treatment. To assess the role of FRAP in PHAS-I phosphorylation in PC12 cells after NGF and EGF addition, we used the inhibitor rapamycin (Fig. 4). NGF- as well as EGF-induced PHAS-I phosphorylation was abolished by the addition of rapamycin (Fig. 4A,B).

Rapamycin did not influence the MAPK-shift induced by NGF (Fig. 4C). This is in agreement with data from the literature describing that rapamycin had no effect on MAPK activity in 3T3-L1 adipocytes as measured by MAPK-mediated phosphorylation of myelin basic protein [12].

3.5. NGF- and EGF-induced activation of $p70^{S6K}$

The role of FRAP in PHAS-I phosphorylation prompted us to investigate the activation of $p70^{S6K}$, which is downstream of FRAP. The activity of $p70^{S6K}$ was measured by its ability to phosphorylate the ribosomal S6 protein (Fig. 5). Addition of NGF led to a 350% increase in $p70^{S6K}$ activity, which was prevented by staurosporine and rapamycin, and strongly diminished by wortmannin (Fig. 5A). EGF addition led to a 50% increase in $p70^{S6K}$ activity with a maximum at 30 min (Fig. 5B). The $p70^{S6K}$ activation was completely blocked by wortmannin, staurosporine, and rapamycin. The activation of

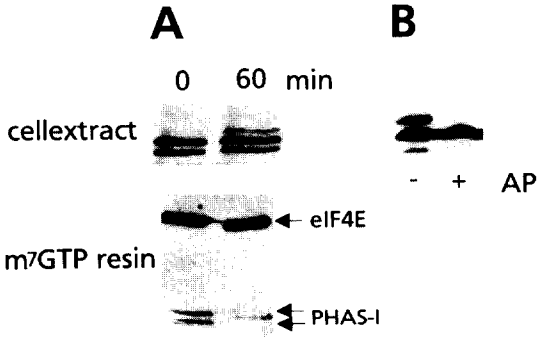

Fig. 2. Dephosphorylation of PHAS-I and its association with eIF4E. (A) PC12 cells were treated for 0 or 60 min with NGF, and eIF4E was purified to determine the association of PHAS-I with eIF4E. (Upper panel) Total cell extract. (Lower panel) m^7GTP Sepharose purified. (B) Untreated cell extracts were incubated with alkaline phosphatase as described in Section 2. The resulting Western blot is shown. AP, alkaline phosphatase treated.

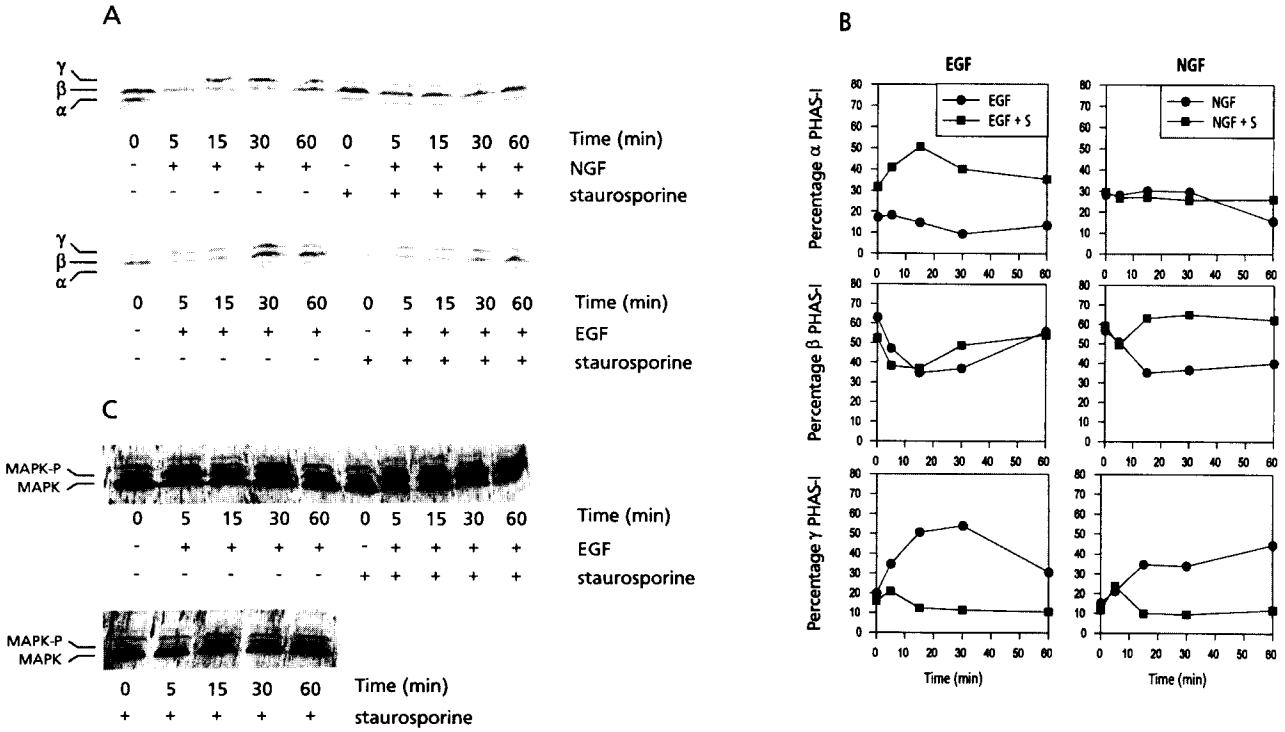

Fig. 3. Effect of staurosporine on NGF- and EGF-induced PHAS-I and MAPK phosphorylation. PC12 cells were treated without (−) or with (+) NGF or EGF in the absence (−) or presence (+) of staurosporine for the times indicated. (A) Analysis of the different forms of PHAS-I. (B) Quantification of panel A was performed as in Fig. 1. (C) Analysis of MAPK phosphorylation.

$p70^{S6K}$ by NGF treatment was more severe and prolonged than by EGF.

3.6. *Pathways leading to PHAS-I phosphorylation*

Wortmannin, staurosporine, and rapamycin were also tested for their effect on PHAS-I phosphorylation when incubated in the absence of growth factors (Fig. 6). In control cells, 8% of PHAS-I was in the α-form, 34% in the β-form and 60% in the γ-form. After 1 h of incubation with wortmannin, the α-form increased to 20%, the β-form to 78%, and the γ-form declined to 2%. The effects were similar after 2 and 3 h, but less apparent. Addition of staurosporine hardly influenced the phosphorylation state of PHAS-I. Rapamycin on the other hand, had the strongest effect of the three inhibitors. After 3 h, 80% of PHAS-I was present in the α-form, and only a small amount in the β-form (14%), or γ-form (6%).

4. Discussion

The activity of eIF4E can be regulated in at least two different manners. First, association of eIF4E with PHAS-I, or the eIF4E-binding protein, prevents assembly of the eIF4F complex and thereby initiation of translation [6,8]. Second, the activity of eIF4E is positively correlated with its phosphorylation state, as described in Section 1. NGF and EGF influence protein synthesis in different manners, as NGF is able to induce eIF4E phosphorylation in PC12 cells, whereas EGF is not [19]. Therefore, the role of PHAS-I phosphorylation in the regulation of eIF4E activity in PC12 cells was determined.

Both EGF and NGF induced phosphorylation of PHAS-I (Fig. 1), which released eIF4E from PHAS-I (Fig. 2A), making eIF4E available for translation [5,13]. Regulation of eIF4E activity exclusively by PHAS-I phosphorylation and not by eIF4E phosphorylation, has been described for insulin treatment of diabetic rats [29], resembling our result with EGF and PC12 cells (Figs. 1, 3 and 4).

The slower migrating forms of PHAS-I are known as the phosphorylated β- and γ-forms [13]. That β and γ are the phosphorylated forms is supported by several literature data and results shown in this report. First, the γ-form of PHAS-I can be converted to the β-form by alkaline phosphatase treatment (Fig. 2B), showing that the γ-form is indeed a phosphorylated form of PHAS-I. The inability of alkaline phosphatase to convert the β-form may be due to inaccessibility of the phosphate residue or a difference in amino acid residues that are dephosphorylated. It has been shown that PHAS-I can be phosphorylated at serine as well as threonine residues [10]. Second, ^{32}P labeling of cells showed two phoshorylated PHAS-I forms [4,9,30], and their migration on SDS-PAGE indicates that these phosphorylated forms are the β- and γ-form.

NGF- and EGF-induced PHAS-I phosphorylation was also studied in the presence of the PI-3 kinase inhibitor wortmannin, the PKC inhibitor staurosporine, and the FRAP inhibitor rapamycin, to determine the involvement of these signal transduction components in the pathway leading to PHAS-I phosphorylation (Figs. 1, 3 and 4). All three inhibitors abolished the effect of NGF and EGF on PHAS-I phosphorylation, showing that PI-3 kinase, PKC, and FRAP are components of the PHAS-I phosphorylation pathway(s).

Inhibition of PI-3 kinase blocked NGF- and EGF-induced PHAS-I phosphorylation in PC12 cells. The involvement of PI-3 kinase in PHAS-I phosphorylation has also been shown for insulin treatment of 32D cells overexpressing the insulin receptor [30], and for serum treatment of 293 cells [31].

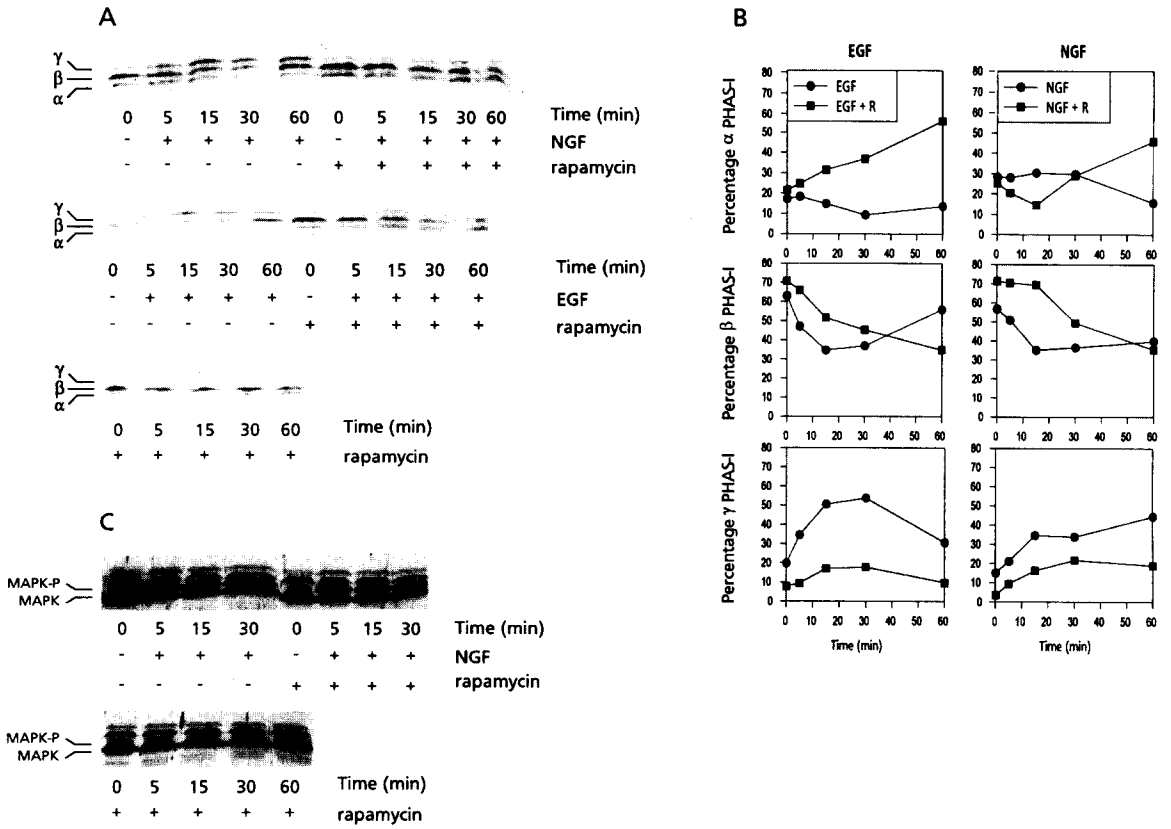

Fig. 4. Effect of rapamycin on NGF- and EGF-induced PHAS-I and MAPK phosphorylation. PC12 cells were treated without (−) or with (+) NGF or EGF in the absence (−) or presence (+) of rapamycin for the times indicated. (A) Phosphorylation state of PHAS-I. (B) Quantification of the phosphorylation state of PHAS-I. The three forms of PHAS-I were analyzed as described in Fig. 1. (C) Analysis of MAPK phosphorylation.

Although wortmannin also inhibited MAPK phosphorylation partially, the results with staurosporine and rapamycin showed that MAPK activation is not involved in the NGF- and EGF-induced PHAS-I phosphorylation (as discussed below).

Our results and literature data suggest that PHAS-I phosphorylation is most likely downstream of $p70^{S6K}$. This is strongly supported by the kinetics of $p70^{S6K}$ activation and PHAS-I phosphorylation after NGF or EGF treatment of PC12 cells, which were very similar (Figs. 1 and 5). Also, rapamycin, staurosporine, as well as wortmannin blocked PHAS-I phosphorylation and $p70^{S6K}$ activation. Furthermore,

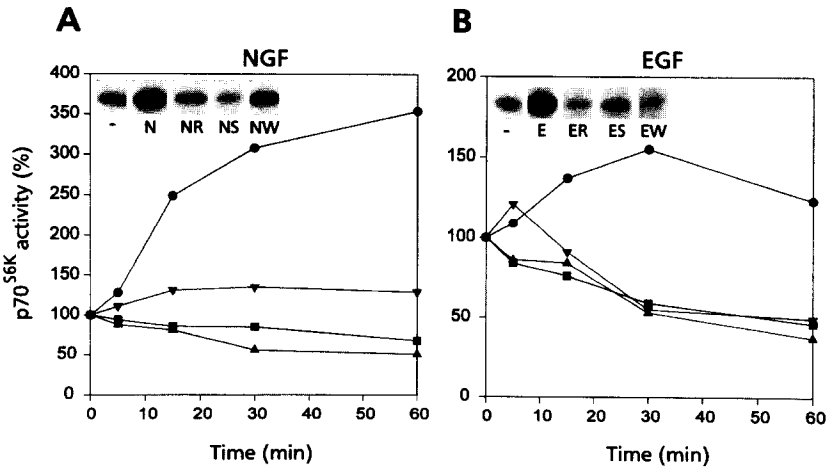

Fig. 5. Activation of $p70^{S6K}$ by EGF and NGF. PC12 cells were treated with EGF or NGF for the times indicated. Cell extracts were incubated in the presence of 80S ribosomes and [γ-^{32}P]ATP and analyzed by SDS-PAGE and autoradiography. Phosphorylation of the ribosomal S6 protein (as shown in the insets for the 30 min time point) was quantified with the phospho-imager (Molecular Dynamics). −, no addition; N, 30 min NGF; NR, 30 min NGF + rapamycin; NS, 30 min NGF + staurosporine; NW, 30 min NGF + wortmannin. In the case of EGF, N is replaced by an E. (A) NGF-induced $p70^{S6K}$ activity. (B) EGF-induced $p70^{S6K}$ activity. (●) NGF or EGF, (■) growth factor + rapamycin, (▲) growth factor + staurosporine, (▼) growth factor + wortmannin.

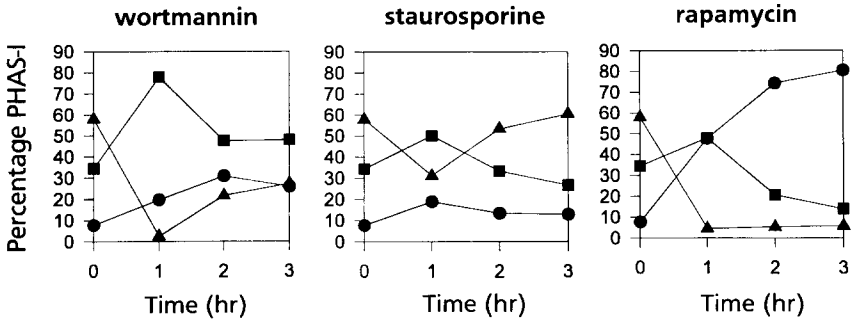

Fig. 6. Effect of wortmannin, staurosporine, and rapamycin on PHAS-I phosphorylation. PC12 cells grown in the presence of 7.5% serum were treated with wortmannin, staurosporine, or rapamycin for 1, 2, or 3 h. PHAS-I phosphorylation was analyzed and quantified. The α-form (●), the β-form (■), and the γ-form (▲) are given as a percentage of the total amount of PHAS-I.

similar results with rapamycin were obtained with insulin treatment of adipocytes, and PDGF and IGF-I treatment of aortic smooth muscle cells [11,12], and concomitant activation of $p70^{S6K}$ and phosphorylation of PHAS-I was also reported for insulin and serum stimulation of 293 cells [31]. However, bifurcation of the signalling pathway downstream of FRAP and upstream of $p70^{S6K}$ cannot be excluded; $p70^{S6K}$ activation and PHAS-I phosphorylation might occur in parallel.

Treatment of cells with the inhibitors in the absence of growth factors gave us more insight into the regulation of PHAS-I phosphorylation in normal growing cells. Wortmannin and rapamycin induced PHAS-I dephosphorylation (Fig. 6), showing that PI-3 kinase and FRAP are components of the signalling pathway involved in the basal level of PHAS-I phosphorylation. On the other hand, phosphorylation of PHAS-I by PKC, as found in vitro [9] and after NGF or EGF stimulation (Fig. 3), does not seem to be involved in the basal level of PHAS-I phosphorylation, as staurosporine showed no effect on PHAS-I phosphorylation (Fig. 6).

A possible role of MAPK activation in PHAS-I phosphorylation was also determined. MAPK was phosphorylated after NGF and EGF addition, coinciding with PHAS-I phosphorylation. However, only wortmannin was able to inhibit MAPK phosphorylation (Fig. 1C), whereas all three inhibitors abolished the effect of NGF and EGF on PHAS-I phosphorylation. This clearly showed that the MAPK pathway is not involved in NGF- and EGF-induced PHAS-I phosphorylation in PC12 cells. This finding is not very surprising, because MAPK only phosphorylated the α-form of PHAS-I [12], which is hardly changed after NGF and EGF addition to PC12 cells (Figs. 1, 3 and 4), and no involvement of MAPK in the β-γ conversion has been shown. So far, no involvement of MAPK in a growth factor-induced PHAS-I phosphorylation has been found [11,12,26,31], although MAPK very efficiently phosphorylates PHAS-I in vitro [9,12,13].

Regulation of PHAS-I phosphorylation by multiple pathways is supported by several literature data. It has been shown that PHAS-I is mostly phosphorylated at serine residues in control cells, but at threonine residues in insulin-treated cells [10]. This suggests that more than one kinase is involved in PHAS-I phosphorylation. In vitro, three different kinases, MAPK, PKC, and casein kinase II have been shown to phosphorylate PHAS-I at distinct sites [9]. Furthermore, an increase in cAMP levels induced by forskolin was able to decrease activation of $p70^{S6K}$ induced by PDGF and IGF-I, thereby decreasing PHAS-I phosphorylation [11]. This suggests an inhibitory role for cAMP-dependent protein kinase in the PHAS-I phosphorylation pathway.

In summary, this paper provides evidence that PI-3 kinase, PKC, as well as FRAP are involved in the regulation of NGF- and EGF-induced PHAS-I phosphorylation in PC12 cells. Furthermore, PI-3 kinase, FRAP, but not PKC are involved in the regulation of the basal level of PHAS-I phosphorylation.

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