The regulation of protein synthesis and of eukaryotic initiation factor eIF2B was studied in PC12 cells. An increase in protein synthesis was observed after nerve growth factor (NGF) and epidermal growth factor (EGF) treatment of PC12 cells, and this increase coincided with activation of eIF2B. Growth factor addition in the presence of the phosphatidylinositol-3'-OH kinase inhibitor wortmannin showed that both NGF- and EGF-induced protein synthesis and eIF2B activation were phosphatidylinositol-3'-OH kinase dependent. The EGF-induced stimulation of protein synthesis and activation of eIF2B was dependent upon FK506-binding protein-rapamycin-associated protein, as shown with the immunosuppressant rapamycin, whereas NGF induction was partially dependent upon FK506-binding protein-rapamycin-associated protein.

The activities of two kinases that act on eIF2B, glycogen synthase kinase-3 and casein kinase II, were measured to assess their potential roles in the activation of eIF2B in PC12 cells. Inactivation of glycogen synthase kinase-3 was not rapamycin-sensitive, in contrast to the activation of eIF2B. This indicates the involvement of another protein kinase or regulatory mechanism in the eIF2B activation. Both growth factors activated casein kinase II. However, the time course of its activation and its insensitivity to wortmannin and rapamycin suggest that casein kinase II does not play a major regulatory role in eIF2B activation under these conditions.

Translation initiates with the binding of eukaryotic initiation factor eIF4E to the cap structure on the mRNA (1, 2). After assembly of the eIF4F complex by association of eIF4E with eIF4A and eIF4G, unwinding of the secondary structure of the 5'-untranslated region (3) facilitates the assembly of the 48 S initiation complex and by formation of the 80 S ribosomal complex. Translation initiation can be regulated at different steps (4). Two of the initiation factors with important roles in this regulation are eIF4E, the cap-binding protein, and eIF2B, the guanine nucleotide exchange factor for eIF2. Both factors are present in limiting amounts in the cell. Although the amount of eIF4E present in reticulocytes is 10-fold higher than previously assumed (5), it is lower than any other initiation factor (6, 7). Furthermore, in the cell types tested the amount of eIF2B is limiting compared with eIF2 (8, 9).

The activity of eIF4E is regulated in at least three ways: by its own phosphorylation state (4, 10–12); by binding to eIF4G (13); and by association with the eIF4E binding proteins PHAS-I or eIF4E-BP1 (14, 15). Changes in the phosphorylation state of eIF4E did not always correlate with the changes in overall protein synthesis (16–19). This indicates that other initiation factors are involved in regulating the rate of protein synthesis.

Another well known regulatory translation initiation factor is eIF2B, which can be regulated in several different ways. First, phosphorylated eIF2 is a potent competitive inhibitor of eIF2B exchange activity (8). Second, allosteric effectors regulate eIF2B activity in vitro. For example, binding of NADPH to eIF2B is necessary to maintain nucleotide exchange activity (20, 21). Third, the activity of eIF2B can be regulated in vitro by phosphorylation of its ε-subunit (22, 23). So far, three kinases have been described that can phosphorylate eIF2B in vitro, casein kinase (CK) I and II (22), and glycogen synthase kinase-3 (GSK-3) (23). Phosphorylation of eIF2B by CKI and -II enhanced eIF2B activity in contrast to phosphorylation by GSK-3, which had an inhibitory effect. Antagonism of the CKII-induced eIF2B activation by GSK-3-mediated phosphorylation has also been shown in vitro (24), suggesting a negative regulatory function for GSK-3.

An increase in eIF2B activity has been shown in vivo after T cell stimulation (25), for concanavalin A-treated T lymphocytes (26), after glucose stimulation of isolated rat islets of Langerhans (27), and after treatment of Swiss 3T3 fibroblasts with insulin, serum, EGF, or phorbol esters (28). Inactivation of GSK-3 has been shown to correlate with eIF2B activation in the first of these cases (25). The role of CKI and II in the control of eIF2B in vivo has not yet been addressed.

Although a number of different mechanisms for regulating translation factor activity have now been described, it is not clear what roles they play in vivo in the activation of translation by specific stimuli. In this study the roles of eIF2 and eIF2B in NGF- and EGF-treated PC12 cells were studied in relation to overall protein synthesis to determine their function in the regulation of protein synthesis. Furthermore, the signal transduction pathway involved in eIF2B activation was studied using the phosphatidylinositol-3'-OH (PI-3) kinase inhibitor

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1 The abbreviations used are: CK, casein kinase; GSK, glycogen synthase kinase-3; NGF, nerve growth factor; EGF, epidermal growth factor; FRAP, FK506-binding protein-rapamycin-associated protein; PI-3, phosphatidylinositol-3'-OH; PKB, protein kinase B; MAPK, mitogen-activated protein kinase; TOP, 5'-terminal oligopyrimidine tract; Tricine, N-tris(hydroxymethyl)methylglycine.
wortmannin and the FK506-binding protein-rapamycin-associated protein (FRAP) inhibitor rapamycin. The activities of the two kinases, GSK-3 and CKII, potentially involved in eIF2B phosphorylation were determined.

**EXPERIMENTAL PROCEDURES**

**Materials**—Wortmannin and NGF were from Sigma, rapamycin from Calbiochem, DF medium (a 1:1 mixture of Ham F-12 and Dulbecco modified Eagle’s medium) from Life Technologies, Inc., EGF from Harlan, [3H]GDP, [32P]ATP and [γ-32P]ATP from Amersham.

**Cell Culture**—Rat pheochromocytoma (PC12) cells were grown to a confluency of 70% in DF medium supplemented with 7.5% fetal calf serum. Cells were deprived of serum for 2 h before treatment with NGF (30 ng/ml) or EGF (50 ng/ml). Wortmannin (100 nM) and rapamycin (25 ng/ml) were added to the cells 10 min prior to addition of growth factors. Cells were washed twice with phosphate-buffered saline before harvesting.

**Protein Synthesis Measurement in PC12 Cells**—PC12 cells were grown in 24-well plates in 200 μl of medium. Cells were treated with NGF, EGF, wortmannin, and rapamycin as described above and labeled with 12.5 μCi [32P]methionine/cysteine per ml for 15 min before harvesting. Cells were harvested in 0.5% Nonidet P40, and equal amounts of protein were used for measuring protein synthesis by hot trichloroacetic acid precipitation.

**Measurement of eIF2B Activity**—Cells grown in 30-mm dishes 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 Na3VO4, and 7 mM β-mercaptoethanol. eIF2-γGDP complexes were formed as described (29). Briefly, 1 pmol of rabbit reticulocyte eIF2 (30) was incubated with 0.2 μCi [3H]GDP (about 15 pmol with a specific activity of 30,000 dpm/pmol) in the presence of 20 mM Tris-HCl, pH 7.6, 120 mM KCl, 1% bovine serum albumin, and 1 mM dithiothreitol. After 15 min at 30 °C, 5 mM MgCl2, and 1 mM GTP were added. The eIF2-γGDP/GTP complex was added to approximately 10 μg of cell extract and incubated for another 15 min at 30 °C. The [3H]GDP/GTP exchange reaction was terminated by adding 1 ml of a cold wash buffer containing 50 mM Tris-HCl, pH 7.6, 5 mM MgCl2, 100 mM KCl, and 7 mM β-mercaptoethanol. The mixture was filtered through nitrocellulose filters, and the filters were rinsed three times with the same wash buffer.

**GSK-3 Kinase and CKII Assay**—About 10 μg of cell extract, prepared as for the eIF2B assay, was incubated with a peptide in 20 mM Tris-HCl, pH 7.5, 2 mM MgCl2, 0.1 mM ATP, and 1 μCi of [γ-32P]ATP (final specific activity of 880 dpm/pmol) in a volume of 25 μl. In the GSK-3 assay, 6 μg of RRAAELDRAGAG/PQQL peptide was used as a substrate and 6 μg of RRAAELDRAGAPQL as a negative control (31). In the CKII assay no peptide or 6 μg of RRDDDDDDDD (32, 33) was used. After 15 min at 30 °C, 10 μl of the sample was spotted onto P81 phosphocellulose paper. The P81 paper was rinsed five times with 3% (v/v) phosphoric acid for the GSK-3 assay and with 0.5% (v/v) phosphoric acid for the CKII assay. The filters were rinsed twice with 96% ethanol, dried, and counted. Another 10 μl of the sample was analyzed by electrophoresis on Tris-Tricine gels (34).

**S6 Phosphorylation Assay**—Approximately 30 ng of cell extract was incubated with 4 μg of 80 S ribosomes, and 0.1 mM [γ-32P]ATP (final specific activity of 2200 dpm/pmol), as described in Kleijn et al. (35). The reaction was stopped by adding Laemmli sample buffer. Samples were heated for 5 min at 95 °C, and analyzed by SDS-polyacrylamide gel electrophoresis (12.5%) and autoradiography. S6 phosphorylation was quantified with a PhosphorImager (Molecular Dynamics). This assay was shown to be specific for p70S6K (36).

**Phosphorylation State of eIF2α**—About 50 μg of cell extract, prepared as for the eIF2B assay, was loaded on an SDS-gel and blotted to Immobilon-P. Phosphorylated eIF2α was detected with a rabbit antibody specific for the phosphorylated form of eIF2α (kindly provided by Dr. G. Krause) (37). eIF2α was detected with a monoclonal antibody (made by Dr. Henshaw, and obtained via Dr. Sarre, Freiburg, Germany). Blots were developed with the alkaline phosphatase method.

**RESULTS**

Protein synthesis in PC12 cells was monitored during NGF and EGF treatment. After 2 h of serum starvation PC12 cells were treated with the differentiation stimulus NGF or the growth stimulus EGF, and protein synthesis was followed for 90 min (Fig. 1). Both growth factors stimulated protein synthesis approximately 1.7-fold. The rate of protein synthesis still increased after 90 min of NGF treatment (Fig. 1A), whereas EGF-induced stimulation of protein synthesis peaked between 30 and 60 min (Fig. 1B). Similar experiments were carried out in the presence of the PI-3 kinase inhibitor wortmannin and the FRAP inhibitor rapamycin. Wortmannin was able to block the activation of protein synthesis by NGF as well as by EGF, indicating that PI-3 kinase is required for the increase in protein synthesis. Inhibition of FRAP by rapamycin did not influence the NGF-induced increase in protein synthesis, although a slight reduction occurred when the effect of rapamycin alone (Fig. 1C) was included. The EGF response was reduced to the rapamycin control level. Clearly, FRAP activation is involved in growth factor-induced protein synthesis in PC12 cells. Wortmannin alone did not influence the basal level of protein synthesis. Rapamycin stimulated basal protein synthesis slightly, which is in contrast to data in which a 10–15% decrease was found during the same time period of treatment (38, 39). Although the severity of inhibition differs between the cell types, an increase in protein synthesis has never previously been reported.

It has been shown that phosphorylation of eIF4E occurred after stimulation with NGF, but not after addition of EGF (40). Therefore, eIF4E phosphorylation is not a general mechanism in PC12 cells to increase protein synthesis.
Fig. 2. NGF- and EGF-induced eIF2B activation in the absence or presence of rapamycin and wortmannin. PC12 cells were serum-starved for 2 h and treated with NGF (A), EGF (B), or no growth factor (C) in the absence or presence of wortmannin (W) and rapamycin (R). The activity of eIF2B was measured by determining the GDP/GTP exchange activity as described under “Experimental Procedures.” Cells were harvested after 0, 30, 60, and 90 min. The basal level of eIF2B activity represents 10,000 dpm [3H]GDP released in 15 min. The results are obtained from four experiments and the percentages are calculated based on the value at t = 0.

An increase in eIF2B activity might occur in various ways, as described in the Introduction. One of the possible regulatory mechanisms is a decrease in the phosphorylation state of eIF2α. Therefore, we determined the phosphorylation state of eIF2α after NGF and EGF treatment. PC12 cells were treated with NGF and EGF for 0, 30, and 60 min, and cell extracts were analyzed by SDS-polyacrylamide gel electrophoresis and Western blotting (Fig. 3). Detection with an antibody that exclusively recognizes the phosphorylated form of eIF2α showed that eIF2α was not dephosphorylated during NGF and EGF treatment (Fig. 3, upper panel). To check whether changes in eIF2α phosphorylation could be detected using this antibody, cell extract was incubated in the absence or presence of the hemin-regulated protein kinase HRI (Fig. 3, lanes 7 and 8). The lower panel shows that equal amounts of cell extract were loaded onto the gel. Concluding, these results showed that the increase in eIF2B activity was not caused by changes in the phosphorylation state of eIF2α. Similar results were obtained earlier (25–28).

The NGF- and EGF-induced increase in protein synthesis and activation of eIF2B was partially dependent upon the rapamycin-sensitive FRAP pathway. Therefore, we determined whether p70S6K, a FRAP-dependent kinase, might be involved in eIF2B activation. NGF induced a 3.5-fold increase in p70S6K activity, which could be blocked by wortmannin as well as by rapamycin (Fig. 4A). The inhibitors also abolished the EGF-induced 1.5-fold increase in p70S6K activity (Fig. 4B). However, the extent of stimulation as well as the kinetics of p70S6K activation suggest that this kinase is not involved in the activation of eIF2B. Also, stimulation of translation is apparently not exclusively due to p70S6K activation (Fig. 1). However, as increased S6 phosphorylation coincides with an increase in translation of 5′-oligopyrimidine sequence-containing mRNAs (38, 50, 51), it may well be that NGF as well as EGF activates translation of this class of mRNAs (discussed below).

The activities of two protein kinases that can regulate eIF2B activity in vitro, GSK-3 (23) and CKII (22), was measured during NGF and EGF stimulation to assess their involvement in the NGF- and EGF-induced eIF2B activation. GSK-3 activity was measured using a peptide containing amino acids 531–542 from the ε-subunit of eIF2B (31, 52). A decrease in GSK-3 activity of 30–36% was found after EGF or NGF addition (Fig. 5). After 90 min, GSK-3 activity in NGF-treated cells was still declining (Fig. 5A), whereas in EGF-treated cells a maximal inhibition was obtained after 60 min (Fig. 5B). The kinetics of GSK-3 inactivation correlated with activation of eIF2B (Fig. 2), as well as with the increase in protein synthesis (Fig. 1). A correlation between changes in the activities of GSK-3 and eIF2B was also seen after T cell activation (25). Wortmannin abolished the effects of NGF and EGF on inactivation of GSK-3 completely (Fig. 5), which indicates that GSK-3 inactivation is PI-3 kinase-dependent as previously reported. Inhibition of
Fig. 3. NGF and EGF do not affect eIF2α phosphorylation. PC12 cells were treated with NGF or EGF for the times indicated in the figure. Cell extracts were incubated with 100 S ribosomes and [γ-32P]ATP and analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. Phosphorylation of the ribosomal S6 protein (as shown in the insets for the 30 min time point) was quantified with the PhosphorImager (Molecular Dynamics). –, no addition; N, NGF; E, EGF; R, rapamycin; W, wortmannin. A, NGF-induced p70S6K activity. B, EGF-induced p70S6K activity. (●) NGF or EGF, (■) growth factor + rapamycin, (▲) growth factor + wortmannin.

FRAP by rapamycin did not influence the effect of NGF or EGF on GSK-3 activity, although rapamycin inhibited eIF2B activation (Fig. 2). Treatment of the cells with the inhibitors alone did not influence GSK-3 activity (data not shown). Analysis of the kinase assays by electrophoresis (Fig. 5C) confirmed that the eIF2Be-peptides were labeled, and that labeling was decreased after NGF and EGF treatment.

The activity of CKII was determined in a kinase assay with a peptide containing the consensus sequence of CKII (32). Both NGF and EGF stimulated the CKII activity in PC12 cells, but the kinetics of activation of CKII were quite different in the two cases (Fig. 6). EGF induced a fast but modest 1.3-fold increase in CKII activity with a maximum at 10 min, and CKII activity was back to basal level after 30 min. In contrast, NGF induced a more prolonged and substantial stimulation of CKII; the activation increased 1.5-fold up to 30 min before a slow decline of kinase activity occurred. Neither wortmannin nor rapamycin had any effect on CKII activation by NGF (Fig. 6A) or EGF (Fig. 6B).

DISCUSSION

Regulation of protein synthesis in PC12 cells by NGF and EGF cannot solely be explained by increased eIF4E phosphorylation or PHAS-I phosphorylation. Phosphorylation of eIF4E after NGF addition is p21ras-dependent, which does not agree with the wortmannin results, and the eIF4E phosphorylation state does not change after EGF treatment (40). However, PI-3 kinase-dependent eIF4E phosphorylation has been described in 32D cells (47). Furthermore, NGF- and EGF-induced PHAS-I phosphorylation is sensitive to rapamycin, whereas NGF-stimulated protein synthesis is grossly independent from FRAP, and only EGF-induced protein synthesis is completely dependent upon FRAP.

We found three lines of evidence that suggest the involvement of eIF2B in the regulation of protein synthesis after growth factor addition. First, the kinetics of protein synthesis activation (Fig. 1) and eIF2B activation (Fig. 2) are similar. Second, the effects of wortmannin and rapamycin on protein synthesis and eIF2B activation are identical. And third, the kinetics of GSK-3 inactivation, and the influence of wortmannin on GSK-3 support the link between eIF2B activity and protein synthesis after growth factor addition.

The increase in eIF2B activity after NGF or EGF treatment was not due to dephosphorylation of eIF2α (Fig. 3). The involvement of PI-3 kinase and FRAP in NGF- and EGF-induced activation of eIF2B suggests that eIF2B activity may be regu-
lated through phosphorylation. This is supported by the correlation between the kinetics of GSK-3 inactivation (Fig. 5) and eIF2B activation (Fig. 2) (see also below).

The activities of two kinases that are able to phosphorylate eIF2B in vitro, GSK-3 and CKII, were studied in NGF- and EGF-treated PC12 cells. The NGF- and EGF-induced inactivation of GSK-3 (Fig. 5) coincided with activation of eIF2B. Although CKII was activated after NGF as well as after EGF addition (Fig. 6), the kinetics of activation drastically differed from the kinetics of eIF2B activation. A role for CKII in regulation of eIF2B activity in vitro is not obvious from these results, but rapid activation of CKII may be involved in the more prolonged control of eIF2B activity. An increase in CKII activity was also shown after addition of insulin, insulin-like growth factor I, EGF, and serum (53–56), although the validity of some early data has recently been questioned (57). The activity of CKII, a serine/threonine kinase, is regulated by phosphorylation (58). Changes in CKII have been reported in several cases and more than a hundred substrates are known for CKII. So far, no signal transduction pathways have been described that might involve CKII. Our results with wortmannin and rapamycin showed that PI-3 kinase and FRAP are not upstream components of the CKII signaling pathway. Therefore, it is unlikely that CKII is a major regulator of eIF2B in this system, despite its ability to increase eIF2B activity in vitro (22). It is interesting to note that the effects of NGF and EGF on CKII activation are similar to those of MAPK phosphorylation (35, 59), transient for EGF and sustained for NGF.

Two lines of evidence support a role for GSK-3 in the regulation of eIF2B. First, the time courses of NGF- and EGF-induced GSK-3 inactivation in vivo matched that of eIF2B activation. GSK-3 phosphorylation and thereby inactivation has been reported after treatment of cells with insulin, insulin-like growth factor 1, and EGF (23, 60, 61). A correlation between GSK-3 inactivation and eIF2B activation has previously been shown after T cell activation (25). And second, the signal transduction pathway involved in eIF2B activation and GSK-3 inactivation is wortmannin sensitive. The PI-3 kinase-dependent eIF2B activation and GSK-3 inactivation is consistent with a regulatory role for GSK-3 in NGF- and EGF-induced eIF2B activation.

Activation of eIF2B was partially dependent on FRAP, whereas GSK-3 inactivation was independent. This indicates that a FRAP-dependent and most likely a GSK-3-independent signaling pathway is involved in eIF2B activation. Regulation of eIF2B activity by a different kinase or phosphatase is supported by the result that GSK-3 activity did not change after glucose stimulation of the islet of Langerhans, whereas eIF2B activity did (27).

Three different protein kinases are able to phosphorylate GSK-3 in vitro, namely p70S6K (62), MAPKAPK-1 (p90rsk) (63), and protein kinase B (PKB) (64), although current evidence favors PKB as playing a major role in the physiological control of GSK-3 activity (64). The signal transduction pathway leading to GSK-3 inactivation in PC12 cells after NGF and EGF treatment still needs to be elucidated. Rapamycin did not affect GSK-3 inactivation, which rules out a role for p70S6K in the control of GSK-3. It is unlikely that GSK-3 is inactivated via the MAPK pathway since MAPK phosphorylation was only partially inhibited by wortmannin in PC12 cells (35), whereas this agent completely abolished the effects of NGF and EGF on eIF2B and GSK-3. Cross et al. (64) have also provided data showing that the MAPK pathway is not required for the inactivation of GSK-3 by insulin in L6 myocytes. Taken together with the data of Kleijn et al. (35), the present findings support a role for PKB in GSK-3 inactivation, since activation of PKB, like that of eIF2B, is sensitive to wortmannin but not to rapamycin and is independent of the MAPK pathway (64–66). The role of PKB in eIF2B activation has not yet been addressed.

Inhibition by rapamycin of the stimulation of protein synthesis could be explained by a reduction in translation of specific mRNAs containing a 5′-terminal oligopyrimidine tract (TOP) at the 5′-end. The family of TOP mRNAs encodes ribosomal proteins, translation elongation factors, and other proteins. All these proteins play a prominent role in cell growth (38). Recruitment of TOP mRNAs is correlated with phosphorylation of the 40S ribosomal protein S6 by p70S6K (38) in response to several mitogens (67). Because NGF induced a higher level of p70S6K activity (Fig. 4) one would expect translation of mRNAs after NGF addition. However, p70S6K-dependent translation does not seem to play an important role in NGF-induced protein synthesis. Translation of TOP mRNAs needs to be studied after NGF and EGF treatment of PC12 cells to determine whether there is a difference in translation of these mRNAs after a differentiation (NGF) or a growth (EGF) stimulus.

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