Initiation Factor 2B Activity Is Regulated by Protein Phosphatase 1, Which Is Activated by the Mitogen-activated Protein Kinase-dependent Pathway in Insulin-like Growth Factor 1-stimulated Neuronal Cells*

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We have previously demonstrated that insulin-like growth factor 1 (IGF1) induces eukaryotic initiation factor 2B (eIF2B) activity in neuronal cells through the phosphatidylinositol 3′ kinase/glycogen synthase kinase 3′ pathway as well as by activation of the mitogen-activated protein kinase (MAPK)-activating kinase (MEK)/MAPK signaling pathway (Quevedo, C., Alcázar, A., and Salinas, M. (2000) J. Biol. Chem. 275, 19192–19197). This paper addresses the mechanism involved in IGF1-induced eIF2B activation via the MEK/MAPK cascade in cultured neurons treated with IGF1 and demonstrates that extracellular signal-regulated MAP kinase 1 and 2 (ERK1 and -2) immunoprecipitates of IGF1-treated neuronal cells promote this activation. This effect did not directly result from eIF2B phosphorylation by ERK immunoprecipitates. In addition, recombinant ERK1 and -2 neither activate eIF2B nor phosphorylate it. Endogenous phosphorylated and -regulated kinase (10–13). eIF2B is a heteropentameric protein that catalyzes the exchange of bound GDP from eIF2 for GDP. The eIF2-GTP complex is then available to undergo further interaction with Met-tRNAi, leading to a new round of initiation. eIF2B activity therefore plays a key role in regulating translation initiation. eIF2B factor can be mainly regulated by two mechanisms. First, eIF2 phosphorylation of the α subunit (eIF2α) inhibits eIF2B because phosphorylated eIF2α is a competitive inhibitor of eIF2B (8, 9). Secondly, eIF2B activity can be regulated by phosphorylation of its ε subunit. Four kinases have been described to phosphorylate the ε subunit of eIF2B (eIF2Be); they are casein kinase (CK) 1 and 2, glycogen synthase kinase 3 (GSK3), and dual specificity tyrosine-phosphorylated and -regulated kinase (10–13). eIF2Be phosphorylation by CK1 and -2 enhances eIF2B activity, whereas phosphorylation by GSK3 has an inhibitory effect (14–17). The phosphorylation by GSK3 requires previous eIF2Be phosphorylation, which is catalyzed in vitro by dual specificity tyrosine-phosphorylated and -regulated kinase (13).

The transalational inhibition caused by eIF2B inhibition through eIF2α phosphorylation is a well known cellular mechanism that triggers in response to different stress situations (18–20). However, in growth factor-treated cells and in response to other different treatments, changes in eIF2B activity independent of eIF2α phosphorylation have been described in vitro (21–26). Increased eIF2B activity paralleling GSK3 inactivation in response to nerve and epidermal growth factors (26), insulin (27), and insulin-like growth factor 1 (IGF1) have been reported (17). IGF1 exerts its action by activating multiple signal transduction pathways, notably the mitogen-activated protein kinase (MAPK)–activated protein kinase 1 and 2 (PP1C, PP1 catalytic subunit; PP1Cα, PP1Cβ isoforms; PP2A, protein phosphatase 2A; PP2AC, protein phosphatase 2AC catalytic subunit; RIPA buffer, radioimmuno precipitation assay buffer.

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protein kinase (MAPK)-activating kinase (MEK)/MAPK and phosphoryldilinolinositol 3 kinase (PI3K) pathways (28–31). In neuronal cells, we previously showed that eIF2β activation by IGF1 depends on both GSK3 inactivation, via a mechanism mediated by PI3K, and MAPK activation (32). The link between IGF1-induced GSK3 inactivation and PI3K activity is provided by protein kinase B, which is located downstream of PI3K and phosphorylates GSK3 at a conserved serine inhibitory site (33, 34). Nevertheless, the signaling pathway or the mechanism through which IGF1-induced MAPK activation leads to eIF2β activation remains unknown.

The phosphorylation status of proteins depends on the relative activities of both kinases and phosphatases. However, the possible role of protein phosphatases in eIF2β regulation has not been established. Protein phosphatases 1 (PP1) and 2A (PP2A) are two major and structurally related families of serine/threonine phosphatases that regulate a large number of cellular processes, including neuronal signaling (35). The regulation of PP1 and PP2A catalytic subunits by extracellular signals seems to be mediated mainly by association with non-catalytic regulatory subunits which inhibit, modulate, or target catalytic subunits to various subcellular structures and substrates (36, 37).

The aim of the present work was to investigate the mechanism of eIF2β regulation by IGF1-induced MAPK activation in cultured neurons. By studying extracellular signal-regulated kinase (ERK) 1 and 2, MAP kinases, and PP1, a novel transduction pathway leading to eIF2β activation in neurons was discovered. Evidence is provided that IGF1-induced eIF2β activation, promoted via MAPK signaling, is exerted by PP1 activation.

**EXPERIMENTAL PROCEDURES**

**Materials**—IGF1, inhibitor 2 (I2), tautomycin, purified recombinant PP1 catalytic subunit (PP1C) α isofrom (PP1Cα), anti-ERK1 and -2 polyclonal antibody, and anti-diphospho-ERK1 and -2 (Thr 183 and Tyr 185) in ERK2 (the active forms of the kinases) monoclonal antibodies were provided by Sigma. Purified recombinant PP1Cγ, 4E-BP1, and fothricin were from Calbiochem, purified recombinant ERK1 and GSKβ3 and anti-PP2A catalytic subunit (PP2AC) polyclonal antibody were purchased from Upstate Biotechnology, and purified recombinant ERK2 and anti-phospho-GSK3β/α (Ser17/19) (the inactive form of the kinases) polyclonal antibodies were provided by New England Biolabs. PP2Aβδ/γ was obtained from Bionol, LYS249062 was from Alexis, anti-ERK2 and anti-ERKα polyclonal antibodies were from Santa Cruz Biotechnology, and anti-PP1C and anti-GSKβ3 monoclonal antibodies were from Transduction Laboratories. Leiboitiz L-15, Ham’s F-12 and high glucose Dulbecco’s media were purchased from Invitrogen. [3H]GDP and [γ-32P]ATP were supplied by Amersham Biosciences, and synthetic peptides were supplied by Mimotopes. eIF2 and eIF2β were purified from calf brain (38).

**Primary Neuronal Cultures**—Primary cultures of cells from cerebral cortex were prepared from 16 day-old fetuses removed from timed-pregnant Sprague-Dawley rats. The fetuses were placed in Leiboitiz L-15 medium for brain dissection. The cerebral cortex was separated from the rest of the brain using iridectomy scissors, and the meningeal membranes were carefully removed. The remaining piece was then dissociated using a Pasteur pipette and 20–21-gauge needles to make a homogenous cell suspension. Trypan blue exclusion was used to count the living cells. Neurons were seeded on plastic multidiwishes precoated with 0.05 mg/ml poly-l-lysine/a density of 2–2.5 × 10^5 cells/cm^2 and cultured at 37 °C with 7.5% CO₂ in air in high glucose Dulbecco’s medium with 15% fetal calf serum. After 24 h, cultures were then treated with a 20% trypsin-EDTA (2 mg/ml) solution, centrifuged at 1000 × g for 5 min, and resuspended in Leiboitiz L-15 medium with 10% FCS before plating. The cells were allowed to attach to the culture dishes at 37 °C for 2 h. After washing and centrifugation, the cells were maintained in serum-free Leiboitiz L-15 medium with 10% FCS before treatment and then placed in the same medium in the absence or presence of additives. When inhibitors were used, cells were treated with them for 1 h (or 2 h in the case of tautomycin) before and during IGF1 treatment. Cells were washed with ice-cold phosphate-buffered saline before harvesting.

**eIF2β Activity Measurement**—Both untreated and treated cells cultured on 35-mm multidishes were lysed for 10 min in hypotonic buffer (10 mM Tris-HCl, pH 7.6, 10 mM KCl, 1 mM dithiothreitol, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidin, 10 µg/ml leupeptin, pepstatin and antipain, 2 mM β-glycerophosphate, 2 mM sodium molybdate, and 0.2 mM sodium orthovanadate). The lysate was made up to 4 mM magnesium acetate and 10 mM potassium acetate, centrifuged for 10 min at 12,000 × g, and saved as cell extract. All steps were carried out at 4 °C. A binary complex, eIF2β-HIGDP, was formed as described (17). The eIF2β activity of purified eIF2β (0.1–0.35 µg) and of cell extracts (40 µg of protein) were measured by the capacity to replace non-radioactive [γ-32P]ATP for free GDP during 3- or 5-min incubations, respectively (17). The substrate used was 1 pmol of [γ-32P]ATP. eIF2β activity was expressed as a percentage of pmol of [γ-32P]GDP released from the binary complex with respect to controls.

**eIF2β Phosphorylation and Activation by ERK Immunoprecipitates and Reconstituted ERK**—Cell extracts (500 or 75 µg) from both untreated and IGF1-treated cells were immunoprecipitated with either 5 µl of anti-ERK2 antibody or 3 µl of anti-diphospho-ERK1 and -2 antibody, respectively, and with 25 µl of protein A-Sepharose or protein G-Sepharose, respectively (Amersham Biosciences) following previously described procedures (39). For the eIF2β phosphorylation assay, ERK immunoprecipitates obtained with the two different antibodies were washed and centrifuged for 5 min at 2200 × g at 4 °C. The supernatants were then collected, and the protein content, as determined by immunocytochemistry with antibodies to neuron-specific protein content, as determined by immunocytochemistry with antibodies to neuron-specific protein content, was quantified using the image analyzer as for the assessment of ERK activity.

To measure eIF2β activity, ERK immunoprecipitates were incubated with eIF2β (0.5–1 µg) as described above for the phosphorylation assay but in the absence of radioactive ATP. After incubation, the reaction was stopped, and aliquots of the supernatants (corresponding to 0.1–0.35 µg of eIF2β) were taken for eIF2β activity analysis. In some cases, the immunoprecipitates were preincubated with 200 mM I2 or 20 nM tautomycin for 12 min before incubation with eIF2β. In other experiments purified recombinant ERK1 and ERK2 (3 and 10 units, respectively) instead of ERK immunoprecipitates from cell extracts were used to phosphorylate eIF2β, and eIF2β activity was assayed following the same protocol as above.

As a positive control of ERK activity, ERK immunoprecipitates as well as purified ERK1 and -2 were incubated with 4E-BP1 (3 µg), a known substrate for these MAPKs in vitro, under the same conditions described for eIF2β phosphorylation. 4E-BP1 phosphorylation was measured using the image analyzer as for the assessment of ERK activity.

**Protein Phosphatase Detection in ERK Immunoprecipitates**—To detect the presence of protein phosphatases that coimmunoprecipitate with ERK1 and -2, both ERK2 and diphospho-ERK1 and -2 immunoprecipitates were analyzed by SDS-PAGE and Western blot. The membranes were developed with antibodies against diphosphorylated ERK1 and -2, ERK1 and -2, PP1C, and PP2AC proteins. In other experiments, diphospho-ERK1 and -2 immunoprecipitates (from 75 µg cell extracts) were washed either with 0.4 M potassium acetate in buffer A, 1% (v/v) Triton X-100 in buffer A, or RIPA buffer (50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM dithiothreitol, 125 mM KCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS) and then centrifuged. The resulting precipitates were washed again three times with buffer A, incubated with purified eIF2β, and eIF2β activity was assessed as described above.

**PP1 and PP2A Phosphatase Activity Assays**—PP1 and PP2A phosphatase activity was determined according to the method of Cohen et al. (40) using purified [3H]-labeled phosphorylase a as the substrate. [3H]-Labeled phosphorylase a was incubated with buffer A (10 mg/ml) with phosphorylase kinase (0.2 mg/ml) as previously described (41). Cells extract (0.5 µg) prepared as described for eIF2β activity assessment in hypotonic buffer but in the absence of phosphatase inhibitors were preincubated for 12 min at 30 °C without and with 12 or 30 µl of tautomycin in 50 mM Tris-HCl, pH 7.6, 1 mM dithiothreitol, 0.1 mM EDTA, 10 mM sodium molybdate. Sodium orthovanadate was added by the addition of 10 µl of [γ-32P]labeled phosphorylase a (60,000 cpm). After 20 min of incubation at 30 °C, the reaction was stopped by the addition of 180 µl of 20% (wt/v) trichloroacetic acid. The tubes were left on ice for 10 min and then centrifuged at 12,000 × g for 5 min at 4 °C. Aliquots

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(180 µl) of the clear supernatant were counted to determine the phosphatase activity as the amount of 32P released.

Phosphorylation a is a substrate for both PP1 and PP2A. Therefore, to differentiate between these two protein phosphatases, assays were performed in the presence of either I2 or fostriecin, specific inhibitors of PP1 and PP2A, respectively. The doses of PP1 and PP2 inhibitors used were predetermined by a set of dose-response experiments in which the effect of each inhibitor was independently measured. The PP1 activity inhibited by I2 (100–500 nm) was comparable with the activity remaining in the presence of the PP2A inhibitor fostriecin (400–1000 nm). Thus, the sum of the PP1 and PP2A activities represents the total phosphorylase a phosphatase activity of the cell extracts. Accordingly, a concentration of 200 nm I2 was chosen to assess phosphatase activity in further experiments. PP1 activity was defined as the phosphorylase a phosphatase activity inhibited by I2. PP2A activity was defined as the remaining activity. PP1 and PP2A phosphatase activities were also assayed in ERK immunoprecipitates from 35 µg of cell extracts.

PP1C and PP1Cγ Phosphatase Activity Assays—PP1Ca and PP1Cγ phosphatase activities were assayed using the peptide RRAAEELD-PQL based on eIF2Be131-146 rat sequence (42) phosphorylated by GSK3β. Purified GSK3β (100 milliunits) was immunoprecipitated with 2 µl of anti-GSK3β and 25 µl of protein G-Sepharose as described elsewhere (39). GSK3β immunoprecipitates were incubated for 20 min at 30°C with 15 µg of eIF2Be peptide in 55 mM Tris-HCl, pH 7.5, 5 mM magnesium acetate, 0.1 mM ATP, and 0.5 µCi of [γ-32P]ATP in a volume of 25 µl. The radioactivity incorporated into the peptide was determined by liquid scintillation.

To study the effect of recombinant PP1C on eIF2Be activity, purified eIF2Be factor (0.5 µg) was phosphorylated with GSK3β immunoprecipitates using 30 µM ATP and 4.5 µCi of [γ-32P]ATP as described above. An aliquot of [32P]-labeled eIF2Be (1.35 µCi) was then used instead of the peptide in the PP1Ca assay activity assay. The reaction was stopped by adding 12.5 µl of SDS sample buffer and analyzed by SDS-PAGE and autoradiography.

To determine the effect of recombinant PP1C on eIF2Be, purified eIF2Be factor (0.5–1 µg) was incubated with PP1Ca or PP1Cγ under the same conditions as described for the peptide. After incubation, an aliquot containing about 0.1–0.35 µg of eIF2Be was used for assessing eIF2Be phosphatase activity.

**Determination of eIF2Be, GSK3β, and ERK1 and -2 Phosphorylation—**

Cell extracts, prepared in the same way as for the eIF2Be assay, were analyzed using horizontal isoelectric focusing slab gels to detect eIF2Be phosphorylation and SDS-PAGE to detect GSK3β and ERK phosphorylation. After electrophoresis, the gels were transferred to a polyvinylidene difluoride membrane (Amersham Biosciences), and the blots were visualized by specific anti-eIF2Be, anti-phospho-GSK3β, and anti-phospho-ERK1 and -2 antibodies. The bands corresponding to eIF2Be and phosphorylated eIF2Be proteins were quantified as described above.

**Statistical Analysis—** Results are expressed as means ± S.E. for independent experiments. Statistical analysis was performed using the t test for paired and unpaired data versus control values or analysis of variance and Dunnett’s post-test for comparisons between treated groups.

**RESULTS**

**ERK1 and -2 Do Not Phosphorylate eIF2Be—** Recently we reported that IGF1 induces MAPK activation, mainly of ERK2, and that this signaling pathway is involved in eIF2Be activation in neuronal cultures (17). eIF2Be contains Pro-Leu-Thr-Pro and Ser-Pro consensus sequences for recognition by ERK1 and -2 MAP kinases. To determine whether eIF2Be was a substrate for ERK2 kinase in vitro, we incubated eIF2Be with ERK2 immunoprecipitates from untreated controls and IGF1-treated cells in an eIF2Be phosphorylation assay. The results showed that ERK2 immunoprecipitates from IGF1-treated cells did not increase eIF2Be phosphorylation and even slightly decreased eIF2Be phosphorylation (68.6 ± 6.5% versus 100% of control cells; Fig. 1A, top panel). To test whether MAP kinases were active in the immunoprecipitates, parallel experiments were performed with the known substrate 4E-BP1. As shown, the observed 4E-BP1 phosphorylation with ERK2 immunoprecipitates from IGF1-treated cells (15.5 ± 0.53 in arbitrary units) was greater than that seen with immunoprecipitates from untreated control cells (6.0 ± 2.1 in arbitrary units, p < 0.05; Fig. 1A, bottom panel). This finding confirms the previously reported MAPK activation induced by IGF1 (17). Furthermore, and supporting the results obtained with the immunoprecipitates, eIF2Be was not phosphorylated by either recombinant ERK1 or ERK2 in vitro (data not shown).

**eIF2Be Activation by ERK1 and -2 Immunoprecipitates—** To
The participation of MAP kinase in IGF1-induced eIF2B activation, we tested the effect of ERK2 immunoprecipitates from IGF1-treated and untreated neurons on purified eIF2B activity. Interestingly, eIF2B activity was significantly increased after incubation with immunoprecipitates from IGF1-treated cells (168 ± 20%; Fig. 1A). A similar result was found when diphospho-ERK1 and -2 immunoprecipitates, obtained with an antibody against diphospho-ERK1 and -2 (active form of the kinases), were incubated with eIF2B factor (150 ± 13%; Fig. 1B). eIF2B activation induced by ERK immunoprecipitates in IGF1-induced neurons was inhibited by cell treatment with the MAPK inhibitor PD98059, whereas the treatment of cells with the PKA inhibitor LY294002 had no effect (Fig. 1B).

Besides, incubation of purified eIF2B with purified recombinant ERK1 or -2 did not change eIF2B activity (not shown). All these findings demonstrate that ERK1 or -2 MAP kinases does not directly modify eIF2B phosphorylation status or activity. Conversely, the above findings suggest that an unknown factor that co-immunoprecipitates with activated ERK1 and -2 may be responsible for eIF2B activation in IGF1-stimulated neurons. Furthermore, when ATP was omitted in incubations with immunoprecipitates, eIF2B activation occurred (not shown), indicating that this effect might not be mediated by kinase activity.

**Detection of PP1C in IGF1-activated ERK1 and -2 Immunoprecipitates**—To further investigate the nature of the unknown factor that activates eIF2B, we studied the potential involvement of PP1 and PP2A, the two main protein phosphatases involved in cell growth and signaling in eukaryotic cells (36, 37). Accordingly, we searched for PP1 and PP2A catalytic subunits in ERK1 and -2 immunoprecipitates effectuated with four different antibodies: anti-ERK1 and -2 diphosphorylated, anti-ERK1 and -2, anti-PP1C, and anti-PP2AC. As shown in Fig. 2A, both PP1C and PP2AC proteins were detected. Interestingly, PP1C levels found in ERK2 immunoprecipitates from IGF1-treated neurons were higher than those from untreated cells, whereas PP2AC levels showed no difference (Fig. 2A). Increased PP1C levels were also detected in diphospho-ERK1 and -2 of IGF1-treated cells, whereas PP2AC was poorly detected (Fig. 2B). These findings further support a close relationship between PP1 and the diphosphorylated active form of the kinases (mainly ERK2) in immunoprecipitates from IGF1-stimulated neuronal cells (Fig. 2, A and B).

To find out whether the presence of PP1C and PP2AC in the immunoprecipitates correlated with phosphatase activity, we measured PP1 and PP2A activities in ERK immunoprecipitates. Although PP1 and PP2A activities were found in the immunoprecipitates, only PP1 phosphatase activity was significantly increased in diphospho-ERK1 and -2 immunoprecipitates from IGF1-treated cells (179 ± 21%) compared with immunoprecipitates from untreated control cells (100%, p < 0.05; Fig. 2C). On the contrary, PP2A phosphatase activity, although present, underwent no change upon IGF1 treatment in these immunoprecipitates (94 ± 8% from IGF1-treated cells versus 100% of control cells; Fig. 2D). Similar results were obtained when measuring PP1 and PP2A phosphatase activities in ERK2 immunoprecipitates (not shown). For further experiments, only ERK immunoprecipitates obtained with antiphospho-ERK1 and -2 antibodies were used.

**PP1C Associated with Diphosphorylated ERK1 and -2 Activates eIF2B Factor**—To determine whether PP1C associated to ERK1 and -2 MAP kinases was responsible for the eIF2B activation induced by ERK immunoprecipitates, the diphospho-ERK1 and -2 immunoprecipitates from IGF1-treated cells were washed before incubation with eIF2B. When the immunoprecipitates were washed with buffer containing 0.4 M potassium acetate or 1% (v/v) Triton X-100, no changes in eIF2B activation were observed. However, when the immunoprecipitates were washed in more stringent conditions using RIPA buffer, eIF2B activity dropped from 156 ± 2.5 to 114 ± 5.3%, the latter being a value close to that obtained with diphospho-ERK1 and -2 immunoprecipitates from untreated control cells (100%) (Fig. 3A). Western blot analysis of washed diphospho-ERK1 and -2 immunoprecipitates revealed that only RIPA buffer removed PP1C from immunoprecipitates (Fig. 3B). To further assess the involvement of PP1 in eIF2B activation, the eIF2B assay was performed with the specific PP1 inhibitor I2 (200 nM). I2 blocked the eIF2B activation induced by diphospho-ERK1 and -2 immunoprecipitates from IGF1-treated neurons (104 ± 5.1% versus 100% of untreated control cells; Fig. 3C). Similar results were obtained using another specific PP1 inhibitor, tautomycin (20 nM, not shown). PP1 inhibitors II and tautomycin at 100–1000 and 20–100 nM concentrations, respectively, produced no effects on eIF2B activity in the absence of immunoprecipitates (not shown). These findings provide evidence that PP1C, either by itself or by forming a complex with some other regulatory proteins.
elicits eIF2B dephosphorylation, *in vitro* studies using recombinant α and γ PP1C isoforms were performed. The activities of PP1Ca and PP1Cy phosphatases were assayed using a peptide based on the eIF2Bε rat sequence (containing the well-characterized GSK3-regulated phosphorylation site Ser358) as a substrate. 0.05 units of PP1Ca released more than 50% of 32P (6,480 cpm) from the 32P-labeled peptide, whereas 1.0 units of PP1Cy were necessary to release a similar amount of 32P (Fig. 4A). This suggests that PP1Cy dephosphorylates the peptide much more efficiently than did PP1Ca. In addition, as shown in Fig. 4B, eIF2B factor phosphorylated by GSK3β was also efficiently dephosphorylated by PP1Ca, confirming that, at least *in vitro*, eIF2B is a PP1C substrate. Interestingly, only PP1Ca was able to stimulate purified eIF2B activity; PP1Cy had no effect (127 ± 4.9 and 94 ± 6.3%, respectively, *versus* 100% in the absence of phosphatase; Fig. 4C). On the other hand, higher concentrations of PP1Ca inhibited eIF2B, suggesting that it might dephosphorylate other residues required for optimal eIF2B activity (Fig. 4D). At the concentrations tested, PP1Ca had no effect on eIF2B activity when the assay was performed without preincubation of the two together and they were only incubated for 3 min in the eIF2B assay (not shown). This result indicates that the binary complex eIF2β3H/GDP is not affected by PP1Ca.

IGF1 Induces PP1 Activation through the MEK/ERK2 Pathway—We have previously demonstrated that IGF1-induced eIF2B activation in neurons is promoted through PI3K and GSK3 kinases (17). We also reported that the IGF1-induced MEK/
MAPK activation pathway was involved in eIF2B activation. This mechanism has been found operative in other cell types as well (43). The findings of the present investigation suggest that IGF1-activated ERK1 and -2 MAP kinases are not directly responsible for eIF2B activation and that IGF1 promotes eIF2B activation through protein phosphatase PP1, which is activated by IGF1 in a MEK/MAPK-dependent fashion.

To determine whether IGF1-activated ERK1 and -2 are directly responsible for eIF2B activation, purified eIF2B was incubated with ERK immunoprecipitates from untreated control and IGF1-treated neurons. eIF2B incubation with ERK immunoprecipitates from IGF1-treated cells activates eIF2B, whereas preincubation of cells with MEK inhibitor PD98059 abolished eIF2B activation. This suggests that MEK activation is required for this to occur. Treatment of cells with PI3K inhibitor LY294002 had no effect on eIF2B activation. This result is not in disagreement with previously reported results (17) because these studies used cell extracts, whereas the present study used ERK immunoprecipitates. The fact that both ERK immunoprecipitates and recombinant ERK failed to phosphorylate eIF2B together with the failure of recombinant ERK to activate eIF2B reasonably supports the idea that ERK1 and -2 are not directly involved in eIF2B activation. Besides, eIF2B activation by ERK immunoprecipitates was also observed in the absence of ATP, which discards any
Further co-immunoprecipitated kinase activity as being responsible for this effect.

Because eIF2B activity is regulated by phosphorylation/dephosphorylation reactions (44), it was considered appropriate to study the potential role of phosphatase activity in IGF1-induced eIF2B activation via MEK. The presence of PP1 and PP2A catalytic subunits in ERK1 and -2 immunoprecipitates was investigated (i) because dephosphorylation was found in eIF2Be subunit when incubated with ERK immunoprecipitates, (ii) because of the aforementioned ATP independence of eIF2B activation of the immunoprecipitates, and (iii) because PP1 participates in glycogen synthase regulation, a protein whose GSK3-recognized sequence is also present in eIF2B.

The results show that phosphatases PP1C and PP2AC are found in ERK1 and -2 immunoprecipitates, suggesting a potential association of such phosphatases with ERK1 and -2. PP1C was eliminated from ERK immunoprecipitates only when they were subjected to a stringent wash, supporting the idea of its specific interaction with ERK. A clear relationship was seen between IGF1 stimulation, ERK1 and -2 phosphorylation on the one hand, and the amount of PP1C in the immunoprecipitated complex on the other. Additionally, only PP1 activity in immunoprecipitates increased after IGF1 treatment. Furthermore, using specific PP1 inhibitors, co-immunoprecipitated PP1 was responsible for the eIF2B activation induced by ERK immunoprecipitates. It is not surprising that PP2A is mostly present in ERK2 immunoprecipitates because it has been identified as one of the physiological ERK2 phosphatases (46).

These findings suggest that IGF1 promotes eIF2B activation through PP1 protein phosphatase via its association with phosphorylated ERK.

Several additional in vivo investigations were included in this work that further clarify the role of PP1 in eIF2B regulation; (i) PP1 activity was induced by IGF1 treatment, and using the specific MEK inhibitor PD98059, PP1 activation was found to depend on MEK activation, and (ii) experiments carried out with tautomycin, a permeable-specific PP1 inhibitor, showed that specific IGF1-induced PP1 activation is essential for eIF2B factor activation by IGF1. The fact that tautomycin did not modify the phosphorylation status of either eIF2α or GSK3 suggests that eIF2B regulation by PP1 is independent of these regulatory mechanisms in IGF1-stimulated neurons. These in vivo findings together with those demonstrating in vitro regulation of eIF2B by PP1 (specifically PP1Ca) might also suggest direct in vivo eIF2B regulation by PP1. Because tautomycin did not modify ERK1 and -2 phosphorylation either, indicating that PP1 does not regulate these kinases, and because PP1 is activated in a MEK-dependent manner, it might be concluded that PP1 is likely to act downstream of the MEK/MAPK sig-
phosphorylation in Ser^535 by GSK3 and that this phosphorylation exerts an inhibitory effect on eIF2β activity (44). Conversely, removing the phosphate in this residue would activate eIF2β. In the present work, using recombinant PP1Co, we demonstrate that PP1 is able to both dephosphorylate eIF2β by GSK3 and activate eIF2β factor in vitro. When higher concentrations of PP1Co were used, eIF2β inactivation was observed. Other phosphorylated residues in eIF2β have been described in vitro, some of which are phosphorylated in vitro by CK2 and are essential for eIF2B activity (12). Excess PP1Co might also release the phosphates in those essential residues required for eIF2β activity to effect its inhibition. According to these findings eIF2B activation would require both GSK3 inactivation and PP1 activity to maintain Ser^535 in a dephosphorylated form in response to IGFl in neuronal cells. A similar type of regulation involving both GSK3 and PP1 pathway) and an association between activated MAPK (notably ERK2) and PP1C. This complex results in enhanced P1 activity, which is efficient for eIF2β activation. All these processes are closely related to one another and run in parallel with levels comparable with those of eIF2β activation by IGFl in neuronal cells.

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