Modulation of Rabbit Reticulocyte Guanine Nucleotide Exchange Factor Activity by Casein Kinases 1 and 2 and Glycogen Synthase Kinase 3†

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ABSTRACT: The *in vitro* phosphorylation of the guanine nucleotide exchange factor (eIF-2B) by casein kinase 2 (CK-2) was previously shown to stimulate the binding of GTP to eIF-2B and increase nucleotide exchange [Singh, L. P., Aroor, A. R., & Wahba, A. J. (1994) *Biochemistry 33*, 9152−9157]. The present study examines the *in vitro* phosphorylation of the 82-kDa subunit of eIF-2B by CK-1 and glycogen synthase kinase 3 (GSK-3) and the effects of this covalent modification on nucleotide exchange. Phosphorylation with CK-1 adds approximately 0.27 mol of phosphate/mol of eIF-2B and doubles guanine nucleotide exchange activity. Treatment of the phosphorylated eIF-2B with alkaline phosphatase reduces its activity by a factor of 4, and rephosphorylation with CK-1 (0.49 mol of phosphate/mol of eIF-2B) restores its specific activity to that of the phosphorylated protein. GSK-3 phosphorylates the 82-kDa subunit of both isolated and alkaline phosphatase-treated eIF-2B; however, the stoichiometry of phosphorylation is much less (approximately 0.12 mol/mol of eIF-2B in both preparations) than that obtained with CK-1 or CK-2. Phosphorylation of eIF-2B with GSK-3 neither stimulates nor inhibits GDP/GTP exchange. The results of this study indicate that phosphorylation of eIF-2B with CK-1 and/or CK-2 is required for GTP binding to the protein. Evidence is also presented for a mechanism of regulation of eIF-2B activity whereby phosphorylation by GSK-3 influences the activity of the protein and partially suppresses phosphorylation by CK-1 or CK-2.

The guanine nucleotide exchange factor (eIF-2B)[1] is a key protein in the control of polypeptide chain initiation (Hershey, 1991). The first step in polypeptide chain initiation is the formation of a ternary complex (eIF-2·GTP·Met-tRNA$_f$ where eIF-2 is eukaryotic initiation factor 2) which is then transferred to a 40S ribosomal subunit. Upon formation of the 80S initiation complex, GTP is hydrolyzed and eIF-2 is released as a binary complex of eIF-2·GDP. Regeneration of the ternary complex requires eIF-2B which facilitates nucleotide exchange and recycling of eIF-2 (Dholakia & Wahba, 1989; Hershey, 1991). The characteristics of eIF-2B, a five subunit protein (82, 65, 55, 40, and 32 or 34 kDa; also known as ϵ, δ, γ, β, and α), suggest that this factor may be composed of several distinct structural and functional domains. The activity of eIF-2B is subject to regulation directly by allosteric effectors (Dholakia et al., 1986; Gross et al., 1988; Singh et al., 1995; Kimball & Jefferson, 1995) and by phosphorylation of its 82-kDa subunit (Dholakia & Wahba, 1988; Singh et al., 1994) or indirectly by phosphorylation of the α-subunit of eIF-2 (Hershey, 1991; Pain, 1986). We have previously demonstrated that phosphorylation of the 82-kDa subunit of eIF-2B *in vitro* by casein kinase (CK)-2 results in the stimulation of nucleotide exchange, and treatment of the protein with alkaline phosphatase decreases its activity by a factor of 5 (Dholakia & Wahba, 1988; Singh et al., 1994), thus providing evidence for a regulatory role for this subunit in guanine nucleotide exchange.

We have recently shown that eIF-2B is phosphorylated *in vivo* (Aroor et al., 1994) and that phosphopeptide mapping of the *in vivo* phosphorylated 82-kDa subunit compared well with the mapping of the 82-kDa subunit phosphorylated *in vitro* with CK-2. This suggests that CK-2 or a kinase with similar specificity is probably involved in the phosphorylation of eIF-2B *in vivo*. Putative CK-2 sites on the 82-kDa subunit were identified at ^{174}Ser, ^{703}Ser, and/or ^{704}Ser and possibly at ^{340}Ser. An additional region of the 82-kDa protein which is also phosphorylated *in vivo* did not match with any of the CK-2-targeted regions. This result suggests that an additional kinase or kinases may be involved in the phosphorylation of the protein *in vivo* (Aroor et al., 1994). Bushman et al. (1993) cloned the 82-kDa subunit of eIF-2B, and analysis of the amino acid sequence using the MOTIF program reveals several putative phosphorylation sites along the entire length of the protein for CK-1, CK-2, GSK-3, cAMP-dependent kinase, protein kinase C, and proline-directed kinase (Kennelly & Krebs, 1991; Pearson & Kemp, 1991; Roach, 1991). Since the latter three enzymes do not phosphorylate eIF-2B *in vitro* (Aroor et al., 1994; Dholakia & Wahba, 1988; Oldfield & Proud, 1992), we investigated the effect of CK-1 and GSK-3 phosphorylation of the 82-kDa subunit on guanine nucleotide exchange activity.

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[1] Abbreviations: ALP, alkaline phosphatase; CK, casein kinase; CNBr, cyanogen bromide; eIF-2, eukaryotic initiation factor 2; eIF-2B, guanine nucleotide exchange factor (also designated as GEF); GSK-3, glycogen synthase kinase 3; PAGE, polyacrylamide gel electrophoresis.

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CK-1 together with CK-2 (Tuazan & Traugh, 1991) and GSK-3 (Fiol et al., 1987) comprise the majority of Ser/Thr kinases present in cell extracts that recognize acidic rather than basic residues in their substrates (Kennelly & Krebs, 1991; Pearson & Kemp, 1991; Roach, 1991). CK-1 may be stimulated directly by hormones (Durand, 1981; Perez et al., 1987) or viral transformation (Elias et al., 1981). The enzyme was purified from various tissues (Ahmed et al., 1984; Dahmus, 1981; Hathaway et al., 1979) and is known to phosphorylate a wide variety of substrates that play a critical role in metabolism and protein synthesis (Haas & Hagedorn, 1991; Rapuano & Rosen, 1991; Vila et al., 1990). It may be distinguished from CK-2 by its inability to utilize GTP as a phosphate donor (Hathaway et al., 1983) and eIF-2 as a substrate (Hathaway et al., 1979). We have recently demonstrated that phosphorylation of the 82-kDa subunit of eIF-2B and the β-subunit of eIF-2 by CK-2 controls the rate and the extent of GDP/GTP exchange during polypeptide chain initiation (Singh et al., 1994).

The *in vitro* phosphorylation of the 82-kDa subunit of eIF-2B with GSK-3 was recently reported (Welsh & Proud, 1993; Welsh et al., 1994). However, the effect of this phosphorylation on eIF-2B activity was not determined. In this paper we demonstrate that the *in vitro* phosphorylation of the 82-kDa subunit of eIF-2B by CK-1 and CK-2 results in a 4−5-fold increase in the activity of the factor, and that phosphorylation with GSK-3 will block the CK-1- and CK-2-dependent stimulation of eIF-2B activity.

MATERIALS AND METHODS

Materials. CK-2 was isolated from reticulocyte lysates as described before (Aroor et al., 1994) or was purchased from Promega Corp., Madison, WI. Recombinant CK-1α and GSK-3β were provided by Paul R. Graves and Peter J. Roach, Indiana University School of Medicine, Indianapolis. All other reagents and chemicals were as previously described (Singh et al., 1994; Aroor et al., 1994).

Purification of eIF-2B. The nucleotide exchange factor was purified from rabbit reticulocyte lysates to apparent homogeneity as described earlier (Dholakia & Wahba, 1988). The purified preparation was free of eIF-2 and kinase(s) that phosphorylate eIF-2B. Dephosphorylation of the isolated eIF-2B with mammalian alkaline phosphatase and the assay for guanine nucleotide exchange were previously described (Dholakia & Wahba, 1988). Protein concentrations were determined by using the Bio-Rad protein assay reagent and bovine serum albumin as the standard (Bradford, 1976).

In Vitro Phosphorylation of eIF-2B by CK-1, CK-2, or GSK-3. eIF-2B (1.0 μg) was incubated at 30 °C with 100 μM [γ-^{32}P]ATP with varying amounts of enzyme or for different time intervals in the presence of 10 milliunits (mU) each of CK-1, CK-2, or GSK-3 in 20 μL containing 20 mM Tris-HCl, pH 7.5, 2 mM dithiothreitol (DTT), 100 mM KCl, and 2.5 mM Mg^{2+} for CK-1 or 10 mM Mg^{2+} for CK-2 and GSK-3. The kinases were added simultaneously when eIF-2B was phosphorylated with CK-1 and/or CK-2 in the presence of GSK-3, and the reaction was started by the addition of [γ-^{32}P]ATP. Otherwise, eIF-2B was preincubated with GSK-3 and [γ-^{32}P]ATP for 10 min before the addition of CK-1 or CK-2. With [γ-^{32}P]ATP (100 μM) as the phosphate donor, 1 unit of each enzyme will transfer to phosvitin (2.5 mg/mL) at 30 °C 1 nmol of phosphate per minute. After 30 min incubation, 5 μL of sample buffer containing SDS was added to stop the reaction, and samples were run on a 10% SDS−PAGE (Wahba & Dholakia, 1991). The gels were stained with Coomassie Brilliant Blue R-250, destained, and dried. Before autoradiography, the gels were exposed at −70 °C to Kodak XR-5 film. The amount of radioactivity incorporated into the 82-kDa subunit was analyzed by cutting the band and counting in a nonaqueous scintillation fluid.

Treatment of Phosphorylated eIF-2B with Mammalian Alkaline Phosphatase. Dephosphorylated eIF-2B (5 μg) was incubated at 30 °C for 1 h with 10 mU each of CK-1, CK-2, or GSK-3 and 100 μM [^{32}P]ATP as previously described (Singh et al., 1994). Each reaction mixture was diluted 5-fold with buffer containing 20 mM Tris-HCl, pH 7.5, 2 mM DTT, 100 mM KCl, 50 units/mL alkaline phosphatase (Boehringer Mannheim), and various protease inhibitors: phenylmethanesulfonyl fluoride, pepstatin, leupeptin, and chymostatin at a final individual concentration of 0.3 mM. After incubation at 30 °C for specific time intervals, an aliquot (20 μL) was removed, and the reaction was stopped by the addition of 5 μL of a 5-fold concentrated SDS sample buffer. The mixture was boiled for 5 min at 100 °C and then applied on SDS−PAGE. The radioactivity associated with the 82-kDa subunit of eIF-2B was determined by cutting the band and counting in a nonaqueous scintillation fluid as described above.

Nucleotide Binding to eIF-2B. Binding of [^{3}H]GTP (6650 cpm/pmol) to eIF-2B, which was previously phosphorylated with CK-1 and/or GSK-3, was carried out in a 20 μL mixture containing 20 mM Tris-HCl, pH 7.5, 100 mM KCl, 2 mM DTT, 1 mM Mg^{2+}, 1 μg of eIF-2B (3.5 pmol), and [^{3}H]-GTP (14 μM). After 10 min at 30 °C, 1 mL of reaction buffer with 1 mM Mg^{2+} was added to each incubation. The reaction mixture was filtered through a nitrocellulose membrane, washed 3 times with the same stop buffer, and dried before measuring the amount of radioactivity retained on filters.

RESULTS

In Vitro Phosphorylation of eIF-2B by CK-1 and CK-2. We have previously shown that phosphorylation of the 82-kDa subunit of eIF-2B *in vitro* by CK-2 and ATP results in increased activity of the factor (Dholakia & Wahba, 1988; Singh et al., 1994). In the present study, we compare the kinetics of eIF-2B phosphorylation by CK-1 and CK-2 under two different conditions. Isolated eIF-2B was phosphorylated with varying amounts of CK-1 or CK-2 at 2.5 mM Mg^{2+} (Oldfield & Proud, 1992) and at 10 mM Mg^{2+} (Dholakia & Wahba, 1988; Singh et al., 1994). We find that both kinases are active at 2.5 mM Mg^{2+}, but the activity of CK-1 is greater than that of CK-2, in terms both of the initial rate and the extent of phosphorylation. Per mole of eIF-2B, 0.27 mol of phosphate is incorporated in 30 min with CK-1, whereas with CK-2 only 0.19 mol of phosphate is incorporated. However, 0.43 mol of phosphate/mol of eIF-2B is incorporated with CK-2 when phosphorylation is carried out at 10 mM Mg^{2+}. With CK-1, the same amount of phosphate is incorporated at 2.5 mM as at 10 mM Mg^{2+} (data not shown). The extent of phosphorylation of isolated eIF-2B with CK-2 is greater than with CK-1 (Figure 1A). For the first 5−10 min, the initial rate of phosphorylation is

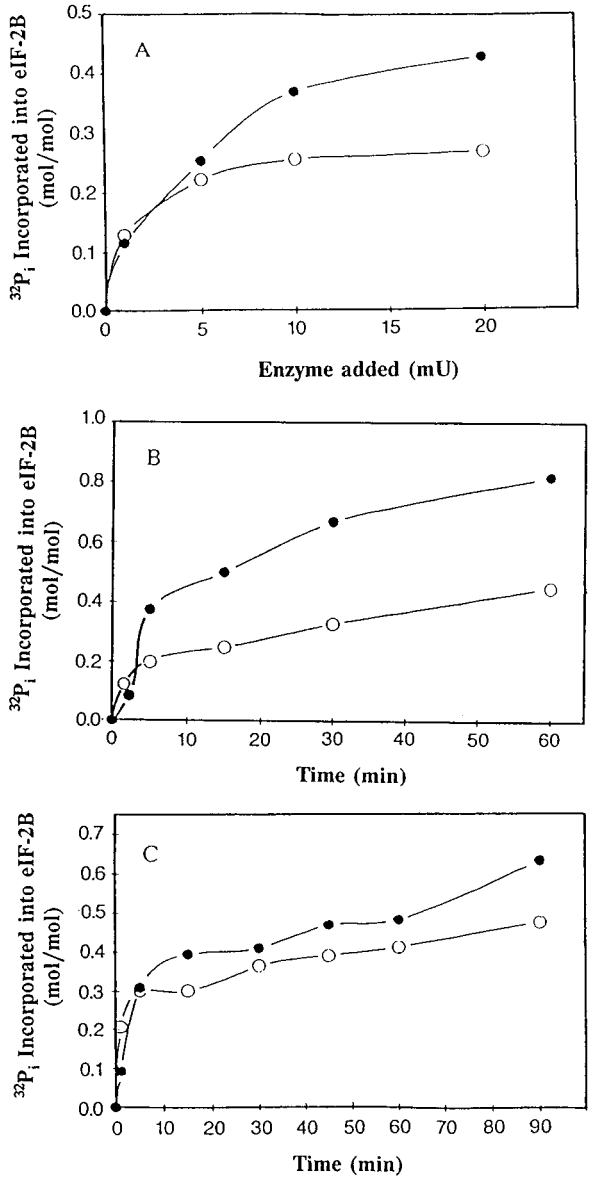

FIGURE 1: Phosphorylation of eIF-2B by CK-1 and CK-2. eIF-2B (1.0 μg) was incubated at 30 °C with 100 μM [γ-^{32}P]ATP (2000 cpm/pmol) for 30 min or for different time intervals in the presence of CK-1 (○) and CK-2 (●) as described under Materials and Methods. (A) Concentration-dependent phosphorylation of the isolated eIF-2B at 2.5 mM Mg^{2+} for CK-1 and 10 mM Mg^{2+} for CK-2. (B) Kinetics of phosphorylation of the alkaline phosphatase-treated eIF-2B with 10 mU of either CK-1 (○) and CK-2 (●), and Mg^{2+} concentrations as in (A). (C) Alkaline phosphatase-treated eIF-2B was first phosphorylated with CK-2 and cold ATP. The factor was then reisolated on a P-11 column, and the kinetics of phosphorylation with CK-1 of this material (●) and the alkaline phosphatase-treated eIF-2B (○) were monitored over a period of 90 min.

slightly faster with CK-1 than with CK-2. Thereafter, the rate and the extent of phosphorylation of the alkaline phosphatase-treated eIF-2B are greater with CK-2 than with CK-1 (Figure 1B). After 60 min, approximately 0.82 mol of phosphate is incorporated with CK-2 but only 0.49 mol of phosphate/mol of eIF-2B with CK-1. Prior phosphorylation of the alkaline phosphatase-treated eIF-2B with CK-2 does not interfere with CK-1 phosphorylation (Figure 1C) and suggests that the two kinases phosphorylate the factor at different sites. When alkaline phosphatase-treated eIF-2B is first phosphorylated with CK-1 and [γ-^{32}P]ATP and subsequently with CK-2, the extent of phosphorylation of the 82-kDa subunit is the sum of phosphorylation of eIF-2B by CK-1 and CK-2 individually (data not shown). This result further suggests that CK-1 and CK-2 phosphorylate the 82-kDa subunit of eIF-2B independently of each other.

Table 1: Effect of CK-1 Phosphorylation of eIF-2B on Nucleotide Exchange[a]

eIF-2B	[^{3}H]GDP released (mol/mol of eIF-2B)	relative activity (x-fold)
isolated eIF-2B	4.0	1.9
phosphorylated eIF-2B	7.9	3.8
dephosphorylated eIF-2B	2.2	1.0
rephosphorylated eIF-2B	8.2	3.9

[a] The activity of eIF-2B was measured by its ability to exchange eIF-2-bound [^{3}H]GDP for GTP in a two-stage assay (Dholakia & Wahba, 1988). For stage 1, eIF-2B (1.5 μg, 5.25 pmol) was incubated at 30 °C for 30 min in a 20−μL reaction mixture containing 20 mM Tris-HCl (pH 7.6), 0.1 M KCl, 2.5 mM Mg^{2+}, 2 mM DTT, 20% (v/v) glycerol, and 100 μM ATP and in the presence or absence of 10 mU of CK-1. One microliter (0.26 pmol of eIF-2B) of this reaction mixture was assayed in the second stage (75 μL) for the release of [^{3}H]GDP from the eIF-2·[^{3}H]GDP binary complex (6.1 pmol, 4900 cpm/pmol), in the presence of 100 μM GTP. eIF-2B was treated with alkaline phosphatase as previously described (Dholakia & Wahba, 1988) and rephosphorylated as in stage 1. Nucleotide exchange activity ([^{3}H]GDP release) was determined with an excess of isolated eIF-2·[^{3}H]GDP binary complex and plotted against eIF-2B concentration (in the range of 0.1−0.4 pmol). Relative activity, normalized to the dephosphorylated form of eIF-2B, is the average of four different experiments. The values fall within 5−10% of each other in each experiment.

Effect of Phosphorylation by CK-1 on eIF-2B Activity. We measured the ability of eIF-2B to exchange eIF-2-bound [^{3}H]-GDP for GTP in a two-stage assay as described previously (Dholakia & Wahba, 1988). Isolated eIF-2B releases 4 mol of [^{3}H]GDP/mol of eIF-2B in a 5 min time period. This activity is doubled when eIF-2B is phosphorylated with CK-1 (Table 1). Treatment of phosphorylated eIF-2B with alkaline phosphatase decreases its activity approximately 4-fold, and rephosphorylation by CK-1 restores it to the level of the phosphorylated factor (from 2.2 to 8.2 pmol of [^{3}H]GDP released/pmol of eIF-2B).

In Vitro Phosphorylation of eIF-2B by GSK-3. We also examined the rate and the extent of phosphorylation of eIF-2B by GSK-3. Our results show that GSK-3 phosphorylates the isolated as well as the alkaline phosphatase-treated eIF-2B (Figure 2). However, the stoichiometry of phosphorylation with GSK-3 is only 0.12 mol/mol of eIF-2B for both the isolated and dephosphorylated material. The initial rate of phosphorylation is faster with GSK-3 than with CK-1 or CK-2 (Figure 1B and Figure 2B), and the reaction is completed in 5−10 min. The extent of phosphorylation with GSK-3 and CK-1 together is only 0.25 mol/mol of eIF-2B as compared to 0.49 mol/mol of eIF-2B with CK-1 alone (Figure 3, lanes 2 and 4). Similarly, when GSK-3 and CK-2 are included in the reaction mixture, phosphorylation is less than that attained with CK-2 alone (0.57 vs 0.82 mol/mol of eIF-2B), indicating that phosphorylation at GSK-3 site(s) will also inhibit some CK-2 sites on the 82-kDa subunit (data not shown).

Reversal of CK-1- or CK-2-Dependent Stimulation of eIF-2B Activity by GSK-3. The activity of eIF-2B after phosphorylation with GSK-3 was also investigated. As shown in Table 2, the activity of the alkaline phosphatase-treated eIF-2B is not changed after rephosphorylation with GSK-3. Phosphorylation of the alkaline phosphatase-treated eIF-2B

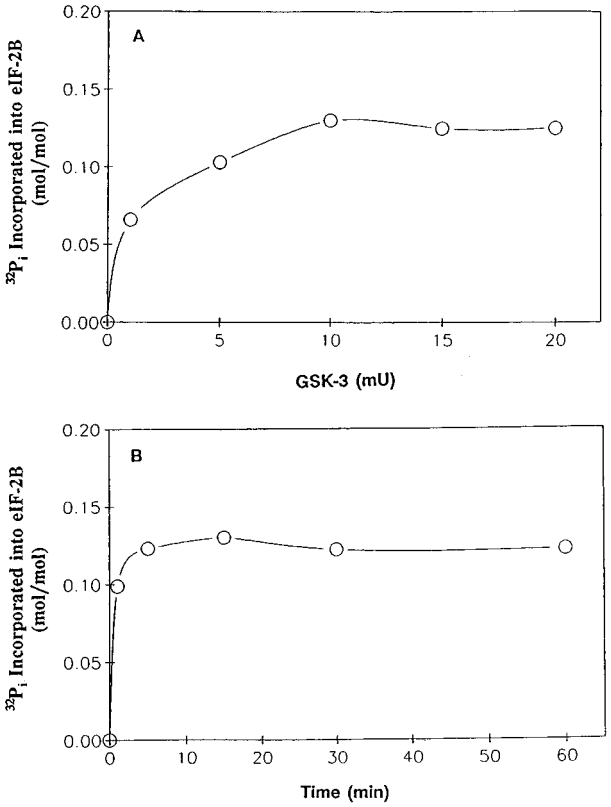

FIGURE 2: Phosphorylation of eIF-2B by GSK-3. eIF-2B (1.0 μg) was incubated at 30 °C with 100 μM [γ-^{32}P]ATP (2000 cpm/pmol) and 10 mM Mg^{2+} for 30 min or for different time intervals in the presence of GSK-3 as described under Materials and Methods. (A) Concentration-dependent phosphorylation of the isolated eIF-2B, and (B) kinetics of phosphorylation of the alkaline phosphatase-treated eIF-2B with 10 mU of GSK-3.

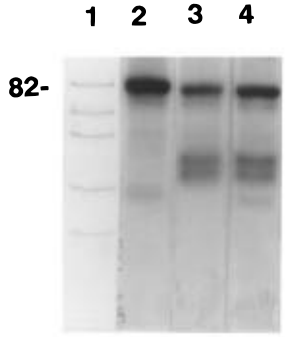

FIGURE 3: Effect of GSK-3 on the phosphorylation of eIF-2B by CK-1. Alkaline phosphatase-treated eIF-2B (1.0 μg) was incubated at 30 °C for 60 min with 100 μM [γ-^{32}P]ATP (2000 cpm/pmol) and CK-1 in the presence or absence of GSK-3. When eIF-2B was phosphorylated with CK-1 in the presence of GSK-3, eIF-2B was preincubated with GSK-3 and [γ-^{32}P]ATP at 30 °C for 10 min before the addition of CK-1. The samples were subjected to SDS−PAGE and analyzed by autoradiography. Lane 1 is a gel of eIF-2B stained with Coomassie Brilliant Blue. Lanes 2−4 are autoradiograms. Lane 2, eIF-2B+CK-1; lane 3, eIF-2B+GSK-3; lane 4, eIF-2B+GSK-3+CK-1. The two-digit number alongside the gel corresponds to the molecular weight ($\times 10^{-3}$) of the 82-kDa subunit of eIF-2B. The other two bands in lanes 3 and 4 are associated with the recombinant GSK-3 preparation. After autoradiography, the 82-kDa subunit of eIF-2B was cut and counted in order to estamate the stoichiometry of phosphorylation.

with CK-1 or CK-2 results in a 4−5-fold stimulation of eIF-2B activity. There is a further increase in nucleotide exchange in the presence of both CK-1 and CK-2; however, this increase is not additive. When GSK-3 is included in the phosphorylation reaction along with CK-1 and/or CK-2, the activity of the phosphorylated eIF-2B is reduced to that of the dephosphorylated factor (from 9.5 to 2.1 mol of [^{3}H]GDP released/mol of eIF-2B) (Table 2). Phosphorylation of the isolated eIF-2B with GSK-3 does not alter eIF-2B activity (data not shown). Similarly, the activity of CK-1 or CK-2 rephosphorylated eIF-2B is not inhibited by subsequent phosphorylation of the protein with GSK-3 (data not shown), implying that GSK-3 site(s) on the 82-kDa subunit need to be phosphorylated first to inhibit CK-1- or CK-2-dependent stimulation of eIF-2B activity.

Table 2: Effect of GSK-3 Phosphorylation of eIF-2B on Nucleotide Exchange[a]

additions	[^{3}H]GDP released (mol/mol of eIF-2B)
ALP-eIF-2B	1.9
ALP-eIF-2B+ATP+GSK-3	2.0
ALP-eIF-2B+ATP+CK-1	7.7
ALP-eIF-2B+ATP+CK-2	8.5
ALP-eIF-2B+ATP+CK-1+CK-2	9.5
ALP-eIF-2B+ATP+CK-1+GSK-3	2.1
ALP-eIF-2B+ATP+CK-2+GSK-3	2.0
LP-eIF-2B+ATP+CK-1+CK-2+GSK-3	2.2

[a] The activity of eIF-2B was measured by its ability to exchange eIF-2-bound [^{3}H]GDPfor GTP in a two-stage assay similar to that described in Table 1 except that 10.0 mM $Mg^{2+}$was used in step one in the presence or absence of 10 mU of CK-1, CK-2, or GSK-3 as indicated. One microliter (0.26 pmol of eIF-2B) of the reaction mixture was assayed in the second stage (75 μL) for the release of [^{3}H]GDP from eIF-2·[^{3}H]GDP binary complex (4.9 pmol, 4900 cpm/pmol), in the presence of 100 μM GTP. The kinases when added alone had no effect on nucleotide exchange. The results are representative of three different experiments. In each experiment, the values fall within 5−10% of each other.

Kinetics of [^{32}P]P_i Release with Alkaline Phosphatase from CK-1-, CK-2- or GSK-3-Phosphorylated eIF-2B. We have previously demonstrated that alkaline phosphatase removes more than 85% of [^{32}P]P_i from CK-2-phosphorylated eIF-2B (Singh et al., 1994). In this study, we compared the rate and the extent of removal of radiolabeled phosphate from CK-1-, CK-2-, or GSK-3-phosphorylated eIF-2B. As illustrated in Figure 4, the rate of [^{32}P]P_i release from GSK-3-phosphorylated eIF-2B by alkaline phosphatase is 3−4-fold faster than that of the CK-1-phosphorylated material. The reaction is complete in 10−15 min for GSK-3-phosphorylated eIF-2B, and more than 90% of the radioactivity is released. After 45 min, alkaline phosphatase releases only 55% [^{32}P]P_i from CK-1-phosphorylated eIF-2B. The rate as well as the extent of dephosphorylation of CK-2-phosphorylated eIF-2B is 78% after 45 min and falls between those for CK-1- or GSK-3-phosphorylated factors. In the presence of protein phosphatase 1 (Zhang et al., 1992), no release of [^{32}P]P_i is observed with ^{32}P-labeled eIF-2B previously phosphorylated with CK-1, CK-2, or GSK-3 (data not shown).

Effect on GTP Binding after Rephosphorylation of Alkaline Phosphatase-Treated eIF-2B with CK-1 or GSK-3. eIF-2B is a GTP binding protein, and this binding is on the 40-kDa subunit (Dholakia & Wahba, 1989; Dholakia et al., 1989). There is no displacement of labeled GDP from eIF-2·[^{3}H]-GDP unless both eIF-2B and GTP are present. The eIF-2B-dependent GDP/GTP exchange occurs by a sequential mechanism in which GTP binding to eIF-2B is followed by eIF-2B−eIF-2 interaction and GDP/GTP exchange (Dholakia

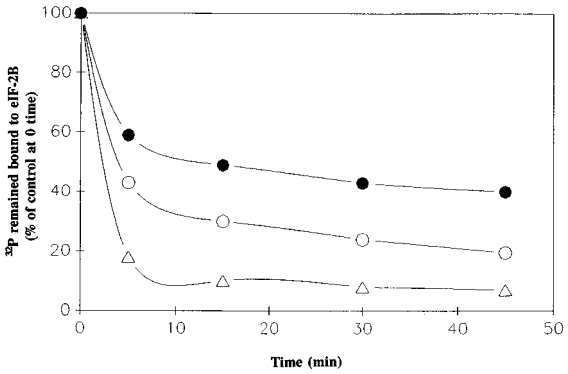

FIGURE 4: Time course of the release of [^{32}P]P_i from CK-1-, CK-2-, and GSK-3-phosphorylated eIF-2B by mammalian alkaline phosphatase. Dephosphorylated eIF-2B (5.0 μg) was incubated with 10 mU each of CK-1 (●), CK-2 (○), or GSK-3 (△) and 100 μM [γ-^{32}P]ATP (specific activity, 4550 cpm/pmol) in a 20 μL reaction mixture as described under Materials and Methods. After 45 min at 30 °C, each reaction mixture was diluted 5-fold with buffer containing 20 mM Tris-HCl, pH 7.5, 2 mM DTT, 100 mM KCl, 50 units/mL alkaline phosphatase, and various protease inhibitors: phenylmethanesulfonyl fluoride, pepstatin, leupeptin, and chymostatin at a final individual concentration of 0.3 mM. After incubation at 30 °C for specific time intervals, an aliquot (20 μL) was removed, and the reaction was stopped by the addition of 5 μL of a 5-fold concentrated SDS sample buffer. The mixture was boiled for 5 min at 100 °C and then applied on SDS−PAGE. After being stained with Coomassie Brilliant Blue R-250, the gels were exposed at −70 °C to Kodak XR-5 film. Radiolabeled subunits were cut and counted in order to estimate the amount of ^{32}P that remained bound to eIF-2B.

Table 3: Binding of [^{3}H]GTP to CK-1- or GSK-3-Phosphorylated eIF-2B[a]

type of eIF-2B	[^{3}H]GTP bound (pmol/3.5 pmol of eIF-2B)
isolated eIF-2B	0.3
dephosphorylated eIF-2B	0.1
CK-1-rephosphorylated eIF-2B	0.4
GSK-3-rephosphorylated eIF-2B	0.1
CK-1-rephosphorylated GSK-3-eIF-2B	0.1

[a] The binding of [^{3}H]GTP (6650 cpm/pmol) to eIF-2B was carried out at 30 °C in 20 μL reaction mixtures containing 20 mM Tris-HCl, pH 7.5, 100 mM KCl, 2 mM DTT, 1 mM Mg^{2+}, 3.5 pmol (1 μg) of eIF-2B, and 14 μM [^{3}H]GTP. The reaction was started with [^{3}H]GTP and stopped after 10 min by the addition of 1 mL of buffer (20 mM Tris-HCl, pH 7.5, 100 mM KCl, 2 mM DTT, and 1 mM Mg^{2+}). The amount of GTP bound to eIF-2B was determined as described under Materials and Methods. Alkaline phosphatase-treated eIF-2B was first phosphorylated with GSK-3 and cold ATP at 30 °C for 20 min, and the factor was then reisolated on a P-11 column. The GSK-3-phosphorylated eIF-2B was subsequently phosphorylated with CK-1 and is designated as CK-1-rephosphorylated GSK-3-eIF-2B.

& Wahba, 1989). Recently, we demonstrated that treatment of eIF-2B with alkaline phosphatase reduces GTP binding to eIF-2B, and rephosphorylation with CK-2 restores the binding of the nucleotide to eIF-2B (Singh et al., 1994). In the present study, we investigated [^{3}H]GTP binding to the alkaline phosphatase-treated eIF-2B after rephosphorylation with CK-1 or GSK-3. After treatment with alkaline phosphatase, the amount of [^{3}H]GTP bound to eIF-2B is reduced from 0.3 pmol to 0.1 pmol/3.5 pmol of eIF-2B (Table 3). Rephosphorylation with CK-1 increases [^{3}H]GTP binding to 0.4 pmol/3.5 pmol of eIF-2B. However, phosphorylation with GSK-3 does not alter GTP binding, and subsequent phosphorylation of GSK-3-phosphorylated eIF-2B with CK-1 does not enhance GTP binding to the protein. We also tested the activity of the GSK-3- or CK-1-rephosphorylated eIF-2B after isolation from P-11 columns. The activity of eIF-2B is not changed after phosphorylation with GSK-3 but increases approximately 4-fold after phosphorylation with CK-1 (data not shown). These results parallel the stimulation of nucleotide exchange activity with alkaline phosphatase-treated eIF-2B after its phosphorylation with CK-1 but not with GSK-3.

DISCUSSION

The regulation of eIF-2B activity by phosphorylation of the 82-kDa subunit by different kinases is the focus of the present study. A 4−5-fold increase in nucleotide exchange activity is obtained when alkaline phosphatase-treated eIF-2B is phosphorylated *in vitro* by CK-1 or CK-2. Phosphorylation of the alkaline phosphatase-treated eIF-2B with GSK-3 does not stimulate or inhibit the exchange of eIF-2-bound GDP for GTP. However, GSK-3 can nullify the effect of CK-1 or CK-2 phosphorylation and activation of eIF-2B activity (Table 2). These results go hand in hand with a lower GTP binding capacity of GSK-3-phosphorylated eIF-2B when compared to that of the CK-1-rephosphorylated eIF-2B. Our results further suggest that GSK-3 interferes with the phosphorylation of the 82-kDa subunit by CK-1 and CK-2 (Figure 3) and that the activity of eIF-2B is influenced by the covalent modification of the protein with GSK-3 (Table 3). Protein phosphatases may, therefore, be equally important in the regulation of eIF-2B activity. So far, a phosphatase acting on eIF-2B, other than alkaline phosphatase, has not been identified.

The stoichiometry of phosphorylation with CK-1 is less than that with CK-2 when isolated and dephosphorylated preparations of eIF-2B are used. The extent of phosphorylation of eIF-2B by CK-1 is approximately 0.27 mol of phosphate/mol of isolated eIF-2B and 0.49 mol of phosphate/mol of dephosphorylated eIF-2B. With CK-2, the extent of phosphorylation is 0.43 mol/mol for the isolated and 0.82 mol/mol for the alkaline phosphatase-treated factor. On the other hand, phosphorylation with GSK-3 results in the incorporation of only 0.12 mol of phosphate/mol of isolated and alkaline phosphatase-treated eIF-2B. It is not clear why stoichiometric phosphorylation does not occur. It is not unusual to detect less than stoichiometric phosphorylation of protein complexes studied *in vitro* (Katada et al., 1985; Venema et al., 1991; Zick et al., 1986). One possibility is that the targeted subunit of the holoprotein may be tied up in a nonconducive conformational state or blocked stearically by interaction with the other subunits, making it inaccessible in the *in vitro* assay used. Alternatively, target sites may be already naturally phosphorylated when the complex is isolated, and they may not be fully dephosphorylated by alkaline phosphatase. Our data indicate that CK-1 and CK-2 target sites are distinct since the *in vitro* phosphorylation of eIF-2B with CK-2 does not block successive phosphorylation with CK-1.

Oldfield and Proud (1992) observed that eIF-2B was more efficiently phosphorylated with CK-1 than with CK-2 at 2.5 mM Mg^{2+} and there was no effect of phosphorylation on nucleotide exchange. We have established that the extent of phosphorylation of eIF-2B with CK-1 is identical at 2.5 and 10 mM Mg^{2+}, whereas phosphorylation with CK-2 is greatly reduced at 2.5 mM Mg^{2+}. The discrepancy between our results and those of Oldfield and Proud (1992) may be

due to the purity of eIF-2B. The possible association of kinase activity with isolated eIF-2B would interfere with the stimulation of nucleotide exchange by CK-1 or CK-2 (Aroor et al., 1994).

eIF-2B is phosphorylated *in vivo*, and this phosphorylation is mediated by CK-2 or a similar kinase (Aroor et al., 1994). Phosphopeptide map analysis of the *in vivo* labeled 82-kDa subunit reveals the presence of additional bands other than those found when CK-2 was used to phosphorylate eIF-2B *in vitro* and suggests an additional kinase(s) may be involved *in vivo*. The *in vitro* phosphorylation of eIF-2B with CK-1 and its effect on guanine nucleotide exchange activity suggest the involvement of this kinase in the *in vivo* phosphorylation of the factor. GSK-3 was shown to phosphorylate *in vitro* the 82-kDa subunit of eIF-2B (Welsh & Proud, 1993). However, the effect of this phosphorylation on nucleotide exchange was not determined. An increase in eIF-2B activity, upon treatment of Chinese hamster ovary cells with insulin, was associated with a decrease in GSK-3 activity. Apparently, GSK-3 can phosphorylate both Ser and Thr residues (Welsh & Proud, 1993). If this kinase has a role *in vivo*, it must only be able to target Ser residues, since our phosphoamino acid analysis indicates only Ser residues are phosphorylated in intact reticulocytes (Aroor et al., 1994). GSK-3 phosphorylates a number of other substrates such as glycogen synthase, inhibitor 2, the regulatory subunit of the ATP-Mg-dependent protein phosphatase 1_i, the microtubule-associated protein (tau) (De-Paoli-Roach, 1984; Giruli et al., 1989; Wang et al., 1994; Woodgett, 1993), and transcriptional factors like c-jun, c-myc, c-myb, etc. (de Groot et al., 1993; Woodgett, 1993). Phosphorylation with GSK-3 of the ATP-Mg-dependent protein phosphatase 1_i stimulates its activity (De-Paoli-Roach, 1984; Giruli et al., 1989; Henry & Killilea, 1993) while phosphorylation of other substrates leads to decreased activity (Giruli et al., 1989; Wang & Roach, 1993; Woodgett, 1993). It is tempting to speculate that stimulation of eIF-2B upon treatment of cells with insulin or growth factors may occur via activation of CK-1 and/or CK-2 (Ackerman et al., 1990; Durand, 1981; Karinch et al., 1993; Perez et al., 1987) and suppression of GSK-3 (Eldar-Finkelman et al., 1995; Hughes et al., 1992; Welsh & Proud, 1993).

At present, the regulation of eIF-2B activity by phosphorylation with different kinases is not completely understood. Although CK-1 and CK-2 may phosphorylate the 82-kDa subunit of eIF-2B at multiple sites (Denslow et al., 1994; Aroor et al., 1994), only phosphorylation at a particular site(s) may be responsible for the stimulation of eIF-2B activity. The results of the present study suggest that prior phosphorylation of the alkaline phosphatase-treated eIF-2B with GSK-3 results in partial suppression of certain CK-1 and CK-2 phosphorylation sites on the 82-kDa subunit and that these sites may be critical for maintaining the active conformation of eIF-2B. In other systems, GSK-3, CK-1, and CK-2 phosphorylate muscle glycogen synthase at multiple sites. However, GSK-3 phosphorylation of primarily only one site correlates with inactivation of glycogen synthase by GSK-3 (Wang & Roach, 1993; Skurat et al., 1994). In order to understand the underlying mechanism of regulation of eIF-2B activity by different kinases, CK-1, CK-2, and GSK-3 phosphorylation sites on the 82-kDa subunit of eIF-2B as well as CK-1 and CK-2 sites that are suppressed by GSK-3 have to be identified. We have previously determined putative CK-2 phosphorylation sites on the 82-kDa subunit at ^{174}Ser, ^{703}Ser, and/or ^{704}Ser and possibly at ^{340}Ser (Aroor et al., 1994).

In conclusion, this study demonstrates that the *in vitro* phosphorylation of alkaline phosphatase-treated eIF-2B either by CK-1 or by CK-2 results in a 4−5-fold increase in nucleotide exchange activity. However, the action of CK-1 and CK-2 on eIF-2B activity is not additive. Phosphorylation of eIF-2B with GSK-3 does not stimulate or inhibit nucleotide exchange, and impedes the activation of eIF-2B by CK-1 and CK-2. Under various physiological and pathological conditions, eIF-2B activity may be modulated directly through the action of different kinases, such as CK-1, CK-2, and/or GSK-3 (Garcia et al., 1994; Hu & Wieloch, 1993; Karinch et al., 1993; Welsh & Proud, 1993; Welsh et al., 1994).

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