Phosphorylation of the Guanine Nucleotide Exchange Factor and Eukaryotic Initiation Factor 2 by Casein Kinase II Regulates Guanine Nucleotide Binding and GDP/GTP Exchange†

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ABSTRACT: In mammalian cells, chain initiation factor (eIF) 2 and guanine nucleotide exchange factor (GEF) play a major role in the regulation of polypeptide chain initiation. Since guanine nucleotide exchange is the rate-limiting step in the recycling of eIF-2, we examined the effects of phosphorylation of GEF and eIF-2 on guanine nucleotide binding and the rate of GDP/GTP exchange. Phosphorylation of the 82-kDa subunit of GEF *in vitro* by casein kinase (CK) II results in the stimulation of guanine nucleotide exchange [Dholakia, J. N., & Wahba, A. J. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85,* 51–54]. CK-II also phosphorylates the β-subunit of eIF2, but the significance of this phosphorylation has not previously been investigated. In this study we demonstrate that treatment of CK-II-phosphorylated GEF or eIF-2 with alkaline phosphatase specifically removes more than 85% of the phosphate incorporated into the factors and alters guanine nucleotide binding to these proteins. In the presence of 1 mM Mg^{2+}, the amount of GTP bound to dephosphorylated GEF is reduced 3.8-fold as compared to that of the CK-II-phosphorylated GEF. Rephosphorylation with CK-II restores GTP binding and increases 4–5-fold the activity of GEF in the exchange of eIF-2-bound GDP for free GTP. On the other hand, the extent of GDP binding to dephosphorylated eIF-2 is increased 2.3-fold as compared to that to the isolated eIF-2. The rate of GEF-catalyzed exchange of dephosphorylated eIF-2-bound GDP for GTP is approximately 2-fold slower than that with the isolated eIF-2. These results suggest that phosphorylation of GEF and eIF-2 with CK-II provides a mechanism for the regulation of nucleotide binding and GDP/GTP exchange during polypeptide chain initiation.

The first step in polypeptide chain initiation is the formation of a ternary (eIF-2·GTP·Met-$tRNA_f$) complex which is then transferred to a 40S ribosomal subunit (Dholakia & Wahba, 1989; Dholakia & Wahba, 1990; Hershey, 1991). Subsequently, in the presence of mRNA, other initiation factors, and the 60S ribosomal subunit, the 80S initiation complex is formed, thereby setting the stage for polypeptide chain elongation. Upon formation of the 80S initiation complex, GTP is hydrolyzed and eIF-2 is released as a binary complex with GDP. This complex is stable in the presence of Mg^{2+} and is functionally inactive. The rate-limiting step in the recycling of eIF-2 is the dissociation of the binary (eIF-2·GDP) complex to make eIF-2 available for ternary complex formation (with GTP and Met-$tRNA_f$) and a further round of initiation. The new cycle of initiation requires the guanine nucleotide exchange factor (GEF),[1] which promotes the recycling of eIF-2 by catalyzing the dissociation of GDP from eIF-2·GDP in the presence of GTP (Dholakia & Wahba, 1989). Phosphorylation of the α-subunit of eIF-2 at Ser^{51} by HCR or dsRNA-activated kinase (Pathak et al., 1988) inhibits GEF activity indirectly by forming a tight complex with eIF-2(αP) and leaves no GEF to promote the recycling of eIF-2 (Clemens et al., 1982; Matts & London, 1984; Hershey, 1991).

The mechanism of regulation of GEF activity under various physiological conditions, namely, during starvation (Kimball & Jefferson, 1988), viral infection (Schneider & Schenk, 1987), and stimulation of cells by insulin and growth factors (Welsh & Proud, 1992) is not well understood. Under these conditions, a different mechanism(s) other than eIF-2α phosphorylation seems to operate for regulating guanine nucleotide exchange rate during polypeptide chain initiation. We have previously demonstrated a direct regulation of GEF activity by phosphorylation/dephosphorylation of the 82-kDa subunit of GEF (Dholakia & Wahba, 1988) and by the redox state of the cell (Dholakia et al., 1986; Colin et al., 1987). Phosphorylation of the 82-kDa subunit of GEF by CK-II stimulates GEF activity, and dephosphorylation by alkaline phosphatase decreases its activity 5-fold. Recently, we demonstrated the *in vivo* phosphorylation of GEF by CK-II in rabbit reticulocytes (Aroor et al., 1994). Putative phosphorylation sites for *in vitro* CK-II phosphorylation of the 82-kDa subunit were identified at Ser residues 174, 703, and/or 704 and also probably at 340 (Aroor et al., 1994). The amino acid sequence for the rabbit reticulocyte 82-kDa subunit was deduced from the cDNA (Bushman et al., 1993), and amino acids in the protein are numbered to start with arginine as the first residue. CK-II also phosphorylates *in vitro* only the β-subunit of eIF-2 (Tuazon et al., 1980; Mehta et al., 1986; Alcazar et al., 1988; Clark et al., 1989). The β-subunit of eIF-2 is phosphorylated in intact cells, and this phosphorylation changes under conditions where the rate of protein synthesis is modulated (Duncan & Hershey, 1985). However, the functional significance of eIF-2(β) phosphorylation is still not understood. CK-II is a ubiquitous cAMP- and Ca^{2+}-independent Ser/Thr kinase (Tuazon & Traugh, 1991) which

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[1] Abbreviations: CK, casein kinase; eIF-2, eukaryotic initiation factor 2; GEF, guanine nucleotide exchange factor (also known as eIF-2B); HCR, heme-controlled repressor; DAI, double-stranded RNA-activated inhibitor (also designated as dsRNA-activated eIF-2α kinase); PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate.

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is highly conserved among eukaryotic organisms. It is located in the nucleus as well as in the cytosol and phosphorylates a broad spectrum of regulatory proteins (Tuazon & Traugh, 1991).

Both GEF and eIF-2 are guanine nucleotide-binding proteins. The binding of nucleotides occurs on the 40-kDa subunit of GEF and the β- and γ-subunits of eIF-2 (Dholakia & Wahba, 1989; Dholakia et al., 1989; Anthony et al., 1990). In the absence of Mg^{2+}, GDP binds to eIF-2 and is freely exchanged for GTP. However, when Mg^{2+} is present, an inactive eIF-2·GDP complex is formed. In this article, we provide evidence that the *in vitro* phosphorylation with CK-II of the 82-kDa subunit of GEF and the β-subunit of eIF-2 modulates the guanine nucleotide-binding properties of these proteins and regulates the rate of GEF-dependent GDP/GTP exchange during polypeptide chain initiation.

MATERIALS AND METHODS

Redivue [γ-^{32}P]ATP (6000 Ci/mmol) was obtained from Amersham, and [8,5-^{3}H]GDP (8.0 Ci/mmol) and [8,5-^{3}H]-GTP (9.4 Ci/mmol) were purchased from Du Pont–New England Nuclear. Initiator [^{35}S]Met-tRNA$_f$ and internal [^{35}S]Met-tRNA$_m$ were generously provided by Dr. Dolph Hatfield of the National Cancer Institute, Bethesda, MD. They were prepared by aminoacylating bovine tRNA with the labeled amino acid in the presence of rabbit reticulocyte synthatases under limiting conditions and subsequent fractionation of the labeled aminoacyl-tRNA by RPC-5 column chromatography as previously described (Hatfield et al., 1979). Mammalian alkaline phosphatase (bovine intestinal mucosa) and spermine-agarose were from Sigma, and nitrocellulose membrane filters (0.45 μm) were from Millipore. DEAE-cellulose (DE-52) and phosphocellulose (P-11) were purchased from Whatman. Mono Q, Mono P, and Mono S HPLC columns were obtained from Pharmacia LKB Biotechnology. The ISCO high-pressure liquid chromatography system was used with the Pharmacia columns. GDP and GTP were obtained from Boehringer Mannhein. GTP was further purified by chromatography on a Pharmacia FPLC polyanionic SI column as described previously (Dholakia & Wahba, 1987). All other reagents and chemicals were of reagent or analytical grade.

Purification of Initiation Factors and CK-II. Reticulocytes were obtained from phenylhydrazine-treated New Zealand white rabbits (Dholakia & Wahba, 1987). GEF and eIF-2 were purified from rabbit reticulocyte lysates to apparent homogeneity as previously described (Dholakia & Wahba, 1988). The purified preparation of GEF was free of eIF-2 and of kinases that phosphorylate GEF. CK-II was isolated from reticulocyte lysates by sequential chromatography on DEAE-cellulose, phosphocellulose (Hathaway & Traugh, 1983), and spermine agarose (Filhol et al., 1991). During purification, GEF activity was determined by monitoring the release of [^{3}H]GDP from an isolated eIF-2·[^{3}H]GDP binary complex (Dholakia & Wahba, 1987) and CK-II activity was assayed with casein and [γ-^{32}P]ATP (Hathaway & Traugh, 1983). Protein concentration was determined by using the Bio-Rad protein assay reagent with bovine serum albumin as the standard (Bradford, 1976). Mammalian alkaline phosphatase treatment of the isolated GEF and eIF-2 and rephosphorylation with CK-II of the dephosphorylated factors were carried out as previously described (Dholakia & Wahba, 1988). Dephosphorylated GEF and eIF-2 were reisolated on Mono S and Mono Q columns, respectively. After treatment of eIF-2 with alkaline phosphatase or CK-II, a 15% SDS-PAGE was run to ensure that the β-subunit of eIF-2, which is prone to degradation, is intact.

In Vitro Phosphorylation of eIF-2 and GEF with CK-II. GEF (0.5 μg) or eIF-2 (0.5 μg) was incubated at 30 °C for 30 min with 100 μM [γ-^{32}P]ATP (2600 cpm/pmol) and varying amounts of CK-II (1–20 mU) in 20 μL containing 20 mM Tris·Cl, pH 7.5, 2 mM DTT, 100 mM KCl, and 10 mM Mg^{2+}. One unit of enzyme will transfer to α-casein (2.5 mg/mL) in the presence of ATP (100 μM) 1 nmol of phosphate per min at 30 °C. After incubation 5 μL of a 5-fold concentrated SDS sample buffer was added to stop the reaction and the mixture was applied to SDS-PAGE. For the analysis of alkaline phosphatase activity, 2.5 μg of either dephosphorylated GEF or eIF-2 was incubated at 30 °C for 1 h with 10 mU of CK-II and 100 μM [^{32}P]ATP. Each reaction mixture was diluted 5-fold with buffer containing 20 mM Tris·Cl, pH 7.5, 2 mM DTT, 100 mM KCl, 50 units/mL alkaline phosphatase, and various protease inhibitors, phenylmethylsulfonyl 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.

Gel Electrophoresis and Autoradiography. One-dimensional gel electrophoresis in sodium dodecyl sulfate (SDS) was performed as previously described (Dholakia & Wahba, 1987; Wahba & Dholakia, 1991). For the analysis of GEF subunits, the concentration of acrylamide was 5% in the stacking gel and 10% in the resolving gels (Wahba & Dholakia, 1991). The resolving gel contained 15% polyacrylamide when eIF-2 was analyzed (Wahba & Dholakia, 1991). The acrylamide/bisacrylamide ratio in both stacking and resolving gels was 37:1 (w/w). After being stained with Coomassie Brilliant Blue R-250, gels were exposed at −70 °C to Kodak XR-5 film. Radiolabeled subunits were cut and counted as described (Dholakia & Wahba, 1988) in order to estimate the stoichiometry of phosphorylation.

Nucleotide Binding Assay. Binding of radiolabeled GTP and GDP to the factors was performed as described (Mehta et al., 1983; Dholakia & Wahba, 1987; Wahba & Dholakia, 1991). Binding of [^{3}H]GTP (6650 cpm/pmol) to GEF was carried out in a 55-μL mixture containing 20 mM Tris·Cl, pH 7.5, 100 mM KCl, 2 mM DTT, and 1 μg of GEF (3.5 pmol) and GTP over the range 1–20 μM either in the presence or absence of 1 mM Mg^{2+}. After 10 min at 30 °C, 1 mL of reaction buffer with or without 1 mM Mg^{2+} was added to each incubation. The reaction mixture was filtered through a nitrocellulose membrane, washed three times with the same stop buffer, and dried before measuring the amount of radioactivity retained on the filters. K_D was estimated by nonlinear least square fits of the binding data using the equation $r = nK[GTP]/(1 + K[GTP])$, where $r = [GTP]_{bound}/GEF$, K is the association constant ($K_D = 1/K$), and n is the binding ratio at saturation. The program FitAll (MTR Software, Toronto) was used for the analysis. The binding of [^{3}H]GDP (12 168 cpm/pmol) to eIF-2 was carried out in a 55-μL mixture containing the buffer described above as well as 1 mM Mg^{2+} and 0.5 μg of eIF-2 (3.57 pmol) and GDP over the range 1–200 nM. Determination of K_D for GDP binding to eIF-2 was described previously (Dholakia & Wahba, 1987; Panniers et al., 1988).

eIF-2 Activity as Measured by Ternary (eIF-2·GTP·[^{35}S]-Met-tRNA$_f$) Complex Formation. Comparison of activities

Table 1: Stoichiometry of Phosphorylation of eIF-2 and GEF CK-II[a]

initiation factor	^{32}P incorporated (mol/mol)	relative phosphorylation
isolated eIF-2	0.90	1.00
dephosphorylated eIF-2	1.87	2.10
isolated GEF	0.43	1.00
dephosphorylated GEF	0.82	1.90

[a] Phosphorylation of eIF-2 and GEF with CK-II and analysis of the stoichiometry of phosphorylation were carried out as described under Materials and Methods.

by ternary (eIF-2·GTP·[^{35}S]Met-tRNA$_f$) complex formation with isolated, alkaline phosphatase-treated, and CK-II-rephosphorylated eIF-2 (0.2 μg or 1.43 pmol) was carried out in the presence of 1 mM Mg^{2+}, cold GTP (200 μM), and [^{35}S]Met-tRNA$_f$ (42 890 cpm/pmol) as previously described (Mehta et al., 1983; Wahba & Dholakia, 1991). After a 10-min incubation at 30 °C, the reactions were terminated by the addition of 1 mL of cold wash buffer (20 mM Tris·Cl, pH 7.8, 100 mM KCl, and 1 mM Mg^{2+}). Bound Met-tRNA$_f$ was measured by counting the amount of radioactivity retained on nitrocellulose filters (Mehta et al., 1983).

Determination of GEF Activity. The binary (eIF-2·[^{3}H]-GDP) complex was formed with highly purified preparations of eIF-2 from rabbit reticulocytes as previously described (Mehta et al., 1983; Wahba & Dholakia, 1991) and used in GEF assays. The release of [^{3}H]GDP from eIF-2·[^{3}H]GDP was used to measure GEF activity as previously described (Rowlands et al., 1988; Wahba & Dholakia, 1991). Briefly, a 75-μL reaction mixture contained 20 mM Tris·Cl, pH 7.5, 100 mM KCl, 2 mM Mg^{2+}, 75 μg of bovine serum albumin, 2 mM dithiothreitol, 100 μM GTP or 4 μM GDP, and appropriate concentrations of the binary complex (eIF-2·[^{3}H]GDP) and GEF. The reaction was carried out at 30 °C for the specified time intervals and terminated by the addition of 1 mL of cold buffer (20 mM Tris·Cl, pH 7.5, 100 mM KCl, 2 mM DTT, and 2 mM Mg^{2+}). Radioactivity remaining on the filters after filtration was measured as described (Dholakia & Wahba, 1988).

RESULTS

Phosphorylation and Dephosphorylation of GEF and eIF-2. The 82-kDa subunit of GEF (Dholakia & Wahba, 1988; Oldfield & Proud, 1992) and the β-subunit of eIF-2 (Tuazon et al., 1980; Mehta et al., 1986; Alcazar et al., Clark et al., 1989) are phosphorylated *in vitro* by CK-II. In the present study, we determined the stoichiometry of phosphorylation of both eIF-2 and GEF by CK-II, and investigated the alkaline phosphatase-dependent removal of radiolabeled Pi from CK-II-phosphorylated factors. In the presence of CK-II, [γ-^{32}P]-ATP, and 10 mM Mg^{2+}, the amounts of phosphate incorporated into isolated GEF and alkaline phosphatase-treated factor are 0.43 and 0.82 pmol of Pi/pmol of GEF, respectively (Table 1). Under similar conditions, the incorporation of Pi is 0.9 pmol/pmol of isolated eIF-2 and 1.87 pmol/pmol of alkaline phosphatase-treated eIF-2. These results are in agreement with those reported earlier (Tuazon et al., 1980; Dholakia & Wahba, 1988). Therefore, it appears that approximately 50% of CK-II phosphorylation sites, both in the 82-kDa subunit of GEF and the β-subunit of eIF-2, are phosphorylated in the isolated factors. Treatment of the CK-II-phosphorylated GEF with alkaline phosphatase removes more than 85% of the Pi from the factor (Figure 1). The amount of ^{32}P remaining in the CK-II-phosphorylated eIF-2

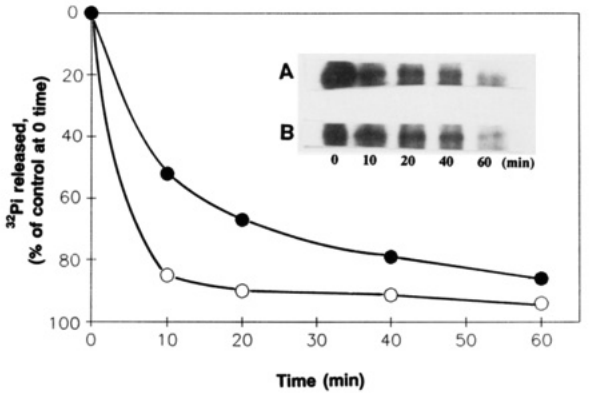

FIGURE 1: Time course of the release of ^{32}Pi from CK-II-phosphorylated eIF-2 and GEF by alkaline phosphatase. Isolated eIF-2 and GEF were treated with alkaline phosphatase and reisolated by chromatography on Mono Q and Mono S columns, respectively. These preparations were used in the phosphorylation studies with CK-II and [^{32}P]ATP. Radiolabeled factors were then treated with alkaline phosphatase (50 units/mL) in the presence of protease inhibitors as described under Materials and Methods. Release of ^{32}Pi from the radiolabeled eIF-2 (○) and GEF (●), expressed as percent of control of 0 time, is plotted against time of incubation. The inset shows an autoradiogram of the gels corresponding to the β-subunit of eIF-2 (A) and the 82-kDa subunit of GEF (B).

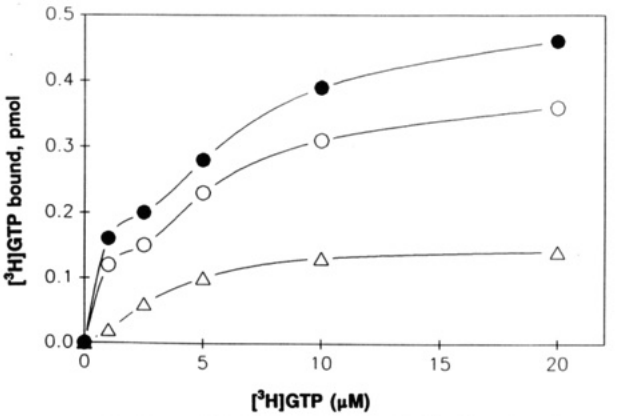

FIGURE 2: Binding of [^{3}H]GTP to isolated, alkaline phosphatase-treated, and CK-II-rephosphorylated GEF. GTP binding to either isolated, dephosphorylated, or CK-II-rephosphorylated factor was determined in a 55-μL reaction mixture containing 20 mM Tris·Cl, pH 7.5, 100 mM KCl, 2 mM DTT, 1 mM Mg^{2+}, 3.5 pmol (1 μg) of GEF, and varying concentrations of [^{3}H]GTP (6650 cpm/pmol) as indicated. After a 10-min incubation at 30 °C, the reaction was stopped by the addition of 1 mL of stop buffer (20 mM Tris·Cl, pH 7.5, 100 mM KCl, 2 mM DTT, and 1 mM Mg^{2+}) and the amount of GTP bound on nitrocellulose membrane was determined as described under Materials and Methods. A background value was subtracted from each determination. Each value is represented by a mean of three identical experiments. The symbols ○, △, and ● represent the binding of GTP to isolated, dephosphorylated, and CK-II-rephosphorylated GEF, respectively.

upon treatment with alkaline phosphatase is reduced 94% from 1.87 to 0.11 mol/mol of eIF-2. The rate of Pi released from phosphorylated eIF-2 after treatment with alkaline phosphatase is faster than that with phosphorylated GEF. These results clearly demonstrate that alkaline phosphatase specifically removes the majority of phosphate incorporated into the 82-kDa subunit of GEF and the β-subunit of eIF-2 after phosphorylation with CK-II.

Effect on GTP Binding after Treatment of GEF with CK-II or Alkaline Phosphatase. Earlier studies from our laboratory have shown that GEF is a GTP-binding protein and this binding is on the 40-kDa subunit of GEF (Dholakia & Wahba, 1989; Dholakia et al., 1989). In the present study, we investigated the ability of GEF to bind GTP with three

Table 2: Summary of Binding Parameters of [^{3}H]GTP to Different Preparations of GEF in the Presence of Mg^{2+} [a]

type of GEF	[^{3}H]GTP bound (pmol/3.5 pmol of GEF)	K_D (μM)
isolated GEF	0.33	4.0
dephosphorylated GEF	0.12	5.0
CK-II-rephosphorylated GEF	0.46	4.0

[a] The binding of [^{3}H]GTP (6650 cpm/pmol) to GEF was carried out at 30 °C in 55-μL reaction mixtures containing 20 mM Tris·Cl, pH 7.5, 100 mM KCl, 2 mM DTT, 1 mM Mg^{2+}, 3.5 pmol (1 μg) of GEF, and different concentrations of [^{3}H]GTP (1–20 μM). The reaction was started with [^{3}H]GTP and stopped after 10 min by the addition of 1 mL of buffer (20 mM Tris·Cl, pH 7.5, 100 mM KCl, 2 mM DTT, and 1 mM Mg^{2+}). The amount of GTP bound to GEF was determined as described under Materials and Methods.

different preparations of GEF, (i) isolated GEF, (ii) alkaline phosphatase-treated GEF, and (iii) rephosphorylated GEF with CK-II. Figure 2 illustrates the binding of [^{3}H]GTP to the three types of GEF in the presence of 1 mM Mg^{2+}. After treatment of the isolated GEF with alkaline phosphatase, total GTP binding to GEF is reduced approximately 2.8-fold. A summary of the binding data is presented in Table 2. The K_D values for GTP binding with the isolated, alkaline phosphatase-treated, and CK-II-rephosphorylated factors remained unchanged. The amount of GTP bound to the isolated GEF is 0.33 pmol/3.5 pmol of GEF and decreases to 0.12 pmol/3.5 pmol of GEF after treatment of the protein with alkaline phosphatase. Upon rephosphorylation with CK-II, total GTP bound is increased to 0.46 pmol/3.5 pmol of GEF. The alterations in the amount of GTP bound to GEF upon treatment with alkaline phosphatase or rephosphorylation by CK-II go hand in hand with the changes in GEF-catalyzed release of eIF-2-bound GDP (Dholakia & Wahba, 1988). In confirmation of the previous results, we observed upon phosphorylation by CK-II of the alkaline phosphatase-treated GEF a 4–5 fold increase in GEF activity (data not shown). Similarly, the binding of [^{3}H]GTP to either the isolated, the alkaline phosphatase-treated, or the CK-II-rephosphorylated GEF in the absence of Mg^{2+} was also examined. The amount of GTP bound to isolated or CK-II-rephosphorylated GEF is 1.2 or 1.3 pmol/3.5 pmol of GEF, respectively, which is higher than that obtained in the presence of Mg^{2+}. We have previously demonstrated that, at 1 mM Mg^{2+}, GTP binding to GEF is diminished and photoinsertion of the 8-azido analog of GTP to the 40-kDa subunit is inhibited (Dholakia et al., 1989). Dephosphorylation of the 82-kDa subunit of GEF reduces or abolishes the accessibility of GTP binding to the 40-kDa subunit of GEF. In this case, we have assumed that GTP binding to the alkaline phosphatase-treated GEF occurs with the residual phosphorylated species with no alteration in the K_D value. Similar results were also obtained when hormone-stimulated CK-II was treated with alkaline phosphatase and the maximal velocity was reduced 4-fold but the apparent K_m of the enzyme remained unchanged (Ackerman et al., 1990).

Effect of Phosphorylation and Dephosphorylation of eIF-2 on GDP Binding. We investigated the binding of [^{3}H]GDP to eIF-2 following treatment of the isolated factor with alkaline phosphatase and after rephosphorylation with CK-II. Total GDP binding to the alkaline phosphatase-treated eIF-2 is increased 2.3-fold as compared to that to the isolated eIF-2. At saturation, the amounts of [^{3}H]GDP bound to the alkaline phosphatase-treated and isolated eIF-2 are 0.33 and 0.14 pmol/pmol of eIF-2, respectively (Table 3). The observed K_D values, as determined from Scatchard plot analysis of the binding data for the alkaline phosphatase-treated eIF-2 and the isolated

Table 3: Effect of Alkaline Phosphatase Treatment of eIF-2 on Binary (eIF-2·[^{3}H]GDP) and GTP-Dependent Ternary (eIF-2·GTP·[^{35}S]Met-tRNA$_f$) Complex Formation[a]

type of eIF-2	[^{3}H]GDP bound (mol/mol of eIF-2)	K_D[b] (nM)	[^{35}S]Met-tRNA$_f$ bound (mol/mol of eIF-2)
isolated eIF-2	0.14	4.5	0.45
dephosphorylated eIF-2	0.33	2.1	0.46
CK-II-rephosphorylated eIF-2	0.16	4.8	0.49

[a] Binding of [^{3}H]GDP (12 168 cpm/pmol) and [^{35}S]Met-tRNA$_f$ (42 890 cpm/pmol) to eIF-2 was carried out as described under Materials and Methods in the presence of 1 mM Mg^{2+} and 0.5 μg (3.57 pmol) or 0.2 μg (1.43 pmol) of eIF-2, respectively. For ternary complex formation, a control experiment was performed with internal [^{35}S]Met-tRNA$_m$ and was subtracted as background from each value. [b] K_D, dissociation constant for GDP binding to eIF-2.

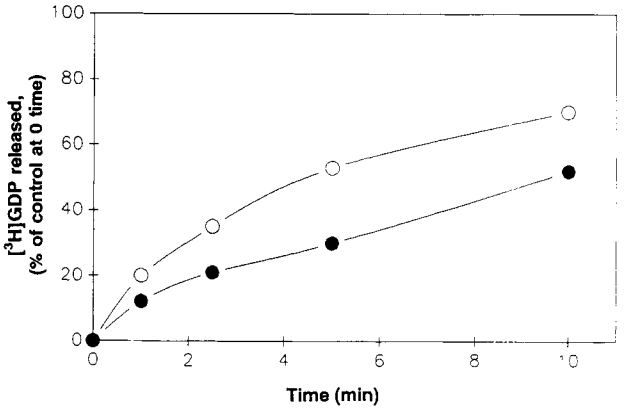

FIGURE 3: Time-dependent release of [^{3}H]GDP from the eIF-2·[^{3}H]GDP binary complex by GEF. The reaction mixture contained (in 75 μL) 20 mM Tris·Cl, pH 7.5, 0.5 mg/mL BSA, 100 mM KCl, 2 mM DTT, 2 mM Mg^{2+}, 100 μM GTP, 22 000 cpm (4.2 pmol) of preformed isolated eIF-2·[^{3}H]GDP (○) or alkaline phosphatase-treated eIF-2·[^{3}H]GDP (●) (5200 cpm/pmol), and 150 ng of isolated GEF (0.52 pmol). The reaction was started by the addition of the binary complex, and the reaction mixture was incubated at 30 °C for the specified time intervals. The amount of radioactivity remaining on the filters was determined as described under Materials and Methods. In the absence of GEF, GTP alone had no effect on nucleotide exchange.

eIF-2, are 2.1 and 4.5 nM, respectively. In the presence of 1 mM Mg^{2+}, a similar K_D value (4.5 nM) for GDP binding to isolated eIF-2 was previously reported (Panniers et al., 1988). Upon rephosphorylation with CK-II of the alkaline phosphatase-treated eIF-2, the affinity as well as the amount of GDP bound are restored to that of the isolated eIF-2 (Table 3). eIF-2 phosphorylated at 1 or 2 mol of Pi/mol of β-subunit may have the same conformational state and, therefore, a similar extent and affinity of GDP binding.

Comparison of eIF-2 Activity by Ternary (eIF-2·GTP·Met-tRNA$_f$) Complex Formation. We compared the activities of the isolated, alkaline phosphatase-treated, and CK-II-rephosphorylated eIF-2 to form GTP-dependent ternary (eIF-2·GTP·[^{35}S]Met-tRNA$_f$) complexes in the presence of 1 mM Mg^{2+}. There were no significant differences in the amount of [^{35}S]Met-tRNA$_f$ bound to the different preparations of eIF-2 except for a marginal increase (10–15%) in the binding of the initiator tRNA to CK-II-rephosphorylated eIF-2 (Table 3). The higher amount of Met-tRNA$_f$ bound to eIF-2 as compared to GDP (Table 3) may be due to stabilization of GTP binding to eIF-2 in the presence of Met-tRNA$_f$ (Gross et al., 1991). It appears that the effect of phosphorylation/dephosphorylation of the β-subunit of eIF-2 is more pro-

Table 4: Effect of Alkaline Phosphatase Treatment of eIF-2 on GEF-Dependent Release of [^{3}H]GDP from the Preformed eIF-2·[^{3}H]GDP Complex[a]

nucleotide used	GEF (ng)	[^{3}H]GDP released from eIF-2·[^{3}H]GDP (cpm) (%)	[^{3}H]GDP released from (ALP)eIF-2·[^{3}H]GDP[b] (cpm) (%)	fold decrease in nucleotide exchange
expt 1	25	3359 (17)[c]	1976 (10)	1.7
(GTP)	50	5137 (26)	3612 (16)	1.6
	100	6718 (34)	3952 (20)	1.7
	150	11065 (56)	5533 (28)	2.0
expt 2	25	4149 (21)	2371 (12)	1.8
(GDP)	50	7509 (38)	3612 (16)	2.4
	100	10473 (53)	5335 (27)	2.0
	150	13634 (69)	7706 (39)	1.8

[a] Each reaction mixture contained (in 75 μL) 20 mM Tris·Cl, pH 7.5, 0.5 mg/mL BSA, 100 mM KCl, 2 mM DTT, 2 mM Mg^{2+}, 100 μM GTP (expt 1) or 4 μM GDP (expt 2), 3.8 pmol of the preformed isolated eIF-2·[^{3}H]GDP or dephosphorylated eIF-2·[^{3}H]GDP (5200 cpm/pmol), and isolated GEF as indicated. The reaction was performed as described under Materials and Methods. [b] Binary complex formed between alkaline phosphatase-treated eIF-2 and [^{3}H]GDP. [c] Values in parentheses represent percent of [^{3}H]GDP released from the binary complex.

nounced in binary (eIF-2·GDP) than in ternary (eIF-2·GTP·Met-tRNA$_f$) complex formation.

Effect of Alkaline Phosphatase Treatment of eIF-2 on GEF-Dependent Release of eIF-2-Bound GDP. We further investigated (Figure 3) the rate and extent of release of [^{3}H]-GDP bound to both isolated and dephosphorylated forms of eIF-2 in the presence of GEF (150 ng) and GTP (100 μM). The $t_{1/2}$ for the release of [^{3}H]GDP from alkaline phosphatase-treated or isolated eIF-2 is 4.2 or 2.5 min, respectively. A concentration-dependent release of [^{3}H]GDP from the binary complex was also studied with varying amounts of GEF with either GTP or GDP as the exchange nucleotide. As shown in Table 4, the activity of GEF to exchange eIF-2-bound [^{3}H]-GDP with 100 μM GTP or 4 μM cold GDP is reduced approximately 2-fold in the case of alkaline phosphatase-treated eIF-2. A 2-fold slower rate of [^{3}H]GDP release from the alkaline phosphatase-treated eIF-2 correlates with a 2.2-fold increase (K_D of 2.1 vs 4.5 nM) in the affinity of alkaline phosphatase-treated eIF-2 for GDP.

DISCUSSION

Covalent modification of proteins, primarily by phosphorylation, is one of the ways of regulating rapidly the rate of protein synthesis during growth and development as well as after treatment of cells with insulin and growth factors. Several proteins involved in the initiation and elongation steps of translation are phosphorylated both *in vitro* and *in vivo* (Hershey, 1991; Proud, 1992; Rhoads, 1993); however, the significance of phosphorylation and the kinases involved are yet to be elucidated. We have previously shown that alkaline phosphatase treatment of GEF reduces its activity 5-fold in the exchange of eIF-2-bound GDP for free GTP. Rephosphorylation of the alkaline phosphatase-treated factor with CK-II restores its guanine nucleotide exchange activity (Dholakia & Wahba, 1988). Until the present study, a mechanism for the regulation of GEF activity by phosphorylation/dephosphorylation of the 82-kDa subunit has not been elucidated. The important observation made in this study is that dephosphorylation of GEF decreases GTP binding to the factor and, consequently GEF activity. Rephosphorylation with CK-II restores GTP binding and GEF activity. The increase in the extent of total GTP-binding to phosphorylated GEF, as demonstrated here, goes hand in hand with increased rates of GDP/GTP exchange upon phosphorylation with CK-II of the 82-kDa subunit of GEF (Dholakia & Wahba, 1988). In other systems, phosphorylation of the filamin-like 250-kDa membrane protein in human platelet with cAMP-dependent protein kinase A increases the binding of GTP to this protein and dephosphorylation with alkaline phosphatase reduces GTP binding activity (Yada et al., 1990). Similarly, phosphorylation of the ras-like GTP-binding protein rab4 at Ser^{196} leads to the translocation of the protein into the cytosol of mitotic cells while dephosphorylation results in the reassociation of rab4 with membranes upon exit of cells from mitosis (van der Sluijs et al., 1992). It, therefore, appears that phosphorylation of the 82-kDa subunit of GEF is crucial in maintaining a favorable conformation for GTP binding to the 40-kDa subunit. Phosphorylation by CK-II may represent one mechanism operating *in vivo* for the regulation of GEF activity during polypeptide chain initiation.

The β-subunit of eIF-2 is involved in guanine nucleotide binding (Dholakia & Wahba, 1987; Dholakia et al., 1989; Gonsky et al., 1990), interaction with mRNA (William et al., 1989; Harary and Kaempfer, 1990), and initiation site selection (Donahue et al., 1988). This subunit can be phosphorylated *in vitro* by several kinases, with CK-II and protein kinase C phosphorylating distinct sites (Clark et al., 1988). However, no functional significance has been ascribed to these phosphorylations. Our results demonstrate that phosphorylation of the β-subunit of eIF-2 with CK-II decreases the affinity of GDP binding to eIF-2 approximately 2.2-fold and results in a faster rate of exchange of eIF-2-bound GDP for free GTP in the presence of both GTP and GEF. Alkaline phosphatase treatment of eIF-2 slows down the rate of GDP/GTP exchange. The manners in which the α- and β-subunits regulate eIF-2 function by phosphorylation and dephosphorylation are also not identical. First, the enzymes involved in the phosphorylation of the α- and β-subunits of eIF-2 are different (Mehta et al., 1986; Proud, 1992). Second, the affinity (K_D) of eIF-2 for GDP is independent of the phosphorylation state of the α-subunit (Konieczny & Safer, 1983) whereas dephosphorylation of the β-subunit by alkaline phosphatase increases the extent and affinity of GDP binding to eIF-2 (this study). Our observations that alkaline phosphatase treatment of eIF-2 reduces guanine nucleotide exchange and does not alter ternary (eIF-2·GTP·Met-tRNA$_f$) complex formation suggest that GDP/GTP exchange is the rate-limiting step in chain initiation.

The affinity of eIF-2 for GDP is more than 400-fold greater than that for GTP (Panniers et al., 1988). A much higher GTP/GDP ratio (or ATP/ADP energy levels) will be required to facilitate the exchange of eIF-2-bound GDP for GTP even in the presence of GEF. An economical way to conserve energy but maintain efficiently higher rates of protein synthesis may be achieved through covalent modification of eIF-2 and GEF via a CK-II-dependent protein phosphorylation pathway. The β-subunit of eIF-2 and the 82-kDa subunit of GEF are readily phosphorylated by CK-II, which utilizes either ATP or GTP as a phosphate donor. A slight increase in the energy level will be sufficient to increase the extent of phosphorylation for both eIF-2 and GEF. CK-II may be involved in the regulation of GDP/GTP exchange during polypeptide chain initiation by phosphorylating the 82-kDa subunit of GEF and the β-subunit of eIF-2 in a coordinate fashion. Recently, we provided evidence for the *in vivo* phosphorylation of the 82-kDa subunit of GEF by CK-II or by a kinase with similar specificity (Aroor et al., 1994). There are now several instances

where the rate of GDP/GTP exchange is altered without significant changes in the level of eIF-2(α) phosphorylation (Gross et al., 1988; Kimball & Jefferson, 1988; Welsh & Proud, 1992). It is possible that regulation of guanine nucleotide exchange activity either directly by phosphorylation of the 82-kDa subunit of GEF or indirectly by phosphorylation of the β-subunit of eIF-2 may occur independently of eIF-2(α) phosphorylation.

In summary, we demonstrate that phosphorylation of the 82-kDa subunit of GEF is crucial in maintaining the GTP-binding property of GEF and subsequently the guanine nucleotide exchange activity. Similarly, phosphorylation of the β-subunit of eIF-2 will determine the extent and affinity of GDP binding to the factor, thereby controlling the rate of release of eIF-2-bound GDP for free GTP in the presence of GTP and GEF. In intact cells, under various physiological conditions, CK-II may phosphorylate both eIF-2 and GEF simultaneously and regulate the rate of GDP/GTP exchange during polypeptide chain initiation. During growth arrest (Telfer & Green, 1993) and differentiation (Wei et al., 1993), alkaline phosphatase may slow down the rate of protein synthesis because both eIF-2 and GEF will undergo dephosphorylation.

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