Phosphorylation of Rabbit Reticulocyte Guanine Nucleotide Exchange Factor *in Vivo*. Identification of Putative Casein Kinase II Phosphorylation Sites†

Annayya R. Aroor,‡ Nancy D. Denslow,§,‖ Lalit P. Singh,‡ Thomas W. O'Brien,§ and Albert J. Wahba*,‡

Department of Biochemistry, University of Mississippi Medical Center, Jackson, Mississippi 39216, and Department of Biochemistry and Molecular Biology and Interdisciplinary Center for Biotechnology Research, University of Florida, Gainesville, Florida 32610

Received November 4, 1993; Revised Manuscript Received January 5, 1994®

ABSTRACT: The guanine nucleotide exchange factor (GEF) is a multi-subunit protein which catalyzes the exchange of GDP for GTP in eukaryotic chain initiation factor 2. Phosphorylation of the 82-kDa subunit of GEF *in vitro* by casein kinase II (CK-II) is associated with a 5-fold increase in nucleotide exchange activity. However, phosphorylation of GEF *in vivo* has not been studied, and the kinase(s) that phosphorylate GEF have not been identified. The 82-kDa subunit of GEF was partially sequenced, and a synthetic peptide was used to generate polyclonal anti-peptide antibodies that react specifically with this subunit. To examine the phosphorylation of GEF in intact cells, the protein was isolated and purified extensively from metabolically ^{32}P-labeled rabbit reticulocytes. Only the 82-kDa subunit was found to be phosphorylated, and on Western blots the anti-peptide antisera reacted specifically with the labeled subunit. Phosphoamino acid analysis indicated that phosphorylation occurred exclusively on Ser residues. Digestion with cyanogen bromide of *in vivo* labeled protein and GEF phosphorylated *in vitro* by CK-II produced comparable phosphopeptide maps. However, additional phosphopeptide bands were also observed with GEF derived from intact cells. Sequence analysis obtained by Edman degradation of the phosphopeptides was compared with the deduced amino acid sequence of a cloned 82-kDa subunit of GEF [Bushman, J. L., Asuru, A. I., Matts, R. L., & Hinnenbusch, A. G. (1993) *Mol. Cell. Biol. 13*, 1920–1932]. Putative sites of phosphorylation were identified at Ser 703 and/or 704, which contain the sequence S(P)XXD, a CK-II consensus recognition motif. In addition, the Ser residue at position 174 could also be phosphorylated by CK-II in a hierarchical manner. These results suggest that the 82-kDa subunit of GEF is phosphorylated *in vivo* by CK-II or a similar enzyme, and this covalent posttranslational modification is a potential mechanism for the regulation of GEF in eukaryotic cells.

In mammalian cells, the guanine nucleotide exchange factor (GEF)[1] plays a major role in regulating protein synthesis (Wahba & Woodley, 1984; Dholakia & Wahba, 1990). 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 (eIF) 2], which is then transferred to a 40S ribosomal subunit (Wahba & Woodley, 1984; Dholakia & Wahba, 1990; Hershey, 1991). Upon formation of the 80S initiation complex, GTP is hydrolyzed and eIF-2 is released as eIF-2·GDP (Clemens et al., 1982; Traschel & Staehelin, 1987). This binary complex is stable in the presence of Mg^{2+} and is functionally inactive (Siekierka et al., 1982; Panniers & Henshaw, 1983). Regeneration of the ternary complex (eIF-2·GTP·Met-tRNA$_f$) for a new cycle of initiation requires GEF, which catalyzes the exchange of eIF-2-bound GDP for GTP (Siekierka et al., 1982; Mehta et al., 1983; Salimans et al., 1984; Dholakia & Wahba, 1989). GEF consists of five polypeptides of M_r 82 000, 65 000, 55 000, 40 000 and 34 000. Activity of the nucleotide exchange factor may be influenced indirectly by the phosphorylation of the α-subunit of eIF-2. Phosphorylation of eIF-2(α) by either the heme-controlled repressor (HCR) or the double-stranded RNA induced kinase (DAI) is associated with the cessation of protein synthesis and is due to the inability of GEF to catalyze the GDP/GTP exchange from eIF-2(αP) (Pain, 1986; Hershey, 1991). We have previously demonstrated that the 82-kDa subunit of GEF can be phosphorylated *in vitro* by CK-II. This is accompanied by a 5-fold increase in the specific activity of GEF (Dholakia & Wahba, 1988). CK-II is a ubiquitous Ser/Thr kinase which is located in the cytosol as well as the nucleus of eukaryotic cells (Pfaff & Anderer, 1988; Filhol et al., 1990). Although the detailed function of the kinase has yet to be delineated both *in vivo* and *in vitro*, it is capable of phosphorylating a number of proteins which play critical roles in regulating cellular growth and metabolism (Pinna, 1990; Tuazon & Traugh, 1991). Its activity rapidly and transiently increases in response to several mitogens, such as the tumor-promoting phorbol 12-myristate 13-acetate (Carroll et al., 1988), hormones (Sommercorn et al., 1987; Klarlund & Czech, 1988), and growth factors (Sommercorn et al., 1987; Ackerman et al., 1990).

In this paper, we describe the preparation of rabbit polyclonal antibodies directed against synthetic peptides derived from the 82-kDa subunit of GEF. With these antibodies we confirm the phosphorylation of the 82-kDa subunit of GEF in intact cells. In addition, we demonstrate,

† This work was supported by NIH Grant GM 25451.

* Author to whom correspondence should be addressed: Department of Biochemistry, University of Mississippi Medical Center, Jackson, MS 39216. Telephone: 601-984-1512. Fax: 601-984-1501.

‡ University of Mississippi Medical Center.

§ University of Florida.

‖ Interdisciplinary Center for Biotechnology Research.

® Abstract published in *Advance ACS Abstracts,* February 15, 1994.

[1] Abbreviations: CK, casein kinase; eIF-2, eukaryotic initiation factor 2; GEF, guanine nucleotide exchange factor (also designated as eIF-2B); HCR, heme-controlled repressor; DAI, double-stranded RNA-activated inhibitor (also designated as dsRNA-activated eIF-2α kinase); MES, 2(*N*-morpholino)ethanesulfonic acid; PVDF or Immobilon-P, poly(vinylidene difluoride) membrane; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; TFA, trifluoroacetic acid.

0006-2960/94/0433-3350$04.50/0

by phosphoamino acid analysis and amino acid microsequencing of phosphopeptides, that phosphorylation occurs only at Ser residues and that CK-II or a similar kinase is involved in the phosphorylation of the 82-kDa subunit of GEF in intact reticulocytes.

MATERIALS AND METHODS

[γ-^{32}P]ATP and carrier-free [^{32}P]orthophosphoric acid were purchased from DuPont-New England Nuclear. Orthophosphoserine, orthophosphotyrosine, orthophosphothreonine, sodium vanadate, sodium pyrophosphate, sodium fluoride, aprotonin, alkaline phosphatase (bovine intestinal mucosa), spermine–agarose, and *o*-phenylenediamine were obtained from Sigma. Imject maleimide-activated ovalbumin was from Pierce Chemical Co., and poly(vinylidene difluoride) membranes (Immobilon-P) were from Millipore. S-Sepharose, Q-Sepharose, FPLC Mono Q, Mono P, and Mono S were obtained from Pharmacia LKB Biotechnology. The ISCO high-pressure liquid chromatography system was used with the Pharmacia columns. All other reagents and chemicals were of reagent or analytical grade.

Purification of GEF and CK-II. Reticulocytes were obtained from phenylhydrazine-treated New Zealand white rabbits (Dholakia & Wahba, 1987). GEF was purified from rabbit reticulocyte lysates to apparent homogeneity as previously described (Dholakia & Wahba, 1988). The purified preparation was free of eIF-2 and kinase(s) that phosphorylate GEF. eIF-2 was isolated from the 0.5 M KCl wash of ribosomes as previously described (Dholakia & Wahba, 1987). 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 and bovine serum albumin as the standard.

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. The acrylamide:bis(acrylamide) ratio was 37:1 (w/w) in both stacking and resolving gels. The gels were stained with Coomassie Brilliant Blue R-250. Before autoradiography, gels were exposed at −70 °C to Kodak XR-5 film.

Chemical Cleavage with CNBr and Microsequencing of Fragments. The 82-kDa subunit of purified rabbit reticulocyte GEF was isolated by SDS–PAGE. For microsequencing experiments, *in situ* cleavage was performed directly on 300 pmol (85.7 μg) of the 82-kDa subunit blotted on a PVDF membrane (Scott et al., 1988). Briefly, the Coomassie Blue stained protein band on the membrane was cut into small strips and placed in 360 μL of 70% trifluoroacetic acid (TFA). A solution (50 μL) of 5 M CNBr in acetonitrile was added, and the reaction was allowed to proceed for 16 h at room temperature in the dark. After cleavage, the TFA solution was dried in a Speed Vac to remove the acid and CNBr. The membrane was extracted with a solution containing 70% 2-propanol and 5% TFA, and the extract was dried as above. After resuspension in water and precipitation with 9 volumes of ice-cold acetone, the fragments were then separated by electrophoresis on a large Tris–Tricine gel (Schagger & von Jagow, 1987) and electroblotted to a second PVDF membrane using 10 mM 2(*N*-morpholino)ethanesulfonic acid (MES), pH 6, and 20% methanol. The protein fragments were stained with 0.01% Coomassie Blue R-250 in 50% methanol, destained in 50% methanol, and rinsed several times with water. Bands of interest were excised and sequenced in a gas-phase protein sequenator (Applied Biosystems, Model 473A). Sequence data were analyzed by using the software package of the Genetics Computer Group (GCG, University of Wisconsin, Madison, WI). These data were compared to the National Biomedical Research Foundation and GenBank Genetic Sequence data bases by using the FASTA and TFASTA programs of Pearson and Lipman (Pearson & Lipman, 1988) and BLAST (Attschul et al., 1990).

Preparation of Synthetic Peptides and Anti-Peptide Antibodies. A synthetic peptide, covering a region of sequence with reasonable immunogenic potential, was synthesized in solid phase using *t*-BOC chemistry. The peptide contained an additional N-terminal cysteine for coupling to the carrier protein, Imject maleimide-activated ovalbumin. The coupling was carried out as described by the manufacturer. Specifically, 2 mg of peptide was mixed with 2 mg of activated ovalbumin in 10 mM phosphate buffer, pH 7.4, and 150 mM NaCl and allowed to react for 2 h at room temperature. The product was then purified by gel filtration. New Zealand white rabbits were immunized with the peptide–protein conjugate. Rabbits developed high titers to the peptide as assayed by ELISA (Dholakia & Wahba, 1987). The peptide was bound to microtiter plates at a concentration of 10 μg/mL in Tris–saline plating buffer (10 mM Tris-HCl, pH 8.3, and 150 mM NaCl; 50 μL per well) at room temperature. Plates were blocked and washed in 10 mM phosphate buffer, pH 7.4, 150 mM NaCl, 0.03% (v/v) Tween 20, and 3% (w/v) BSA before the addition of serial dilutions of antisera. They were then incubated for 1 h before washing and the addition of a 1:10 000 dilution of the second antibody, horseradish peroxidase conjugated to an anti-rabbit IgG. The plates were developed with *o*-phenylenediamine.

Western Blots. SDS–PAGE of purified GEF was performed as described (Dholakia & Wahba, 1991). The separated subunits were transferred to an Immobilon-P membrane with MES buffer as described above. Narrow strips of membranes were cut for testing with the different batches of the anti-peptide serum. One control strip was stained with Coomassie Brilliant Blue R-250 to visualize the position of the individual subunits. After being blocked with 5% nonfat dry milk in Tris-buffered saline, the membrane was incubated with anti-peptide antibody. This was followed by incubation with the anti-rabbit antibody conjugated to horseradish peroxidase (Dholakia & Wahba, 1987) and detection of immunoblots with the ECL detection reagent from Amersham.

In Vitro Phosphorylation of GEF by CK-II. The phosphorylation of GEF (50 μg) was carried out in a reaction mixture of 150 μL containing 20 mM Tris-HCl, pH 7.5, 0.1 M KCl, 10 mM Mg^{2+}, 2 mM dithiothreitol, 100 μM [γ-^{32}P]-ATP (2000 cpm/pmol), and 0.5 unit of CK-II (Dholakia & Wahba, 1988). One unit of enzyme will transfer 1 nmol of phosphate per min at 30 °C to α-casein (2.5 mg/mL) with [γ-^{32}P]ATP (100 μM) as the phosphate donor. After 30 min of incubation at 30 °C, the sample was dialyzed against the same buffer to remove free ATP. The phosphorylated sample was analyzed by SDS–PAGE followed by autoradiography.

In Vivo Phosphorylation of GEF. Rabbit reticulocytes from 20 mL of blood were washed three times with cold phosphate-

buffered saline and resuspended in the original volume. After dilution with an equal volume of nutritional medium (Bitte & Kabat, 1974) containing [^{32}P]orthophosphate (0.62 mCi/mL), reticulocytes were incubated at 37 °C in a shaking water bath. After 60 min, 4 volumes of ice-cold phosphate-buffered saline was added. Cells were washed two times with ice-cold phosphate-buffered saline and lysed with a hypotonic buffer containing phosphatase inhibitors (20 mM NaF, 20 mM Na_2MoO_4, 20 mM glycerol β-phosphate, and 20 mM sodium pyrophosphate) and protease inhibitors (1 mg/mL leupeptin, 1 mg/mL pepstatin, 1 mg/mL aprotonin, and 0.33 mM PMSF). The postribosomal supernatant was used for the isolation of GEF. Purified GEF was subjected to SDS–PAGE, autoradiography, and immunoblotting as described above.

Phosphoamino Acid Analysis. The ^{32}P-labeled 82-kDa polypeptide digested with CNBr as described above was used for phosphoamino acid analysis. The acetone precipitate from the extract was resuspended in 25 μL of constantly boiling 6 N HCl and incubated under N_2 in a microfuge tube for 2 h at 100 °C. The acid was removed under vacuum, and the pellet was resuspended in 10 μL of distilled water containing 50 nmol each of phosphoserine, phosphothreonine, and phosphotyrosine and spotted on Whatman 3MM chromatography paper. Phosphoamino acids were separated by electrophoresis at 1000 V for 1.5 h in pyridine:glacial acetic acid:H_2O (5:50:945, v/v). Phosphoamino acid standards were visualized by spraying the Whatman 3MM paper with a 0.3% solution of ninhydrin in *n*-butanol followed by 5 min of heating at 65 °C. The ^{32}P-labeled experimental sample was visualized by autoradiography, and the location of the signal was directly compared with that of the phosphoamino acid standards (Kroll & Rowe, 1991).

Phosphopeptide Mapping and Analysis of the 82-kDa Subunit of GEF Phosphorylated in Intact Cells and in Vitro by CK-II. Partial cleavage with CNBr was carried out as described above for microsequencing of the 82-kDa subunit of GEF phosphorylated *in vivo* or *in vitro* with CK-II. Briefly, 10 μg of *in vivo* phosphorylated GEF or 25 μg of GEF phosphorylated *in vitro* with CK-II was subjected to SDS–PAGE and blotted on a PVDF membrane. The 82-kDa band was excised from the membrane, dissolved in 70% TFA, and cleaved with CNBr for 16 h at room temperature. After evaporation of the TFA, the sample was redissolved in SDS sample buffer, and subjected to Tris–Tricine gel electrophoresis (Schagger & von Jagow, 1987), stained with silver, and subjected to autoradiography. A companion gel on the *in vitro* labeled, CNBr-cleaved 82-kDa subunit was blotted to a PVDF membrane. Bands were visualized by Coomassie Blue staining, and phosphorylated peptides were localized by autoradiography. Bands of interest from the *in vitro* CK-II phosphorylated material were excised, and five amino acid residues from the N-terminus were determined as described above.

RESULTS

Preparation and Characterization of Anti-Peptide Antibodies. We determined portions of the amino acid sequence of the 82-kDa subunit of GEF in order to characterize this protein and to allow us to raise specific antibodies. The 82-kDa subunit of GEF is blocked at the N-terminus, rendering amino acid sequencing of the intact protein by Edman degradation chemistry impossible. We resorted to cleavage with CNBr in order to generate large internal fragments of the 82-kDa subunit that could subsequently be used for sequencing. For this experiment, 300 pmol (85.7 μg) of a highly purified preparation of GEF was used. The subunits were separated by SDS–PAGE and transblotted to a PVDF membrane. The 82-kDa band was excised and digested with CNBr, generating five major fragments, which were separated by Tris–Tricine gel electrophoresis and blotted to a second PVDF membrane. In order of staining intensity, the bands were labeled as follows: 20 kDa, 6.5 kDa, 11 kDa, 5.5 kDa and 14 kDa. The five fragments were subjected to N-terminal sequencing. The 20-kDa fragment could not be sequenced, indicating that it contained the blocked N-terminus. The sequence of the 6.5-kDa fragment was GYQLEILAEETILSXFGQRDVTDKGRQLRKNQQL, and that of the 11-kDa fragment was IFKESXPSHP. The 5.5 and 14-kDa fragments had the same sequence, DSPLEANRYXALLLPLLKAXSPVF, indicating that the 5.5-kDa fragment was a partial cleavage product of the 14-kDa fragment. The sequence for the 5.5-kDa fragment listed above varies from the published sequence deduced from the cDNA (Bushman et al., 1993) at position 608 where we find a Leu instead of an Ile. We were able to identify a total of 66 residues in all.

Sequence data were compared to reported National Biomedical Research Foundation and GenBank sequences using both FASTA and TFASTA data base search programs (Pearson & Lipman, 1988). In addition, the BLAST program (Attschul et al., 1990) was used to search for similarities. The longest sequence, the 6.5-kDa fragment, was analyzed by the Peptide Structure program in the GCG Software package to reveal regions of immunogenic potential. One region, QRDVTDKGRQLRKN, corresponding to amino acid residues 673–686 of the cDNA-derived amino acid sequence of the 82-kDa subunit of GEF (Bushman et al., 1993), was predicted to be highly immunogenic. A synthetic peptide was constructed containing this stretch of amino acids, and polyclonal antibodies were produced in rabbits against this peptide. The specificity of the antibody is revealed in Western blots of purified GEF. It reacted specifically with the 82-kDa subunit of GEF at a 1:5000 dilution, indicating that this would be a useful reagent for the identification of *in vivo* labeled GEF.

In Vivo Phosphorylation of GEF. Rabbit reticulocytes were incubated in the presence of $^{32}P_i$ to investigate the *in vivo* phosphorylation of GEF. Following cell lysis, the postribosomal supernatant was used for the purification of GEF. The protein was analyzed by SDS–PAGE and autoradiography. The results show that the 82-kDa subunit is phosphorylated in intact reticulocytes (Figure 1, lane 2), and immunoblotting with the anti-peptide antibody (Figure 1, lane 3) confirmed that the 82-kDa phosphoprotein was indeed phosphorylated *in vivo*.

Phosphoamino Acid Analysis. Ser or Thr residues are involved in CK-II-dependent phosphorylation (Pinna, 1990). However, Tyr phosphorylation resulting from other kinases may occur *in vivo*. The 82-kDa subunit of GEF isolated from ^{32}P-labeled reticulocytes was acid-hydrolyzed, and the resulting phosphoamino acids were resolved by high-voltage electrophoresis. As is evident from Figure 2a, the vast majority of radioactivity migrated with authentic phosphoserine with no detectable signal migrating at phosphothreonine or phosphotyrosine, suggesting that the 82-kDa polypeptide is exclusively phosphorylated at Ser *in vivo*. Identical results were also obtained with the 82-kDa subunit of GEF phosphorylated *in vitro* by CK-II (Figure 2b).

Phosphopeptide Map Analysis of the 82-kDa Subunit of GEF Phosphorylated in Intact Cells and in Vitro by CK-II. In order to demonstrate whether the region of the 82-kDa subunit that is phosphorylated in intact cells corresponds to

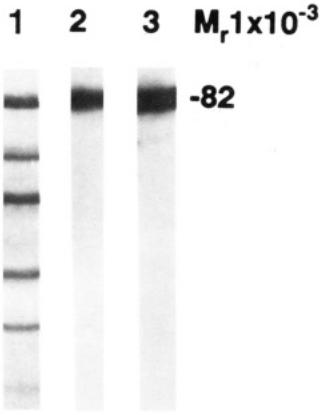

FIGURE 1: Immunoblot analysis of reticulocyte GEF, metabolically labeled with [^{32}P]-orthophosphate. Reticulocytes were metabolically labeled with [^{32}P]orthophosphate, and phosphorylated GEF was isolated as described under Materials and Methods. The sample was subjected to SDS–PAGE and analyzed by autoradiography and Western immunoblotting. Lane 1, Coomassie Blue R-250 stained gel; lane 2, autoradiogram of phosphorylated GEF; lane 3, immunoblot of phosphorylated GEF. The two-digit number alongside the gel corresponds to the molecular weight (×10^{-3}) of the 82-kDa subunit of GEF.

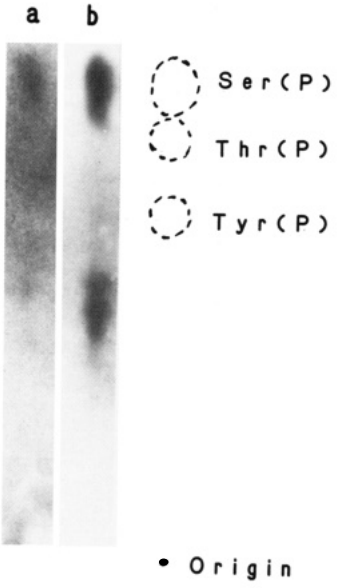

FIGURE 2: Phosphoamino acid analysis of the ^{32}P-labeled 82-kDa subunit of GEF. The 82-kDa subunit was isolated from GEF metabolically labeled and *in vitro* labeled with CK-II. Each preparation was subjected to acid hydrolysis, and the labeled amino acids were analyzed by high-voltage paper electrophoresis as described under Materials and Methods. Lane a, autoradiogram of the metabolically labeled sample; lane b, autoradiogram of the sample labeled *in vitro* with CK-II. The mobility of each authentic phosphoamino acid is denoted by the broken circles.

that phosphorylated *in vitro* by CK-II, we compared one-dimensional phosphopeptide maps produced by partial digestion with CNBr of the 82-kDa subunit. The number and size distribution of phosphopeptides produced from *in vitro* and *in vivo* phosphorylated material were examined by autoradiography (Figure 3). Phosphopeptides of 42, 37, 30, 25, 23, 14, 11, and 6.5 kDa were visualized in both cases. However, the quantitative differences in intensity observed in the otherwise similar phosphopeptide patterns of GEF phosphorylated *in vitro* by CK-II and GEF phosphorylated *in vivo* might be due to conditions required to purify GEF and perform phosphorylation assays. They might also be a result of the involvement of other kinase(s) in the phosphorylation of GEF *in vivo*. Indeed, the metabolically labeled GEF had additional

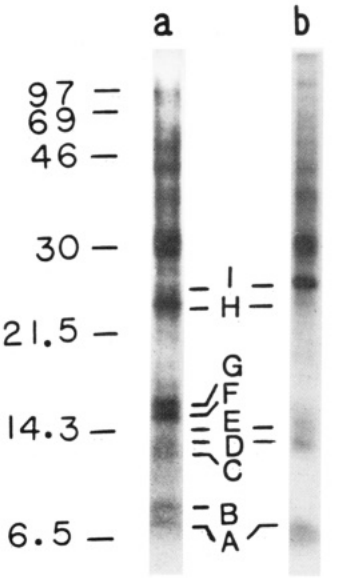

FIGURE 3: Autoradiogram of the partial peptide map of phosphorylated GEF obtained by cleavage with CNBr. The protein was phosphorylated *in vivo* (lane a) by metabolic labeling of reticulocytes with ^{32}P and *in vitro* (lane b) by CK-II. The 82-kDa subunit of GEF from each preparation was isolated by SDS–PAGE and cleaved with CNBr as described under Materials and Methods. Fragments were separated by high-resolution Tris–Tricine gel electrophoresis and visualized by autoradiography. The gels were calibrated with molecular weight markers in an adjacent lane. Numbers to the left of the gels correspond to the molecular weights (×10^{-3}) of the fragments, and the letters correspond to fragments of importance to the discussion.

phosphopeptides of 16, 15, 10.5, and 7.3 kDa. The presence of extra bands in the *in vivo* material suggests the possible involvement of additional kinase(s) in the phosphorylation of GEF.

Localization of CK-II Phosphorylation Sites. The 82-kDa subunit of rabbit reticulocyte GEF has been recently cloned and sequenced (Bushman et al., 1993). By using the MOTIF program in the GCG software package, we located several phosphorylation recognition motifs. Since Ser is the only residue phosphorylated both *in vivo* and *in vitro*, we limited our search to recognition motifs involving only Ser residues. This is indicated by the smaller solid circles positioned above Ser residues in the linear map of the 82-kDa subunit in Figure 4 (top panel); Ser residues in CK-II phosphorylation site motifs are indicated by the larger solid circles. Thus, of the 27 potential sites identified by this program, nine are possible CK-II sites. We analyzed our peptide map data (Figure 3) to determine if we could pinpoint any of these sites as putative phosphorylation sites *in vitro* and *in vivo*. The smaller fragments, especially those that correspond to completely cleaved proteins, are the most discriminating and the ones we have used in this analysis (fragments A–I, Figure 3). For unequivocal identification of the fragments, we sequenced five amino acid residues from the N-terminus of each of several radiolabeled and Coomassie Blue stained bands transferred to an Immobilon-P membrane (Table 1). This sequence information was used to position the fragments in the primary sequence diagram for both the CK-II-labeled GEF (Figure 4, middle panel) and the metabolically labeled GEF (Figure 4, bottom panel). Characterization of these fragments is summarized in Table 1.

The two most discriminating fragments for the CK-II experiment, fragments A and D (fragments 6.5 and 11 kDa, respectively), point to at least two disparate regions of the protein containing phosphorylation sites: segments between

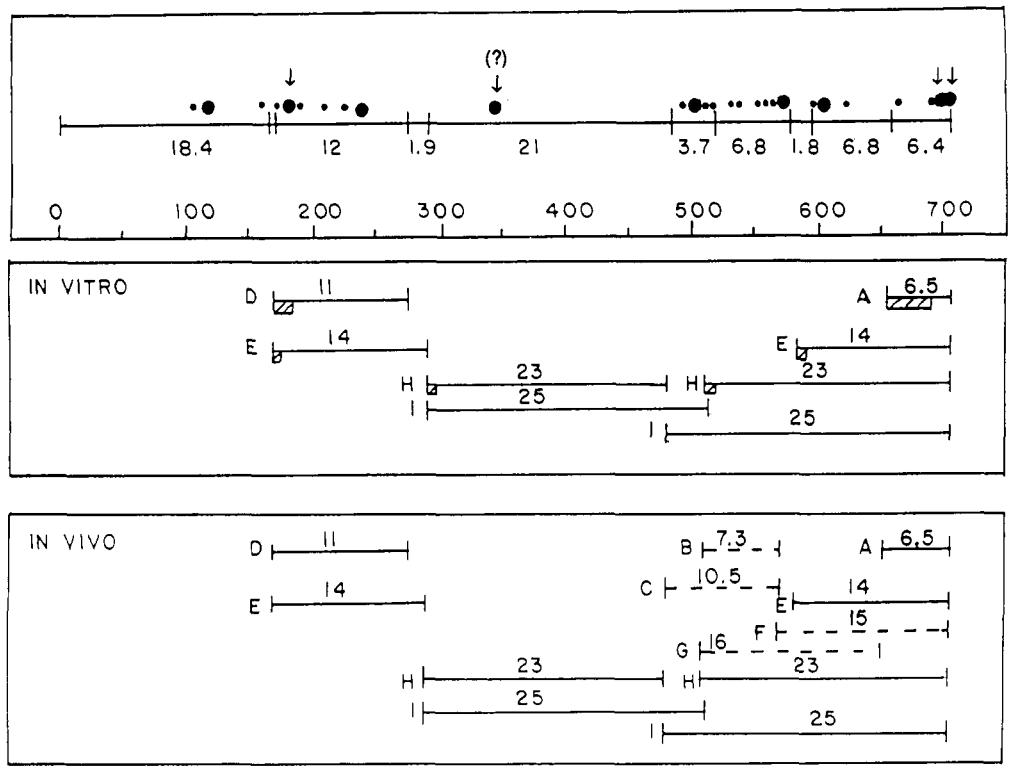

FIGURE 4: Analysis of potential phosphorylation sites in the linear map of the 82-kDa protein. Top panel: The position of each Met residue in the sequence is indicated by a vertical line. Potential phosphorylation sites for Ser residues determined by the MOTIF program in GCG for CK-II motifs are indicated by the smaller solid circles, and the subset of these sites corresponding to CK-II motifs are indicated by the larger solid circles. The arrows above some of the circles represent putative CK-II phosphorylation sites identified by microsequencing of the radiolabeled CNBr fragments, and (?) denotes uncertainty regarding phosphorylation at this site. Fragment sizes $\times 10^{-3}$ Da are indicated by numbers below each segment. The scale at the bottom of the panel refers to the number of amino acids in the primary sequence. Middle and bottom panels: The radiolabeled fragments (labeled with uppercase letters to correspond to Figure 3) empirically obtained from partial cleavage with CNBr are positioned below the linear map for GEF phosphorylated *in vitro* with CK-II (middle panel) and for GEF metabolically labeled *in vivo* (bottom panel). Dashed lines are used to accentuate radiolabeled fragments found exclusively in the *in vivo* experiment. Regions that have been determined by direct N-terminal sequencing are boxed below fragments in the middle panel. Two fragments, corresponding to segments 595–654 (5.5 kDa) and 520–654 (16 kDa), which are not phosphorylated by CK-II but for which we have N-terminal sequence information, are not indicated in this panel. We have sequenced 24 residues of segment 595–654 and 5 residues of segment 520–654.

Table 1: Analysis of Radiolabeled Fragments from a CNBr Digest of the 82-kDa Subunit of GEF Phosphorylated *in Vitro* with CK-II[a]

fragment size[b] (by gel), kDA	theoretical[c] fragment size, kDa	N-terminal[d] sequence of fragment	position[e] in sequence
A/ 6.5	6.4	GFYQL	655–707
D 11	12	IFKES	170–275
E 14	13.2	DSPLE	595–707
	13.9	IFKES	170–291
H 23	21	YSAVC	292–488
	21.8	DSEEL	520–707

[a] The subunits of purified reticulocyte GEF were separated by SDS–PAGE and electroblotted to Immobilon-P. The 82-kDa band was excised and digested with CNBr, and the fragments were separated by Tris–Tricine gel electrophoresis and blotted to Immobilon-P as described under Materials and Methods. The major bands which correspond to the *in vivo* digest were analyzed by automated Edman degradation. [b] Fragment sizes determined by high-resolution Tris–Tricine gels. [c] Closest sized fragment determined by calculation of a CNBr partial digest of the known sequence of the 82-kDa subunit. [d] N-Terminal amino acid sequence of the radiolabeled fragments cut from a Coomassie Blue stained PVDF membrane. [e] Numerical position of the fragment in the deduced sequence of the 82-kDa subunit. [f] Letters correspond to phosphorylated fragments in Figure 3.

Table 2: CK-II Recognition Motifs[a]

motif	fragment[b]
SXXD	116SLGD
	703SSED
	704SEDD
SXXE	340SILE
	511SETE
	574SLKE
	596SPLE
SXXS(P)	174SSPS(P)
	236SICS(P)

[a] The consensus sequence for CK-II can be summarized as S*/T*-(D/E/S(P)$_{1-3}$,X$_{2-0}$) (Kenelly & Krebs, 1991). [b] The numerical position represents the putative phosphorylation site in the deduced sequence of the 82-kDa subunit (Bushman et al., 1993).

residues 655 and 707 and between residues 170 and 275 (Figure 4, middle panel). In the first segment (655–707) there are two putative CK-II phosphorylation sites at positions 703 (SSED) and 704 (SEDD) (Table 2). Similarly, in the second segment, there are two potential CK-II phosphorylation sites at positions 174 (SSPS(P)) and 236 (SICS(P)), both sites requiring phosphorylated Ser as the acidic specificity determinant.

The 14-kDa phosphopeptide (fragment E) contained a mixture of two phosphopeptides, one fragment (170–291) containing fragment D and the other (595–707) bearing fragment A, as shown in Table 1. Although the latter fragment contains an additional CK-II recognition motif at position 596 (SPLE), this position does not appear to be phosphorylated since the 5.5-kDa gel fragment (6.8 kDa by calculation from the gene sequence corresponding to amino acid residues 595–654) comprising this motif was not radiolabeled. Thus, radiolabeling of these fragments confirms results obtained from analysis of the fragments A and D (above) and adds no additional putative phosphorylation sites for consideration.

The analysis of the 23-kDa phosphopeptide (fragment H) also includes a mixture of two phosphopeptides, one comprising residues 292 to 488 and another 520–707. The former peptide has CK-II motifs at position 340 (SILE), and the latter contains CK-II motifs at positions 574 (SLKE) and 596 (SPLE) and at positions 703 and 704 as described for the 6.5-kDa fragment (fragment A). The 25-kDa phosphopeptide (fragment I) contains two phosphopeptides covering the same general region as the 23-kDa fragments except that each includes the additional segment from residues 489–519. The 23- and 25-kDa fragments result from partial cleavage, which appears to vary somewhat from one experiment to another.

In the case of the metabolically labeled GEF (Figure 4, bottom panel), we can point to another potential region targeted by other kinases, besides the potential CK-II sites. This region falls within the segment 489–707 and is defined by fragments B, C, F, and G. The position of fragment B can be clearly differentiated from the other fragment of the same size (595–654). The latter band moves anomalously in this gel system at 5.5 kDa as determined by direct sequencing of a Coomassie Blue stained band (not radiolabeled). Fragment F (580–707) includes the carboxyl-terminal segment (655–707), which bears the two CK-II sites and two CK-I target sites approximately at the same location. Other possible phosphorylation sites identified by the MOTIF program in the region covered by segments B, C, and G are five putative CK-I sites, three possible proline-directed kinase sites, and two putative protein kinase C sites.

DISCUSSION

Phosphorylation plays a major role in the regulation of protein synthesis. Initiation factors, ribosomal proteins, messenger ribonucleoprotein particles, and aminoacyl-tRNA synthetases have been shown to be modified by phosphorylation (Farrell et al., 1977). Current evidence suggests that regulation of GEF activity is important in translational control. Phosphorylation of eIF-2(α) represents a well-known mechanism by which GEF activity may be modulated. Covalent modification of eIF-2(α) by either HCR (Farrell et al., 1977; Pal et al., 1991) or DAI (Hunter et al., 1975; Minks et al., 1979) is associated with cessation of protein synthesis and is due to the inability of GEF to catalyze the GDP/GTP exchange with eIF-2(αP)·GDP (Matts et al., 1983; Goss et al., 1984). We have previously shown that the *in vitro* phosphorylation of GEF by CK-II results in a 5-fold increase in the specific activity of GEF (Dholakia & Wahba, 1988). Recently, Oldfield and Proud (1992) isolated GEF by a modified procedure involving S-Sepharose, Q-Sepharose, and Mono Q chromatography. Phosphorylation of the factor by CK-I and CK-II was observed; however, no increase in nucleotide exchange activity was obtained (Oldfield & Proud, 1992). The discrepancy between the two sets of results may be due to the type of GEF preparation used. After Mono Q chromatography, we found that GEF is still associated with kinase activity. The presence of ATP in the control reaction allows the phosphorylation of the 82-kDa subunit, thereby reflecting no difference in activity between control and experimental values (Oldfield & Proud, 1992). Moreover, 34 nM GEF was used in the exchange assay (Oldfield & Proud, 1992). Under these conditions more than 70–80% of the labeled GDP is released from the eIF-2·[^{3}H]GDP binary complex, and a further increase in activity would be difficult to observe. We have previously demonstrated that GEF activity is linear in a reaction containing 1.3–5.3 nM GEF and 5–6 pmol eIF-2·[^{3}H]GDP (Dholakia & Wahba, 1988).

In the present study we provide definitive evidence for the phosphorylation of GEF *in vivo* and demonstrate that this phosphorylation is mediated by at least two kinases, one of which has the specificity of CK-II. First, when isolated from ^{32}P-labeled reticulocytes, the 82-kDa subunit of GEF is found to be labeled and reacts specifically with the anti-peptide antibody (Figure 1, lane 3). This is the same subunit phosphorylated *in vitro* by CK-II. Second, only Ser residues are modified in both the *in vivo* and the *in vitro* phosphorylated GEF (Figure 2). Third, phosphopeptide maps of *in vivo* and *in vitro* CK-II phosphorylated GEF are similar, with the exception of additional phosphopeptide bands in the *in vivo* phosphorylated material (Figure 3). Fourth, after digestion of the phosphorylated 82-kDa subunit with CNBr, the analysis of the derived phosphopeptide fragments reveals the presence of CK-II recognition motifs. Thus, the isolation of phosphorylated GEF from intact reticulocytes, together with immunoblot analysis, phosphoamino acid analysis, phosphopeptide mapping, and sequence analysis, establishes the covalent modification of GEF.

The minimal consensus for CK-II phosphorylation sites is recognized as S(or T)-X-X-acidic amino acid. The crucial specificity determinant in this sequence is the acidic amino acid, which can be Asp, Glu, or Ser(P) (Marin et al., 1986; Kuenzel et al., 1987; Meggio et al., 1988; Litchfield et al., 1990). Sequence analysis of phosphopeptides and a comparison with the deduced amino acid sequence of the 82-kDa subunit of GEF (Bushman et al., 1993) indicate the localization of CK-II putative phosphorylation sites throughout the protein. Analysis of our data (6.5-kDa fragment) indicates that the two putative sites at Ser residues 703 and 704 may indeed be phosphorylated. There are precedents for the phosphorylation by CK-II of adjacent Ser residues (Pinna, 1990; Luscher et al., 1990).

Other direct putative CK-II recognition sites may be identified at Ser 116, 340, 511, 574, and 596. The phosphorylation site at Ser 596 can be excluded since the 5.5-kDa CNBr peptide (595–654) is not radiolabeled. Sequence analysis of the 23-kDa phosphopeptide (fragment H) indicates the presence of two fragments of the same size. One encompasses residues 292–488, and the other, residues 520–707, which implicates Ser residues at positions 340, 574, and 596. We can exclude Ser 574 and 596 from consideration since we cannot detect radiolabeling of the 16-kDa fragment (corresponding to residues 520–654) or the 5.5-kDa gel fragment (595–654). Phosphorylation by CK-II of Ser 340, localized in the 292–488 fragment, is not conclusive, and further analysis is required to determine whether the site is targeted.

Fragment I (25-kDa phosphopeptide) represents two segments, one containing residues 292–519, and the other, residues 489–707 (Figure 4, middle panel). In addition to the sites described above for fragment H, both 25-kDa fragments share a 3.7-kDa fragment (489–519) which has a putative CK-II recognition motif at Ser 511. However, we can exclude this site from consideration since we detect no radioactivity in the lower region of our gel, indicating that the 3.7-kDa fragment is not radiolabeled. The other possible phosphorylation site to consider is at Ser 116. This site, however, can also be excluded because the 20-kDa CNBr fragment with the blocked N-terminus is not radiolabeled. Taken together, these results suggest that Ser residues 703 and 704, and possibly 340, are phosphorylated directly by CK-II *in vitro*.

Additional Ser phosphorylation sites containing CK-II recognition motifs are present in the 11-kDa amino-terminal

phosphopeptide (170–275) at positions 174 and 236. The recognition motif in these sites is represented by SXXS(P) where the C-terminal Ser must be phosphorylated to meet the requirement for an acidic determinant for CK-II recognition (Kennelly & Krebs, 1991; Roach, 1991). The sequence near Ser 174 appears to be a CK-I motif (173ESSPS*177), and the one near Ser 236 appears to be the proline-directed kinase motif (236SICS*PQ241). Since the latter enzyme does not phosphorylate GEF *in vitro* (P. Richard Vulliet, personal communication), we can localize the radioactivity found in this fragment to Ser 174. This finding suggests that the GEF used for labeling experiments *in vitro* with CK-II already contains a phosphate at residue 177. For these experiments we used GEF that had been isolated directly from rabbit reticulocytes and that had not been dephosphorylated. It is likely, then, that the CK-II recognition motif at Ser 174 represents a common motif for two enzymes involved in a hierarchical protein phosphorylation (Hemmings et al., 1982; Flotow & Roach, 1989). Indeed, CK-II has been shown to participate in multisite and hierarchical phosphorylation involving glycogen synthase 3 (Hemmings et al., 1982). The interaction between these kinases and their effect on GEF phosphorylation and nucleotide exchange may explain the regulation of GEF activity in mammalian cells.

Phosphopeptide map analysis of metabolically labeled GEF also suggests that GEF is a substrate for other kinase(s) in intact cells. This is evident by the presence of additional phosphopeptides after CNBr cleavage of the *in vivo* phosphorylated GEF. Fragments of 16, 15, 10.5, and 7.3 kDa are overlapping (Figure 4, bottom panel) and comprise residues 489–707. Since Ser residues are exclusively phosphorylated *in vivo* (Figure 2), only kinases such as CK-I, protein kinase C, and cAMP-dependent kinase that target Ser should be considered (Kennelly & Krebs, 1991; Pearson & Kemp, 1991). Because protein kinase C and cAMP-dependent kinase (Dholakia & Wahba, 1988; Oldfield & Proud, 1992) do not phosphorylate GEF *in vitro,* CK-I is left as the most likely kinase involved in the *in vivo* phosphorylation of GEF in this region. Studies are underway to examine phosphorylation sites on GEF by CK-I and other putative kinase(s).

Identification of GEF as a phosphoprotein and a substrate of CK-II has a twofold significance. First, it may well be possible to regulate GEF activity through phosphorylation of GEF itself rather than phosphorylation of eIF-2. In other systems where GDP/GTP exchange plays an important regulatory role (e.g., hormone receptor associated G-proteins), it is the exchange factor rather than the guanine nucleotide binding component whose activity is subject to control (Freissmuth et al., 1989). Recent studies indicate that phosphorylation of eIF-2(α) may not always influence the rate of protein synthesis and GEF activity. The addition of 2-aminopurine was shown to prevent host mRNA shut-off following adenovirus infection (Huang & Schneider, 1990). Under these conditions, DAI remains active and eIF-2(α) is phosphorylated to a relatively high extent. In extracts from glutamine-starved Ehrlich ascites cells, GEF activity cannot be reversed by the addition of exogenous eIF-2·GDP (Rowland et al., 1988). After starvation or under diabetic conditions, GEF activity is decreased in skeletal muscle without any detectable change in the phosphorylation of eIF-2(α) (Kimball & Jefferson, 1988). These observations may be interpreted as evidence for the regulation of GEF activity by mechanisms other than eIF-2(α) phosphorylation. Second, CK-II-dependent phosphorylation of the 82-kDa subunit of GEF *in vivo* will provide new insights into the translational control of gene expression. It is noteworthy to mention that CK-II may be involved in the phosphorylation of regulatory proteins involved in DNA metabolism, RNA synthesis, and protein synthesis (Pinna, 1990; Tuazon & Traugh, 1991). We were able to synthesize a peptide with immunogenic potential and prepare an anti-peptide antibody with high titer and specificity. This will allow us to examine the changes in the phosphorylation state of GEF under various conditions of translational repression. Moreover, the modulation of CK-II activity by stimulators (Sommercorn et al., 1987; Ackerman et al., 1990) will allow us to study the phosphorylation of GEF under different conditions.

ACKNOWLEDGMENT

We thank Hung P. Nguyen for expert technical assistance with the peptide maps and Benne Parten for microsequencing.

REFERENCES

Ackerman, P., Glover, C. V. C., & Osheroff, N. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 821–825.

Attschul, S. F., Gish, W., Miller, W., Mayers, E. W., & Lipman, D. J. (1990) *J. Mol. Biol. 215*, 403–410.

Bitte, L., & Kabat, D. (1974) *Methods Enzymol. 30*, 563–590.

Bushman, J. L., Asuru, A. I., Matts, R. L., & Hinnenbusch, A. G. (1993) *Mol. Cell. Biol. 13*, 1920–1932.

Carroll, D., Santoro, N., & Marshak, D. R. (1988) *Cold Spring Harbor Symp. Quant. Biol. 53*, 91–95.

Clemens, M. S., Pain, V. M., Wong, S., & Henshaw, E. C. (1982) *Nature 297*, 93–95.

Dholakia, J. N., & Wahba, A. J. (1987) *J. Biol. Chem. 262*, 10164–10170.

Dholakia, J. N., & Wahba, A. J. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 51–54.

Dholakia, J. N., & Wahba, A. J. (1989) *J. Biol. Chem. 264*, 546–550.

Dholakia, J. N., & Wahba, A. J. (1990) in *Post-Translational Control of Gene Expression* (McCarthy, J. E. G., & Tuite, F. M., Eds.) NATO Advanced Study Institutes Series H49, pp 539–548, Springer-Verlag, Secaucus, NJ..

Farrell, P. J., Balkow, K., Hunt, T., Jackson, R. J., & Trachsel, H. (1977) *Cell 11*, 187–200.

Filhol, O., Cochet, C., & Chambaz, E. M. (1990) *Biochemistry 29*, 9928–9936.

Filhol, O., Cochet, C., Delagoutte, T., & Chambaz, E. M. (1991) *Biochem. Biophys. Res. Commun. 180*, 945–952.

Flotow, H., & Roach, P. J. (1989) *J. Biol. Chem. 264*, 9126–9128.

Freissmuth, M., Casey, P. J., & Gilman, A. J. (1989) *FASEB J. 3*, 2125–2131.

Goss, D. J., Parkhurst, L. J., Mehta, H. B., Woodley, C. L., & Wahba, A. J. (1984) *J. Biol. Chem. 259*, 7374–7377.

Hathaway, G. M., & Traugh, J. A. (1983) *Methods Enzymol. 99*, 317–331.

Hemmings, B. A., Aitken, A., Cohen, P., Rymond, M., & Hofmann, F. (1982) *Eur. J. Biochem. 127*, 473–481.

Hershey, J. W. B. (1991) *Annu. Rev. Biochem. 60*, 717.

Huang, J., & Schneider, R. J. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 7115–7119.

Hunter, T., Hunt, T., Jackson, R. J., & Robertson, H. D. (1975) *J. Biol. Chem. 250*, 409–417.

Kennelly, P. J., & Krebs, E. G. (1991) *J. Biol. Chem. 266*, 15555–15558.

Kimball, S. R., & Jefferson, L. S. (1988) *Biochem. Biophys. Res. Commun. 156*, 706–711.

Klarlund, J. K., & Czech, M. P. (1988) *J. Biol. Chem. 263*, 15872–15875.

Kroll, D. J., & Rowe, T. C. (1991) *J. Biol. Chem. 266*, 7957–7961.

Kuenzel, E. A., Mulligan, J. A., Sommercorn, J., & Krebs, E. G. (1987) *J. Biol. Chem. 262*, 9136–9140.
Litchfield, D. W., Lozeman, F. J., Piening, C., Sommercorn, J., Takio, K., Walsh, K. A., & Krebs, E. G. (1990) *FEBS Lett. 261*, 117–120.
Luscher, B., Christenson, E., Litchfield, D. W., Krebs, E. G., & Eisenman, R. N. (1990) *Nature 344*, 517–522.
Marin, O., Meggio, F., Marchiori, F., Borin, G., & Pinna, L. A. (1986) *Eur. J. Biochem. 160*, 239–244.
Matts, R. L., Levin, D. H., & London, I. M. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 2559–563.
Meggio, F., Perish, J. W., Johns, R. B., & Pinna, L. A. (1988) *FEBS Lett. 237*, 225–228.
Mehta, H. B., Woodley, C. L., & Wahba, A. J. (1983) *J. Biol. Chem. 258*, 3438–3441.
Minks, M. A., West, D. A., Benvin, S., & Baglioni, C. (1979) *J. Biol. Chem. 254*, 10180–10183.
Oldfield, S., & Proud, C. G. (1992) *Eur. J. Biochem. 208*, 73–81.
Pain, V. M. (1986) *Biochem. J. 235*, 625–637.
Pal, J. K., Chen, J.-J., & London, I. M. (1991) *Biochemistry 30*, 2555–2562.
Panniers, R., & Henshaw, E. C. (1983) *J. Biol. Chem. 258*, 7928–7934.
Pearson, R. B., & Kemp, B. E (1991) *Methods Enzymol. 200*, 63–81.
Pearson, W. R., & Lipman, D. J. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 2444–2448.
Pfaff, M., & Anderer, F. A. (1988) *Biochim. Biophys. Acta 969*, 100–109.
Pinna, L. A. (1990) *Biochim. Biophys. Acta 1054*, 267–284.
Roach, P. J. (1991) *J. Biol. Chem. 266*, 14139–14142.
Rowland, A. G., Montine, K. S., Henshaw, E. C., & Panniers, R. (1988) *Eur. J. Biochem. 175*, 93–99.
Salimans, M., Goumans, H., Amesz, H., Benne, R., & Voorma, H. O. (1984) *Eur. J. Biochem. 145*, 91–98.
Schagger, H., & von Jagow, G. (1987) *Anal. Biochem. 166*, 368–379.
Scott, M. G., Crimmins, D. L., McCourt, D. W., Tarrand, J. J., Eyerman, M. C., & Nahm, M. H. (1988) *Biochem. Biophys. Res. Commun. 155*, 1353–1359.
Siekierka, J., Mauser, L., & Ochoa, S. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 2537–2540.
Sommercorn, J., & Krebs, E. G. (1987) *J. Biol. Chem. 262*, 3839–3843.
Sommercorn, J., Mulligan, J. A., Lozeman, F. J., & Krebs, E. G. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 8834–8838.
Traschel, H., & Staehelin, T. (1987) *Proc. Natl. Acad. Sci. U.S.A. 75*, 204–208.
Tuazon, P. T., & Traugh, J. A. (1991) *Adv. Second Messenger Phosphoprotein Res. 23*, 123–164.
Wahba, A. J., & Woodley, C. L. (1984) *Prog. Nucleic Acid Res. Mol. Biol. 31*, 221–265.
Wahba, A. J., & Dholakia, J. N. (1991) in *Methods in Nucleic Acid Research* (Karam, J. D., Chao, L., & Warr, G. W., Eds.) pp 335–357, CRC Press, Boca Raton, FL.