MOLECULAR AND CELLULAR BIOLOGY, Apr. 1993, p. 2332–2341
0270-7306/93/042332-10$02.00/0

Vol. 13, No. 4

Autoactivation of Catalytic (Cα) Subunit of Cyclic AMP-Dependent Protein Kinase by Phosphorylation of Threonine 197

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Received 26 May 1992/Returned for modification 29 July 1992/Accepted 16 January 1993

We recently found, using cultured mouse cell systems, that newly synthesized catalytic (C) subunits of cyclic AMP-dependent protein kinase undergo a posttranslational modification that reduces their electrophoretic mobilities in sodium dodecyl sulfate (SDS)-polyacrylamide gels and activates them for binding to a Sepharose-conjugated inhibitor peptide. Using an *Escherichia coli* expression system, we now show that recombinant murine Cα subunit undergoes a similar modification and that the modification results in a large increase in protein kinase activity. Threonine phosphorylation appears to be responsible for both the enzymatic activation and the electrophoretic mobility shift. The phosphothreonine-deficient form of C subunit had reduced affinities for the ATP analogs *p*-fluorosulfonyl-[^{14}C]benzoyl 5′-adenosine and adenosine 5′-*O*-(3-thiotriphosphate) as well as for the Sepharose-conjugated inhibitor peptide; it also had markedly elevated K_ms for both ATP and peptide substrates. Autophosphorylation of C-subunit preparations enriched for this phosphothreonine-deficient form reproduced the changes in enzyme activity and SDS-gel mobility that occur in intact cells. A mutant form of the recombinant C subunit with Ala substituted for Thr-197 (the only C-subunit threonine residue known to be phosphorylated in mammalian cells) was similar in SDS-polyacrylamide gel electrophoresis mobility and activity to the phosphothreonine-deficient form of wild-type C subunit. In contrast to the wild-type subunit, however, the Ala-197 mutant form could not be shifted or activated by incubation with the phosphothreonine-containing wild-type form. We conclude that posttranslational autophosphorylation of Thr-197 is a critical step in intracellular expression of active C subunit.

It has been known for some time that catalytic (C) subunit of cyclic AMP (cAMP)-dependent protein kinase purified from animal tissues is phosphorylated at two sites, Thr-197 and Ser-338 (21, 22); these sites are also apparently phosphorylated in *Escherichia coli* (results cited in reference 32). A third site (Ser-10) can be phosphorylated by C subunit in vitro (29). Dephosphorylation of native C subunit has been difficult to achieve (22), and as a result, the importance of phosphorylation to C subunit function has remained unknown. Because of its specific labeling by a peptide-based affinity reagent, Thr-197 has been localized to a region in or near the active site of C subunit (17); recent crystallographic data substantiate this localization and suggest that the phosphate on Thr-197 might contribute to stabilization of the active conformation of C subunit (11, 12). Nevertheless, although mutations at the homologous threonine (Thr-241) of yeast C subunit reduced the protein's ability to bind regulatory (R) subunit, they had only small effects on its enzymatic properties (14).

In recent studies of the expression of C subunits in cultured mouse cells, we found that both Cα and Cβ subunits underwent at least two posttranslational maturation steps. The first step enhanced solubility of the newly synthesized subunits and was defective in kinase-negative mutants of S49 mouse lymphoma cells; the second step reduced mobilities of the subunits in sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and activated the proteins for binding to an inhibitory pseudosubstrate peptide (25). Using an inducible *E. coli* expression system for murine Cα subunit, we now show that this second step can be attributed to phosphorylation on Thr-197 and that it results in a marked stimulation of enzymatic activity. The phosphothreonine-deficient, faster-migrating (fast) form of C subunit has reduced affinities for inhibitory analogs of both peptide and ATP substrates and elevated K_ms for the substrates themselves. Purified preparations of C subunit enriched for the fast form can undergo an autophosphorylation reaction that converts the protein to the more active, slower-migrating (slow) form. C subunits with Ala substituted for Thr-197 have SDS-PAGE mobility and substrate K_ms similar to those of the fast form of wild-type C subunit, but incubation of these mutant C subunits with active, wild-type C subunit results in neither a shift to slower SDS-PAGE mobility nor an increase in activity.

MATERIALS AND METHODS

Expression of Cα subunits in *E. coli*. The system for bacterial expression of Cα subunit was similar to that described by Slice and Taylor (24). The coding region of murine Cα subunit from pCαEV (30) was modified by polymerase chain reaction (PCR) to contain an *Nco*I site at the initiation codon and introduced into the T7 promoter-based expression vector pET-8c (27) to produce a full-length, nonfusion product. A Thr-197→Ala mutation was introduced into this construct from the mammalian expression plasmid MtCαT197A (9) after PCR-mediated amplifica-

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tion to introduce an *Xba*I cloning site in the 3′ flanking region. The C subunit sequences of both bacterial expression plasmids were verified in their entireties by double-stranded sequencing with a Sequenase II kit (U.S. Biochemical), using a collection of C-subunit-specific oligonucleotides distributed throughout the gene (7). Oligonucleotides for PCR and sequencing were synthesized by the Molecular Biology Resource Facility of the University of Oklahoma Health Sciences Center. The bacterial host for C-subunit expression studies was *E. coli* BL21(DE3) (27), which carries a chromosomal gene for T7 bacteriophage RNA polymerase under *lac* control. Cells were cultured in TZB medium (10 g of Bacto Tryptone [Difco], 5 g of sodium chloride, 1 g of ammonium chloride, 3 g of monobasic potassium phosphate, 6 g of dibasic sodium phosphate, 4 g of glucose, and 1 ml of 1 M magnesium sulfate in 1 liter of water) at 37°C; overnight cultures were grown without shaking from highly diluted inocula to prevent outgrowth of plasmid-cured cells. For induction of C subunits, cultures at an optical density at 550 nm (OD_{550}) of ~0.7 were cooled to 23 to 24°C and incubated with shaking in the presence of 0.4 mM isopropylthiogalactoside (IPTG).

After inductions, cells were chilled, harvested by centrifugation, washed by resuspension in ice-cold 0.9% sodium chloride, and recentrifuged; drained cell pellets were frozen and stored at −70°C. For purification of C subunits from cells induced for 50 min (T50min preparations), cells were chilled rapidly by harvesting into an equal volume of 0.9% sodium chloride that was partially frozen.

$^{32}P_i$ labeling of recombinant C subunits in *E. coli*. For labeling, bacteria were grown in TES-glucose medium (25 mM *N*-trishydroxymethyl-methyl-2-aminoethanesulfonic acid [pH 7.2], 92 mM sodium chloride, 40 mM potassium chloride, 21 mM ammonium chloride, 0.1 mM calcium chloride, 1 mM magnesium chloride, 0.01 mM ferric chloride, 0.16 mM sodium sulfate, 0.4% glucose) supplemented with 1% Bacto Tryptone (Difco) from which phosphorus had been reduced by precipitation as the magnesium ammonium phosphate (5). A mid-log-phase culture of C-subunit expression cells was diluted to an OD_{550} of 0.42 with medium containing $^{32}P_i$ (carrier-free; Dupont-NEN) to give a final concentration of 1 mCi/ml, and the culture was shaken at 23.5°C. After 1 h, a portion of the culture was induced with 0.4 mM IPTG, and incubation was continued. At various times, 17- to 36-μl samples (to give about 5.5 μg of cell protein) were taken into clear microtubes containing 0.3 ml of ice-cold 0.9% NaCl and centrifuged for 10 s at 10,000 × *g*; supernatant fractions were aspirated, and cell pellets were frozen and stored at −70°C.

Preparation of cell extracts. For analysis of C-subunit induction, frozen cell pellets were thawed, suspended in EB (10 mM Tris-HCl [pH 7.5], 2 mM dithiothreitol, 0.1 mM EDTA), and lysed by sonication. Extracts were separated into supernatant and pellet fractions by centrifugation in a microcentrifuge for 15 min at 12,000 × *g*; for the samples shown in Fig. 1B, pellet fractions were washed by resuspension in EB, recentrifuged, and suspended in EB. Radiolabeled preparations were extracted directly into SDS-gel sample buffer (25).

Assays of protein kinase activity. For most studies, protein kinase was assayed by the transfer of ^{32}P from [γ-^{32}P]ATP to the heptapeptide substrate kemptide as described previously (23) or, for the experiment shown in Fig. 8, modified by using 100 mM morpholinepropanesulfonic acid (MOPS) buffer (pH 7.0), 150 mM potassium chloride, 10.1 mM magnesium sulfate, and 100 μM each ATP and kemptide substrates. For K_m determinations (Table 1), an enzyme-linked spectrophotometric assay modified slightly from that of Roskoski (20) was used; these reactions (0.7 ml) were performed at 25°C and contained various concentrations of kemptide and ATP in a mixture of 100 mM MOPS buffer (pH 7.0), 150 mM potassium chloride, 10 mM magnesium sulfate, 0.1 mg of bovine serum albumin per ml, 1 mM phosphoenolpyruvate, 0.2 mM NADH, 15 U of lactate dehydrogenase per ml, 11 U of pyruvate kinase per ml, and about 0.4 to 1.0 μg of C subunit. (ATP was added as a 1:1 mixture with magnesium sulfate to keep a constant concentration of free magnesium.) Data were fitted to the Michaelis-Menten equation by using the nonlinear curve-fitting module of Fig.P software (BioSoft).

Purification of C subunits. Cell pellets from 1- to 3-liter cultures of induced bacteria were thawed and suspended in DE buffer (10 mM Tris-HCl [pH 7.5], 10 mM 2-mercaptoethanol, 0.1 mM EDTA) plus 100 mM sodium chloride, using 16 ml/1,000 OD_{550} units of cells. The cells were lysed by two passages through a French pressure cell at 16,000 lb/in^2, and lysates were centrifuged for 1 h at 100,000 × *g*. Supernatant fractions were dialyzed against two changes of DE buffer containing 50 mM sodium chloride and then passed through columns of either DEAE-cellulose (DE52; Whatman) or Accell Plus QMA (Millipore/Waters) equilibrated with the same buffer (using about a 1-ml bed volume per 10 mg of extract protein). The flowthrough material generally resolved into at least two peaks containing significant amounts of C subunit; these were pooled, diluted twofold with 0.2 M potassium phosphate in HA buffer (10 mM morpholineethanesulfonic acid [MES; pH 6.5], 10 mM 2-mercaptoethanol, 0.1 mM EDTA), and loaded onto columns of hydroxylapatite (Bio-Gel HTP) equilibrated with 0.1 M potassium phosphate in HA buffer. (Hydroxylapatite columns were one-fifth the bed volumes of anion-exchange columns.) Hydroxylapatite columns were washed with 10 volumes of equilibration buffer, and bound C subunits were eluted with 10-volume linear 0.1 to 0.4 M gradients of potassium phosphate in HA buffer. C subunits eluted at about 0.3 M potassium phosphate, with slow form eluting slightly sooner than fast form; C subunits from cells induced for 5 h (T5h preparations) were almost pure at this stage, while T50min C subunits were generally only about 30 to 50% pure (by SDS-PAGE analysis). For several experiments, C subunits were purified further by gel filtration on Sephacryl S-200HR (Pharmacia), using HA buffer containing 0.1 M potassium phosphate. Purified T50min or Ala-197 C subunits were concentrated by using Centriprep-10 and/or Centricon-10 units (Amicon) and stored at 4°C; purified T5h C subunits were concentrated in the same way, dialyzed against storage buffer (100 mM MES, 100 mM potassium phosphate, 2 mM dithiothreitol, 0.1 mM EDTA [pH 6.5]), and stored at −20°C. Concentrations of protein in extracts or purified preparations of C subunit were determined by the method of Lowry et al. (15), using bovine serum albumin as a standard; concentrations of C subunit in impure preparations were estimated by densitometry of silver-stained SDS-PAGE patterns (see below), using standard curves of pure (Sephacryl-purified) T5h C subunits for reference.

ATP affinity labeling of C subunits. Reaction mixtures (10 μl) contained about 350 ng of C subunit in a mixture of 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.0), 5 mM magnesium sulfate, 10 mM 2-mercaptoethanol, 0.1 mM EDTA, and *p*-fluorosulfonyl-[^{14}C]benzoyl 5′-adenosine ([^{14}C]FSBA; Dupont-NEN) to concentrations indicated in the legend to Fig. 3 (added in 1.5 μl of

dimethyl sulfoxide). [In control experiments (not shown), magnesium was omitted from the reaction, kemptide was included at a concentration of 100 μM, and/or competing ATP or adenosine 5′-*O*-(3-thiotriphosphate) (ATP-γ-S) was included in the reactions. Labeling was independent of magnesium or kemptide, but inhibition with ATP or ATP-γ-S required magnesium.] After incubation for 2 h at 30°C, reactions were stopped by addition of 10 μl of twice-concentrated SDS-gel sample buffer. Incorporation into slow and fast forms of C subunit was determined by SDS-PAGE, fluorography, and densitometry.

Affinity binding of C subunits by a peptide inhibitor. For the experiment shown in Fig. 4, columns (40 μl) of protein kinase inhibitor peptide (PKIP; Thr-Thr-Tyr-Ala-Asp-Phe-Ile-Ala-Ser-Gly-Arg-Thr-Gly-Arg-Arg-Asn-Ala-Ile-His-Asp)-Sepharose or control-Sepharose were prepared in 1-ml syringe barrels and blocked with nonfat dry milk, using resins and methods described previously (25). Columns were washed five times (at 4°C) with 200 μl of PKI buffer (10 mM MES [pH 6.6], 0.5 mM dithiothreitol, 2 mM magnesium sulfate, 0.25 M sodium chloride, 0.1 mM EDTA, 50 μM ATP, 0.25 mg of bovine serum albumin per ml, 0.25% Nonidet P-40), liquid was removed from below the column beds, and 15-μl samples containing about 22 μg of T50min C subunit (in PKI buffer without bovine serum albumin or sodium chloride) were loaded. The samples were overlaid with ~10 μl of PKI buffer and allowed to bind for 4 h; the columns were then washed five times with 400 μl of PKI buffer (collecting the first 2 drops as unbound fractions) and four times with 200 μl of PKI buffer without bovine serum albumin or sodium chloride before elution with 120 μl of 200 mM L-arginine (pH 6.6) in 10 mM MES–0.2 mM dithiothreitol–0.1 mM EDTA. Unbound fractions were diluted with concentrated SDS-gel sample buffer (for gel analysis) or with HA buffer containing 1 mg of bovine serum albumin per ml (for protein kinase assays). The bound fractions were precipitated with 5% trichloroacetic acid, and the precipitates were washed twice with 95% ethanol before being dissolved in SDS-gel sample buffer for gel analysis. To purify wild-type fast-form C subunits for K_m determinations (Table 1), two hydroxylapatite subfractions of T50min C subunits (differing in amount of slow form) were adsorbed to PKIP-Sepharose as described above but using 200-μl columns in 3-ml syringe barrels and 80-μl samples containing 150 to 250 μg of C subunits; the first 0.4 ml of unbound material was collected, dialyzed against two changes of buffer containing 100 mM MOPS (pH 7.0), 150 mM potassium chloride, 10 mM 2-mercaptoethanol, and 0.1 mM EDTA, and stored at 4°C (see Fig. 7, lanes b to e, for gel patterns of these C subunits before and after adsorption).

Autoactivation and autophosphorylation of fast-form C subunit. For the experiments shown in Fig. 5 and 6, C subunits in purified T50min preparations were diluted to about 100 μg/ml in 10 mM HEPES (pH 7.0)–6 mM magnesium sulfate–10 mM 2-mercaptoethanol–1 mg of bovine serum albumin per ml–1 mM ATP and incubated for various times at 30°C. For the experiments shown in Fig. 8 and 9, T50min or Ala-197 C subunits were mixed with purified T5h C subunits as described in the figure legends, and incubations were for 5 or 6 h at 30°C; for the experiment shown Fig. 8 (only), ATP was increased to 2.5 mM and magnesium sulfate was increased to 7.5 mM. Samples were diluted with either SDS-gel sample buffer (for gel analysis) or ice-cold buffer containing 1 mg of bovine serum albumin per ml (for protein kinase assays). For control reactions, ATP was omitted (Fig. 5 and 8) or substituted with ATP-γ-S (data not shown). To monitor autophosphorylation, the ATP in the autoactivation reaction was spiked with [γ-^{32}P]ATP (Dupont-NEN) to give a specific radioactivity of 50 or 200 μCi/μmol.

SDS-PAGE, silver staining, autoradiography, and fluorography. Protein separations were on SDS-polyacrylamide gels (0.8-mm thick) containing 10% polyacrylamide as described previously (25); good resolution of slow and fast forms of C subunit required that the pH of the lower gel buffer be 8.8 to 8.9. Silver staining was done by the procedure of Merril et al. (16); fluorography was done by the method of Bonner and Laskey (1); autoradiography was either unenhanced or enhanced with Lightning-Plus intensifying screens (Dupont).

Silver-stained gels and autoradiographs were scanned with a Molecular Dynamics model 300A computing densitometer. Patterns were expanded and normalized with the aid of Fig.P software (BioSoft), and integrated ODs in C-subunit forms were estimated by weighing excised peaks from printed patterns.

Phosphoamino acid analysis. ^{32}P-labeled protein bands were excised from dried SDS-PAGE gels and transferred to 1.5-ml screw-cap microcentrifuge tubes (Sarstadt); 0.2 ml of 2 N hydrochloric acid was added to each tube, tubes were purged with nitrogen gas, and samples were hydrolyzed for 4.5 to 5 h at 110°C. Hydrolysates were transferred to fresh tubes, dried under vacuum, and analyzed by electrophoresis on cellulose thin-layer plates at pH ~1.9 as described previously (26).

RESULTS

Posttranslational modification and concomitant activation of recombinant C subunit. Figure 1 shows a time course for induction of recombinant C subunit protein and activity in *E. coli* at ~23.5°C. Figures 1A and B show silver-stained SDS-PAGE patterns from cells induced for up to 6 h with IPTG (lanes 0 to 6). In the soluble protein fraction (Fig. 1A), an induced C-subunit band (about 39 kDa) was prominent by 1 h of induction; a slower-migrating band was visible by 2 h and became the predominant induced species at later times. A small proportion of the induced C subunit was insoluble in cell extracts (Fig. 1B), and the majority of this insoluble material remained in the fast-migrating form. (Note that four times more cell equivalents of insoluble than soluble material was loaded per gel lane.) Induction of soluble protein kinase activity (Fig. 1C) paralleled the increase in the slow form of C subunit, reaching a maximum at about 3 h of induction. Negligible amounts of activity were associated with the insoluble fraction and might reflect a small amount of contamination with soluble C subunit. A portion of the culture was treated with chloramphenicol to inhibit protein synthesis after 2 h of induction. Both the shift of C-subunit protein from fast to slow form and the increase in protein kinase activity proceeded normally in the absence of further protein synthesis. The inhibitor also allowed depletion of C-subunit protein from the insoluble pool, suggesting either that the insoluble material accumulated up to 2 h of induction was degraded or that it had not been denatured irreversibly.

Since the only posttranslational modification of C subunit known to occur in *E. coli* is phosphorylation, we investigated whether phosphorylation could account for the posttranslational shift in C subunit from fast to slow form. Figure 2A shows autoradiographic patterns from an SDS-PAGE gel of $^{32}P_i$-labeled bacteria induced for various times with IPTG. Labeling of C subunit could be detected after 1 h of induction and increased over the next several hours; the labeling was

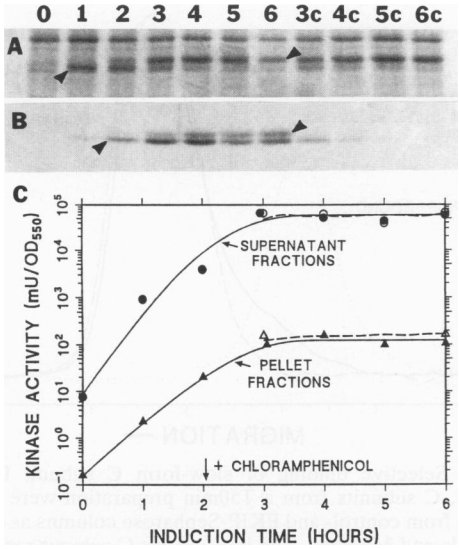

FIG. 1. Expression and posttranslational activation of murine Cα subunit in *E. coli*. A culture of Cα subunit-expressing bacteria was induced with IPTG, and after 2 h, a portion of the culture was treated with 100 μg of chloramphenicol per ml to inhibit further protein synthesis; samples were harvested at hourly intervals for extraction, fractionation, and analysis (Materials and Methods). (A) Silver-stained SDS-PAGE patterns from supernatant fractions of extracts; (B) silver-stained SDS-PAGE patterns from pellet fractions of extracts; (C) results from protein kinase assays. Samples for panel A contained about 0.7 μg of supernatant protein corresponding to ~0.002 OD_{550} unit of bacteria; those for panel B contained particulate material from ~0.008 OD_{550} unit of bacteria. For panels A and B, numbers indicate hours of induction, c's designate samples from the chloramphenicol-treated culture, upward-pointing arrowheads indicate the position of the fast-form C subunit, and downward-pointing arrowheads indicate the position of the slow-form C subunit. Symbols in panel C are as follows: circles, supernatant fractions; triangles, pellet fractions; and open symbols, samples from the chloramphenicol-treated culture.

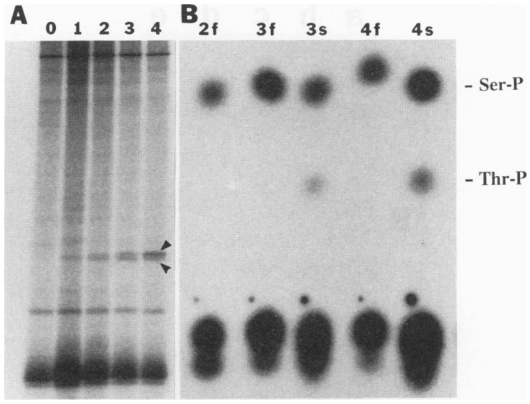

FIG. 2. Phosphorylation of C subunit on serine and threonine in *E. coli*. C-subunit-expressing bacteria were preincubated for 1 h with $^{32}P_i$ and then induced with IPTG as described in Materials and Methods. (A) SDS-PAGE patterns of radiolabeled proteins from cells harvested at 0, 1, 2, 3, or 4 h of induction (0 sample was from cells incubated for the entire labeling period without IPTG). Samples contained about 0.5 μg of cell protein and 1.6×10^6 cpm of incorporated phosphate; patterns shown are from an 18-h autoradiographic exposure. Arrowheads indicate positions of fast and slow forms of C subunit as for Fig. 1. (B) Bands corresponding to fast (f) or slow (s) form of C subunit were excised from a gel run in parallel with that of panel A; the excised bands were hydrolyzed with hydrochloric acid, and the resulting phosphoamino acids were resolved by thin-layer electrophoresis (Materials and Methods). Samples for panel B contained four times as much radiolabeled material as did those for panel A; patterns shown are from an autoradiogram of the thin-layer plate exposed to X-ray film for 15 days with an intensifying screen. Ser-P and Thr-P indicate the positions of phosphoserine and phosphothreonine standards in the electrophoresis pattern.

initially of fast-form C subunit but shifted progressively to slow form. Phosphoamino acid analysis of gel-purified, radiolabeled C subunits revealed phosphoserine label in both slow and fast forms but phosphothreonine label only in the slow form (Fig. 2B). It appeared, therefore, that both the electrophoretic shift and the increase in catalytic activity might be attributable to threonine phosphorylation.

Slow-form C subunit binds preferentially to analogs of ATP and peptide substrates. To better analyze the properties of slow and fast forms of the protein, C subunits were purified from bacteria induced for either long or short times. T5h preparations were greater than 90% pure and contained only slow-form C subunit; T50min preparations were somewhat less pure but contained predominantly fast-form C subunit. (See Fig. 7 for SDS-PAGE patterns of C subunits from representative preparations.)

Figure 3 shows labeling of recombinant C subunits with the ATP affinity reagent [^{14}C]FSBA at various concentrations. The slow-form C subunit from a T5h preparation gave a single radiolabeled band after reaction with the affinity reagent; labeling was half-maximal at less than 62.5 μM FSBA and maximal at 125 μM or above (Fig. 3A). Since modification with FSBA reduces the mobility of C subunits in SDS-PAGE (data not shown), the appearance of a single labeled adduct with the T5h C subunit suggested that labeling was at a single site. In control experiments, labeling of slow and fast forms of C subunit was inhibited by both ATP and its hydrolysis-resistant congener ATP-γ-S (data not shown). In a subfraction of T50min C subunits that was highly enriched for fast form, affinity labeling was mostly of this species; labeling of fast-form C subunit was half-maximal at about 125 μM [^{14}C]FSBA and maximal at 250 μM or above (Fig. 3B). Figure 3C shows labeling of a more typical T50min preparation. Although only about 13% of the C subunit in this preparation was in slow form (by densitometry of silver-stained gels; data not shown), more than 30% of incorporated label was in the slow-form adduct at 500 μM [^{14}C]FSBA; labeling of slow form increased to about 67% of the total as the concentration of [^{14}C]FSBA was reduced stepwise to 31.3 μM. In complementary experiments testing the ability of ATP-γ-S to inhibit labeling with 500 μM [^{14}C]FSBA, slow-form C subunit from a T5h preparation was about 10 times more sensitive than fast-form C subunit from a T50min preparation (4).

Figure 4 shows the selective binding of slow-form C subunit by an affinity resin containing a pseudosubstrate peptide inhibitor of C subunit (PKIP). Samples of the T50min C-subunit preparation used for the experiment shown in Fig. 3C (Fig. 4, inset, lane a) were loaded onto columns of either PKIP- or control-Sepharose and incubated for 4 h in the cold before washing (see Materials and Methods). The unbound material was collected (Fig. 4, inset, lanes b and c), as was material that could be eluted with arginine-containing buffer after extensive washing (Fig. 4, inset, lanes d and e). No C subunit could be detected in the

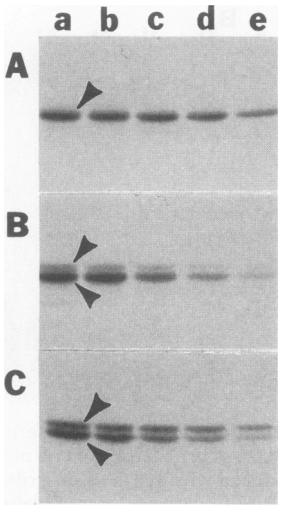

FIG. 3. Affinity labeling of slow- and fast-form C subunit with the ATP analog [^{14}C]FSBA. Purified preparations of recombinant C subunit were incubated with [^{14}C]FSBA at concentrations of 500 (lane a), 250 (lane b), 125 (lane c), 62.5 (lane d), or 31.3 nM (lane e) as described in Materials and Methods. Patterns shown are from a 5-day fluorographic exposure; arrowheads indicate the positions of adducts from slow- and fast-form C subunits. C subunits for panel A were from a T5h preparation and were mostly in slow form, those for panel B were from a hydroxylapatite column fraction of T50min C subunits that was about 96% enriched for fast form, and those for panel C were from a T50min preparation containing about 13% slow form.

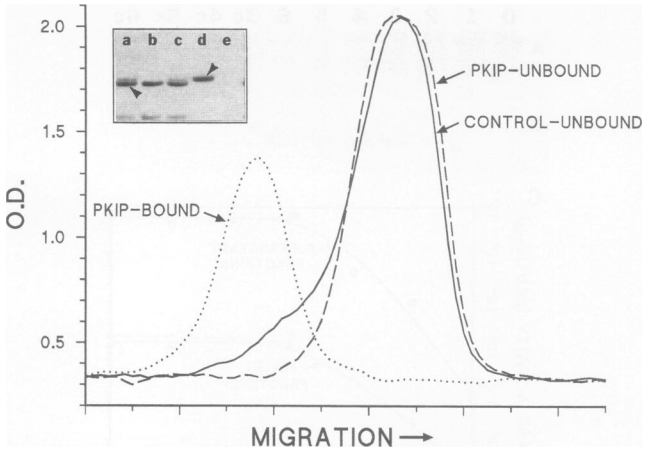

FIG. 4. Selective binding of slow-form C subunit by PKIP-Sepharose. C subunits from a T50min preparation were bound to and eluted from control- and PKIP-Sepharose columns as described in Materials and Methods. The inset shows C-subunit regions from silver-stained SDS-PAGE patterns of the material loaded onto the columns (lane a), the unbound, flowthrough fractions from PKIP-Sepharose (lane b) and control-Sepharose (lane c) columns, and the concentrated, bound fractions from PKIP-Sepharose (lane d) and control-Sepharose (lane e) columns. About 150 ng of C-subunit protein was run for lanes a to d, and an equivalent portion of the sample was run for lane e. Arrowheads indicate positions of slow and fast forms of C subunit. The main figure shows densitometer tracings from silver-stained SDS-PAGE patterns of unbound fractions from the two columns and the bound fraction from the PKIP-Sepharose column. Tracings for the unbound fractions were from lanes loaded with about 300 ng of C-subunit protein to visualize more clearly the small amount of slow-form C subunit in the preparation.

control column eluate (Fig. 4, inset, lane e). Assays of the unbound fractions from control and affinity columns suggested that more than 90% of protein kinase activity was removed specifically by the PKIP-containing resin (data not shown). Nevertheless, as shown by silver-stained SDS-PAGE patterns of the fractions (Fig. 4, inset and densitometer tracings), unbound fractions from both columns contained about equivalent amounts of the fast-form C subunit that was predominant in the T50min C-subunit preparation. Only the small shoulder of slower-migrating material was removed specifically by the PKIP-containing resin. When eluted from the column with arginine and concentrated, the PKIP-bound material (Fig. 4, inset, lane d) was indistinguishable on SDS-PAGE from T5h slow-form C subunit (data not shown).

Fast-form C subunit undergoes phosphorylation-dependent autoactivation *in vitro*. Figure 5 shows results from an experiment in which a T50min C-subunit preparation was incubated for various times with or without ATP. Silver-stained SDS-PAGE patterns revealed an ATP- and time-dependent conversion of C subunit from fast to slow form (Fig. 5A). Densitometry suggested that the slow form of C subunit was increased by about seven- to ninefold after 6 h of incubation; the half-time for the conversion was about 1.6 h (Fig. 5B). Concomitant with the increase in slow-form C subunit was an increase in protein kinase activity; this increase reached a maximum of about fivefold at 6 h (Fig. 5C). The concentration of ATP required for half-maximal stimulation of the fast-to-slow conversion was about 0.5 mM, and the rate of conversion appeared to depend on the initial concentration of slow-form C subunit in the preparation (4).

By using [γ-^{32}P]ATP in an incubation similar to that for Fig. 5, we found that the mobility shift and activation were accompanied by (and presumably resulted from) incorporation of phosphate into the protein (Fig. 6). Although most of the incorporated phosphate was in the position of slow-form C subunit, there was also label in the position of fast-form C subunit (Fig. 6, inset); this finding suggested that serine as well as threonine residues were phosphorylated in these incubations. The presence of both phosphothreonine and phosphoserine in autophosphorylated C subunit was confirmed by thin-layer electrophoresis of acid hydrolysates of the in vitro-phosphorylated material (see Fig. 9).

Phosphorylation at Thr-197 is responsible for both the SDS-PAGE mobility shift and enzymatic activation of recombinant C subunit. In view of evidence that Thr-197 is the major site for threonine phosphorylation of C subunit, it seemed likely that phosphorylation at this residue was responsible for the changes in SDS-PAGE mobility and activity described above. To test this conjecture, we modified our recombinant plasmid to express a mutant C subunit with alanine substituted for Thr-197. In contrast to the wild-type protein, the Ala-197 subunit gave a single band in SDS-PAGE at all times of induction (data not shown). Figure 7 compares the SDS-PAGE mobility of purified mutant protein (from bacteria induced for 4 h) with mobilities of wild-type C subunits; migration of the Ala-197 subunit (Fig. 7, lane f) was identical to that of the phosphothreonine-deficient, fast form of wild-type C subunit. These results suggested that phosphorylation of Thr-197 was responsible for the electrophoretic shift observed in expression of wild-type C subunit, but it remained possible that the Ala-197

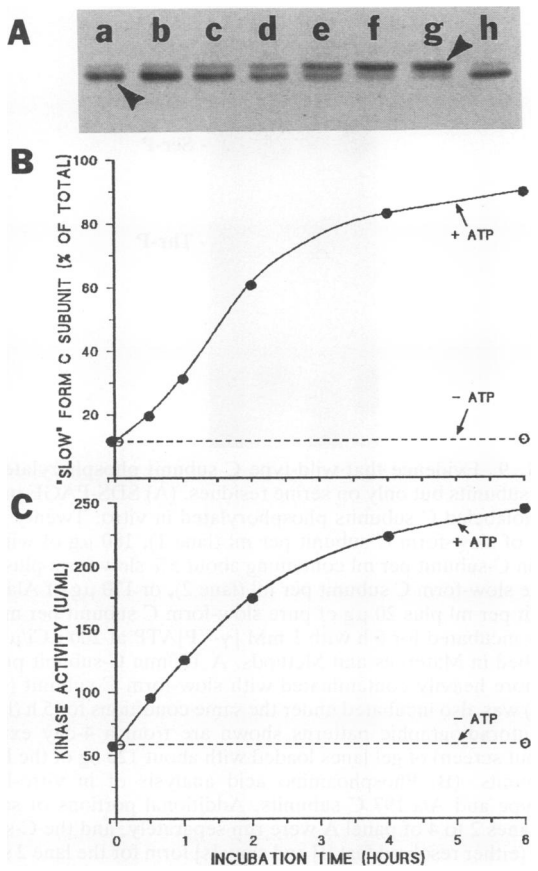

FIG. 5. Evidence that autoactivation of fast-form C subunit accompanies its ATP-dependent conversion to slow form. A preparation of T50min C subunit was incubated for various times in the presence or absence of ATP, and samples were analyzed by SDS-PAGE or protein kinase assays as described in Materials and Methods. (A) C-subunit regions of silver-stained SDS-PAGE patterns from samples incubated without ATP for 0 (lane a) or 6 (lane h) h or with 1 mM ATP for 0 (lane b), 0.5 (lane c), 1 (lane d), 2 (lane e), 4 (lane f), or 6 (lane g) h. About 100 ng of C-subunit protein was loaded per lane; arrowheads indicate positions of slow and fast forms of C subunit. (B) The patterns of panel A were quantified by densitometry (Materials and Methods) to plot the time course of C-subunit conversion from fast to slow form. (C) Results of protein kinase assays on the incubated samples (in nanomoles of phosphate incorporated per minute per milliliter). (Activities in these samples were underestimated because of partial degradation of the tracer [^{32}P]ATP used in the assay [unpublished results]. Nevertheless, the magnitude of ATP-dependent activation in this and replicate experiments was consistent with a final enzyme activity equivalent to that of pure C subunit in T5h preparations [Fig. 8 and data not shown].)

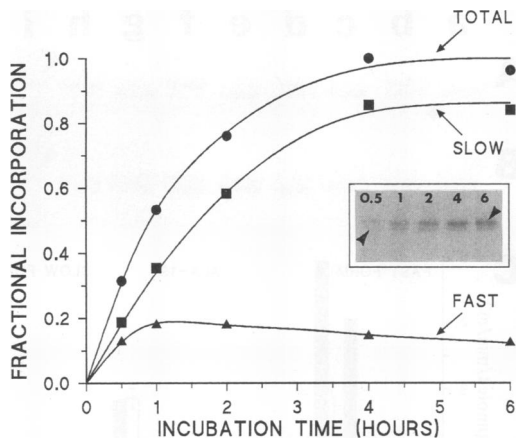

FIG. 6. Autophosphorylation of fast-form C subunit. A T50min preparation of C subunit was incubated as for Fig. 5 but with the inclusion of [γ-^{32}P]ATP at 50 μCi/μmol. The inset shows SDS-PAGE patterns of label incorporated into ~100 ng of C-subunit protein from a 5-day autoradiographic exposure; arrowheads indicate positions of fast and slow forms of C subunit. The plots show phosphate incorporation into slow and fast forms of C subunit (and their sums) determined by integrating densitometer scans of these patterns (and averaging them with scans from duplicate samples); values were normalized to the average of totals for 4- and 6-h samples).

substitution inactivated the enzyme (see also below) and thereby prevented autophosphorylation at some other site. To examine this possibility, we incubated the mutant subunit with wild-type slow-form C subunit under autophosphorylation conditions.

Figure 8 shows data from an experiment in which fixed concentrations of Ala-197 or wild-type fast-form C subunits were incubated with increasing concentrations of wild-type slow-form C subunits to give matched samples containing up to about 40% slow-form C subunits; control samples were either incubated without ATP or taken before incubation. The wild-type fast-form preparation, which contained about 5% slow-form C subunit, was only partly converted to slow form after 5 h of incubation; the addition of 60 μg or more of pure slow-form C subunit per ml stimulated the process to near completion (Fig. 8A). On the other hand, slow-form C subunit concentrations of up to 106 μg/ml were unable to promote detectable conversion of the Ala-197 protein to a slower-migrating form (Fig. 8B). (The slight shift of the Ala-197 subunits to faster mobility after incubation with active C subunit was reproducible and might reflect phosphorylation at one or more serine residues [see below].) Consistent with the mobility shift data, kinase assays (at 100 μM each ATP and kemptide substrates) revealed ATP-dependent activation of the wild-type fast-form C subunits to a maximum of about 2.5 μmol/min/ml over control values but no detectable activation of the Ala-197 C subunits; activity of pure slow-form C subunit was unaffected by incubation (Fig. 8C). (Assays with 5 mM ATP and kemptide confirmed that both Ala-197 and wild-type fast-form prepa-

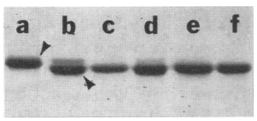

FIG. 7. Relative SDS-PAGE mobilities of wild-type and Ala-197 C subunits. Wild-type C subunits were purified from bacteria induced for 5 h (lane a) or 50 min (lanes b and d) as described in Materials and Methods. (The T5h purification included a Sephacryl S-200HR step; the T50min C subunits eluted from hydroxylapatite were divided into early-eluting [lane b] or late-eluting [lane d] pools.) Lanes c and e show fast-form C subunits purified from the two T50min C-subunit pools by adsorption on PKIP-Sepharose. Ala-197 subunits from bacteria induced for 4 h (lane f) were purified by chromatography on Accell Plus QMA and hydroxylapatite as described in Materials and Methods. About 200 ng of C subunit was loaded per lane for these silver-stained patterns; arrowheads show positions of slow and fast forms of wild-type C subunit.

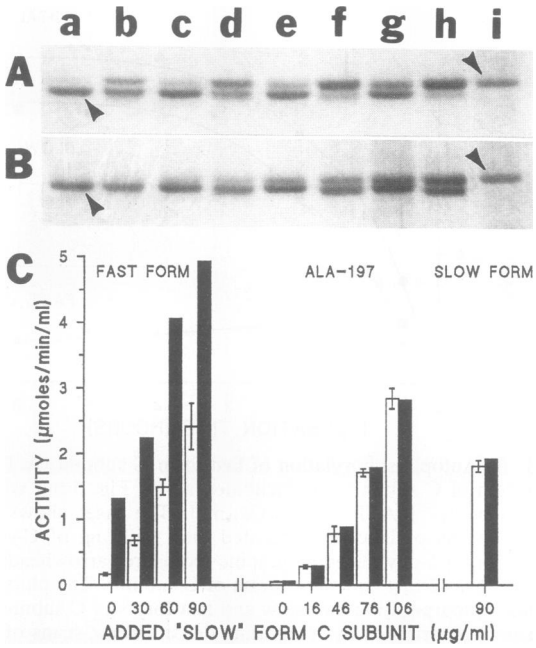

FIG. 8. Evidence that Ala-197 C subunits are neither converted to slow form nor activated by incubation with active wild-type C subunit. Ala-197 C subunits at ~170 μg/ml were incubated alone or with pure wild-type slow-form C subunit (Fig. 7, lane a) for 5 h in the absence or presence of 2.5 mM ATP as described in Materials and Methods. For comparison, ~180 μg of wild-type T50min C subunits (containing 94 to 96% fast form [Fig. 7, lane d]) per ml was incubated with slightly less added slow-form C subunit. Before and after incubation, samples were diluted into appropriate buffers (Materials and Methods) for gel analysis (A and B) or protein kinase assays (C). (A) SDS-PAGE patterns of wild-type fast-form C subunits incubated with various amounts of slow-form C subunit. Patterns are from samples taken before (lanes a, c, e, and g) or after (lanes b, d, f, and h) 5 h of incubation with ATP; reaction mixtures included 0 (lanes a and b), 30 (lanes c and d), 60 (lanes e and f), or 90 (lanes g and h) μg of added slow-form C subunit per ml. Lane i is from a reaction mixture containing 90 μg of slow-form C subunit alone per ml. Samples were diluted identically to give about 130 ng of fast-form C subunit in the nonincubated samples; patterns shown are from silver-stained gels with arrowheads indicating positions of fast- and slow-form C subunits as for previous figures. (B) SDS-PAGE patterns of Ala-197 C subunits incubated with various amounts of wild-type slow-form C subunits. Patterns are from samples taken before or after incubation (or containing slow-form C subunit alone [lane i]) as for panel A, but samples included 16 (lanes a and b), 46 (lanes c and d), 76 (lanes e and f), or 106 (lanes g and h) μg of added slow-form C subunit per ml. Ala-197 C subunits alone were unchanged by incubation (data not shown). (C) Effect of slow-form C subunit on the activity of wild-type fast-form and Ala-197 C subunits. Samples described above were diluted in protein-containing buffer to give activities of between 100 and 600 nmol/min/ml and then diluted threefold further into 30-μl reaction mixtures containing 100 μM kemptide, 100 μM [γ-^{32}P]ATP at ~150 μCi/μmol, and other components as described in Materials and Methods; reactions were for 30 min at 30°C. Open bars show average protein kinase activity values (with standard deviations) for controls (incubated without ATP or taken before incubation with and without ATP); filled bars show values for samples incubated with ATP.

rations had comparable amounts of high-K_m kinase activity [data not shown, but see below].)

Despite its failure to either reduce the SDS-PAGE mobility or increase the activity of Ala-197 mutant C subunits, wild-type slow-form C subunit could phosphorylate the

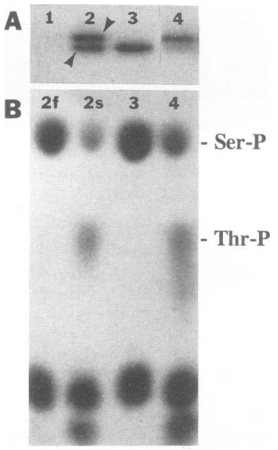

FIG. 9. Evidence that wild-type C subunit phosphorylates Ala-197 C subunits but only on serine residues. (A) SDS-PAGE patterns of radiolabeled C subunits phosphorylated in vitro. Twenty micrograms of slow-form C subunit per ml (lane 1), 180 μg of wild-type T50min C-subunit per ml containing about 5% slow form plus 10 μg of pure slow-form C subunit per ml (lane 2), or 170 μg of Ala-197 C subunit per ml plus 20 μg of pure slow-form C subunit per ml (lane 3) was incubated for 6 h with 1 mM [γ-^{32}P]ATP at 200 μCi/μmol as described in Materials and Methods. A T50min C-subunit preparation more heavily contaminated with slow-form C subunit (Fig. 7, lane b) was also incubated under the same conditions for 5 h (lane 4). The autoradiographic patterns shown are from a 4-day exposure (without screen) of gel lanes loaded with about 120 ng of the labeled C subunits. (B) Phosphoamino acid analysis of in vitro-labeled wild-type and Ala-197 C subunits. Additional portions of samples from lanes 2 to 4 of panel A were run separately, and the C-subunit bands (either resolved fast [f] and slow [s] form for the lane 2 sample or the entire C-subunit region for the lane 3 and 4 samples) were excised, hydrolyzed, and analyzed by thin-layer electrophoresis as described in Materials and Methods. The SDS-PAGE samples for this analysis had five times as much labeled material as did those for panel A, and the pattern shown is from a 6-day autoradiographic exposure (with intensifying screen) of the thin-layer electrophoresis plate; Ser-P and Thr-P indicate positions of unlabeled markers (phosphoserine and phosphothreonine) as for Fig. 2B.

mutant protein (Fig. 9). Mixtures of Ala-197 or wild-type fast-form C subunits with small amounts of endogenous and/or added wild-type slow-form C subunits were incubated with [γ-^{32}P]ATP as for Fig. 6. The labeled Ala-197 protein gave a single band in the position of fast-form C subunit (Fig. 9A, lane 3), while the labeled T50min wild-type C subunits gave bands in positions of both slow and fast forms (Fig. 9A, lanes 2 and 4). (The sample for Fig. 9A, lane 2, was the highly enriched fast-form C subunit used for Fig. 8 [and also Fig. 7, lane d], with 10 μg of added pure slow-form C subunit per ml; the sample for lane 4 was from a subfraction of the same T50min C-subunit preparation more heavily contaminated with slow-form C subunit [Fig. 7, lane b].) Slow-form C subunit alone gave negligible labeling (Fig. 9A, lane 1). Phosphoamino acid analysis (Fig. 9B) of the in vitro-labeled subunits gave results similar to those for C subunits labeled with $^{32}P_i$ in bacteria (Fig. 2): the slower-migrating species from autophosphorylated wild-type preparations contained both phosphothreonine and phosphoserine, but the faster-migrating subunits from wild-type and mutant preparations contained only phosphoserine.

Activation of C subunit by phosphorylation at Thr-197 is manifested through decreases in K_ms for both ATP and peptide substrates. Table 1 summarizes K_m data from assays of the

TABLE 1. Kinetic parameters of slow and fast forms of wild-type and of mutant C subunits

Recombinant C subunit	K_m (mM) for:	
	Kemptide[a]	ATP[b]
Wild type		
Slow form[c]	0.029	0.022
Fast form A[d]	0.94	1.14
Fast form B[d]	0.85	0.92
Ala-197[e]	1.37	1.42

[a] For Kemptide K_m determinations, ATP concentrations of 100 μM for slow-form C subunit and 5.0 mM for mutant and fast-form C subunits were used.

[b] For ATP K_ms, kemptide concentrations were fixed at about five times K_m (150 μM for slow form, 5.2 mM for fast form A, 4.1 mM for fast form B, and 7.1 mM for Ala-197).

[c] Homogeneous slow-form C subunit from a T5h preparation purified through the gel filtration step described in Materials and Methods (see Fig. 7, lane a).

[d] Hydroxylapatite-purified C subunits from a T50min preparation were divided into early (A)- and late (B)-eluting fractions (Fig. 7, lanes b and d), which were then freed of contaminating slow-form C subunit by adsorption with PKIP-Sepharose (Fig. 7, lanes c and e).

[e] Mutant C subunits were purified through the hydroxylapatite step from bacteria induced for 4 h (Fig. 7, lane f).

substrate dependence of purified slow and fast forms of wild-type C subunit and the purified Ala-197 mutant C subunit. Fast-form C subunits were purified by PKIP-Sepharose affinity adsorption from early- and late-eluting pools of hydroxylapatite-purified T50min C subunit; the adsorption appeared to have removed all slow-form contamination (Fig. 7, lanes c and e). K_m values for kemptide were determined by using ATP concentrations of 100 μM for the slow-form enzyme and 5 mM for the other preparations; K_m values for ATP were determined by using kemptide concentrations about five times these measured kemptide K_ms. K_m values for the slow-form enzyme were around 25 μM for both substrates, and the apparent V_{max} was about 44 μmol/min/mg of C subunit. K_ms for the fast-form C subunits (both wild-type and mutant) were about 30- to 65-fold higher than those for the slow-form enzyme, with values for the Ala-197 mutant enzyme slightly higher than those for the wild type. Although impurities in the fast-form preparations precluded precise protein determinations, estimates based on stained SDS-PAGE patterns (see Materials and Methods) suggested that both mutant and wild-type fast-form enzymes had V_{max} values comparable to that of the slow-form enzyme.

DISCUSSION

In a previous study using metabolic labeling of C subunits in cultured mammalian cells, we showed that newly synthesized C subunits undergo a posttranslational modification that reduces their mobility in SDS-PAGE and increases their ability to bind to PKIP-Sepharose columns (25). The present results confirm and extend these observations, using C subunits expressed in *E. coli* under *lac* repressor (and T7 bacteriophage RNA polymerase) control. Since endogenous protein kinase activity in the uninduced *E. coli* system is negligible, it was possible to show that the posttranslational modification correlated with a large increase in enzymatic activity (Fig. 1). Although both unmodified (fast-form) and modified (slow-form) C subunit were phosphorylated, only the slow form contained phosphothreonine (Fig. 2); this finding suggested that threonine phosphorylation was responsible for both the mobility shift and the increase in activity. Thr-197 was the likely site for this phosphorylation-dependent activation, since it is the only threonine residue known to be phosphorylated both in mammalian C subunits (21) and in bacterially expressed recombinant C subunits (12, 32). Mutational substitution of alanine for this threonine yielded a C subunit with SDS-PAGE mobility and activity nearly identical to those of the phosphothreonine-deficient, fast form of wild-type C subunit (Fig. 7; Table 1). These results strongly suggested that phosphorylation of Thr-197 underlies posttranslational activation of wild-type C subunit.

In a recent review article, Taylor et al. (28), citing unpublished studies with mutant C subunits defective in ATP binding (Lys-72→His or Arg), proposed that phosphorylation of the recombinant protein on both Ser and Thr residues was catalyzed by the activity of C subunit itself. This would account for the persistence of the less active fast-form C subunit during the early phase of induction in *E. coli* (Fig. 1); at later times, when kinase activity levels had increased, conversion of newly synthesized (radiolabeled) C subunit to slow form proceeded more rapidly (4). Consistent with these results, preparations of purified C subunit enriched for fast form could be autophosphorylated by incubation with ATP (Fig. 6 and 9); concomitant with this phosphorylation were a shift to the mobility of slow-form C subunit and an increase in kinase activity to levels approaching those of pure slow-form C subunit (Fig. 5 and 8). The Ala-197 C subunit could be phosphorylated by incubation with slow-form wild-type C subunit, but only on serine residues (Fig. 9); this finding confirmed that Thr-197 is the only detectable site for threonine autophosphorylation in the recombinant protein. Serine phosphorylation of the Ala-197 protein resulted in neither a shift to slower SDS-PAGE mobility nor a change in activity (Fig. 8). Studies on the mechanism of autophosphorylation are under way. Preliminary results suggest that the observed reaction was an intermolecular reaction catalyzed by the slow form of C subunit in the preparations: the rate of fast-to-slow form conversion depended on the relative amount of slow form C subunit in the preparation, and the rate of conversion was reduced markedly by dilution (4).

The ATP-mimetic covalent affinity reagent [^{14}C]FSBA labels specifically a single residue (Lys-72) near the ATP-binding site of porcine C subunit (11, 33, 34). The appearance of a single labeled adduct in SDS-PAGE patterns of FSBA-modified slow-form C subunit (Fig. 3A) and inhibition of labeling by both ATP and ATP-γ-S confirmed that labeling was also specific to a single site in the recombinant C-subunit preparations used here. Both fast and slow forms of C subunit were labeled by the affinity reagent, but slow form had a higher apparent affinity for the compound (Fig. 3); slow-form C subunit also appeared to have a higher affinity for the thiophosphate analog ATP-γ-S (4). (The ability of ATP to promote autophosphorylation compromised interpretation of its relative effects on affinity labeling of fast and slow forms by FSBA.) The slow and fast forms of C subunit differed more dramatically in their apparent affinities for a pseudosubstrate peptide inhibitor. In the experiment shown in Fig. 4, there was essentially quantitative binding of slow-form C subunit to PKIP-Sepharose, with no detectable binding of the fast form species. In retrospect, we suspect that this difference in binding of the two forms to PKIP-Sepharose was magnified by positive interactions between ATP- and peptide-binding sites (31); the ATP concentration used was probably near saturating for the slow-form enzyme but subsaturating for the fast-form enzyme.

Enzyme kinetic studies revealed that the phosphothreonine-deficient fast form of wild-type C subunit and the

Ala-197 mutant protein had K_ms for both ATP and peptide substrates about 30- to 65-fold higher than those for the phosphothreonine-containing slow-form enzyme. V_{max}s were roughly the same for all forms of the protein and were in the range of published values for purified mammalian C subunits (e.g., reference 10). The magnitude of the effect of the Ala-197 mutation on activity of the recombinant murine C subunit was markedly higher than that reported for the equivalent mutation in yeast C_1 subunit (Thr-241→Ala); in the yeast protein, this substitution had only a two- to threefold effect on K_ms for ATP or kemptide substrates (14).

Thr-197 lies in a region of the consensus protein kinase core structure (subdomain VIII) containing actual or potential sites for phosphorylation in most protein kinases (8). Enhanced catalytic activity has been associated with phosphorylation or autophosphorylation of these sites in several protein-tyrosine kinases and in the protein-serine/threonine kinases $p34^{cdc2}$ and $p42^{mapk}$ (2, 8, 19). It is unsurprising, therefore, that autophosphorylation of Thr-197 should also be stimulatory. In the crystal structure of C subunit complexed with PKIP, Thr-197 flanks the P+1 site of the bound peptide, and its phosphate is buried among side chains of Thr-195 and the basic residues His-87, Arg-165, and Lys-189; these interactions should promote stability of a conformation that ensures proper orientation of substrate peptides relative to the site of phosphotransfer (12). Data in support of this supposition come from mutants of the yeast C_1 subunit in which alanine was substituted for the residues equivalent to His-87 and Arg-165; these mutations each caused increases in K_ms for ATP and kemptide (6). Peptide binding also appears to stabilize a more compact structure of the C-subunit catalytic cleft that might be equivalent to the active conformation (discussed in reference 11). From these observations, we favor a dynamic model for C-subunit structure involving equilibria among two or more conformational states, only one of which binds substrates with high affinity and is competent for the phosphotransfer reaction. By shifting the equilibrium toward this active state, phosphorylation reduces K_ms for both ATP and peptide substrates; the interactions of the phosphate with surrounding residues in this structure (see above) presumably account for its resistance to phosphatase-mediated removal. With a greater tendency to assume a more open conformation, the nonphosphorylated C subunit would be accessible for phosphorylation of Thr-197; its high-K_m activity could be explained by low-affinity substrate binding and alternative stabilization of the active conformation by ternary complex formation. Since V_{max}s of the nonphosphorylated and Ala-197 mutant enzymes were comparable to that of the phosphorylated, low-K_m enzyme, neither substrate binding nor the conformational change leading to phosphate transfer should be rate limiting for the kinase reaction; this notion is consistent with results showing that release of ADP is rate limiting for phosphotransfer by the bovine C subunit (13). The relatively small effect of the Ala-241 mutation on activity of the yeast protein suggests that phosphorylation is less important for stabilization of the active form in this enzyme than it is in the mammalian enzyme. Under our model, the greater effect of this yeast mutation on binding of R subunit (14) implies that the phosphate has an additional, perhaps direct, role in R-subunit binding. In this regard, it is interesting to note that although substitution of aspartate or glutamate instead of alanine at position 241 of the yeast subunit ameliorated somewhat the defective binding of R subunit, they had little or no effect on the elevated K_ms for kemptide and ATP (14). We have not yet been able to test for possible differential effects of the Thr-197 phosphate on binding of substrate or pseudosubstrate peptides and of R subunit in the recombinant mammalian protein.

The results described here demonstrate clearly that phosphorylation of Thr-197 is necessary for the low-K_m activity of recombinant murine C subunit; we believe, but cannot yet prove, that this phosphorylation is also sufficient for the low-K_m activity. At least one serine site is phosphorylated efficiently in *E. coli*, and the kinetics of $^{32}P_i$ incorporation into slow and fast forms suggest that a second serine site may be phosphorylated at later times of induction (Fig. 1 and 2 and data not shown); either or both of these phosphorylations could contribute to C-subunit activation. Nevertheless, several observations suggest that serine phosphorylation is not important for low-K_m C-subunit activity: (i) although phosphate incorporation into slow and fast forms of C subunit was substantially slower than appearance of these proteins in silver-stained SDS-PAGE patterns (Fig. 1 and 2 and data not shown), increases in protein kinase activity during induction correlated well with amounts of slow-form C subunit present (Fig. 1 and data not shown); (ii) incubation of the Ala-197 mutant protein with active wild-type C subunit resulted in serine phosphorylation without apparent effect on activity (Fig. 8 and 9); and (iii) subfractions of slow-form C subunit separated by cation-exchange chromatography and most likely differing in phosphoserine content had comparable specific enzyme activities (4).

The present studies were undertaken to elucidate the posttranslational modification in cultured mouse cells that reduced the SDS-PAGE mobility of newly synthesized C subunits and increased their ability to bind to PKIP-Sepharose columns (25). We have shown here that Thr-197 phosphorylation of recombinant murine C subunit reproduces both of these behaviors and that failure to bind to PKIP-Sepharose can reflect a deficit in enzyme activity. Further evidence that Thr-197 phosphorylation activates C subunit in mammalian cells comes from two transfection studies comparing wild-type murine C subunit with the Ala-197 mutant in transactivation of reporter genes under cAMP-responsive promoter elements. Huggenvik et al., using a chloramphenicol acetyltransferase reporter gene driven by the human proenkephalin promoter in CV-1 cells, reported virtually no activity of the Ala-197 mutant protein (9); Orellana and McKnight, using a luciferase reporter gene driven by the human chorionic gonadotropin α promoter in JEG-3 cells, found that the Ala-197 mutant had fivefold less activity than wild-type C subunit and diminished response to coexpressed regulatory subunit (18). If, as shown here for the recombinant protein, the effect of the Ala-197 mutation is on substrate K_ms, the apparent differences between these studies can be rationalized as the result of differences in concentration or substrate properties of the phosphorylateable factor(s) responsible for the observed transcriptional activations. The diminished response to R subunit may be another reflection of the reduced affinity of the peptide-binding site of nonphosphorylated C subunit or an additional effect of the Thr-197 phosphate on R-subunit binding as discussed above. Mammalian expression of an inactive mutant C subunit (with a Lys-72→Met mutation in its ATP-binding domain) gave a protein with faster than wild-type mobility (3), consistent with autophosphorylation being responsible for the mobility shift accompanying maturation of the mammalian protein. Furthermore, the Ala-197 mutant protein expressed in CV-1 cells also had faster SDS-PAGE mobility than did the wild-type C subunit (29a); in view of evidence (Fig. 7) that nonphosphorylated wild-type and

Ala-197 mutant C subunits have equivalent mobilities, this observation confirms that Thr-197 phosphorylation is responsible for the mobility shift observed in cultured mouse cells as well as that seen in bacteria and in vitro.

ACKNOWLEDGMENTS

We thank Michael D. Uhler for graciously providing plasmids containing wild-type and mutant mouse C-subunit genes and sharing unpublished observations on expression of these genes in mammalian cells. We also thank Karen B. Gorman for sequencing the C-subunit gene in our wild-type and mutant expression plasmids.

These studies were supported by grants DK37583 from the National Institute of Diabetes and Digestive and Kidney Diseases and PHF674 from the Presbyterian Health Foundation.

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