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A Kinase-Negative Mutant of S49 Mouse Lymphoma Cells Is Defective in Posttranslational Maturation of Catalytic Subunit of Cyclic AMP-Dependent Protein Kinase

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Kinase-negative mutants of S49 mouse lymphoma cells, which lack detectable catalytic (C) subunit of cyclic AMP-dependent protein kinase, nevertheless contain cytoplasmic mRNAs for the two major forms of C subunit, Cα and Cβ. Investigation of the metabolism of C subunits in wild-type and mutant cells was undertaken to identify the step(s) at which C subunit expression was defective in kinase-negative cells. [^{35}S]methionine-labeled C subunits from cytosolic fractions of wild-type S49 cells or C subunit-overexpressing cell lines were visualized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis after purification by either affinity chromatography using a peptide inhibitor of C subunit as the ligand or immunoadsorption with an anti-C subunit antiserum. Immunoadsorption revealed electrophoretic forms of Cα and Cβ subunits that migrated faster than those detected in affinity-purified samples; this unexpected heterogeneity suggested that functional activation of C subunit may require posttranslational modification. Immunoadsorption of cytosolic fractions from wild-type cells labeled for various times with [^{35}S]methionine revealed an additional posttranslational maturation step. The bulk of immunoadsorbable C subunit label in cells pulse-labeled for 5 min or less was in an insoluble fraction from which it could be solubilized with a detergent-containing buffer; solubilization of the newly synthesized material proceeded over an incubation period of about 10 min. The primary defect in kinase-negative cells appeared to be in this solubilization step, since about equal C subunit radioactivity was found in detergent extracts of wild-type and kinase-negative cells but very little was found in mutant cytosols. I speculate that an accessory factor required for proper folding of newly synthesized C subunit is defective in the kinase-negative cells.

Among mutants of S49 mouse lymphoma cells that are resistant to cytolysis by cyclic AMP (cAMP) analogs is a class, designated kinase negative, that lacks detectable catalytic (C) subunit of cAMP-dependent protein kinase (7, 22). Although most mutations causing deficiencies of enzyme activities are recessive to wild-type alleles, the kinase-negative phenotype is fully dominant in somatic cell hybrids with wild-type S49 cells (22). This implies that the mutation lies outside of C subunit structural genes and affects rather some sort of regulator of C subunit expression. cAMP-sensitive revertants of kinase-negative cells have been isolated and include sublines in which restoration of enzyme activity is either partial or temperature dependent (25). The behaviors of these revertants support the argument that the kinase-negative lesion is external to C subunit structural genes and suggest further that the target for kinase-negative mutations is a protein-encoding regulatory gene. In previous studies, neither C subunit activities nor immunoreactive C subunit could be detected in extracts from kinase-negative cells (22, 25). Orellana and McKnight showed recently, and I confirm here, that kinase-negative cells contain C subunit-specific mRNAs of normal abundance and size; cDNA derived from kinase-negative cell mRNA encoded a functional C subunit (15). These results point to a mutational lesion affecting some aspect of C subunit protein metabolism, either synthesis, modification/maturation, or degradation.

At least two genes encode C subunit proteins in mouse and other mammalian species. The products of these genes, designated Cα and Cβ subunits, are similar in size and enzymatic properties; their sequences are about 91% identical, with most differences concentrated in a small region near their amino-terminal ends (17, 23). When isolated from animal tissues, C subunits are found modified by N-terminal myristoylation and phosphorylation at two internal sites, threonine 197 and serine 338 (16). Whether or not the phosphates affect enzymatic activity is not clear (4), but studies with recombinant enzymes show that the myristate is not necessary for activity (3, 18). In a recent study by Olsen and Uhler (14), Cα and Cβ subunits were purified from overexpressing cell lines by affinity chromatography, using a peptide inhibitor of C subunit as the affinity ligand. The purified species each ran as a single band of M_r about 40,000 on sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), but Cα subunit had slightly faster electrophoretic mobility than did Cβ subunit.

In this study, using affinity chromatography or immunoadsorption of metabolically labeled C subunits, I confirm the mobility difference between Cα and Cβ subunits and show, in addition, that at least two immunoreactive forms of these subunit isotypes are found in cells. Both C subunit isoforms are synthesized at about normal rates in kinase-negative cells. Maturation of newly synthesized C subunits into functional species apparently involves both a rapid step that renders the protein soluble in cytosolic extracts and a slower modification step that activates the protein for binding to peptide substrates. The primary defect in kinase-negative S49 cells is a failure in the first of these steps.

MATERIALS AND METHODS

Cell culture. Wild-type (subline 24.3.2) or kinase-negative (subline 24.6.1) S49 cells were grown in suspension culture

in Dulbecco modified Eagle medium (DME) with 3 g of glucose per liter, 2.24 g of sodium bicarbonate per liter, and 10% heat-inactivated horse serum as described previously (20, 22). NIH 3T3 cells stably transfected with metallothionein promoter-containing expression plasmids for Cα (line P4) or Cβ (line B1) subunit (24) and a stably transfected L cell constitutive for high expression of Cα subunit (line Cα12) were obtained from Michael Uhler (Mental Health Research Institute, University of Michigan, Ann Arbor). The transfected cell lines were maintained in monolayer culture in DME as described above but with Geneticin (GIBCO/BRL) at 750 μg/ml and with horse serum that had not been heat inactivated.

Hybridization probes. Complementary DNA plasmids containing the entire coding sequences of murine Cα (pCα-2) or Cβ (pCβ-45) in pGEM-4 (Promega Biotec) were obtained from Michael Uhler, and a cDNA plasmid containing about 92% of the coding sequence for murine α-tubulin in pUC-8 (MAT 1.1) was obtained from Gary Johnson (National Jewish Center for Immunology and Respiratory Medicine, Denver, Colo.). The inserted cDNAs were excised from these plasmids with appropriate restriction endonucleases and purified by electrophoresis on gels of low-melting-temperature agarose. Agarose slices containing the purified inserts were mixed with water, melted, and stored for subsequent labeling as described by Feinberg and Vogelstein (5).

RNA preparation, gel electrophoresis, blotting, and hybridization. Poly(A)-containing RNA was purified by oligo(dT)-cellulose chromatography from either total guanidine hydrochloride-extracted S49 cell RNA or postnuclear supernatant fractions as described previously (8) except that RNasin (Promega Biotec) at 1,000 U/ml was incubated in the extraction buffer for preparation of postnuclear supernatants.

RNAs were separated by electrophoresis on formaldehyde-containing 1% agarose gels as described by Maniatis et al. (12) and transferred onto nylon membranes by capillary blotting, using 10× SSC (SSC is 0.15 M NaCl plus 0.015 M sodium citrate). Membranes were dried for 2 h in a vacuum oven at about 80°C, prehybridized for 4 h at 42°C in hybridization solution (5× SSPE, 50% formamide, 5× Denhardt solution, 0.5% SDS, 100 μg of denatured herring sperm DNA per ml), and then hybridized for 48 h at 42°C in hybridization solution containing ^{32}P-labeled DNA probes (see below). After hybridization, filters were washed five times with 2× SSPE containing 0.1% SDS and then incubated at 60°C with two changes (30 min each) of 0.1× SSPE containing 0.1% SDS. Compositions of SSC, SSPE, and Denhardt solution were from Maniatis et al. (12). For the experiment shown in Fig. 1, 2×10^6 cpm each of Cα and Cβ subunit probes and 2×10^5 cpm of α-tubulin probe per ml were used in a single hybridization mixture; in experiments not shown, filters were hybridized with individual probes. Filters were subjected to autoradiography, and the resulting films were quantified by using a Molecular Dynamics model 300A computing densitometer.

Probe DNAs (see above) were labeled with [^{32}P]dCTP by second-strand synthesis, using Klenow fragment of DNA polymerase as described elsewhere (5); the Cα and Cβ subunit probes were labeled to about 5×10^9 dpm/μg of DNA, and the α-tubulin probe was labeled to about 2×10^8 dpm/μg.

Cell labeling and extraction. Cells were labeled in low-methionine DME (DME with 3 g of glucose per liter, 2.5 μM L-methionine, 0.5 g of sodium bicarbonate per liter, and 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES] buffer [pH 7.0]) supplemented with either 10% dialyzed, heat-inactivated horse serum (for S49 cell experiments) or 15% dialyzed fetal bovine serum (for overexpressing cell experiments). S49 cells were washed, concentrated, and preincubated in low-methionine DME before incubation with [^{35}S]methionine as described previously (19) and in the figure legends. For chase experiments, labeled cells were diluted 25-fold with conditioned medium prepared by sterile filtration of culture supernatants, and incubation was continued. Transfected cells were labeled at about 85 to 95% confluence in 35- or 60-mm dishes after rinsing and preincubating for 20 to 30 min with low-methionine DME; for chase experiments (Fig. 3), labeling medium was aspirated and replaced with conditioned medium prepared as described above. Before addition of [^{35}S]methionine, volumes of medium were reduced to about 0.5 or 1.5 ml, respectively, for 35- or 60-mm dishes. For the zinc-inducible cell lines P4 and B1, cells were incubated for 4 h with fresh growth medium containing 15% fetal bovine serum and then treated for 20 to 22 h with 90 μM zinc sulfate (tissue culture grade; Sigma Chemical) before labeling protocols were begun. After labeling (or label-chase), S49 cells were diluted with phosphate-buffered saline containing 2 mM L-methionine and harvested by centrifugation. Supernatant fractions were aspirated; cell pellets were suspended in the appropriate extraction buffer (see below), frozen on dry ice, and stored at −70°C. Medium from labeled monolayer cells was aspirated, cells were rinsed with phosphate-buffered saline containing 2 mM L-methionine, and dishes were placed on dry ice to freeze the attached cells; dishes were stored at −70°C and then thawed before scraping into extraction buffer. Cells were extracted with either CE buffer (10 mM morpholineethanesulfonic acid [MES] buffer [pH 6.6], 100 mM sodium chloride, 2 mM dithiothreitol, 10 mM magnesium sulfate, 0.1 mM EDTA, 10 μM ATP, 10 μM cAMP, 2 mM L-methionine) or RIPA buffer (10 mM Tris hydrochloride [pH 7.4], 158 mM sodium chloride, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS). Extracts were centrifuged for 12 min at 180,000 × *g* in an Airfuge microcentrifuge (Beckman Instruments), and supernatant fractions were used for subsequent purification.

Preparation of affinity resin. The 20-amino-acid 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 (2), was synthesized by the Molecular Biology Resource Facility of the University of Oklahoma Health Sciences Center, using an Applied Biosystems model 430A peptide synthesizer; the crude peptide was dissolved in 15% acetic acid and passed through a column of Sephadex G-10 (Pharmacia LKB Biotechnology) in 10% acetic acid before coupling to epoxy-activated Sepharose 6B (Pharmacia LKB) by incubating about 62 mg of peptide in 0.1 M sodium carbonate buffer (pH 10.5) with 2 g of washed resin for 48 h at 40°C. The resin mixture was then diluted 1:1 with 1 M ethanolamine in 0.1 M sodium carbonate buffer (pH 9.5), and incubation was continued overnight to block any unreacted epoxide groups. A coupling efficiency of 22% gave a final concentration of about 1.9 mg of peptide per packed ml of Sepharose. Control-Sepharose was prepared in parallel by using the same procedure but omitting the peptide.

Affinity column purification. Columns (30 μl) of PKIP- or control-Sepharose were prepared in 1-ml syringe barrels as described previously for cAMP affinity columns (19). Columns at room temperature were blocked by incubation with 5% nonfat dry milk (Carnation) in 10 mM MES buffer (pH 6.6) for 30 min and washed three times with 150 μl of C

column buffer (10 mM MES buffer [pH 6.6], 250 mM sodium chloride, 0.2 mM dithiothreitol, 2 mM magnesium sulfate, 0.1 mM EDTA, 5 μM ATP, 1 mM L-methionine, 0.5 mg of bovine serum albumin per ml, 0.5% Nonidet P-40) before loading of supernatant fractions from extracts made in CE buffer (see above). Extracts were allowed to enter column beds and incubated for 2 h at room temperature; columns were then washed eight times with 300 μl of C column buffer and three times with C column buffer lacking sodium chloride, methionine, and bovine serum albumin. C subunits were eluted with 90 μl of arginine buffer (200 mM L-arginine [pH 6.6] in 10 mM MES buffer [pH 6.6]–0.2 mM dithiothreitol–0.1 mM EDTA) after removal of liquid from below column beds (19). Samples were mixed with 50 μg of bovine serum albumin and equal volumes of 10% trichloroacetic acid and then were incubated overnight at 4°C to precipitate the eluted proteins. Precipitates were collected by centrifugation for 15 min at 12,000 × *g* in a microcentrifuge (Beckman Microfuge II) at 4°C, washed once with 100 μl of 95% ethanol, and dried before being dissolved with 22 μl of SDS-gel sample buffer (13). Portions of 2 μl were counted in 6 ml of ACS (Amersham) to determine volumes to load on gels.

Immunoadsorption. Supernatant fractions from extracts in RIPA buffer or fractions in CE buffer diluted with equal volumes of 2× RIPA buffer were preadsorbed with control serum (from a rabbit immunized against keyhole limpet hemocyanin) by incubating 15 μl of extract with 4 μl of serum for 30 min at 0°C, adding 10 μl of a 20% suspension of Pansorbin (Calbiochem) that had been washed once with RIPA buffer and resuspended in RIPA buffer containing 1% bovine serum albumin, incubating the mixture for 10 min more at 0°C, and centrifuging it for 2 min at 12,000 × *g*. For immunoadsorptions, 25-μl portions from the preadsorbed supernatant fractions were incubated with 4 μl of control serum, pooled normal rabbit serum (KC Biological), or a rabbit antiserum against recombinant murine Cα subunit (provided by Wes Yonomoto and Susan Taylor, Department of Chemistry, University of California at San Diego) for 1 h at 0°C, adsorbed with 10 μl of 20% Pansorbin as described above, and centrifuged for 1 min at 12,000 × *g*. (For the experiment of Fig. 3, the preadsorption step was omitted; for the experiment of Fig. 9, an additional 4-μl sample of anti-C subunit antiserum was preincubated with 2.4 μg of purified bovine C subunit [Promega Biotec] for 30 min on ice before addition of the preadsorbed material.) Supernatant fractions were aspirated carefully (using a Pasteur pipette whose end was drawn out to a fine point), and Pansorbin pellets were washed three times by resuspension in 200 μl of RIPA buffer (centrifuging for 1 min at 12,000 × *g* after each resuspension). Pellets were finally resuspended with 22 μl of SDS-gel sample buffer and centrifuged for 2 min at 12,000 × *g*. Supernatant samples were transferred to fresh tubes, and 2-μl portions were counted as described above for column eluates.

SDS-PAGE and fluorography. SDS-polyacrylamide gels (0.8 mm thick) containing 10% polyacrylamide were prepared and run as described by Laemmli (9). Gels were either dried and subjected to direct autoradiography or impregnated with 2,5-diphenyloxazole in dimethyl sulfoxide before drying for fluorography as described by Bonner and Laskey (1). Quantitation of selected gel patterns was performed by densitometry, using the Molecular Dynamics instrument mentioned above. The different plots shown in Fig. 9B were generated from the densitometric data by using Fig. P software (Biosoft).

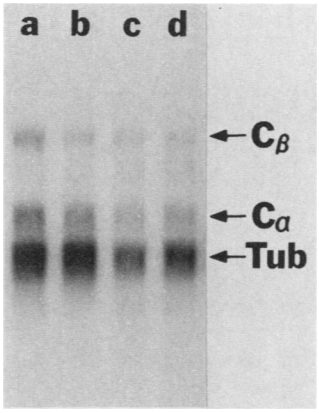

FIG. 1. Northern blot analysis of C subunit-specific poly $(A)^+$RNAs from wild-type and kinase-negative S49 cells. Poly(A)-containing RNAs were purified from postnuclear supernatant fractions (a and b) or from total cellular RNAs (c and d) of wild-type (a and c) or kinase-negative (b and d) cells, electrophoresed in a formaldehyde-containing agarose gel, blotted onto a nylon membrane, and hybridized to a mixture of ^{32}P-labeled DNA probes specific for murine Cα subunit (Cα), murine Cβ subunit (Cβ), or murine α-tubulin (Tub) as described in Materials and Methods. The pattern shown is from a 5-day autoradiographic exposure of the washed membrane. Relative to murine rRNA markers, migrations of the major species indicated corresponded to sizes of about 4.6 (Cβ), 2.2 (Cα), and 1.6 (Tub) kb.

RESULTS

Kinase-negative cells have wild-type levels of mRNAs for both α and β isoforms of C subunit. Figure 1 shows Northern (RNA) blot hybridization patterns of poly(A)-containing RNAs from either cytoplasmic fractions or total extracted RNAs of wild-type or kinase-negative cells. Filter-bound RNA was hybridized to a mixture of murine structural gene probes for Cα subunit, Cβ subunit, and α-tubulin (as a normalization control). Identification of the major bands at about 4.6, 2.2, and 1.6 kb as mRNAs for Cβ subunit, Cα subunit, and α-tubulin mRNAs, respectively, was based on experiments in which filters were hybridized with individual probes (data not shown) and are consistent with published values for these murine mRNA species (10, 23). In this and several other experiments, there was no major deficit of mRNA for the two C subunit isoforms in preparations from kinase-negative cells. Furthermore, C subunit mRNA sizes were identical in preparations from the two cell lines. By densitometry, the ratio of Cα to Cβ subunit mRNA signals was about 3:1 for both preparations from the two S49 sublines.

C subunits from wild-type, but not from kinase-negative, cells can be visualized after affinity purification on PKIP-Sepharose. The finding of apparently normal C subunit mRNAs in kinase-negative cells suggested that the kinase-negative lesion affected some aspect of C subunit protein metabolism. Therefore, I undertook a series of metabolic labeling and label-chase experiments, using [^{35}S]methionine to assess rates of C subunit synthesis and turnover in wild-type and mutant cells. Since C subunits constitute only about 0.02% of S49 cell proteins (assuming that they are slightly less than equimolar with R subunits), considerable purification was required to estimate radiolabeling of C subunit proteins. Initial attempts relied on affinity purifica-

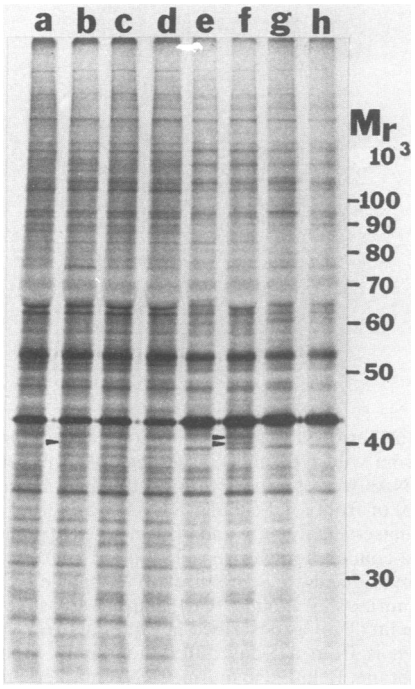

FIG. 2. Affinity purification of C subunits from extracts of [^{35}S]methionine-labeled wild-type or kinase-negative S49 cells. Wild-type (a, b, e, and f) or kinase-negative (c, d, g, and h) cells were pulse-labeled with [^{35}S]methionine for 10 min (a to d) or chased for 3 h after a 10-min pulse (e to h). Samples of cell extracts were loaded onto control-Sepharose (a, c, e, and g) or PKIP-Sepharose (b, d, f, and h) columns; the columns were washed and eluted, and eluted fractions were concentrated and subjected to SDS-PAGE (Materials and Methods); 12,000 cpm from each sample was loaded onto the gel. The autoradiographic exposure shown was for 20 days. Arrowheads indicate the positions of species specific to PKIP-Sepharose eluates from wild-type cell extracts.

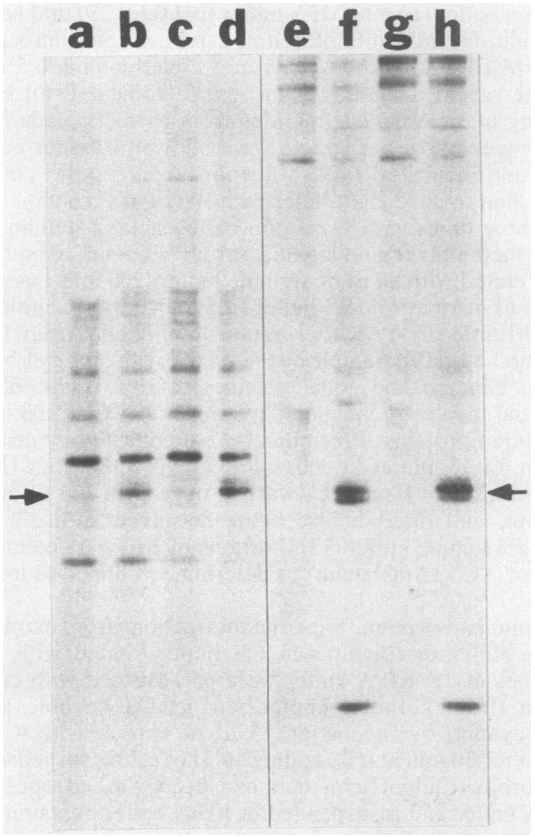

FIG. 3. Comparison of radiolabeled C subunit species purified by affinity chromatography or by immunoadsorption from extracts of a Cα subunit-overexpressing cell line. Zinc-induced P4 cells were pulse-labeled for 10 min with [^{35}S]methionine (a, b, e, and f) or chased for 15 min after a 10-min pulse (c, d, g, and h). Samples of extracts in CE buffer were purified either by affinity chromatography on control-Sepharose (a and c) or PKIP-Sepharose (b and d) columns or by immunoadsorption with control (e and g) or anti-C subunit (f and h) serum; 300 cpm from each of the purified fractions was subjected to SDS-PAGE. Patterns shown are from a 22-day fluorographic exposure; arrows indicate positions in the gel patterns corresponding to proteins of M_r about 40,000.

tion using a column-immobilized peptide inhibitor of C subunit activity (Materials and Methods).

Figure 2 shows SDS-PAGE patterns from eluates of control- or PKIP-Sepharose columns loaded with extracts of wild-type or kinase-negative cells that had been pulse-labeled with [^{35}S]methionine and either harvested immediately or chased for 3 h before harvesting. The column purifications reduced sample radioactivities by about 200-fold, but results from parallel columns with unlabeled wild-type cell extracts suggested that incomplete binding of C subunit reduced the effective purification to about 40-fold (data not shown). Consistent with this estimate, the gel patterns were complex and nearly identical in samples from control and PKIP columns. Nevertheless, in PKIP-purified samples from wild-type extracts (Fig. 2, lanes b and f), specific bands could be identified (arrowheads) that migrated with apparent molecular weights of about 40,000 to 41,000 characteristic for C subunit polypeptides. The faster-migrating band, which was also the better resolved, comigrated with labeled Cα subunit purified in a similar manner from overproducing P4 cells (see Fig. 3), and the slower-migrating band had a mobility identical to that of labeled Cβ subunit purified by affinity chromatography from overproducing B1 cells (data not shown). No labeled C subunit species were detected in affinity-purified extracts from kinase-negative cells (Fig. 2, lanes d and h) in this or in three similar experiments in which labeled wild-type subunits could be detected.

Multiple forms of C subunit proteins are detected in immunoadsorbed fractions from extracts of labeled S49 or C subunit-overexpressing cells. The question of whether or not C subunit synthesis was defective in kinase-negative cells was confounded by the consistently poor recovery of labeled C subunit in affinity column eluates from extracts of pulse-labeled wild-type cells (e.g., Fig. 2, lane b). A comparison of column-bound and immunoadsorbed C subunit fractions from Cα-overproducing cells suggested that the apparent requirement of extended incubation times for efficient recovery of labeled C subunit by PKIP affinity purification involved posttranslational processing (Fig. 3). Samples were either pulse-labeled for 10 min with [^{35}S]methionine or chased for 15 min after a 10-min pulse. A single protein species of M_r about 40,000 was specifically purified by affinity purification from the pulse-labeled sample (Fig. 3, lane b), and its relative intensity was nearly doubled in affinity-bound material from the chase sample (Fig. 3, lane d). An anti-C subunit antiserum capable of immunoadsorb-

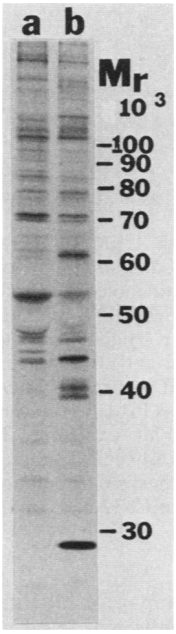

FIG. 4. Identification of immunoadsorbed C subunits from extracts of radiolabeled S49 cells. Wild-type S49 cells were labeled for 20 min with [^{35}S]methionine and extracted with CE buffer. The extract was centrifuged at high speed and preadsorbed with control serum, and portions were immunoadsorbed with either pooled normal rabbit serum (a) or anti-C subunit serum (b); 1,600 cpm from the adsorbed fractions was subjected to SDS-PAGE. Patterns shown are from a 14-day fluorographic exposure.

ing both Cα and Cβ activity from appropriate cell extracts (data not shown) specifically adsorbed two species of M_r about 40,000 from the Cα-enriched cell extracts (Fig. 3, lanes f and h). The more slowly migrating species comigrated with the form recovered after affinity purification, and its increase in the chase sample was paralleled by a decrease in the faster-migrating form. (For reasons unknown, the extent of apparent conversion from fast- to slow-migrating form was unusually high in this experiment [e.g., see Fig. 5].)

The anti-C subunit antibody can be used to detect labeled C subunit species in extracts of wild-type S49 cells (Fig. 4 and 5). Figure 4 shows SDS-PAGE patterns from a pulse-labeled S49 cell extract adsorbed with control or anti-C subunit serum; two major bands (and at least one minor band) of M_r about 40,000 were specifically enriched by the anti-C subunit serum. Immunoadsorption of these species was blocked by preincubating the serum with purified bovine C subunit, and the same species were also immunoadsorbed by a rabbit anti-bovine C subunit antiserum that exhibited a different pattern of nonspecific cross-reactions (data not shown; see Fig. 9). Figure 5 (lanes a to c) compares the C subunit species immunoadsorbed from labeled S49 cells with those immunoadsorbed from Cα or Cβ subunit-overexpressing cells. The two major C subunit bands from S49 cells corresponded to the major forms of Cα and Cβ subunits purified from the overexpressing cells. Because of the post-translational modification described above, both C subunit isoforms ran as doublets (more easily discerned in shorter fluorographic exposures of this gel). Since the minor, slow-migrating form of Cα comigrated with the major, faster-migrating form of Cβ, only three forms were resolved from the S49 cell preparation. A longer labeling duration in this

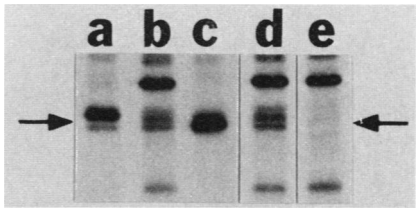

FIG. 5. Comparisons of immunoadsorbed C subunits from wild-type S49 cells with those from C subunit-overexpressing cells or with immunoadsorbed material from kinase-negative S49 cells. Wild-type (b and d) or kinase-negative S49 (e) cells were labeled for 4 h and zinc-induced B1 (a) and P4 (c) cells were labeled for 2.5 h with [^{35}S]methionine. Supernatant fractions of extracts in CE buffer were preadsorbed with control serum and adsorbed with anti-C subunit serum; the resulting samples were resolved by SDS-PAGE. About 4600 cpm from the S49 cell samples (b, d, and e) and 2,400 cpm from the C subunit-overexpressing cell samples (a and c) were loaded onto the gel. Patterns shown are from a 2-day fluorographic exposure. Arrows indicate the C subunit portion of the gel patterns (corresponding to proteins of M_r about 40,000).

experiment than in that of Fig. 4 resulted in increased labeling of the slower-migrating S49 cell forms, but they still remained relatively minor species (see also Fig. 9). Lanes d and e of Fig. 5 compare immunoadsorbed samples from wild-type and kinase-negative cells labeled for 4 h with [^{35}S]methionine. Consistent with results from Western immunoblotting (25), the C subunit bands identified in wild-type S49 cell extracts were not detectable in the kinase-negative cell sample. (Faint bands visible in the C subunit region from the immunoadsorbed kinase-negative sample (Fig. 5, lane e) were also seen in gel patterns of material adsorbed with a control serum [data not shown].)

Immunoadsorption of pulse-labeled cell extracts reveals C subunit synthesis in kinase-negative cells. To determine whether C subunit isoforms were synthesized in kinase-negative cells, the immunoadsorption experiment was repeated by using extracts from pulse-labeled cells. For the experiment of Fig. 6, cells were labeled with [^{35}S]methionine for 10 min. As before, two major radiolabeled C subunit bands were immunoadsorbed from wild-type cells. Furthermore, for the first time in these investigations, similar species were enriched from the mutant cells. The intensities

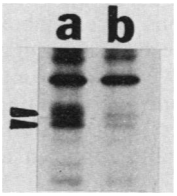

FIG. 6. Appearance of newly synthesized C subunits immunoadsorbed from CE buffer extracts of pulse-labeled wild-type or kinase-negative cells. Wild-type (a) or kinase-negative cells (b) were pulse-labeled for 10 min with [^{35}S]methionine and extracted with CE buffer. High-speed supernatant fractions were then preadsorbed and immunoadsorbed with anti-C subunit serum as for Fig. 4 and 5. About 2.8×10^6 cpm of protein radioactivity was added to each immunoadsorption, and equal fractions of the resulting samples (about 4,000 and 2,900 cpm, respectively, for wild-type and kinase-negative preparations) were loaded onto a gel for SDS-PAGE. Patterns shown are from a 6-day fluorographic exposure; arrowheads indicate positions of the two major forms of S49 cell C subunits.

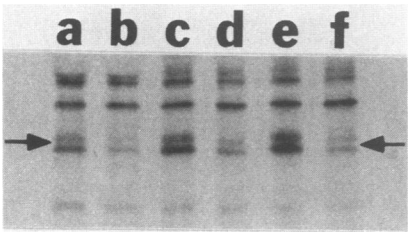

FIG. 7. Immunoadsorbed C subunits from wild-type or kinase-negative cells pulse-labeled for very short intervals. Wild-type (a, c, and e) or kinase-negative (b, d, and f) cells were pulse-labeled with [^{35}S]methionine for 2.5 (a and b), 5 (c and d), or 10 (e and f) min. Extracts in CE buffer were immunoadsorbed, and the C subunit-enriched fractions subjected to SDS-PAGE as for Fig. 4 to 6; 1,800 cpm of immunoadsorbed samples was loaded onto the gel. Patterns shown are from a 16-day fluorographic exposure. Arrows indicate the C subunit portion of the gel patterns.

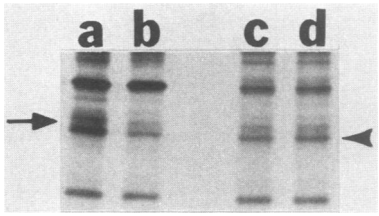

FIG. 8. Extraction with RIPA buffer, revealing differential solubility of newly synthesized C subunits from wild-type and kinase-negative cells. Wild-type (a and c) or kinase-negative (b and d) cells were labeled for 10 min with [^{35}S]methionine and extracted with either CE or RIPA buffer. High-speed supernatant fractions from CE (a and b) or RIPA (c and d) buffer extracts were immunoadsorbed and subjected to SDS-PAGE (Materials and Methods); 3,500 cpm from each sample was loaded onto the gel. Patterns shown are from a 14-day fluorographic exposure. The arrow indicates the doublet of major forms of S49 cell C subunits; the arrowhead shows the position of newly synthesized Cα subunit immunoadsorbed from an extract of pulse-labeled Cα12 cells and run in a parallel lane.

of C subunit bands in the kinase-negative sample (corrected for differences in sample loading [see figure legend]) were about fivefold less than in the wild-type samples.

Newly synthesized C subunit is not immediately degraded in kinase-negative cells. The difference in C subunit labeling between mutant and wild-type cells in the experiment of Fig. 6 appeared to be consistent with the speculation of Orellana and McKnight (15) that newly synthesized C subunits were degraded rapidly in kinase-negative mutant cells. To account for the fivefold difference in 10-min pulse-labeled cells, half-lives of the proteins would have to be less than 5 min in kinase-negative cells. To test for this possibility, the experiment of Fig. 6 was repeated with even shorter pulse times. Figure 7 shows immunoadsorbed C subunit patterns from wild-type or kinase-negative cells that were labeled for 2.5, 5, or 10 min with [^{35}S]methionine. Although still not identical, the intensities of bands in the C subunit region were closer for wild-type and kinase-negative cells labeled for 2.5 min. Contrary to predictions of a rapid turnover model, however, the increased difference in C subunit labeling between wild-type and mutant cells with longer labeling times resulted not from decreased labeling in the mutant preparations but from increased labeling in the wild-type preparations. A similar increase in C subunit labeling specific to wild-type cells was observed when a 2-min pulse-labeling period was followed by a 10-min chase; relative labeling of wild-type C subunits was not appreciably different from preparations of cells labeled for 10 or for 30 min (data not shown). These results suggested that newly synthesized C subunits were mostly either inaccessible to or unreactive with antibody; wild-type, but not kinase-negative, cells could convert some portion of this cryptic material to an immunoadsorbable form on a time scale of a few minutes.

Newly synthesized C subunit is mostly insoluble in S49 cell extracts. Since the antibody used for these studies was prepared against a nonnative form of recombinant C subunit protein and all immunoadsorptions were performed in RIPA buffer containing both ionic and nonionic detergents, it seemed unlikely that failure to detect newly synthesized C subunit protein resulted from an inability of the antibody to recognize an immature protein. This left the possibility that the newly synthesized protein was insoluble in the CE buffer used for preparing extracts. To test this hypothesis, immunoadsorptions were repeated by using pulse-labeled cells extracted with either CE buffer or RIPA buffer.

The differences in apparent labeling of C subunits from wild-type and kinase cells extracted with CE buffer (Fig. 6, 7, and 8, lanes a and b) disappeared when extraction was with RIPA buffer (Fig. 8, lanes c and d). Since RIPA buffer extracted more than twice as much labeled protein as did CE buffer and equal amounts of immunoadsorbed protein radioactivity were loaded for both types of preparation, the reduced apparent intensities of wild-type C subunit bands in the RIPA buffer sample probably reflect a higher proportion of nonspecific background proteins. Nevertheless, there was concern that bands in positions of C subunit might be spurious.

To ensure that the apparent labeling of C subunit species in samples immunoadsorbed from RIPA buffer extracts was truly C subunit and not a contaminant specifically enriched by the RIPA buffer extraction, two kinds of control adsorption were performed on RIPA buffer extracts. Figure 9A shows SDS-PAGE patterns of adsorbed species from wild-type or kinase-negative cells that were labeled for 30 min with [^{35}S]methionine, extracted with RIPA buffer, and adsorbed with either control rabbit serum, anti-C subunit serum, or anti-C subunit serum that had been blocked by preincubation with pure bovine C subunit. Background labeling in the C subunit region was more apparent in control serum adsorptions from these extracts (Fig. 9A, lanes a and d) than in control adsorptions from CE buffer extracts (Fig. 4 and unpublished experiments). Nonspecific labeling in this region was even more intense in the blocked antibody controls (Fig. 9A, lanes c and f). Nevertheless, the anti-C subunit antiserum alone (Fig. 9A, lanes b and e) clearly adsorbed more radioactivity in this region than was adsorbed by either control preparation. Difference plots of optical densities in C subunit regions of the fluorographic patterns for species adsorbed by the antiserum alone or by the C subunit-blocked antiserum are shown in Fig. 9B. (Original base lines for wild-type and kinase-negative samples were equivalent, but the wild-type plot was moved upward for clarity.) The relative displacement of wild-type radioactivity toward the gel origin is consistent with the posttranslational modification described above; this modification is apparently reduced or absent in the kinase-negative sample. When integrated by cutting and weighing, the areas under peaks from the two plots were identical to within 10%; this finding suggested that synthesis of C subunits in wild-type and

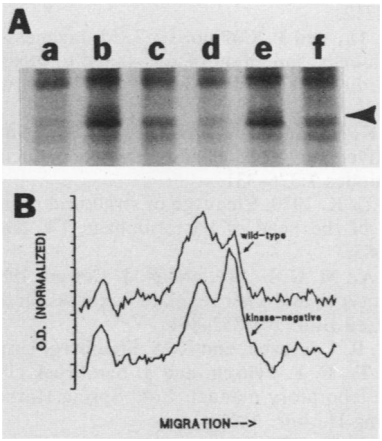

FIG. 9. Immunoadsorption of RIPA buffer extracts, showing that C subunit synthesis is equivalent in wild-type and kinase-negative cells. Wild-type or mutant cells were labeled for 30 min with [^{35}S]methionine and extracted with RIPA buffer. Extracts were centrifuged and preadsorbed as for Fig. 8, and equal portions of the preadsorbed extracts were adsorbed with either normal rabbit serum (a and d), anti-C subunit serum (b and e), or anti-C subunit antiserum that had been preincubated with pure bovine C subunit (c and f) as described in Materials and Methods; 17,500 cpm from each sample was subjected to SDS-PAGE, and the gel patterns were developed by fluorography. (A) Patterns from a 4-day fluorographic exposure. Lanes a to c are from wild-type and lanes d to f are from kinase-negative cell samples. The position of bovine Cα subunit is indicated by the arrowhead. (B) Quantitation the radioactivity in wild-type and mutant C subunits by subtracting the patterns for C subunit-blocked antibody controls from those for antibody alone. Appropriate lanes of the fluorogram of panel A were scanned with a computing densitometer (Materials and Methods), and the digitized data were normalized by subtracting film background and then multiplying by factors calculated to equalize values for a prominent contaminating species that runs just above C subunits (visible at the tops of gel patterns shown in panel A). The values for control lanes (c and f) were then subtracted from those for antibody alone (b and e). To separate the two plots, the normalized wild-type data points were incremented by 0.2 optical density (O.D.) units.

kinase-negative cells was indeed equal and that degradation of the subunits was not appreciably greater in the kinase-negative cells over the 30-min labeling period. In a pulse-chase experiment using RIPA buffer extraction, apparent half-lives for C subunits were in excess of 2 h in both wild-type and mutant cells (data not shown).

DISCUSSION

The experiments described in this report show that kinase-negative S49 cells not only have mRNA for C subunits but also synthesize C subunit proteins at rates comparable to those of wild-type cells. Furthermore, turnover of C subunit proteins is not sufficiently different to explain the nearly 100-fold reduction in C subunit activity in kinase-negative cells (22). The mutational defect in kinase-negative cells apparently prevents an early posttranslational maturation step that promotes solubility of C subunit proteins and permits their activation by further modification. Despite the intense biochemical scrutiny to which C subunits have been subjected over the past 2 decades, these complexities in production of active C subunits were entirely unanticipated; their full elucidation will require additional study.

The conclusions of this report rely critically on the identification of C subunit species in SDS-PAGE patterns of metabolically labeled S49 cell proteins. Two major species of about C subunit size were immunoadsorbed from extracts of pulse-labeled S49 cells; minor forms with slightly slower mobilities became stronger in samples from cells labeled for longer intervals (or chased after pulse-labeling), although resolution was insufficient to visualize all of these forms clearly. Identification of the major species of M_r about 40,000 as well as one minor species as C subunit proteins was supported by their specific adsorption by two unrelated anti-C subunit antisera and their failure to be adsorbed by control sera; immunoadsorption of the major forms was also blocked by preincubating the antiserum with pure bovine C subunit. The major species from S49 cells corresponded, in mobilities, to newly synthesized forms of Cα (faster) and Cβ (slower) subunits. In extracts from overexpressing cells, these major forms of Cα and Cβ subunits also were accompanied by slower-migrating forms; both forms of the appropriate C subunit isotype were induced by zinc sulfate in the zinc-inducible P4 and B1 cell lines (data not shown).

Although the faster-migrating forms of Cα and Cβ subunits were the major labeled C subunit species in extracts of pulse-labeled cells, only the slower-migrating forms were recovered by affinity purification on PKIP-Sepharose columns. Since the affinity ligand is a pseudosubstrate peptide whose binding properties parallel those for peptide substrates, it seems probable that the modification that alters gel mobilities of C subunit proteins also activates them for substrate binding. The nature and consequences of this modification and factors regulating its rate or extent are currently under investigation. Both electrophoretic forms of Cα and Cβ are phosphorylated in the overexpressing cells. Preliminary experiments with Cα subunits expressed in *Escherichia coli* suggest both that the mobility shift is caused by phosphorylation on threonine and that this modification causes significant increases in both PKIP-binding and phosphotransferase activities (21).

Newly synthesized C subunits in S49 cells were mostly in an insoluble compartment from which they could be extracted with buffer containing detergents (Triton X-100, deoxycholate, and SDS). In wild-type but not kinase-negative cells, a significant fraction of this sedimentable C subunit was solubilized over the course of a few minutes. With available reagents, it was not possible to assess accurately the proportion of total C subunit label extracted from pulse-labeled cells with RIPA buffer. An attempt to extract additional C subunit label by boiling cells in 1% SDS (containing 0.1 M 2-mercaptoethanol) resulted in even less retention of C subunit forms, but this could be attributed to a threefold-greater dilution required to reduce the SDS concentration to a level compatible with immunoadsorption (unpublished experiment). The nature and fate of the small amount of soluble C subunit proteins detected in extracts of pulse-labeled mutant cells remain unclear. Preliminary studies suggest that the insoluble pool of C subunit proteins in the mutant cells turns over faster than the soluble enzyme in wild-type cells, but this remains to be proven.

The solubilization of newly synthesized C subunit proteins appeared to precede and to be independent of the modification that could be detected by altered mobility in SDS-PAGE. Although a variety of explanations for this change in solubility remain possible, I favor the view that it reflects a slow folding of the proteins into their mature conformations. Its failure in kinase-negative cells implies that, whatever the process, it is facilitated or regulated by some gene product that is defective in the mutant. Dominance of the mutant

phenotype suggests that the putative maturation factor may contain multiple copies of this gene product, one defective copy of which is sufficient to inactivate the complex. A possible requirement of an accessory protein for correct folding of newly synthesized C subunit, although unprecedented for a monomeric cytoplasmic protein, could help explain the observation that expression of mammalian C subunit proteins in *E. coli* is mostly of inactive, insoluble forms (11, 18). The apparent specificity of the kinase-negative defect for accumulation of C subunit protein would distinguish this system from the chaperones and other proteins thought to facilitate protein folding in eucaryotic cells (reviewed in reference 6), but this specificity must be reexamined in light of the results described here.

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