Studies on the Phosphorylation and Synthesis of Type I Regulatory Subunit of Cyclic AMP-dependent Protein Kinase in Intact S49 Mouse Lymphoma Cells*

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Phosphorylation and synthesis of type I regulatory subunit (R\textsubscript{II}) of cAMP-dependent protein kinase were studied using two-dimensional polyacrylamide gel electrophoresis of \textsuperscript{35}S-methionine-labeled proteins from intact S49 mouse lymphoma cells. \textsuperscript{32}P\textsubscript{32}PPhosphate labeling, peptide mapping, and acid hydrolysis confirm that charge heterogeneity in R\textsubscript{II} results from phosphorylation of a single serine residue. In drug-free cells, phosphorylation proceeds to a steady state proportion of 90 to 95% of total R\textsubscript{II} with a half-time of about 25 min. The rate and steady state extent of R\textsubscript{II} phosphorylation are reduced by some, but not all, agents causing intracellular kinase activation. These results suggest that R\textsubscript{II} might assume different conformations in association with different amounts of cAMP or different analogs of cAMP. Endogenous kinase activation has no immediate effect on R\textsubscript{II} synthesis but leads to a moderate increase in R\textsubscript{II} synthesis after several hours; this induction occurs with all agents tested. Mutants of S49 cells lacking catalytic activity of cAMP-dependent protein kinase exhibit reduced phosphorylation and synthesis of R\textsubscript{II}. Comparative studies suggest that the phosphorylation of R\textsubscript{II} and its induction by kinase activation are fairly general phenomena: the extent of R\textsubscript{II} phosphorylation and the relative rate of R\textsubscript{II} synthesis are variable among cell types.

Cyclic AMP-dependent protein kinases (EC 2.7.1.37) from cytosols of virtually all mammalian tissues are tetrmeric enzymes consisting of a regulatory subunit dimer and 2 catalytic subunits in an inactive holoenzyme complex (1, 2). Activation is thought to occur by binding of cAMP to R\textsuperscript{I} and subsequent release of free C (1, 2). But the possibility of an active holoenzyme-cAMP intermediate has not been ruled out. R monomers carry two sites for binding cAMP (3–5) which can be distinguished experimentally by different dissociation rates for cAMP and different relative affinities for a variety of analogs of cAMP (6).

Two general classes of cAMP-dependent kinases, designated types I and II, have been characterized and appear to differ primarily in their R subunits (7–9). S49 cells have predominantly, if not exclusively, type I kinase (10, 11). R\textsubscript{II} from a variety of tissue sources can be phosphorylated by incubation of holoenzyme or dissociated subunits with MgATP (12, 13); this autophosphorylation has become one criterion for distinguishing between protein kinases I and II (6, 14). Freshly isolated R\textsubscript{II} from bovine heart is mostly in a phosphorylated form (15), suggesting that phosphorylation might be an important aspect of kinase metabolism. Phosphorylated R\textsubscript{II} has a reduced affinity for C in the presence or absence of cAMP (12, 16) and the rate of reassociation of R\textsubscript{II} with C is enhanced by dephosphorylation (17). Free phosphorylated R\textsubscript{II} is more susceptible to phosphatase action than is holoenzyme-associated R\textsubscript{II} (18). These observations suggest the possibility that phosphorylation and dephosphorylation play facilitating roles in dissociation and reassociation of kinase subunits following elevation and depression of intracellular cAMP levels (12, 19).

Although autophosphorylation of R has not been observed in purified preparations of protein kinase I (8, 14), there is evidence for physiological phosphorylation of R\textsubscript{II}. We have shown that R\textsubscript{II} purified from S49 mouse lymphoma cells labeled with \textsuperscript{35}S-methionine can be resolved into two isoelectric forms differing by a single unit of charge and that the more acidic form co-migrates in two-dimensional gel electrophoresis with a cAMP-binding species which labels with \textsuperscript{32}P\textsubscript{32}P-phosphate (10, Fig. 1 below). Several "K\textsubscript{r}" mutants of S49 cells with lesions affecting R\textsubscript{II} activity contain 4 isoelectric forms of R\textsubscript{II}, 2 of which label with \textsuperscript{32}P\textsubscript{32}P-phosphate. The electrophoretic and labeling behaviors of these forms are most consistent with a mutation in a single R\textsubscript{II} allele affecting both nonphosphorylated and phosphorylated forms of R\textsubscript{II} (10). "Kinase-negative" mutants of S49 cells lacking detectable C activity but retaining some R\textsubscript{II} exhibit greatly reduced phosphorylation of R\textsubscript{II}, suggesting that C activity is required for R\textsubscript{II} phosphorylation (11, and below).

Electrophoretic heterogeneity of R\textsubscript{II} similar to that seen in S49 cells has also been observed in R\textsubscript{II} purified from bovine, rabbit, and rat skeletal muscle (10, 20). In R\textsubscript{II} immunoprecipitated from Y1 mouse adrenocortical tumor cells (21), in affinity-labeled R\textsubscript{II} from rat ovarian cells (22), and in cAMP affinity-purified material from mouse embryo fibroblasts (4), GH3 rat pituitary tumor cells (5), and mouse neuroblastoma cells (7). In some cases, two forms of R\textsubscript{II} have been observed in purified preparations (6–9, 12), and in other cases, only one form has been seen (14). A possible explanation is that the two forms are phosphorylated at different charge sites and that the forms are different in phosphorylation state but are indistinguishable by electrophoresis. The cAMP-dependent protein kinase B from bovine heart has three forms of molecular weight 190,000, 215,000, and 240,000 and two forms of molecular weight 204,000 and 225,000, which differ by one or two phosphorylation sites (23). A similar situation may apply to R\textsubscript{II}.

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The abbreviations used are: R, regulatory subunit of cAMP-dependent protein kinase; C, catalytic subunit of cAMP-dependent protein kinase; R\textsubscript{I} and R\textsubscript{II}, regulatory subunits from protein kinase isozyme types I and II; R\textsubscript{c} and R\textsubscript{d}, nonphosphorylated and phosphorylated forms of R\textsubscript{II} kinase; kinase-negative, B\textsubscript{t}cAMP, dibutyryl-cAMP; 8-Br-cAMP, 8-bromo-cAMP.

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(23), so R1 phosphorylation might be a quite general phenomenon. In the case of R1 from bovine and rat skeletal muscle, Georg and Kraus have shown recently that the charge heterogeneity results from phosphorylation (20). R1 can be phosphorylated in vitro by cGMP-dependent protein kinase (24), but there is no evidence that cGMP-dependent kinase is involved in in vivo phosphorylation.

In this report we present data confirming that the isoelectric point heterogeneity of S49 cell R1 results from phosphorylation and suggesting that a single serine residue is phosphorylated. We analyze the kinetics and extent of R1 phosphorylation in intact cells and assess the effect of endogenous kinase activation on R1 phosphorylation. In view of the reduction in R subunit activity in kin- cells (11), we assess the effects of kinase activation on R1 synthesis and compare rates of R1 synthesis in wild type and kin- cells.

Since activation of kinase involves complex formation between R and cAMP (or one of its analogs), labeling or purification of R with cAMP affinity reagents is complicated, if not excluded, as an approach for visualization and quantification of R1 in cells stimulated with analogs or inducers of cAMP. To avoid this problem and to minimize possible artifacts of differential extraction, purification, or lability of the two forms of R1, we have extracted cells directly in a strongly denaturing solvent and subjected this total cell extract to high resolution two-dimensional gel electrophoresis (25). Since resolution of R1 is not always complete in gel patterns of whole cell extracts, we have developed and used a procedure of computer-assisted densitometry and image enhancement to quantify the radioactivity in R1 spots from autoradiographs of the two-dimensional gel patterns (26).

**EXPERIMENTAL PROCEDURES**

**Materials**

Dibutyryl-cAMP, 8-bromo-cAMP, methyl isobutylxanthine, n-isoproterenol, Tris base, and 4-morpholinethanesulfonic acid were purchased from Sigma; Amphotericin B was from LKB. Electrophoresis grade acrylamide and methylenebisacrylamide were from Bio-Rad and Eastman; sodium dodecyl sulfate (specially pure) was from BDH; Nonidet P-40 was from The Aldrich Corp. uroselectin and cholera enterotoxin (a gift from Dr. C. Johnson) were from Schenken/Mann; trypsin (tosylphenylalanin chloromethyl ketone-treated) was from Worthington; Phastglocculin aures strain V8 protease was from Miles; and RO 20-1724 (4-[3-hutoxy-4-methoxybenzyl]-2-imidazolidine) was from H. Sheppard of Hoffman-La Roche. N(2-Aminoethyl)-cAMP-Sepharose was prepared as described by Dils et al. (27) and control Sepharose was prepared by reacting CNBr-activated Sepharose 4B (Pharmacia) with ethanamine (10). [35S]Methionine (>800 Ci/mmol) and scintillation mixtures (PACS and ACS) were from Amersham; [32P]orthophosphate (carrier-free in water) was from New England Nuclear. X-ray film and photographic chemicals were from Kodak and intensifying screens from Dupont. Cellulose thin layer plates (0.1 mm thickness) were from E. Merck. Other chemicals were reagent grade and used without further purification.

S49 mouse lymphoma cells (28) were grown in suspension culture as described previously (11). Wild type cells were from subclone 243.2 (11, 29), kin- cells (subclone 246.6.1) from a spontaneous kinase-negative mutant clone (11, 29), and kim- cells (subclone 1200.19) from a kinase-negative clone which arose after mutagenesis with the frameshif mutagen ICR 191 (11, 30).

**Methods**

**Radiolabeling and Preparation of Cell Extracts—Cells were labeled with [35S]methionine or [32P]orthophosphate after preincuba-

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1 Cyclic AMP-binding proteins from mouse neuroblastoma cells have two-dimensional gel mobilities extremely similar to the two forms of S49 cell R1, but the isoelectric point heterogeneity has been attributed, thus far, to peptide differences, rather than to phosphorylation (23).

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**RESULTS**

Fig. 1 illustrates the heterogeneity in isoelectric point of R1 isolated from wild type S49 cells. Fig. 1a shows a portion of a two-dimensional gel pattern of [35S]methionine-labeled proteins purified from S49 cells by affinity chromatography on N(2-aminoethyl)-cAMP-Sepharose; spots designated by arrows have been shown to correspond to R1 (10). Fig. 1b and c shows the same portions of gel patterns from extracts of cells.
labeled with \([^{32}P]\)phosphate which were either affinity-puriﬁed (Fig. 1c) or mock-puriﬁed on a control Sepharose column (Fig. 1b): the spot designated by the right-hand arrow in Fig. 1c was the only \([^{32}P]\)-labeled species speciﬁcally puriﬁed by the cAMP afﬁnity resin. Fig. 1d shows that this \([^{32}P]\)-labeled species co-migrates with the more acidic form of \([^{35}S]\)methionine-labeled \(R_0\).

Figs. 2 and 3 show strong similarities in peptide maps of the two forms of \([^{35}S]\)methionine-labeled \(R_0\) and the species labeled with \([^{32}P]\)phosphate. The maps for both these ﬁgures were generated by a modiﬁcation of the partial proteolysis procedure of Cleveland et al. (33) in which proteins separated in a ﬁrst dimension isoelectric focusing gel are subjected to proteolysis in a second dimension sodium dodecyl sulfate gel. Fig. 2a shows peptides from the \(R_0\) region of a gel from a preparation similar to that shown in Fig. 1a, but from cells labeled for only 2 h to give increased relative labeling of the more basic spot (see below); Fig. 2b shows peptides from a control Sepharose-puriﬁed preparation to allow discrimination between peptides from the two \(R_0\) spots and those from contaminating proteins. (Several contaminating peptides are indicated by arrows to aid in alignment.) The two forms of \(R_0\) have similar, but nonidentical, maps; most of the differences appear to involve peptides from the more acidic form which have slightly slower mobilities than corresponding peptides from the more basic form. Comparable results were also obtained using papain to generate peptides (not shown). Fig. 3 compares peptides from the more acidic form of \(R_0\) labeled with either \([^{35}S]\)methionine (Fig. 3a) or \([^{32}P]\)phosphate (Fig. 3b); peptides from a mixture of \([^{35}S]\)methionine- and \([^{32}P]\) phosphate-labeled material are shown in Fig. 3c. The near identity of these peptide patterns conﬁrms the view that the same protein is labeled by both isotopes. Furthermore, many of the misaligned peptides between the basic and acidic forms of \([^{35}S]\)methionine-labeled \(R_0\) seen in Fig. 2 are those containing phosphate label, suggesting that the nonidentity of maps of the two forms of \([^{35}S]\)methionine-labeled \(R_0\) results from an effect of the phosphate on sodium dodecyl sulfate gel mobility.

Taken with the genetic evidence reported previously (10), the results of the peptide mapping experiments of Figs. 2 and 3 provide strong conﬁrmation that the two isoelectric forms of S49 cell \(R_0\) are nonphosphorylated and phosphorylated forms of the same protein; these will be referred to as \(R_N\) and \(R_P\), respectively, throughout the rest of this paper.

To ascertain the nature of the phosphate in S49 cell \(R_0\), \([^{32}P]\)phosphate-labeled \(R_0\) was excised from two-dimensional gels (similar to that of Fig. 1c), hydrolyzed with hydrochloric acid, and subjected to electrophoresis as described under “Experimental Procedures.” Unlabeled phosphoserine and phosphothreonine markers were run in parallel lanes. In two experiments using slightly different hydrolysis conditions, a single labeled anodic species was observed which migrated identically to phosphoserine (not shown—free orthophosphate was run off the electrophoretograms). \(R_N\) and \(R_P\) differ by a single charge unit (10) which is consistent with a difference of one and only one serine phosphate. Fig. 4b shows that when gel-puriﬁed \([^{32}P]\)phosphate-labeled \(R_P\) is digested exhaustively with trypsin, a single \([^{32}P]\)-labeled peptide is found. Fig. 4a shows tryptic peptides from \([^{35}S]\)methionine-labeled \(R_0\) to illustrate the resolution obtained in this chromatograph: Fig. 4c shows a more intense exposure of the track shown in Fig. 4b: the faster migrating, faintly labeled peptide observed in this track is a product of incomplete digestion. Preliminary results using partial proteolysis of native S49 kinase with
thermolysin or chymotrypsin suggest that the phosphorylation site is about 8,000 to 11,000 daltons from the NH₂ terminus of R₁.

Figs. 5 and 6 show results from an experiment investigating the time course of R₁ phosphorylation in intact S49 cells. Cells were pulse-labeled with [³⁵S]methionine, washed, and resuspended in medium with or without BrCAMP and RO 20-1724, an inhibitor of phosphodiesterase. At various times after resuspension, cells were harvested by centrifugation and frozen for later analysis: initial samples were taken at about 30 min after the addition of label. Duplicate samples were taken from the drug-free control culture and either extracted under non-denaturing conditions for affinity purification of R₁ or dissolved in gel sample buffer for whole extract electrophoresis. Drug-treated samples were analyzed only by the second method since R₁ from treated cells did not bind efficiently to the affinity resin. Fig. 5 shows the R₁ portions of two-dimensional gel patterns from these samples and Fig. 6 shows densitometric data from these autoradiograms. Autoradiograms from purified R₁ samples (left-hand column in Fig. 5) were scanned manually with a Joyce-Loebl microdensitometer while those from crude cell extracts (middle and right-hand columns in Fig. 5) were scanned with a Syntec computer-assisted microdensitometer; an image enhancement procedure was used to separate optical density in R from that in contaminating spots for the crude extract patterns (26). The two

methods for estimating the extent of R₁ phosphorylation gave similar results (Fig. 6): the small differences at early times may result from exclusion of BrCAMP complexes from the affinity-purified material. In control cells, phosphorylation proceeded with a half-time of about 25 min until about 95% of R₁ label was in R₁. In cells treated with BrCAMP, the proportion of R₁ in R₁ reached only about 75%. The kinetics of R₁ phosphorylation in the presence of BrCAMP cannot be determined from this experiment because it takes nearly 15 min for exogenously added BrCAMP to exert its full effect on endogenous protein kinase (31). Label-chase experiments carried out over longer time courses suggest that the differences in extent of R₁ phosphorylation seen in the experiment of Figs. 5 and 6 reflect steady state differences; in two experiments in which BrCAMP was added to cultures labeled for 1 h in its absence, the proportion of labeled R₁ in R₁ dropped from more than 80% to about 75% after 6-7 h of chase with only slight degradative loss of total R₁ label. Portions of these same cultures untreated with the analog maintained about 90% of their labeled R₁ in R₁ for more than 10 h of chase.

The experiment of Fig. 7 uses labeling of intact cells with [³²P]phosphate and two-dimensional gel electrophoresis of whole cell extracts to confirm the BrCAMP-mediated inhibition of R₁ phosphorylation. Wild type cells labeled in the absence of BrCAMP exhibited relatively strong labeling of R₁ (Fig. 7a) compared with cells labeled in the presence of the analog (Fig. 7b). There was only slight labeling of R₁ in kin- cells (Fig. 7c) consistent with previous results using affinity-

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Fig. 5. R1 patterns from cells labeled for 15 min with [35S] methionine and chased in the presence and absence of Bt2cAMP. Wild type cells labeled for 15 min with 250 μCi/ml of [35S] methionine were washed by centrifugation through a layer of horse serum and resuspended in conditioned growth medium with or without 1 mM Bt2cAMP and 30 μM RO 20-1724. At the times indicated to the right of the figure (in minutes), cells were harvested and quantitated were prepared as described under "Experimental Procedures"; the initial time point was about 30 min after the addition of label. The left-hand column shows two-dimensional gel patterns from affinity-purified material from drug-free control cells; the center column shows patterns from whole cell extract of the control cells; and the right-hand column shows patterns from whole cell extracts of cells exposed to Bt2cAMP and RO 20-1724 during the case. 2.5 x 10^5 cpm of the affinity-purified material and 10^5 acid-precipitable cpm of the whole cell extracts were loaded per gel and autoradiographic exposures were for 10 days and 4 days, respectively. Arrows indicate the positions of R3 and R5.

purified R1 from these mutants (11). Reduced phosphorylation of R1 in kin− cells has also been confirmed by quantitation of label in the positions of R4 and R5 in gels of whole extracts of kin− cells pulse-labeled with [35S]methionine (see below, Table 1). R5 spots from the gels of Fig. 7 were excised and subjected to tryptic mapping as in Fig. 4; all three preparations gave identical phosphopeptides (not shown).

Table 1 presents data from several experiments in which wild type or kin− cells were pulse-labeled with [35S]methionine for 10 to 30 min after preincubation with or without Bt2cAMP. Equal amounts of acid-precipitable radioactivity from whole cell extracts were subjected to two-dimensional gel electrophoresis and radioactivity in R8 and R9 was quantified from the resulting autoradiograms using computer-assisted densitometry and image enhancement. In cells labeled without drugs, the proportion of R1 in R5 varied with labeling time in a manner consistent with the 25-min phosphorylation half-time determined above (Fig. 6). Bt2cAMP treatment caused a 50 to 80% reduction in the relative labeling of R1; the greater inhibitions were seen in cells treated for several hours with the analog. In the experiments of Table I, labeling durations were sufficiently short that phosphorylation was well below its steady state levels. Therefore, the proportion of R1 in R5 is largely a reflection of the phosphorylation rate. Bt2cAMP is thus seen to slow R1 phosphorylation by as much as 4- to 5-fold. In kin− cells, R1 phosphorylation is very low and not significantly affected by Bt2cAMP.

The data of Table I also show the effects of Bt2cAMP and kin− mutations on the rate of synthesis of R1 relative to total cellular protein synthesis. Kinase activation by Bt2cAMP (Table I) or a variety of other agents (see below, Fig. 8) had no significant immediate effect on R1 synthesis, but after several hours of drug treatment an increase in R1 synthesis was seen consistently. The extent of induction has been quite variable, ranging from about 10% to almost 2-fold (Table I, Fig. 8). In Kin− cells, which lack functional C subunits (11), the relative rate of R1 synthesis was about 50% of that in wild type cells and was unaffected by Bt2cAMP.

Although Bt2cAMP has invariably inhibited R1 phosphorylation in more than a dozen experiments, such inhibition was not observed with 8-Br-cAMP or isoproterenol in an experiment performed to compare different agonist effects on

Fig. 6. Time course of R1 phosphorylation in intact S49 cells and the effect of Bt2cAMP on this phosphorylation. Radioactivity in R8 and R9 was quantified from autoradiographs of the gels shown in Fig. 5 using the densitometric procedures described under "Experimental Procedures" and the data were expressed as the proportion of total R1 in R5. For the 0- and 5-min time points of affinity-purified material, additional gels were run and quantified because of slight artifacts in the gels of Fig. 5. △—△, affinity-purified R1 from drug-free control cells; O—O, whole extract R1 from control cells; □—□, whole extract R1 from cells treated with Bt2cAMP and RO 20-1724 during the chase.
### Table 1

<table>
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<tr>
<th>Experiment</th>
<th>Subline*</th>
<th>Drug pretreatment</th>
<th>Labeling duration'</th>
<th>$A$ in R$_i$, spote$^d$</th>
<th>R$_c$</th>
<th>R$_p$</th>
<th>$\Sigma R_i$</th>
<th>R$_c$/R$_p$</th>
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<tr>
<td>I</td>
<td>Wild type</td>
<td>Bt$_c$AMP + HO, 2 h</td>
<td>10 min</td>
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<td>&lt;0.04</td>
<td>0.63</td>
<td>&lt;0.06</td>
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<td>0.29</td>
<td>1.00</td>
<td>0.29</td>
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<td>0.09</td>
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<td>0.26</td>
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*Subline refers to the genetic background of the cells.

Effects of Bt$_c$AMP and a kin$^+$ mutation on labeling of phosphatase. Wild type or kin$^+$ cells were labeled for phosphatase at 4 mCi/ml in the presence or absence of P$_i$, washed once with ice-cold phosphate-buffered saline, and precipitated cpm from whole cell extracts were subjected to two-dimensional gel electrophoresis as described under Experimental Procedures. Autodigest graphs were exposed for 4 h. a, wild type cells without drug; b, wild type cells with kin$^+$ cells without drug. Arrows indicate the position of substrate phosphorylations (31): this same experiment showed an inhibited labeling of R$_i$ phosphorylation with both Bt$_c$AMP and cholera toxin, but not with sodium butyrate. Gel patterns of labeled R$_i$ from an experiment in dyes have been repeated and extended. Cells for 30 min with $^{35}$Smethionine after short or long treatments with analogs or inducers of cAMP. Long treatments were 6 h plus the labeling interval and short treatments varied from 5 min for isoproterenol to 90 min for Bt$_c$AMP to give a maximal response at the time of labeling (31, 34, 35). Drug-free control cells exhibited nearly equal labeling of R$_c$ and R$_p$ in 30 min (Table 1, Fig. 8, a and b). As seen previously, Bt$_c$AMP inhibited phosphorylation relative to that in control cells and the inhibition was greater in cells treated for 6 h after labeling (Fig. 8d) than in those treated for only 15 min (Fig. 8c). Bt$_c$AMP had no apparent effect on the relative proportion of R$_c$ and R$_p$ after long treatment (Fig. 8a), although the dose used is physiologically equivalent to the 1 mm Bt$_c$AMP used for Fig. 8, c and d (34). Cholera toxin inhibited R$_i$ phosphorylation with short treatment (Fig. 8g), but not after longer treatment (Fig. 8h). Isoproterenol in combination with the phosphodiesterase inhibitor methyl isobutyxanthine behaved similarly to cholera toxin (Fig. 8, k and l). Methyl isobutyxanthine alone had no effect on R$_i$ phosphorylation (Fig. 8, j and l). Judging by electrophoretic shifts in endogenous substrate proteins (31, 35), all agents except methyl isobutyxanthine alone caused complete activation of endogenous kinase during the short treatment protocols (not shown); methyl isobutyxanthine caused a significant but incomplete, relative phosphorylation. The relative phosphorylations of endogenous substrates were lower after long treatments with all agents, but the effects of Bt$_c$AMP, 8-Br-cAMP, and cholera toxin were approximately equal and nearly maximal. Isoproterenol plus methyl isobutyxanthine-treated cells exhibited somewhat lower relative phosphorylation of endogenous substrates after long treatment and cells treated for 6 h with methyl isobutyxanthine alone exhibited an even smaller, but still significant, response. As mentioned above, prolonged treatment with all of these agents led to a significant induction of R$_i$ synthesis (Fig. 8, d, f, h, j, and l).

Studies on the effects of cAMP analogs on two-dimensional gel patterns from a variety of cultured cell types (35) provide comparative data on R$_i$ metabolism. Three additional murine cell lines of lymphoid origin (two lymphomas and a myeloma, Ref. 35) were virtually identical with S49 cells in relative

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1. R. A. Steinberg, unpublished results.
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Fig. 8. Effects of several analogs and inducers of cAMP on R₁ synthesis and phosphorylation. Wild type cells were labeled for 30 min with 200 μCi/ml of [³⁵S]methionine after a 6-h preincubation in low methionine medium. Drugs were added at various times before labeling as specified below and were present throughout the labeling interval. Labeling was terminated by adding 11 volumes of ice-cold phosphate-buffered saline. Cells were harvested by centrifugation, and whole cell extracts were prepared as described under "Experimental Procedures." ¹⁰⁶ acid-precipitable cpm were subjected to two-dimensional gel electrophoresis and autoradiographs were exposed for 2 days. a and b, no drug controls; c, 1 mM Br₆-cAMP, 15 min; d, 1 mM Br₆-cAMP, 6 h; e, 2 mM 8-Br-cAMP, 15 min; f, 2 mM 8-Br-cAMP, 6 h; g, 100 ng/ml of cholera toxin, 90 min; h, 100 ng/ml of cholera toxin, 6 h; i, 50 μM methyl isobutylxanthine, 5 min; j, 50 μM methyl isobutylxanthine, 6 h; k, 10 μM isoproterenol plus 50 μM methyl isobutylxanthine, 5 min; l, 10 μM isoproterenol plus 50 μM methyl isobutylxanthine, 6 h. Arrowheads indicate the positions of R₆ and R₇.

labelling of R₁, extent of R₁ phosphorylation, inhibition of phosphorylation by Br₆-cAMP, and induction by prolonged treatment with Br₆-cAMP. More disparate cell types, including mouse embry fibrolasts, primary cultures of rat adrenal cells, and GH₂ rat pituitary tumor cells, exhibited greater variations in relative labeling and phosphorylation of R₇. Labeling of R₁ varied from a level near that of S₀ in GH₂ cells to about a quarter of this level in mouse fibroblasts; induction of R₁ synthesis after 6 h of exposure to Br₆-cAMP was observed in all but the GH₂ cells. R₁ phosphorylation was less pronounced in the nonlymphoid cells than in the lymphoid cell lines studied, ranging from undetectable phosphorylation in rat adrenal cells to about half the relative phosphorylation seen in S₀ cells for mouse fibroblasts and GH₂ cells. An inhibitory effect of Br₆-cAMP on R₁ phosphorylation was observed in GH₂ cells; results were inconclusive for the other cell types.

DISCUSSION

The experiments presented in Figs. 1 to 3, 5, and 6 confirm our earlier suggestions (10, 11) that the isoelectric point heterogeneity observed in [³⁵S]methionine-labeled R₁ from S₀ cells results from phosphorylation. Nearly identical peptide maps are generated by partial proteolysis of the two [³⁵S]methionine-labeled forms of R₁ or of the form labeled with [³²P]phosphate: the slight differences observed in peptides generated from the two [³⁵S]methionine-labeled forms can be attributed to effects of the phosphate group on peptide moieties in the sodium dodecyl sulfate gel system. Pulse-labeling and label-chase experiments indicate that R₁ phosphorylation is a post-translational modification; interconversion of the two [³⁵S]methionine-labeled forms of R₁ has been observed in both phosphorylation and dephosphorylation directions. Phosphorylation appears to be on a single serine residue located in the NH₂-terminal quarter of the protein. R₁ phosphorylation proceeds to an extent of 90 to 95% with a half-time of about 25 min in drug-free wild type cells and is inhibited by some, but not all, treatments causing endogenous activation of cAMP-dependent protein kinase. R₁ phosphorylation is also greatly reduced in kin⁻ mutants of S₀ cells which lack detectable C subunit activity (11).

The difference in steady state phosphorylation of R₁ in basal and Br₆-cAMP-treated cells can be explained fully by a 3- to 6-fold inhibition of the rate of phosphorylation assuming that phosphorylation and dephosphorylation are first order (or pseudo-first order) processes; this is about the range of inhibition seen in pulse-labeling experiments (Table I). These same assumptions predict that the half-time for phosphorylation and dephosphorylation of R₁ during treatment with Br₆-cAMP should be 2.5 to 5 times greater than the half-time for phosphorylation in the absence of the analog. Consistent with this prediction, label-chase experiments in which Br₆-cAMP was added to cells with more than 80% of labeled R₁ in R₇ revealed a dephosphorylation half-time on the order of 1 to 2 h. The relative small difference in steady state phosphorylation of R₁ under basal and activated conditions and the slow dephosphorylation rate in the presence of Br₆-cAMP render unlikely the notion that R₁ phosphorylation plays an important role in modulating the rapid dissociation or reassociation of kinase subunits in S₀ cells. Such a role has been postulated for R₂ phosphorylation on the basis of studies with purified kinase (12, 19), but evidence is lacking for the rapid cycling of R₁ in vivo as required by this model. Whether or not phosphorylation of R₁ affects parameters for the activation of kinase remains unknown; the phosphatase appears to be located in a region of the molecule which has been implicated in B-C interaction (36), so an effect on this interaction would not be surprising. If this is indeed the case, the differences in relative R₁ phosphorylation observed in different cell types may reflect a fine-tuning mechanism in cellular responsiveness to cAMP elevation.

The reduction of R₁ phosphorylation in kin⁻ sublines of S₀ cells strongly suggests that C subunit plays a role in the phosphorylation, but the nature of that role remains unclear. Four possibilities we have considered are that C is catalytic in an autophosphorylation, that C activates another kinase which, in turn, phosphorylates R₁, that C inactivates a phosphatase active against R₁, or that C plays a structural role facilitating the phosphorylation of R₁ by another kinase. The second and third of these possibilities are apparently ruled out by the inhibition of R₁ phosphorylation under some conditions which activate kinase; catalytic cascade or phosphatase inhibition mechanisms predict greater phosphorylation in activated cells. Some phosphorylation of R₁ by another kinase is suggested by the low, but detectable, phosphorylation of R₁ in kin⁻ cells at the same site which is phosphorylated in wild type cells. A model in which C facilitates R₁ phosphorylation by another kinase would easily accommodate this observation as well as the failure of purified type I kinase to undergo autophosphorylation (8). On the other hand, the

- A similar, but more dramatic, effect of phosphorylation on protein mobility in this gel system has been reported for bovine R₂ (15).
- R. A. Steinberg and D. A. Agard, unpublished results.
proportion of Rₚ in labeled Rₜ from kin⁻ cells remains low even after several hours of labeling or in a chase of several hours (11) in contrast to the significant phosphorylation of Rₜ in wild type cells labeled in the presence of Br⁻cAMP: this result suggests that phosphorylation of “free” Rₜ is greater in wild type than in kin⁻ cells and thus implicates C in a catalytic role.

Studies on the autophosphorylation of type II kinase indicate that Rₜ phosphorylation is unimolecular in the absence of cAMP and bimolecular in its presence (37), presumably as a result of cAMP-mediated subunit dissociation. If C subunit plays a catalytic role in Rₜ phosphorylation, this sort of behavior could explain the inhibition of phosphorylation by agents which activate kinase. It should be noted, however, that inhibition of Rₜ phosphorylation by cAMP has been reported only at very low concentrations of kinase (37) or in the presence of Zn²⁺ (13). If C plays a structural role facilitating Rₜ phosphorylation, inhibition of phosphorylation upon kinase activation is expected so long as activation proceeds by subunit dissociation.

Although Rₜ phosphorylation has been inhibited by Br⁻cAMP, chola toxin, and isoproterenol plus methyl isobutylxanthine, such inhibition is not an obligatory result of intracellular kinase activation. 8-Br-cAMP had no apparent effect on Rₜ phosphorylation and the effects of chola toxin and isoproterenol were limited to times when their effects on intracellular cAMP levels were maximal. These results suggest that, in intact cells, kinase can distinguish between analogs of cAMP, and, perhaps, between physiological and superphysiological levels of cAMP. A basis for such discrimination is provided by the presence of two cAMP binding sites per Rₜ monomer (4, 5); recent studies of Rannels and Corbin suggest that these sites differ in their affinities for cAMP analogs (6).

To explain our results with cholera toxin and isoproterenol plus methyl isobutylxanthine, one must postulate that the two sites also differ in their affinities for cAMP; purified R exhibits apparently equal cAMP binding to the two sites (6), but the situation in holoenzyme has not yet been investigated. If the inhibition of Rₜ phosphorylation by Br⁻cAMP or high levels of intracellular cAMP results from subunit dissociation as discussed above, the absence of inhibition by 8-Br-cAMP or submaximal levels of intracellular cAMP suggests that kinase can be activated without dissociation. Such a possibility has been entertained previously (2, 38), but, to our knowledge, there is no evidence in its support. Alternatively, activation might always involve subunit dissociation, but the conformation and, hence, phosphorylability of the released Rₜ might depend upon the agent used to activate. Both of these models predict that binding of butyryl-cAMP and 8-Br-cAMP to Rₜ result in different conformations, and this prediction is supported by differences in the stability of Rₜ in cells activated with Br⁻cAMP or 8-Br-cAMP (39). Nevertheless, we cannot at present rule out alternative explanations for our results involving effects of cAMP metabolites on Rₜ phosphorylation or effects mediated by some unknown system affected differentially by various analogs and inducers of cAMP.

We have reproducibly observed a moderate induction of Rₜ synthesis in S49 cells and in a variety of other cell types after several hours of treatment with analogs or inducers of cAMP; the variability in magnitude of this effect has prevented our obtaining good kinetic data for the response. Two- to five-fold increases in Rₜ have been reported in mouse neuroblastoma cells (23) and in hybrids between mouse neuroblastoma and rat glioma cells (40), after prolonged incubation in Br⁻cAMP. At least part of the increase of Rₜ in neuroblastoma cells has been attributed to increased synthesis (41). Kin⁻ S49 cells exhibit reduced synthesis of Rₜ which is opposite to the effect of “freeing” Rₜ by kinase activation. The reduced synthesis of Rₜ in kin⁻ cells and the induction of Rₜ by kinase activation can be reconciled by assuming either a direct or indirect role of C in modulating Rₜ gene expression where both basal and activated kinase have positive effects, but the activated enzyme is more effective. The regulation of cAMP phosphodiesterase in S49 cells is similar in that it is induced by kinase activation and its basal expression is reduced about 2-fold in kin⁻ cells (29, 34). The 2-fold reduction of Rₜ synthesis in kin⁻ cells is insufficient in explaining the 5- to 7-fold decrease in cAMP-binding activity found in extracts of these cells (11); we have results suggesting that degradation of Rₜ is also affected in these mutants (39).

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