

Turnover of Regulatory Subunit of Cyclic AMP-dependent Protein Kinase in S49 Mouse Lymphoma Cells

REGULATION BY CATALYTIC SUBUNIT AND ANALOGS OF CYCLIC AMP*

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Turnover of regulatory subunit (R) of type I cAMP-dependent protein kinase in intact S49 mouse lymphoma cells was studied using two-dimensional gel electrophoresis to analyze [³⁵S]methionine label in R during label-chase experiments. R decays exponentially with a half-life of about 8.4 h in drug-free, wild type cells. In mutant cells lacking functional kinase catalytic subunit, R is about 10 times more labile than in wild type cells. 8-bromo-cAMP, isoproterenol, and cholera toxin destabilize R in wild type cells to an extent comparable to the "kinase-negative" mutation. In contrast, dibutyryl-cAMP stabilizes R in both wild type and kinase-negative cells. Sodium butyrate has no significant effect on R stability. These results are discussed in terms of R structure and the regulation of R expression.

Cyclic AMP-dependent protein kinases (EC 2.7.1.37) play critical roles in intercellular communication in higher animals and have, therefore, been subjected to intensive study (1-3). Nevertheless, very little is known about the metabolism of these enzymes and its physiological regulation. At least two structurally similar isozymes of cAMP-dependent protein kinase have been identified in mammalian tissues (4, 5). They each consist of two catalytic subunits and a dimer of regulatory subunits in an inactive holoenzyme tetramer (1, 3, 5). Isozyme specificity resides in the R¹ subunits (3, 5, 6). Activation of kinase appears to proceed by a concerted reaction involving binding of up to 2 cAMP molecules/R monomer and release of free C subunits (3, 7-9).

Our interest in the metabolism of kinase subunits derives

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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_N and R_P, nonphosphorylated and phosphorylated forms of R; kin⁻, kinase-negative; Bt₂cAMP, dibutyryl-cAMP; MIX, methyl isobutylxanthine; 8-Br-cAMP, 8-bromo-cAMP; BtcAMP, N¹-monobutyryl cAMP.

from studies on cAMP-resistant mutants of S49 mouse lymphoma cells which have alterations in the structure and/or expression of these subunits (10, 11). The cAMP-dependent protein kinase in S49 cells is predominantly the type I isozyme and its R subunits are found in nonphosphorylated (R_N) and phosphorylated (R_P) forms which can be separated and visualized by high resolution two-dimensional gel electrophoresis (10-12). One class of mutants, designated "kinase-negative," lacks detectable C subunit activity and has reduced levels of apparently wild type R subunit (11). The R subunits of kin⁻ cells behave chromatographically like wild type R subunits dissociated from C by cAMP but show a significantly reduced level of phosphorylation (11, 12). To investigate whether the reduced level of R expression in kin⁻ cells is a direct consequence of the putative regulatory mutation (11) or a secondary effect of the absence of functional C subunit, we asked how kinase activation and kin⁻ mutations affects R metabolism. The present report shows that R turnover is markedly stimulated by kinase dissociation or the kin⁻ mutation except when cells have been exposed to dibutyryl cAMP (Bt₂cAMP). The effects of kinase activation and kin⁻ mutations on R synthesis and phosphorylation are reported elsewhere (12).

EXPERIMENTAL PROCEDURES

Chemicals and Radiochemicals—Cyclic nucleotides, MIX, and DL-isoproterenol were from Sigma, [³⁵S]methionine (>900 Ci/mmol) and ACS scintillation mixture were from Amersham, and electrophoresis grade acrylamide and methylenebisacrylamide were from Eastman. Additional two-dimensional gel reagents were obtained as described elsewhere (12), and other chemicals were reagent grade and used without further purification.

Cell Culture—Wild type (subclone 24.3.2) and kin⁻ (subclone 24.6.1) S49 mouse lymphoma cells (11, 13, 14) were grown in suspension culture in a 5% carbon dioxide atmosphere at 37 °C in Dulbecco's modified Eagle's medium (Gibco) with 3 g/liter of glucose, 2.24 g/liter of sodium bicarbonate and were supplemented with 10% heat-inactivated horse serum (Microbiological Associates).

Radiolabeling and Chase Protocols—Cells were labeled with [³⁵S]methionine after 2 h of preincubation in low methionine medium as described previously (15). To establish chase conditions, labeled cells were centrifuged through two or more volumes of heat-inactivated horse serum (at 37 °C), medium and serum were aspirated, and cells were resuspended in conditioned growth medium prepared by centrifuging cells from a log-phase culture of S49 cells, adding fresh glutamine to 4 mM, and filtering to sterilize. This procedure removed more than 95% of unincorporated radioactivity and resulted in a decrease in methionine-specific activity of about 5000-fold. For the experiments of Figs. 1 and 2, labeling was for 1 h at 100 μCi/ml, all components were sterile filtered, penicillin (200 units/ml) and streptomycin (200 μg/ml) were included in chase media, drugs were added at the time of chase, and initial samples were taken after 1 h of chase. For the experiment of Fig. 3, labeling was for 30 min at 200 μCi/ml, drugs were added 15 min before labeling and also included in chase media, and initial samples were taken immediately after labeling. In the experiments of Figs. 1 and 2, cells were resuspended at about 6 × 10⁵/ml for the chase and 1-ml portions were taken for each experimental point; in the experiment of Fig. 3, cells were resuspended at 1.2 × 10⁶/ml in 0.3-ml portions for the chase. Samples were harvested by centrifugation in a Fisher microcentrifuge (5 s at 10,000 × g), media were aspirated, cell pellets were lysed with gel sample buffer, and extracts were frozen and stored at -70 °C as described previously (15). To monitor loss of incorporated radioactivity during chase conditions, 5-μl culture samples were dissolved in 50 μl of 1 N sodium hydroxide, precipitated with 4 ml of ice-cold 5% trichloroacetic acid in the presence of 0.6 mg of bovine serum albumin as carrier, and filtered onto Whatman GF/C filters. Tubes and filters were rinsed twice with 4 ml of 5% trichloroacetic acid, and filters were counted in

6 ml of ACS scintillation mixture. Samples were counted for gel loading as described previously (15) but using ACS scintillation mixture.

Two-dimensional Gel Electrophoresis, Autoradiography, and Densitometry—The O'Farrell two-dimensional gel electrophoresis procedure (16) was used with modifications described previously (15). First dimension isoelectric focusing gels were run for 7000 volt-hours and second dimension sodium dodecyl sulfate gels were 7.5% in polyacrylamide. Dried gels were exposed to Kodak No-Screen film for autoradiography; 3×10^5 acid-precipitable cpm were loaded for the gels of Fig. 1, and densitometry was performed on autoradiograms exposed for 5 or 25 days. 10^5 acid-precipitable cpm were loaded for the gels of Figs. 2 and 3, and exposures shown were for 6 (Fig. 2) or 10 (Fig. 3) days. All gel patterns are shown with the acidic end of the first dimension at the right and the low molecular weight region of the second dimension at the bottom. Densitometry was performed using a Syntex AD-1 autodensitometer, and absorbance in R was resolved from that in contaminating spots by a constrained deconvolution procedure described elsewhere (12, 17). Data shown in Fig. 1 represent the sums of absorbance in R_N and R_P spots normalized to the value for drug-free wild type cells at the initial time point. Spots whose absorbances were within the linear response range of the film for both short and long exposures were used to generate a normalization constant for interpolating data from short and long autoradiographic exposures.

RESULTS AND DISCUSSION

Turnover rates of R were determined in the absence of metabolic inhibitors using a label-chase protocol as described under "Experimental Procedures." To avoid potential artifacts due to differential extraction, differential purification, or postlysis degradation, cells were extracted under highly denaturing conditions, and entire cell extracts were subjected to high resolution two-dimensional gel electrophoresis. Within an experiment, all gels were loaded with equal amounts of protein radioactivity, and the relative proportion of radioactivity in R was determined by the intensities of spots corresponding to R_N and R_P in gel autoradiographs. Changes in total acid-precipitable radioactivity/ml of cell culture were negligible over at least 15 h of chase under the conditions of these experiments (data not shown).

Fig. 1 compares the turnover of R in wild type cells chased in the presence and absence of Bt_2cAMP with that in kin^- cells chased in the absence of drugs. Label in R_N and R_P was quantified using a computer-assisted densitometry procedure (17) and the sums were normalized to the initial value for drug-free wild type cells. In drug-free wild type cells, R decay was exponential with a half-life of 8.4 h; Bt_2cAMP caused significant stabilization of the subunit. In kin^- cells, R turnover was about 10 times faster than in wild type cells.

The opposite effects of Bt_2cAMP and kin^- mutations on R degradation were unexpected and raised the possibility that C subunit activity inhibited R degradation. If Bt_2cAMP -mediated stabilization were the result of kinase activation, other agents that activated kinase would be expected to cause a similar stabilization. Fig. 2 shows the R regions of gel patterns from a label-chase experiment in which Bt_2cAMP , 8-Br-cAMP, or endogenous cAMP was used to activate kinase during the chase period. Cells were chased for 1, 8, or 15 h in the absence of drugs (Fig. 2, a-c), or in the presence of Bt_2cAMP (Fig. 2, d-f), 8-Br-cAMP (Fig. 2, g-i), MIX, (Fig. 2, j-l), or isoproterenol plus MIX (Fig. 2, m-o). Although drug-free control cells exhibited R turnover consistent with that seen in the experiment of Fig. 1 and Bt_2cAMP stabilized R as before, both 8-Br-cAMP and isoproterenol plus MIX destabilized R to the extent that it could not be detected in gel patterns from samples taken at 8 and 15 h of chase; MIX alone had negligible effect. Cholera toxin plus MIX (data not shown) had an effect identical to that of 8-Br-cAMP or isoproterenol plus MIX. These results suggested that the half-

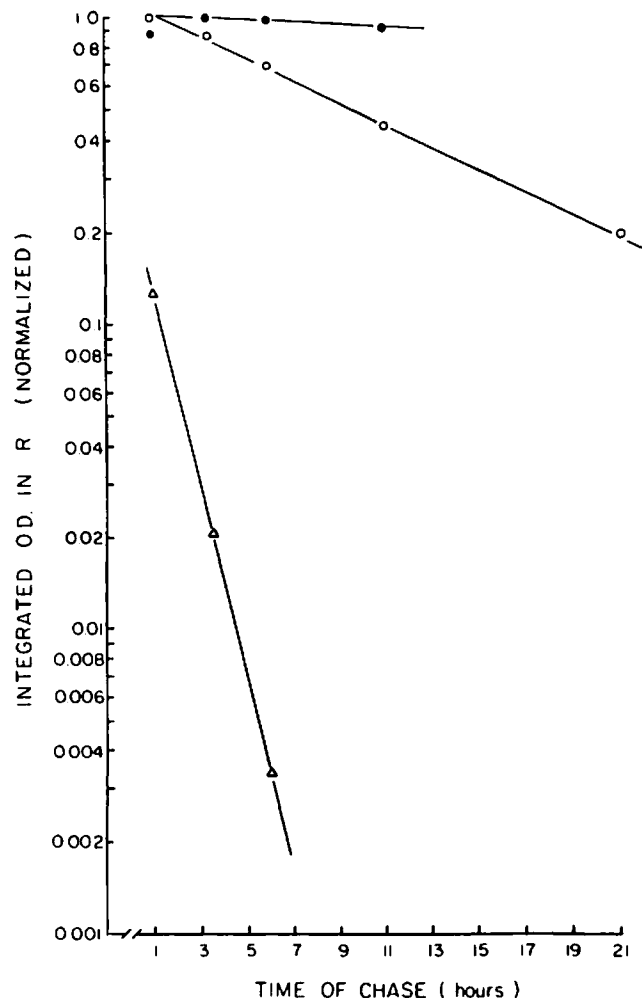


FIG. 1. Time courses for degradation of R in wild type and kin^- S49 cells and in wild type cells treated with Bt_2cAMP . Cells were labeled for 1 h with [^{35}S]methionine in the absence of drugs and chased in the presence or absence of 1 mM Bt_2cAMP . At various times of chase, cells were harvested and lysed, and the lysates were subjected to two-dimensional gel electrophoresis. Radioactivity in R_N and R_P was quantified from autoradiographs as described under "Experimental Procedures" and the sums were normalized to the value for untreated wild type cells at 1 h of chase. (○), untreated wild type cells; (●), Bt_2cAMP -treated wild type cells; (△), untreated kin^- cells. R could not be detected in gel patterns from kin^- cells after 7 h of chase. The lethal effect of prolonged Bt_2cAMP exposure on wild type cells precluded data for treated cells at 21 h of chase.

life of R in wild type cells activated by elevation of endogenous cAMP or by treatment with 8-Br-cAMP was similar to that in untreated kin^- cells. This expectation was confirmed in an experiment using shorter chase times with 8-Br-cAMP or isoproterenol plus MIX (data not shown).

The differential effects of Bt_2cAMP and cAMP (or 8-Br-cAMP) on R turnover are specific to this protein. Several of the protein species resolved by the gels used in these experiments have shorter than average half-lives ranging from about 15 min to more than 8 h.² Turnover of these proteins was unaffected by any of the treatments used in these studies (data not shown). We report elsewhere that R_N and R_P are only slowly interconvertible in wild type S49 cells (12). The similar degradation rates of "free" R in kin^- cells (mostly R_N) and in wild type cells activated with cAMP or 8-Br-cAMP (mostly R_P) suggest that the phosphate does not play a

² R. A. Steinberg, unpublished results.

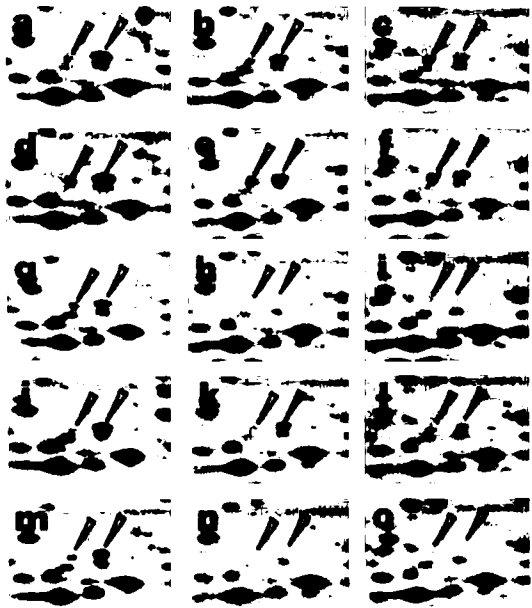


FIG. 2. Effects of several analogs and inducers of cAMP on R degradation in wild type S49 cells. Cells were labeled with [35 S] methionine and chased in the presence or absence of drugs as for the experiment of Fig. 1. Samples were harvested after 1 (a, d, g, j, and m), 8 (b, e, h, k, and n), or 15 (c, f, i, l, and o) h of chase, cells were lysed, and lysates were subjected to two-dimensional gel electrophoresis. Portions of gel autoradiographs are shown from extracts of cells chased in the absence of drugs (a-c), or in the presence of 1 mM Bt₂cAMP (d-f), 2 mM 8-Br-cAMP (g-i), 50 μ M MIX (j-l); or 10 μ M isoproterenol plus 50 μ M MIX (m-o). Arrows indicate the positions of R_N (left) and R_P (right).

significant role in the metabolic stability of R.

Since the stabilizing effect of Bt₂cAMP was apparently not a consequence of kinase activation yet was specific to R protein, we sought to determine whether or not the analog would stabilize R in kin⁻ cells. Furthermore, since butyrate is released concomitantly with intracellular conversion of Bt₂cAMP to its active derivative, BtcAMP, (18, 19), we tested the effects of butyrate as well as Bt₂cAMP on R degradation in kin⁻ cells. Fig. 3 shows the results of an experiment in which kin⁻ cells were pulse-labeled with [35 S]methionine in the absence (Fig. 3, a and b) or presence of Bt₂cAMP (Fig. 3, c and d) or sodium butyrate (Fig. 3, e and f), then washed and chased in growth medium with or without the same drugs. Samples were taken immediately after labeling (Fig. 3, a, c, and e) or after 2.5 h of chase (about 3 half-lives for R in untreated kin⁻ cells; Fig. 3, b, d, and f). In kin⁻ cells, R was clearly stabilized by Bt₂cAMP and unaffected by sodium butyrate. These results confirm that Bt₂cAMP-mediated R stabilization is independent of kinase activation and imply that it is an effect of BtcAMP binding to R. The difference in effects of 8-Br-cAMP and Bt₂cAMP on R stability is consistent with the preferential binding of these analogs at different sites on R (20) and suggests that different conformations might be associated with occupation of these two cAMP-binding sites. It remains possible, however, that BtcAMP has a more direct inhibitory effect on R degradation. We have also observed differential effects of Bt₂cAMP and 8-Br-cAMP on the inhibition of *in vivo* phosphorylation of R in S49 cells (12); in these studies, superphysiological levels of intracellular cAMP appeared to mimic the effects of Bt₂cAMP. Whether or not R stability is also affected differently by different levels of intracellular cAMP has not yet been satisfactorily determined.

The stabilization of R by Bt₂cAMP might explain why

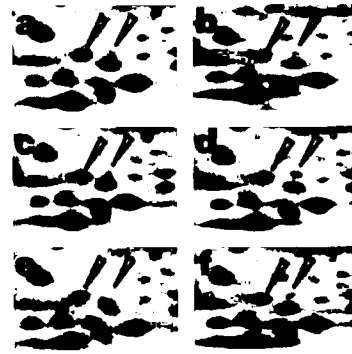


FIG. 3. Effects of Bt₂cAMP and sodium butyrate on R degradation in kin⁻ S49 cells. Kin⁻ cells were labeled for 30 min with [35 S]methionine in the presence or absence of drugs; samples were taken immediately (a, c, and e) or after 2.5 h of chase with the same drugs (b, d, and f). Cell extracts were subjected to two-dimensional gel electrophoresis, and the gels were dried and autoradiographed. Portions of gel patterns are shown from extracts of drug-free cells (a and b), cells treated with 1 mM Bt₂cAMP (c and d), and cells treated with 1 mM sodium butyrate (e and f). Arrows indicate the positions of R_N and R_P as in Fig. 2.

neuroblastoma cells and neuroblastoma/glioma hybrids stimulated for several hours with Bt₂cAMP show a marked increase in type I R levels (21, 22) but only a moderate increase in R synthesis (23). 8-Br-cAMP and agents that elevate intracellular cAMP also promote increased synthesis of R in a variety of cell types (12), but the destabilization of R by these agents tends to counteract the effect on R synthesis. In S49 cells, the balance between these two effects favors degradation such that the concentration of R is expected to fall by more than 5-fold with a half-time of about 1 h in the presence of activating levels of cAMP or 8-Br-cAMP. Depending on the relative stability of activated C subunit, this loss of R subunit might result in either persistent activation of kinase after cAMP levels have returned to basal values or a refractory period during which cellular responsiveness to all agents acting through kinase would be diminished. A decrease in kinase activity has been reported for Leydig cells incubated for several hours with human chorionic gonadotropin (24) and we have preliminary evidence for decreased kinase activity and effectiveness in S49 cells activated with cAMP analogs for extended periods of time.² Determination of the relative rates and extents of decrease of R and C subunits awaits further studies.

The relative instability of free R not only serves to explain the deficiency of cAMP-binding activity in extracts of kin⁻ cells (11) but also suggests a mechanism for coordinating the relative levels of R and C subunits in normal cells. So long as R is replenished at a sufficient rate, the greater lability of free than of holoenzyme-associated R will maintain R levels in slight excess of C levels over a range of C subunit concentrations. Such coordination of R and C subunit levels has been reported for a variety of rabbit tissues (25) and appears to hold as well for mutant sublines of S49 cells varying over about a 10-fold range in levels of cAMP-dependent protein kinase.³

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³ R. A. Steinberg and T. van Daalen Wetters, unpublished results.

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