Alvin M. Malkinson and Martin S. Butley

School of Pharmacy, University of Colorado, Boulder, Colorado 80309

ABSTRACT

The regulatory molecule, cyclic adenosine 3':5'-monophosphate (cAMP), can inhibit cell proliferation, reverse the neoplastic phenotype, and promote tumor regression. Since the biological effects of cAMP are mediated by an activation of protein kinase enzymes, the interaction of cAMP with the regulatory subunits of type I and II isozymes (Ri and RII) was studied in normal and neoplastic tissues. Regulatory subunits in extracts from normal adult and neonatal lung, urethane-induced lung adenoma, and uninvolved lung tissue were covalently labeled with 8-azidoadenosine cyclic 3':5'-monophosphate (8-N3-[32P]cAMP), a specific photoaffinity analog of cAMP. The ability of Ri to bind 8-N3-[32P]cAMP is the same in all tissues, while RII binding characteristics depend on both developmental stage and neoplastic state. Binding curves for Ri from normal adult lung suggest two 8-N3-[32P]cAMP binding sites, one which saturates at 125 nM and one which saturates at 800 nM. The RII from tumor tissue appears to have only the low-affinity site, while RII from neonatal lung shows greatly reduced binding to both sites. These changes in 8-N3-[32P]cAMP binding characteristics correlated with changes in the cAMP dependence of RII autophosphorylation. In adult lung, cAMP can either stimulate or inhibit endogenous phosphorylation of RII, depending on the divalent cation (Mg2+ or Zn2+), respectively) present during the reaction. In the tumor, cAMP has little stimulatory effect but is strongly inhibitory. The amount of 32P that can be incorporated into neonatal RII is very slight with either cation. Thus, both the 8-N3-[32P]cAMP binding sites on RII and the phosphorylatable sites on RII change during normal and neoplastic development. Tumor growth is unregulated by normal physiological constraints. A decreased responsiveness to alterations in intracellular cAMP concentrations in lung adenomas may increase the proliferative rate and help maintain the neoplastic state.

INTRODUCTION

The reversible phosphorylation of proteins is a sensitive index of perturbations in the cellular environment and is thought to regulate many of the resulting changes in cell metabolism (15). The pleiotropic effects of protein kinase enzymes which catalyze these reactions may play a role in tumor development. The product of the transforming gene of many oncogenic viruses is a protein kinase (5), and altered or additional forms of kinase molecules may also regulate the neoplastic conversion of normal tissues in vivo. Indeed, unique patterns of phosphorylated proteins have been found in tumors (32, 47).

Many types of protein kinase exist, including those activated by the second messengers cAMP, guanosine cyclic 3':5'-monophosphate, and Ca2+ (15). The effects of these informational molecules on cells are totally or partially mediated by kinase activation. cAMP-dependent kinases have been studied in tumors, and increased (29), decreased (27), and unaltered (12) kinase specific activities have been described. cAMP-dependent kinases fall into 2 major structural classes, types I and II (6). The tetrameric holoenzymes contain 2 catalytic subunits and a regulatory subunit dimer; the classes vary only in the nature of the regulatory subunit dimer (18). The isozymes have identical catalytic specificities but differ in their regulatory responses to the nucleotides cAMP and ATP. For example, only the type II isozyme normally undergoes autophosphorylation in which RII is phosphorylated in a reaction catalyzed by the catalytic subunit (18). The relative amounts of the type I and type II isozymes change during alterations in physiological status such as normal development (23, 30), neoplastic transformation (13), cell cycle traverse (8), and mitogenic stimulation (11). Since the intracellular functions of these 2 enzyme classes have not been established, the physiological consequences of such changes are unclear.

The mouse pulmonary adenoma is an excellent system for the study of biochemical changes which regulate tumor development. Inbred strains of mice vary in whether or not they develop these tumors spontaneously or by chemical induction, and these genetic differences may be exploited to study mechanisms of tumorigenesis (10). The number of tumors arising can be modified by inhibitors (43) and promoters (46) of carcino genesis. Tumor development follows a consistent series of changes, from hyperplastic foci to benign adenoma, to malignant adenocarcinoma, to metastasis; each successive progression occurs in a decreasing fraction of the preceding population (39). Readily dissectable adenomas derived from type 2 alveolar stem cells are present within 2–3 months following urethane injection. We have measured the specific activity of cAMP-dependent protein kinases in the lung adenoma (29), during postnatal lung development (30), and following pulmonary injury caused by the antioxidant, butylated hydroxytoluene (28). The kinase specific activity increased in the adenoma relative to uninvolved lung, while no changes occurred during normal development or following butylated hydroxytoluene treatment. We also measured the kinase isozyme composition by column chromatography and found no differences between neonatal and adult lung (30).

A sensitive means of characterizing protein kinases is to covalently label the regulatory subunits with 8-N3-[32P]cAMP, a photoaffinity analog of cAMP (31). Photolysis with UV generated A sensitive means of characterizing protein kinases is to covalently label the regulatory subunits with 8-N3-[32P]cAMP, a photoaffinity analog of cAMP (31). Photolysis with UV generated

3 The abbreviations used are: cAMP, adenosine cyclic 3':5'-monophosphate; RII, regulatory subunit of type II isozyme; 8-N3-[32P]cAMP, 8-azidoadenosine cyclic 3':5'-(32P)monophosphate; SDS, sodium dodecyl sulfate; RII, regulatory subunit of type II isozyme; HTCT, hepatoma tissue culture; dibutyryl cAMP, dibutyryl adenosine cyclic 3':5'-monophosphate.

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2 To whom requests for reprints should be addressed.

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erates a nitrene group which can then react with any amino acid residue within the active site (16). The analog mimics the binding properties and ligand specificity of cAMP (34). We report here that when the regulatory subunits from lung adenoma are compared with regulatory subunits from lungs taken at representative stages of normal lung development, differences among the Rn's are observed. These differences include both the ability of Rn to bind 8-N3-[32P]cAMP and the ability of cAMP to modify Rn autophosphorylation. These results suggest a mechanism for the decreased responsiveness of pulmonary adenoma to environmental stimuli.

 MATERIALS AND METHODS

Urethan-Induction of Lung Adenoma. Male A/J mice (The Jackson Laboratory, Bar Harbor, Maine), 6 to 12 weeks old, were given i.p. injections of 1 mg urethan, dissolved as a 10% solution (w/v) in 0.85% NaCl. The mice were killed by cervical dislocation 4 to 6 months after injection, and tumors, which were benign by histological analysis, were collected. The tumor yield per mouse for the 21 mice used was 16.8 ± 1.4 (S.E.). Lung adenomas and pieces of lung tissue in which no tumors were evident (uninvolved tissue) were dissected from the same mice and maintained at -85°C until further analysis. Lungs were also collected from neonatal A/J mice (mixed sexes; 3 to 10 days old) and adult males (3 to 5 months old) and stored at -85°C.

Photoaffinity Labeling. Lungs and tumors were homogenized in 5 volumes (w/v) of 0.32 M sucrose, and cytosolic fractions were prepared by centrifugation at 20,000 × g for 45 min. Twenty μl of extract and 1 μl of 0.5 M 2-(N-morpholino)ethanesulfonate buffer, pH 6.5, were added to a 5-μl aliquot of 8-N3-[32P]cAMP obtained from Dr. Boyd E. Haley, University of Wyoming, or purchased from ICN Corp., Irvine, Calif.). The final concentration of 8-N3-[32P]cAMP in the reaction mixture ranged from 6 to 1000 nM. These components were mixed with an airstream on a spot plate. The samples were incubated at 35°C for 30 min in the dark to promote exchange between the ligand and any endogenous cAMP already bound to regulatory subunits (16) and were further incubated for 30 min at 4°C in the dark. Photolysis with UV (254 nm) was done at 4°C using a UVSL-25 Mineralight handlamp held at 8 cm for 7 min, and the reaction was terminated with 26 μl of a stop solution containing SDS (21).

Phosphorylation Reaction. The procedure of Malkin et al. (31) for phosphorylating endogenous proteins was slightly modified. The 100-μl reaction mixture contained 50 μg soluble lung protein, 50 mM 2-(N-morpholino)ethanesulfonate (pH 6.5), either 5 mM ZnCl2 or 10 mM MgCl2, 1.5 μM [γ-32P]ATP (5 to 20 Ci/mmol), with or without 10 μM CAMP. After incubation for 20 sec at 30°C, the reaction was terminated with 50 μl of SDS stop solution (21).

Denaturing Gel Electrophoresis. The labeled cytosolic proteins were separated on one-dimensional SDS slab gels (21), using 8 to 12% linear gradient gels. After the gels were dried, autoradiograms were prepared on Kodak X-5 X-ray film (Eastman Kodak Co., Rochester, N. Y.) with Dupont Cronex Quanta III intensifying screens (Dupont Instruments, Wilmington, Del.). The films were exposed for 1 to 2 days at -85°C. Apparent molecular weights were estimated using the following protein markers: phosphorylase A (m.w. 92,500), bovine serum albumin (m.w. 66,000), pyruvate kinase (m.w. 57,000), ovalbumin (m.w. 45,000), DNase (m.w. 31,000), β-lactoglobulin (m.w. 18,400), and lysozyme (m.w. 14,300). To quantitate the amount of 8-N3-[32P]cAMP bound to Rn and Rn, the dried gels were marked with radioactive ink and placed on film. This allowed precise alignment of the gel with the film in order to determine the locations of the radioactive proteins. Sections of the gel corresponding to Rn and Rn on the autoradiogram were cut out, placed in scintillation fluid, and counted in a Beckman LS 8000 counter (Beckman Instruments, Inc., Fullerton, Calif.).

Two-Dimensional Gel Electrophoresis. After photolabeling by the above procedure, 26 μl of lysis buffer (33) were added to each sample. The samples were then saturated with urea and electrophoresed according to the method of O'Farrell (33) with minor modifications. First-dimension gels were run for 6400 volt-hr. Samples were overlayed with 5% Nonidet P-40, 8 M urea, and 1% ampholines, composed of 0.8% of the pH 5 to 7 ampholine and 0.2% of the pH 3.5 to 10 ampholine, both from LKB Instruments, Inc. (Rockville, Md.). Second-dimension gels were run to 12% linear gradients.

Limited Proteolysis of Labeled Proteins. Trypsin (Worthington Biochemical Corp., Freehold, N. J.), at concentrations ranging from 20 to 200 μg/ml, was added to 8-N3-[32P]cAMP-labeled extracts prior to the addition of the lysis buffer. After incubation at 30°C for 15 min, 1200 μg of soybean trypsin inhibitor per ml (Millipore Corp., Bedford, Mass.) was added to stop digestion. Samples were mixed with lysis buffer and fractionated by 2-dimensional gel electrophoresis.

RESULTS

8-N3-[32P]cAMP Binding. The major proteins which are stained by Coomassie blue in the urethan-induced pulmonary adenoma, uninvolved tissue, and neonatal and adult lung are very similar (Fig. 1A). The main differences appear to be quantitative rather than qualitative, with the concentrations of certain proteins higher in uninvolved and adult tissues than in tumors and neonatal tissues. The staining patterns of the tumor and neonatal tissues are nearly identical.

Extracts from these tissues were radiolabeled with 8-N3-[32P]cAMP, and Fig. 1B is the resultant autoradiogram of the gel shown in Fig. 1A. Three molecular weight classes of proteins were photolabeled, corresponding to Rn (m.w. 54,000), Rn (m.w. 49,000), and a m.w. 37,000 protein which corresponds in size to a commonly observed proteolytic fragment of regulatory subunits (41). More of the labeled m.w. 37,000 protein is consistently present in neonatal lung cytosol than in the other samples. Samples photolabeled in the presence of 40 μM cAMP show no incorporation of 8-N3-[32P]cAMP into proteins, demonstrating the specificity of the analog for cAMP binding sites. To ensure that the m.w. 54,000 and 49,000 proteins corresponded to Rn and Rn, respectively, lung cytosols were fractionated by DEAE-cellulose chromatography (6, 30). The m.w. 49,000 binding protein coelutes with type I kinase activity (low salt), and the m.w. 55,000 protein coelutes with type II kinase activity (high salt) (data not shown).

Rn is photolabeled in all of the samples, but Rn does not incorporate label in neonatal and tumor tissues (Fig. 1B). These results suggest an absence of type II kinase, but from our previously published studies with young mice (30) we know...
this is not the case. Fractionation of lung cytosol from Swiss-Webster mice by DEAE-cellulose chromatography showed that most of the kinase present in both neonatal and adult mice was the type II isozyme. We repeated these chromatographic experiments with the A/J mice used in the present studies and obtained identical results; i.e., the reason for a lack of binding in neonatal mice is not due to a lack of the type II kinase isozyme. In other systems, photoincorporation of $8-N_3-[^{32}P]cAMP$ into R, and Rn is in close quantitative agreement with the relative amounts of the type I and type II isozymes (42).

The lack of such a correlation here suggests that the ability of R, to bind $8-N_3-[^{32}P]cAMP$ is regulated during the course of normal and possibly neoplastic development.

To determine the age at which $8-N_3-[^{32}P]cAMP$ labeling of lung R, begins, the lungs from mice of various ages were subjected to photolabeling (Fig. 2). R, incorporation of $8-N_3-[^{32}P]cAMP$ increased from a lack of any visible labeling at 10 and 23 days of age, gradually rose to a peak at 125 days, and then decreased with age. R, labeling was approximately constant until 243 days of age, after which time the binding of $8-N_3-[^{32}P]cAMP$ began to decrease. Labeling of the m.w. 37,000 proteolytic fragment was highest in the youngest mice and then declined. Mice of 3 to 5 months of age were chosen to represent the adult labeling pattern in subsequent experiments.

To investigate this phenomenon of an apparently blocked $8-N_3-[^{32}P]cAMP$ binding site on R, from tumors and neonatal lung, extracts were photolabeled using a wide range of ligand concentrations. After the proteins were separated by denaturing gel electrophoresis, the amount of radioactivity incorporated into each band was determined. The resulting binding curves are shown (Chart 1). In the adult animal (Chart 1A), R, appears to have one high-affinity binding site which saturates at 125 nm $8-N_3-[^{32}P]cAMP$, while Rn has equal numbers of high- and low-affinity binding sites. The R, high-affinity site saturates at about 125 nm, while the low-affinity site saturates at about 800 nm. An equivalent amount of binding to 2 sites differing in their affinity for $8-N_3-[^{32}P]cAMP$ is consistent with recent findings demonstrating the presence of 2 cAMP binding sites/ regulatory subunit monomer, each with characteristic affinities (7, 22, 45). The sigmoidal shape of the high-affinity portion of the R, binding curve suggests positive cooperative binding. In the neonatal animal (Chart 1A), the R, binding curve is essentially identical to that seen in the adult. In contrast, the neonatal R, binding curve reflects either a great reduction in the affinity of all R, binding sites for $8-N_3-[^{32}P]cAMP$ or a complete inhibition of $8-N_3-[^{32}P]cAMP$ binding at a majority of the sites. The mechanism by which Rn binding is specifically regulated during lung development is unknown. Passage of neonatal lung cytosol through a Sephadex column does not significantly increase the ability of Rn to bind $8-N_3-[^{32}P]cAMP$, implying that the masking effect is not mediated through a freely diffusible small molecule.4

In the tumor extract (Chart 1B), appreciable binding to Rn is seen only at relatively high ligand concentrations. The shape of the tumor binding curve suggests that the high-affinity binding site is missing, while the low-affinity site is intact. The binding of $8-N_3-[^{32}P]cAMP$ to Rn from uninvolved lung is similar to that of adult lung, with 2 apparent classes of Rn binding sites present. The minor differences between the normal adult and uninvolved curves probably result from experimental manipulations, e.g., the time required to dissect the uninvolved lung, heat from the microscope light, etc. In control experiments where normal adult lung was subjected to these conditions, the adult lung Rn binding curve was identical to that of uninvolved lung.

Structural differences between tumor and uninvolved lung Rn were analyzed by separating photolabeled proteins by 2-dimensional gel electrophoresis. Three molecular weight classes of $8-N_3-[^{32}P]cAMP$-binding proteins are seen in the resulting autoradiograms (Fig. 3), corresponding to Rn, R,, and the m.w. 37,000 proteolytic fragment. R, at a molecular weight of 49,000, is separated into 4 distinct protein species, ranging in pi from 5.42 to 5.61. These same species are seen in both tumor and uninvolved lung cytosols. The number and relative intensities of R, spots vary in different tissues and between species, but the pattern for a single organ from the same species is very reproducible (33a). This suggests that these heterologously charged R, proteins reflect in vivo microheterogeneity and do not arise from artifacts created during experimental manipulations. Different concentrations of $8-N_3-[^{32}P]-cAMP$ do not change the relative intensities of the R, spots, suggesting that the relative affinities of each charge variant for

4 M. S. Butley, D. S. Beer, and A. M. Malkinson, unpublished results.
8-N3-[32P]cAMP are similar. A single Rn spot (m.w. 54,000; pl 5.1) is observed when uninvolved lung cytosol is labeled with 125 nM 8-N3-[32P]cAMP (Fig. 3B). This spot is not present in the corresponding tumor extract, which is consistent with the results of the binding curves (Chart 1). Autoradiograms resulting from the 2-dimensional electrophoretic separation of 8-N3-[32P]cAMP-labeled neonatal lung proteins are identical to that of the tumor extract in Fig. 3A, and the adult lung pattern is identical to the uninvolved lung pattern shown in Fig. 3B.

**Limited Proteolysis.** It was of interest that, even though there were multiple charge species of Rn and Rh, only a single m.w. 37,000 proteolytic fragment was observed in both the tumor and the uninvolved lung samples (Fig. 3). This suggested that either limited proteolysis of all forms of the regulatory subunits resulted in an identical m.w. 37,000 digestion product or that only a single regulatory subunit form was sensitive to endogenous proteolysis. To distinguish between these possibilities, 8-N3-[32P]cAMP-labeled adult lung was treated with increasing increments of the exogenous protease, trypsin (Fig. 4). At the lowest trypsin concentration, the label disappeared from the Rn and Rh positions and reappeared as a series of heterologously charged proteins of m.w. 37,000. (Fig. 4B). This implies that the m.w. 37,000 protein observed in the absence of exogenously added protease (Fig. 4A), results from the specific cleavage of a single regulatory subunit charge variant by endogenous proteases. The exogenously applied trypsin apparently cleaves all regulatory subunits, giving rise to additional m.w. 37,000 proteolytic fragments with slightly different charge. Additional trypsin wipes out any residual undigested regulatory subunits (Fig. 4C). At the highest trypsin concentration, the m.w. 37,000 charge variants are converted into multiple m.w. 32,000 fragments (Fig. 4D).

Having established the digestion pattern for adult lung, digestion of the regulatory subunits from the lung tumor was investigated. Undigested, 8-N3-[32P]cAMP-labeled regulatory subunits were mixed with labeled extracts treated with various amounts of trypsin so that the native proteins and the partially digested species could be examined simultaneously. The resultant 2-dimensional electrophoretic patterns (Fig. 5) are very reproducible. The tumor pattern is very similar to the neonatal mixture of native proteins and proteolytic fragments. The uninvolved and adult patterns are similar to each other but somewhat different from the tumor-neonatal mixtures. The various m.w. 37,000 peptides in the tumor-neonatal series arose from digestion of labeled Rh, since only Rh had been labeled. The main difference between the tumor-neonatal and the uninvolved-adult mixtures can thus be attributed to the Rh digestion products present in the latter samples.

**Autophosphorylation of Rn.** Among the protein phosphorylation reactions stimulated by cAMP is the intramolecular phosphorylation of Rn by the catalytic subunit of the type II kinase isozyme. cAMP stimulates adult lung Rn phosphorylation when Mg2+ is the divalent cation present during the reaction (Fig. 6). When this reaction is carried out in the presence of Zn2+, however, the addition of cAMP inhibits Rn phosphorylation, as has also been found with other tissue extracts (31) and with purified type II kinase (25).

In the tumor, the stimulatory effect of cAMP on Rn phosphorylation seems to be negligible. cAMP strongly inhibits tumor Rn phosphorylation in the presence of Zn2+, however. The extent to which neonatal lung proteins were endogenously phosphorylated was so slight that the film exposure period had to be greatly increased for any phosphoproteins to become visible. Stimulation of neonatal Rn phosphorylation by cAMP in the presence of Mg2+ did occur, but no Zn2+-dependent inhibitory effect was observed. The effects of cAMP on Rn phosphorylation thus vary during normal and neoplastic development.

**DISCUSSION**

We have herein described 2 classes of binding sites on adult lung Rn, which differ in their affinity for 8-N3-[32P]cAMP. The ability of Rn to bind 8-N3-[32P]cAMP varies with age and neoplastic state. Neonatal Rn binds 8-N3-[32P]cAMP only slightly, while tumor Rn lacks the higher-affinity binding site. In addition to altered 8-N3-[32P]cAMP binding, tumor and neonatal lung extracts differ from adult lung in their ability to phosphorylate Rh and in the effects of cAMP on this autophosphorylation reaction. Each of these findings is discussed below, as are the functional implications of these changes in Rn with respect to the regulation of growth by cAMP.

Prior to a few years ago, it was believed that each regulatory subunit monomer bound one molecule of cAMP, and interpretation of the regulation of kinase activity by cAMP was based on this assumption (38). Very few earlier studies had provided evidence for more complex binding of cAMP to regulatory subunits (24). The stoichiometry of cAMP binding was typically measured using a filtration assay through a cellulose acetate filter (14). More recently, 3 laboratories have independently measured cAMP binding by other techniques and found that 2 molecules of cAMP were bound to each Rh and Rn monomer (7, 22, 45). Binding of regulatory subunits to the cellulose acetate filter caused the release of one-half of the total bound cAMP (7, 22, 45). Within the past year, Rannels and Corbin (37) measured the rates of cAMP dissociation from regulatory subunits in the presence of various cAMP derivatives. The 2 intrachain cAMP binding sites differ in their affinity for the various cAMP analogs, including 8-azidoadenosine cyclic 3':5'-monophosphate. This difference between the 2 cAMP binding sites on each regulatory subunit monomer is consistent with our results on the covalent labeling of Rn from adult mouse lung by 8-N3-[32P]cAMP. Why multiple affinities of Rn for 8-N3-[32P]cAMP were not apparent and whether 8-N3-[32P]cAMP binding can discriminate between multiple sites on regulatory subunits from other tissues is not known.

Our results suggest that the 2 intrachain cAMP binding sites may have unique physiological roles because of the differential regulation of these sites during normal and neoplastic development. This is the first evidence for a functional difference between these Rn binding sites. The absence of the high-affinity class in the tumor implies a physiological loss or a decreased availability of these sites. The greatly reduced binding in the neonate suggests that both the high- and low-affinity sites are blocked. This reduction in neonatal Rn binding is not due to a decreased Rn concentration, since neonatal mouse lung contains the same amount of type II kinase activity as does adult lung (30). ATP and ionic strength have been shown to affect the ability of regulatory subunits to bind 8-N3-[32P]cAMP (19), but the masking of neonatal Rn binding sites is probably not caused by a freely diffusible small molecule since the sites remain blocked after Sephadex filtration. The nearly complete absence of 8-N3-[32P]cAMP incorporation into neonatal lung Rn...
may be due to a structurally altered Rn protein. One of the early studies suggesting multiple intrachain cAMP binding sites also described different cAMP binding characteristics between normal liver and HTC hepatoma cells (24). While liver Rn had both high- and low-affinity sites for binding cAMP, the HTC cells lacked the high-affinity sites. This difference was ascribed to structural alterations in the Rn of the HTC cells (25). This is very analogous to our results, even though we used a different tumor system, a cAMP derivative rather than cAMP, and a different type of binding assay.

In addition to first demonstrating 2 cAMP binding sites on each regulatory subunit, Corbin and colleagues (11) also were the first to demonstrate that each Rn monomer contains 2 phosphorylatable sites. These sites differ in their rate of phosphate incorporation. They suggest the presence of 2 functional domains/Rn monomer (11). Each domain contains one cAMP binding site and one phosphorylatable site, and each domain has its own characteristic rate of phosphorylation and affinity for cAMP. Our results suggest a functional correlation between cAMP binding and phosphorylation in a given domain. Adult and uninvolved tissues contain both high- and low-affinity 8-N3-32P-cAMP binding sites and display both the stimulatory and inhibitory effects of cAMP on Rn phosphorylation. Neonatal lung has greatly reduced 8-N3-32P-cAMP binding to both Rn sites and can phosphorylate Rn only slightly. The low-affinity 8-N3-32P-cAMP binding site and the phosphorylatable site which is inhibited by cAMP in the presence of Zn2+ are both present in the tumor. Thus, the 8-N3-32P-cAMP binding sites and the phosphorylatable sites vary coordinateably with age and with neoplastic state.

The cellular function of Rn autophosphorylation is not known. Hoyer et al. (19) have presented a model of type II kinase activation by cAMP in which the ability of the kinase subunits to reassociate depends upon the phosphorylation state of Rn. This follows the demonstration that unphosphorylated Rn reassociates with the catalytic subunit more readily than does phosphorylated Rn (36). Autophosphorylation may serve other regulatory functions as well. Phosphorylation of Rn modifies its sensitivity to endogenous proteolysis and, thus, its conversion to a m.w. 37,000 proteolytic fragment (35). Our finding that probably only one of the multiple regulatory subunit forms is sensitive to endogenous proteolysis implies that limited proteolysis of regulatory subunits may be extremely specific in vivo. Since the m.w. 37,000 fragment can bind cAMP but cannot inhibit catalytic subunit activity, it may modulate the free intracellular cAMP concentration and prolong the activation state of catalytic subunits (44). Others have reported more endogenous regulatory subunit proteolysis in malignant tumors than in their normal tissues of origin (20), but we observed no analogous increase in the pulmonary adenoma (Figs. 1 and 4). More of the endogenously produced m.w. 37,000 fragment was present in neonatal lung than in adult lung (Figs. 1 and 2). Finally, at least one of the phosphorylated serines on Rn is at the site which interacts with the catalytic subunit (7). The presence or absence of phosphate at this site may influence this Rn-catalytic subunit interaction.

Alterations in cAMP binding by Rn and Rb in neoplastic tissues could provide an escape from growth regulation by cAMP. Changes in Rn from Walker mammary carcinoma at least partially determine the responsiveness of cell growth to hormonal manipulations. Clones can be isolated from these tumors which do not stop growing in response to dibutyryl cAMP, and these cells have cAMP binding characteristics different from responsive cells (2). The regulatory subunit in the insensitive cells has an altered sensitivity to heat, suggesting a structural alteration (3). Increased type II kinase activity can be observed in responsive cells prior to hormonally induced changes in tumor size (4). Another example of a correlation between decreased responsiveness to growth control by cAMP and altered kinase regulatory subunits can be observed in the S49 lymphoma system. Dibutyryl cAMP normally inhibits the growth of these cells, but cAMP-resistant S49 cells have been induced with mutagens (9). The genetic lesion in nearly all of the independently derived variants was a structural change in the protein kinase regulatory subunits (40). The dose-response curve of the mutant lymphoma cells to growth inhibition by dibutyryl cAMP was consistent with the altered dissociation constants of the regulatory subunits for cAMP binding (17).

In summary, we have found functional changes in the regulatory subunits from the type II isozyme of cAMP-dependent protein kinases during normal and neoplastic lung development. These are manifested as altered 8-N3-32P-cAMP binding and changes in the regulation and/or amount of Rn phosphorylation. Such changes may affect the responsiveness of the tumor to the regulation of growth by cAMP.

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Fig. 1. Major soluble proteins separated by SDS-gel electrophoresis (A) and autoradiogram (B) showing those soluble proteins which incorporated 8-N3-[32P]cAMP in extracts from urethan-induced lung adenoma (T), uninvolved lung (U), whole neonatal (N), and whole adult (A) lung. The lane on the left of A contains proteins used as molecular weight markers. Samples were photolabeled with 60 nM 8-N3-[32P]cAMP in the absence (−) or presence (+) of 40 μM cAMP. After electrophoresis, the samples were stained with Coomassie blue (A), and the gel was dried and then exposed to X-ray film to produce the autoradiogram shown on B. The molecular weights of the labeled proteins in the autoradiogram are shown: Rα, 54K; Rβ, 49K, and a proteolytic fragment, 37K.

Fig. 2. Autoradiogram showing the effect of age on 8-N3-[32P]cAMP binding to proteins of mouse lung. Lung extracts were made from mice ranging in age from 10 to 363 days and photolabeled with 100 nM 8-N3-[32P]cAMP as described in “Materials and Methods.” The molecular weights of the labeled proteins, as determined using protein markers of known molecular weights, are indicated.

Fig. 3. Autoradiogram of proteins from lung tumor (A) and uninvolved tissue (B) labeled with 100 nM 8-N3-[32P]cAMP and fractionated by 2-dimensional gel electrophoresis.
Fig. 4. Autoradiogram of adult lung proteins labeled with 100 nM 8-N3-[32P]cAMP and digested with varying amounts of trypsin prior to 2-dimensional gel electrophoresis. A, no trypsin; B, 20 µg trypsin per ml; C, 60 µg trypsin per ml; D, 200 µg trypsin per ml.

Fig. 5. Autoradiogram of mixtures of 8-N3-[32P]cAMP-labeled proteins digested with various amounts of trypsin. Extracts of tumor (A), uninvolved lung (B), whole neonatal lung (C), and whole adult lung (D) were labeled with 100 nM 8-N3-[32P]cAMP. Aliquots of labeled proteins were digested with either 20 or 60 µg of trypsin per ml. Undigested samples were mixed with samples which had been treated with each of the 2 trypsin concentrations. These mixtures were fractionated by 2-dimensional gel electrophoresis, and autoradiograms were made. The molecular weights of the native (54K and 49K) and digested products (37K and 32K) are shown, along with relevant pH values.

Fig. 6. Autoradiogram of soluble proteins from adult lung, tumor, and neonatal lung which were phosphorylated with [γ-32P]ATP in the presence of either 10 mM MgCl₂ or 5 mM ZnCl₂, with or without 10 µM cAMP. The location of R₆ (54K) on the gel is shown.
Alterations in Cyclic Adenosine 3′:5′-Monophosphate-dependent Protein Kinases during Normal and Neoplastic Lung Development

Alvin M. Malkinson and Martin S. Butley


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