Cyclic AMP Receptor Triggers Nuclear Protein Phosphorylation in a Hormone-Dependent Mammary Tumor Cell-Free System

Abstract. *Adenosine 3',5'-monophosphate (cyclic AMP) receptor protein of 56,000 daltons increases markedly in mammary tumors induced by 7,12-dimethylbenz[a]anthracene (DMBA) after incubation of tumor slices with cyclic AMP, benzamidine, and arginine. Incubation of cytosol from these tumor slices with nuclei from unincubated tumors results in nuclear uptake of the 56,000-dalton cyclic AMP receptor and in phosphorylation of the 76,000-dalton nuclear protein. Binding of the 56,000-dalton receptor and phosphorylation of the 76,000-dalton protein also occur in DMBA tumor nuclei when protein kinase type II of bovine heart is used. The results suggest that cyclic AMP receptor is involved in the nuclear events of a hormone-dependent mammary tumor.*

We recently reported (*1*) that during growth arrest of rat mammary carcinoma induced by 7,12-dimethylbenz[*a*]-anthracene (DMBA) after either hormonal deprivation (ovariectomy) or treatment with $N^6,O^{2'}$-dibutyryl adenosine 3',5'-monophosphate (dibutyryl cyclic AMP), a nonhistone basic protein species, regression-associated protein (RAP), becomes the predominant endogenous substrate for cyclic AMP-dependent protein kinase in the tumor nuclei. Phosphorylation of RAP occurs in vitro after incubation of tumor slices with cyclic AMP, and this cyclic AMP effect is abolished by the simultaneous addition of 17β-estradiol in the incubation medium (*2*). The exogenous substrate specificity and the effect of protein kinase inhibitor protein (*1, 3*) on the nuclear kinase activity suggested that the phosphorylation of RAP is probably due to cyclic AMP-dependent protein kinase that translocates from the cytoplasm to the nucleus. This occurs in vivo during tumor regression after ovariectomy or dibutyryl cyclic AMP treatment (*1*) and in vitro during treatment of tumor slices with cyclic AMP (*2, 3*). It has been postulated that the translocation of cytoplasmic cyclic AMP-dependent protein kinase to the nucleus influences gene expression in other tissues (*4*).

In the work reported here, we explored the role of cyclic AMP receptor, the regulatory subunit of cyclic AMP-dependent protein kinase (*5*), in the nuclear protein phosphorylation of DMBA-induced mammary carcinoma in a cell-free system (we use the term receptor to denote a high-affinity cyclic AMP binding protein). Our results support the following conclusions: (i) cyclic AMP receptor of 56,000 daltons from both DMBA-induced tumor and protein kinase of bovine heart can bind to DMBA tumor nuclei; (ii) concomitant with the nuclear binding of the 56,000-dalton protein is the appearance of new phosphorylation of the 76,000-dalton nuclear protein of DMBA tumor; and (iii) nuclear translocation of protein kinase type II, which influences nuclear events of DMBA tumor, may have occurred by means of the protein kinase holoenzyme–cyclic AMP complex.

Slices from growing DMBA-induced tumors were incubated at 30°C with $10^{-7}M$ cyclic AMP in the presence of benzamidine and arginine. Cytosols, prepared from either incubated or unincubated tumor slices, were incubated at 0°C for 1 hour with whole nuclei of unincubated tumors in the presence of $10^{-8}M$ cyclic AMP; then the washed nuclei were incubated with [γ-^{33}P]adenosine triphosphate (ATP) and the nuclear protein phosphorylation was measured.

New phosphorylation of RAP (*1*) (Fig. 1C) was induced when the "activated" cytosols (cytosols prepared from incubated tumor slices) were incubated with the nuclei, whereas new phosphorylation of RAP was not seen when the cytosols (data not shown) and the nuclei (Fig. 1A) were incubated separately or the nuclei were incubated with control cytosols (cytosols prepared from unincubated tumor slices) (Fig. 1B). Figure 1 also shows that phosphorylation of growth-associated protein (GAP) (*1*), the major radioactivity peak of growing DMBA tumor nuclei (Fig. 1A), was appreciably decreased concomitant with the appearance of RAP phosphorylation (Fig. 1C). The peak radioactivity of RAP coincided with the molecular species of 76,000 daltons (Fig. 1C), as we previously observed with both regressing DMBA tumors and DMBA tumor slices incubated with cyclic AMP in vitro (*1, 2*). Stimulation of RAP phosphorylation and inhibition of GAP phosphorylation in DMBA tumor nuclei also occurred with protein kinase type II (*6*) holoenzyme from bovine heart (Fig. 1F). The nuclear protein phosphorylation could not be reproduced with only the catalytic subunit of protein kinase, C (*5*) (Fig. 1E), probably because it required a special (activated) form of the kinase holoenzyme, RC (*5*). We found that the apparent activation of the kinase was dependent on the concentration of cyclic AMP (optimum concentration, $10^{-8}M$), the presence of the proteolysis inhibitors benzamidine and polyarginine, and temperature (*7*).

It is not likely that RAP phosphorylation was due to activation of the endogenous protein kinase by either cyclic AMP or proteolysis inhibitor, since no RAP phosophorylation occurred when the nuclei were incubated with cyclic AMP, benzamidine, polyarginine, or arginine in the absence of the activated kinase or the activated cytosol (data not shown). Thus it appears that the phosphorylation of RAP may be due to new penetration of cyclic AMP-dependent protein kinase into the nucleus. In fact, the kinase extracted [with Triton X-100 (*3*)] from the nuclei that exhibit RAP phosphorylation was stimulated by cyclic AMP and preferentially utilized histone as an exogenous substrate, whereas the kinase extracted from the nuclei that show no RAP phosphorylation exhibited no cyclic AMP stimulation and preferentially utilized casein (data not shown), as demonstrated in studies in vivo (*1*).

The data of Fig. 1 also show that the radioactive peak of the 76,000-dalton protein species in DMBA tumor nuclei was always accompanied by other radioactive protein species, as shown in studies in vivo (*1*). One such radioactive protein species coincided with the 56,000-dalton protein (Fig. 1, C and F), which is the major phosphorylated protein of protein kinase type II (Fig. 1D) (*8*) as well as the regulatory subunit—that is, the cyclic AMP binding protein of the kinase (*6, 8*). This observation suggested that cyclic AMP binding protein is involved in nuclear protein phosphorylation. More conclusive evidence that cyclic AMP binding protein penetrates the nuclei was shown by the experiments using the photoaffinity cross-linking technique (*9*). When covalent attachement of cyclic 8-N_3-[^{32}P]AMP (*10*) to protein kinase type II from bovine heart and cross-linking of the cyclic AMP–receptor complex to the nucleus were followed by [^{33}P]-ATP incorporation into the nuclear proteins, both ^{32}P and ^{33}P radioactivity peaks coincided with the 56,000-dalton protein species, with the simultaneous appearance of a ^{33}P peak that coincided with the 76,000-dalton protein species (*7*).

That phosphorylation of these nuclear proteins is a physiological event rather than an experimental artifact was suggested by the following results (data not shown). First, phosphorylation patterns similar to those shown in Fig. 1 were ob-

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served with highly purified nuclei (*11*) before and after incubation with cytosol. Second, activated cytosol from a hormone-independent tumor (one that does not regress following ovariectomy or dibutyryl cyclic AMP treatment) with a high cyclic AMP binding activity (*12*) failed to stimulate nuclear protein phosphorylation. Third, more than 80 percent of the radioactivity was released from the phosphorylated nuclei only after extraction with a high-salt solution (1.0*M* KCl, 0°C, 2 hours) or Triton X-100 (0.1 percent, 0°C, 2 hours). Unlike the nonspecific binding to the particulate fraction of catalytic subunits of cytosol protein kinase, which can be inhibited by hypertonic (*13*) or isotonic (*14*) media, the nuclei-bound radioactivity was not resolved by 0.3*M* KCl. Fourth, when the phosphorylated nuclear proteins were treated with hydroxylamine before electrophoresis, no loss of incorporated ^{33}P was observed; however, all radioactivity incorporated was released when the nuclei were treated with 1*N* NaOH at 95°C for 5 minutes. This suggested that ^{33}P incorporation probably occurred in the nuclear proteins by a phospho-*O*-amino acid linkage.

Figure 2 shows a comparison of cyclic AMP binding proteins derived from DMBA tumor cytosol and bovine heart protein kinase type II by the use of the photoaffinity label cyclic 8-N_3-[^{32}P]AMP (*10*) and endogenous phosphorylation (*15*). By incorporation of this label, it was shown that the cytosol from growing DMBA tumor (Fig. 2a) contained a major cyclic AMP binding protein of 39,000 daltons and a minor binding species of 56,000 daltons, whereas the protein kinase type II from bovine heart contained mostly the 56,000-dalton receptor (Fig. 2c). However, after incubation of DMBA tumor slices with cyclic AMP, benzamidine, and arginine, a marked increase of only the 56,000-dalton receptor protein occurred in the cytosol (Fig. 2b). Benzamidine and arginine enhanced the increment of 56,000-dalton receptor in

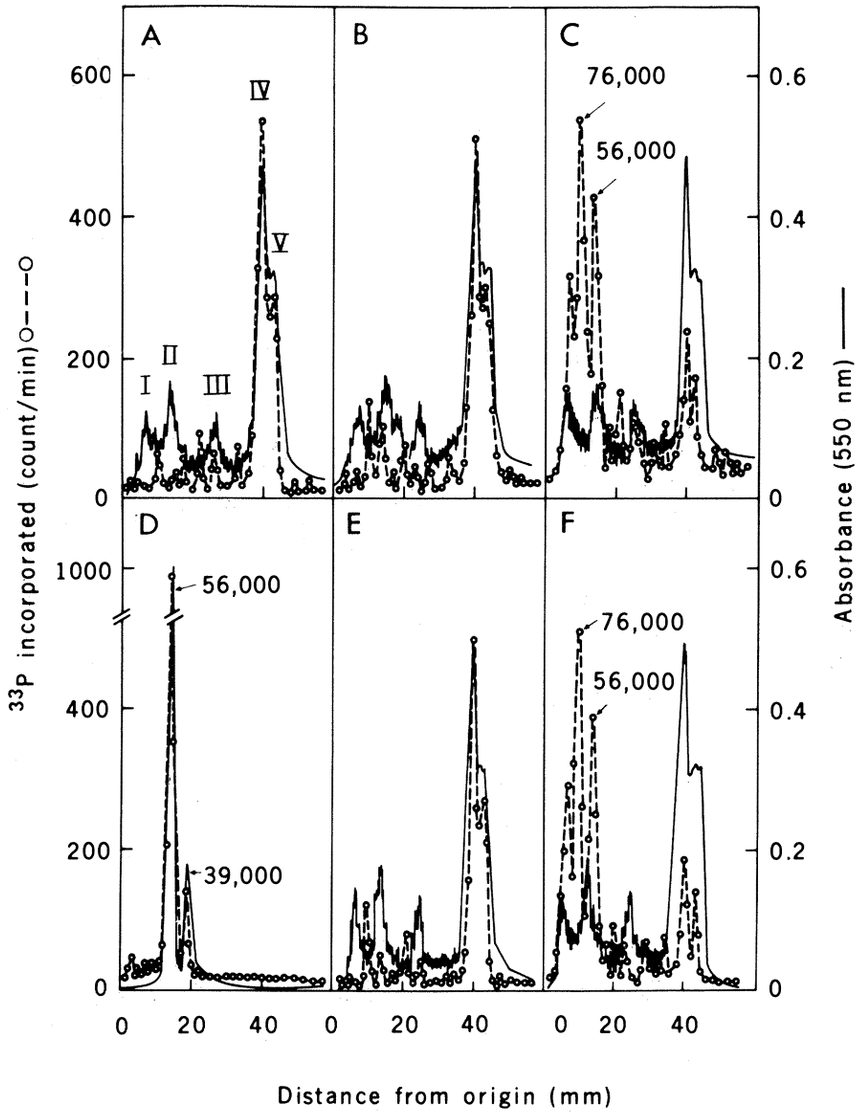

Fig. 1. Cyclic AMP receptor binding and protein phosphorylation in DMBA-induced mammary tumor nuclei in a cell-free system. Slices of six pooled tumors (0.2 g) were incubated in five volumes of buffer A (0.25*M* sucrose, 2 m*M* $MgCl_2$, 1 m*M* $CaCl_2$, 10 m*M* KCl, and 20 m*M* tris-HCl, *p*H 7.5) containing 50 m*M* L-arginine at 30°C for 15 minutes; then $10^{-7}M$ cyclic AMP and 50 m*M* benzamidine were added to the mixture. Incubation reactions were stopped after 15 minutes by diluting the mixtures with 2.5 volumes of cold buffer A. Samples were immediately centrifuged (755*g*, 10 minutes), washed once with cold buffer A, homogenized with ten volumes of buffer A, centrifuged at 105,000*g* for 60 minutes, and the resulting supernatants designated as activated cytosols. Control cytosols were prepared from slices of six pooled unincubated tumors or tumor slices incubated in the absence of cyclic AMP, benzamidine, arginine as described. Nuclei were prepared from unincubated tumor slices as previously described (*1*). Purified protein kinase type II from bovine heart (Sigma Chemical Co.) (35 μg) in 0.5 ml of buffer A containing 0.1 percent bovine serum albumin was incubated at 23°C for 60 minutes with $10^{-8}M$ cyclic AMP, 50 m*M* benzamidine, and polyarginine (10 μg per 0.1 ml), and designated as activated PK type II. A 0.5-ml sample (1.2 mg of protein) of control or activated cytosol (from a 0.06-g tumor) was then incubated with 0.2 ml (0.15 mg of protein) of nuclear suspension (in buffer A, from a 0.2-g tumor) and 0.3 ml of buffer A containing $10^{-8}M$ cyclic AMP at 0°C. In parallel incubations, nuclei were incubated alone in 1.0 ml of buffer A containing $10^{-8}M$ cyclic AMP and also in the presence of 35 μg of either activated PK type II or catalytic subunit of kinase (Sigma Chemical Co.) in the manner described for incubation of nuclei with cytosol. After 1 hour at 0°C, incubation mixtures were centrifuged (755*g*, 10 minutes) and nuclear pellets were washed once with five volumes of cold buffer A. The nuclear pellets (consisting mostly of morphologically intact nuclei with slight contamination by cell debris as seen by phase-contrast microscopy) were incubated with 12.6 μM [γ-^{33}P]ATP (25 Ci/mmole, ICN Pharmaceuticals) to phosphorylate the nuclear proteins via endogenous protein kinase (*1*). Nuclear pellets were then treated with 1 percent SDS and 1 percent mercaptoethanol, dialyzed, and subjected to electrophoresis in 0.1 percent SDS–10 percent polyacrylamide gels (*1*). Protein kinase type II was self-phosphorylated and subjected to electrophoresis in the same manner. Samples containing 40 μg of protein were loaded onto all gels. Parallel gels were stained in Coomassie blue (*19*) and scanned at 550 nm (*20*). Incorporation of ^{33}P into nuclear proteins was measured in 1-mm gel slices (*1*). Maximum phosphorylation was reached within 15 minutes after incubation (*1*). Radioactivity recovery after electrophoresis was 60 percent in all gels. Protein concentrations were determined by the method of Lowry *et al.* (*21*). Molecular weights of polypeptides were estimated from a standard curve of log molecular weight versus mobility (*19*), prepared from polypeptide chains of known molecular weight.

the cytosol after incubation of tumor slices with cyclic AMP (data not shown). This may have been due to inhibition of proteolysis-induced degradation of the 56,000-dalton receptor, as has been shown with purified protein kinase type II (*8*). The 56,000-dalton cyclic AMP receptor protein comigrated on sodium dodecyl sulfate (SDS)–polyacrylamide gels with an endogenously phosphorylated protein of 56,000 daltons, as shown by both tumor cytosols and purified preparations of protein kinase type II from bovine heart (Fig. 2). The endogenous phosphorylation of 56,000-dalton protein may represent autophosphorylation of protein kinase type II—that is, phosphorylation of the regulatory subunit by the catalytic subunit (*15*). This is suggested by the fact that phosphorylation occurred in the presence of either Mg^{2+} or Zn^{2+} (Fig. 2), which has been described as characteristic of autophosphorylation of purified protein kinase type II (*16*). Endogenous phosphorylations of other proteins were considerably inhibited in the presence of Zn^{2+} (Fig. 2), as demonstrated with other tissues (*17*). Thus the cyclic AMP receptor protein of 56,000 daltons that is present in DMBA tumor cytosol appears to be the regulatory subunit of protein kinase type II which binds to the nuclei.

In studies with the purified regulatory subunit, R (*5*), of protein kinase from bovine heart, Corbin *et al.* (*8*) showed that cyclic AMP binding protein of 56,000 daltons can be broken down by proteolysis to a 39,000-dalton protein that retains the intact cyclic AMP binding sites but has a reduced ability to bind to the catalytic subunit. This finding together with our present data showing nuclear binding of 56,000-dalton but not of 39,000-dalton cyclic AMP receptor and inability of the protein kinase catalytic subunit to stimulate nuclear protein phosphorylation in DMBA tumor nuclei (Fig. 1) suggest that the penetration of protein kinase type II into DMBA tumor nuclei may have occurred by means of the activated holoenzyme (RC)–cyclic AMP complex, as previously postulated in other tumor systems (*18*). New phosphorylation of the nuclear protein RAP may be due to the subsequent release of the catalytic subunit from the complex.

We have described the regulatory mechanism of a specific nuclear protein phosphorylation of a hormone-dependent mammary carcinoma in a cell-free system. Interaction of the 56,000-dalton cyclic AMP receptor protein of cytosol with both the catalytic subunit of cyclic AMP–dependent protein kinase and cyclic AMP in the form of an activated ternary complex is proposed. This complex may penetrate into the nuclei and stimulate new phosphorylation of a nuclear protein. Of particular significance is our finding that purified protein kinase type II holoenzyme from bovine heart mimicked the action of kinase type II from DMBA-induced tumor in that the regulatory subunit of the 56,000-dalton protein binds to tumor nuclei and concomitantly stimulates a specific nuclear protein phosphorylation. Our results suggest a universal role for the 56,000-dalton cyclic AMP receptor, which appears to be species-nonspecific among eukaryotic organisms.

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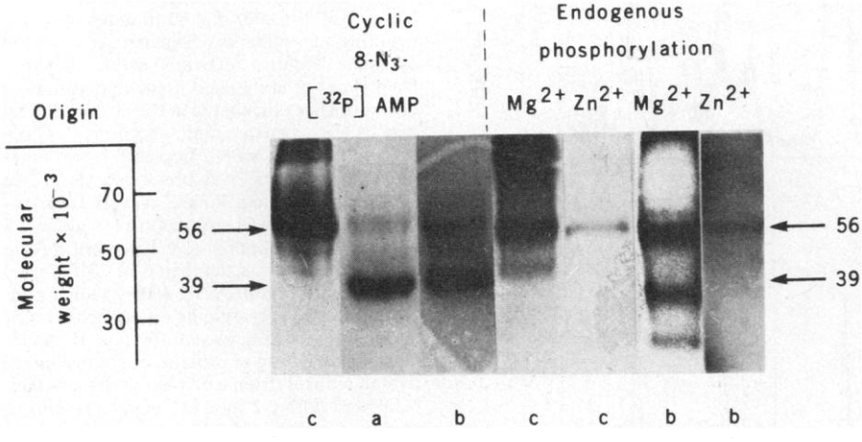

Fig. 2. Autoradiograph showing cyclic 8-N_3-[^{32}P]AMP labeling and endogenous phosphorylation of proteins in (a) control and (b) activated cytosols from DMBA tumor slices and (c) protein kinase type II from bovine heart. Control and activated cytosols were prepared as in Fig. 1. Photoactivated incorporation of cyclic 8-N_3-[^{32}P]AMP was performed by the method of Pomerantz *et al.* (*10*) with a slight modification. The reaction mixtures (final volume, 30 μl) contained $4 \times 10^{-7}M$ cyclic 8-N_3-[^{32}P]AMP (60 Ci/mmole, ICN Pharmaceuticals) and various amounts of cytosol in buffer A (see Fig. 1); preincubation was carried out at 23°C for 60 minutes in the dark. After irradiation, the samples were mixed with 15 μl of SDS-containing "stop" solution (*10*) and heated at 65°C for 30 minutes. Samples containing 40 μg of protein were then subjected to 0.05 percent SDS–10 percent polyacrylamide slab gel electrophoresis modified according to Maizel (*22*). Proteins were stained with Coomassie blue (*19*) or slab gels were soaked in acetic acid (10 percent) solution containing glycerol (3 percent) for 60 minutes and dried; autoradiography was then carried out (*23*). Apparent molecular weights of polypeptides were estimated as described in Fig. 1. Radioactive bands were localized by autoradiography, cut out of the dried gels, and counted by liquid scintillation. Incorporation of cyclic 8-N_3-[^{32}P]AMP was proportional to the amount of cytosol protein up to 200 μg. Endogenous phosphorylation was measured (*17*) in reaction mixtures (final volume, 33 μl) containing 10 mM $MgCl_2$ or 5 mM $ZnCl_2$, $3.6 \times 10^{-5}M$ [γ-^{32}P]ATP (25 Ci/mmole, ICN Pharmaceuticals) and various amounts of cytosol in buffer A (*p*H 7.6), placed in a Dubnoff metabolic shaker. After incubation at 30°C for 5 minutes the reactions were stopped by the addition of 15 μl of the SDS-containing stop solution and the samples were heated at 60°C for 30 minutes. Samples containing 40 μg of protein were then subjected to slab gel electrophoresis and autoradiography as described above.

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