Effect of Retinoic Acid Treatment of F9 Embryonal Carcinoma Cells on the Activity and Distribution of Cyclic AMP-dependent Protein Kinase*

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Treatment of F9 teratocarcinoma cells with all trans-retinoic acid previously has been shown to induce differentiation to parietal endoderm and to enhance the responsiveness of these cells to the addition of cyclic AMP. Evidence is presented that retinoic acid treatment of F9 cells causes an increase in both cytosolic and plasma membrane-associated cyclic AMP-dependent protein kinase activities. Retinoic acid elicits a progressive increase in cytosolic kinase activity through 72 h. Cyclic AMP-dependent kinase activity of the plasma membrane fraction is enhanced within 3 h of retinoid treatment of F9 cells and reaches a maximum at 15 h after the addition of retinoic acid. The amount of regulatory subunits of cyclic AMP-dependent protein kinases I (RI) and II (RII) present in the subcellular fractions was quantitated by photoaffinity labeling with 8-azido-cyclic [32P]AMP. In the cytosol, the amount of RI and RII both increase with the period of retinoid treatment through 24 h, and remain elevated through 5 days; the ratio of RI/RII (~2.5) found in the cytosol remains constant following retinoic acid treatment. RI is much more prevalent than RII (RI/RII = 7.5) in the plasma membrane prepared from untreated F9 cells. Retinoic acid treatment elicits a preferential increase in the amount of the RII regulatory subunit associated with the membrane fraction. After 15 h of retinoid treatment, a 1.6-fold increase in RI is observed, while membrane-associated RII is increased 4.5-fold. After 24 h of retinoic acid treatment, the level of RI returns toward the level noted with untreated cells, while the amount of RII remains significantly elevated through 5 days of retinoid treatment. These findings suggest that the increase in cyclic AMP-dependent protein kinase activity and the specific accumulation of RII associated with the plasma membrane fraction may be early events of retinoic acid action to mediate eventual cellular differentiation.

Cyclic AMP has been implicated as a modulator of cellular differentiation in a variety of cell types (5, 6). In F9 cells treated with retinoic acid, the addition of cyclic AMP increases the production of plasminogen activator (4), and as described by Kuff and Fewell (7), cyclic AMP also causes the appearance of neural-like cells with elongated processes and elevated acetylcholinesterase activity. Recently, Strickland et al. (8) presented evidence to suggest that this cyclic AMP effect on F9 cells pretreated with retinoic acid served only to promote differentiation to the endodermal state. Nonetheless, it is of interest that control F9 cells do not respond in this manner to the addition of cyclic AMP (4, 7). This indicates that retinoic acid treatment of F9 cells induces changes which alter the response to this cyclic nucleotide. It is generally believed that cyclic AMP-dependent protein kinases are the major, if not the only receptors responsible for carrying out the biological effects of cyclic AMP (for reviews, see Refs. 9 to 11). The cyclic AMP-dependent protein kinases have been found in both soluble and particulate fractions of mammalian tissues (9, 10). Two major forms of the cyclic AMP-dependent protein kinase have been identified and are referred to as types I and II. These two forms of the kinase differ in the nature of their regulatory subunits (RI and RII) since their catalytic subunits appear to be identical (10, 12).

The primary cyclic AMP binding proteins present in mammalian cells are the RI and RII regulatory subunits of the cyclic AMP-dependent protein kinase (11, 13). Studies have shown that the photoaffinity label 8-azido-cyclic [32P]AMP can be utilized to accurately measure cyclic AMP-binding to these regulatory subunits (10-15). To determine what effect retinoic acid is having on F9 cells to enhance the response to cyclic AMP, we have investigated the effect of retinoic acid treatment of these cells on protein kinase activity. These studies include photoaffinity labeling of the RI and RII regulatory subunits with 8-azido-cyclic [32P]AMP to examine the effect of retinoic acid treatment of F9 cells on the relative distribution and amount of these cyclic AMP binding proteins in the cytosolic, nuclear, and plasma membrane subcellular fractions. Results of these studies are presented in this communication.

EXPERIMENTAL PROCEDURES

Materials—Histone type II-A (calf thymus), protein kinase inhibitor type III (porcine heart), and adenosine 3':5'-monophosphate (cyclic AMP) were from Sigma. All trans-retinoic acid was purchased from Eastman and 8-azido-cyclic [32P]AMP from ICN Pharmaceuticals. [γ-32P]ATP was obtained from Amersham and sodium 2-mercaptoethanol.

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Cells—The F9 line of embryonal carcinoma cells was kindly provided by Dr. Shoshana Segal (National Institutes of Health, Bethesda). The cells were grown as described elsewhere (16). Where indicated, retinoic acid, dissolved in absolute ethanol, was added 1 day after plating to give a final concentration of 0.1 μM.

Preparation of Cellular Fractions—All procedures were performed at 0-4 °C. Cells were washed 3 times with ice-cold buffer A (20 mM Tris, pH 7.4/0.28 M sucrose/2 mM MgCl2/1 mM CaCl2/10 mM KCl), removed from the dish by scraping, and homogenized with a Dounce homogenizer. Crude nuclei were obtained by centrifuging the homogenate at 800 × g for 10 min. The nuclei were further purified by resuspending the pellet in 5 to 10 volumes of buffer A containing 0.2 M sucrose and again centrifuging at 800 × g for 10 min. The nuclear pellet obtained was washed once by resuspending in buffer A. Purity of the nuclear fraction was monitored by electron microscopy.

Cytosolic and plasma membrane subcellular fractions were obtained as follows. The 800 × g supernatant from the initial step in nuclei preparation was centrifuged at 10,000 × g for 10 min. The pellet was retained for plasma membrane preparation. The supernatant was centrifuged at 100,000 × g for 45 min, and the resulting supernatant was used as the cytosolic fraction. The 10,000 × g pellet was resuspended in 1 ml of buffer A and further purified by centrifugation through a 10% Ficoll cushion as described by Pinkett and Anderson (17). The purified plasma membranes were washed once with buffer A and utilized experimentally on the same day as preparation.

Protein Kinase Assay—Protein kinase activity was determined by measuring the transfer of 32P from [γ-32P]ATP to histone type II-A (casein) as described by Corbin et al. (18). The reaction was initiated by the addition of 25 μl of cell extract (~800 μg of protein) to a mixture containing 25 mM morpholinoethanesulfonic acid, pH 7.0, 0.5 mM ethylene glycol bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid, 2.5 mM sodium fluoride, 5 mM magnesium acetate, 600 pg of histone, and 0.1 mM [γ-32P]ATP (~900 cpm/pmol) in a total volume of 100 μl. After incubating at 37 °C for 10 min, 25-μl aliquots of the reaction mixture were spotted onto filter paper strips (Whatman No. 3MM) and dropped into cold 5% trichloroacetic acid. A blank value, obtained by stopping the reaction at time zero, was subtracted from total 32P incorporation. Results are expressed as pmol of 32Pi incorporated/min/mg of cellular protein. When autophosphorylation of the isolated subcellular fractions was measured, histone was omitted from the reaction mixture.

Photoaffinity Labeling With 8-Azido-Cyclic [32P]AMP—Photoaffinity labeling of the RI and RII regulatory subunits of the cyclic AMP-dependent protein kinase was performed as described by Walter et al. (13). The reaction mixture (80 μl) contained 50 mM 4-morpholinoethanesulfonic acid, pH 6.2, 10 mM MgCl2, 0.4 or 1 μM 8-azidocyclic [32P]AMP, and 33 to 80 μg of protein from the subcellular fractions. Where indicated, 0.1 μM cyclic AMP was included to determine nonspecific labeling. Incubation was carried out for 30 to 60 min in the dark at 4 °C, and the samples then irradiated for 10 min with a UV lamp at a distance of 10 cm. The irradiated samples were pipetted into 20 μl of a stop solution containing 9% sodium dodecyl sulfate, 15% glycerol, 3 mM EDTA, and 30 mM Tris buffer, pH 8, and heated at 100 °C for 20 s. Two μl of 2-mercaptoethanol and 5 μl of 0.1% bromphenol blue in 50% glycerol were then added. The sample was then subjected to 10% polyacrylamide slab gel electrophoresis in the presence of sodium dodecyl sulfate. Molecular weight standards were run with each gel. Electrophoresis was carried out overnight at a constant current of 20 mA/gel. The slab gels were then stained for measuring the transfer of 32P from [γ-32P]ATP to histone type 11-A contained as follows. The 800 μl from the reaction mixture were spotted onto filter paper strips (Whatman No. 3MM) and dropped into cold 5% trichloroacetic acid. A blank value, obtained by stopping the reaction at time zero, was subtracted from total 32P incorporation. Results are expressed as pmol of 32Pi incorporated/min/mg of cellular protein. When autophosphorylation of the isolated subcellular fractions was measured, histone was omitted from the reaction mixture.

RESULTS

Effect of Retinoic Acid Treatment of F9 Cells on Cyclic AMP-dependent Protein Kinase Activity of Subcellular Fractions—There is at least a 2- to 3-day lag between initial treatment of F9 cells with retinoic acid and induced changes in cell morphology, growth rate, and adenylyl cyclase activ-

![Fig. 1 (left). Time course of the effect of retinoic acid treatment of F9 cells on cyclic AMP-dependent protein kinase activity present in the cytosol. F9 cells were treated with 0.1 μM retinoic acid for the time indicated and then cytosol was prepared as described. Phosphotransferase activity was measured in the presence and absence of 1 μM cyclic AMP as described under "Experimental Procedures." Cyclic AMP-dependent protein kinase activity was determined by subtracting the activity measured in the absence of cyclic AMP from that measured in the presence of cyclic AMP.](http://www.jbc.org/)
approximately twice as much type I as type II regulatory subunit (R_I/R_{II} = 2.3) (Fig. 3). R_I is much more predominant than R_{II} (R_I/R_{II} = 7.5) in the plasma membranes prepared from control cells. Differentiation of F9 cells to the endodermal cell type by treatment with retinoic acid for 3 days causes a 2- to 3-fold increase in both R_I and R_{II} present in the cytosolic fraction, with the ratio of R_I to R_{II} (1.9) remaining constant. Interestingly, a marked increase (3-fold) in the amount of R_{II} associated with the plasma membranes is observed following retinoic acid treatment of F9 cells. This results in a dramatic change in the ratio of R_I to R_{II} (R_I/R_{II} = 1.9) found in the plasma membrane fraction of retinoic acid-treated cells (Fig. 3).

Figs. 4 and 5 depict the results of studies to determine the time course for retinoic acid treatment to increase 8-azido-cyclic [\textsuperscript{32}P]AMP binding to R_I and R_{II} in the cytosolic and plasma membrane fractions. The data presented in Figs. 4 and 5 have been calculated to give fmol of 8-azido-cyclic AMP bound/mg of protein. The concentration of 8-azido-cyclic AMP (1 \mu M) used is saturating for total photoaffinity labeling.

In the cytosol, the amounts of R_I and R_{II} increase with increasing time of retinoic acid treatment through 24 h, and remain elevated through 5 days of such treatment (Fig. 4). The results shown in Fig. 5 again demonstrate a predominant increase in membrane-associated R_{II} following retinoic acid treatment of F9 cells. Both R_I and R_{II} are maximally increased after 15 h of retinoid treatment. At 24 h after the addition of retinoic acid to F9 cells, the level of R_I appears to regress back toward the level noted in untreated cells. The amount of R_{II} (though somewhat lower than the level found at 15 h of retinoid treatment) remains significantly elevated through 5 days of incubation of F9 cells with retinoic acid.

**Fig. 3.** Effect of retinoic acid treatment on the amount of 8-azido-cyclic [\textsuperscript{32}P]AMP specifically bound to proteins present in plasma membrane and cytosolic fractions of control F9 cells and of cells treated for 3 days with 0.1 \mu M retinoic acid. A and B, plasma membrane fractions from retinoic acid-treated and control F9 cells; C and D, cytosolic fraction from retinoic acid-treated and control F9 cells, respectively. Upper panel, autoradiograph showing the photoactivated incorporation of 8-azido-cyclic [\textsuperscript{32}P]AMP into cytosol and plasma membrane cyclic AMP binding proteins analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described under "Experimental Procedures." The subcellular fractions were photoaffinity-labeled with 0.4 \mu M 8-azido-cyclic [\textsuperscript{32}P]AMP in the presence and absence of 10 \mu M cyclic AMP as indicated. Eighty \mu g of cytosolic or plasma membrane protein was applied to each gel lane. Lower panel, quantitation of amount of radioactivity incorporated into each of the labeled bands detected by autoradiographic analysis in the upper panel. The \textsuperscript{32}P incorporated was determined by slicing the gel into 2-mm segments and counting each slice. Results are expressed as specific counts/min/slice. Peak I, M, \sim 49,000; peak II, M, \sim 56,000.

**Fig. 4.** Time course of the effect of retinoic acid treatment of F9 cells on 8-azido-cyclic [\textsuperscript{32}P]AMP photoaffinity labeling of the R_I (○—○) and R_{II} (■—■) regulatory subunits of cyclic AMP-dependent protein kinase present in the cytosolic fraction. Cytosol was prepared from control F9 cells and from cells treated with 0.1 \mu M retinoic acid for the time indicated. Fifty \mu g of cytosolic protein were labeled with 1 \mu M 8-azido-cyclic [\textsuperscript{32}P]AMP (50 counts/min/pmol) and then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The gels were sliced and counted to determine \textsuperscript{32}P bound, as described under "Experimental Procedures." Results are expressed as fmol of R_I or R_{II}/mg of protein applied to the gel, and the ratio (Δ—Δ) given is the fmol of R_I divided by fmol of R_{II} present.
fraction. In both cell types, there was membrane fraction. Plasma membranes were prepared from F9 cells and then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Panel of R₁ and R₂ and the ratio of R₁/R₂ (Δ—Δ) were determined as described in Fig. 4.

Other studies were carried out to determine the 8-azido-cyclic AMP photoaffinity labeling pattern of the nuclear fractions prepared from control F9 cells and from cells treated with retinoic acid for 3 days. Autoradiographic analysis indicated that treatment of F9 cells with retinoic acid did not appear to alter the amount of R₁ or R₂ found in the nuclear fraction. In both cell types, there was ~30 to 35 pmol of 8-azido-cyclic [³²P]AMP bound to each of R₁ and R₂/mg of nuclear protein applied to each gel lane. Very little cyclic AMP-dependent histone phosphorylation was detected in nuclei prepared from control and retinoic acid treated cells.

**DISCUSSION**

Retinoid treatment of numerous cell systems has been shown to alter important biochemical and cellular properties. For example, retinoids retard tumorigenesis in many experimental systems (20, 21) and inhibit the growth of several cell types in culture (22, 23). It also has been suggested that retinoic acid plays an important role in early development. Wolbach and Howe (24) reported that retinoic acid is required for normal epithelial cell differentiation. Relative to the present study, retinoids have been shown to induce the differentiation of embryonal carcinoma cells to parietal endoderm (4, 7, 8, 25) and to retard teratocarcinoma tumor development (26). Thus, there is considerable interest in elucidating the mechanism by which retinoic acid mediates cell growth and differentiation. In this regard Ludwig et al. (27) recently reported that retinoic acid treatment of B16-F₁ murine melanoma cells significantly increased the activity of cyclic AMP-dependent protein kinase, and suggested that this effect on protein kinase may be involved in mediating some of the actions of retinoic acid. However, these authors did not look for possible changes in the subcellular distribution of protein kinase activities following retinoid treatment.

In this article, we report on changes in the activity and subcellular distribution of cyclic AMP-dependent protein kinase in response to treatment of F9 embryonal carcinoma cells with retinoic acid. Retinoid treatment of F9 cells causes an increase in cyclic AMP-dependent histone phosphorylation both in the soluble and plasma membrane fractions. No increase in protein kinase activity could be detected in the nuclear fraction following incubation of F9 cells with retinoic acid.

The amounts of the R₁ and R₂ regulatory subunits of the cyclic AMP-dependent protein kinase present in various subcellular fractions were also quantitated by determining specific binding of the photoaffinity label, 8-azido-cyclic [³²P]AMP to these cyclic AMP binding proteins. Similar amounts of R₁ and R₂ are observed in the nuclear fraction, and retinoic acid treatment does not alter either the quantity of these regulatory cyclic AMP binding proteins or the relative ratio of R₁ to R₂ present in purified nuclei. However, retinoic acid treatment provokes a 2- to 3-fold increase in the amount of both R₁ and R₂ present in the cytosolic fraction; the relative ratio of R₁/R₂ (~2) present in the cytosol remained constant following retinoid treatment.

Of particular interest is the effect of retinoic acid treatment of F9 cells on the cyclic AMP-dependent protein kinases associated with the plasma membrane fraction. Along with the increase in histone protein kinase activity, retinoid treatment elicits a preferential increase in the amount of the R₂ regulatory subunit associated with plasma membranes. After 15 h of retinoic acid treatment, there is only a 1.6-fold increase in the amount of R₂, but a 4.5-fold increase in the amount of R₁ associated with the membrane.

This change in membrane-associated R₂ takes on added significance when one considers that incubation of cells with retinoic acid previously has been shown to alter such membrane functions as cell-to-substratum adhesiveness (28), increase in fibronectin (29), modification of cell surface glycosylation (30), and alteration of adenylate cyclase hormonal responsiveness (16). Yet, it is not clear if the alteration of cyclic AMP-dependent protein kinase activity and distribution is involved in mediating any of the actions of retinoic acid. The present results seem to indicate that the increase in cyclic AMP binding protein and histone protein kinase activity is an early event of retinoic acid treatment of F9 cells, rather than a result of differentiation to the endodermal cell type. The changes in kinase occur within 3 to 15 h after retinoid addition, whereas differentiation to the endodermal cell type requires 2 to 3 days (4, 7, 8).

The subcellular localization or compartmentalization of the type I and type II cyclic AMP-dependent protein kinases between the soluble and particulate fractions has been observed to differ in different tissues (10, 11). For example, R₁ is found in the cytoplasm and R₂ in the membrane of red blood cells (31). In contrast, rat and rabbit heart cytosol contains primarily R₂, while R₁ is found associated with the membrane fraction (32). BALB/3T3 cells contain predominantly the type II protein kinase, and transformation with simian virus 40 leads to the appearance of the type I enzyme (33). Other studies have shown that the relative amounts and localization of the two kinases can be modified within a given cell type. The amounts of the type I and type II kinases have been shown to change at different stages of the cell cycle (34), and also during development in rat testis (35). Fuller et al. (36) have reported that the amount of type I cyclic AMP-dependent protein kinase in the prostate is influenced by testosterone. Further, treatment of 7,12-dimethylbenz[a]-anthracene-induced mammary tumors with dibutyryl cyclic AMP has been shown to cause a translocation of the type II cyclic AMP-dependent protein kinase to the nucleus (37, 38). This latter increase in type II kinase in the nucleus is repressed by estrogen.

Cyclic AMP-stimulated phosphorylation of membrane proteins may play a role in the regulation of a variety of cell surface and intracellular phenomena (10). Much of this phos-
phorylation is probably catalyzed by kinases specifically associated with the membrane fraction. Since the catalytic subunits of the type I and type II cyclic AMP-dependent protein kinases are identical, it has been suggested that the two forms of the regulatory cyclic AMP binding subunits determine the intracellular localization of the two kinase types. Thus, the increase in the amount of R1 associated with the plasma membrane fraction in response to retinoic acid treatment of F9 cells perhaps serves to localize the catalytic subunit of the kinase near specific membrane protein substrates. The retinoic acid-induced changes in protein kinase activities and in their relative distribution probably allow preferential cyclic AMP-mediated phosphorylation of specific proteins. Conceivably, the altered distribution of the kinases might result in changes in the phosphorylation pattern which may be responsible for the dramatic cyclic AMP-mediated change in cell morphology observed following retinoic acid treatment of embryonal carcinoma cells.

REFERENCES


A Plet, D Evain and W B Anderson