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RELATIONSHIP BETWEEN CHANGES IN THE CALCIUM DEPENDENT REGULATORY PROTEIN AND ADENYLATE CYCLASE DURING VIRAL TRANSFORMATION

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SUMMARY

The levels of the calcium dependent regulatory protein in transformed chicken embryo fibroblasts are higher both in soluble fractions and membrane fractions compared to untransformed cells. The kinetics for changes in the calcium dependent regulatory protein, hexose transport, and adenylate cyclase were compared using a temperature sensitive mutant of Rous sarcoma virus. Decreases in adenylate cyclase activity and increased hexose transport accompanying transformation occurred with half-lives of approximately 7 to 8 hours. Increases in the calcium dependent regulatory protein occurred much slower with a half-life of seventeen hours. It is concluded that the increase in calcium dependent regulatory protein levels is a late event during viral transformation and that the decline in adenylate cyclase activity cannot be due to changes in the amount of calcium dependent regulatory protein.

Adenylate cyclase activity is decreased in many malignant cell types including chicken embryo fibroblasts (CEF)[1] transformed by Rous sarcoma virus (RSV)(1). Recent studies have shown that adenylate cyclase activities in mammalian brain (2,3) and glial tumor cells (4) are modulated by the calcium dependent regulatory protein (CDR). In addition, Watterson et al. (5) have reported that CDR levels are higher in transformed than normal fibroblasts and Brostrom et al. (4) have reported that high levels of CDR inhibit adenylate cyclase (4). Because of these observations, we have examined the kinetics for changes in CDR levels and adenylate cyclase activity using a temperature dependent mutant of RSV.

MATERIALS AND METHODS

Growth and Harvesting of Cells

Chicken embryo fibroblasts were grown as previously described (6). Secondary cultures were plated at cell densities of 6.25×10^5 cells/100-mm plate for normal cells and 1.25×10^6 cells/100-mm plate for cells transformed by the Schmidt-Ruppin strain of Rous sarcoma virus, subgroup A (RSV-SR-A). Because fewer of the trans-

[1] The abbreviations used are: CEF, chicken embryo fibroblasts; RSV, Rous sarcoma virus; CDR, calcium dependent regulatory protein.

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formed cells attached and grew this resulted in similar cell densities at the time of the experiment. Cells were grown at 36°C in Dulbecco's Modified Eagle's Medium, high glucose formula containing penicillin and streptomycin, supplemented with 10% tryptose phosphate broth, 4% calf serum, and 1% heat-inactivated chicken serum. Fibroblasts were harvested on the 3rd day without an intervening culture medium change. In the temperature shift experiment CEF were infected with the tsNY68 mutant of RSV-SR-A or with wild type RSV-SR-A and grown initially at 42°C. Cells were shifted from 42°C to 36°C at various times so that all cells could be harvested within a two hour period. Cells were harvested as previously described (7).

Membrane Preparation and Solubilization

Crude membranes were prepared as previously described (7) except in the experiment with tsNY68 where the crude homogenate was not diluted prior to centrifugation. Supernatants were assayed for CDR activity. CDR depleted membranes were prepared by a modification of the method of Brostrom et al. (8). One volume crude membranes was homogenized with 9 volumes 1 mM EDTA, 10 mM imidazole pH 7.5 using a tight fitting dounce homogenizer. The suspension was incubated at 4°C for 0.5 hr. with periodic vortexing and then centrifuged at 100,000 x g for 0.75 hr. The pellet was washed twice more with EDTA-Tris buffer and the pellet was finally resuspended in 500 μM EDTA, 1 mM $MgCl_2$, 10 mM imidazole pH 7.5. Crude membranes were solubilized in a buffer containing 25 mM Tris·Cl pH 7.5, 0.25 M sucrose, 1 mM $MgCl_2$, 1 mM EDTA and 1% Brij 96. The ratio of detergent/membrane protein (w/w) was 2:1. The suspension was incubated at 4° for 0.5 hours with periodic vortexing and then centrifuged at 100,000 x g for one hour. The 100,000 x g supernatant was used for adenylate cyclase assays.

Adenylate Cyclase Assay

Adenylate cyclase was assayed by the method of Salomon et al. (9) using [α^{32}P]ATP as a substrate and [^{3}H]cAMP to monitor recovery. ATP used in assays was purified by DEAE Sephadex A-25 chromatography followed by Dowex AG-50 chromatography.

Calcium Dependent Regulatory Protein Assay

CDR was assayed by its ability to stimulate CDR depleted phosphodiesterase as previously described (10).

Polyacrylamide Gel Electrophoresis

Supernatant protein was subjected to electrophoresis on non-denaturing discontinuous polyacrylamide slab gels using the procedure of Davis (11). EGTA at a concentration of 100 μMolar was present in the electrophoresis buffers.

Transport of [^{3}H]-2-Deoxyglucose

Transport of 2-deoxyglucose was determined as previously described (6).

RESULTS AND DISCUSSION

The CDR content of normal and RSV-SR-A transformed CEF are reported in Table I. The soluble fraction from transformed cells contained approximately twice as much CDR activity as normal cells. Watterson et al. reported that the CDR content of CEF transformed by the Prague strain of Rous sarcoma virus was approximately four times higher than normal CEF (5). Our results are qualitatively consistent with the data reported by Watterson et al. The quantitative differences may reflect the different Rous sarcoma strains used or the methods employed for quantitating

TABLE I
CDR in Normal and Transformed CEF

Sample	CDR (units/mg)*		
	Soluble†		Membrane‡
	36°	42°	36°
Normal	1351	1272	341
RSV-SR-A	2857	2754	443
RSV-tsNY68	2750	1125	ND§

Cells were grown at 36° or 42° and harvested as previously described (6, 7). In the temperature shift experiment, CEF were infected with the temperature sensitive mutant of RSV (RSV-tsNY68) or with wild type RSV-SR-A and grown initially at 42°. Cells were then shifted from 42° to 36°.

‡ Membranes were prepared as previously described (7).
† Soluble refers to the supernatant obtained after centrifuging homogenized cells for one hr at 100,000 x g.
* CDR was assayed by stimulation of the CDR-depleted Ca^{2+} sensitive phosphodiesterase (10). One unit of CDR caused 50% stimulation of 10 units of the phosphodiesterase. The CDR content of membranes was determined after solubilization of membranes with lubrol PX (7).

CDR levels. The CDR content of normal and RSV-SR-A transformed membranes were also determined since adenylate cyclase is a membrane associated enzyme. As shown in Table I, the CDR content of transformed membranes was comparable to normal membranes. Similar results were obtained with a temperature sensitive mutant of Rous sarcoma virus, RSV-tsNY68. When CEF infected with RSV-tsNY68 were grown at the nonpermissive temperature, 42°C, the CDR content was comparable to normal CEF. When these cells were grown at the permissive temperature, 36°C, the CDR content was approximately twice as high as the normal cells. These data indicate that either there is twice as much CDR in transformed CDF or the intrinsic activity of the protein is higher compared to CDR in normal cells. However, results obtained by gel electrophoresis, discussed below, show that there is actually more CDR in the transformed cells in agreement with the results of Watterson et al. (5). Although the 100,000 x g supernatant from normal cells contained half as much CDR activity as the normal cells, both

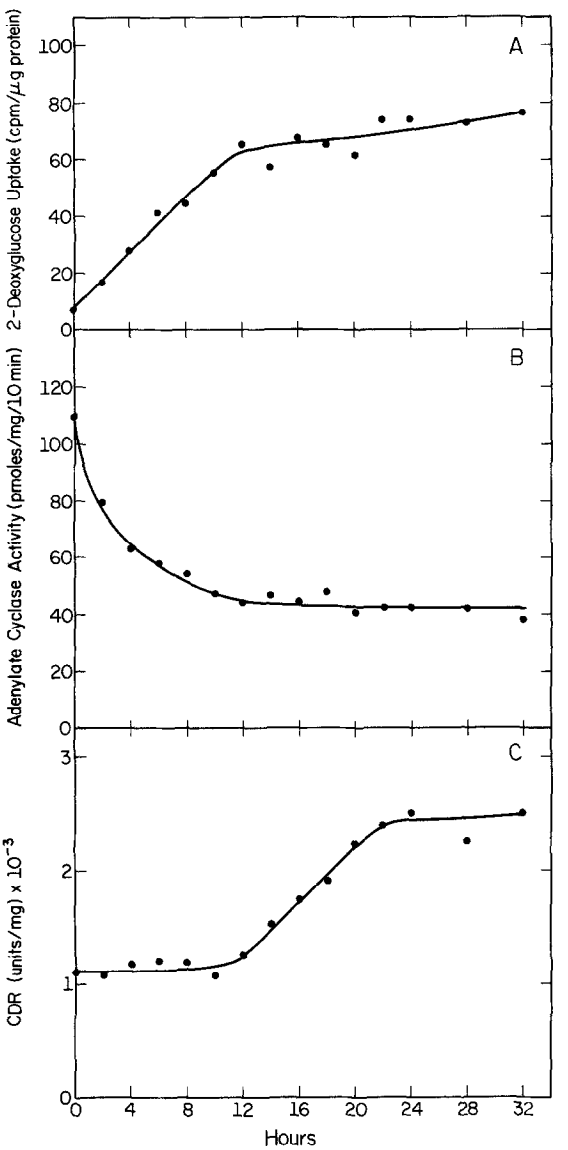

Fig. 1. Time course of change in (A) 2-deoxyglucose uptake, (B) adenylate cyclase activity, and (C) CDR activity in CEF infected with RSV-tsNY68 following a shift in temperature from 42° to 36°. CEF were grown and harvested as previously described (6, 7). Cells were infected with RSV-tsNY68, grown at 42° and then shifted to 36°C (time zero). Membranes were prepared as previously described (7). Transport of 2-deoxyglucose by whole cells was assayed using [^{3}H]-2-deoxyglucose (6). Adenylate cyclase was assayed by the method of Salomon using [α^{32}P]ATP as a substrate and [^{3}H]cAMP to monitor recovery (9). CDR activity in the soluble fraction was assayed by stimulation of CDR depleted phosphodiesterase (10).

supernatants stimulated CDR-depleted PDE to the same maximum level. This same level of stimulation was obtained with saturating amounts of purified brain CDR and all stimulations required Ca^{2+}.

In order to more fully define the relationship between changes in CDR levels and adenylate cyclase activity which occur upon transformation, the time course for these changes were compared. Cells infected with RSV-tsNY68 are phenotypically normal when grown at 42°, but rapidly become transformed when shifted to 36°. Adenylate cyclase and CDR activities were measured in membrane and soluble fractions obtained from the same cells while 2-deoxyglucose uptake (which increases approximately 6 fold upon transformation) was measured using sister cells at various times after shifting from 42° to 36° (Fig. 1). Adenylate cyclase and 2-deoxyglucose uptake changed with the same time course. 50% change in activity occurred at approximately 7 to 8 hours after the temperature shift. By 12 hours, the changes were essentially complete. No differences in CDR activity, adenylate cyclase activity or hexose transport occurred in normal cells or cells transformed by wild type virus following a temperature shift. Morphological changes typical of transformed cells were apparent in the temperature shifted tsNY68 cultures within four hours and were also essentially complete by 12 hours. In contrast, changes in CDR levels did not begin to occur until 12 hours after the temperature shift. Seventeen hours were required for 50% change in CDR levels and more than 21 hours were required for complete change in activity. The time course of increase in CDR activity corresponded with the increase in the amount of CDR as quantitated on polyacrylamide gels (Fig. 2). These data indicate that the increase in CDR is a relatively late event during transformation and it is unlikely that the changes in the amount of CDR are directly responsible for the decrease in adenylate cyclase activity.

Because of the observations cited above and the reports that CDR mediates Ca^{2+} stimulation of brain adenylate cyclase (2, 3), the calcium sensitivity of adenylate cyclase in normal and RSV transformed membranes was examined. Adenylate cyclase activities were assayed as a function of Ca^{2+} concentration in the

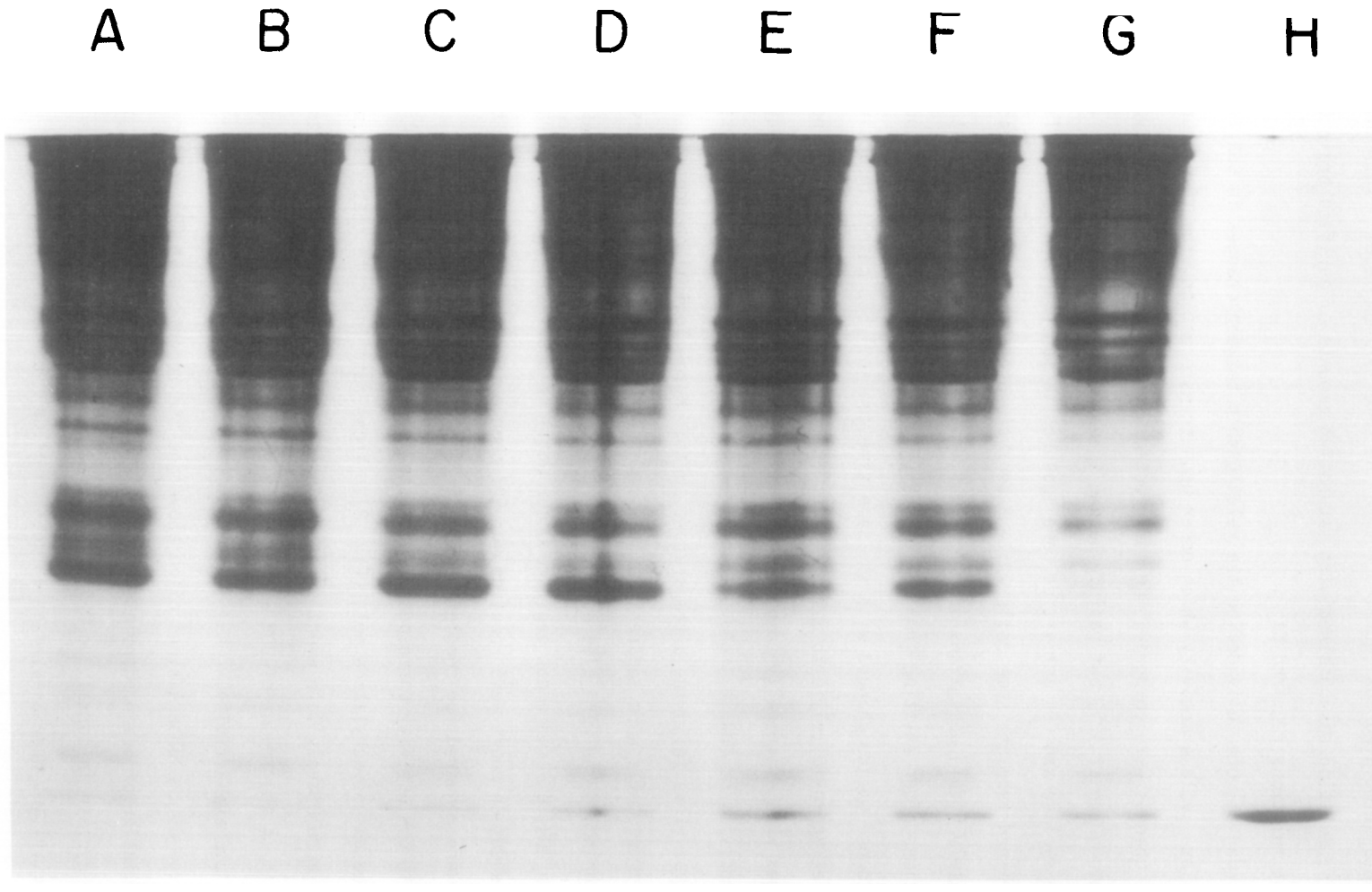

Fig. 2. Polyacrylamide slab gel of 100,000 x g supernatant from CEF infected with RSV-SR-tsNY68 shifted from 42° to 36° at (A) 0, (B) 6, (C) 12, (D) 18, (E) 24, (F) 30 hours following temperature shift, (G), cells grown at 36°, (H) 3 µg of purified bovine brain CDR (10). Supernatant protein was subjected to electrophoresis on non-denaturing discontinuous polyacrylamide slab gels (11). Slots A-G each contained 250 µg of protein.

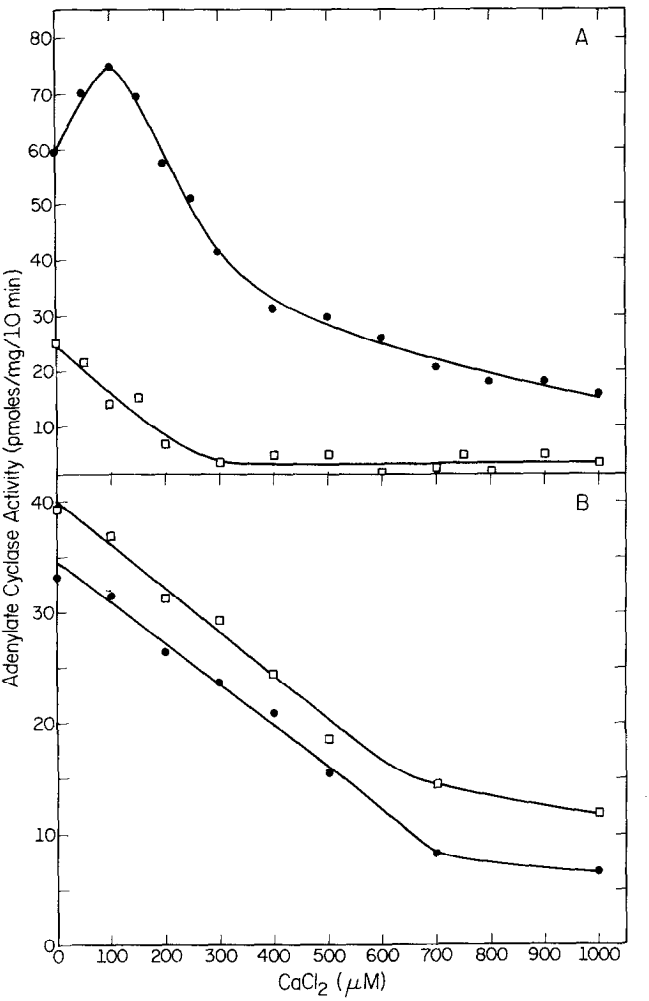

Fig. 3. Effect of $CaCl_2$ on adenylate cyclase activity from normal (●) and RSV-SR-A infected CEF (□). Membranes were prepared as previously described (7). Adenylate cyclase was assayed by the method of Salomon (9) using 1 mM [α^{32}P]ATP 3 mM $MgCl_2$, 200 μM EGTA, and indicated amounts of $CaCl_2$ in the assay cocktail. A, membranes; B, membranes solubilized with Brij 56 (7).

presence of 3 mM Mg^{2+} and 200 μM EGTA (Fig. 3A). Adenylate cyclase in normal membranes showed a biphasic response to calcium. This activity was stimulated 40% at low calcium concentrations, followed by inhibition at higher concentrations. In contrast, adenylate cyclase activity in transformed membranes was inhibited at all concentrations of calcium tested. Similar calcium dependencies were observed when 1 mM EGTA was used to complex endogenous Ca^{2+}. Solubilization of

adenylate cyclase from normal membranes abolished calcium stimulation and the calcium sensitivity of the enzyme solubilized from normal and transformed membranes was comparable (Fig. 3B). When normal membranes were washed extensively with EDTA containing buffers to remove CDR, calcium stimulation of adenylate cyclase was lost suggesting that the calcium stimulation of adenylate cyclase was mediated by CDR. Since there are sufficient levels of CDR present in transformed membranes, these data imply that adenylate cyclase in transformed membranes is unable to interact with Ca^{2+}·CDR.

The data presented in this report demonstrate that the increase in CDR levels accompanying transformation of CEF by RSV is a late event which occurs after the decrease in adenylate cyclase activity and increased hexose transport. Therefore, the decline in adenylate cyclase activity cannot be due to changes in the amount of CDR present. The differences in adenylate cyclase calcium sensitivity in normal and transformed membranes are not attributable to changes in the amount of CDR since these membrane preparations contained comparable levels of CDR. It seems likely that Ca^{2+}·CDR cannot modulate adenylate cyclase activity in transformed membranes because of alterations in the adenylate cyclase system or membrane environment.

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