Differences in Calmodulin Levels of Normal and Transformed Cells as Determined by Culture Conditions

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ABSTRACT

Several studies have suggested that calmodulin (CAM) levels increase in cells as a consequence of transformation by RNA tumor viruses. This study examines factors affecting CAM levels in normal and transformed chick embryo fibroblasts. Significant differences in CAM levels of normal and transformed cells were observed as cells grew from subconfluent to confluent densities. These changes were not due to cell cycle dependent, nor did they correlate with the growth rate of the cultures. The most significant difference between normal and transformed cultures was a lack of down-regulation of CAM levels in transformed cells as compared to normal chick embryo fibroblasts. This decrease in CAM levels in normal cells occurred in high density cultures that were allowed to grow undisturbed for several days without trypsinization and reseeding. These experiments do not support the contention that differences in the growth potential of cycling cells grow from subconfluent to confluent densities. This decrease in CAM levels was observed as cells grew from subconfluent to confluent densities. The results of this study indicate that CAM levels respond differently to regulation signals in normal versus transformed cells. These differences develop as cultures become densely populated, however, and appear to depend on the differing abilities of normal and transformed cultures to respond to cell-to-cell contact and/or transmediated cellular regulation signals.

MATERIALS AND METHODS

Materials. Tissue culture medium (F-10), calf serum, penicillin-streptomycin, and trypsin were purchased from Grand Island Biological Co. Tryptose phosphate broth was obtained from Difco. Fungizone was purchased from Squibb. Dimethyl sulfoxide was purchased from Fisher Scientific. [3H]dThd (specific activity, 77.2 Ci/mmol) was obtained from New England Nuclear. Plastic tissue culture dishes were obtained from Falcon. CAM was purified by phenothiazine-Sepharose 6B affinity-based procedures (12) coupled with the standard isolation procedures of Watson et al. (31).

Tissue Culture. CEF were prepared from 11-day-old SC (Hyline International, Dallas Center, IA) embryos as described previously (30). Primary CEF were trypsinized and reseeded, and portions of the culture at second passage were infected with either PR-C (Rous sarcoma virus, Prague strain, Subgroup C) or SR-A (Rous sarcoma virus, Schmitt-Ruppin strain, Subgroup A). Secondary cultures were then grown to confluence, trypsinized, and replated at least 3 additional times before being used in the experiments described. The stocks of both normal and transformed cells were maintained in 150-mm plastic culture dishes in GM consisting of Medium F-10 supplemented with tryptose-phosphate broth (10% v/v), 1% dimethyl sulfoxide, antibiotics (50 units penicillin/ml, 50 μg streptomycin/ml, and 2 μg Fungizone/ml), and 5% (v/v) calf serum. For experiments, cells were seeded in the presence of 2% fetal calf serum and 8% calf serum to promote attachment. Attached cells were fed daily with GM and, in some experiments, shifted to SM containing only 1% calf serum, which is adequate to maintain normal cell viability but not to promote cell growth.

Protocol for Cell Synchrony Experiments. Cells were synchronized by a nontraumatic method developed recently in our laboratories (28, 29). Briefly, the cells were trypsinized and replated. The cultures, which have a 24-hr division cycle, were fed precisely every 24 hr with GM. The degree of synchrony established was evaluated by monitoring cell number, [3H]dThd incorporation into trichloroacetic acid-precipitable material, and labeling index by autoradiography and by cytofluorography as described below.

Determination of Labeling Index. Approximately 10^6 cells which had been incubated with 1 μCi of [3H]dThd/ml in GM or SM for 24 hr were centrifuged onto a microscope slide. The cells were fixed and extensively washed with 70% methanol to remove free [3H]dThd. The slides were then coated with Kodak NTB-2 emulsion and exposed for 3 weeks at room temperature in the dark. The cell nucleus and cytoplasm were stained with hematoxylin-eosin, and the slides were scored for the percentage of labeled cells during each labeling period.

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2 Supported by Grant CA 11265 from the NIH.
3 The abbreviations used are: CAM, calmodulin; dThd, thymidine; CEF, chick embryo fibroblasts; GM, growth medium; SM, maintenance medium.

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**Cell Sorter Analysis.** Progression of CEF through the cell cycle was monitored with an Ortho Model 50H cyttofluorograph system fitted with a Lexal 95 four-watt argon ion laser at 250 milliwatts. Increasing green fluorescence was measured at 515 to 530 nm as an indicator of DNA content in cells on an arbitrary scale of 0 to 512 units. Cells for analysis were collected after trypsinization of cell layers with 0.025% trypsin in Ham's F-10 medium without fetal calf serum. They were then pelleted, resuspended at 2 x 10⁷ cells/ml in Ham's F-10 medium containing 0.01 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.2, and 10% glycerol, and stored at -20°C until analysis. This procedure was found to give identical results to those obtained by carrying out analysis of cells at the time of collection. Before sorting, cells were diluted 10-fold, and 1 ml of cells was mixed into a solution containing 0.1% Triton X-100, 0.05 M HCl, and 0.15 M NaCl at 4°C for 15 sec. Cells were then adjusted with staining solution at room temperature to 12 μg acridine orange/ml, 1 mM EDTA, and 0.15 M NaCl in phosphate citrate buffer at pH 6.0, essentially as described by Richman (20).

Preparation of Cell Extracts. Frozen cell pellets were resuspended in 1 ml of lysis buffer (100 mM sodium acetate-10 mM β-mercaptoethanol-10 mM EDTA-20 mM phenylmethylsulfonyl fluoride-1% Trasylol, pH 7.4) according to the method of Watterson et al. (32). An aliquot was disrupted with a Branson sonifier (four 15-sec bursts), the disrupted cells were centrifuged in a microfuge, and the resulting supernatant was isolated and diluted with 75% glycerol (2 parts supernatant and 1 part glycerol). These samples were stored at -20°C until analyzed for CAM and protein content.

Quantitation of CAM. A highly sensitive quantitative procedure for assay of CAM was developed for this study by modification of the discontinuous polyacrylamide tube gel electrophoretic procedure described by Watterson et al. (31), as shown in Chart 1. In the current study, a 1.5-mm-thick slab gel was used instead of tube gels, and proteins were visualized using silver staining (18) instead of Coomassie blue, thus increasing the sensitivity of the assay approximately 100-fold. CAM was quantitated by densitometry as described in the legend to Chart 1. As shown in Chart 1, standard curves obtained with purified CAM were highly reproducible and linear with concentrations from 1 to 100 ng of protein applied to the gel.

In this study, the separated band was confirmed to contain greater than 95% CAM by electrophoresing the sample in 1 mM Ca²⁺ versus 1 mM ethyleneglycol bis(β-aminoethyl ether)-N,N’N’N’-tetraacetic acid. Under these conditions, the entire CAM band exhibited a unique mobility shift of more than 0.5 cm within the 10-cm gel track.

Protein Content. The protein concentration of the cellular supernatants in 25% glycerol was determined by the method of Bradford (4) using a solution of bovine serum albumin (the concentration of which was quantified by amino acid analysis) as a protein standard. The concentration of stock solutions of purified CAM was also determined by amino acid analysis.

**RESULTS**

CAM Levels in Synchronized Cells. Previous results suggested that CAM levels were elevated as a consequence of cell transformation (6, 7, 14, 15, 32, 33). Therefore, it was important to determine whether there were any major differences in CAM levels through the 24-hr growth cycle of normal and virus-transformed CEF. Chart 2 demonstrates the synchronous cell division (A) and DNA synthesis (B) obtained in SR-A-transformed cells by the synchrony methodology described in "Materials and Methods." Cells were found to double over a 4- to 6-hr period, which closely followed DNA synthesis in the cultures. Progression of normal CEF through the cell cycle was monitored by [³H]dThd incorporation into DNA (see Chart 3, inset) and acridine orange staining of sequentially collected replicate cultures followed by analysis of DNA content by cell sorting as described in "Materials and Methods." Chart 3 illustrates the progression of CEF through 2 cycles of division at Days 3 and 4 after seeding of the culture dishes. By quantitating the CAM concentration per cell in the synchronized, transformed cultures, it was demonstrated that CAM is synthesized shortly after medium addition and remains elevated until the cells divide, at which time the CAM level per cell decreases and is maintained at one-half of its premitosis value (Chart 2A). Similar results were demonstrated in normal cultures, with CAM levels per cell changing in proportion to the number of dividing cells in the cultures (data not shown). However, it is important to note that calculation of CAM levels per μg of protein as shown in Chart 4 revealed that CAM is synthesized in concert with the bulk of other cellular cytoplasmic proteins in both normal and transformed cells. Thus, the concentration of this Ca²⁺ receptor protein remains constant through the cell cycle.

CAM Levels during Growth to Confluent Densities. Since
Chart 2. CAM content per cell in synchronized transformed CEF. Cells were trypsinized from confluent secondary cultures, replated at a density of 3.2 x 10^6 cells in 100-mm culture dishes, and fed daily for 4 days with GM. During the last 24 hr (from Day 4 to Day 5), cells were pulse labeled for 3-hr intervals with [3H]dThd (1 μCi/ml). After the 3-hr pulse, cells were trypsinized and harvested using a rubber policeman, centrifuged, and washed with standard phosphate-buffered saline (0.145 M NaCl, 10 mM sodium phosphate). Protein and CAM content were measured as described in "Materials and Methods." A. ng CAM/10^6 cells (A) and cell number (C) in synchronized SR-A-transformed cultures. B. [3H]dThd incorporated into DNA in these SR-A-transformed cultures after sequential 3-hr pulses.

no cell cycle-dependent variation was observed in CAM levels in normal and transformed cells, parallel studies were performed to monitor the response of normal and virus-transformed cultures as they grew from subconfluent densities to confluence. Chart 5 depicts the results of a study in which synchronized cultures of normal and SR-A-transformed cells were monitored for CAM content for 10 days following trypsinization. CAM levels in normal cells (Chart 5E) increased over the first 3 days following trypsinization and decreased as the culture became confluent. In contrast, CAM content in SR-A-transformed cells remained relatively constant for the first 6 days in culture and exhibited an increase 6 to 10 days following trypsinization (Chart 5F). Viability of all cultures was greater than 95%.

Similar 1-week studies were performed on cells obtained from other embryos. The initial increase in CAM content in normal cultures (discussed above) was not always observed, suggesting that, if enough CAM is present in normal cells, the cultures do not exhibit this initial elevation. However, in every case, the CAM content in freshly seeded normal cultures (greater than 20 ng/μg) decreased with time to less than 10 ng/μg as cultures achieved maximum densities following trypsinization. In parallel studies, the CAM levels in SR-A- as well as PR-C-transformed cells remained constant (10 to 15 ng/μg) for the first week following trypsinization.

These experiments demonstrated no obligatory increase in CAM levels as a consequence of transformation by RNA tumor viruses, but they did suggest that CAM concentrations are controlled differently in transformed and normal cells. In normal cells, CAM concentrations always decreased with time in culture. (This decrease, however, was not attributable simply to cessation of division as shown below.) Conversely, CAM levels in transformed cells remained the same or increased.

The cultures in these comparative growth experiments were all synchronized, and for each time point, the cells were analyzed 22 to 24 hr following feeding, a time well removed from CAM synthesis and cell division. Taken together, the experiments in Charts 2, 4, and 5 suggest that, although CAM levels appear to
normal cells into SM immediately resulted in a 3-fold reduction in the percentage of dividing cells (Chart 5A, ▲). However, while CAM content did decrease immediately following shift (Chart 5E, ◊), the rate of decrease was substantially slower than observed in GM (Chart 5E, ◊). Likewise, no correlation between growth rate and CAM levels could be demonstrated in transformed cultures (Chart 5, B, D, and F). Thus, there does not appear to be an obligatory correlation between CAM levels and the percentage of dividing cells in the cultures.

**Effect of Cell Density on CAM Levels.** Although the experiments above indicated that CAM levels were regulated differently by normal and transformed cells as cultures grew to high density, it was not clear whether CAM content was affected directly by culture density or by other differences that might be expected to develop as cells are maintained for long periods of time in culture without trypsinization. Therefore, a series of transformed and normal cultures was seeded over a 100-fold range in density, and the CAM levels in these cultures were determined 1 and 3 days after seeding.

As shown, CAM levels per 10⁶ cells increased in transformed cultures as cell density increased (Chart 6, ◊ and □), while it remained constant in normal CEF (Chart 6, ●). Chart 7 shows that, between Days 1 and 3 in culture, CAM concentration increased in PR-C- and SR-A-transformed cells at both low and high density. In contrast, normal cells seeded at high density decreased their CAM content with time in culture, results consistent with the observations discussed above (Chart 5).

**DISCUSSION**

A number of laboratories over the past several years, including our own, have reported that CAM levels are higher in transformed

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**Figure 5**

**A** CAM levels in normal and transformed CEF. Growth curves, labeling index, and CAM content of normal CEF and SR-A-transformed CEF maintained in culture 7 to 10 days were measured. Cells were trypsinized from 150-mm stock dishes and seeded in 100-mm culture dishes at the following densities: CEF, 1.5 x 10⁶ cells/dish; SR-A, 1.8 x 10⁶ cells/dish. The cells were fed at exactly 24-hr intervals with GM. Under these conditions, after 5 days in culture, the normal cells became too dense, and the cell layer peeled off the dish, limiting further experimentation. On Day 3, some of the dishes were transferred into SM and fed subsequently at 24-hr intervals with this medium. Six plates were harvested for each time point. Four plates were scraped using a rubber policeman. The cells were combined, and the resulting cell suspension was centrifuged, washed with phosphate-buffered saline, recentrifuged, and frozen at −20°C for CAM and protein determinations. The remaining 2 plates were labeled with [³H]dThd (1 μCi/ml) for the 24 hr prior to harvesting. The cells were then trypsinized and washed as separate pellets with phosphate-buffered saline, and aliquots were removed to determine cell number and labeling index. The cell number and labeling index values are averages of triplicate determinations. The CAM and protein values are an average of triplicate measurements on each homogenate supernatant. A, growth curve for CEF; B, growth curve for SR-A-transformed CEF. C, labeling index for CEF; D, labeling index for SR-A-transformed CEF. E, cultures maintained in GM; F, cultures maintained in SM. E, CAM levels in CEF; F, CAM levels in SR-A-transformed CEF. ●, cells fed with GM; ▲, cells fed with SM; ◊, theoretical cell number assuming 100% of the cells are dividing every 24 hr.

**Figure 6**

**A** CAM Levels in Normal and Transformed Cells. Cells were trypsinized from confluent stocks and seeded into 100-mm culture dishes at 5 different cell densities spanning a 10-fold range (10⁶ to 10⁷ cells/plate). Because of differences in attachment and growth rate, the normal cells did not achieve as high a density as did the transformed cells. The cultures were fed at 24-hr intervals for 3 days. One-half of the plates were harvested on Day 1; the remaining plates were harvested on Day 3. Four plates at each density were trypsinized. The cells from 2 plates were combined, and the resulting 2 cell suspensions were washed with phosphate-buffered saline-calf serum to neutralize the trypsin and then washed extensively with phosphate-buffered saline to remove calf serum and cell debris. An aliquot was removed for determining Cell number, and the remaining cells were frozen as a cell pellet for subsequent protein and CAM analyses. This chart depicts ng CAM/10⁶ cells as a function of cell density obtained on Day 3. Each determination represents an average of single values from duplicate homogenates. ●, normal CEF; ◊, PR-C-transformed CEF; and □, SR-A-transformed CEF.
than in normal cells (6, 7, 14, 15, 32, 33), giving impetus to the suggestion that CAM could be an important direct mediator of the transformation process. This suggestion has been supported by the observation that transformed cells have the ability to grow in concentrations of Ca\(^{2+}\) that are 10-fold lower than that required for proliferation of normal CEF (1). However, the experiments reported here do not support the conclusion that CAM levels are obligatorily elevated in transformed cells. Further, no evidence for changes in CAM levels during cell cycle traverse of normal and transformed cultures was found. Our results also do not support the premise, suggested in our earlier studies (32), that CAM levels of unperturbed cultures are tightly linked to cell growth rate. On the other hand, these investigations do indicate that CAM levels respond differently to regulation signals in transformed versus normal cells.

Chart 5 depicts an experiment in which CAM levels were initially lower in sparsely seeded cultures of transformed versus normal cells (note well: these levels were generally similar in such cultures). Further, CAM levels tended to remain constant or to increase with cell density at 5 to 6 days after reseeding transformed cells in new culture dishes. In contrast, normal cells, assayed 4 to 6 days after trypsinization, demonstrated a decrease in CAM levels relative to bulk cellular protein. In conclusion, the lack of down-regulation of CAM levels in transformed cultures was the most consistent observation in these experiments.

This conclusion stands in apparent conflict with several earlier studies. For instance, CAM levels have been reported previously to be 2- to 3-fold higher in Rous sarcoma virus- and SV40-transformed 3T3 and normal rat kidney cells (6, 7). However, the steady-state levels of CAM in this study were quantitated on a per cell basis. Such comparisons may be misleading because of potential cell volume differences in transformed versus normal cultures. In our hands, for instance, Rous sarcoma virus-transformed cells were approximately 50% larger in volume than were normal CEF (data not shown). Therefore, although transformed CEF contain more CAM per cell, they do not contain a significantly higher concentration of CAM in the soluble fraction of the cell.

Furthermore, many of the studies comparing CAM levels in normal and transformed cultures have used radioimmunoassay or activation of phosphodiesterase to quantify CAM (6, 7, 10, 25, 27). These studies have led to reports of CAM levels which are much lower than those obtained in studies from our labora-

tory (29, 32), where CAM was quantified by densitometric scans of electrophoretic gels. At least in some cases, these discrepancies cannot be attributed to differences in the cell types studied and appear to be assay dependent (25, 27, 29, 32). The assay, modified for our study, has been carefully characterized for its ability to accurately quantify CAM. Supporting the efficacy of this technique are the following observations. The CAM band, quantitated in nondenaturing polyacrylamide gels, exhibited a unique Ca\(^{2+}\)-dependent mobility shift. In previous studies, this band was also extracted, electrophoresed on sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and shown to contain a single protein with a molecular weight identical to CAM (32). Furthermore, this extracted fraction activated phosphodiesterase to the same extent as did chicken brain CAM (32). In other studies, Van Eldik and Watterson (26) have also isolated CAM from normal and transformed CEF and shown that CAMs from these 2 sources are identical. This demonstrates that transformed and normal cells do not contain different CAM isozymes. On the other hand, recent reports do suggest that transformed cells may contain a novel Ca\(^{2+}\)-binding protein, oncomodulin (16).

The possibility exists in the present study that some CAM may not be detected due to differential binding of this protein to insoluble components of transformed and normal cells. However, in the chick system, soluble and particulate fractionation (14) and detailed subcellular localization studies (25, 27) indicate that 80 to 90% of cellular CAM is found in the soluble protein fraction of the cells. Furthermore, no differences in distribution of CAM were observed between normal, SR-A-, and PR-C-transformed CEF. It should be noted, however, that, in other cell types, distributional differences in CAM have been reported (10, 24).

Changes in CAM levels have also been investigated in order to determine whether modulation of this protein is pivotal in regulating the progression of cells through their division cycle (5, 29). Our data demonstrate that, although CAM per cell increases markedly early in the S phase of the cell cycle, CAM does not appear to be uniquely synthesized but increases in concert with the bulk of other cellular proteins (29); i.e., CAM concentration/mg of total cellular protein remains quite constant throughout the growth cycle of both transformed and normal cells. The best demonstration of a cell cycle-dependent spike in CAM concentration has been in cultures of cells that were growth inhibited by starvation for Ca\(^{2+}\) and subsequently reinitiated into growth by addition of this cation (2).

It is important to emphasize that CAM regulation may be mediated by not only changes in CAM levels but also changes in the interaction of CAM with specific CAM-binding proteins. Although alterations in CAM levels do not seem to play a critical role in cell cycle progression, cell cycle regulation by CAM has been suggested by other studies. Naphthalene sulfonamide derivatives, which act as CAM antagonists, have been used to block division of Chinese hamster ovary cells, arresting them at the G1-S interface (11). Similar results have also been obtained by simply culturing cells in Ca\(^{2+}\)-free medium (3).

From our studies, we concluded that changes in CAM levels per se are not linked to metabolic processes attendant to DNA synthesis or division in cycling cells or cell transformation. However, our study provides strong evidence that CAM is in a state of flux in cultures and that the levels of this protein are differentially mediated in normal and transformed cells.

Although changes in CAM levels do not appear to underly control of phenotypic differences between normal and trans-
formed cells, CAM does appear to regulate many of the processes that distinguish these cell types. For example, CAM has been implicated in regulation of various aspects of cytoskeletal function (22, 23; for a review, see Ref. 13) that are perturbed upon transformation. In addition, trifluoperazine, a potential CAM antagonist, has been shown to inhibit pinocytosis (21) as well as to block spreading and migration of a variety of cell strains and lines (9, 19) but not to affect attachment of these cells to substratum (9). The decrease in CAM levels observed in normal fibroblasts in the present study may also be controlled by late-developing processes of cell-to-cell contact (gap junctions or tight junctions) that distinguish these cells from their transformed counterparts.

In conclusion, modulation of CAM levels probably does not provide a primary regulatory signal, distinguishing initial growth potentials of cycling cultures of normal and transformed cells. However, CAM remains an attractive potential mediator of changes induced by transformation, since transformation results in perturbation of second messenger and major cascade regulation systems and involves many structural changes mediated by this protein. The changes in CAM levels observed in this and previous work coincide with development of many of the membrane structures and potentials for cell-cell contact or communication that distinguish normal from transformed cultures. Thus, the apparent inability of transformed cells to down-regulate CAM levels could be related to the altered morphology indicative of this phenotype. Clearly, a more detailed understanding of the role of CAM in these systems and of the possible abrogation of its normal function, as a consequence of transformation, is an important focus of future studies.

ADDENDUM


REFERENCES


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