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Synthesis and Accumulation of Calmodulin in Suspension Cultures of Carrot (*Daucus carota* L.)[1]

Evidence for Posttranslational Control of Calmodulin Expression

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ABSTRACT

The expression of calmodulin mRNA and protein were measured during a growth cycle of carrot (*Daucus carota* L.) cells grown in suspension culture. A full-length carrot calmodulin cDNA clone isolated from a λgt10 library was used to measure steady-state calmodulin mRNA levels. During the exponential phase of culture growth when mitotic activity and oxidative respiration rates were maximal, calmodulin mRNA levels were 4- to 5-fold higher than they were during the later stages of culture growth, when respiration rates were lower and growth was primarily by cell expansion. Net calmodulin polypeptide synthesis, as measured by pulse-labeling in vivo with [^{35}S]methionine, paralleled the changes in calmodulin steady-state mRNA level during culture growth. As a consequence, net calmodulin polypeptide synthesis declined 5- to 10-fold during the later stages of culture growth. The qualitative spectrum of polypeptides synthesized and accumulated by the carrot cells during the course of a culture cycle, however, remained largely unchanged. Calmodulin polypeptide levels, in contrast to its net synthesis, remained relatively constant during the exponential phases of the culture growth cycle and increased during the later stages of culture growth. Our data are consistent with increased calmodulin polypeptide turnover associated with periods of rapid cell proliferation and high levels of respiration.

CaM[2] is universally distributed among eukaryotes and is one of the best characterized Ca^{2+}-binding proteins (27). The primary structure of CaM is highly conserved between both plants and animals, which is interpreted to mean that it plays similar important roles in regulating Ca^{2+} homeostasis and transducing the primary signals of hormones and environmental cues (24, 27). Several reports from animal systems indicate that CaM plays a key role in growth and the cell division cycle. Net synthesis of CaM protein and accumulation of CaM mRNA in chicken embryo fibroblasts is higher in Rous sarcoma virus-transformed cells, in which the growth rate is increased compared with normal cells. Cycling of partially synchronized Chinese hamster ovary cells is specifically inhibited at the G_1/S boundary when cells are treated with the CaM antagonist W7 (16). Increases in both CaM and CaM mRNA levels are associated with G_1/S transition in Chinese hamster ovary-K1 cells (6). Furthermore, in mouse cells induced to overproduce CaM by transformation with a chicken CaM gene, the time required to traverse the cell cycle was shown to decrease due to a reduction in length of the G_1 phase (25).

There is also evidence of involvement of CaM in plant growth and cell division. CaM levels are typically elevated in actively growing regions of plants (1, 15, 22, 33). Immunofluorescence microscopy of pea and onion root cells showed that CaM is localized at the spindle poles during mitosis (29). We previously demonstrated (33) that CaM mRNA levels are 3-fold higher in the barley leaf meristematic zone compared with regions of the leaf where fewer cells are dividing. One major drawback to the monocot leaf system employed in these studies, however, was that it contains at least three major cell types: mesophyll, epidermal, and vascular, which account for approximately 59, 9, and 32% of the cells in the leaf, respectively (18). A further complication in our original work is that the three barley leaf cell types were progressing through a program of terminal differentiation, most likely at somewhat different rates, as they were displaced from the meristem region. To investigate the relationship between CaM gene expression and growth in a simpler plant cell system, we used a nonregenerable line of carrot cells in culture to extend our earlier observations. Our results show that steady-state CaM mRNA levels are elevated in rapidly respiring, asynchronously dividing plant cell populations compared with cells growing primarily by cell expansion. These measurements, together with estimates of net CaM protein synthesis and accumulation, indicate that posttranslational controls play an important role in regulating CaM expression in cultured plant cells.

MATERIALS AND METHODS

Plant Material

Carrot (*Daucus carota* L. var Danvers) suspension cultures obtained from Dr. J. Widholm (Department of Agronomy, University of Illinois) were grown on basal Murashige and Skoog medium supplemented with 1.8×10^{-6} M 2,4-D (30).

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[2] Abbreviation: CaM, calmodulin.

Suspensions were maintained at 27°C on a rotary shaker and subcultured every 8 d (0.5 g fresh weight/50 mL medium).

Growth Curves

Three parameters were used to monitor growth of the suspension culture over a period of 12 d: fresh weight, cell number, and protein content. For fresh weight measurements, 5-mL aliquots were filtered under suction, air dried for 5 min, and weighed. Cell numbers were measured after vigorously mixing a known fresh weight of cells with 8% (w/v) chromic acid. Cell aggregates were disrupted by repeated passages through a 22-gauge needle. Dilutions were prepared and cells were counted on a Fuchs-Rosenthal counting chamber. Protein content was estimated (21) in 200-μL aliquots of cell suspension, which were precipitated with 4 volumes of acetone and resuspended in 0.1 N NaOH. Lysozyme was used as a standard. Culture viability was monitored daily by staining a small aliquot of cells with 0.1% (w/v) phenosafranine (31).

Preparation of Soluble Protein Extracts

At three different time points during a growth cycle of the carrot cell suspension, 2-mL aliquots of cells were withdrawn from the culture and collected by centrifugation. The cells were resuspended in 1 mL of 20 mM Tris-HCl (pH 7.5), 1 mM Na_2EDTA, and 10 mM DTT and were lysed by sonication (Branson model 185 sonifier equipped with a micro tip, in five 10-s bursts, at a power setting of 5). Soluble proteins were collected after clarifying the extract in a microfuge for 10 min. Protein concentration was estimated (21) using lysozyme as a standard. In one set of experiments, steady-state CaM levels were estimated by a competition radioimmunoassay using a commercially available kit with nonheated bovine brain CaM as a standard (Dupont-New England Nuclear).

Gel Electrophoresis and Western Blotting

To enrich for CaM, soluble protein extracts were fractionally precipitated with ethanol (23, 32, 33). CaM-enriched, 50 to 80% ethanol-precipitated protein fractions derived from equal amounts of soluble protein were subjected to SDS-PAGE on 15% (w/v) polyacrylamide gels and transferred to nitrocellulose filters. After transfer, the filter was fixed in 0.2% (v/v) glutaraldehyde in Tris-buffered saline (10 mM Tris/HCl, pH 7.5, 150 mM NaCl) for 45 min (26). The filter was then blocked in Tris-buffered saline containing 0.05% (v/v) Tween-20 supplemented with 0.5% (w/v) gelatin. The primary antibody was rabbit anti-soybean CaM (14) (1:100 dilution). Immune complexes were detected using alkaline phosphatase-linked anti-rabbit immunoglobulin G secondary antibody as described (34).

Respiration

The respiration rate of the suspension cells was measured polarographically at different times during the growth cycle using a YSI model 53 biological oxygen monitor. Aliquots of cell suspension were withdrawn from the culture and their O_2 consumption measured within 1 min directly in culture medium at the culture growth temperature. In every case, the initial dissolved O_2 concentration in the culture medium was between 40 and 60% of that of air-saturated H_2O, which was assumed to be 255 μM at 26°C.

Protein Synthesis In Vivo

On the 3rd, 6th, and 10th d of a typical growth cycle, 10 mL of carrot suspension were withdrawn from the culture and incubated with 100 μCi L-[^{35}S]methionine (1091 Ci/mmol) for 1 h. Labeled samples were centrifuged, the pelleted cells were resuspended in 20 mM Tris-HCl (pH 7.5), 1 mM Na_2EDTA, and 10 mM DTT (0.1 g/ml based on fresh weight), and the soluble proteins were extracted by sonication as described above. Ten-microliter aliquots of labeled extract were collected on Whatman filters and the percentage of isotopic incorporation was measured after precipitation with TCA by liquid scintillation counting.

Labeled protein samples, enriched for CaM by ethanol fractionation, were analyzed on nondenaturing gels, in which CaM is readily identified by its unique migration (28, 32, 33). The gels were loaded on the basis of either total incorporated ^{35}S cpm or by corrected specific activity. The corrected specific activity for the three time points was obtained by adjusting the ^{35}S cpm incorporation per unit protein by a factor that accounted for the differences in the free methionine pool sizes (measurement described below). Duplicate gels were electrophoresed and stained. One gel was treated with fluor, dried, and exposed to x-ray film. The CaM bands from the second gel were excised, minced, and dissolved by incubating them in 30% hydrogen peroxide at 77°C for 3 to 4 h. The ^{35}S cpm incorporated into CaM was determined by liquid scintillation counting. To confirm that this gel system could be used to assay carrot cell extracts for net CaM synthesis, we eluted the ^{35}S-labeled CaM zone from a nondenaturing gel. The eluted protein was then subjected to SDS-PAGE in the presence or absence of Ca^{2+}. We observed a single species of ^{35}S-labeled protein whose mobility showed the Ca^{2+}-induced mobility increase characteristic of CaM (ref. 4, data not shown).

On days 3, 6, and 10 of a culture cycle, cells were harvested on a Buchner funnel and 0.5 g of cells (by fresh weight) were processed for amino acid analysis as described (12). Individual amino acids were measured using a TSM Technicon automatic amino acid analyzer. The pools of free methionine were found to increase during the time course of culture growth by a factor of 5 and 7, respectively, at days 6 and 10 compared with day 3 (on a per μg soluble protein basis). This value was similar to the 7.8-fold increase in methionine pool size (measured on a fresh weight basis) in the same cell line between days 3 and 10 of culture reported previously (12). To compensate for the resulting decrease in methionine specific activity during in vivo labeling experiments, gels used for analyzing labeled proteins in these experiments were loaded on the basis of ^{35}S cpm incorporated/mg protein multiplied by the measured pool size of methionine relative to that of day 3.

RNA Extraction

Total carrot cell RNA was isolated as described previously (13) with the following modifications. Frozen plant tissue was ground to a fine powder under liquid N_2 followed by homogenization in 3 volumes of 0.2 M $Na_2B_4O_7 \cdot 10\ H_2O$-NaOH (pH 7), 30 mM Na_2EDTA, 5 mM DTT, and 1% (w/v) SDS heated to 90°C. Homogenates were treated with proteinase K (200 μg/ml) by incubation for 1 h at 37°C, and after filtration through miracloth, each was adjusted to 0.125 M KCl and incubated for 10 min on ice. Following centrifugation at 10,000*g* for 15 min at 4°C, the homogenates were extracted with equal volumes of phenol:chloroform:isoamyl alcohol (25:24:1), followed by chloroform:isoamyl alcohol (24:1). RNA was precipitated with LiCl and further purified by ethanol precipitation. Poly(A^+)-enriched RNA was prepared by affinity chromatography on oligo(dT)-cellulose (2).

cDNA Library Construction and Screening

Poly(A^+) RNA was isolated from a 3-d-old culture and used for cDNA synthesis as directed in a Pharmacia-LKB cDNA synthesis kit. Following addition of *Eco*RI linkers, the cDNA was cloned in λgt10. Approximately 3×10^4 unamplified recombinant phage were screened using a barley CaM cDNA (20) as a heterologous probe as described (19). A single positively hybridizing phage was plaque purified and the *Eco*RI insert subcloned into pBS-SK^+ plasmid (Stratagene, La Jolla, CA). Progressive deletions were made by the exonuclease III/S1 nuclease method and the insert was sequenced as described previously (20).

Northern and Slot Blot Hybridizations

Total RNA was electrophoresed on formaldehyde-agarose gels and transferred to nitrocellulose filters (33). Filters were prehybridized in 50% (v/v) formamide, 5× SSPE (1× SSPE is 0.135 M NaCl, 0.015 M NaH_2PO_4/Na_2HPO_4, pH 7.4), 10× Denhardt's solution (1× Denhardt's solution is 0.02% w/v each of BSA, Ficoll, and PVP), and 1% (w/v) SDS for 12 to 24 h at 45°C. Hybridizations were carried out at 45°C for 24 h in 50% formamide, 5× SSPE, 1× Denhardt's, and 0.1% (w/v) SDS with 20 ng/mL ^{32}P-labeled CaM cDNA insert probe (3.0×10^6 cpm/mL). The probe was prepared by oligolabeling the *Eco*RI carrot CaM insert as described (17). After hybridization, filters were washed for about 15 min in each of the following: 2× SSC, 0.2% (w/v) SDS at room temperature; 1× SSC, 0.1% (w/v) SDS at 45°C; 0.1× SSC, 0.1% (w/v) SDS at 45°C; and finally in 0.1× SSC, 0.1% (w/v) SDS at 50°C. CaM mRNA was detected by exposing the filter to x-ray film at −80°C using an intensifying screen. Total RNA was slot blotted onto nitrocellulose using a BioDot microfiltration apparatus (Bio-Rad) according to the manufacturer's instructions. Blots were hybridized with carrot CaM and flax rRNA (9) probes as described above for northern blots. Blots were probed for extensin mRNA using a carrot extensin cDNA insert (7) as described above for CaM mRNA, except that the hybridization solution contained 40% formamide at 42°C.

RESULTS

Growth Kinetics of the Carrot Suspension Cells

The growth kinetics of the carrot cell culture line used in our experiments is shown in Figure 1a. The cultures exhibited an initial lag phase, followed by a period of exponential growth due to asynchronous cell divisions (beginning at day 2), expansion (beginning at about day 8), and saturation (beginning at day 10) over a 12-d period. Cell culture growth kinetics were monitored during all subsequent experiments and were always consistent with this general pattern. The viability of the cell cultures was high (typically >90–95%) throughout the experimental period, as measured by phenosafranine staining (31).

Microscopic examination of the cells, as shown in Figure 1, qualitatively confirmed the general observation that growth during the exponential phase was attributable primarily to asynchronous cell divisions, and that expansion growth predominated during a period of about 4 d prior to the cessation of all growth in the culture. During exponential growth as seen in culture samples examined at day 3 (Fig. 1b), most cells were isodiametric and newly divided cells remained together in small clumps. As the growth cycle progressed from days 6 through 12, cells expanded and

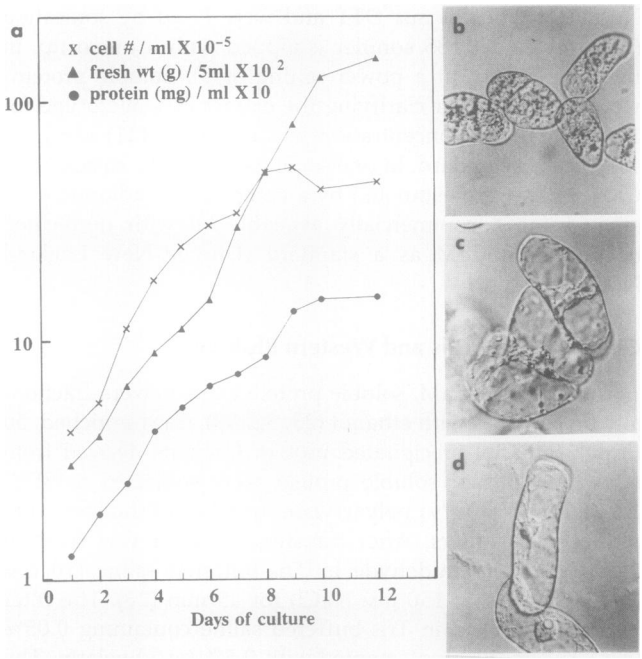

Figure 1. Growth kinetics and morphology of carrot cell suspension culture cells. The growth curve exhibits an initial lag phase followed by exponential growth, cell expansion and saturation (panel a). Fifty milliliters of Murashige and Skoog medium were inoculated with 0.5 g (by fresh weight) of carrot cells from an 8-d-old suspension culture. Five-milliliter aliquots were weighed daily, cell numbers counted in 8% chromic acid, and protein content measured by Lowry assay: ▲, Fresh weight ($g \cdot 5\ mL^{-1}$ culture); X, cell number ($cells \cdot mL^{-1}$); ●, protein ($mg \cdot mL^{-1}$). Panels b, c, and d show Nomarski optics micrographs of typical carrot suspension culture cells taken at days 3, 6, and 12, respectively, after transfer to fresh medium (×250 magnification).

reached up to 3 times their original length and became increasingly vacuolated (Fig. 1, c and d). It should be noted that the cell line used in our work was not regenerable (J.M. Widholm, personal communication), and thus, the changes in cell size and shape were assumed not to indicate cell differentiation.

Based on these observations, for all further experiments, cultures were sampled at times corresponding to early exponential, midexponential, and saturation phases (days 3, 6, and 10 or beyond, respectively) to draw comparisons between asynchronously dividing, mixed populations of asynchronously dividing and expanding cells, and cells growing by expansion, respectively. We note that the measurements of relative CaM accumulation, net CaM synthesis, and relative mRNA accumulation were each performed in three independent experiments.

Steady-State CaM Protein Levels

Steady-state CaM levels were examined over the course of a growth cycle in total soluble protein extracts prepared from aliquots of cultured cells at different times after transfer to fresh medium. In two independent experiments, extracts containing equal amounts of soluble protein (200 μg) were analyzed for their relative CaM content, after ethanol fractionation to enrich for CaM (23, 32, 33), by SDS-PAGE and western blotting followed by densitometric scanning of the immunostained blots. In a separate experiment, duplicate total soluble protein samples were analyzed by a competition radioimmunoassay for their CaM content. Table I shows that the level of CaM relative to total soluble protein in the cells did not change significantly during the growth cycle as visualized by desitometry of stained western blots. However, radioimmunoassays revealed an increase in CaM content of the cells at day 10 from 1 ng/μg soluble protein, which was observed through the first 8 d of culture, to 1.6 ng/μg soluble protein. It should also be noted that no major qualitative changes in the polypeptide compositions of the carrot cells at different times following transfer were detected using one-dimensional SDS-PAGE in these experiments (data not shown).

Table I. *Steady-State Accumulation of CaM in Soluble Protein Extracts Prepared from Carrot Cell Suspension Cultures*

Days after Subculturing	Calmodulin Accumulation	
	Western blot[a]	Radioimmunoassay[b]
3	0.97	1.00
6	1.00	0.98
8	0.98	1.16
10	0.99	1.60

[a] Measured in relative units by densitometric scanning of two independent western blot experiments. Measurements were conducted by separating the CaM-enriched, 50 to 80% ethanol fractions derived from 200 μg of total soluble protein by SDS-PAGE, and detecting the CaM using an anti-soybean CaM antibody and alkaline phosphatase-linked secondary antibody. [b] Measured as ng CaM/μg total soluble protein by radioimmunoassay with a commercially available kit (DuPont-NEN) using nonheated bovine brain CaM as a standard. The values presented are the averages of duplicate measurements whose values agreed within ± 12%.

It is not clear why the two methods we used gave different results for the relative CaM levels in day 10 cells. One possibility is that because the protein extracts prepared from day 10 cells were more dilute, the ethanol fraction protocol we used to enrich CaM for western blotting did not quantitatively precipitate the protein in those samples. Another possibility is that there may have been a different spectrum of CaM-binding proteins in these cells that bound CaM and removed it from solution at a lower concentration of ethanol in the day 10 cells compared with younger cells. However, the discrepancy was not due to saturation of the western blots with CaM, because standards of partially purified wheat germ CaM that gave 2- to 3-fold higher signals were fractionated in parallel with our test samples in these experiments.

Net Synthesis of CaM Protein In Vivo

In contrast to the steady-state CaM polypeptide levels, net CaM synthesis was markedly elevated during the exponential phase of the growth cycle. Net protein synthesis was followed by labeling aliquots of cultured cells with L-[^{35}S]methionine for 1 h at different times after transfer to fresh medium. Relative net CaM synthesis for each time point was compared by assaying ethanol-fractionated, labeled soluble protein extracts by nondenaturing gel electrophoresis (described in "Materials and Methods") and autoradiography or scintillation counting.

Total net protein synthesis was highest in the carrot cells on day 3, which corresponded to early exponential growth phase. Total net protein synthesis declined by day 6 to 65% of that on day 3. By the end of proliferative growth at day 10, total protein synthesis was reduced to 20% of that observed on day 3. Although total net protein synthesis declined over the course of the carrot cell culture cycle, there were no major qualitative changes observed in the labeling pattern when total soluble proteins from ^{35}S-labeled cells were compared by SDS-PAGE as shown in Figure 2a. Net CaM synthesis was inferred by observing the level of ^{35}S labeling associated with gel-fractionated CaM after enrichment of the protein by ethanol fractionation. This was measured both by densitometric scanning of autoradiographs of dried gels and by liquid scintillation counting of gel slices dissolved in hydrogen peroxide. Figure 2b shows the autoradiographic signals observed for CaM in duplicate labeling experiments. Net CaM synthesis was highest at early and midexponential phases of culture growth (days 3 and 6), but was reduced at day 10 to 20 to 30% of the level of ^{35}S incorporation into CaM observed on days 3 and 6.

It should be noted that the autoradiographic comparisons of CaM labeling shown in Figure 2b were made on the basis of equivalent, total ^{35}S incorporation into protein: i.e., CaM fractions were prepared from soluble protein extracts having equal ^{35}S cpm. We also assessed the possibility that the differences we observed in CaM labeling were attributable to changes in the endogenous pools of methionine in the carrot cells at different stages of the culture cycle. When gel loadings for measuring net CaM synthesis at different stages of the

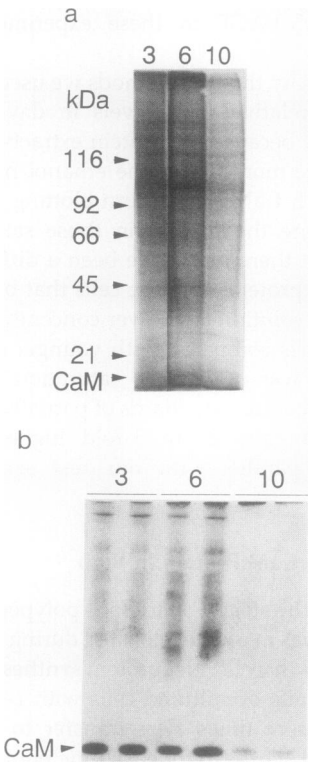

Figure 2. Net CaM synthesis is increased during the exponential phase of the carrot suspension cell growth cycle. On days 3, 6, and 10 after transfer to fresh medium, 10 mL of cells were labeled for 1 h with 100 μCi L-[^{35}S]methionine. a, Fluorograph of total soluble proteins (10^5 cpm/lane) extracted from each of the labeled cultures. b, Fluorograph of CaM-enriched 50 to 80% (v/v) ethanol fractions made from the soluble protein extracts analyzed on a nondenaturing gel and detected by fluorography; samples were prepared from aliquots of total soluble proteins having equal incorporated ^{35}S cpm. The paired lanes represent samples of CaM fractions prepared from duplicate, ^{35}S-labeled cultures. The migration of carrot CaM, detected by staining the gel prior to fluorography, is indicated in the figure.

culture cycle were corrected for the difference in methionine specific activities of the cells used in the labeling experiments, our results were identical to those presented in Figure 2b; i.e. CaM labeling at day 10 was 20 to 30% as great as that observed on day 3.

Isolation and Nucleotide Sequence of a Carrot CaM cDNA and Expression of CaM mRNA in Cultured Carrot Cells

To determine whether the greater net CaM protein synthesis we observed in mitotically proliferating cells is due to higher levels of CaM mRNA, we isolated a carrot CaM cDNA clone so that steady-state CaM mRNA levels could be assayed by northern blot hybridization. A carrot cDNA library was screened using a ^{32}P-labeled barley CaM cDNA (19) as a probe at a hybridization stringency that would allow for 15 to 20% nucleotide sequence mismatch (20). A single strongly hybridizing plaque was isolated, subcloned, and sequenced completely. The cDNA clone (pCcam-1) was 704 bp in length and contained an open reading frame starting at position 39 encoding the complete amino acid sequence of CaM, with a predicted molecular mass of 16.848 kD. Figure 3A is a restriction map of pCcam-1 and also shows the sequencing strategy; Figure 3B shows the complete nucleotide and amino acid sequence of the pCcam-1 insert. The nucleotide sequence of Ccam-1 shares 70 to 75% identity with those of barley (20), alfalfa (3), and *Arabidopsis* (19) CaM cDNAs. The derived amino acid sequence is very similar to other plant CaM proteins, with only one conservative amino acid substitution compared with alfalfa (Asp for Glu at position 7) and barley (Ser for Ala at position 10). These amino acid changes did not occur within the highly conserved Ca^{2+}-binding loop regions (indicated with asterisks in Fig. 3B).

To examine CaM mRNA levels, total RNA fractions were prepared from carrot suspension culture cells at different

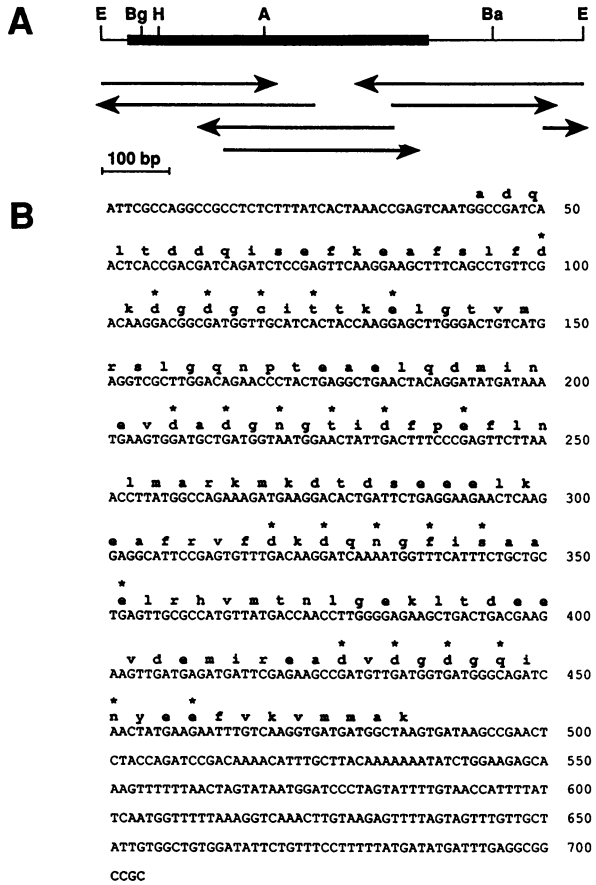

Figure 3. Physical map, sequencing strategy, and nucleotide sequence of a full-length carrot CaM cDNA clone. A, Solid bar represents the protein coding region. Restriction enzyme sites are indicated above the map: E, *Eco*RI; Bg, *Bgl*II; H, *Hin*dIII; A, *Apa*I; Ba, *Bam*HI. The overlapping arrows below the restriction map indicate the direction and extent of nucleotide sequence determined using subcloned restriction fragments. B, Nucleotide sequence and derived amino acid sequence of the *Eco*RI restriction fragment comprising Ccam-1. Amino acids are shown in the single-letter International Union of Pure and Applied Chemistry nomenclature. Residues marked with asterisks are those most likely to act as Ca^{2+}-binding ligands as deduced by comparison with rat testis CaM.

times after transfer to fresh growth medium. After separating equal amounts of RNA in a formaldehyde-agarose gel and transferring the RNA to nitrocellulose, CaM mRNA was visualized by hybridizing with ^{32}P-labeled pCcam-1 insert DNA sequences followed by autoradiography. Figure 4 shows that this probe strongly hybridized with a single, 750-nucleotide RNA species under stringent conditions. The size of the hybridizing RNA was similar to CaM mRNAs previously detected in several other plant species (19, 20, 33), and it was comparable in size to the pCcam-1 insert. By densitometry of the autoradiographic signals, we found that steady-state CaM mRNA levels were highest during the exponential growth (days 3 and 6). They declined to 60% of the day 3 level by day 8, when the culture growth rate was decreasing. CaM mRNA levels further declined to 25% of the day 3 level by day 10, when proliferative growth had ceased. In contrast, the right panel of Figure 4 shows that levels of extensin mRNA increased by 40 to 50% over the same time course. Extensin, which encodes a glycoprotein component of the plant cell wall, is thought to play a role in maintaining the structural integrity of plant cell walls throughout development (5). Hybridization with a rRNA probe confirmed that the gel and slot blot loadings used in these experiments were comparable for each of the RNA fractions.

DISCUSSION

In this report, we utilized a carrot suspension culture system to measure the relative levels of CaM protein, its net synthesis, and the levels of CaM mRNA during the course of plant cell growth in the apparent absence of cell differentiation. Figure 5 summarizes these experimental data, together with the rates of respiration and total net [^{35}S]methionine incorporation during the culture growth cycle. The pattern of CaM mRNA expression was very closely reflected in the net

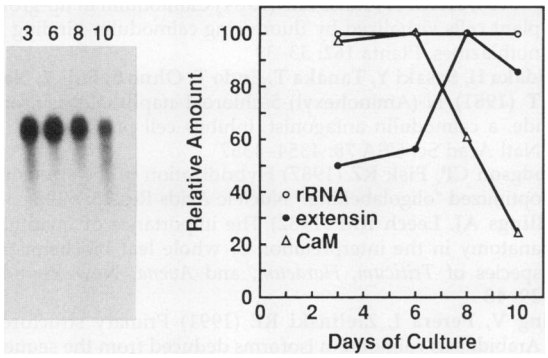

Figure 4. CaM mRNA levels are elevated during the exponential growth phase of carrot suspension cells. Total RNA was extracted from cells on days 3, 6, 8, and 10 after transfer to fresh medium. Left, RNA (4 μg/lane) was fractionated in a formaldehyde-agarose gel and transferred to a nitrocellulose filter. The northern blot was hybridized with 16 ng/mL of a ^{32}P-labeled carrot CaM cDNA probe (3.4×10^6 cpm/mL), washed, and detected by autoradiography as described in "Materials and Methods." Right, Densitometric quantitation of northern (CaM) and slot (extensin and rRNA) blot assays of specific RNA levels in total RNA fractions isolated from suspension cultured carrot cells.

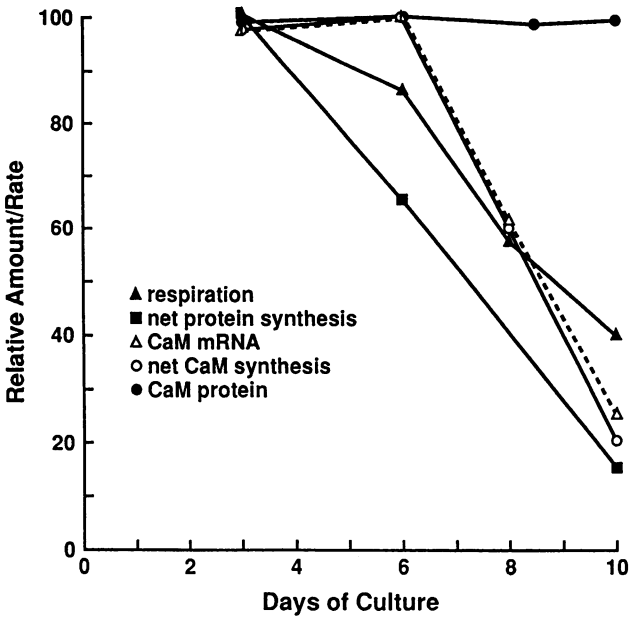

Figure 5. Summary of the relative levels of CaM protein (●), net CaM synthesis (○), and steady-state CaM mRNA (Δ) during the growth cycle of suspension cultured carrot cells. These values are compared with the total net protein synthesis (■) and O_2 consumption (▲). The values for CaM mRNA content and net CaM synthesis represent densitometric scans of autoradiographs, similar to the ones presented in Figures 2 and 4, for a single experiment. In three independent experiments, these values varied by less than 10%. Steady-state CaM levels were measured by densitometric scanning of western blots as described in Table I.

CaM protein synthesis levels. Both steady-state CaM mRNA levels and net CaM synthesis were highest during periods of asynchronous cell proliferation and high oxidative metabolism and declined by 75 to 80% by day 10, when respiration rates declined and cell division had ceased. Overall, there is a good correlation between these two measurements and the general trend of metabolic activity (reflected in the levels of net protein synthesis and respiration) during the growth cycle. However, at day 6 during the midexponential phase, CaM synthesis and CaM mRNA were maintained at maximal levels, whereas net protein synthesis and respiration declined to 65 and 85% of their respective values at day 3. It is difficult to assess the physiological significance of this observation.

In sharp contrast to the patterns of CaM mRNA accumulation and net CaM synthesis, steady-state CaM protein levels remained constant over the period of the culture growth cycle in which respiration rates were high and cells were dividing, and they increased during the latter phases of the culture cycle, which were characterized by expansion growth. From these results, we infer that CaM production is regulated, in this system, at the posttranslational level with a rapid turnover of CaM protein occurring during the exponential phase of growth. This explanation is consistent with the idea that increases in steady-state CaM levels in dividing plant cells are localized and transient (15, 22; 29). However, since the carrot cell cultures used in our experiments were nonsynchronous and also contained a small proportion of noncycling

cells, no data on cell cycle stage-specific CaM protein or mRNA levels could be obtained. Further experiments using synchronized plant cells will determine whether CaM levels are altered at specific times during the cell division cycle in cultured carrot cells.

Our results indicate that posttranslational mechanisms play an important role in regulating CaM expression in cultured plant cells. Posttranslational degradation of many intracellular proteins is regulated in vivo by the ubiquitin-dependent proteolytic pathway, in which proteins marked for degradation are covalently conjugated with ubiquitin and then rapidly degraded by specific proteases (8). The Lys^{115} residue of CaM appears to be a specific site for ubiquitin conjugation in vitro, where it prevents the formation of ubiquitin conjugates and protects the protein from ubiquitin-dependent degradation (10, 11). These in vitro findings, however, have yet to be confirmed in vivo. Nevertheless, if the trimethylation state of Lys^{115} inversely reflects the rate of CaM turnover, our results predict that the pool of CaM in cells at stationary phase should be more highly trimethylated than is CaM from cells at exponential phase. Consistent with this prediction is the observation that the level of Lys^{115} trimethylation in CaM from pea roots varies with the developmental state of the tissue (23). In this system, the levels of CaM trimethylation were higher in mature (nondividing) regions of pea roots compared with younger, more actively growing regions near the root apex.

The carrot CaM cDNA isolated in this report facilitated the measurement of CaM mRNA in the carrot suspension culture system, but results similar to those shown in Figure 4 were also obtained using a barley CaM cDNA probe (results not shown). Thus, it is reasonable to conclude that our measurements accurately reflected the total CaM mRNA content of RNA extracted from the carrot suspension cells. It should be noted that at least one other higher plant, *Arabidopsis thaliana*, has been shown to encode a CaM multigene family (ref. 20, and M.C. Gawienowski, I.Y. Perera, D. Szymanski, and R.E. Zielinski, unpublished data). Southern blot experiments suggested that a similar situation exists in the carrot genome (data not shown). An interesting question is whether CaM genes are differentially expressed during the plant cell division cycle. However, this question could not be addressed with the asynchronously dividing cell cultures used in the experiments reported in this study.

NOTE ADDED IN PROOF

Our prediction that CaM isolated from rapidly proliferating carrot cells should be less highly trimethylated at Lys^{115} than CaM isolated from nondividing carrot cells was recently confirmed by Oh et al. (Arch Biochem Biophys **297:** 28–34).

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