

Two Calmodulins in *Naegleria* Flagellates: Characterization, Intracellular Segregation, and Programmed Regulation of mRNA Abundance during Differentiation

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Abstract. Flagellates of *Naegleria gruberi* contain two calmodulins that differ in apparent molecular weight and intracellular location. Calmodulin-1, localized in flagella, has an apparent molecular weight of ~16,000, approximately the size of other protozoan calmodulins, whereas calmodulin-2, localized in cell bodies, is 15,300. Both proteins, purified, are calmodulins by several criteria, including Ca²⁺-dependent stimulation of calmodulin-dependent cyclic nucleotide phosphodiesterase and affinity for antibodies to vertebrate calmodulin. The finding of two calmodulins is unusual. Since the only known difference is apparent molecular weight, one calmodulin could be derived from the

other, except that both calmodulins are synthesized in a wheat germ, cell-free system directed by RNA from differentiating *Naegleria*. Translatable mRNAs encoding calmodulins 1 and 2, not detected in amebas, appear and subsequently disappear concurrently during the 100-min differentiation of *Naegleria* from amebas to flagellates. Furthermore, these mRNAs increase and then decrease in abundance concurrently with those for flagellar tubulins, which suggests the possibility that the expression of the unrelated genes for calmodulin and tubulin may be under coordinate control during differentiation.

CELLS of the unicellular eukaryote *Naegleria gruberi* can be induced, individually or in suspended populations, to change from the vegetative form, amebas, to flagellates with a streamlined body contour and two flagella (13). This rapid 1:1 conversion offers opportunities to study events of a dramatic phenotypic change in a uniform, synchronous cell population. A first analysis of the intracellular regulation of this cell shape morphogenesis indicated a change from the actin-based motility system of amebas to the tubulin-based motility system of flagellates, and implicated changes in intracellular free calcium ions in the process (12). In addition, there are major changes in gene expression during differentiation. Actin synthesis ceases, and translatable actin mRNA rapidly disappears (37), whereas the tubulin for flagella is synthesized during differentiation and its translatable mRNA markedly increases in abundance and subsequently decreases in parallel with the rate of flagellar tubulin synthesis *in vivo* (28). The likely role of intracellular free calcium ions in this morphogenesis led us to examine the synthesis, and translatable mRNA, for a major receptor of calcium signals in eukaryotic cells, calmodulin.

Calmodulin, ubiquitous and conserved among eukaryotes, serves as the calcium-dependent regulator of diverse enzymes and proteins (9, 24, 30). It has also been implicated in cell motility, in the assembly and function of both actin-based and tubulin-based motility systems, and it has been found in flagella (24, 30), although in most cases these roles of calmod-

ulin are not yet precisely defined. As a multifunctional target for calcium, calmodulin is a logical starting point for an inquiry into the role of intracellular free calcium ions in cell morphogenesis.

If calmodulin were important in *Naegleria* differentiation it, like flagellar tubulin, might be synthesized during differentiation. In this study, we first developed a simple assay to determine whether translatable mRNA for a calmodulin-like calcium-binding protein changes in abundance during differentiation. We found, to our surprise, that translatable mRNAs for not one but two such polypeptides, which differ in apparent molecular weight, appear during differentiation. A preliminary account of this finding has been presented (14). The unexpected finding of two calcium-binding proteins motivated us to determine whether one or both of these polypeptides is indeed calmodulin; here we show that both are calmodulin by several standard criteria. In addition, we find that they are segregated in flagellates, one in flagella and the other in the cell bodies. These results indicate that two different calmodulins are synthesized during differentiation and serve distinct roles in the flagellate. Finally, we report that the translatable mRNAs for these two calmodulins increase in abundance and subsequently decrease concurrently during differentiation and, further, that these changes in abundance occur contemporaneously with the changes in abundance of mRNAs for flagellar tubulin.

Materials and Methods

Differentiation, RNA Preparation, and Cell-free Translation

Total RNA was isolated from cells of *N. gruberi* NEG undergoing synchronous differentiation at 25°C and then translated in the wheat germ, cell-free system, using L-[³⁵S]methionine as label, as described (28). To enrich for calmodulin-like proteins among the translation products, at the end of translation the extract was heated for 2 min at 90°C, cooled to 4°C, and the coagulated material removed by centrifugation (4°C, 10 min at 10,000 g). The supernatant was divided into two aliquots, one of which received CaCl₂ to 1 mM and the other EGTA to 1 mM. After addition of Laemmli sample buffer (27), mixing, and 2 min at 100°C, the translation products were separated by SDS PAGE (see below) and visualized by autoradiography. The radioactivity in individual gel bands was determined as described (28).

Purification of Calmodulin

Amebas of *N. gruberi* NEG were grown in 50 baking trays (25), which yielded 5×10^{10} cells. To obtain differentiation to flagellates, amebas were washed to 2 mM Tris-HCl, pH 7.2, and incubated at 28°C in suspension (25). At 110 min, when flagellates had full-length flagella (15 μm), the flagellates were centrifuged (4°C, 4 min at 900 g), and either processed directly or fractionated into cell bodies and flagella. For this fractionation, flagella were detached by a 15-s pH shock, cell bodies were collected and washed by low-speed centrifugation, and the flagella were purified through discontinuous sucrose gradients (25). Subsequent steps were at 0–4°C unless otherwise stated. Whole flagellates, cell bodies, or flagella were finally resuspended in 100 ml or less of lysis buffer (10 mM Tris-HCl, pH 7.5, 40 mM sodium pyrophosphate, 0.1 mM 2-mercaptoethanol, 1 mM EGTA, 30% [wt/vol] sucrose) containing protease inhibitors (leupeptin, 40 μg/ml; aprotinin, 0.1 trypsin inhibitor units/ml; and phenylmethylsulfonyl fluoride, 0.1 mM; all from Sigma Chemical Co., St. Louis, MO). Triton X-100 was added to 0.5% (vol/vol) and the suspension was shaken vigorously for 40 s. The lysate was transferred quickly to a pre-chilled Beckman SW27 rotor and centrifuged (2 h at 25,000 rpm). The supernatant was heated at 90°C for 5 min with gentle swirling, cooled to 0°C for 30 min, and the denatured proteins were removed by centrifugation (30 min at 40,000 g, unless otherwise specified). This supernatant was dialyzed against buffer D (20 mM Tris-HCl, pH 7.5, 0.1 mM EGTA, 0.1 mM MgCl₂, 0.1 mM 2-mercaptoethanol), and centrifuged to remove any precipitate. This heat-stable extract was fractionated on three successive chromatographic columns (11, 18). The eluted fractions were monitored by measuring A₂₈₀ of each fraction and by taking 0.5-ml aliquots of selected fractions to assay their ability to stimulate phosphodiesterase activity. (These aliquots were dialyzed against 1 mM 2-mercaptoethanol, lyophilized, resuspended in 50 μl 10 mM Tris-HCl, pH 7.5, and assayed with phosphodiesterase as described below.) The heat-stable extract was loaded on a DEAE-cellulose (DE-52, Whatman Inc., Clifton, NJ) column (2.5 × 30 cm) equilibrated with buffer D. The column was washed with buffer D until A₂₈₀ reached background, and then bound proteins were eluted with buffer D containing 0.4 M NaCl. The fractions that stimulated phosphodiesterase were pooled and dialyzed against buffer C (20 mM sodium acetate, pH 5.0, 1 mM CaCl₂, 0.1 mM 2-mercaptoethanol), centrifuged, and the supernatant loaded on a DEAE-Sephadex A-25 (Pharmacia Fine Chemicals, Piscataway, NJ) column (1.5 × 25 cm) pre-equilibrated with buffer C. The column was eluted with a 400-ml linear gradient of 0 to 0.6 M NaCl in buffer C. The active fractions were pooled and dialyzed against buffer P (20 mM Tris-HCl, pH 7.5, 1 mM CaCl₂, 0.1 mM 2-mercaptoethanol), centrifuged, and loaded onto a phenyl-Sepharose (Pharmacia Fine Chemicals) column (1 × 10 cm) at room temperature, pre-equilibrated with buffer P. The column was washed with buffer P until A₂₈₀ reached background, and then bound proteins were eluted with buffer E (20 mM Tris-HCl, pH 7.5, 10 mM EGTA, 0.1 mM 2-mercaptoethanol). Pooled fractions were dialyzed against 1 mM 2-mercaptoethanol, lyophilized, dissolved in 1 mM Tris-HCl, pH 7.5, 0.1 mM CaCl₂, and 0.1 mM 2-mercaptoethanol, and stored in small aliquots at –80°C.

Protein Determination and Electrophoresis

Routine protein determinations used the Lowry method (29) with bovine serum albumin as standard. Purified calmodulins were quantified by densitometry of polyacrylamide tube gels stained with Fast Green FCF (15), using purified bovine brain calmodulin as standard. SDS PAGE on discontinuous slab gels (27) used either a 15 to 20% exponential gradient of acrylamide (28) (Figs. 1 and 7) or 15% acrylamide (Figs. 3–4); the gels were stained with Coomassie Brilliant Blue R-250. Size standards were as follows: bovine brain calmodulin,

prepared as described (3), a gift of S. Rudnick (New York Medical College); *Dictyostelium* calmodulin (11), a gift of M. Clarke (Albert Einstein College of Medicine); rabbit skeletal muscle troponin C and troponin I (20), a gift of C. Cohen (Brandeis University); and egg white lysozyme (Worthington Biochemical Corp., Freehold, NJ).

Phosphodiesterase Assay

The ability of extracts and proteins to stimulate calcium- and calmodulin-dependent cyclic nucleotide phosphodiesterase were determined as described (11), except that bovine heart phosphodiesterase (Sigma Chemical Co., P0520, 5 μg/50 μl) was used. In the absence of calmodulin, ~10% of the cyclic [³H]-AMP in a reaction mixture was hydrolyzed. The addition of calmodulin resulted in a maximal increase of fivefold.

Radioimmune Assay

Competitive radioimmunoassays of calmodulin, similar to published procedures (5, 7), were done using New England Nuclear (Boston, MA) kit NEK-018. Serial dilutions of purified protein or heat-stable cell extracts were assayed in duplicate for their ability to compete with ¹²⁵I-labeled calmodulin (bovine brain) for binding to affinity-purified anti-calmodulins (prepared to rat testis calmodulin in sheep). All samples were heated 5 min at 90°C just before assay. Control tubes without competing antigen were used to calculate 100% bound, which averaged ~6,000 cpm/sample.

Results

Assay for Translatable mRNA Encoding Calmodulin-like Proteins

A simple assay was developed to test for the presence and relative abundance of translatable mRNA encoding calmodulin-like calcium-binding proteins during *Naegleria* differentiation. This assay uses two attributes of calmodulin: heat stability and calcium-dependent mobility shift in SDS PAGE (3, 9, 19). [³⁵S]Methionine-labeled translation products were heated to denature most of the protein in the extract. The supernatant was then analyzed by SDS PAGE in the presence or absence of Ca²⁺, using paired samples on single gels (17, 19), followed by autoradiography.

Translatable mRNA for Two Calcium-binding Proteins Is Present in Differentiating Cells But Not in Amebas

The above assay was applied to translation products directed by total RNA extracted from cells at various times during differentiation. Under the conditions used, differentiation is initiated in early stationary-phase amebas at 0 min, the cells begin to form flagella at 57 ± 2 min, and by 100 min the flagella reach full length and the cells have assumed their streamlined body contour (16, 28). Translatable mRNAs that directed the synthesis of two calcium-binding polypeptides were found in differentiating cells (20–80 min); these mRNAs reached peak abundance at ~60 min of differentiation (see below). A sample result is shown in Fig. 1. The two polypeptides in the translation product of 60-min RNA that migrated faster in the presence of Ca²⁺ than in its absence are designated products 1 and 2. Product 1 was the major heat-stable translation product. An inventory of these products (Table I) shows that they accounted for ~1.5 and 0.6% of the methionine radioactivity in the translation product. In spite of these amounts, the two products were not detected when the unfractionated translation product was examined by one-dimensional electrophoresis and autoradiography without the enrichment procedure.

The major and apparently larger product, product 1, migrated faster in SDS PAGE than bovine brain calmodulin,

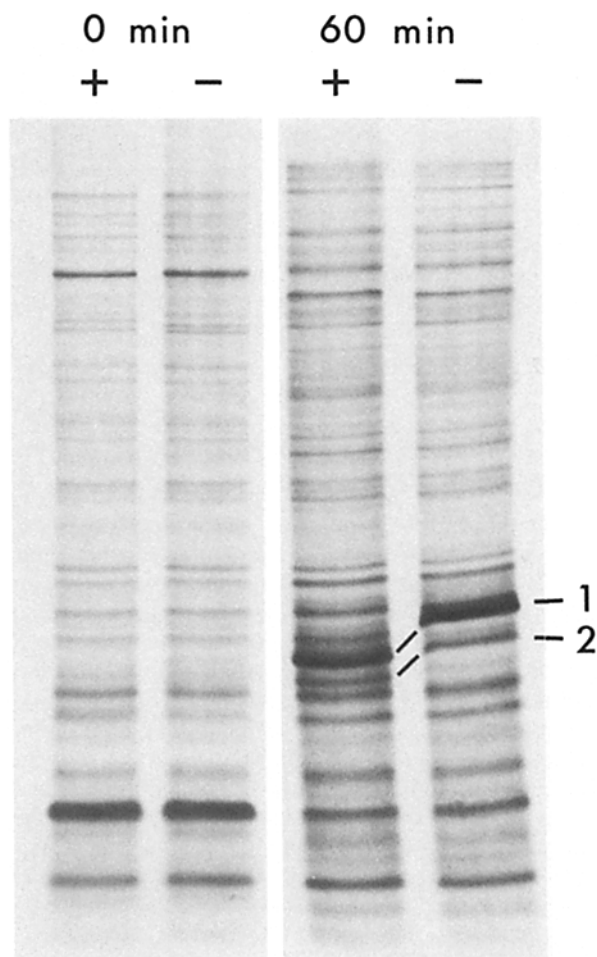


Figure 1. Calcium-binding proteins among the [³⁵S]methionine-labeled translation products directed by total RNA extracted after 0 and 60 min of differentiation. The supernatants, after heating, were resolved on SDS PAGE in the presence of 1 mM CaCl₂ (+) or in the absence of Ca²⁺ produced by 1 mM EGTA (-). Each lane was loaded with 1.5 × 10⁶ cpm of heat-stable translation product. The two calcium-binding proteins are indicated on the right.

but co-migrated with *Dictyostelium* calmodulin. This product had an apparent molecular weight, in the absence of Ca²⁺, of ~16,000, close to a recent estimate for *Dictyostelium* calmodulin (21), as compared to 16,800 for vertebrate calmodulin (24). Product 2 migrated still faster, with an apparent molecular weight of ~15,300. These polypeptides all showed a calcium-dependent mobility shift. In our early experiments, this shift was reproducibly 4–6 mm (as Fig. 1), with brain calmodulin showing a shift of 6 mm; *Naegleria* product 1 and *Dictyostelium* calmodulin, 5 mm; and *Naegleria* product 2, 4 mm. In later experiments, using new lots of all PAGE components, we were unable to obtain shifts greater than 2 mm, but all still showed the shift. We conclude that the two products synthesized from mRNA of differentiating *Naegleria* are calcium-binding proteins that migrate similarly to protozoan calmodulins and show the calcium-dependent anodal mobility shift characteristic of calmodulin.

A most striking result is that translatable mRNAs for these two calcium-binding polypeptides could not be detected in 0-min RNA, i.e., in RNA from amoebas (Fig. 1). As described previously (28, 37), and as shown in the comparison of heat-

Table I. Inventory of Calcium-binding Proteins in a Cell-free Translation Directed by RNA Isolated at 60 min of Differentiation

Sample	Product %
Translation product (659,000, trichloroacetic acid insoluble, cpm/μl)	100
Heat-stable supernatant	17
Calcium-binding protein-1 (9% of heat stable)	1.5
Calcium-binding protein-2 (37.5% of -1)	0.6

Table II. Purification of *Naegleria* Calmodulins: Scheme and Example of Yields

Fraction	mg protein (% of initial)		
	Flagellate	Cell body	Flagella
Extract	3,062 (100)	2,800 (100)	60 (100)
5 min at 90°C supernatant	567 (18.5)	435 (15.5)	22 (36.7)
DEAE-cellulose active fractions	150 (4.9)	94 (3.4)	10 (16.7)
DEAE-Sephadex A-25 active fractions	20 (0.65)	15 (0.54)	3 (5.0)
Phenyl-Sepharose EGTA eluate	0.15 (0.005)	0.01 (0.0003)	0.07 (0.12)
Specific activity of final product*	2.36	2.30	2.25
Calmodulin found	CaM-1 and CaM-2	CaM-2	CaM-1
Ratio CaM-1/CaM-2	3.4 [†]	—	—

* Units of calmodulin activity/μg protein. One unit is the amount required to increase the rate of cAMP hydrolysis by 1 nmol/min under the conditions of phosphodiesterase assay.

[†] The average of values obtained by scanning gels, such as Figs. 3b and 4a, and determining the relative peak areas.

stable translation products in Fig. 1, translatable mRNAs for some products remain relatively constant during differentiation, others increase or decrease in abundance, and others show such marked changes that they appear or disappear. The apparent absence of translation products for calmodulin-like proteins in the 0-min sample has been reproduced with several different preparations of 0-min RNA.

Isolation of Calmodulin-like Proteins from Flagellates

The presence of mRNA for two calmodulin-like polypeptides in differentiating cells, but not in amoebas, indicated that these polypeptides probably are synthesized during differentiation. This motivated us to attempt to purify and characterize these proteins from flagellates. Successful isolation was dependent on the presence of protease inhibitors during extraction. Our final purification scheme is outlined in Table II. Fractions of the heat-stable extract that activated calmodulin-dependent phosphodiesterase eluted from each of the DEAE columns as described by others for calmodulins (see, for example, references 3, 11, 24). As shown in Fig. 2, a small active fraction bound to phenyl-Sepharose (18) in the presence of Ca²⁺ but eluted in the absence of Ca²⁺. This fraction, when examined by electrophoresis, showed two bands that gave a calcium-

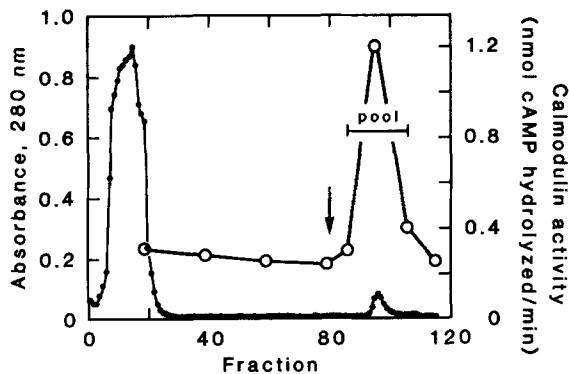


Figure 2. Phenyl-Sepharose hydrophobic affinity chromatography, the final step in purification of calmodulins from flagellates. The initial sample and elution buffer contained 1 mM CaCl_2 . 1-ml samples were collected, and their A_{280} determined (●). At fraction 80 (arrow), after the A_{280} reached background, the elution was continued with a buffer containing 10 mM EGTA. At intervals, aliquots were assayed for their ability to stimulate calmodulin-dependent phosphodiesterase (○). The indicated fractions were pooled.

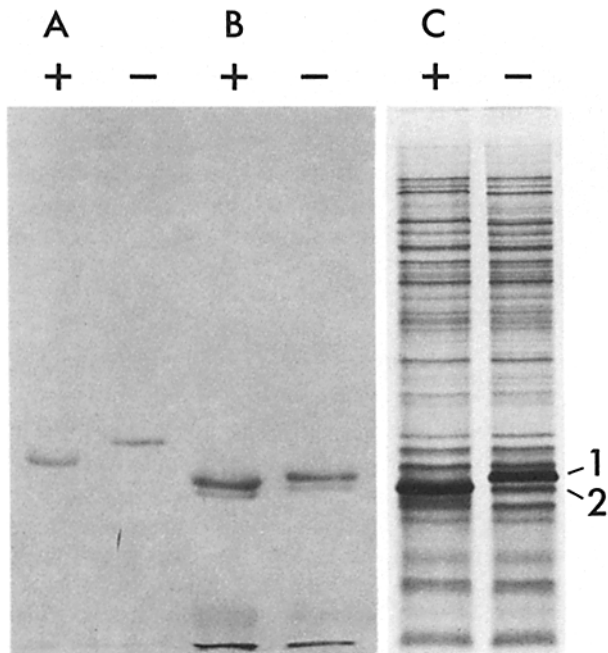


Figure 3. Electrophoretic mobility of calmodulins from flagellates. SDS PAGE in the presence (+) or absence (-) of Ca^{2+} (5 mM CaCl_2 or EGTA, respectively) of (A) 2 μg bovine brain calmodulin, and (B) 5 μg of calmodulins purified from flagellates. (C) Autoradiograph of translation products directed by 60-min RNA.

dependent mobility shift (Fig. 3b), co-migrated with the two calcium-binding polypeptides in translation products directed by 60-min RNA (Fig. 3c), and migrated faster than vertebrate calmodulin (Fig. 3a). No polypeptides other than these two have been detected in the purified fraction, but we have not had sufficient amounts of the proteins for a proper assessment of homogeneity. The steps in isolation indicate that these two polypeptides share the following attributes of calmodulin: thermal stability, binding to and elution from DEAE columns (which also indicates that they are acidic polypeptides), hydrophobic interaction with phenyl-Sepharose in the presence but not in the absence of Ca^{2+} , and a calcium-dependent

mobility shift in SDS PAGE. Two more decisive tests, presented below, justify designation of these proteins as calmodulins, specifically as CaM-1 and CaM-2.

In several comparisons the mobilities of these two polypeptides, in the presence and absence of Ca^{2+} , have been indistinguishable between the proteins purified from flagellates and the products of translation of 60-min RNA in a cell-free system. We conclude that these are the same polypeptides, and that their duality occurs both in *Naegleria* and in the wheat germ system. In purified preparations, the ratio of CaM-1/CaM-2 was 3.4 (Table II); in translation products it was 2.7 (Table I). In view of the uncertainties in both measurements, these results are considered similar and indicative of a 3:1 ratio.

The Two Calmodulin-like Proteins Are Segregated, One in Flagella and the Other in Cell Bodies

Preliminary observations indicated that only CaM-1 was found in heat-stable extracts of flagella. To examine this localization further, we separated flagellates into cell bodies and flagella, purified calmodulin-like proteins as described above, and compared them by electrophoresis. The results (Table II and Fig. 4) indicate that flagella contain CaM-1 and cell bodies contain CaM-2. This segregation has been observed in all experiments. Mixtures of the calmodulins purified from the cell body (Fig. 4b) and from the flagella (Fig. 4c) restored the doublet of CaM-1 and -2 seen in calmodulin purified from whole flagellates (Fig. 4a) (data not shown). Since the amount of protein, particularly CaM-2, is small, we cannot conclude that the segregation is absolute, but we have never detected CaM-1 in cell bodies or CaM-2 in flagella.

The Two Proteins Are Calmodulins

Two particularly decisive tests for calmodulins, as opposed to related calcium-binding proteins, are the calcium-dependent

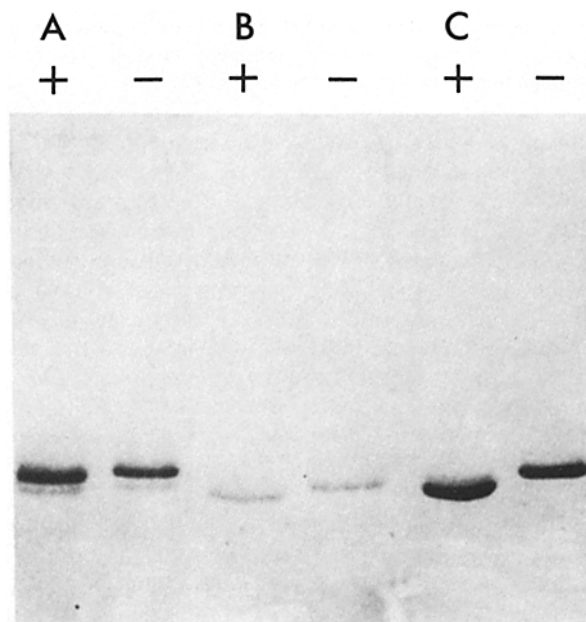


Figure 4. Segregation of two calmodulins in flagellates. SDS PAGE in the presence (+) and absence (-) of Ca^{2+} (as in Fig. 3). (A) 5 μg of flagellate calmodulins, (B) 2 μg of cell body calmodulin, and (C) 5 μg of flagellar calmodulin.

stimulation of calmodulin-dependent enzymes, especially phosphodiesterase, and immunoreactivity to anti-calmodulin sera. These tests are specific to calmodulin (4, 24), and both are used to measure calmodulin in crude extracts (7, 40). The calmodulin-like proteins of *Naegleria* flagellates were isolated on the basis of their ability to activate calmodulin-dependent phosphodiesterase (Table II and Fig. 2). A direct comparison of the ability of the purified proteins to activate phosphodiesterase is shown in Fig. 5. The three *Naegleria* preparations tested—the mixture of CaM-1 and -2 from whole flagellates, CaM-1 from flagella, and CaM-2 from cell bodies—were all equally effective in activating the phosphodiesterase, and indistinguishable from bovine brain calmodulin. The activation required Ca^{2+} and the extent of activation was dose dependent (Fig. 5). Similar quantities of CaM-1 and CaM-2 gave comparable activation, which indicates that both these polypeptides have calmodulin activity, i.e., that minor contamination of one with the other cannot be responsible for the stimulation of phosphodiesterase. These results are typical of those obtained with other calmodulins. Pure *Dictyostelium* calmodulin, for example, is also indistinguishable from vertebrate calmodulin in the activation of phosphodiesterase (21, 31). The sensitivity to EGTA and titration of activity with increasing calmodulin rule out artifactual activation such as can be caused by proteolytic cleavage of phosphodiesterase (24, 40).

Diverse antisera to vertebrate calmodulin have been found to react only with calmodulin. We assessed the immunoreactivity of flagellate calmodulin-like proteins in a competitive radioimmuno assay using antibodies to rat testis calmodulin and bovine brain calmodulin as the competing antigen. All three preparations of flagellate proteins reacted with the antisera (Fig. 6). CaM-1 from flagella, or the mixture of CaM-1 and -2 from flagellates, competed similarly, and gave 50%

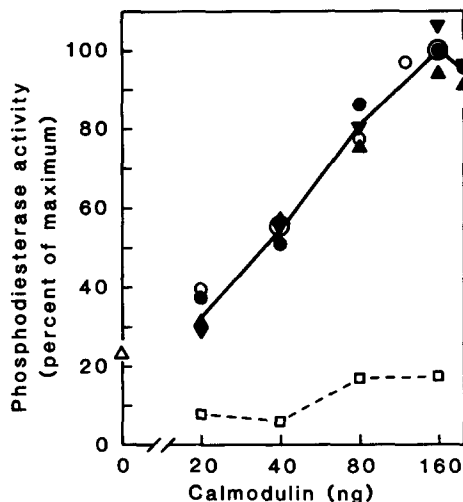


Figure 5. Dose-dependent activation of cyclic nucleotide phosphodiesterase by *Naegleria* and bovine calmodulins. Each reaction mixture contained the indicated amounts of (○) bovine brain calmodulin, (●) calmodulins from whole flagellates, (▲) CaM-1 from flagella, and (▼) CaM-2 from cell bodies. For the three samples from *Naegleria*, the solid line connects the average of the data points. The basal phosphodiesterase activity is indicated (Δ), as is the activity when calmodulins from flagellates were assayed in the presence of 10 mM EGTA (□).

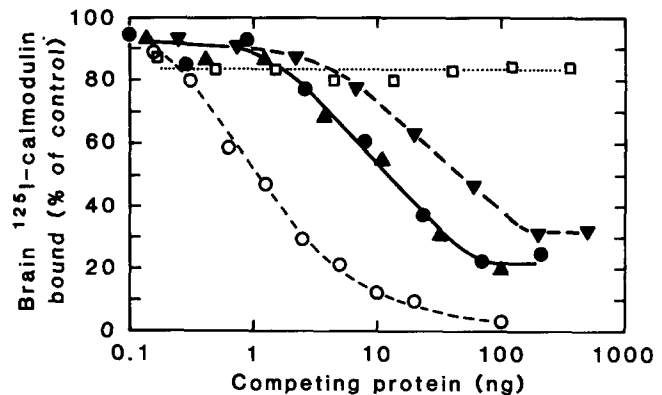


Figure 6. Immunoreactivity of *Naegleria* calmodulins. Dilutions of each of the following proteins were assayed for their ability to compete for the binding of bovine brain calmodulin to antibodies against rat testis calmodulin. (○) Bovine brain calmodulin; (●) calmodulins from whole flagellates; (▲) CaM-1 from flagella; (▼) CaM-2 from cell bodies; and (□) troponin C.

inhibition of binding at ~12 ng protein. CaM-2 from cell bodies reproducibly reacted somewhat less, and in this experiment required 46 ng for 50% inhibition. Both were less immunoreactive than homologous vertebrate calmodulin, which gave 50% inhibition at 1.1 ng. In a similar radioimmuno assay, *Dictyostelium* calmodulin was found to require ~25-fold more protein than brain calmodulin for 50% inhibition (1). In our assays, the brain calmodulin also competed more completely than did either of the *Naegleria* calmodulins (Fig. 6), which suggests that perhaps 20–30% of the anti-calmodulins in this polyclonal antiserum were antibodies that did not recognize *Naegleria* calmodulins. Troponin C, most closely related to calmodulin among the known calcium-binding proteins (24), did not compete in the radioimmuno assay, even at 360 ng.

The radioimmuno assay was used to estimate the amount of calmodulin in flagellates. The soluble proteins in a heat-stable extract of flagellates required 26 μg of protein for 50% inhibition, which is equivalent to 0.12 μg calmodulin per mg of total flagellate protein, or to calmodulin as ~0.012% of the total protein of a flagellate. The final yield of calmodulin from whole flagellates was 0.005% of the total protein (Table II), so based on the value obtained from radioimmuno assay, the recovery of calmodulin was roughly 40%. Radioimmuno assay of heat-stable extracts of amoebae indicated that they contain ~20% as much calmodulin antigen as do extracts of flagellates. Extracts of both amoebae and flagellates were able to compete 70–80% of the vertebrate calmodulin, as were the purified *Naegleria* calmodulins.

Translatable mRNAs for the Two Calmodulins Increase and Decrease in Abundance during Differentiation Concurrently with Those for Flagellar Tubulin

To assess the relative abundance of translatable mRNAs for CaM-1 and CaM-2 during differentiation, total RNA isolated at successive times during differentiation was translated at a rate-limiting concentration of RNA to minimize competition (28), and the heat-stable products were resolved by SDS PAGE under the conditions used for Fig. 1. After the methionine-labeled radioactive products were localized by autoradiogra-

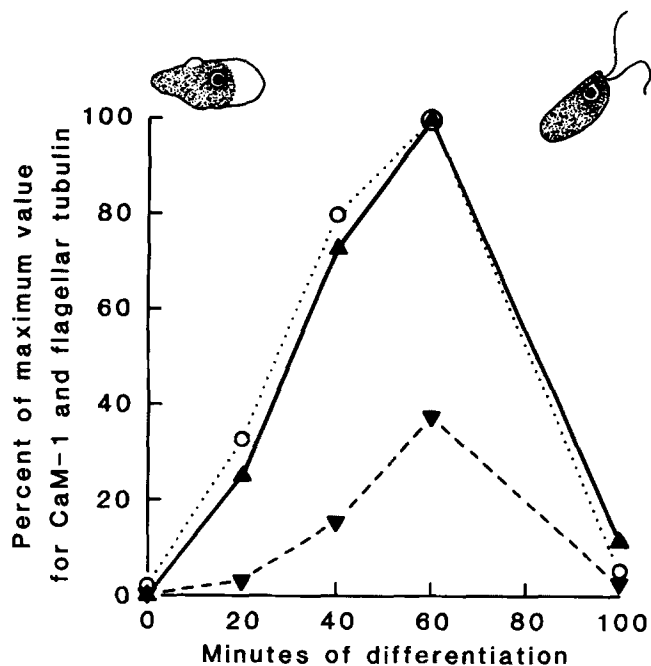


Figure 7. Relative abundance of translatable mRNAs for calmodulins and for flagellar tubulin during differentiation. (○) Flagellar tubulin, (▲) CaM-1, (▼) CaM-2. Quantitative translation of total RNA extracted at successive times during differentiation and assay of flagellar tubulin in the product were as described (28). The calmodulins were assayed by heating the translation products, analyzing the supernatants as in Fig. 1, and then determining the amount of radioactivity in the bands formed by CaM-1 and -2 in the presence of Ca^{2+} . Each lane was loaded with 1.5×10^6 cpm of heat-stable translation product. The lane containing the 0-min translation product showed no detectable CaM-1 or -2 (see Fig. 1), but contained 21,910 and 21,625 cpm, respectively, in the excised bands. These background counts, low for any region of lanes containing translation product, were subtracted from all other counts so the 0-min values for translatable calmodulin mRNAs are 0%, and no accurate estimate can be made of the extent of increase. If these background values were not subtracted, translatable mRNA for CaM-1 increased at least 6.2-fold, and CaM-2 at least threefold. The two major translation products are normalized to 100% at 60 min, when flagellar tubulin amounts to ~5% of the radioactivity in the product (28) and CaM-1 to ~1.5% (Table I).

phy, the calmodulin bands were excised from the lanes containing Ca^{2+} and their radioactivity was determined. The same RNA samples were translated and quantitatively immunoprecipitated using polyclonal antibodies reactive to both subunits of flagellar tubulin, as described previously (28). The results are presented in Fig. 7. The amounts of the translation products for the two calmodulins, and therefore the abundance of their translatable mRNAs, reached a maximum at ~60 min—just as the flagella were being assembled—and then declined markedly by 100 min. The rise and fall in abundance of translatable mRNAs for these two calmodulins was contemporaneous with the similar change in abundance of translatable mRNAs for the flagellar tubulin subunits.

Discussion

The evidence is compelling that the two polypeptides, isolated from flagellates, are both calmodulins. Some attributes are shared with other small calcium-binding proteins, including

thermal stability, acidity, Ca^{2+} -dependent mobility shift in SDS PAGE, and binding to phenyl-Sepharose. No protein other than calmodulin, however, is known to activate calmodulin-dependent phosphodiesterase in a Ca^{2+} - and dose-dependent manner, or to react with antibodies to vertebrate calmodulin. All these criteria, considered together, are sufficient to define CaM-1 and CaM-2 as calmodulins. Because these proteins amount to such a minute fraction of the total cell protein—~0.01% together—preparing the proteins for more extensive characterization, such as amino acid sequencing, would be a major challenge. We think it more promising to first approach matters such as the extent to which the sequences of these calmodulins match other calmodulins via DNA cloning and sequencing.

The two calmodulins differ in apparent molecular weight which, along with the difference in intracellular location, could easily arise from posttranslational processing of a single polypeptide. However, two calcium-binding proteins with the same electrophoretic mobilities are synthesized in the wheat germ, cell-free system directed by mRNA from differentiating cells. In addition they are synthesized in about the same proportion, 3:1, that CaM-1 and CaM-2 are found in flagellates. If one proposed that their differences arise by posttranslational modification of a single polypeptide, then such processing must occur, to a comparable extent, in the wheat germ extracts and in differentiating *Naegleria*. It is more likely that they are the independent products of different transcripts, although one can only conjecture whether such transcripts arise from one gene or two. DNA cloning and sequencing also offer a promising way to dissect the nature of the two calmodulins and their gene or genes.

Calmodulins migrate anomalously in SDS PAGE (3, 24). Protozoan and plant calmodulins appear smaller than vertebrate calmodulins (4, 24), but in at least *Dictyostelium*, *Tetrahymena*, and spinach calmodulins, the change in apparent molecular weight is not due to a comparable change in polypeptide chain length (31, 41, 42). We conclude that the difference in apparent molecular weight of CaM-1 and -2 does not justify inferences about possible differences in polypeptide chain length.

Considerable evidence supports the conclusion that a eukaryote usually contains only a single calmodulin, even among all the different tissues of a vertebrate (24). The chicken has only a single calmodulin gene (34), although recently a related but considerably diverged intronless gene has been expressed in bacteria to produce a calmodulin-like protein (35). *Xenopus* has two calmodulin genes, possibly the consequence of total genome duplication during evolution, but these two genes encode a single calmodulin (10). The ciliate *Tetrahymena* and the alga *Chlamydomonas* each appear to share a single calmodulin between cell bodies and flagella (17, 22, 23, 33, 36, 39, 42), although a calmodulin-like protein, which failed to activate phosphodiesterase, has also been reported in *Chlamydomonas* flagella (39). The only clear exception to one calmodulin per organism, other than our report, is the finding of two calmodulins in the egg of the sea urchin *Arbacia punctulata* (2). These two calmodulins differ in electrophoretic mobility and in other attributes, including extent of immunoreactivity and amino acid composition. Sperm of the same species, and eggs and sperm of another sea urchin, *Strongylocentrotus purpuratus*, contain a single

calmodulin (2); it is unclear why *Arbacia* eggs contain two. Our finding is the first report of two calmodulins in a unicellular organism. Since the only readily detected difference between CaM-1 and -2 is in apparent molecular weight, it is quite possible that other organisms may have multiple calmodulins which do not show a difference in electrophoretic mobility, i.e., that have not been detected.

The roles of these two calmodulins in the flagellate are not known. Calmodulins have been found in cilia and flagella, and are presumed to be important in some aspect of regulation of flagellar motility (24, 30); perhaps CaM-1 has such a role. The cell body calmodulin, CaM-2, possibly might be involved in the calcium-regulation of cell morphogenesis described previously (12). Since both are found in flagellates, roles for each either in differentiation or in the flagellate phenotype are likely.

Differential localization of these two calmodulins in flagellates is provocative, and raises questions not only about the role of the two calmodulins but also about how the segregation arises during differentiation. The segregation places the major concentration of calmodulin in the flagella. High concentrations of calmodulin have also been found in *Tetrahymena* cilia (32).

The evidence strongly suggests that these two calmodulins are synthesized during differentiation. The increase in abundance of translatable calmodulin mRNA during the first hour of differentiation, and the subsequent decrease, both parallel the changes in abundance of flagellar tubulin mRNA, which has been rigorously shown to be synthesized during differentiation (15, 26). Estimates by radioimmune assay indicate roughly a fivefold increase in calmodulin antigen during differentiation of amebas to flagellates. At the peak, 2% of the methionine radioactivity in the translation product is in CaM-1 and -2, but at the end of this rapid differentiation, these proteins amount to only 0.01% of the protein of the flagellate. This discrepancy of quantities is to be expected if these calmodulins are synthesized during this brief interval.

In numerous attempts, we have failed to isolate calmodulin from amebas. The radioimmune assay indicates that some calmodulin is present in amebas, as is likely from the ubiquitous distribution of calmodulin among eukaryotes. The matter of the presence of calmodulin in *Naegleria* amebas and especially whether, if present, it is CaM-1, -2, or an undescribed CaM-3, remain unresolved.

There have been few studies of calmodulin synthesis and mRNA abundance. Van Eldik et al. (38) described an assay for translatable mRNA for spinach calmodulin, using a different method to enrich for the product. Calmodulin (6, 30, 43) and calmodulin mRNA (43) are elevated about twofold in transformed versus normal vertebrate cells. Changes in intracellular calmodulin levels during the cell cycle of mammalian cells is accompanied by a 50% decrease and then a fourfold increase in calmodulin mRNA (8). Ours is the first report of a major developmental change in abundance of calmodulin mRNA.

In addition to our finding of two calmodulins in this unicellular organism, their segregation in flagellates, and the programmed change in abundance of their mRNAs during differentiation, we are excited by the contemporaneous rise and fall in abundance of translatable mRNAs for the two subunits of flagellar tubulin and for the two calmodulins. This

result suggests the possibility that the expression of the genes for these unrelated proteins are under coordinate control during differentiation.

We thank Dr. M. Clarke for teaching us the phosphodiesterase assay and for initial advice and encouragement, and Drs. M. Clarke, C. Cohen, and S. Rudnick for generous gifts of proteins.

This work was supported by grants from the National Science Foundation (DCB-8216357) and the National Institutes of Health (GM24444).

Received for publication 17 September 1985, and in revised form 17 December 1985.

References

1. Bazari, W. L., and M. Clarke. 1981. Characterization of a novel calmodulin from *Dictyostelium discoideum*. *J. Biol. Chem.* 256:3598-3603.
2. Burgess, W. H. 1982. Characterization of calmodulin and calmodulin isotypes from sea urchin gametes. *J. Biol. Chem.* 257:1800-1804.
3. Burgess, W. H., D. K. Jemiolo, and R. H. Kretsinger. 1980. Interaction of calcium and calmodulin in the presence of sodium dodecyl sulfate. *Biochim. Biophys. Acta.* 623:257-270.
4. Burgess, W. H., M. Schleicher, L. J. Van Eldik, and D. M. Watterson. 1983. Comparative studies of calmodulin. In *Calcium and Cell Function*. W. Y. Cheung, editor. Academic Press, Inc., New York. 4:209-261.
5. Chafouleas, J. G., J. R. Dedman, R. P. Munjaal, and A. R. Means. 1979. Calmodulin. Development and application of a sensitive radioimmunoassay. *J. Biol. Chem.* 254:10262-10267.
6. Chafouleas, J. G., R. L. Pardue, B. R. Brinkley, J. R. Dedman, and A. R. Means. 1981. Regulation of intracellular levels of calmodulin and tubulin in normal and transformed cells. *Proc. Natl. Acad. Sci. USA.* 78:996-1000.
7. Chafouleas, J. G., M. E. Riser, L. Lagace, and A. R. Means. 1983. Production of polyclonal and monoclonal antibodies to calmodulin and utilization of these immunological probes. *Methods Enzymol.* 102:104-110.
8. Chafouleas, J. G., L. Lagace, W. E. Bolton, A. E. Boyd III, and A. R. Means. 1984. Changes in calmodulin and its mRNA accompany reentry of quiescent (G0) cells into the cell cycle. *Cell.* 36:73-81.
9. Cheung, W. Y. 1980. Calmodulin plays a pivotal role in cellular regulation. *Science (Wash. DC).* 207:19-27.
10. Chien, Y.-H., and I. B. Dawid. 1984. Isolation and characterization of calmodulin genes from *Xenopus laevis*. *Mol. Cell. Biol.* 4:507-513.
11. Clarke, M., W. L. Bazari, and S. C. Kayman. 1980. Isolation and properties of calmodulin from *Dictyostelium discoideum*. *J. Bacteriol.* 141:397-400.
12. Fulton, C. 1977. Intracellular regulation of cell shape and motility in *Naegleria*. First insights and a working hypothesis. *J. Supramol. Struct.* 6:13-43.
13. Fulton, C. 1977. Cell differentiation in *Naegleria gruberi*. *Annu. Rev. Microbiol.* 31:597-629.
14. Fulton, C., and E. Y. Lai. 1980. Programmed appearance of translatable mRNAs for two calmodulin-like proteins during cell differentiation in *Naegleria*. *J. Cell Biol.* 87 (2, Pt. 2):282a. (Abstr.)
15. Fulton, C., and P. A. Simpson. 1976. Selective synthesis and utilization of flagellar tubulin. The multi-tubulin hypothesis. *Cold Spring Harbor Conf. Cell Proliferation 3 (Book C):987-1005.*
16. Fulton, C., and C. Walsh. 1980. Cell differentiation and flagellar elongation in *Naegleria gruberi*: dependence on transcription and translation. *J. Cell Biol.* 85:346-360.
17. Gitelman, S. E., and G. B. Witman. 1980. Purification of calmodulin from *Chlamydomonas*: calmodulin occurs in cell bodies and flagella. *J. Cell Biol.* 87:764-770.
18. Gopalakrishna, R., and W. B. Anderson. 1982. Ca²⁺-induced hydrophobic site on calmodulin: application for purification of calmodulin by phenyl-Sepharose affinity chromatography. *Biochem. Biophys. Res. Commun.* 104:830-836.
19. Grab, D. J., K. Berzins, R. S. Cohen, and P. Siekevitz. 1979. Presence of calmodulin in postsynaptic densities isolated from canine cerebral cortex. *J. Biol. Chem.* 254:8690-8696.
20. Greaser, M. L., and J. Gergely. 1973. Purification and properties of the components from troponin. *J. Biol. Chem.* 248:2125-2133.
21. Jamieson, G. A., Jr., and W. A. Frazier. 1983. Dictyostelium calmodulin: affinity isolation and characterization. *Arch. Biochem. Biophys.* 227:609-617.
22. Jamieson, G. A., Jr., T. C. Vanaman, and J. J. Blum. 1979. Presence of calmodulin in *Tetrahymena*. *Proc. Natl. Acad. Sci. USA.* 76:6471-6475.
23. Kakiuchi, S., K. Sobue, R. Yamazaki, S. Nagao, S. Umeki, Y. Nozawa, M. Yazawa, and K. Yagi. 1981. Ca²⁺-dependent modulator proteins from *Tetrahymena pyriformis*, sea anemone, and scallop and guanylate cyclase activation. *J. Biol. Chem.* 256:19-22.
24. Klee, C. B., and T. C. Vanaman. 1982. Calmodulin. *Adv. Protein Chem.* 35:213-321.

25. Kowit, J. D., and C. Fulton. 1974. Purification and properties of flagellar outer doublet tubulin from *Naegleria gruberi* and a radioimmune assay for tubulin. *J. Biol. Chem.* 249:3638-3646.
26. Kowit, J. D., and C. Fulton. 1974. Programmed synthesis of tubulin for the flagella that develop during cell differentiation in *Naegleria gruberi*. *Proc. Natl. Acad. Sci. USA.* 71:2877-2881.
27. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)*. 227:680-685.
28. Lai, E. Y., C. Walsh, D. Wardell, and C. Fulton. 1979. Programmed appearance of translatable flagellar tubulin mRNA during cell differentiation in *Naegleria*. *Cell*. 17:867-878.
29. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* 193:265-275.
30. Manalan, A. S., and C. B. Klee. 1984. Calmodulin. *Adv. Cyclic Nucleotide Protein Phosphorylation Res.* 18:227-278.
31. Marshak, D. R., M. Clarke, D. M. Roberts, and D. M. Watterson. 1984. Structural and functional properties of calmodulin from the eukaryotic microorganism *Dictyostelium discoideum*. *Biochemistry*. 23:2891-2899.
32. Nagao, S., Y. Banno, Y. Nozawa, K. Sobue, R. Yamazaki, and S. Kakiuchi. 1981. Subcellular distribution of calmodulin and calmodulin-binding sites in *Tetrahymena pyriformis*. *J. Biochem. (Tokyo)*. 90:897-899.
33. Ohnishi, K., Y. Suzuki, and Y. Watanabe. 1982. Studies on calmodulin isolated from *Tetrahymena* cilia and its localization within the cilium. *Exp. Cell Res.* 137:217-228.
34. Putkey, J. A., K. F. Ts'ui, T. Tanaka, L. Lagace, J. P. Stein, E. C. Lai, and A. R. Means. 1983. Chicken calmodulin genes. A species comparison of cDNA sequences and isolation of a genomic clone. *J. Biol. Chem.* 258:11864-11870.
35. Putkey, J. A., G. R. Slaughter, and A. R. Means. 1985. Bacterial expression and characterization of proteins derived from the chicken calmodulin cDNA and a calmodulin processed gene. *J. Biol. Chem.* 260:4704-4712.
36. Schleicher, M., T. J. Lukas, and D. M. Watterson. 1984. Isolation and characterization of calmodulin from the motile green alga *Chlamydomonas reinhardtii*. *Arch. Biochem. Biophys.* 229:33-42.
37. Sussman, D. J., E. Y. Lai, and C. Fulton. 1984. Rapid disappearance of translatable actin mRNA during cell differentiation in *Naegleria*. *J. Biol. Chem.* 259:7355-7360.
38. Van Eldik, L. J., A. R. Grossman, D. B. Iverson, and D. M. Watterson. 1980. Isolation and characterization of calmodulin from spinach leaves and *in vitro* translation mixtures. *Proc. Natl. Acad. Sci. USA.* 77:1912-1916.
39. Van Eldik, L. J., G. Piperno, and D. M. Watterson. 1980. Similarities and dissimilarities between calmodulin and a *Chlamydomonas* flagellar protein. *Proc. Natl. Acad. Sci. USA.* 77:4779-4783.
40. Wallace, R. W., E. A. Tallant, and W. Y. Cheung. 1983. Assay of calmodulin by Ca²⁺-dependent phosphodiesterase. *Methods Enzymol.* 102:39-47.
41. Watterson, D. M., D. B. Iverson, and L. J. Van Eldik. 1980. Spinach calmodulin: isolation, characterization, and comparison with vertebrate calmodulins. *Biochemistry*. 19:5762-5768.
42. Yazawa, M., K. Yagi, H. Toda, K. Kondo, K. Narita, R. Yamazaki, K. Sobue, S. Kakiuchi, S. Nagao, and Y. Nozawa. 1981. The amino acid sequence of the *Tetrahymena* calmodulin which specifically interacts with guanylate cyclase. *Biochem. Biophys. Res. Commun.* 99:1051-1057.
43. Zendejui, J. G., R. E. Zielinski, D. M. Watterson, and L. J. Van Eldik. 1984. Biosynthesis of calmodulin in normal and virus-transformed chicken embryo fibroblasts. *Mol. Cell. Biol.* 4:883-889.