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Detection of calmodulin-binding proteins and calmodulin-dependent phosphorylation linked to calmodulin-dependent chemotaxis to folic and cAMP in *Dictyostelium*

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Abstract

Calmodulin (CaM) antagonists, trifluoperazine (TFP) or calmidazolium (R24571), dose-dependently inhibited cAMP and folic acid (FA) chemotaxis in *Dictyostelium*. Developing, starved, and refed cells were compared to determine if certain CaM-binding proteins (CaMBPs) and CaM-dependent phosphorylation events could be identified as potential downstream effectors. Recombinant CaM ([^{35}S]VU-1-CaM) gel overlays coupled with cell fractionation revealed at least three dozen Ca^{2+}-dependent and around 12 Ca^{2+}-independent CaMBPs in *Dictyostelium*. The CaMBPs associated with early development were also found in experimentally starved cells (cAMP chemotaxis), but were different for the CaMBP population linked to growth-phase cells (FA chemotaxis). Probing Western blots with phosphoserine antibodies revealed several phosphoprotein bands that displayed increases when cAMP-responsive cells were treated with TFP. In FA-responsive cells, several but distinct phosphoproteins decreased when treated with TFP. These data show that unique CaMBPs are present in growing, FA-chemosensitive cells vs. starved cAMP-chemoresponsive cells that may be important for mediating CaM-dependent events during chemotaxis.

Keywords: Calmodulin; CaM-binding proteins; Chemotaxis; Protein phosphorylation; *Dictyostelium*

1. Introduction

Chemotaxis, the directed movement of cells towards or away from a chemical source, occurs during neurogenesis, metastasis, the inflammatory and immune responses, fertilization, and embryogenesis. In transiting from the proliferative, unicellular, feeding phase to the starved, multicellular developmental phase, the cells of *Dictyostelium* switch from folic acid (FA) chemoresponsiveness to cAMP chemotaxis [1–4]. Starvation initiates development leading to an increase in $[Ca^{2+}]_i$ levels and changes in expression of several genes including *car1*, which encodes cAMP receptor 1 (cAR1) [2,5–7]. Among other things, cAR1 mediates the G protein independent influx of Ca^{2+} [8]. More is known about chemotaxis in *Dictyostelium* than any other organism and the role of calcium in chemotaxis has long been recognized [7,9–14]. Calcium-dependent events are central to cytoskeletal rearrangement, membrane ruffling, receptor recycling, filopodial extension, and secretion. One approach to understanding the role of Ca^{2+} in chemotaxis is to examine downstream calcium-binding proteins (CaBPs). Genes encoding eight different four-EF-hand CaBPs, including calmodulin (CaM), have been identified in *Dictyostelium*, of which calcium-binding protein 1 (CBP1) appears to play an essential role in regulating the actin cytoskeleton during aggregation [15,16]. The role of CaM in chemotaxis has also come under scrutiny.

CaM is a primary sensor and effector of intracellular calcium fluxes in all eukaryotic cells and has changed little throughout evolution, such that *Dictyostelium* and mammalian CaM have only a few amino acid differences, yet they are functionally identical [17,18]. This small protein (149 amino acids) is essential in *Dictyostelium*, as it is in all other eukaryotes, since gene disruption results in a lethal phenotype [17]. In *Dictyostelium*, cell and pronuclear fusion, secretion, spore germination, and cell motility are all CaM-dependent events [18–22]. More specifically, cell

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motility and/or contraction in *Dictyostelium* cells is mediated by CaM-dependent effectors including myosin light-chain kinase [23,24], myosin heavy-chain kinase [25], actin-binding protein fodrin [26], IQGAP-related protein DGAP1 [27], plus the myosin I isoforms myoA–E [28] and myoJ [29]. Calcineurin, the sole eukaryotic CaM-dependent protein phosphatase, has also been implicated in cAMP chemotaxis [30,31]. In spite of this knowledge, the role of CaM in chemotaxis may be more complex than previously envisioned, since at least four dozen Ca^{2+}-dependent and -independent CaM-binding proteins (CaMBPs) have been shown to exist in *Dictyostelium* with some of them likely mediating some of subtleties of behavior exhibited by cells responding to different chemoattractants. To date, comparative studies of the signal transduction events that mediate FA (growth phase) and cAMP (developmental phase) chemotaxis in *Dictyostelium* have received little attention and no direct study has been carried out on the overall importance of CaM during chemotaxis to either FA or cAMP [8,32–35]. Similarly, no comparative or experimental analyses of the subcellular CaMBP populations or of their changes during asexual development or chemotaxis have been done in this organism. We have been characterizing cDNAs from an expression library from cAMP-responsive, developing cells of *Dictyostelium* in an attempt to understand their role in development and chemotaxis.

In the present study, the importance of CaM for chemotaxis to both FA and cAMP chemotaxis was demonstrated through the use of the antagonists trifluoperazine (TFP) and calmidazolium (R24571), each of which dose-dependently inhibited chemotaxis in a bioassay system. A subsequent analysis of the CaMBPs that were present in cytosolic, nuclear, and membrane fractions of *Dictyostelium* cells during development and in starved and refed cells was carried out revealing that certain CaMBPs could be specifically linked to either FA or cAMP chemotaxis. Finally, certain proteins that underwent CaM-dependent phosphorylation or dephosphorylation were observed in feeding (FA chemotaxis) or starved (cAMP chemotaxis) cells. In total, this work has shown that while chemotaxis to FA and cAMP are both CaM-dependent, CaM mediates different events in FA- and cAMP-responsive cells by operating through different CaMBP populations and by affecting the level of phosphorylation of different serine-containing phosphoproteins that may underlie these chemotactic processes.

2. Material and methods

2.1. Culture conditions and synchronous morphogenesis

Dictyostelium strain JH10 (from R. Firtel, University of California, San Diego) was grown as shaking culture using HL5 medium at 22°C supplemented with thymidine at a final concentration of 100 μg/ml [36,37]. To initiate multicellular development, cells in the exponential phase of growth (1×10^6 to 2×10^6 cells/ml) were washed three times in LPS (25 mM KCl, 0.5 g/ml streptomycin sulfate, 2.5 mM $MgCl_2$, 27 mM NaH_2PO_4, 24 mM Na_2HPO_4, pH 6.4), resuspended at the same density in LPS, and allowed to starve in this nonnutrient buffer for 2 h. After this time, the cells were collected and resuspended in LPS at a concentration of 1×10^8 cells/ml [36]. A fraction of this suspension (500 μl) was uniformly plated onto 47-mm Millipore filters over LPS-saturated Whatman No. 3 filters in a petri dish. The petri dishes were then incubated in a humidity chamber and the cells were harvested every 6 h for subcellular fractionation.

2.2. Radial chemotaxis assay

Dictyostelium strain JH10 was grown on Sussman's (SM) agar with *Escherichia coli* as a food source [38]. Spores from newly developed sorocarps were harvested and inoculated with *E. coli* and allowed to germinate for 40–42 h in the presence of bacteria on new SM agar plates. Amoebae were harvested, washed three times in KK_2-phosphate buffer (16.5 mM KH_2PO_4, 3.8 mM K_2HPO_4, pH 6.2), resuspended at 5×10^6 cells/ml in the same buffer, and shaken at 100 rpm for 2 and 4 h. Cells starved for 4 h were refed bacteria at 1.5 A_{595} and shaken at 100 rpm for 2 and 4 h. An aliquot of vegetative amoebae, starved cells (2 and 4 h), and refed cells (2 and 4 h) were harvested, concentrated to 1×10^8 cells/ml, and spotted at regular intervals on agar plates using a 5–10-μl micropipette tip [35,39]. Each plate contained 1 ml of 0.4% agar, 10 μM cAMP (Sigma, St. Louis, MO) or 50 μM FA (Sigma) made with KK_2-phosphate buffer. Where indicated, TFP (Sigma) or calmidazolium (R24571; Biomol Research Laboratories, Plymouth Meeting, PA) were dispensed from stock solutions prepared in ddH_2O or ethanol, respectively, added to the warm cAMP or FA agar plates and mixed uniformly before allowing the agar to cool. Cooled agar plates where then spotted with the concentrated cell suspension and incubated at room temperature. After 3 h, outwardly migrating cells produced a visible halo; the diameter of this halo was subtracted from the diameter of the original drop of cells allowing for the distance migrated to be calculated. Images were captured at 3 h using a Zeiss inverted microscope equipped with a Sony 950 digital camera and analyzed via the Northern Eclipse image analysis system (Empix Imaging, Mississauga, ON).

2.3. Subcellular fractionation and SDS-PAGE

Cells were harvested at the times indicated above and separated into cytosolic, nuclear, and membrane fractions. For cytosolic fractions, cell extracts were centrifuged at 3500 rpm for 30 s, lysed with SDS sample buffer containing 250 mM Tris–HCl, pH 6.8, 50% glycerol, 10% SDS, 25% 2-β-mercaptoethanol, 0.25% bromophenol blue, boiled for 3–4 min, and centrifuged at 14,500 rpm for 10 min. The

supernatant was removed and stored at −20°C. Preparation and isolation of nuclear extracts was performed as previously detailed [40]. Cells were collected by centrifugation at 3500 rpm for 30 s and washed with ice-cold KK_2 buffer. Cells were then resuspended in 1:10 original volume with lysis buffer containing 50 mM HEPES (pH 7.5), 40 mM $MgCl_2$, 20 mM KCl, 0.15 mM spermidine, 5% sucrose, 14 mM β-mercaptoethanol, 0.2 mM PMSF, 10% Percoll, and 1% NP-40. The nuclei were pelleted by centrifugation at 4900 rpm for 5 min. The nuclear-enriched fraction was again resuspended in 1:10 original volume of lysis buffer and centrifuged at 1050 rpm for 5 min to pellet unlysed cells. The supernatant was then centrifuged at 4900 rpm for 5 min to pellet the nuclei that were subsequently suspended in SDS sample buffer. Crude membrane extraction was performed as previously described [41]. Cells were collected by centrifugation at 3500 rpm for 30 s and resuspended in sucrose lysis buffer containing 10 mM Tris–HCl (pH 7.6), 30% sucrose, 40 mM sodium pyrophosphate, 2 mM EDTA, 1 mM EGTA, 0.4 mM DTT, 0.5% ethanol, 5 mM PMSF. The cells were lysed through three cycles of freeze (with liquid nitrogen)/thaw (at room temperature). Crude membranes were collected by centrifugation at 17,000 rpm for 15 min and subsequently washed in the same lysis buffer. The membranes were resuspended in 20 mM sodium phosphate (pH 6.8) and layered onto a gradient containing 35% sucrose over 55% sucrose in phosphate buffer. The membranes were centrifuged through this gradient at 61,000 rpm for 1 h. Crude membrane fractions were collected from the 35%/55% sucrose interface and washed twice in 20 mM phosphate buffer (pH 6.8) at 14,500 rpm for 10 min. The pellet was then suspended in SDS sample buffer. Protein from all enriched fractions was quantified spectrophotometrically (Bio-Rad Laboratories, Mississauga, ON). Equal amounts of protein (15–20 μg) were added to each lane and electrophoretically separated by 10% SDS-PAGE [42].

2.4. Gel overlay with [^{35}S]VU-1-CaM and Western blotting

Production and purification of recombinant [^{35}S]VU-1-CaM was performed as previously described [18]. Proteins separated by 10% SDS-PAGE were immediately fixed in 40% methanol/7% acetic acid for 30 min and washed in 10% ethanol overnight. Gels were again washed with fresh 10% ethanol for 1 h and 100 mM imidazole (pH 7.0) for 30 min. The gels were then equilibrated in probe buffer containing 20 mM imidazole (pH 7.0), 200 mM KCl, 0.1% BSA, and either 1 mM $CaCl_2$ or 2 mM EGTA for 30 min. Finally, gels were incubated in probe buffer containing 1 μCi/ml [^{35}S]VU-1-CaM overnight at 4°C. Probed gels were washed three times in probe buffer at 2-h intervals and fixed in 40% methanol/7% acetic acid for 1 h. Fixed gels were then stained with Coomassie blue, destained, dried, and exposed to Beta-Max autoradiographic film (Amersham).

Phosphoserine-containing proteins were visualized by Western analysis [43]. Proteins were separated by 10% SDS-PAGE and electrophoretically transferred onto polyvinylidene difluoride membranes (Boehringer Mannheim). Membranes containing transferred proteins were blocked at room temperature for 1 h in Tris-buffered saline (TBS) with 4% BSA. Blocked membranes were then incubated with antiphosphoserine antibodies (Sigma) at a 1:1000 dilution in blocking buffer for 1 h. Probed blots were washed three times for 10 min each in TBS containing 0.05% Tween-20 (TTBS). Washed blots were incubated with horseradish peroxidase-coupled antimouse antibodies (Bio/Can Scientific, Mississauga, ON) at 1:60,000 dilution in blocking buffer for 30 min, subsequently washed four times for 15 min each with TTBS, and visualized using the ECL detection system (Amersham).

3. Results

3.1. Subcellular localization of CaMBPs

Earlier research had shown that cells of *Dictyostelium* were rich in a diversity of CaMBPs but no study had compared the subcellular populations of these CaM-dependent proteins or examined their changes during asexual development or under any other conditions associated with chemotaxis. This work is an important step towards understanding the role of CaM and identifying potential CaMBPs that may mediate events of chemotaxis and/or asexual development (cell differentiation, morphogenesis). Approximately three dozen Ca^{2+}-dependent CaMBPs (i.e., bind CaM in the presence of Ca^{2+}) were resolved during asexual, multicellular development ranging in relative molecular weight from ~12 to 220 kDa (Fig. 1). Most of them localized to the cytosol with about one-half as many present in the nuclear fraction. Only seven Ca^{2+}-dependent CaMBPs were distinctly evident in the membrane fraction. In total, only about one dozen different Ca^{2+}-independent CaMBPs (i.e., can bind CaM in the absence of Ca^{2+}) appeared to be present. The Ca^{2+}-independent CaMBPs comprised a group of proteins with molecular weights of $\sim 40 \times 10^3$ Da and below but one large Ca^{2+}-independent CaMBPs at 220+ kDa was detected. Many Ca^{2+}-independent CaMBPs existed in the cytosol with an equivalent number appearing to localize to the nuclear fraction, while fewer numbers were detected in the membrane fraction (Fig. 1). Of particular interest was the apparent presence of a single, unique Ca^{2+}-inhibited CaMBP (i.e., can bind CaM in the absence of calcium but not when it is present) detected only in the nuclear fraction at ~164 kDa (Fig. 1).

3.2. Developmental changes in CaMBP profiles

Numerous Ca^{2+}-dependent CaMBPs appeared to undergo changes during asexual development. In the cytosol, six Ca^{2+}-dependent CaMBPs ranging in size from 12 to 78 kDa (CaMBPs 78, 66, 56, 46, 43, and 12 kDa)

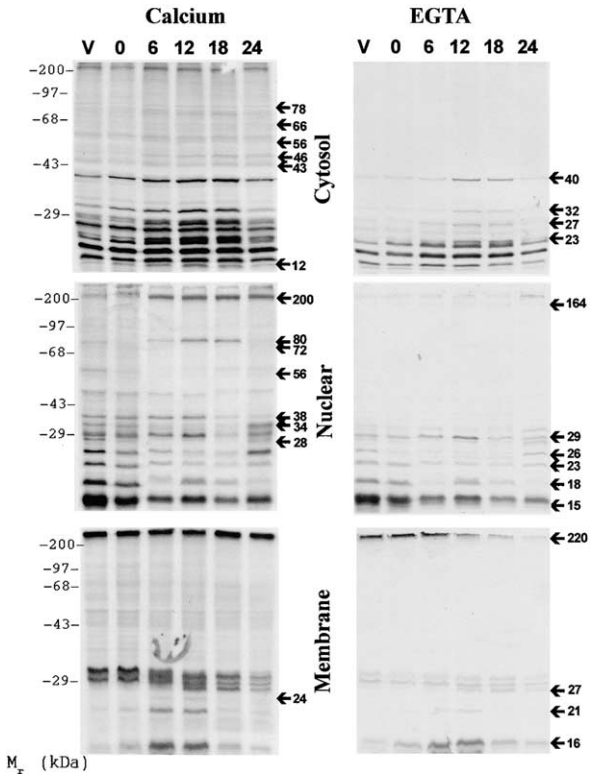

Fig. 1. Ca^{2+}-dependent and -independent CaMBPs from cytosolic-, nuclear-, and membrane-enriched fractions during *Dictyostelium* asexual, multicellular development. Samples collected every 6 h during asexual development were fractionated as per Material and Methods and probed with [^{35}S]VU-1-CaM in the presence of 1 mM calcium or 2 mM EGTA. Molecular weight markers run in parallel lanes are indicated in the left column.

underwent clear developmental changes. Of these, four (CaMBPs 78, 66, 46, and 43 kDa) were low or undetectable in vegetative and 0-h starved cells but increased markedly in amount by 6 h, after which with minor variations in amount they were still present after 24 h (Fig. 1). The Ca^{2+}-dependent CaMBP at 56 kDa was not detected in vegetative or 0-h starved cells but was present in 6- and 12-h cells before decreasing to undetectable levels by 24 h. A very low molecular weight Ca^{2+}-dependent CaMBP at 12 kDa was barely detected in vegetative cells but immediately increased upon starvation to peak at about 18 h before decreasing.

In the nuclear fraction, a large number of Ca^{2+}-dependent CaMBP changes occur throughout asexual development (Fig. 1). A large molecular weight CaMBP (200 kDa) was detected at low levels during vegetative growth but increased dramatically at 6 h and remained high throughout development. Two Ca^{2+}-dependent CaMBPs, one present in high amounts (CaMBP 56 kDa) and one low (CaMBP 72 kDa) both disappeared immediately upon starvation. Three other Ca^{2+}-dependent CaMBPs that were present at high levels in vegetative cells decreased at various times (CaMBPs 38 and 34 kDa) or disappeared completely (CaMBP 28 kDa) before increasing in amount again at the end of development. In the membrane fraction, only one Ca^{2+}-dependent CaMBP (24 kDa) underwent developmental changes (Fig. 1). This protein was low or undetectable in vegetative or 0-h starved cells but increased slightly by 6 through 12 h after which it decreased again.

The Ca^{2+}-independent CaMBPs, while fewer in total number, still demonstrated some interesting developmental shifts in various subcellular compartments (Fig. 1). In the cytosol, bands (CaMBPs 40, 32, 27, and 23 kDa) of varying intensities were low in amount in vegetative and 0-h starved cells, appeared to increase to varying degrees in the 6- through 18-h cell samples and then decreased by 24 h. In the nuclear fraction, many minor bands in the low molecular weight range made assessments of band changes during development difficult. In spite of this, some specific changes were evident. CaMBP 164 was detected at low levels in 8–18-h cells and disappeared by 24 h. Interestingly, this protein appears to be a Ca^{2+}-inhibited CaMBP since it is not detected in the presence of Ca^{2+}. Another Ca^{2+}-independent CaMBP (29 kDa) was low in vegetative and 0-h starved cells but increased in 6–12-h cells before again decreasing. In contrast, CaMBP 26 was present only in vegetative cells and terminally differentiated fruiting bodies (24 h). Two other CaMBPs (23 and 15 kDa) were present in vegetative cells decreased at different times. CaMBP 18 decreased by 6 h from originally high levels in vegetative and starved cells before peaking again in 12-h cells prior to again diminishing in amount. In the membrane fraction, the high molecular weight CaMBP ($\sim$220+ kDa) was present in high amounts early in development through 6 h then steadily decreased. Two Ca^{2+}-independent CaMBP bands also changed in the membrane fraction: one (27 kDa) appeared at 12 h and then decreased slowly in amount; a second (21 kDa) appeared at 6–12 h and then disappeared. One other low molecular weight membrane-localized Ca^{2+}-

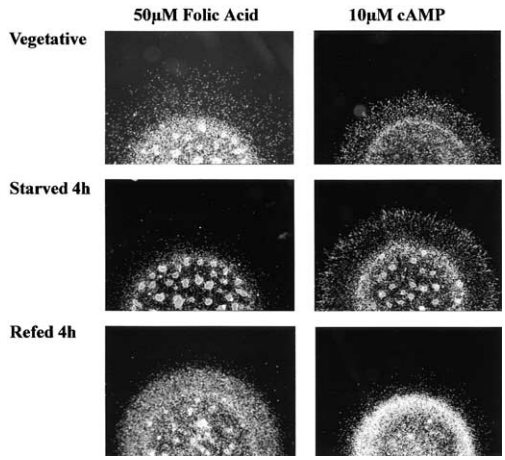

Fig. 2. Cell migration in the presence of FA or cAMP. Vegetative cells, cells starved for 4 h in phosphate buffer, and cells refed bacteria for 4 h were concentrated to a cell density of 10^8 cells/ml, an aliquot of cells was placed on a thin agar surface containing 50 μM FA or 10 μM cAMP. Vegetative cells differentially respond to both chemoattractants, while starved cells and refed cells respond maximally to cAMP and FA, respectively.

independent CaMBPs (16 kDa) increased steadily through 6–12 h before again decreasing in amount.

3.3. Chemotaxis to FA and cAMP

While numerous CaMBPs have been shown to exist and undergo developmental changes that implicate them in different aspects of development, their relationship to and importance during chemotaxis remains to be demonstrated. The goal of the following experiments was to define a protocol for analyzing shifts in CaMBPs during changes in cell responsiveness to the two chemoattractants, cAMP and FA. The radial bioassay is an effective system for assessing chemotaxis in *Dictyostelium*, yielding chemotactic rates that are essentially identical to other bioassay methods [35,39]. In this study, the original radial method was used, which allows cells to move as individuals within the agar substratum forming large halos that can easily be measured [39]. We used bacterially grown JH10 cells since they display more efficient chemotaxis than cells grown in axenic, shaking culture. Bacterially grown cells, however, represent a heterogeneous population of cells probably, in part, because of differences in stages of the cell cycle and in physiology due to spatial separation of some amoebae from the food source. As a result, where some cells in the population are capable of chemotactically responding to FA (feeding cells) others can respond to cAMP (starving cells; Figs. 2 and 3A,B). However, these vegetative cells move faster towards FA (3.5 μm/min) than cAMP (2.0 μm/min). Starvation of these cells leads to full chemotactic competence to cAMP with the concomitant loss in the ability to move towards FA (Figs. 2 and 3A,B). While starving for 2 h lead to a slight decrease (∼15%) in FA chemotaxis and a similar increase in chemotaxis to cAMP, starvation for 4 h resulted in an even greater loss of folate chemotaxis (∼50%) with a concomitant smaller increase (∼20%) in chemotaxis of cells towards cAMP. Refeeding reversed these patterns. Refeeding of the cells with bacteria results in the loss of cAMP-responsiveness (0.75 μm/min) as the cells gain full responsiveness to FA (2.1 μm/min; Fig. 3A,B). Refeeding with bacteria for 2 h

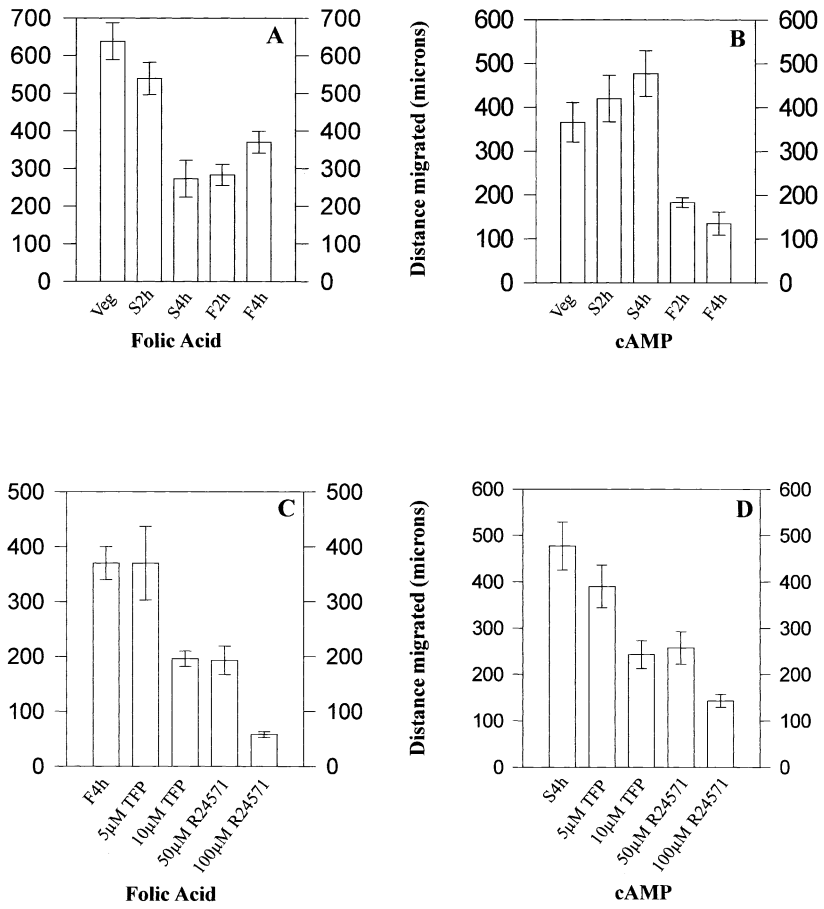

Fig. 3. Starved and refed cell migration toward cAMP and FA in the absence and presence of CaM antagonists, TFP or calmidazolium (R24571). Vegetative cells (Veg), cells starved for 2 h (S2 h) and 4 h (S4 h) in phosphate buffer, and 4-h starved cells refed bacteria at 1.5 A_{595} for 2 h (F2 h) and 4 h (F4 h) were plated onto a thin agar surface containing 50 μM FA (A) or 10 μM cAMP (B). The effects of TFP or calmidazolium (R24571) on maximally migrating refed cells (F4 h) toward FA (C) and starved cells (S4 h) toward cAMP (D) were quantified after 3 h of incubation. The distance migrated by cells refed bacteria at 1.5 A_{595} for 4 h (F4 h) was dose-dependently inhibited in the presence of 5 and 10 μM TFP or 50 and 100 μM calmidazolium. Similarly, TFP at 5 and 10 μM or calmidazolium at 50 and 100 μM dose-dependently inhibited the distance migrated by cells starved for 4 h (S4 h) in phosphate buffer. The distance migrated (μm) was measured using the Northern Eclipse image analysis system.

had a little effect on the ability of cells to move chemotactically towards FA but markedly decreased their chemoresponsiveness to cAMP. Extending the feeding time to 4 h led to an increase in FA chemotaxis and a further minor reduction in cAMP chemotaxis.

3.4. Effects of trifluoperazine and calmidazolium on chemotaxis

Without any direct evidence that CaM mediates chemotaxis in *Dictyostelium*, we carried out pharmacological analyses to determine if inhibitors of CaM function would interfere with this process. TFP and calmidazolium, two known antagonists of CaM, both dose-dependently inhibit chemotaxis to FA (Fig. 3C). While 5 μM TFP had no evident effect on chemotaxis to FA, 10 μM TFP inhibited it by approximately 50% (Fig. 3C). At 50 μM calmidazolium, chemotaxis to FA was inhibited by 50% and increasing the concentration of the drug to 100 μM resulted in an approximately 70% reduction in the rate of chemotaxis (Fig. 3C). The results were similar for chemotaxis to cAMP (Fig. 3D). At 5 μM, TFP inhibited cAMP chemotaxis by 20% while at 10 μM the level of inhibition was ~50%. Calmidazolium, at 50 μM, reduced the rate of chemotaxis to cAMP by 50% while at 100 μM calmidazolium chemotaxis to cAMP was inhibited by over 70%.

3.5. CaMBP changes during starvation and refeeding

With the knowledge that CaM was essential for normal rates of chemotaxis and that different CaMBPs were present in vegetative (FA-responsive cells) vs. starved/developing cells (cAMP-chemosensitive cells), our approach was to carry out starvation and refeeding experiments to discern if any specific CaMBPs were temporally linked to either of these processes (Fig. 4). In the cytosol, two Ca^{2+}-dependent CaMBPs (78 and 66 kDa) remained present through 4 h of starvation but completely disappeared upon refeeding. At least two other bands (46 and 43 kDa) that were high in vegetative and starved cells decreased significantly but not completely upon refeeding. In the nuclear fraction, several changes in Ca^{2+}-dependent CaMBPs were associated with either starvation or refeeding. Bands at 200, 38, and 34 kDa were present strongly in vegetative and starved cells but completely disappeared (200 kDa) or significantly decreased (38 and 34 kDa) upon refeeding. In contrast, several Ca^{2+}-dependent CaMBPs that were low or undetectable in starved cells increased upon refeeding (56, 50, and 27 kDa). A very weak Ca^{2+}-dependent band at ~24 kDa in the membrane fraction appeared in low amounts after 4 h of refeeding. In addition, many changes occurred in the Ca^{2+}-independent CaMBPs in the various fractions during starvation and refeeding. In the cytosol, four Ca^{2+}-independent bands of varying intensities (CaMBPs 40, 32, 27, and 18 kDa) appeared to increase upon starvation and decrease again upon refeeding. In the nuclear fraction, all of the changes involved only low molecular weight Ca^{2+}-independent CaMBPs. Several bands present in moderate (28, 23, and 18 kDa) to high (16 kDa) amounts in vegetative cells increased upon starvation and disappeared after 2 or 4 h of refeeding. In the membrane fraction only the 16-kDa Ca^{2+}-independent CaMBP showed any detectable changes, increasing slightly during starvation then disappearing during refeeding.

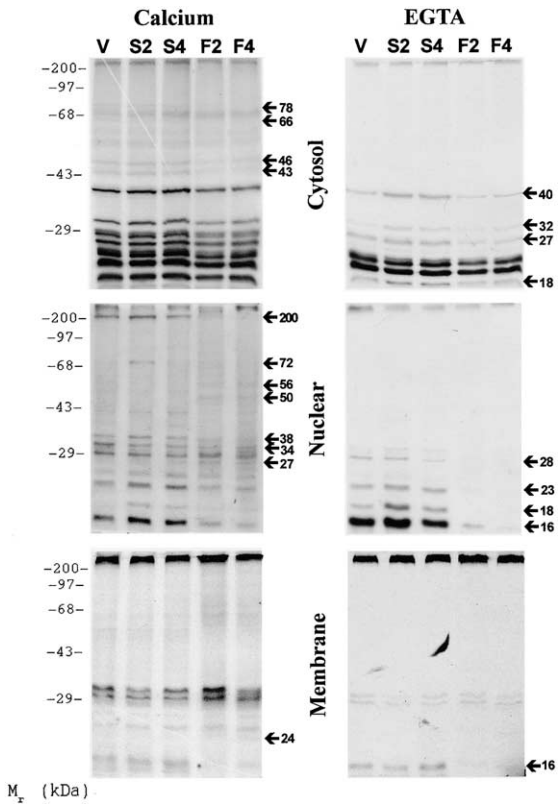

Fig. 4. Ca^{2+}-dependent and -independent CaMBPs from cytosolic-, nuclear-, and membrane-enriched fractions during starvation and refeeding. Samples were collected from vegetative cells, cells starved in phosphate buffer for 2 h (S2) and 4 h (S4), and cells refed bacteria for 2 h (F2) and 4 h (F4). Proteins from fractionated cells were separated by 10% SDS-PAGE and probed using [^{35}S]VU-1-CaM in the presence of 1 mM calcium or 2 mM EGTA. Molecular weight markers run in parallel lanes are indicated in the left column.

3.6. A comparison of CaMBPs visualized during development vs. starvation and refeeding

A cross-comparison of the results in CaMBP profile shifts during development from a vegetative feeding state (chemoresponsive to FA) to a starved developmental state (cAMP-responsive) with the starvation/refeeding experiments gave further insight into those CaMBPs that showed a direct link to each specific type of chemotaxis (Table 1). Four Ca^{2+}-dependent (43, 46, 66, and 78 kDa) and three Ca^{2+}-independent (27, 32, and 40 kDa) CaMBPs that localized to the cytosol were directly linked with cAMP chemotaxis during both development and the starvation–refeeding experiments.

Table 1
Changes in developmental CaMBPs that correlate with CaMBP changes observed during starvation and refeeding experiments

	Developmental changes		Experimental changes	
	Related to chemotaxis to		Starvation	Refeeding
	cAMP	FA	cAMP	FA
CaC^{2+}-dependent CaMBPs				
Cytosol	12, 43, 46, 56, 66, 78	none	43, 46, 66, 78	none
Membrane	24	none	none	24
Nucleus	200	28, 34, 56, 72	200, 72	27, 34, 50, 56
Ca^{2+}-independent CaMBPs				
Cytosol	23, 27, 32, 40	none	18, 27, 32, 40	none
Membrane	16, 21	none	16	none
Nucleus	18, 29	15, 18, 26	16, 18, 23, 28	none

The number refer to the $M_r \times 10^3$ of the CaMBPs identified either during developmental studies or during the starvation/refeeding experiments as associated with either cAMP or FA chemotaxis. The underlined numbers indicate those CaMBPs that are common to the events of chemotaxis detected in both development and the starvation/refeeding experiments.

In addition, one Ca^{2+}-dependent (200 kDa) and one Ca^{2+}-independent (18 kDa) CaMBP that localized to the nucleus were associated with starvation (cAMP chemotaxis). Only one membrane CaMBP that was Ca^{2+}-independent (16 kDa) showed a strong link to starvation (cAMP chemotaxis). In contrast, only two nuclear Ca^{2+}-dependent CaMBPs (34, 56 kDa) showed a recurring association with feeding (FA chemotaxis). No other Ca^{2+}-dependent or -independent CaMBPs could be linked to active feeding (FA chemotaxis) in these comparative analyses.

3.7. Phosphoserine phosphorylation patterns during starvation and refeeding

Several CaM-dependent protein kinases and only a single CaM-dependent phosphatase (calcineurin) have been identified in eukaryotes, suggesting that patterns of CaM-mediated phosphorylation might reveal further insight into the role of CaM and its CaMBPs during vegetative growth and early development (during chemotaxis). While dozens of proteins containing phosphoserine residues were detected in vegetative cells of *Dictyostelium*, only a limited number of changes were associated with either starvation and/or refeeding (Fig. 5). In the cytosolic fraction, of the over two dozen phosphoserine-containing proteins detected only five clearly appeared to undergo changes in phosphorylation related to either starvation or refeeding. Three phosphoproteins of approximately 36, 29, and 27 kDa all appeared to remain relatively unchanged with starvation but disappeared or were barely detectable with refeeding. In the presence of TFP, the levels of these phosphoprotein bands were intensified in starved cells but remained unresolved in refed cells. Unexpectedly, a band at 34 kDa was only observed in starved cells treated with TFP. A TFP-insensitive band at $\sim$20 kDa was present during starvation but disappeared upon refeeding. In the nuclear fraction, a clearly detectable 36-kDa protein moderately increased in intensity with starvation but dramatically increased with refeeding. Treating starved and refed cells with 10 μM TFP resulted in a significant decrease in staining of this band. The appearance of a slightly smaller band (just below the 36-kDa arrow) in refed cells and the concomitant loss of the 36-kDa band may be a posttranslationally modified form of the 36-kDa protein rather than a new protein. The phosphoprotein at 32 kDa appeared to be sensitive to TFP in starved cells, where it was undetected in vegetative or refed cells. In the membrane fraction, just over a dozen clearly evident phosphoserine bands were evident. Of these, a phosphoprotein band at 64

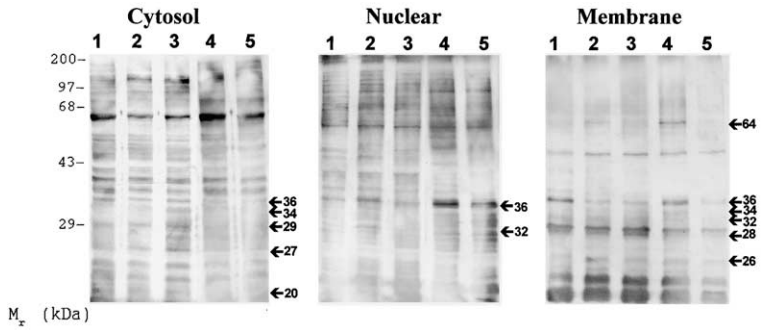

Fig. 5. Phosphoserine-containing proteins from cytosolic-, nuclear-, and membrane-enriched fractions of starved and refed cells in the presence or absence of TFP. Vegetative cells (lane 1), cells starved for 4 h (lane 2), cells starved for 4 h in the presence of 10 μM TFP (lane 3), cells refed bacteria for 4 h (lane 4), refed cells in the presence of 10 μM TFP (lane 5) were fractionated, separated by 10% SDS-PAGE and probed with antiphosphoserine antibodies.

kDa that was barely detectable in starved cells markedly increased with refeeding. The intensity of this band decreased significantly by treating cells with 10 μM TFP. Several other lower molecular weight phosphoproteins also underwent changes in the membrane fraction. One band at around 36-kDa decreased upon starvation and reappeared with refeeding. In the presence of TFP, the band was barely detectable. A 34-kDa TFP-sensitive band was only detected during refeeding. A 32-kDa TFP-insensitive band remained present during starvation but disappeared upon refeeding, while the intensity of a 26-kDa protein increased with starvation then decreased with refeeding. The level of the ~28-kDa band, detected only in vegetative and starved cells, increased dramatically in the presence of TFP.

4. Discussion

Chemotaxis plays essential roles during all aspects of *Dictyostelium* development, from food seeking to multicellular aggregation and cell sorting during morphogenesis [2,44]. This study, coupled with previous research by others, has clearly shown that chemotaxis in *Dictyostelium* is a CaM-dependent process. Furthermore, vegetative/refed cells that are chemotactic to FA and starved/early developing cells that show cAMP-chemoresponsivenes each possess different cytosolic, nuclear, and membrane populations of CaMBPs that reflect differences in the way CaM may work at each stage (e.g., in response to each chemoattractant). Several serine-containing phosphoproteins also showed CaM-dependent, quantitative changes associated with vegetative growth (FA chemotaxis) vs. early development (cAMP chemotaxis). Overall, *Dictyostelium* cells that are chemoresponsive to either FA or cAMP contain several downstream CaM-responsive elements that may be critical to carrying out events in response to increases in intracellular calcium that are induced by the each chemoattractant.

Studying the chemotactic behavior of cells in response to different chemoattractants under various conditions is one initial route to elucidating those events that are common to all chemotactic cells or which mediate chemoattractant-specific processes. For example, different chemoattractants result in different levels of cytosolic calcium that has allowed researchers to define the calcium-dependent steps that are essential for the component events of chemotaxis in neutrophils [45]. It has long been recognized that *Dictyostelium* cells can respond to two different chemoattractants: FA is used during growth to find food while cyclic AMP is used during development first to form a multicellular tissue-like aggregate and second to regulate cell patterning during morphogenesis [1–3,5,44]. Only recently have the differences and similarities between the two chemotactic processes come under close scrutiny. Each of these events is mediated through different receptors and G proteins [46–49]. But are the downstream signalling pathways common to both? Apparently not, since tyrosine kinase activity has been demonstrated to play a role during chemotaxis towards folate but not cAMP and two specific tyrosine phosphorylated proteins (38 and 52 kDa) associated with the FA-responsive cells have been detected [35]. Calcium signalling is critical for chemotaxis to both FA and to cAMP but as yet comparative analyses of the calcium targets are limited [50].

This study has shown that CaM, a major intracellular calcium sensor and effector, is an important mediator of both FA and cAMP chemotaxis in *Dictyostelium*. TFP and calmidazolium each dose-dependently inhibited chemotaxis to both chemoattractants. Work has shown that other CaM antagonists (i.e., Melittin, mastoparan) also inhibit chemotaxis dose-dependently (Gauthier and O'Day, unpublished results). Thus, a diverse number of CaM antagonists with different chemical structures and modes of action all inhibit chemotaxis. Previously, the role for CaM in chemotaxis had not been directly assessed but its importance had been inferred indirectly from research that has shown certain CaM targets are important. The identified CaMBPs include MLCK [23,24], MHCK [25], fodrin [26], DGAP1 [27], myoA–E [28], and myoJ [29]. Similarly, the subcellular localization of CaM supports its role in cellular motility and chemotaxis [50–52]. The CaM-dependent proteins that have been studied so far comprise only a small proportion of the full CaMBP complement of *Dictyostelium* cells. Since CaM is important to some aspect of chemotaxis, it is important to identify the full complement of CaM targets that are present in chemoresponsive cells. Over three dozen Ca^{2+}-dependent and one dozen or so Ca^{2+}-independent CaMBPs are present and undergo specific changes associated with cell and pronuclear fusion, zygote differentiation, and spore germination in *Dictyostelium* [18–22]. In this study, fractionation of cells has allowed a finer dissection of the CaMBPs that may be linked to the process of chemotaxis in *Dictyostelium*.

A comparison of CaMBP profiles of developing cells (shift from FA to cAMP chemoattraction) with those from starvation (cAMP chemotaxis) and refeeding experiments (reacquisition of FA chemotaxis) yielded further insight into those CaMBPs that might be closely associated with each specific event (Table 1). In total, 10 Ca^{2+}-dependent and Ca^{2+}-independent CaMBPs were strongly linked to early development (cAMP chemotaxis). Of these, seven were cytosolic, two were nuclear, and one was a membrane-associated. Other than the Ca^{2+}-independent membrane-associated CaMBP, the Ca^{2+}-dependent and -independent CaMBPs were equally represented. In contrast, only two nuclear Ca^{2+}-dependent CaMBPs could be linked to vegetative growth (FA chemotaxis) under all of the conditions studied. No cytosolic or membrane-bound CaMBPs appeared to be solely linked to growth (FA chemotaxis). This is not surprising considering the complex functions of cAMP vs. folate chemotaxis. *Dictyostelium* cells use FA chemotaxis simply to find a bacterial food source while cAMP chemotaxis mediates morphogenesis that involves the coordinated movements of about 10^5 cells to form a multicellular patterned tissue [44,49,53]. From the very

start, cells not only move towards sources of cAMP, they relay their responsiveness to other cells with the goal of setting up cellular streams that will lead to the formation of the multicellular aggregate [54]. It should be emphasized that while the approach taken here potentially identifies those CaMBPs unique to vegetative growth (FA-responsive cells) and early development (cAMP-responsive cells), it does not shed light on those CaMBPs that are common to both types of chemotaxis. Similarly, some of the CaMBPs may have other cellular functions at each stage.

While it might seem to be premature to assign definitive identities to the CaMBPs singled out here as potential effectors in CaM signalling during the two stage-specific events, the results presented here do serve as a prelude to ongoing studies. Using [^{35}S]VU-1-CaM to probe a cDNA expression library from multicellular development of *Dictyostelium*, a number of clones encoding putative CaMBPs have been isolated and the cDNAs sequenced (DdCaMBP38, GenBank Accession No. AF140042; DdCaMBP46, GenBank Accession No. AF140043; DdCaMBP15, GenBank Accession No. AF140044). The cytosolic Ca^{2+}-dependent CaMBP of 43×10^3 Da observed in this study as being linked to early development (cAMP chemotaxis) migrates to the same position as CaMBP38 in SDS-PAGE, has identical developmental kinetics, and is localized in the cytosol fraction (O'Day and Myre, submitted manuscript). Thus, the methods and data reported here coupled with other approaches can be used not only to identify CaMBPs that are linked to FA or cAMP chemotaxis for further analysis but also to understand how they may interact to effect CaM-dependent events. The methods can also be used to detect the level of expression or absence of CaMBPs in mutants under various experimental and developmental conditions. The latter approach was used to detect the absence of myoJ in knockout mutants for this CaMBP in *Dictyostelium* [29].

Both CaM-dependent protein kinases and the protein phosphatase calcineurin have been identified in *Dictyostelium* with the latter being well characterized [30,55–57]. The present study has identified several potential substrates that undergo experimentally induced, CaM-dependent changes in levels, as revealed by immunostaining with antiphosphoserine, that may reflect alterations in levels of CaM-dependent phosphorylation/dephosphorylation in starved vs. refed cells. Three cytosolic (27, 29, and 36 kDa) and a single membrane (28 kDa) protein increased in staining in the presence of 10 μM TFP in starved cells (cAMP-responsive). This increase might be explained by TFP inhibition of calcineurin. In this study, a cytosolic, Ca^{2+}-dependent CaMBP band migrating just below the 78-kDa marker fits with the developmental pattern of CNA (73 kDa) [56]. Also a cDNA encoding CNA was the first sequence isolated in our probing of the *Dictyostelium* expression library with recombinant CaM (GenBank Accession No. U22397) [22]. Furthermore, the calcineurin antagonist permethrin inhibits chemotaxis to cAMP but not to FA, while its inactive analog, deltamethrin, is totally ineffective [31]. Three membrane proteins (34, 36, and 64 kDa) and a single nuclear (36 kDa) protein decreased in intensity in refed cells treated with 10 μM TFP. In contrast only one nuclear (32 kDa) protein decreased in staining in starved, TFP-treated cells. These decreases in staining of serine phosphoprotein bands in the presence of TFP could indicate that one or more CaM-dependent protein kinases might be involved in mediating phosphorylation in these cells. So far, Ca^{2+}/CaM-dependent kinase activity has only been shown to play a role in *Dictyostelium* culmination during asexual development [55] and in biomembrane fusion during sexual development [19].

In summary, the results presented here provide some basis for differences in calcium-dependent signalling mediated by CaM during vegetative growth and early development in *Dictyostelium*. While FA and cAMP both lead to an increase in intracellular calcium, each mediates chemotactic processes that have different functions and different complexities. Each chemoresponsive cell type has been shown to be sensitive to inhibitors of CaM but each possesses a unique profile of CaMBPs and of CaM-sensitive serine-containing phosphoproteins that could effect the downstream events that CaM mediates in each type of chemotactic process.

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References

[1] Bonner JT, Hall EM, Sashsenmaier W, Walker BL. J Bacteriol 1970;102:682–7.
[2] Loomis W. The development of Dictyostelium discoideum. New York: Academic Press, 1982.
[3] Tillinghast HS, Newell PC. J Cell Sci 1987;87:45–53.
[4] Devreotes PN. Annu Rev Cell Biol 1988;4:649–86.
[5] Pan P, Hall EM, Bonner JT. Nat New Biol 1972;237:181–2.
[6] Abe F, Maeda Y. FEBS Lett 1994;342:239–41.
[7] Tanaka Y, Itakura R, Amagai A, Maeda Y. Exp Cell Res 1998;240:340–8.
[8] Milne JL, Coukell MB. J Cell Biol 1991;112:103–10.
[9] Bominaar AA, Van Haastert PJM. In: O'Day DH, editor. Calcium as an intracellular messenger in eucaryotic microbes. Washington (DC): ASM Press, 1990. pp. 65–78.
[10] Van Duijn B, Van Hasstert PJM. J Cell Sci 1992;102:763–8.
[11] Schlatterer D, Gollnick F, Schmidt E, Meyer R, Knoll G. J Cell Sci 1994;107:2107–15.
[12] Newell PC, Malchow D, Gross JD. Experientia 1995;51:1155–65.
[13] Yumura S, Furuya K, Takeuchi I. J Cell Sci 1996;109:2673–8.
[14] Nebl T, Fisher PR. J Cell Sci 1997;110:2845–53.
[15] Dharamsi A, Tessarolo D, Coukell B, Pun J. Exp Cell Res 2000;258:298–309.
[16] Dorywalska M, Coukell B, Dharamsi A. Biochim Biophys Acta 2000;1496:356–61.
[17] Clarke M. In: O'Day DH, editor. Calcium as an intracellular messenger in eucaryotic microbes. Washington (DC): ASM Press, 1990. pp. 375–91.

[18] Lydan MA, O'Day DH. Exp Cell Res 1993;205:134–41.
[19] Lydan MA, O'Day DH. Biochem Biophys Res Commun 1993; 192:1073–8.
[20] Lydan MA, Cotter DA, O'Day DH. Cell Signalling 1994;6:751–62.
[21] Lydan MA, Cotter DA, O'Day DH. Biochem Biophys Res Commun 1994;201:430–5.
[22] O'Day DH, Lewis KE, Lydan MA. In: Jamieson BGM, Ausio J, Justine JL, editors. Advances in spermatozoal taxonomy and phylogen. Paris: Memoires du Museum National d'Histoire Naturelle, 1995. pp. 23–36.
[23] Manalan AS, Klee CB. In: Greengard P, Robinson GA, editors. Advances in cyclic nucleotide and protein phosphorylation research: Vol. 18. New York: Raven Press, 1984. pp. 227–78.
[24] Warrick HM, Spudich JA. Annu Rev Cell Biol 1987;3:379–421.
[25] Yumura S, Kitanishi-Yumura T. J Cell Sci 1993;105:233–42.
[26] Bennett H, Condeelis J. Cell Motil Cytoskeleton 1988;11:303–17.
[27] Faix J, Clougherty C, Konzok A, Mintert U, Murphy J, Albrecht R, Muhlbauer B, Kuhlmann J. J Cell Sci 1998;111:3059–71.
[28] Cote GP, Abensi JP, Ueno T, Hammer JA, Korn ED. J Biol Chem 1985;260:4543–6.
[29] Hammer J, Lydan M, Jung G. Mol Biol Cell 1996;7:374a.
[30] Kessen U, Schaloske R, Aichem A, Mutzel R. J Biol Chem 1999;274:37821–6.
[31] Polesky AJM, O'Day DH. Mol Biol Cell 1995;6:91a.
[32] Mato JM, Van Haastert PJM, Krens FA, Rhijnsburger EH, Dobbe FCPM, Konijn TM. FEBS Lett 1977;79:331–6.
[33] Van Haastert PJM. Biochem Biophys Res Commun 1983;115:130–6.
[34] Yumura S. Cell Struct Funct 1994;19:143–51.
[35] Browning DD, The T, O'Day DH. Cell Signalling 1995;7:481–9.
[36] Watts P, Ashworth J. Biochem J 1970;119:171–4.
[37] Podgorki G, Deering RA. Mol Cell Biol 1984;4:2784–91.
[38] Sussman M. In: Spudich J, editor. Molecular approaches to cell biology. Methods in cell biology: Vol. 28. *Dictyostelium discoideum*. New York: Academic Press, 1987. pp. 9–29.
[39] O'Day DH. Can J Microbiol 1979;25:1416–26.
[40] Nellen W, Datta S, Reymond C, Sivertsen A, Mann S, Crowley T, Firtel RA. In: Spudich J, editor. Molecular approaches to cell biology. Methods in cell biology: Vol. 28. *Dictyostelium discoideum*. New York: Academic Press, 1987. pp. 67–100.
[41] Goodloe-Holland C, Luna E. In: Spudich J, editor. Molecular approaches to cell biology. Methods in cell biology: Vol. 28. *Dictyostelium discoideum*. New York: Academic Press, 1987. pp. 103–28.
[42] Laemmli J. Nature 1970;227:680–5.
[43] Towbin H, Staehelin T, Gordon J. Proc Natl Acad Sci USA 1979;76:4350–4.
[44] Firtel RA, Meili R. Curr Opin Genet Dev 2000;10:421–7.
[45] Fabbri E, Spisani S, Biondi C, Barbin L, Colamussi ML, Cariani A, Traniello S, Torrini I, Ferretti ME. Biochim Biophys Acta 1997; 1359:233–40.
[46] Klein PS, Sun TJ, Saxe CL, Kimmel AR, Johnson RL, Devreotes PN. Science 1988;241:1467–72.
[47] Kesbeke F, Van Haastert PJM, De Wit RJW. J Cell Sci 1990;96: 669–73.
[48] Devreotes PN. Neuron 1994;12:235–41.
[49] Parent CA, Devreotes PN. Science 1999;274:765–70.
[50] Ulbricht B, Soldati T. Protein Expression Purif 1999;15:24–33.
[51] Bazari WC, Clarke M. Cell Motil Cytoskeleton 1982;2:471–82.
[52] Tanaka Y, Itakura R, Amagai A, Maeda Y. Exp Cell Res 1998; 240:340–8.
[53] VanEs S, Devreotes PN. Cell Mol Life Sci 1999;55:1341–51.
[54] Reymond CD, Schaap P, Veron M, Williams JG. Experientia 1995;51: 1166–74.
[55] Dunbar AJ, Wheldrake JF. FEMS Lett 1994;115:113–8.
[56] Dammann H, Hellstern S, Husain Q, Mutzel R. Eur J Biochem 1996;238:391–9.
[57] Horn F, Gross J. Differentiation 1996;60:269–75.