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Elongation in a *Dictyostelium* in vitro translation system is affected by calmodulin antagonists

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We have previously shown that the *Dictyostelium discoideum* ribosomal protein L19 specifically binds Ca^{2+}/calmodulin [Sonneman et al. (1991) J. Biol. Chem. 266, 23091–23096]. To investigate the role of calmodulin in the regulation of protein synthesis, we have now established an in vitro protein synthesizing system from *Dictyostelium* cells which can elongate polypeptide chains with high efficiency. Various calmodulin antagonists affected translation in this system. The inhibitory effects of the antagonists could be partially reversed by addition of calmodulin. A monoclonal antibody against *D. discoideum* calmodulin also specifically inhibited protein synthesis. Similar effects of calmodulin antagonists were found in a standard wheat germ in vitro translation system.

Ribosomal protein; L19; Calcium; Protein synthesis; Wheat germ

1. INTRODUCTION

Cellular activities can be controlled at three levels: synthesis and post-transcriptional processing of mRNAs, translation of mRNAs into proteins, and post-translational modification of protein activity. Until recently the ribosome itself had been considered too rigid and complex to be subject to fine regulation. However, there are now a number of studies indicating that ribosomal proteins could be involved in cellular regulation. Mitogenic stimulation of ribosomal protein S6 phosphorylation is the best-understood example [1]. Alterations in the levels of mRNAs for ribosomal proteins were found in colon carcinomas [2–4], and in mitogen-activated mouse B-lymphocytes and activated human T-lymphocytes [5]. In *Dictyostelium*, ribosomal proteins could be involved in mRNA stability, since hybrid 40S subunits made from 17S rRNA and 40S ribosomal proteins of different developmental stages can cause differential stability of a developmental marker mRNA (G. Mangiarotti, personal communication). At an even more subtle level alterations in the intracellular Ca^{2+} concentration were reported to affect protein synthesis [6], and a study on the effects of calmodulin (CaM) antagonists on translation in vitro and in vivo in Ehrlich ascites tumor cells indicated that at least part of these effects could be mediated by CaM or a closely related Ca^{2+}-binding protein [7].

CaM is a ubiquitous Ca^{2+}-binding protein which controls a multitude of Ca^{2+}-dependent cellular processes in eukaryotes [8]. During recent studies on the role of Ca^{2+}/CaM-binding proteins in cell communication, cell differentiation, and morphogenesis of the cellular slime mold *Dictyostelium discoideum* we demonstrated that a protein of the large ribosomal subunit that is highly homologous to the mammalian ribosomal protein L19, specifically binds CaM [9]. Our speculation that CaM binding to L19 plays a regulatory role at the level of the ribosome has received indirect support by the recent observation that tumor cells overexpressing the *erb*B-2 oncogene also specifically overexpress an mRNA for ribosomal protein L19 [10].

In order to assess the role of CaM and, more specifically, to prepare a basis for investigations on the role of L19 in the regulation of protein synthesis, we have optimized a previously described in vitro translation system from *D. discoideum* cells [11], and studied the effects of CaM antagonists on the elongation of polypeptide chains in vitro. We show that CaM-antagonists inhibit translation in the *Dictyostelium* extracts, and that similar effects can be observed in a standard cell-free translation system from wheat germ.

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Abbreviations: CaM, calmodulin; W-5, *N*-(6-aminohexyl)naphthalenesulfonamide; W-7, *N*-(6-aminohexyl)5-chloro-1-naphthalenesulfonate; m^7-GMP, 7-methylguanosine 5′-mono-phosphate.

2. MATERIALS AND METHODS

2.1. *Materials*

[^{35}S]Methionine (>1,000 Ci/mmol) was from Amersham. *N*-(6-Aminohexyl)naphthalenesulfonamide (W-5), *N*-(6-aminohexyl)5-chloro-1-naphthalenesulfonamide (W-7), compound 48/80, 7-methylguano-

sine 5'-monophosphate (m^7-GMP), and cycloheximide were from Sigma. Melittin, ATP, GTP, creatine phosphate, creatine phosphokinase, antipain, bestatin, leupeptin, pepstatin, calf liver tRNA, and micrococcal nuclease were from Boehringer-Mannheim. Spermidine was from Merck. RNasin was from Promega. Bovine brain CaM and *Staphylococcus aureus* 'Pansorbin' cells were from Calbiochem. Monoclonal antibodies against *D. discoideum* CaM were generously provided by M. Clarke. Monoclonal antibodies against alkaline phosphatase from *Escherichia coli* were a generous gift from M. Ehrmann. Tobacco mosaic virus RNA was kindly supplied by L. van Vloten-Doting.

2.2. *Cell culture*

D. discoideum strain Ax2 was grown axenically to a density of 4–8 × 10^6 cells/ml and allowed to develop to aggregation competence in suspension as described [12]; t_x designates the time (in h) after the onset of starvation.

2.3. *Preparation of translation extracts*

Cells were washed in ice-cold 25 mM HEPES/KOH, pH 7.5, 25 mM potassium acetate and 5 mM magnesium acetate, and collected at 1,000 × *g* for 3 min. The pellet was resuspended in the same buffer containing 15% glycerol and the cells lysed by freezing and thawing. The homogenate was spun in an Eppendorf centrifuge for 5 min at 4°C, and the supernatant was recentrifuged at 41,000 × *g* in a Beckman TLA 45 rotor for 45 min at 4°C. The pellet was resuspended in the same buffer without glycerol, and 0.25% (w/v) bovine serum albumin was added. For long-term storage 25 mM dithiothreitol was added; at −70°C the extracts remained active for at least six months. The preparation of larger amounts of extract, e.g. 1 litre of cells usually resulted in a reduced translation activity, probably due to prolonged protease attack during longer preparation times.

2.4. *Amino acid incorporation assay*

The procedure of Ntamere and Barkley [11] was followed with minor modifications. Protein synthesis was measured at 23°C for 10–60 min in a total volume of 25 μl containing 0.4 to 2.5 mg of protein extract per ml final reaction volume in the presence of the following components: 20 mM HEPES/KOH, pH 7.5, 100 mM potassium acetate, 8 mM magnesium acetate, 2 mM dithiothreitol, 0.5 mM spermidine, 1 mM ATP, 0.4 mM GTP, 20 mM creatine phosphate, 50 μg/ml creatine phosphokinase, 250 μM each of 19 amino acids (excluding methionine), and also per ml final reaction volume, 0.4 mCi [^{35}S]methionine, 120 μg calf liver tRNA, 1,000 U RNasin and the indicated additions. The radioactivity incorporated into the translation products was measured as described [13]. Several protease inhibitors, added together at the outset of incubation, did not affect protein synthesis at the following concentrations: 100 μg/ml antipain, 25 μg/ml bestatin, 100 μg/ml leupeptin, and 7.5 μg/ml pepstatin. Inclusion of 1 mM cycloheximide completely abolished protein synthesis.

2.5. *Wheat germ translation system*

Wheat germ translation extracts were prepared and in vitro protein synthesis was measured as described [13].

3. RESULTS

3.1. *Optimization of protein synthesis in* Dictyostelium *extracts*

The conditions described by Ntamere and Barkley [11] were used as starting point and gradually modified to yield high incorporation of [^{35}S]methionine. Variations of either pH, the concentrations of added amino acids and exogenous tRNA, or K^+ and Mg^{2+} had only small effects (not shown). However, in vitro translation could be substantially improved by altering the procedure for the preparation of extracts. Fig. 1 shows that protein synthesis was very inefficient when vegetative cells (t_0) were taken as a source of the translation apparatus. We speculated that low incorporation was mainly due to the high protease activities in *Dictyostelium* extracts, and therefore concentrated on reduction of these activities. Aggregative cells in general contain lower protease activities than growing cells. Therefore cells that had been starved for 6 h (t_6) were next chosen for preparation of the extracts, yielding about twice as much incorporation (Fig. 1). Since protease inhibitors did not affect protein synthesis (not shown), further precautions were taken to diminish protease activity. Inclusion of glycerol during lysis of the cells is known to stabilize proteins; indeed, a large increase of methionine incorporation was observed when translation extracts from aggregative cells were prepared in the presence of 15% glycerol (Fig. 1).

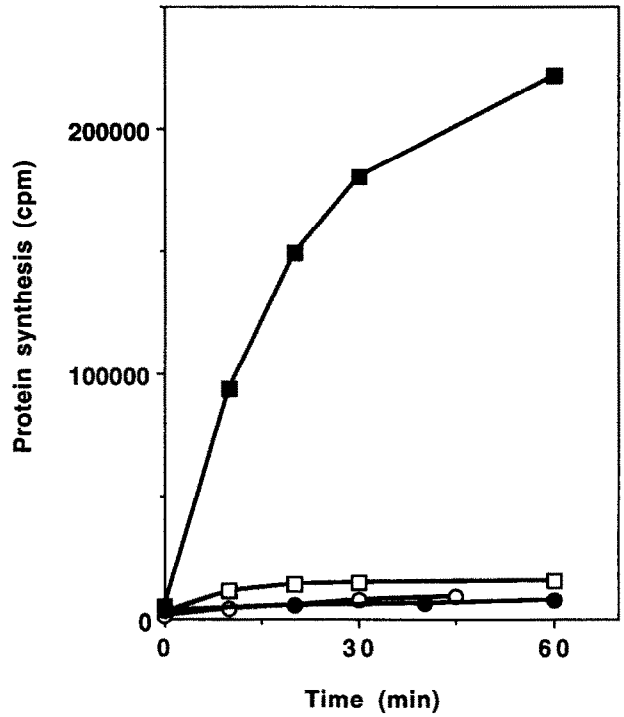

Fig. 1. Effect of different preparation procedures of cell-free extracts from *D. discoideum* on in vitro protein synthesis. The cell-free system was assayed for [^{35}S]methionine incorporation into hot TCA-precipitable material. Each assay contained approximately 1 mg of protein per ml final reaction volume. Cells were harvested at t_0 (circles) or t_6 (squares), either in the absence (open symbols) or presence (closed symbols) of 15% glycerol. Similar results were obtained in several independent experiments.

We next tested whether the system could support protein synthesis from exogenously supplied mRNA. Addition of total RNA from *D. discoideum* or tobacco mosaic virus RNA did not cause higher incorporation of [^{35}S]methionine than control assays without added RNA. When translation extracts were treated with mi-

crococcus nuclease and supplied with either tobacco mosaic virus RNA or *D. discoideum* RNA, no incorporation of [^{35}S]methionine could be observed (data not shown). We examined whether this could be due to the incapability of the system to initiate translation. m^7-GMP, a structural analogue of the 5'-terminal structures of most eukaryotic mRNAs including the mRNAs of *Dictyostelium* [14], inhibits translation by impeding the initiation of protein synthesis [15], but has no effect on chain elongation [16]. When m^7-GMP at concentrations up to 2 mM was added to the *Dictyostelium* system, no inhibition of protein synthesis was observed (not shown). We conclude that the system as it stands, can elongate polypeptide chains, but not initiate translation (see section 4).

3.2. *Effects of CaM antagonists on cell-free protein synthesis in* Dictyostelium *extracts*

Kumar et al. [7] reported that protein synthesis in extracts from Ehrlich ascites tumor cells and rabbit reticulocytes is inhibited by the CaM antagonists, compound 48/80, calmidazolium, trifluoperazine, and W-7. To determine whether CaM antagonists show similar effects on translation in the lower eukaryote *Dictyostelium*, several CaM antagonists belonging to different chemical categories were tested for their effects on the *Dictyostelium* system. Fig. 2 shows that 10 μm compound 48/80 abolished protein synthesis; half maximal inhibition was observed at 2.5 μM. A peptide CaM antagonist, melittin, also inhibited translation in a dose-dependent manner, with half-maximal inhibition at 18 μM. The naphthalene sulfonamide derivatives W-5 and W-7 are closely related compounds of different effectiveness against CaM-dependent processes; W-5, a chlorine-deficient analogue of W-7, has a 5- to 10-fold lower inhibitory potency than W-7, and is therefore considered a suitable control to discriminate between specific effects on CaM and more unspecific effects of hydrophobic compounds [17]. Fig. 2 shows that 50% inhibition was reached at 400 μM for W-7, whereas W-5 even at 1,000 μM inhibited only 13%.

To investigate whether this inhibition reflects a specific interaction of the antagonists with CaM, exogenous CaM was added to the inhibited system, resulting in a partial reversion of the inhibition by all three antagonists (Table I). The concentration of CaM (50–100 μM) required for preventing the inhibition was independent of the concentration of each antagonist required for efficient inhibition (from 10 μM for compound 48/80 to 1,000 μM for W-7; Table I). Addition of 100 μM CaM alone to the system did not affect protein synthesis.

3.3. *Inhibition of protein synthesis by anti-CaM antibodies*

To further confirm that the inhibition by CaM antagonists is caused by a specific interaction with CaM, the effects of anti-CaM antibodies on in vitro translation were examined. Addition of monoclonal antibodies against CaM from *Dictyostelium* [18] caused a 50% inhibition of methionine incorporation (Table II). A control antibody against alkaline phosphatase from *E. coli*

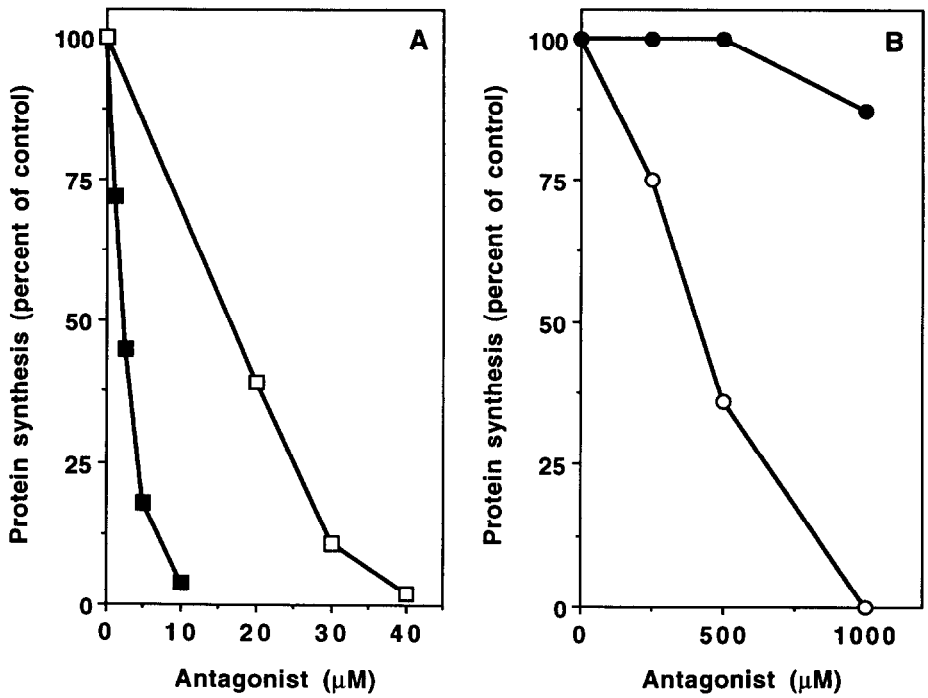

Fig. 2. Inhibition of cell-free translation by CaM antagonists in extracts from *D. discoideum*. The cell-free system was assayed for [^{35}S]methionine incorporation into hot TCA-precipitable material after 60 min. (A) 48/80 (■); melittin (□); (B) W-5 (●) and W-7 (○) were dissolved in dimethyl sulfoxide and equal amounts of the solvent were added to control incubations.

showed no effect, even at higher concentrations. Table II also shows that the inhibitory activity could be removed from the antibody preparation by prior adsorption to Pansorbin.

3.4. *Effects of CaM antagonists on translation in wheat germ extracts*

We also investigated whether the inhibition by CaM antagonists represents a general phenomenon in eukaryotic cells. Using a standard wheat germ translation extract [13] the effects of compound 48/80 and melittin on the incorporation of [^{35}S]methionine into proteins synthesized from tobacco mosaic virus RNA were measured. Like in *Dictyostelium* extracts, compound 48/80 and melittin efficiently inhibited protein synthesis. Again, inhibition of translation could be partially restored by addition of exogenous CaM. Contrary to the observations on the *Dictyostelium* system, addition of CaM itself impeded protein synthesis (Fig. 3).

4. DISCUSSION

This work has two objectives: first, to establish an in vitro translation system from *Dictyostelium* cells, and second, to investigate the possible role of CaM in the regulation of protein synthesis. The *Dictyostelium* system – otherwise a well-recognized simple eukaryotic model for studies of cellular regulation [19] – has so far resisted to the development of an efficient in vitro translation system ([11,20] and T. Dingermann and H. Kersten, personal communication). Proteolytic degradation of components of the translation apparatus appeared to be the major problem in preparations of cell-free extracts of high protein-synthesizing activity. Efforts to control proteolytic activity with protease inhibitors failed, suggesting that additional proteases were not being successfully inhibited. The use of developing cells (t_6) for the preparation of extracts and the inclusion of glycerol in the lysis buffer are well-known possibilities to reduce proteolytic degradation. Separately applied, their usefulness was only restricted, whereas their combination led to a striking improvement of translational activity. Apparently, this procedure stabilized the components required for elongation, but could not support a stable initiation process, as revealed by the observation that the initiation inhibitor m^7-GMP had no effect on methionine incorporation. We hypothesize that this failure is due to a high degree of instability or protease sensitivity of one or several initiation factors, possibly towards proteases that are dependent on divalent cations. Since translation extracts have to be prepared in the presence of high concentrations of Mg^{2+} to conserve the integrity of the ribosomes, this problem cannot be easily solved. Future work on improvement of the in vitro translation system will therefore focus on the reconstitution of the translation apparatus by combination of differentially prepared ribosomal and cytosolic fractions. Another possibility is the design of a hybrid system composed of *Dictyostelium* ribosomes and soluble factors from wheat germ as recently proposed by Ramagopal [21].

A number of CaM antagonists inhibited protein synthesis in a dose-dependent manner. Several criteria support the view that their action reflects a specific influence of CaM on protein synthesis. The antagonists tested belong to widely different chemical classes, and the differential effects of the closely related drugs W-5 and W-7 are consistent with their different ability to affect other CaM-dependent processes. Inhibition by each of the drugs could be reversed by the addition of exogenous CaM. Most important, a monoclonal antibody against CaM specifically repressed protein synthesis, whereas a monoclonal antibody against an *E. coli* protein was completely ineffective. Similar to previous studies with an in vitro system from tumor cells [7] the concentrations of CaM antagonists required to achieve efficient inhibition were fairly high; we explain this by the high concentration of total protein in the assay that could titrate considerable amounts of the drugs by nonspecific binding.

Table I

Restoration of protein synthesis in the inhibited system by exogenous CaM from bovine brain

Antagonist	CaM	Protein synthesis (% of control)
–	100 μM	102
10 μM compound 48/80	–	0
10 μM compound 48/80	20 μM	8
10 μM compound 48/80	40 μM	29
10 μM compound 48/80	100 μM	82
1 mM W-7	–	11
1 mM W-7	100 μM	54
40 μM melittin	–	8
40 μM melittin	50 μM	81

The cell-free *Dictyostelium* system was assayed for [^{35}S]methionine incorporation into translation products after 60 min.

Table II

Effects of antibodies on in vitro protein synthesis

Antibody	Pansorbin	Protein synthesis (% of control)
200 μg/ml anti-CaM	–	54
800 μg/ml anti-alkaline phosphatase	–	113
200 μg/ml anti-CaM	2 mg/ml	95
–	2 mg/ml	103

The cell-free *Dictyostelium* system was assayed for [^{35}S]methionine incorporation into translation products after 60 min. Antibodies were dissolved in phosphate-buffered saline and equal amounts of the buffer were added to control incubations. In the respective experiment, antibodies were incubated with Pansorbin for 20 min and the Pansorbin/antibody complexes were pelleted for 5 min in an Eppendorf centrifuge.

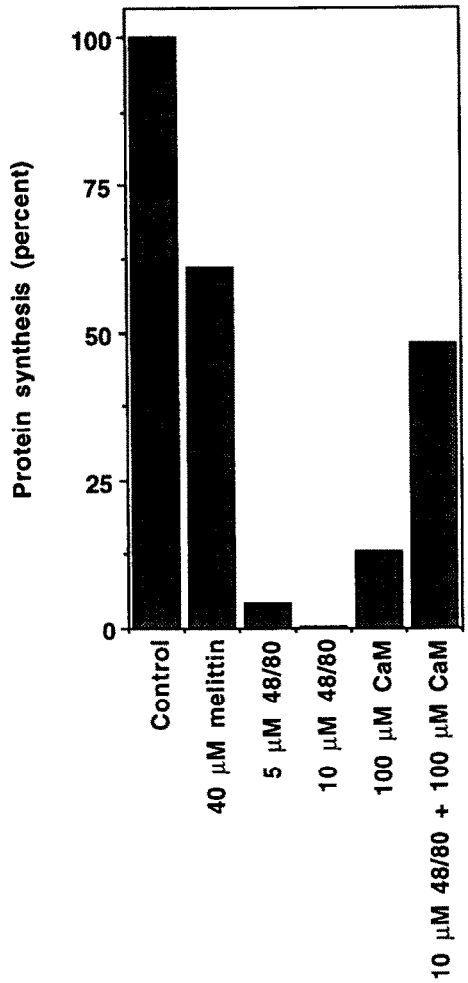

Fig. 3. Effects of CaM and CaM antagonists in the wheat germ system. The cell-free system was assayed for [^{35}S]methionine incorporation into hot TCA-precipitable material after 60 min. The assays contained 80 μg/ml tobacco mosaic virus RNA.

The great majority of regulatory mechanisms involved in the control of protein synthesis appear to modulate translational initiation [22]. Brostrom et al. [23] and Kumar et al. [24] have reported on the sensitivity of initiation of protein synthesis with respect to Ca^{2+} deprivation in cultured cells. Furthermore, Kumar et al. [7] have found that CaM antagonists inhibit translation in Ehrlich ascites tumor extracts at the level of initiation. However, in the *Dictyostelium* system, protein synthesis seemed to be blocked by CaM antagonists at the level of elongation, since the system could not initiate protein synthesis from either homologous or heterologous mRNA.

Similar protein synthesis repressing activities by CaM antagonists were observed in the wheat germ system, possibly resulting from the same underlying mechanisms. A puzzling finding was the inhibitory action of CaM itself on translation in wheat germ extracts; in fact, inhibition of translation by CaM could be partially reversed by addition of the CaM antagonist compound 48/80 (see section 3, Fig. 3). CaM is known to inhibit elongation in mammalian cell extracts via the activation of the CaM-dependent protein kinase III which phosphorylates elongation factor-2 [25,26]. It is not fully clear at present whether a similar mechanism is operational in wheat germ translation extracts [27], and no data are available for *Dictyostelium*. However, since the wheat germ system is strictly initiation-dependent and the *Dictyostelium* extract only supports elongation, an inhibitory activity of CaM on initiation of protein synthesis in wheat germ extracts could also be responsible for the different effects of CaM on the two systems.

Together with previous observations on mammalian cells [7], these results suggest that CaM could be a general regulator of eukaryotic protein synthesis. Its action on CaM-dependent protein kinase III is well established. On the other hand, our findings of a ribosomal CaM-binding protein [9] are compatible with an interaction directly at the ribosome level. In order to address this point, we will now analyze the consequences of genetically altered expression of the *Dictyostelium* ribosomal protein L19 on the regulation of protein synthesis.

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