EVIDENCE FOR THE OCCURRENCE OF CALMODULIN IN THE YEASTS *CANDIDA ALBICANS* AND *SACCHAROMYCES CEREVISIAE*

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1. Introduction

Calmodulin was originally characterised as the calcium-dependent activator of brain cyclic nucleotide phosphodiesterase. Subsequently it was shown to be the modulator of a variety of calcium-dependent cellular activities (reviews [1–3]). Calmodulin is now recognised as being a highly conserved and widely distributed protein, having been demonstrated in many vertebrates and invertebrates [3,4], plants and higher fungi [5,6] as well as several unicellular eukaryotes; e.g., *Dictyostelium discoideum* [7], *Blastocladiella emersonii* [8], *Euglena gracilis* and *Amoeba proteus* [9]. Prokaryotes, in contrast, appear to lack calmodulin [6,7] although (exogenous) calmodulin has been shown to activate the adenylate cyclase of *Bordetella pertussis* [10]. The observed distribution of calmodulin has led to the suggestion that it is ubiquitous in eukaryotes [3,5]. However, the inability of several laboratories to detect calmodulin in yeast [6,7,11] is in conflict with this assertion. This paper reports the presence of a calmodulin-like protein in extracts of the yeasts *Candida albicans* and *Saccharomyces cerevisiae*. The demonstration of this protein appears to be dependent on the use of the protease inhibitor, PMSF.

2. Materials and methods

2.1. *Materials*

Fluphenazine · 2 HCl was kindly supplied by E. R. Squibb and Sons. TFP was a generous gift of Smith, Kline and French Labs. PMSF and bovine brain phosphodiesterase were obtained from Sigma. Protein A–Sepharose was purchased from Pharmacia.

Abbreviations: TFP, trifluoperazine; PMSF, phenylmethylsulfonylfluoride; SDS–PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis

2.2. *Cells*

Yeast cells of *C. albicans* (ATCC 10261), prepared in shake cultures as in [12], were harvested at late exponential phase. Fresh *S. cerevisiae* cells were obtained from a local brewery.

2.3. *Preparation of extracts*

All operations were performed at 4°C. Washed yeast cells were resuspended in 3 vol. homogenisation buffer (10 mM Tris–HCl, 2 mM EDTA (pH 7.5) with 0.6 mM PMSF added from a freshly prepared stock in 95% ethanol) and disrupted in a Braun homogeniser. The homogenate was centrifuged (6000 × *g*, 10 min) and the supernatant heated (85–90°C, 2 min) then chilled. Precipitated proteins were removed by centrifugation (39 000 × *g*, 10 min) and further PMSF (0.3 mM) added. Acid ammonium sulphate fractionation of the supernatant was performed as in [13]. Extracts were dialysed against distilled water and stored at −20°C.

2.4. *Fluphenazine–Sepharose affinity chromatography*

Fluphenazine was coupled to Sepharose-4B (200 ml) as in [5]. Affinity chromatography was performed as for peanut calmodulin [5] with the following modifications: the column buffer was 10 mM Tris–HCl (pH 7.5), 0.5 mM $CaCl_2$; the salt wash was 0.1 M NaCl in column buffer; the elution buffer was 10 mM Tris–HCl, 10 mM EGTA (pH 8.0); chromatography was performed at 4°C. The extracts were made 0.5 mM in free Ca^{2+} and dialysed against column buffer prior to loading. Free Ca^{2+} concentration was computed as in [14].

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2.5. *Antibodies to calmodulin*

Rabbit antiserum to performic acid-oxidised ovine brain calmodulin was raised as in [15] and the IgG fraction isolated by affinity chromatography on protein A–Sepharose [16]. Passive haemagglutination assays were performed as in [17] using glutaraldehyde as the coupling agent.

2.6. *Other methods*

Human erythrocyte Ca^{2+} + Mg^{2+}-ATPase and regulator-deficient bovine brain 3′:5′-cyclic nucleotide phosphodiesterase were assayed for calmodulin-activation as in [18,19]. Calmodulin was isolated from ovine brain using affinity chromatography of heat-treated, acid ammonium sulphate fractionated extracts, with conditions essentially as above. Protein concentration was estimated by microassay [20] with BSA as a standard. Continuous PAGE was performed in 10% gels (2.63% cross-linked) in the presence and absence of SDS (0.1%) [21]. Protein was stained with Coomassie blue.

3. Results and discussion

The heat stability of calmodulin permits rapid preparation of enriched cell-free extracts which can be assessed for calmodulin content by assaying with established calmodulin-regulated enzymes [1–4]. Our preliminary analysis of heat-treated extracts prepared from *C. albicans* in the presence of PMSF indicated that they were capable of activating 2 calmodulin-regulated enzymes, Ca^{2+} + Mg^{2+}-ATPase and brain phosphodiesterase, in a Ca^{2+}-dependent manner. This calmodulin-like activity was not detected in extracts prepared in the absence of PMSF. Accordingly, PMSF was routinely used in all subsequent extraction procedures.

Acid ammonium sulphate fractionation of heat-treated extracts of *C. albicans*, following a procedure used for calmodulin [13], preserved the calmodulin-like activity. SDS–PAGE of this fraction revealed several major protein species, including one than comigrated with ovine brain calmodulin (fig.1). Likewise, in the absence of SDS, PAGE revealed a protein band that comigrated with calmodulin. Protein eluted from this band activated brain phosphodiesterase in the same manner as calmodulin (table 1).

These findings indicated that the yeast activator had both physical and biological similarity to calmodulin. Affinity chromatography was therefore attempted. Fractionation of heat-treated *C. albicans* extracts on fluphenazine–Sepharose following the procedure used for peanut calmodulin [5] resulted in low protein yields at the EGTA elution (calmodulin) step. Modifying the procedure as in section 2 increased

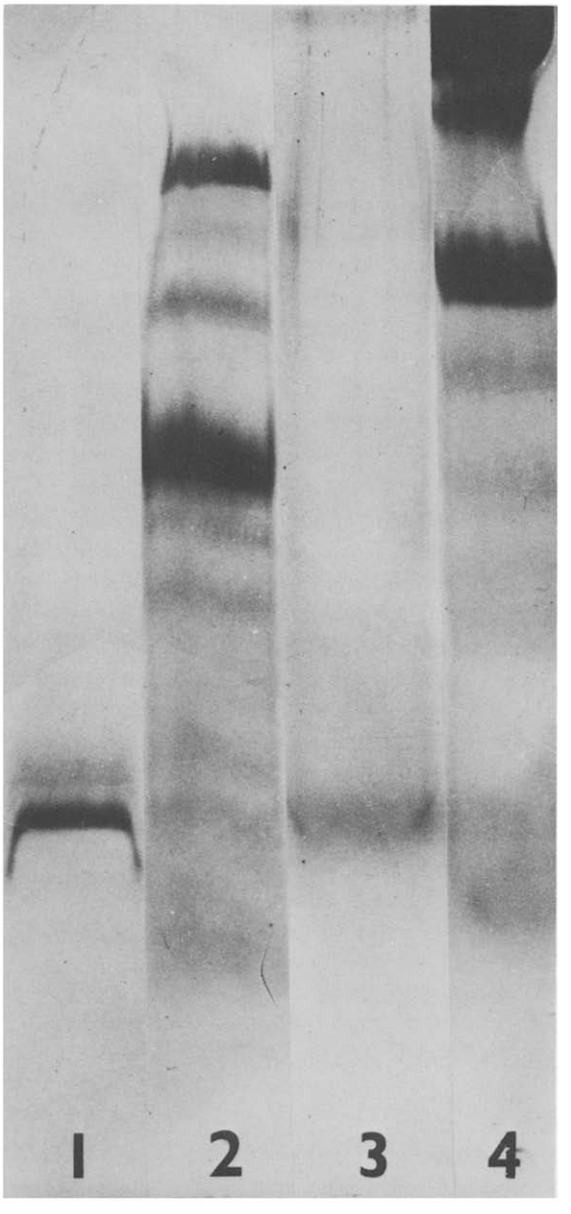

Fig.1. SDS–PAGE of protein fractions: ovine brain calmodulin, 10 μg (1); acid ammonium sulphate fraction of *C. albicans*, 80 μg (2) and *S. cerevisiae*, 80 μg (4); *C. albicans* protein fraction eluted from fluphenazine–Sepharose with EGTA, 15 μg (3).

Table 1
Activation of bovine brain phosphodiesterase by calmodulin and yeast protein fractions

Fraction	Phosphodiesterase activity (nmol cAMP hydrolysed . min^{-1} . mg $enzyme^{-1}$)				
	Basal activity[a] (A)	A + protein fraction[b]	A + protein fraction + Ca^{2+}	Activation (-fold)	A + protein fraction + Ca^{2+} + TFP
Ovine brain calmodulin	0.90	0.90	2.90	3.2	0.95
C. albicans protein[c]	0.80	0.85	3.15	3.7	1.10
PAGE eluate[d]	0.70	0.70	2.20	3.1	0.90

[a] Activity of phosphodiesterase under standard assay conditions [19] in the absence of activator fraction. Values are the mean of at least duplicate experiments with variation of <10%
[b] Where indicated the following were added: protein fraction, 5 μg; free Ca^{2+}, 1 mM; TFP, 20 μM
[c] Protein fraction eluted from fluphenazine–Sepharose with EGTA
[d] Protein fraction eluted from band corresponding in mobility to calmodulin after PAGE (in absence of SDS) of acid ammonium sulphate fractionated *C. albicans* extract

the yield and the protein fraction obtained gave over a 3-fold activation of brain phosphodiesterase, in the presence of Ca^{2+} (table 1). This activation was inhibited by EGTA and the calmodulin-inhibitory drug [22], TFP. SDS–PAGE revealed one major protein species, that comigrated with calmodulin (fig.1). Preliminary immunologic analysis of this semi-pure calmodulin-like protein, using the passive haemagglutination technique indicated complete immunoreactivity with the anti-calmodulin IgG preparation. Using the procedure described for *C. albicans*, we found that a heat-treated extract of *S. cerevisiae* provided 2.5-fold activation of brain phosphodiesterase. This activation was Ca^{2+}-dependent and TFP-sensitive. SDS–PAGE of the acid ammonium sulphate fraction revealed a protein band that comigrated with calmodulin (fig.1).

These findings indicate the presence of a calmodulin-like protein in the yeasts *C. albicans* and *S. cerevisiae.* The previous inability to detect calmodulin in yeast (*S. cerevisiae*) [6,7,11] may have been due to inadequate precautions being taken against proteolysis. Proteolytic artefacts are a particular problem in the biochemistry of yeast, and are due to their high content of endogenous proteases [23]. PMSF is an effective inhibitor of yeast proteases B and C, while EDTA interferes with exopeptidase activity by chelating divalent cations. Maintenance of neutral pH inhibits protease A activity and, at the same time, favours the formation of endogenous protease–protease inhibitor complexes. Protease content is lowest in actively growing cells [23]. All of these measures were taken as a precaution against proteolysis here; in particular, the use of PMSF appeared to be critical.

The salient characteristics of the *C. albicans* calmodulin-like protein are its: heat stability; acidic nature; activation of 2 calmodulin-regulated enzymes in a Ca^{2+}-dependent, TFP-sensitive manner; Ca^{2+}-dependent binding to fluphenazine; comigration with calmodulin during PAGE; immunoreactivity with anti-calmodulin IgG. Although further criteria are required before this protein can be absolutely classified as a 'calmodulin' [24], the now likely existence of calmodulin in yeast supports the concept [3,5] that calmodulin is ubiquitous in eukaryotes. Apart from being of evolutionary interest, further investigation of yeast calmodulin is warranted because of the obvious advantages associated with the study of these genetically and metabolically well-characterised organisms.

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