Calmodulin and calmodulin-binding proteins of *Rhodospiridium toruloides*, a basidiomycetous yeast

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Calmodulin (CaM)-interacting proteins of *Rhodospiridium toruloides*, a basidiomycetous yeast, were studied. CaM-binding proteins isolated from the soluble cell extract of vegetative cells and gamete cells (mating-pheromone-treated cells) by CaM-Sepharose affinity chromatography were analysed by sodium dodecylsulphate-polyacrylamide gel electrophoresis to compare the molecular species of CaM-binding proteins of the two cell types. The major CaM-binding proteins of the vegetative cells had molecular masses of 103 and 96 kDa. In the pheromone-treated cells, in addition to the bands seen in the vegetative cells, prominent bands of 40 and 38 kDa and minor bands of 92 and 60 kDa were present. The bands of CaM-binding proteins present in both cell types were more intense in the gamete cells. CaM-binding proteins of total cell extracts were also detected on acrylamide gels by a gel overlay method using ^{125}I -CaM as the probe. The results obtained by the two procedures suggested that various CaM-binding proteins are present in the yeast cell, and the expression of many CaM-binding protein genes is altered by the mating pheromone. Ca^{2+} - and CaM-dependent protein kinase activity was detected in the CaM-affinity-purified proteins. After incubation under conditions for protein phosphorylation by the kinase, the enzyme no longer required the activators, indicating that the enzyme activity is modulated by endogenous phosphorylation. A protein of 60 kDa was labelled by endogenous phosphorylation in a Ca^{2+} /CaM-dependent manner, indicating that the labelled protein may be the Ca^{2+} /CaM-dependent protein kinase or else be involved in its regulation.

Introduction

The process of mating between haploid cells of two compatible mating types (*a* and *A*) of *Rhodospiridium toruloides*, a basidiomycetous yeast, is initiated by a mating-type-specific mating pheromone secreted by the haploid cells (Abe *et al.*, 1975). Rhodotorucine *A*, a farnesylated peptide mating pheromone produced by mating type *A* cells (Kamiya *et al.*, 1979) induces a very rapid and transient influx of Ca^{2+} into the cells of mating type *a* from the medium (Miyakawa *et al.*, 1985, 1986). The mating pheromone induces cascade reactions that result in sexual differentiation for mating which could be triggered by the transient rise of intracellular free Ca^{2+} .

Calcium ions play an important role in the regulation of specialized cells in response to diverse extracellular signals such as hormones, growth factors and neurotrans-

mitters. An early event in the action of these regulatory agents is a transient increase in the intracellular free Ca^{2+} level (Weiss *et al.*, 1980). In most cases, the regulatory activities of Ca^{2+} are mediated by calmodulin (CaM), a highly conserved low-molecular-mass Ca^{2+} -binding protein. Upon binding Ca^{2+} , CaM undergoes conformational changes revealing hydrophobic sites that enable binding to a number of target proteins whose activities are regulated by the Ca^{2+} /CaM complex.

In the ascomycetous yeast *Saccharomyces cerevisiae*, Ca^{2+} is suggested to be involved in cell cycle progression (Baum *et al.*, 1986). Recently, CaM genes of *S. cerevisiae* (Davis *et al.*, 1986) and *Schizosaccharomyces pombe* (Takeda & Yamamoto, 1987) have been cloned. It was demonstrated genetically that CaM is essential for the growth of the yeast cells (Davis *et al.*, 1986; Takeda & Yamamoto, 1987; Ohya & Anraku, 1989). Identification and characterization of CaM-interacting proteins will be important to elucidate Ca^{2+} /CaM-mediated regulatory processes. However, little is known about the identity of proteins that interact with CaM in yeast.

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Abbreviations: CaM, calmodulin; TFP, trifluoperazine.

We describe here the isolation and preliminary characterization of CaM-binding proteins in soluble cell extracts of haploid vegetative cells and sexually differentiated cells (mating pheromone-treated cells) of *R. toruloides*. The presence of an autoregulatory Ca^{2+} /CaM-dependent protein kinase in the CaM-binding protein fraction is also described.

Methods

Micro-organisms and growth conditions. The haploid strains of *Rhodospiridium toruloides* IFO 0880-M1057 (mating type *a*) and IFO 0559-M919 (mating type *A*) described previously by Abe *et al.* (1975) were used. The cells were grown aerobically in YPG medium (Miyakawa *et al.*, 1982). Gamete cells were prepared by incubation of 2×10^9 vegetative cells (mating type *a*) with rhodotorucine *A* at concentrations of 1×10^7 cells ml^{-1} and 20 U ml^{-1} , respectively, for 3 h at 28 °C. One unit of activity (U) ml^{-1} is defined as the pheromone concentration which gives the characteristic morphological change in 30% of the assay cells (Miyakawa *et al.*, 1982).

Preparation of CaM. Bovine brain CaM was purified according to the method of Gopalakrishna & Anderson (1982). *R. toruloides* CaM was purified to homogeneity from the soluble fraction by heat treatment (5 min at 100 °C) followed by phenyl Sepharose hydrophobic affinity chromatography.

Radiolabelling of CaM. CaM was labelled with ^{125}I using Bolton-Hunter reagent as described by Bolton & Hunter (1973). Labelled CaM was purified by hydrophobic affinity chromatography.

Preparation of CaM-binding proteins. CaM-binding proteins were prepared from the soluble cell extract of *R. toruloides* (about 2×10^9 cells) by CaM-Sepharose affinity chromatography as described previously for *Saccharomyces cerevisiae* CaM-binding proteins (Miyakawa *et al.*, 1989).

Protein kinase assay. Protein kinase activity was measured in a reaction mixture (100 μl) containing 20 mM-Tris/HCl (pH 7.0), 10 mM- MgCl_2 , 1 mM-dithiothreitol, 0.02 mM- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (2 μCi , 74 kBq) and 10 μg each of κ -casein and bovine serum albumin (BSA). When required, 0.5 mM- CaCl_2 , 1 mM-EGTA, 1.5 μg *R. toruloides* CaM ml^{-1} , or 50 μM -trifluoperazine (TFP) were added. The reaction was initiated by the addition of labelled ATP to the mixture prewarmed at 28 °C. After incubation at 28 °C (usually for 10 min), the mixture was applied quickly onto a piece of filter paper (diameter, 23 mm; Whatman 3MM paper) and immersed immediately in 10% (w/v) trichloroacetic acid (TCA) at 100 °C (10 ml per filter). The filters were then washed three times with ethanol (5 min for each rinse, 10 ml per filter). The radioactivity on the filter was determined by liquid scintillation counting. To determine the effect of protein preincubation on the kinase activity, CaM-binding proteins were prephosphorylated under various conditions similar to those for the kinase assay, except that phosphorylation was carried out with unlabelled ATP (0.02 mM) in the absence of the exogenous substrate (κ -casein). After incubation, the enzyme activities were measured as described above by adding 2 μCi $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (4000 Ci mmol^{-1} , 148 TBq mmol^{-1}). Total and Ca^{2+} /CaM-independent protein kinase activities were measured in the presence of 0.5 mM- CaCl_2 and 1.5 μg CaM ml^{-1} , and 1 mM-EGTA and 50 μM -TFP, respectively.

Analysis of phosphorylated proteins. CaM-binding proteins were phosphorylated with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (4000 Ci mmol^{-1} , 148 TBq mmol^{-1}) by incubation in the presence and absence of Ca^{2+} /CaM as described

above for the protein kinase assay, except that κ -casein was omitted from the incubation mixture. Phosphorylated proteins were precipitated in a microfuge tube, with 20 μg BSA added as carrier, by the addition of 100 μl of 10% (w/v) TCA. The precipitates were collected by centrifugation in a microfuge and rinsed three times with 0.5 ml ethanol. The washed precipitates were then dissolved in sample buffer and analysed by sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (1970), using 12.5% (w/v) gels. The radioactivity on the gel was detected by autoradiography. Gels were calibrated using human erythrocyte membrane proteins and BSA as molecular mass markers.

Gel overlay method. Samples of cell extracts (18 μg protein) were resolved by SDS-PAGE in 12.5% gels and the CaM-binding proteins were detected by the gel overlay method described by Glenney & Weber (1983).

Results and Discussion

CaM from *R. toruloides*

CaM has been purified to homogeneity from various sources in a single step using hydrophobic affinity chromatography (Gopalakrishna & Anderson, 1982). This procedure, which exploits the characteristic Ca^{2+} -dependent change of hydrophobicity of CaM, was effective for the purification of CaM from *R. toruloides*. With an additional step of heat-treatment of the cell extract before the affinity chromatography step, a practically homogeneous preparation of CaM was obtained (Fig. 1). Usually, about 1 mg CaM was obtained from a 5 l culture (4×10^{11} cells or 2.0 g protein in the cell extract). CaM from *R. toruloides* changed its electrophoretic mobility when electrophoresed in the presence and absence of Ca^{2+} , as occurs with CaM from many other sources (Fig. 1).

Fig. 1. CaM isolated from *R. toruloides*. Samples (4 μg protein) purified by hydrophobic affinity chromatography were analysed by SDS-PAGE (12.5% gel). The sample contained 1 mM-EGTA (lane 1) or 1 mM- Ca^{2+} (lane 2).

Fig. 2. CaM-binding proteins of *R. toruloides*. (a) CaM-binding proteins affinity-purified from vegetative cells (V) and gamete cells (G) were separated by SDS-PAGE (7.5% gel) and the gel was stained with Coomassie brilliant blue. 1, Total cell extract of vegetative cells; 2, CaM-binding proteins of vegetative cells; 3, CaM-binding proteins of gamete cells. (b) Soluble extracts purified from vegetative and gamete cells (each containing 60 μg protein) were separated by SDS-PAGE and the CaM-binding proteins were detected by the gel overlay method (12.5% gel) as described in the text. The gels were incubated with [^{125}I]CaM in the presence of 1, 1 mM- Ca^{2+} ; 2, 5 mM-EGTA; or 3, 1 mM- Ca^{2+} plus 5 μM -TFP.

CaM-binding proteins

CaM-interacting proteins in the soluble fraction of vegetative cells and gamete cells (mating-pheromone-treated cells) of *R. toruloides* were identified and the molecular species of CaM-binding proteins of the two cell types were compared. CaM-binding proteins were detected by two different procedures.

In the first procedure, CaM-binding proteins were isolated from soluble cell extracts of the two cell types by CaM-Sepharose affinity chromatography. The cell extracts, containing equal amounts of protein, were applied to the column in the presence of Ca^{2+} , and the CaM-binding proteins were eluted with a buffer containing EGTA. The affinity-purified proteins from the two cell types were analysed by SDS-PAGE (7.5% acrylamide gel) and visualized by Coomassie blue staining (Fig. 2a). The major CaM-binding proteins of the vegetative cells detected by this procedure had molecular masses of 103

and 96 kDa. In the pheromone-treated cells, major bands of 40 and 38 kDa, and minor bands of 92 and 60 kDa, were seen in addition to those present in the vegetative cells. The bands common to the two cell types were more intense in the gamete cells (Fig. 2a).

In the second procedure for the detection of CaM-binding proteins, equal amounts of soluble protein samples prepared from the vegetative and the gamete cells were resolved by SDS-PAGE (12.5% acrylamide), and the CaM-binding proteins were labelled with ^{125}I -CaM after removing SDS from the gel (gel overlay method) (Fig. 2b). The binding of labelled CaM to the proteins in the gel was Ca^{2+} -dependent, and it was completely inhibited by TFP. This technique has been used for detecting CaM-binding proteins of mammalian cells, and is a very sensitive and specific method (Glenny & Weber, 1985). The proteins observed by the gel overlay method were basically similar to those isolated by CaM-affinity chromatography, except that the gel overlay

method gave greater sensitivity of detection. Most of the affinity-purified CaM-binding proteins were detected by the gel overlay method, showing the validity of the gel overlay procedure in the detection of CaM-binding proteins.

The major CaM-binding proteins of the vegetative cells detected by the gel overlay method had molecular masses of 103, 96, 58, 41, 35, 28 and 22 kDa (Fig. 2*b*). The CaM-binding protein bands of the gamete cells were generally more intense than those of the vegetative cells. Among these, the proteins with molecular masses of 103 and 96 kDa were especially more prominent in the gamete cells. In addition, a few CaM-binding proteins (molecular masses of 138, 46, 40, 34, 25, 23 and 21 kDa) were unique to the gamete cells, and others (molecular masses of 58 and 22 kDa) to the vegetative cells. However, correlation of the proteins of the two samples is not clear, since most bands in the two lanes did not share exactly the same mobility. Slight differences in electrophoretic mobility may be due to differences in protein modification such as phosphorylation.

The results indicated that the expression of many CaM-binding protein genes is under the control of the mating pheromone. Part of the differentiation-specific proteins may be involved in the organization of mating tubes, a characteristic morphology of the gamete cell. Our results suggest that CaM may play important roles not only in the cellular processes of the vegetative growth, but also in sexual differentiation. It has been shown that the sexual differentiation of *R. toruloides* is extremely sensitive to the CaM antagonist TFP (Miyakawa *et al.*, 1986).

Some proteins (e.g. 92 and 38 kDa species of the gamete cells) were visible only in the affinity-purified proteins. These proteins might be subunit proteins associated with CaM-binding proteins.

CaM prepared from bovine brain was used for the experiments. The use of CaM from different species for the detection of yeast CaM-binding proteins may cause a problem if there are any differences in the specificity of recognition. It was shown genetically, however, by Ohya & Anraku (1989) that chicken CaM is functional in the yeast *S. cerevisiae*, indicating that CaM from higher organisms can interact with CaM-binding proteins of lower eukaryotes.

Ca²⁺- and CaM-dependent protein kinase

Ca²⁺/CaM-dependent protein kinase activity in the CaM-binding proteins was measured using κ -casein as substrate (Fig. 3). Protein kinase requiring both Ca²⁺ and CaM for activity was present in this preparation. The activity was strongly inhibited by TFP. Mammalian

Fig. 3. Ca²⁺/CaM-dependent protein kinase of the CaM-affinity-purified proteins. Protein kinase activity was measured using κ -casein as substrate and [γ -³²P]ATP as phosphate donor.

Fig. 4. Effect of preincubation conditions on the activity of Ca²⁺/CaM protein kinase of *R. toruloides*. CaM-affinity-purified proteins were preincubated with nonradioactive ATP in the presence (○, ●) or absence (△, ▲) of Ca²⁺/CaM for various periods. [γ -³²P]ATP and κ -casein were added to the mixture and protein kinase activities were determined. The assay was performed in the presence of Ca²⁺/CaM for total kinase activity (○, △) and in the presence of EGTA/TFP (●, ▲) for Ca²⁺/CaM-independent kinase activity.

type II Ca²⁺/CaM-dependent protein kinase has been reported to be regulated by autophosphorylation, changing its Ca²⁺/CaM-dependence (Lai *et al.*, 1986; Lou *et al.*, 1986; Schworer *et al.*, 1986). We tested whether the yeast protein kinase has a similar autoregulatory property. The affinity-purified enzyme was preincubated in the

Fig. 5. Endogenous phosphorylation of CaM-binding proteins. CaM-binding proteins were phosphorylated in the presence (1) and absence (2) of Ca^{2+} /CaM. The labelled proteins were analysed by SDS-PAGE (10.0% gel) and detected by autoradiography.

presence and absence of the kinase activators. After various periods of preincubation, kinase activity was determined in the presence of Ca^{2+} and CaM (for total kinase activity), in the presence of EGTA and TFP (for Ca^{2+} /CaM-independent protein kinase activity). The activity of protein kinase became independent of the activators very rapidly (within 4 min) during the incubation (Fig. 4). For the conversion to occur, all three of Ca^{2+} , CaM and ATP had to be present during the preincubation (data not shown).

Protein species endogenously phosphorylated by the incubation of the CaM-binding proteins were analysed. Labelled proteins were separated by SDS-PAGE after phosphorylation of the CaM-binding proteins in the absence of κ -casein. Protein with a molecular mass of 60 kDa labelled in the Ca^{2+} /CaM-dependent manner was present in the CaM-binding proteins (Fig. 5). All of the above-described properties of the Ca^{2+} /CaM-dependent protein kinase are very similar to those of mammalian type II Ca^{2+} /CaM-dependent protein kinases which have been demonstrated to be regulated by autophosphorylation (Lai *et al.*, 1986; Lou *et al.*, 1986; Schworer *et al.*, 1986). The autoregulated mammalian protein kinase is believed to be involved in the transduction of the Ca^{2+} signal, responding to a transient elevation of cellular free Ca^{2+} induced by various ligands. It is intriguing to speculate that Ca^{2+} /CaM-dependent protein kinase of *R. toruloides* may be involved in the process of sexual differentiation, responding to a transient elevation of cellular Ca^{2+} level induced by the mating pheromone rhodotorucine A

(Miyakawa *et al.*, 1985). The presence of an autoregulatory Ca^{2+} /CaM-dependent protein kinase with properties very similar to those described here has also been found in *S. cerevisiae* (Miyakawa *et al.*, 1985).

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