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Ca^{2+}/calmodulin-dependent protein kinase in *Saccharomyces cerevisiae*

John Londesborough and Marja Nuutinen

Research Laboratories of the Finnish State Alcohol Company (Alko), Box 350, SF-00101 Helsinki 10, Finland

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Ca^{2+}-dependent chromatography of soluble cytosolic proteins on calmodulin-Sepharose gave a fraction that exhibited Ca^{2+}- and calmodulin-dependent phosphorylation of several polypeptides, including 60, 56 and 45 kDa species. At 0.2 μM beef calmodulin the phosphorylation was optimal at 3 μM free Ca^{2+}, and at 80 μM free Ca^{2+} it was half-maximal at about 0.1 μM beef calmodulin. It is concluded that the fraction contains calmodulin-dependent protein kinase(s) which is (are) autophosphorylated or associated with substrates.

Calmodulin-dependent protein kinase; (*Saccharomyces cerevisiae*)

1. INTRODUCTION

In higher eukaryotic organisms, the Ca^{2+}-binding protein calmodulin regulates several enzymes, including adenylate and guanylate cyclases, cyclic nucleotide phosphodiesterases and protein kinases [1]. A calmodulin-like protein has been demonstrated [2,3] and a gene for calmodulin identified [4] in *Saccharomyces cerevisiae*. Here we describe Ca^{2+}/calmodulin-dependent protein kinase activity in *S. cerevisiae*.

2. EXPERIMENTAL

2.1. *Organisms*

The yeasts used were commercial baker's yeast from our Rajamäki factory (here called baker's yeast A), and the *S. cerevisiae* strains Lh225 (a former production baker's yeast from Alko's collection) and X2180. They were grown aerobically at 30°C in shake flasks containing 1% yeast extract/1% peptone/2% glucose medium for 12–19 h and then harvested (8–22 g fresh yeast/l) and broken. The stored baker's yeast A preparation was made from a 1 kg pressed block stored 1 week at 5°C.

Correspondence address: J. Londesborough, Research Laboratories, Alko Ltd, Box 350, SF-00101 Helsinki, Finland

2.2. *Partial purification of Ca^{2+}/calmodulin-dependent protein kinase*

All operations were at 0°C to 10°C. Washed cells (15 g) were broken with a Braun MKII homogeniser in 20 mM Tris-HCl/10 mM benzamidine/3 mM $MgCl_2$/1 mM EGTA/1 mM dithiothreitol, pH 7.5, containing freshly added 1 mM phenylmethylsulphonyl fluoride and pepstatin A (10 μg/ml) (buffer A). The homogenate was centrifuged 15 min at 28000 × *g* and then 60 min at 145000 × *g*. The supernatant was mixed with 1.2 vols of saturated $(NH_4)_2SO_4$ in buffer A. Precipitated proteins were dissolved in 4 ml of buffer A/0.1 M NaCl and then concentrated to about 1 ml and washed with 2 lots of 2.5 ml of buffer A/0.1 M NaCl above an Amicon PM10 membrane. After addition of $CaCl_2$ to

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2 mM and centrifugation for 10 min at 28000 × *g*, the supernatant was run into a calmodulin-Sepharose 4B (Pharmacia) column (3.2 × 0.7 cm) equilibrated with buffer A/0.1 M NaCl/2 mM $CaCl_2$. The column was shut for about 16 h and then washed with about 15 ml of buffer A/0.1 M NaCl/2 mM $CaCl_2$. The column was filled with buffer A/0.1 M NaCl containing an extra 2 mM EGTA and again shut for 45 min. The protein kinase was then eluted with this buffer.

2.3. *Phosphorylation reactions*

Reaction mixtures (100 μl) containing 40 mM Pipes/KOH, pH 7.0, 4 mM $MgCl_2$, 0.1 mM EDTA, 2 mM dithiothreitol and enzyme were equilibrated for 2 min at 30°C. Reactions were started with 10 μl of 10 μM [γ-^{32}P]ATP (6 × 10^6 cpm) and stopped with 50 μl of 3-fold concentrated electrophoresis sample buffer [5] and boiling. Electrophoresis [5] and autoradiography were performed as described [6].

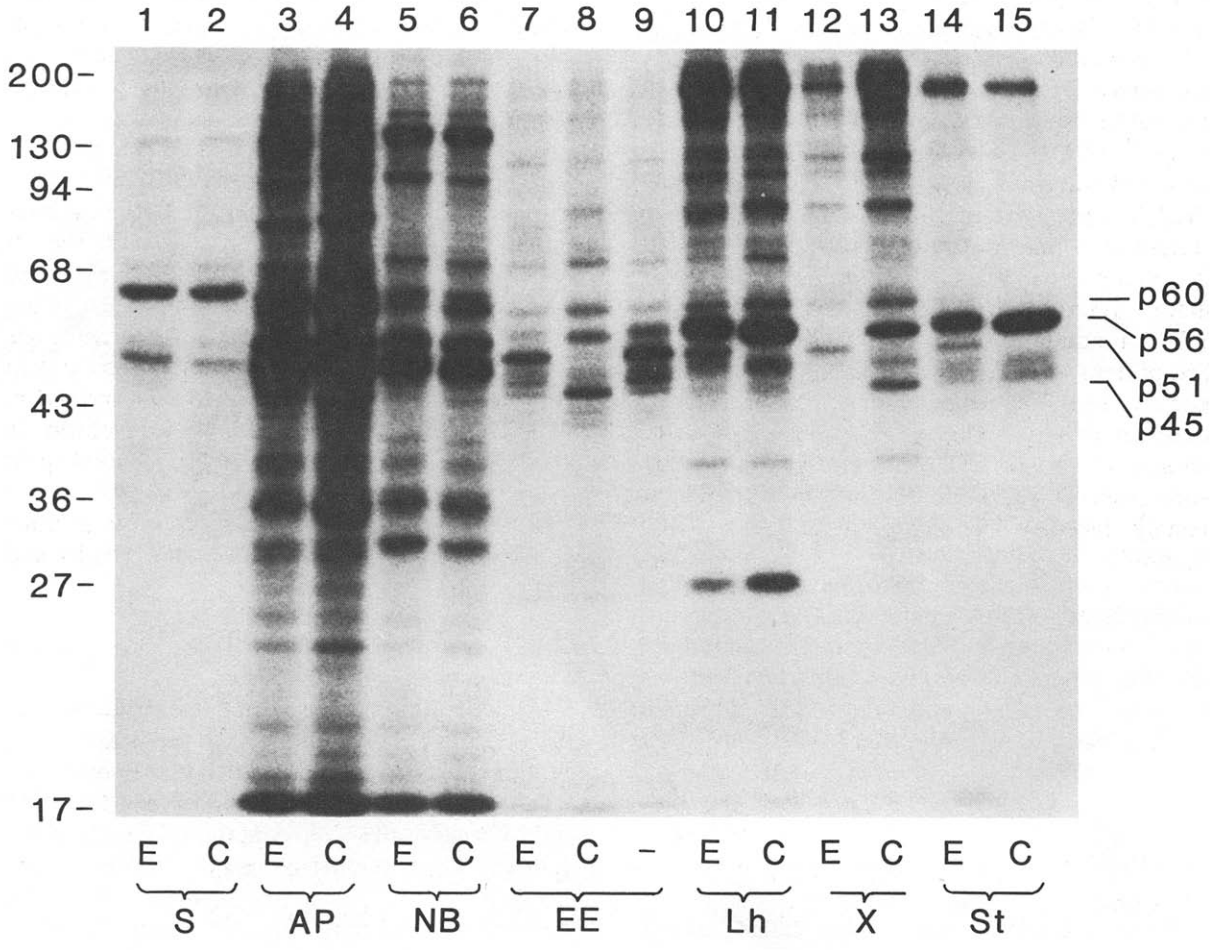

Fig.1. Endogenous phosphorylation of fractions before and after chromatography on calmodulin-Sepharose. The fractions were labelled for 2 min as described in section 2 with 1 mM EGTA (E) or 1 mM $CaCl_2$/1 μM calmodulin (C) as indicated. For lanes 9–15 0.25 mM EGTA was also present. Lanes 1–9 show fractions obtained from baker's yeast A grown to 13 g fresh cells/l (final protein concentration in parentheses): 145000 × *g* supernatant (S; 0.4 mg/ml); $(NH_4)_2SO_4$-precipitated proteins (AP; 0.6 mg/ml); material not bound by calmodulin-Sepharose (NB; 0.2 mg/ml); and material eluted with EGTA from calmodulin-Sepharose (EE; 70 μg/ml). Lanes 10–15 show the EGTA-eluates (assayed at about 20 μg/ml) from Lh225 grown to 19 g fresh cells/l (Lh), X2180 grown to 12 g fresh cells/l (X) and stored baker's yeast A (St). Molecular mass standards are shown on the left and identity of bands on the right.

2.4. *Materials*

Ox brain and spinach calmodulin, mixed histones (type IIA), protamine sulphate (grade X), myosin, trifluoperazine and melittin were from Sigma, [γ-^{32}P]ATP from Amersham, and other chemicals from sources described in [6].

3. RESULTS

The endogenous phosphorylation of crude subcellular fractions from *S. cerevisiae* showed only weak and poorly reproducible responses to Ca^{2+} and calmodulin (not shown). The proteins precipitated by $(NH_4)_2SO_4$ from the 145000 × *g* supernatant were much more extensively labelled by [γ-^{32}P]ATP especially in the presence of $CaCl_2$/calmodulin (fig.1). More than 90% of the $(NH_4)_2SO_4$-precipitated proteins did not bind to calmodulin-Sepharose in the presence of $CaCl_2$. The endogenous phosphorylation of this non-binding fraction was insensitive to EGTA or $CaCl_2$/calmodulin (fig.1). About 1% of the applied protein was eluted with 3 mM EGTA. In this material, p51 (named according to its apparent molecular mass in kDa) was labelled only in the presence of excess EGTA, whereas most other bands, especially p60, p56 and p45 were more strongly labelled in the presence of $CaCl_2$/calmodulin.

The phosphorylation patterns (fig.1, lanes 7–15) of the EGTA-eluates from various *S. cerevisiae* samples differed. Material from stored baker's yeast A was least phosphorylated and least responsive to $CaCl_2$/calmodulin. Labelling at high molecular mass (about 180 kDa) was present in both the X2180 and Lh225 preparations shown, but was dependent on $CaCl_2$/calmodulin only in the X2180 preparation, which was from a younger culture. Experiments (not shown) with 4 cultivations of X2180 grown to between 8 and 22 g fresh cells/l showed that EGTA-eluates from older cultures gave a weaker p45, weaker p51, and labelling at about 180 kDa less dependent on $CaCl_2$/calmodulin than did those from younger cultures.

Strong labelling of p60, p56 and p45 required the presence of both calmodulin and free Ca^{2+}, whereas labelling of p51 at 80 μM Ca^{2+} was suppressed by calmodulin (fig.2). However, the labelling of p51 at 0.2 μM calmodulin seemed to require some Ca^{2+}, and was optimal between 0.1 μM and 0.3 μM free Ca^{2+}. Concentrations within the physiological range of cytosolic free Ca^{2+} increased the labelling of p60, p56 and p45 at 0.2 μM calmodulin. All three bands were optimally labelled at about 3 μM Ca^{2+} and weak (p60, p56) or absent (p45) below 0.1 μM free Ca^{2+}.

Brain calmodulin suppressed p51 by at least 6-fold and 2.5-fold, respectively, at 780 μM and

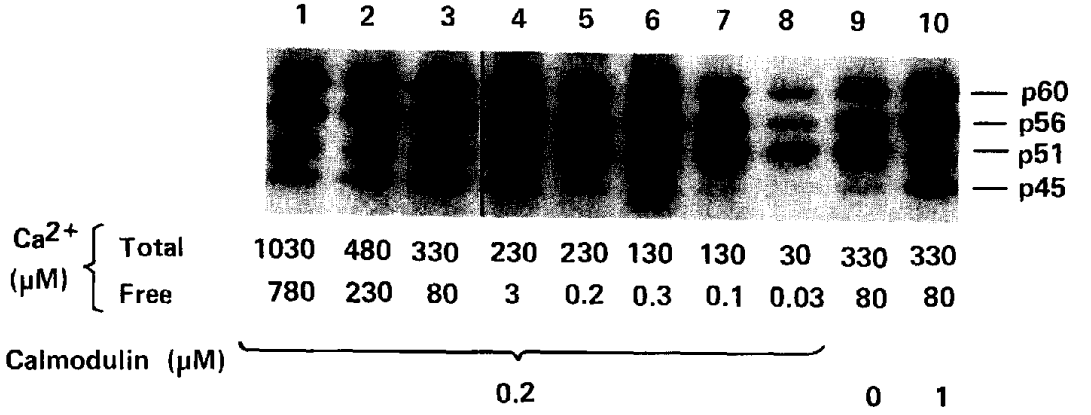

Fig.2. Calmodulin- and Ca^{2+}-dependence of the phosphorylation. EGTA-eluate from X2180 yeast grown to 12 g/l was phosphorylated for 2 min at 15 μg protein/ml with the indicated amounts of calmodulin and $CaCl_2$ and either 0.25 mM or 0.50 mM (lanes 5 and 7) EGTA. Total calcium concentrations include 30 μM adventitious Ca found by atomic absorption spectroscopy. Free Ca^{2+} levels were estimated assuming the apparent stability constant of Ca·EGTA in the buffer used as 4×10^6 M^{-1} (cf. [7]).

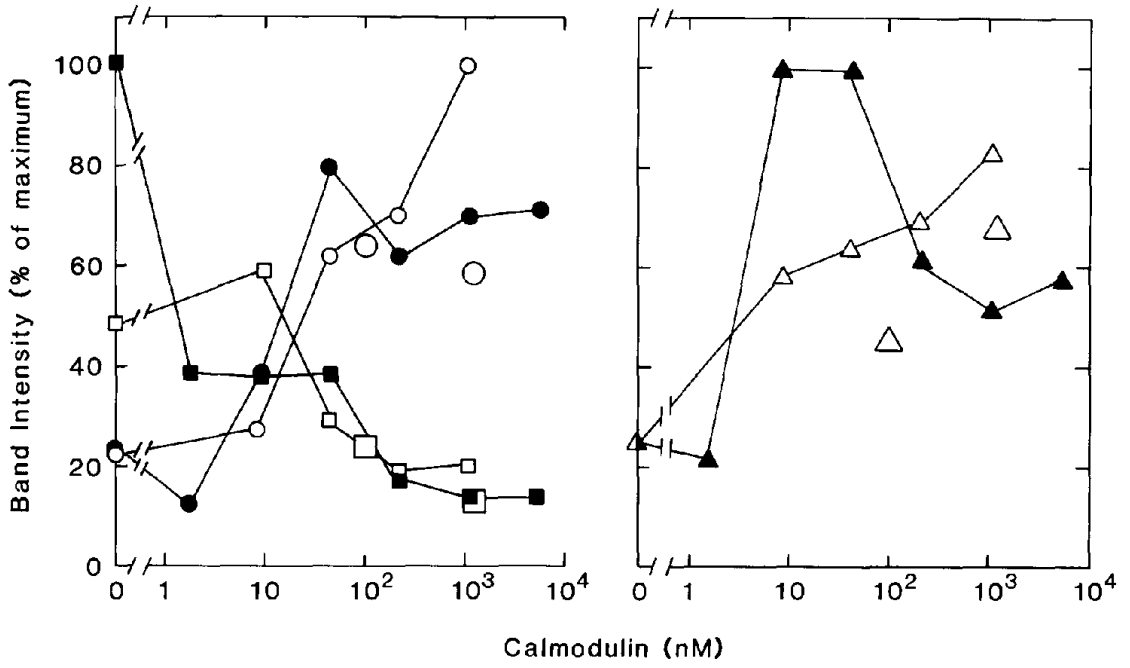

Fig.3. Calmodulin dependence of the phosphorylation. Brain (small symbols) or spinach (large symbols) calmodulin were varied at 780 μM (filled symbols) or 80 μM (open symbols) free Ca^{2+}. The intensities of p45 (●,○,○), p51 (■, □, □) and p56 (▲, △, △) were estimated by densitometry of autoradiograms under conditions where the peak heights were proportional to the exposure time. Experimental details were as in fig.2.

80 μM free Ca^{2+} (fig.3). The suppression is probably greater (fig.2, lanes 9 and 10), but a band at 50 kDa interfered with densitometry of suppressed p51. Calmodulin enhanced p45 and p56 at least 5-fold (fig.3). At 780 μM Ca^{2+} these enhancements were half-maximal at or below 10 nM calmodulin and decreased above 100 nM calmodulin. At 80 μM free Ca^{2+} more calmodulin was needed to cause the same enhancement, and the enhancement increased up to at least 1 μM calmodulin. This is less than the calmodulin concentration in some cells [8]. Spinach calmodulin also enhanced p56 and p45 and suppressed p51.

Two calmodulin antagonists were tested at 1 mM $CaCl_2$ and 1 μM brain calmodulin (not shown). Trifluoperazine at 20 μM partially inhibited p45 and caused p51 to appear, whereas at 0.8 mM it inhibited both p45 and p60 but did not cause the appearance of p51. Melittin (50 μg/ml) partially inhibited p45 and caused p51 to appear.

The EGTA-eluate phosphorylated protamine and mixed histones in a reaction potentiated by $CaCl_2$/calmodulin (not shown). Cytochrome *c* and casein were also phosphorylated but ovalbumin and rabbit muscle myosin were not.

EGTA-eluates were gel-filtered through a Superose 12 FPLC column (Pharmacia) equilibrated with buffer (20 mM Tris-HCl/200 mM NaCl/1 mM EGTA/1 mM dithiothreitol, pH 7.5) containing 0.2 mg of cytochrome *c*/ml, and the fractions phosphorylated. $CaCl_2$/calmodulin-dependent p56 was found in fractions covering an apparent molecular mass range of 60–175 kDa and peaking at 120 kDa (not shown). It must be either autophosphorylated or phosphorylated by a kinase of similar molecular mass. $CaCl_2$/calmodulin-dependent p45 and EGTA-dependent p51 were found together in fractions after p56 corresponding to molecular masses between 40 and 90 kDa.

4. DISCUSSION

The concentration of free Ca^{2+} is between 0.1

and 1 μM in the cytosol of animal cells, and regulates, usually via calmodulin and similar proteins, functions such as motility, secretion and cell division which often involve the cytoskeleton. Ca^{2+} homeostasis applies also in *S. cerevisiae*, where total Ca^{2+} levels are between 5 and 25 μM in the cytoplasm [9]. Free Ca^{2+} is probably an order of magnitude smaller. Work with *S. cerevisiae* including studies of Ca^{2+} uptake [10], calmodulin antagonists [10,11] and high Ca^{2+}-dependent mutants [12] indicates that Ca^{2+}- and calmodulin-dependent processes are involved in cell division.

Our fractionation method was modified from that [13] used to demonstrate Ca^{2+}/calmodulin-dependent protein kinase in *Neurospora crassa*. The results (fig.1) suggest that the EGTA-eluate contains one or more Ca^{2+}/calmodulin-dependent protein kinases. The labelled polypeptides may be either substrates or autophosphorylated components of these kinases. Substrates might be retained by calmodulin-Sepharose because they have affinity for calmodulin or because they bind to calmodulin-binding proteins. The EGTA-eluate may contain other calmodulin-dependent enzymes, including phosphoprotein phosphatases. This might explain the suppression of p51 by $CaCl_2$/calmodulin.

Like the multifunctional Ca^{2+}/calmodulin-dependent protein kinases from brain and skeletal muscle [14], the yeast activity could phosphorylate histone. Animal multifunctional Ca^{2+}/calmodulin-dependent protein kinases are large (~500 kDa) oligomers containing autophosphorylatable subunits of 50–60 kDa. However, gel filtration of the yeast preparation indicated that p56 is a subunit of an approx. 120 kDa molecule whereas p51 and p45 originate from molecules smaller than 90 kDa. Even if these bands are not themselves (subunits of) protein kinases, the kinase(s) acting on them must be relatively small.

The decreased labelling of p45 and p51 in EGTA eluates from cells from older cultures suggests that p45 and p51 are present in smaller amounts or are already heavily phosphorylated in these cells. The latter hypothesis might also explain why the labelling at about 180 kDa no longer requires calmodulin in the preparations from older cultures, since some calmodulin-dependent kinases loose their calmodulin dependence when they are autophosphorylated [15].

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