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Selective ubiquitination of calmodulin by UBC4 and a putative ubiquitin protein ligase (E3) from *Saccharomyces cerevisiae*

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A putative ubiquitin protein ligase (E3-CaM) which cooperates with UBC4 in selectively ubiquitinating calmodulin has been partially purified from *Saccharomyces cerevisiae*. Ca^{2+} was required for this activity and monoubiquitinated calmodulin was the main product of the reaction. The apparent K_m of E3-CaM for calmodulin was approximately 1 μM which is of the same order of magnitude as the concentration of calmodulin in yeast cells. Proteins which are good substrates for other E3s (E3α or E3-R) were not ubiquitinated by E3-CaM. Lower but significant activities of E3-CaM were observed when UBC1 replaced UBC4.

Ubiquitin; Calmodulin; UBC4; Ubiquitin protein ligase; *Saccharomyces cerevisiae*

1. INTRODUCTION

In eukaryotic cells Ca^{2+} fluxes play an important role in modulating diverse cellular functions. One of the mediators of the Ca^{2+} signal is calmodulin (CaM)[1], a small calcium-binding protein. The binding of Ca^{2+} to CaM causes a change in its conformation which promotes its attachment to a wide variety of target proteins whose activity it modulates (reviewed in [1,2]). In *Saccharomyces cerevisiae* the gene encoding CaM (*CMD1*) is essential [3,4], and Ca^{2+}/CaM-dependent protein kinases and phosphatases have been identified [5,6], but the physiological functions of CaM in this organism are still unknown [7].

CaM from *S. cerevisiae*, *Dictyostelium*, *Neurospora*, spinach and mammals can be conjugated to ubiquitin by reticulocyte extracts [8–12]. Enzymes catalyzing the Ca^{2+}-dependent ligation of ubiquitin to CaM from yeast or mammals have also been found in extracts of *S. cerevisiae* [13] as well as in extracts of mammalian tissues other than reticulocytes [14]. Ubiquitin is a 76 residue eukaryotic protein whose multiple cellular functions involve its covalent ligation to other proteins. This is usually the first step in the degradation of the target proteins by an intracellular proteolytic pathway (reviewed in [15,16]). The ligation of ubiquitin to its target proteins involves three enzymatic steps. In the first step ubiquitin is linked via a thioester bond to the ubiquitin activating enzyme E1. The second step is the transfer of ubiquitin from E1 to one of a family of ubiquitin-conjugating enzymes (UBC or E2). Some of the E2 enzymes are able to conjugate ubiquitin to particular target proteins without the participation of additional factors [17–19] but the conjugation of ubiquitin to other target proteins requires the intervention of yet another class of enzymes known as ubiquitin protein ligases or E3s [20–24]. Since previous in vitro studies on CaM ubiquitination [8–14] have been carried out with crude enzyme systems, they do not indicate whether CaM is recognized by an E2 alone or requires the additional participation of an E3 enzyme. Here we present evidence for the presence in *S. cerevisiae* of a putative E3 enzyme which cooperates with the E2 enzyme UBC4 [25] to ubiquitinate CaM in the presence of Ca^{2+}.

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Abbreviations CaM, calmodulin; DTT, dithiothreitol, EGTA, ethylene glycol-bis-(β-aminoethyl ether)*N,N,N'N'*-tetraacetic acid; PMSF, phenylmethyl sulfonyl fluoride.

2. MATERIALS AND METHODS

2.1. *Preparation of Fraction II*

Yeast cells (strain SUB325 [23], kindly provided by D. Finley) grown in a fermentor were suspended in 1 vol. of buffer consisting of 25 mM Tris-HCl pH 7.8, 5 mM EDTA, 1 mM DTT and 0.5 mM PMSF and broken in a cooled Matcu Gaulin Homogenizer at 8000 psi. After adjusting the pH to 7.0 with Tris base, the extract was fractionated by ammonium sulfate precipitation. The fraction precipitating between 25% and 70% saturation was collected by centrifugation, suspended in a minimal volume of 50 mM sodium phosphate buffer pH 7.5 and 2 mM mercaptoethanol and dialyzed against 100 vols. of the same buffer. This fraction was aliquoted and stored at −20°. Aliquots were fractionated on a DEAE-cellulose (Whatman DE52) column by the following modification of previously described methods [17,20]. The sample, containing 3 mg of protein per ml of column, was loaded and the column was washed with 1.5 vols. of 3 mM phosphate buffer pH 7.0 containing 20 mM KCl, 5 mM EDTA and 1 mM DTT. 'Fraction II' was eluted with 3 vols. of 20 mM Tris

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pH 7.2, containing 0.5 M KCl, 5 mM EDTA and 1 mM DTT and was precipitated with ammonium sulfate at 70% saturation. The precipitate was suspended in a minimal volume of a buffer containing 20 mM Tris-HCl pH 7.5, 5% glycerol, 5 mM $MgCl_2$, 5 μM ATP, 0.2 mM DTT and 1 mM EDTA and dialyzed twice against 40 vols. of the same buffer.

2.2. *Phenyl-Sepharose chromatography*

A sample of Fraction II was mixed with an equal volume of 100 mM sodium phosphate pH 7.0 containing 2 M ammonium sulfate, 4 mM DTT and 2 mM EGTA and loaded on a phenyl-Sepharose CL4B column equilibrated with a 50 mM sodium phosphate pH 7.0 and 1 M ammonium sulfate. The column was washed with 3 vols. 50 mM phosphate buffer pH 7.0 containing 1 M ammonium sulfate and 2 mM DTT and eluted with steps of 1.5 vols. each of 50 mM sodium phosphate pH 7.0 plus 2 mM DTT containing successively 0.3 M, 0.15 M and no ammonium sulfate. This was followed by 1.5 vols. of 15 mM phosphate buffer pH 7.0 plus 2 mM DTT and 16.5 vols. of 5 mM phosphate buffer pH 7.0 plus 2 mM DTT. The ubiquitination activity on CaM (dependent on UBC4) was eluted at 5 mM phosphate and the active fractions were dialyzed against 50 mM Tris-HCl pH 7.5, 5% glycerol and 4 mM DTT.

2.3. *Calmodulin-agarose chromatography*

A calmodulin-agarose column (Sigma) was equilibrated with buffer A (50 mM Tris-HCl pH 7.5, 1 mM DTT, 1 μg/ml ovalbumin) supplemented with 0.1 mM $CaCl_2$ and 100 μg/ml ovalbumin. The sample in Buffer A plus 0.1 mM $CaCl_2$ was applied and the column was washed with 10 vols. of buffer A containing 0.1 mM $CaCl_2$ followed by 10 vols. of the same buffer containing 1 M KCl (KCl eluate). After washing with 10 vols. of Buffer A (without $CaCl_2$) the column was eluted with 10 vols. of Buffer A containing 1 mM EGTA (EGTA eluate) followed by 10 vols. of Buffer A supplemented with 1 mM EGTA and 1 M KCl (KCl + EGTA eluate). Fractions were concentrated on Centriprep 30 (Amicon) ultrafilters and washed twice with 10 vols. of Buffer A. Samples of fractions were subjected to electrophoresis and proteins were detected by silver staining [26].

2.4. *FPLC on Mono-Q*

A Mono-Q column (Pharmacia) was equilibrated in a buffer containing 50 mM Tris-HCl pH 7.5, 5% glycerol, 0.2 mM EDTA and 2 mM DTT. The EGTA eluate from the calmodulin agarose column was applied and 0.5 ml fractions were collected in a 0–1 M KCl gradient. Samples were collected into tubes containing 5 μg of ovalbumin. UBC4-dependent activity on CaM was eluted at 150–210 mM KCl. Samples were dialyzed against the buffer used to equilibrate the column and concentrated with Centricon 30 ultrafilters.

2.5. *Preparation of E1 and E2s*

E1 was purified from yeast as described previously [23]. UBC1, UBC4, RAD6 and CDC34 were prepared from extracts of *E. coli* containing the appropriate expression vectors (generously provided by S. Jentsch) essentially as described in an earlier paper [23] except that the UBC4-containing extract was used without further purification.

2.6. *Ubiquitin conjugation assay*

The conjugation of [^{125}I]ubiquitin to bovine CaM (Sigma) was detected as described previously [23]. The standard assay system (12.5 μl) contained 2.5 μg bovine calmodulin (Sigma), and approximately 0.1 pmol E1, 2 pmol of UBC4 and varying amounts of fractions containing E3-CaM. Additional components were: 50 mM Tris-HCl pH 7.6, 5 mM $MgCl_2$, 2 mM DTT, 2 mM ATP, 10 mM creatine phosphate, 2.5 μg creatine kinase, 7 pmol of [^{125}I]ubiquitin (2×10^5 cpm) and 10 μg/ml each of the following peptide protease inhibitors: leupeptin, pepstatin, chymostatin, bestatin, and antipain. After incubation for 1 h at 30°C, the reaction was stopped with SDS electrophoresis sample buffer and the samples were heated for 3 min in a boiling water bath. SDS-PAGE electrophoresis and autoradiography were performed as described earlier [23].

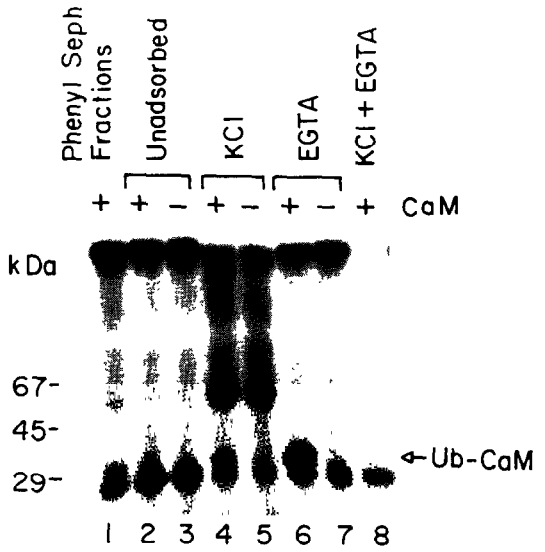

Fig. 1. E3-CaM activity of fractions eluted from a CaM-agarose column. Activity was detected by incubating [^{125}I]ubiquitin and CaM with a sample of the fraction supplemented with E1 and UBC4, followed by SDS-PAGE electrophoresis and autoradiography. Lane 1, pooled phenyl-Sepharose E3-CaM enriched fractions loaded on column; lane 2, unadsorbed fraction plus CaM; lane 3, unadsorbed fraction, without CaM; lane 4, KCl eluate plus CaM; lane 5, KCl eluate without CaM; lane 6, EGTA eluate plus CaM; lane 7, EGTA eluate without CaM; lane 8, KCl plus EGTA eluate plus CaM.

3. RESULTS

We have partially purified a putative E3 (hereafter referred to as E3-CaM) from *Saccharomyces cerevisiae*, which catalyzes the ubiquitination of bovine calmodulin. Since preliminary experiments showed that E3-CaM acts preferentially with UBC4, enzyme activity was assayed in the presence of added E1 and UBC4 and was detected by the formation of a 29 kDa band corresponding to mono-ubiquitinated bovine calmodulin [13]. A lower molecular weight band (25 kDa) which was found in almost all fractions is the putative monoubiquitinated derivative of UBC4. Fraction II, prepared by a modification of previous methods [17,20,23] was subjected to hydrophobic chromatography on phenyl-Sepharose. E3-CaM was strongly bound to the phenyl-Sepharose column and was eluted at low salt concentrations (not shown). It has previously been shown that CaM ubiquitination by yeast extracts is Ca^{2+}-dependent [13]. This was exploited to further purify E3-CaM from pooled phenyl-Sepharose fractions on a CaM-agarose column (Fig. 1). E3-CaM was retained on the CaM-agarose column in the presence of Ca^{2+} and 1 M KCl and was eluted with EGTA. Silver staining showed that the EGTA eluate contained multiple protein bands none of which could be identified as E3-CaM (not shown). The E3-CaM-enriched fractions produced monoubiquitinated CaM as well as high molecular weight ubiquitin conjugates (Fig. 1). Since the formation of these high molecular weight conjugates

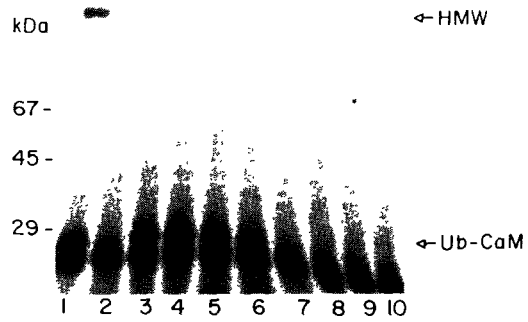

Fig. 2. E3-CaM activity of fractions eluted from a Mono-Q column. E3-CaM activity was detected by the conjugation of [^{125}I]ubiquitin to CaM as described in section 2 and Fig. 1. Lane 1, CaM-agarose EGTA eluate applied to column. The other lanes contained fractions eluted at the following KCl concentrations: lane 2, 60–110 mM; lane 3, 110–150 mM; lane 4, 150–210 mM, lane 5, 210–250 mM; lane 6, 250–310 mM; lane 7, 310–390 mM; lane 8, 390–450 mM; lane 9, 450–510 mM; lane 10, assay system without added fraction.

was independent of added CaM, they apparently represented conjugates of endogenous substrates with ubiquitin. The EGTA eluate from the CaM-agarose column was further purified by FPLC on a Mono-Q column (Fig. 2) from which E3-CaM was eluted between 110– 250 mM KCl. Even the purest UBC4-dependent CaM- E3 fractions contained many different proteins none of which could be identified as CaM-E3 (not shown). The high molecular weight conjugates were greatly reduced in E3-CaM-containing fractions from the Mono-Q column (Fig. 2). We were unable to determine the overall degree of purification of E3-CaM since E3-CaM activity in Fraction II could not be determined because of high background activity. In addition, protein could not be determined in the Mono-Q column fractions which were collected into tubes containing ovalbumin to stabilize the enzyme. E3-CaM was purified about 10-fold in the CaM-Agarose step.

The apparent K_m of E3-CaM for CaM was approximately 1 μM which is of the same order of magnitude as the concentration of CaM in yeast cells [4]. In our experiments only monoubiquitinated CaM could be detected whereas in previously published experiments [13] both singly and multiply ubiquitinated species of CaM were formed. This apparent discrepancy could be due to differences in the reaction conditions but may also indicate that an additional component present in the crude system but not in ours, such as an additional E2, is required for multiubiquitination.

Fig. 3A shows that CaM is not ubiquitinated unless E1, E2 (UBC4) and E3 (E3-CaM) are all present. Weak, but distinct, E3-CaM activity was observed when UBC4 was replaced by UBC1 (Fig. 3B). Like the activity with UBC4 this activity was Ca^{2+}-dependent. Traces of activity were also observed when RAD6 replaced UBC4 (Fig. 3B). This activity was only partially sensitive to EGTA and may have been due, at least in part, to contamination with E3-R. The RAD6-dependent E3 (E3-R) described previously [23] had appreciable CaM-ubiquitinating activity which was insensitive to EGTA (Raboy, unpublished experiments). CDC34 did not cooperate detectably with E3-CaM (Fig. 3B). The ubiquitin conjugates at about 70 kDa which are also formed in the absence of CaM (not shown) are probably various species of self-ubiquitinated CDC34.

E3-CaM activity was inhibited by EGTA and restored by the addition of excess Ca^{2+} (Fig. 4). This could mean that E3-CaM itself requires Ca^{2+} for activity or, more likely, that the substrate of E3-CaM is Ca^{2+}-bound CaM rather than the free form. E3-CaM activity was completely inhibited by 10 μM M5 (kindly donated by Y. Salomon). M5 is an 18 residue synthetic peptide modelled after the CaM-binding domain of rabbit skeletal muscle myosin light chain kinase [27]. Trifluoperazine (30 μM), which is thought to bind to the hydrophobic regions of CaM exposed upon Ca^{2+} binding [1,28], also inhibited CaM ubiquitination by E3-CaM.

E3-CaM had no detectable activity on substrates of E3α [21] or E3-R [23], namely, β-lactoglobulin, β- and κ-casein (Fig. 5), α-casein and oxidized ribonuclease (not shown). Thus, as far as can be deduced from these experiments, E3-CaM seems to be specific for CaM.

4. DISCUSSION

Previous experiments in other laboratories showed that CaM is ubiquitinated in a Ca^{2+}-dependent manner by cell extracts [9–14] but the species of E2 involved as well as the requirement for an E3 were unknown. Here we describe a putative E3 which cooperates with UBC4 in ubiquitinating CaM in the presence of Ca^{2+}. The UBC4 requirement of E3-CaM contrasts with the RAD6 requirement of other E3s described earlier, namely, UBR1 [24], E3α [21], and E3-R [23]. In vivo experiments demonstrate an important role for UBC4 in the degradation of abnormal proteins but neither

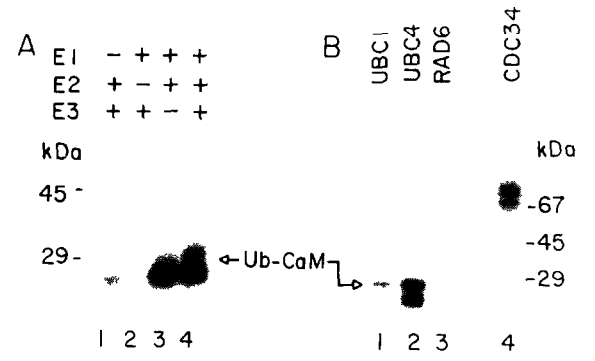

Fig. 3. Requirement for E3-CaM and a specific E2 in the CaM ubiquitination system. E3-CaM was from pooled Mono-Q fractions. (A) Lane 1, E1 omitted; lane 2, E2 (UBC4) omitted; lane 3, E3 (E3-CaM) omitted; lane 4, complete system. (B) E1 plus E3-CaM supplemented with: lane 1, 0.8 pmol UBC1; lane 2 1.9 pmol UBC4; lane 3, 0.9 pmol RAD6; lane 4, 1.9 pmol CDC34.

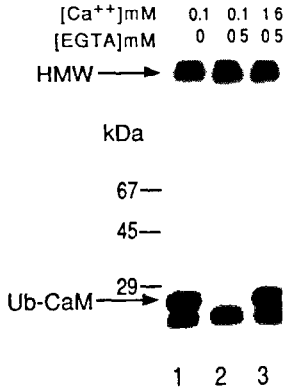

Fig. 4. Dependence of E3-CaM activity on Ca^{2+}. The reaction mixture contained CaM-agarose-purified E3-CaM. Lane 1, 0.1 mM Ca^{2+}, lane 2, 0.1 mM Ca^{2+} plus 0.5 mM EGTA; lane 3, 1.6 mM Ca^{2+} plus 0.5 mM EGTA.

unassisted ubiquitination of specific target proteins by UBC4 in vitro nor ubiquitination assisted by E3 have been observed previously.

Although endogenous CaM has been shown to be ubiquitinated in rabbit tissue extracts in vitro [29], the existence of CaM ubiquitination in vivo has not yet been demonstrated, nor, if it exists, are there any clues as to its function. The rates of decay of CaM levels upon transfer to glucose in *cmd1* cells bearing a galactose-induced *CMD1* gene do not reveal a rapid turnover of CaM in yeast [4]. However, CaM ubiquitination and degradation may be activated in vivo only under special circumstances when intracellular Ca^{2+} levels are elevated. It may be significant that the ubiquitin conjugating enzymes which cooperate with E3-CaM are UBC4 and UBC1 which have overlapping functions in protein degradation [25]. Another interesting possibility is that ubiquitination modulates CaM function rather than

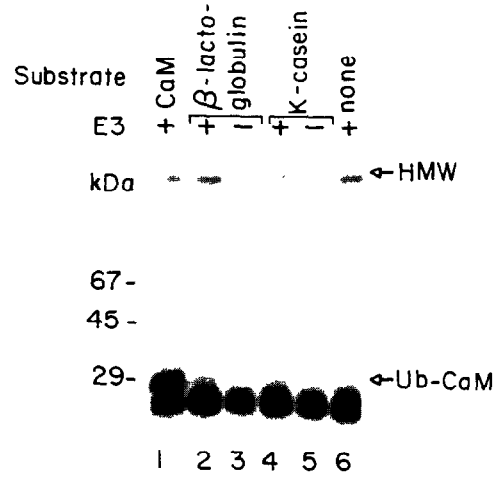

Fig. 5. Substrate specificity. E3-CaM was from pooled Mono-Q column fractions. Substrates, 2 μg each were added as indicated. Lane 1, CaM with E3-CaM; lane 2, β-lactoglobulin with E3-CaM; lane 3, β-lactoglobulin without E3-CaM; lane 4, κ-casein with E3-CaM, lane 5, κ-casein without E3-CaM; lane 6, no additions.

promoting its breakdown. When Ca^{2+}-bound CaM was associated with one of its target proteins, phosphorylase kinase, it was resistant to ubiquitination [10]. CaM ubiquitination was also inhibited by the peptide inhibitor M5 which mimics the CaM-binding domain of rabbit skeletal muscle myosin light chain kinase [27]. Apparently the structural features of Ca^{2+}-CaM recognized by the ubiquitination system were masked by binding to its target molecules. This raises the possibility that E3-CaM may bind to CaM by means of an amphiphatic α-helix in a similar manner to that of CaM target proteins [2,30,31]. Alternatively, E3-CaM may interact by some other mechanism with the hydrophobic pockets of Ca^{2+}-CaM which would be masked by binding to M5 or to trifluoperazine. If the ubiquitination site on CaM is at or near the target-binding groove of CaM [30,31], the bound ubiquitin could modulate the interaction of CaM with its targets. Since CaM ubiquitination did not cause the release of bound Ca^{2+}, ([9] and our experiments, not shown), it is unlikely that ubiquitination affects CaM function by modulating Ca^{2+} binding. The specificity of E3-CaM for Ca^{2+}-CaM strongly suggests an in vivo role for CaM ubiquitination but further investigation is required to determine what this is.

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