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Ca^{2+}-dependent ubiquitination of calmodulin in yeast

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Recently we were able to show that calmodulin from vertebrates, plants (spinach) and the mold *Neurospora crassa* can be covalently conjugated to ubiquitin in a Ca^{2+}-dependent manner by ubiquityl-calmodulin synthetase (uCaM-synthetase) from mammalian sources [R. Ziegenhagen and H.P. Jennissen (1990) FEBS Lett. 273, 253–256]. It was therefore of high interest to investigate whether this covalent modification of calmodulin also occurs in one of the simplest eukaryotes, the unicellular *Saccharomyces cerevisiae*. Yeast calmodulin was therefore purified from bakers yeast. In contrast to calmodulin from spinach and *N. crassa* it does not activate phosphorylase kinase. Crude yeast uCaM-synthetase conjugated ubiquitin Ca^{2+}-dependently to yeast and mammalian (bovine) calmodulin. Yeast calmodulin was also a substrate for mammalian (reticulocyte) uCaM-synthetase. As estimated from autoradiograms the monoubiquitination product (first-order conjugate) of yeast calmodulin has an apparent molecular mass of ca. 23–26 kDa and the second-order conjugate an apparent molecular mass of ca. 28–32 kDa. Two to three ubiquitin molecules can be incorporated per yeast calmodulin. Experiments with methylated ubiquitin in the heterologous reticulocyte system indicate that, as with vertebrate calmodulins, only one lysine residue of yeast calmodulin reacts with ubiquitin so that the incorporation of multiple ubiquitin molecules will lead to a polyubiquitin chain. These results also indicate that the ability of coupling ubiquitin to calmodulin was acquired at a very early stage in evolution.

Yeast; Calmodulin; Ubiquitin; Ubiquityl-calmodulin synthetase; Protein-ubiquitination; Phosphorylase kinase; Trimethyl lysine

1. INTRODUCTION

In eukaryotes and yeast [1] calmodulin is an essential signal transducing molecule for the second messenger Ca^{2+} (for review see [2]). Of all calmodulins yeast calmodulin (M_w=16.1 kDa, from sequence) has the largest sequential deviation from mammalian calmodulin (M_w=16.7 kDa, from sequence) only sharing 60% sequence identity [1]. Still mammalian and yeast calmodulin are functionally interchangeable in yeast [3]. Yeast ubiquitin (M_w = 8.5 kDa) only has three amino acid exchanges in comparison to mammalian and human ubiquitin [4] and has a similar three-dimensional structure as the other ubiquitins [5]. Yeast also possesses a ubiquitin-dependent proteolysis system [6,7] similar to that found in reticulocytes (for review see [8]).

Recently [9–13] we first demonstrated that vertebrate, plant and mold calmodulins can be covalently coupled to ubiquitin in the presence of Ca^{2+} and Mg^{2+}-ATP by ubiquityl-calmodulin synthetase (uCaM-synthetase) from mammalian tissues. At present calmodulin is the only protein where conjugation to ubiquitin is regulated by a second messenger (Ca^{2+}) [9,10]. Multiple ubiquitination at a single site appears characteristic for calmodulins [12,13] which apparently can only be ubiquitinated in the free form [10]. UCaM-synthetase, for which a specific affinity-based assay has been described [10], has been detected at significant levels in nearly all mammalian tissues tested so far [14]. Therefore it was a crucial question whether calmodulin from the unicellular eukaryote *Saccharomyces cerevisiae*, which shows the highest sequence differences to vertebrate calmodulins, can also be covalently modified with ubiquitin.

In this paper it will be shown that uCaM-synthetase capable of conjugating yeast and mammalian calmodulins exists in yeast and that yeast calmodulin can also be covalently linked to ubiquitin by mammalian uCaM-synthetase.

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2. MATERIALS AND METHODS

2.1. *Preparative methods*

Reticulocyte APF II (ATP-dependent proteolysis fraction II) was prepared as described in [15,16]. This fraction also contains high activities of uCaM-synthetase. For the preparation of the yeast enzyme, packages (500 g) of pressed baker's yeast were obtained from Uniferm GmbH (D-4712 Herne). The yeast was ground in a 'Bead Beater' (Biospec Products, Bartlesville, OK, USA) [17]. In a typical case 150 ml glass beads (0.5 mm diameter, Fa. H. Clauss, 6369 Niederau) were mixed with 170 ml 10 mM Tris-HCl, 1 mM EDTA, 14 mM mercaptoethanol, 5 μg/ml leupeptin, 1 mM phenylmethyl sulfonylfluoride (PMSF; stock solution 1 M PMSF in dioxane) pH 7.5 (buffer A) and 80 g pressed yeast broken down to 1–2 g portions. Beads, buffer and yeast were stirred with a spatula until the yeast was evenly dispersed. Then the mixture was homogenized in the Bead Beater 25 times for 15 s followed by a 10 s pause (W.H. Kunau, Bochum) at 0°C respectively. The beaker with the homogenate was decanted and the beads were washed twice with 25 ml buffer A. The homogenate (ca. 250 ml) was first centrifuged at 12 000×g for 20 min

and the supernatant (crude extract) was then ultracentrifuged in a Beckmann 45 Ti rotor at 30 000 rpm (100 000×g) for 90 min yielding ca. 140 ml supernatant (30-S extract). 240 g of yeast were processed in this way yielding 400 ml of the 30-S extract which was applied to DEAE Fractogel EMD (Superformance column 2.6×2 cm gel height; Merck Darmstadt) as described for DEAE-cellulose [15,16]. The column was eluted in one step by addition of 500 mM KCl to the buffer with 1 mM PMSF as described [15] and pooled (ca. 40 ml). The pool was dialyzed against 20 mM Tris-HCl, 1 mM DTE, 5 μg/ml leupeptin, 0.1 mM PMSF, pH 7.6 (buffer B) for 3 h and immediately used (yeast APF II, 21 mg/ml). Ubiquitin was purchased from Sigma (Munich). Ubiquitin was methylated according to [18]. The 8 primary amino groups per mol ubiquitin were reduced to ca. 0.23 groups/mol [12]. [^{125}I]CT-ubiquitin and methylated [^{125}I]CT-ubiquitin ([^{125}I]CT-ubiquitin-m) were synthesized (50–200 cpm/pmol) according to the chloramine-T procedure [15,19]. Bovine testis calmodulin was isolated according to [20] and purified further by affinity chromatography according to [21]. Calmodulin from spinach and *N. crassa* were purchased from Sigma. Yeast calmodulin was also isolated from pressed baker's yeast (see above). The yeast (10 kg) was extracted by autolysis in ethyl acetate as described by [22]. The autolysate was heat treated [1] by placing it in a boiling water bath for 15 min. After spinning down the denatured protein in a centrifuge the supernatant was precipitated by 5% trichloroacetic acid [20] dialyzed against 20 mM Tris-HCl, 1 mM Ca^{2+}, pH 7.0 (buffer C) and a sample (570 ml, 23.2 mg/ml) was applied to fluphenazine-Sepharose (100 ml packed gel) according to [21]. The enriched calmodulin fraction eluted by EGTA was then reprecipitated by TCA and redialyzed as above and applied (sample: 20 ml, 2.4 mg/ml) to butyl-*S*-Sepharose (12 μmol/ml packed gel, 100 ml packed gel) [23] and eluted under identical conditions [21]. Final purification of the butyl-*S*-Sepharose fraction (sample: 0.5 ml, 16 mg/ml) was achieved by gel filtration on Sephadex G-50 fine (1×48 cm, flow rate 5 ml/h) in 50 mM sodium glycerophosphate, 0.2 M NH_4Cl, 1 mM EGTA, pH 7.0 (buffer D) yielding a total amount of 2.5 mg pure yeast calmodulin. Phosphorylase *b* (3rd crystals) was prepared according to Fisher and Krebs [24]. Phosphorylase kinase (180 nkat/mg, pH 6.8/pH 8.2 ratio ca. 0.04) was isolated according to Cohen [25] with the modifications of Jennissen and Heilmeyer [26].

2.2. *Analytical methods*

Reticulocyte uCaM-Synthetase was quantitated with the FP-Test [10]. The incubation mixture contained 50 mM Tris-HCl, 1 mM DTE, 5 mM $MgCl_2$, 1 mM ATP, 10 mM phosphocreatine, 0.1 mg/ml creatine kinase, 500 μg/ml calmodulin, 50 μg/ml [^{125}I]CT-ubiquitin (specific radioactivity 50–200 cpm/pmol), 0.9 mg/ml reticulocyte APF II [10,11]. The mixtures with calcium contained 1.1 mM Ca^{2+} and 1 mM EGTA. The mixtures without calcium only contained 1 mM EGTA. The coating procedure for the microtest tubes with bovine serum albumin (BSA) [10] was simplified: the supernatants of the heat step (1.1 ml) were transferred to microtubes containing 100 μl BSA (30 mg/ml). To this mixture FP-Sepharose was added as described [10]. For the analysis of *yeast uCaM-synthetase* the FP-test was modified to contain 250 μg/ml calmodulin and 1.35 mg/ml [^{125}I]CT-ubiquitin. The final concentration of yeast APF II was 8.5 mg/ml. All other parameters remained unchanged. The incubation mixtures for the autoradiographic analysis of uCaM on polyacrylamide gels also contained 250 μg/ml calmodulin and 1.35 mg/ml [^{125}I]CT-ubiquitin.

Activities of phosphorylase *b* [27] and phosphorylase kinase [28] as modified in [29] were determined on an AutoAnalyzer (Technicon, Tarrytown, USA). The biological activity of the calmodulins was tested in the phosphorylase kinase AutoAnalyzer test. After 3-fold dilution in the AutoAnalyzer at pH 6.8 the sample contained as final concentrations 300 ng/ml phosphorylase kinase and from 0.15 to 100 μg calmodulin in kinase dilutant: 10 mM sodium glycerophosphate, 50 mM mercaptoethanol, 5 mg/ml bovine serum albumin, pH 7.0 (buffer E). The activation of phosphorylase kinase by calmodulin is expressed as the degree of activation [30] which is defined as $E=(n/n_o)-1$ where n is the measured activity with calmodulin and n_o the activity in the absence. The kinetic data was non-linearly fitted to a hyperbola according to [31] on a Commodore 64 computer [10]. Electrophoresis in the presence of SDS was performed on 15% polyacrylamide gels according to [32]. Unless otherwise stated, the sample buffer [32] contained 10 mM EGTA [12]. The molecular weight standards (ovalbumin 45 kDa, glyceraldehyde 3-phosphate dehydrogenase 36 kDa, carbonic anhydratase 29.2 kDa, trypsinogen 25 kDa, trypsin inhibitor 20.1 kDa, lactalbumin 14.2 kDa) were obtained from Sigma. Ca^{2+}-dependent mobility changes of calmodulin [33] during electrophoresis were performed as previously described [9]. For autoradiography the X-ray films were exposed for 24–96 h as required and developed as described [10–11]. Protein [34] was determined on an AutoAnalyzer II (Technicon) employing bovine serum albumin as standard.

3. RESULTS

Purity, apparent molecular mass and Ca^{2+}-dependent mobility change of yeast calmodulin are demonstrated in Fig. 1 in comparison to the mammalian protein. Yeast calmodulin runs with an apparent molecular mass of 14.9 kDa in the absence and 13.5 kDa in the presence of Ca^{2+} respectively. The mammalian counterpart (16.7 kDa) splits up into two bands (ca. 17.5, 18.8 kDa) as previously described [10] in the presence of Ca^{2+} and runs with a mass of 20.1 kDa in the absence of Ca^{2+}.

Yeast calmodulin does not activate phosphorylase kinase as is shown in Fig. 2. The concentration of bovine calmodulin (employed as control) for half-maximal activation (maximal activation 5-fold) is 49 nM as calculated from a non-linear hyperbolic fit of the data (line through data in Fig. 2). In contrast to the yeast

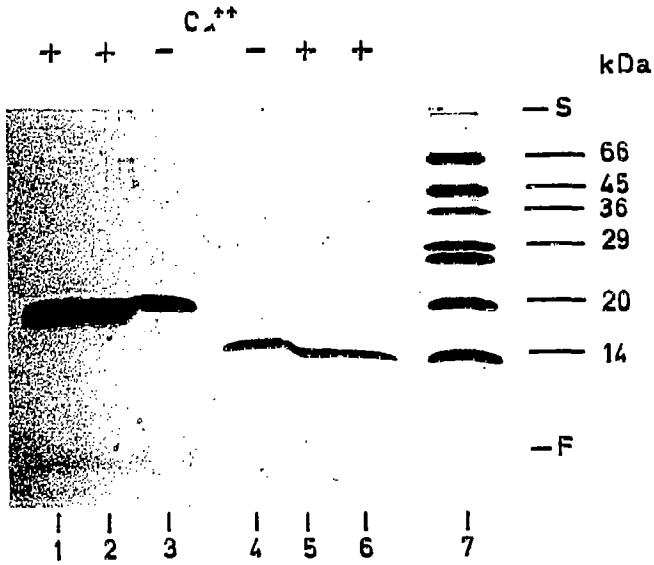

Fig. 1. Electrophoretic characterization and Ca^{2+}-dependent mobility change of calmodulin from yeast. SDS-PAGE was performed according to Laemmli [32] on 15% gels. 5 μg of the respective calmodulin and 10 μg of the standard protein mixture was applied in 60 μl of sample buffer after heating to 100°C. To generate the mobility change the sample buffer contained 800 μM $CaCl_2$ (+Ca^{2+}) or 10 mM EGTA (−Ca^{2+}) as described [9,33]. (Lanes 1–3) bovine testis calmodulin; (lanes 4–6) yeast calmodulin; (lane 7) standard proteins. For further details see [9,32,33], Methods and the text.

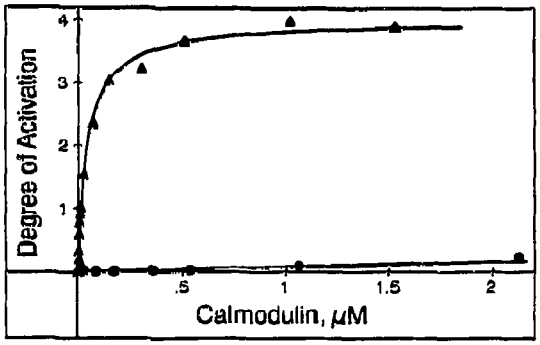

Fig. 2. Influence of yeast calmodulin on the activity of phosphorylase kinase. The activity of phosphorylase kinase was measured as described in Methods on an AutoAnalyzer. The degree of activation (E) [30] is described in Methods. Half-maximal activation of phosphorylase kinase with bovine testis calmodulin occurred at a calmodulin concentration of 49 nM. The maximal degree of activation was 4.0 corresponding to a 5-fold activation of phosphorylase kinase. For further details see Methods, the text and Table I.

modulator calmodulins from spinach and *N. crassa* can activate phosphorylase kinase 2–3-fold at 50 nM concentrations (Table I).

The Ca^{2+}-dependent formation of ubiquityl-calmodulin from yeast calmodulin by uCaM-synthetase from rabbit reticulocytes is shown in Fig. 3A. With control bovine testis calmodulin (Fig. 3, lane 1) shown first three conjugates are formed: first-order (bands Ia and Ib; ca. 27–29 kDa), second-order (II; ca. 35 kDa), and third-order (III; ca. 41 kDa) conjugates corresponding to one, two and three ubiquitin molecules per calmodulin (see [12]). In the absence of Ca^{2+} (Fig. 3A, lane 2) only very little calmodulin is ubiquitinated. In the control (lane 5) lacking exogenous bovine calmodulin faint ubiquitination bands corresponding to the first-order conjugate (Ia, Ib) can be detected originating from the endogenous calmodulin in the reticulocyte APF II [14, 35]. Yeast calmodulin (Fig. 3A, lane 3) also yields 3 ubiquitination bands which are, however, of lower apparent molecular mass: first-order ca. 24–26 kDa, second-order ca. 32 kDa and third-order ca. 40 kDa. The blur between the first- and second-order conjugates is due to the presence of first-order conjugate from the endogenous calmodulin (ca. 29 kDa) in reticulocyte APF II (see above). In the absence of Ca^{2+} (Fig. 3A, lane 4) a larger amount of yeast ubiquityl-calmodulin is formed than in the case of bovine calmodulin (lane 1) indicating a decreased Ca^{2+}-sensitivity of the ubiquitination reaction in the case of yeast calmodulin. This can be confirmed by the enzymatic test (see Table II below).

Ubiquityl-calmodulin conjugate formation with methylated ubiquitin (which cannot form polyubiquitin chains [18]) is shown in Fig. 3B. Ubiquitination of control testis calmodulin (Fig. 3B, lane 1) leads to a single band as has been shown previously [12,13]. An identical

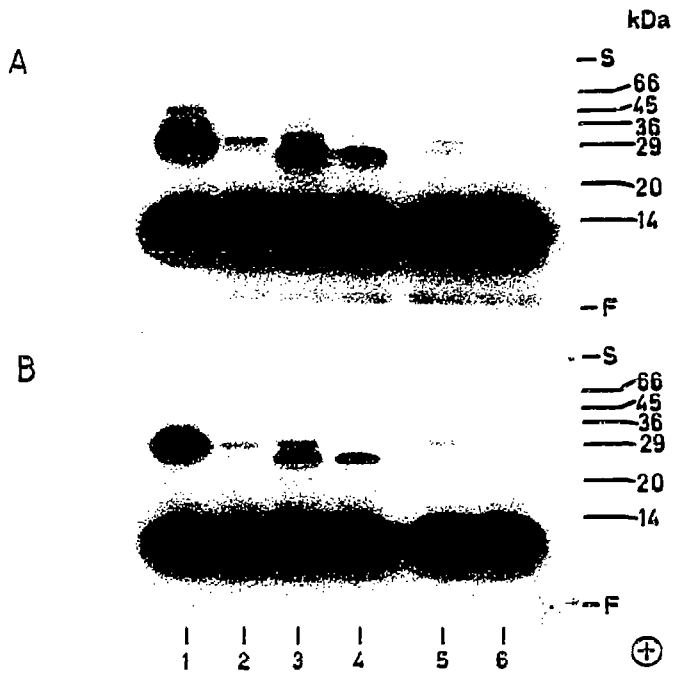

Fig. 3. Ca^{2+}-dependent ubiquitination of yeast and bovine calmodulin with reticulocyte uCaM-synthetase. The incubation mixtures (50 μl) contained 250 μg/ml calmodulin, 1.35 mg/ml [^{125}I]CT-ubiquitin or methylated [^{125}I]CT-ubiquitin-m (ca. 0.75×10^6 cpm/mixture) and 3.6 mg/ml reticulocyte APF II. Mixtures with calcium contained 1.1 mM Ca^{2+} + 1 mM EGTA, mixtures without calcium only contained 1 mM EGTA [10]. After incubation for 1 h the mixtures were boiled for 5 min, immediately placed on ice for 5 min and centrifuged. The supernatants (ca. 40 μl) were added to 60 μl sample buffer with 10 mM EGTA [13,32], heated to 100°C and concentrated to a total volume of ca. 70 μl for ca. 5 min. These samples were applied to 15% polyacrylamide gels [32]. For autoradiography the X-ray film was exposed for 24 h at −80°C and developed at room temperature [10,11]. The sample was applied to the cathodic top of the gel 'S'. Unconjugated [^{125}I]CT-ubiquitin (large dark band ca. 5.5 kDa [15]) runs above the buffer front 'F'. For further details see Methods and the legend to Fig. 1. The incubation mixtures from which the samples were derived contained:

(A)

(Lane 1) bovine testis CaM	+Ca^{2+}	[^{125}I]CT-ubiquitin;
(Lane 2) bovine testis CaM	+EGTA	[^{125}I]CT-ubiquitin;
(Lane 3) yeast CaM	+Ca^{2+}	[^{125}I]CT-ubiquitin;
(Lane 4) yeast CaM	+EGTA	[^{125}I]CT-ubiquitin;
(Lane 5) control without exogenous CaM	+Ca^{2+}	[^{125}I]CT-ubiquitin;
(Lane 6) control without exogenous CaM	+EGTA	[^{125}I]CT-ubiquitin.

(B)

(Lane 1) bovine testis CaM	+Ca^{2+}	[^{125}I]CT-ubiquitin-m;
(Lane 2) bovine testis CaM	+EGTA	[^{125}I]CT-ubiquitin-m;
(Lane 3) yeast CaM	+Ca^{2+}	[^{125}I]CT-ubiquitin-m;
(Lane 4) yeast CaM	+EGTA	[^{125}I]CT-ubiquitin-m;
(Lane 5) control without exogenous CaM	+Ca^{2+}	[^{125}I]CT-ubiquitin-m;
(Lane 6) control without exogenous CaM	+EGTA	[^{125}I]CT-ubiquitin-m.

result is found for the yeast calmodulin (Fig. 3B, lane 3). The additional band seen in lane 3 corresponds to the ubiquitination product of endogenous reticulocyte calmodulin (see control in Fig. 3B, lane 5). The

ubiquitination patterns in the absence of Ca^{2+}, again show the strong Ca^{2+}-dependence of ubiquitination in the case of bovine testis calmodulin and the reduced dependence for yeast calmodulin.

To quantitate the differences in the ubiquitination rates between the various calmodulins fluphenazine-agarose affinity based assays for uCaM-synthetase (i.e. FP-test [10]) were performed in Table II. A specific activity of 87 fkat/mg is obtained for the bovine control calmodulin and a somewhat higher activity for the plant calmodulin. For *N. crassa* a 30% lower and for yeast calmodulin a 50% lower specific activity are found respectively. In addition the $-Ca^{2+}/+Ca^{2+}$-ratio increases by 50% indicating a two-fold higher Ca^{2+}-independent activity of the enzyme with yeast calmodulin as substrate in comparison to the testis calmodulin.

The autoradiogram in Fig. 4 demonstrates the yeast uCaM-synthetase activity obtained from the DEAE Fractogel as described in Methods. Both the bovine control (lane 1) and yeast calmodulins are conjugated to ubiquitin in the presence of Ca^{2+} by the yeast enzyme. First-order (ca. 23 kDa) and second-order (ca. 28 kDa) conjugates are formed from yeast calmodulin. This also demonstrates clearly that the second band in Fig. 3A, lane 3 is the second-order conjugate of yeast calmodulin. In the case of testis calmodulin only a first-order conjugate (ca. 26 kDa) is seen under these conditions. Within the experimental error these conjugate molecular masses are in agreement with those found for the reticulocyte enzyme in Fig. 3A (see above). No conjugate bands indicative of endogenous yeast calmodulin were detected in mixtures to which no exogenous calmodulin was added (Fig. 4, lanes 5,6).

The data of the autoradiogram in Fig. 4 was quantitated with the FP-test in Table II. The specific activities of the yeast enzyme with yeast and bovine calmodulin are 7–10-fold lower than the reticulocyte enzyme. However the Ca^{2+}-dependence is very pronounced. The $-Ca^{2+}/+Ca^{2+}$ ratio increases from 0.06 for yeast to 0.15 for testis calmodulin indicating a reduced Ca^{2+}-sensitivity for mammalian calmodulin.

Table I

Activation of phosphorylase kinase by different calmodulins[a]

Calmodulin	Degree of activation
Bovine testis	3.4
Spinach	1.6
Neurospora crassa	2.2
*Saccharomyces cerevisiae**	0*

[a] The activation of phosphorylase kinase by calmodulin was measured in an automated assay [29]. Maximal activation of phosphorylase kinase was 5.7-fold. The degrees of activation (see Methods) are calculated for a concentration of 50 nM calmodulin from activation curves measured between 3–55 nM concentrations of calmodulin. The value for yeast* was taken from Fig. 2. For further details see Methods, Fig. 2 and the text.

4. DISCUSSION

Although the absolute molecular mass of yeast calmodulin (16.1 kDa, [1]) is only slightly smaller than the mammalian protein (16.7 kDa) it runs with a ca. 4–5 kDa smaller apparent molecular mass on SDS-PAGE. This is in agreement with the observations of Davis et al. [1].

Apparently the 60% sequence difference [1] between yeast and bovine testis calmodulin is so large that phosphorylase kinase cannot be activated by the yeast modulator (see Fig. 2). This is very interesting since a 2–3-fold activation by spinach and *N. crassa* calmodulin is still possible (see Table I). As the C-terminal fragment of calmodulin, 74–148, can fully activate phosphorylase kinase [36], it can be speculated that it is the variance of this portion of yeast calmodulin which leads to a loss of biological activity.

In general the conjugation of yeast calmodulin by reticulocyte uCaM-synthetase is very similar to testis calmodulin (Fig. 1A) and thus also to plant and fungus calmodulins [12,13]. From the generation of only one ubiquitination band from yeast calmodulin with methylated [^{125}I]ubiquitin (Fig. 1B) it can be concluded that as with all previous calmodulins [12,13] there is only a single lysine present which can be conjugated to ubiquitin. From the fact that the most highly conserved part of yeast calmodulin [1] lies in the N-terminal Ca^{2+}-domain (see above) it can be speculated that the putative lysine residue involved in ubiquitination resides in this portion of the molecule.

The ubiquitination of calmodulin is difficult to detect unequivocally in crude yeast extracts. However as shown here the activity of this enzyme as well as the Ca^{2+}-dependence can be clearly demonstrated in yeast APF II (see Fig. 4). The Ca^{2+}-dependent conjugation of calmodulin with ubiquitin, first detected in rabbit reti-

Table II

uCaM-synthetase activity in reticulocyte and yeast APF II as tested with various calmodulins[a]

Calmodulin	Reticulocyte uCaM-synthetase		Yeast uCaM-synthetase	
	Specific activity (fkat/mg)	$-Ca^{2+}/+Ca^{2+}$ ratio	Specific activity (fkat/mg)	$-Ca^{2+}/+Ca^{2+}$ ratio
Bovine testis	87	0.11	6.0	0.15
Neurospora crassa	58	0.15	ND	ND
Saccharomyces cerevisiae	41	0.21	6.5	0.06

[a] UCaM-Synthetase was quantitated with the FP-test [10]. The activity was corrected for activity with endogenous calmodulin. The final protein concentration of reticulocyte APF II was 0.9 mg/ml and that of yeast APF II was 8.5 mg/ml. The preparation of reticulocyte and yeast APF II is described in Methods. For other differences in the FP-test between the reticulocyte and yeast enzyme assay see Methods. For further details see Methods, Figs. 3 and 4, and the text.

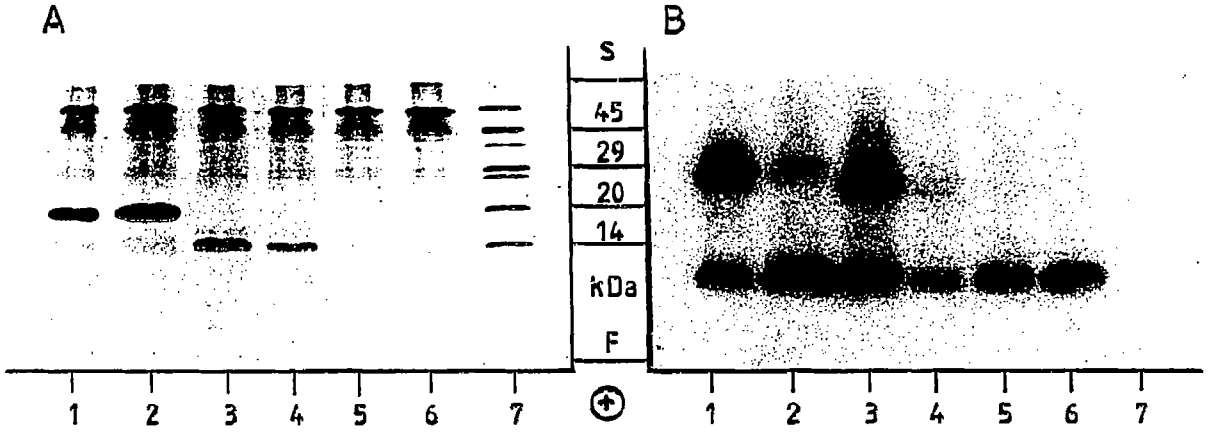

Fig. 4. Ca^{2+}-dependent ubiquitination of yeast and bovine with yeast uCaM-synthetase. The incubation of the yeast enzyme with calmodulin and [^{125}I]CT-ubiquitin (1.4×10^6 cpm/mixture) was performed as described in the legend to Fig. 3 except that the final protein concentration of yeast APF II was 8.5 mg/ml. To increase the sensitivity of the autoradiography the supernatants obtained after the boiling step were adsorbed to FP-sepharose (1 ml packed gel/mg calmodulin) as described for the FP-test. The EGTA eluates of FP-Sepharose were precipitated with 5% trichloroacetic acid and the pellets were solubilized in 80 μl sample buffer (see legend to Fig. 3) with 5 μl 2 M Tris which was concentrated to 60–70 μl by heating at 100°C and applied to the electrophoresis gel [12]. For autoradiography the X-ray film was exposed for 13 days at −80°C. The protein band at 66 kDa on the Coomassie blue-stained gel (A) is BSA from the coating procedure (see Methods), the band at ca. 20 kDa (lanes 1,2) is testis calmodulin and the band at ca. 14 kDa (lanes 3,4) is yeast calmodulin. As shown in Fig. 3 unconjugated [^{125}I]CT-ubiquitin (5.5 kDa) runs above the buffer front 'F'. A small fraction of this molecule (band below 14 kDa in Lanes 1–6) appears to be non-specifically adsorbed and eluted under the given conditions (see also [11]). For further details see Methods, legend to Fig. 3 and the text.
(A) Coomassie blue-stained gel. (B) Autoradiogram.

(Lane 1) bovine testis CaM +Ca^{2+} [^{125}I]CT-ubiquitin;
(Lane 2) bovine testis CaM +EGTA [^{125}I]CT-ubiquitin;
(Lane 3) yeast CaM +Ca^{2+} [^{125}I]CT-ubiquitin;
(Lane 4) yeast CaM +EGTA [^{125}I]CT-ubiquitin;
(Lane 5) control without exogenous CaM +Ca^{2+} [^{125}I]CT-ubiquitin;
(Lane 6) control without exogenous CaM +EGTA [^{125}I]CT-ubiquitin.

culocytes [9], can now be followed all the way back to yeast. The conservation of calmodulin ubiquitination in contrast to the non-conserved activation of phosphorylase kinase (Table I) is an indication for the evolutionary age and importance of this reaction.

The degree of Ca^{2+}-dependence appears to show a species specificity (see $-Ca^{2+}/+Ca^{2+}$ ratios, Table II), where each calmodulin in the heterologous enzyme system shows a reduced Ca^{2+}-dependence. However, a final conclusion on this aspect can only be gained through experiments with the purified enzyme. Whether the ubiquitination of calmodulin in yeast is associated with a degradation of the modulator by a ubiquitin-dependent proteolytic system remains to be shown. This is still an open question in the mammalian system also [13]. One may speculate, however, that the conjugation of ubiquitin with calmodulin, which has been so highly conserved, is of prime biological importance and might be associated with a linked evolution of these two proteins in eukaryotes. Another indication for such a linkage is the location of the calmodulin locus 2.0 kbp upstream of the ubiquitin fusion gene in *Trypanosoma cruzi* [37].

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