Plant and fungus calmodulins are polyubiquitinated at a single site in a Ca^{2+}-dependent manner

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In plants Ca^{2+} plays a crucial role as second messenger. Thus calmodulin is one of the most important signal transducing molecules for metabolic regulation in plants. Previously we showed that bovine testis calmodulin can be covalently coupled at one site to ubiquitin in a Ca^{2+}-dependent manner in the presence of ATP/Mg^{2+} by ubiquityl-calmodulin synthetase. Since calmodulin from spinach has 13 amino acid sequence differences to bovine calmodulin – two of them in Ca^{2+}-binding loops – it was unclear, whether a conjugation of ubiquitin to this molecule would be possible. In this paper it is shown that calmodulin from spinach and a similar calmodulin from the mold *Neurospora crassa* can be covalently conjugated to ubiquitin in a Ca^{2+}-dependent manner. It is shown that higher molecular mass conjugates containing up to three ubiquitin molecules per calmodulin are obtained. Experiments with methylated ubiquitin demonstrate that, as with vertebrate calmodulins, only one lysine residue is linked to ubiquitin and that the incorporation of addditional ubiquitin molecules leads to a polyubiquitin chain.

Plant calmodulin; Ubiquitin; Calmodulin-ubiquitin conjugate; Protein ubiquitination; ATP-dependent proteolysis; Trimethyllysine

1. INTRODUCTION

Calmodulin ($M_r = 16.9$ kDa) is an essential signal transducing molecule for the second messenger Ca^{2+} in enkaryotes (for review see [1]). Recently [2–4] we first demonstrated that mammalian calmodulin can be covalently coupled to ubiquitin in the presence of Ca^{2+} and ATP/Mg^{2+} by ubiquityl-calmodulin synthetase (uCaM-synthetase) [5,6] from various tissues. This functionally links another important eucaryotic signalling protein, namely ubiquitin (an 8.5 kDa heat stable polypeptide cofactor of ATP-dependent proteolysis; for reviews see [7,8]), to the modification and possibly to the metabolism of calmodulin. At present calmodulin is the only protein where conjugation to ubiquitin is regulated by a second messenger (Ca^{2+}) [2,3]. Multiple ubiquitination [3] at a single site [4] appears characteristic. Only the free form of calmodulin is substrate for uCaM-synthetase as was demonstrated by the inhibitory action of phophorylase kinase [3]. UCaM-synthetase, for which a specific affinity-based assay has been described [3], has been determined at significant levels of ca 5 fkat/mg in nearly all mammalian tissues tested so far [6], indicating a lack of tissue specificity for the enzyme.

Similar to vertebrate calmodulins, plant calmodulin contains the rare amino acid trimethyllysine at residue 115 and has a blocked amino terminus [9–11]. Since equimolar amounts of trimethyllysine have been detected in the monoubiquitination product of bovine testis calmodulin [4] a direct regulatory function of this rare amino acid could be excluded (see also [2]). Fungus calmodulin from *N. crassa* (for which the sequence has not been reported), is similar to plant calmodulin but does not contain trimethyllysine [12]. On the other hand calmodulin from spinach [9] has 13 amino acid sequence differences to bovine calmodulin.

In plant cells Ca^{2+} plays a major role as a second messenger whereas such a function could not be established for cAMP (for review see [13]). Thus calmodulin, the mediator of many calcium effects, and calmodulin metabolism are crucial to the viability of plant cells.

We therefore addressed ourselves to the question, if calmodulin from spinach [9], which contains such large sequence differences to bovine calmodulin, can also be modified with ubiquitin in a manner similar to the bovine modulator protein. We found, that up to three ubiquitin molecules (u_3CaM) can be incorporated as a single polyubiquitin chain into spinach as well as into *Neurospora crassa* calmodulin.

2. MATERIALS AND METHODS

Reticulocyte APF II (ATP-dependent proteolysis fraction II) was prepared as described in [14,15]. Ubiquitin was purchased from Sigma (Munich). Ubiquitin was methylated according to [16]. The 8 primary amino groups per mol ubiquitin were reduced to 0.23 groups/mol [4]. [^{125}I]CT-ubiquitin ($1–2 \times 10^8$ cpm/mg) and methylated [^{125}I]CT-ubiquitin ([^{125}I]CT-ubiquitin-m) were synthesized according to the chloramine-T procedure [14,17]. Bovine testis

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calmodulin was isolated according to [18] and purified further by affinity chromatography according to [19]. Bovine brain calmodulin [10,11], spinach calmodulin (*Spinacia oleracea*) [9] and *Neurospora crassa* calmodulin [12] were obtained from Sigma (Taufkirchen, FRG). The amino acid composition of the commercial calmodulins was checked (OPA Method [4,18]) and found to be in agreement with the literature values [9,12] (M. Laub, this laboratory). The biological activity of calmodulins was tested [20] with phosphorylase kinase in the AutoAnalyzer test [21,22]. The concentration of calmodulin necessary for half maximal activation of phophorylase kinase (maximal activation 6–7-fold) was 30–70 nM. Electrophoresis in the presence of SDS was performed on 15% polyacrylamide gels according to [23]. For autoradiography the X-ray film was exposed for 24 h and developed as described [3,5]. Protein [24] was determined on an AutoAnalyzer II (Technicon) employing bovine serum albumin as standard.

3. RESULTS AND DISCUSSION

The Ca^{2+}-dependent formation of ubiquityl-calmodulin from plant and *N. crassa* calmodulins by uCaM-synthetase in rabbit reticulocyte APF II [2,3,5] is shown in Fig. 1. In the control experiment (Fig. 1A, lane 2) with bovine testis calmodulin three conjugates (25–45 kDa) are formed by uCaM-synthetase between calmodulin and [^{125}I]CT-ubiquitin (see legend to Fig. 1). The first-order (I, ca 29 kDa), second-order (II, ca 35 kDa), and third-order (III, ca 41 kDa) conjugates correspond to one, two and three ubiquitin molecules per calmodulin (see [3]). The amount of conjugate formed is so high (ca 4% of total calmodulin), that the novel protein bands can be detected by eye on Coomassie blue stained gels (Fig. 1B, lanes 2, 3, 5, 7). In control lane 1 ($-Ca^{2+}$) of Fig. 1B fainter protein bands of similar molecular mass as the conjugates can also be detected. Affinity purification on fluphenazine-Sepharose [3] however demonstrates that these contaminating bands do not correspond to 'cold' endogenous ubiquitin-calmodulin conjugates (not shown). As can be derived from Fig. 1, a nearly identical conjugate band pattern is found for the control testis calmodulin (lane 2) and for the calmodulins from bovine brain (lane 3), *N. crassa* (lane 5) and spinach (lane 7). In Fig. 1B it can be seen that the bovine calmodulins run with a higher molecular mass (ca 19 kDa) than the plant and fungus calmodulins (ca 17 kDa, see [12]). The strong Ca^{2+}-dependence of conjugation of plant calmodulin is of special interest since two of the amino acids exchange as compared to bovine calmodulin occurring in Ca^{2+}-binding sites (site I, Cys-26/Thr-26; site III, Gln-96/Gly-96) [9].

Ubiquityl-calmodulin conjugate formation with methylated ubiquitin (which cannot form polyubiquitin chains [16]) is shown in Fig. 2. As seen with control testis calmodulin (lane 1) the typical threefold band pat-

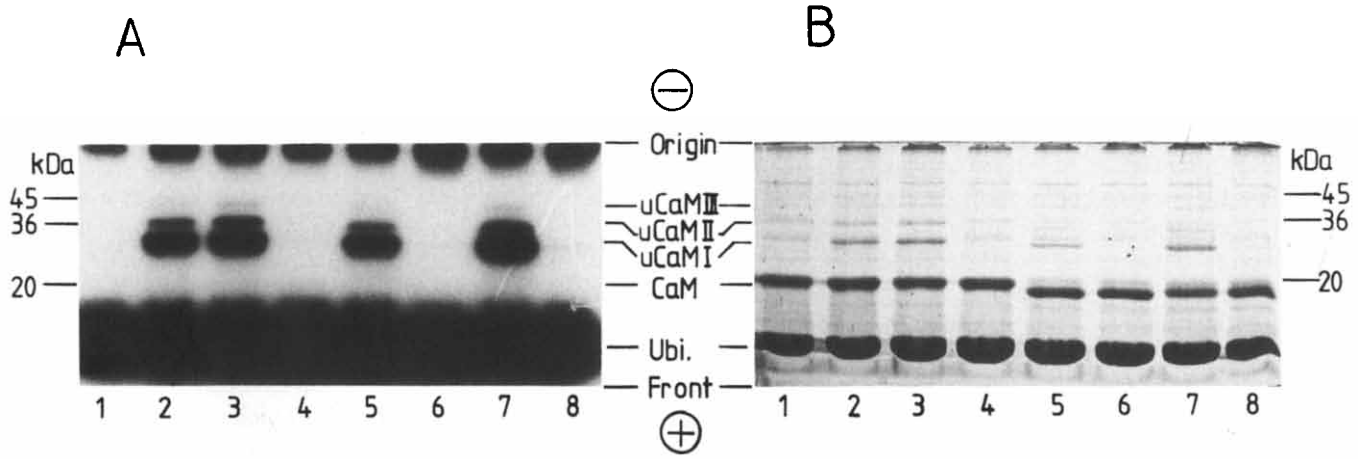

Fig. 1. Ca^{2+}-dependent ubiquitination of spinach and *Neurospora crassa* calmodulins. The incubation mixtures [3,5] contained 50 mM Tris-HCl, 1 mM DTE, 5 mM $MgCl_2$, 1 mM ATP, 10 mM phosphocreatine, 0.1 mg/ml creatine kinase, 250 μg/ml calmodulin (15 μM) 1.35 mg/ml [^{125}I]CT-ubiquitin (159 μM, specific radioactivity 64 cpm/pmol), 3.6 mg/ml DEAE fraction II. All mixtures (0.1 ml) contained 1.0×10^6 cpm (i.e. identical specific radioactivities). Mixtures with calcium contained 1.1 mM $CaCl_2$ + 1 mM EGTA, mixtures without calcium only contained 1 mM EGTA. After incubation for 1 hour the mixtures were boiled for 5 min, centrifuged and the supernatants precipitated with 5% trichloroacetic acid. The pellets were resuspended in 50 μl sample buffer [24] which contained 10 mM EGTA [3] and to which 40 μl 1% SDS had been added and analyzed on 15% polyacrylamide gels according to [24]. The autoradiogram (A) and the Coomassie blue stain (B) of the gel are shown. For autoradiography the X-ray film was exposed for 24 h and developed at room temperature [3,5]. Unconjugated [^{125}I]CT-ubiquitin runs at the buffer front. For further details and the molecular mass standards employed (not shown) see section 2, [3] and the text. The incubation mixtures from which the samples were derived contained:

Lane 1: bovine testis calmodulin + EGTA
Lane 2: bovine testis calmodulin + Ca^{2+}
Lane 3: bovine brain calmodulin + Ca^{2+}
Lane 4: bovine brain calmodulin + EGTA
Lane 5: *Neurospora crassa* calmodulin + Ca^{2+}
Lane 6: *Neurospora crassa* calmodulin + EGTA
Lane 7: Spinach calmodulin + Ca^{2+}
Lane 8: Spinach calmodulin + EGTA

tern is obtained with non-methylated [^{125}I]CT-ubiquitin. In addition, very high molecular mass polyubiquitin conjugates remain at the origin of the gel. The first-order conjugate band is a doublet (not shown, see [3]). A similar finding (doublet) has been made for the first-order conjugate of histone uH2A [25]. With methylated [^{125}I]CT-ubiquitin-m [4,16] only one major band (ca 29 kDa) is formed with calmodulins from bovine testis (lane 2), bovine brain (lane 3), *N. crassa* (lane 4) and spinach (lane 5). However above the first-order conjugate a minor band (ca 1–2% of major band) corresponding to a second-order conjugate can be detected. This appears due to the fact that the primary amino groups of ubiquitin cannot be methylated to 100% [4,16]. A minor band can be expected from the fact that 0.23 mol primary amino groups/mol ubiquitin (i.e. ca one mol amino group/5 mol ubiquitin) remain intact after methylation (see legend to Fig. 2 and [4]). Assuming a statistical distribution of this amino group among the 8 primary amino groups of ubiquitin one second-order conjugate should be found for every 40–50 conjugates (i.e. 2–2.5%). These experiments demonstrate that the three incorporated ubiquitin molecules are linked to a single lysine residue in the form of a polyubiquitin chain.

The fluphenazine-agarose affinity based assay for uCaM-synthetase (i.e. FP-test, [3]) was employed to quantitate differences in the ubiquitiation rates of the various calmodulins. As can be seen in Table I, Ca^{2+}-dependent specific activities of 117–131 fkat/mg are obtained for the bovine calmodulins. This compares to 107 fkat/mg and 70 fkat/mg for the spinach and *N. crassa* calmodulins respectively. From the ratio + Ca^{2+} / − Ca^{2+} it can be seen that Ca^{2+}-independent activity is ca twofold higher for the spinach and *N. crassa* calmodulins. This may indicate some functional difference between vertebrate and non-vertebrate calmodulins.

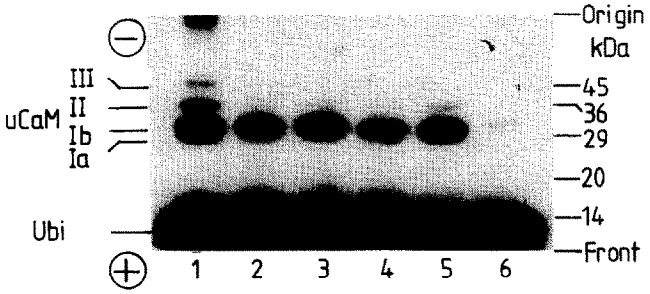

Fig. 2. Inhibition of higher-order spinach and *N. crassa* ubiquityl-calmodulin formation by methylated ubiquitin. The incubation mixtures contained 250 μg/ml calmodulin (15 μM), 250 μg/ml [^{125}I]CT-ubiquitin or methylated [^{125}I]CT-ubiquitin-m (159 μM, specific radioactivity 48 cpm/pmol; 0.75×10^6 cpm/mixture) and 3.6 mg/ml reticulocyte APF II in the presense of calcium. For further details see section 2 and the legend to Fig. 1. The incubation mixtures from which the samples were derived contained:

Lane 1: bovine testis calmodulin + [^{125}I]CT-ubiquitin
Lane 2: bovine testis calmodulin + [^{125}I]CT-ubiquitin-m (methylated)
Lane 3: bovine brain calmodulin + [^{125}I]CT-ubiquitin-m (methylated)
Lane 4: *Neurospora crassa* calmodulin + [^{125}I]CT-ubiquitin-m (methylated)
Lane 5: spinach calmodulin + [^{125}I]CT-ubiquitin-m (methylated)
Lane 6: control without calmodulin + [^{125}I]CT-ubiquitin-m (methylated)

Table I

UCaM-synthetase activity as measured in the presence of calcium by the standard FP-test [3][a]

Calmodulin	UCaM-synthetase Specific activity (fkat/mg)	(+Ca^{2+}/ −Ca^{2+} ratio)
Bovine testis	131	6.5
Bovine brain	117	6.3
Spinach	107	3.1
Neurospora crassa	71	3.9

[a]UCaM-Synthetase was quantitated with the FP-test [3]. The incubation mixture contained 50 mM Tris-HCl, 1 mM DTE, 5 mM $MgCl_2$, 1 mM ATP, 10 mM phasphocreatine, 0.1 mg/ml creatine kinase, 500 μg/ml calmodulin (30 μM), 50 μg/ml [^{125}I]CT-ubiquitin (5.9 μM, specific radioactivity 425 cpm/pmol), 0.9 mg/ml DEAE fraction II [14,15]. The mixtures with calcium contained 1.1 mM Ca^{2+} and 1 mM EGTA. The mixtures without calcium only contained 1 mM EGTA.

All of the data presented here indicate that the ubiquitination of mammalian and plant/fungus calmodulins occur in a very similar manner. Since a ubiquitin-dependent proteolytic system similar to that found in rabbit reticulocytes has been reported for higher plants [26] it seems from the lack of tissue specificity in rabbit [6] and species specificity (as shown here) that the conjugation of calmodulin with ubiquitin and, what remains to be shown, the corresponding enzyme are very old on an evolutionary basis, possibly as old as these highly conserved proteins themselves.

Previously we have suggested a Ca^{2+}-dependent ('irreversible') ubiquitination model for the targeting of calmodulin [5] towards proteolytic degradation. The finding of a polyubiquitin chain linked to plant and *N. crassa* calmodulins lends support to this interpretation for plant and fungus cells also, since this type of ubiquitin structure enhances the proteolytic degradation of ubiquitinated proteins [16]. On the other hand it may also be that calmodulin is targeted to some other protein or to a specific site (e.g. lysosome in mammalian systems [27]) in the cell. A very different possibility is a 'reversible' modification of calmodulin with ubiquitin (see also [2,4]) similar to phosphorylation-dephosphorylation cycles. A possible indication for such a mechanism are the maximum curves obtained for calmodulin ubiquitination in crude tissue extracts [6]. Strong evidence for such a model has been provided for mammalian histone H2A, where it was shown that the ubiquitin moiety has a higher turnover rate than the histone core (for review see [28]). Finally our finding of a Ca^{2+} regulated ubiquitination of plant calmodulin stresses the importance of ubiquitin-dependent reac-

tions in plants, a fact which is underlined by the interesting work on phytochrome ubiquitin conjugates in oat seedlings [29].

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