Multiple ubiquitination of calmodulin results in one polyubiquitin chain linked to calmodulin

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Received 16 July 1990; revised version received 6 August 1990

In the presence of Ca^{2+} and ATP/Mg^{2+} mammalian calmodulin can be covalently coupled to ubiquitin by ubiquityl calmodulin synthetase (uCaM-synthetase). Three ubiquitin derivatives ^{125}I-CT-ubiquitin (prepared by the chloramine-T method), ^{125}I-BH-ubiquitin (prepared by the Bolton-Hunter method) and methylated forms of ubiquitin were tested with native calmodulin. Alternatively native ubiquitin was tested with the Bolton-Hunter derivative of calmodulin (^{125}I-BH-calmodulin). Up to three molecules of ubiquitin can be incorporated into one molecule of calmodulin. Since both native forms of ubiquitin and calmodulin are good substrates of uCaM-synthetase, ubiquitination is not a result of an altered conformation (i.e. denaturation) of either protein. With ^{125}I-BH-calmodulin it is demonstrated that calmodulin is also present in the higher molecular weight ubiquitin conjugates. If methylated ubiquitin is employed as substrate for uCaM-synthetase only one conjugate corresponding to the mono-ubiquitination product of calmodulin is formed. This demonstrates that only a single lysine residue in calmodulin is conjugated to ubiquitin. All other higher molecular weight ubiquitin-calmodulin conjugates must therefore be composed of one molecule of calmodulin to which an oligo- or poly-ubiquitin chain is linked. Since it can be shown that the mono-ubiquitination product of calmodulin still contains ca. 1 mol trimethyllysine/mol calmodulin, the polyubiquitin chain is not linked to lysine 115 of calmodulin. In addition a demethylation of trimethyllysine 115 by enzymes in reticulocyte lysate or the DEAE-enriched enzyme fraction with subsequent ubiquitination at this site of calmodulin can also be excluded.

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

1. INTRODUCTION

The heat stable regulatory polypeptide ubiquitin ($m = 8.5$ kDa), which consists of 76 amino acids is a cofactor of ATP-dependent proteolysis in reticulocytes (for a review see [1]). In this reaction sequence ubiquitin is covalently attached '(ubiquitination') via an isopeptide linkage to the protein to be degraded [1]. Ubiquitin cannot only be coupled to various proteins for subsequent degradation but can also be conjugated with itself forming oligo- or poly-ubiquitin chains [2]. In our hands this cofactor role for proteolysis could not be shown for any other tissue outside reticulocytes [3].

Recently we showed that mammalian calmodulin-containing trimethyllysine 115 is covalently coupled to ubiquitin in a Ca^{2+}-dependent manner in the presence of ATP/Mg^{2+} by ubiquityl calmodulin synthetase (uCaM-synthetase) [4-6]. This conjugation reaction can be inhibited by phosphorylase kinase [5]. The enzyme uCaM-synthetase [6] for which a specific affinity-based assay has been described [5] has been detected at significant levels of ca. 5 fkat/mg in cardiac muscle [6] and nearly all mammalian tissues tested so far [3], indicating a lack of tissue specificity. Up to three molecules of ubiquitin could be incorporated into one molecule of calmodulin [5]. However, up to now it could not be differentiated whether the three molecules of ubiquitin are incorporated into calmodulin at three different sites, i.e. lysine residues or as a poly-ubiquitin chain at a single site. In this paper it will be shown that the latter possibility has been realized in nature.

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2. MATERIALS AND METHODS

2.1. *Preparative methods*

2.1.1. Reticulocytes, reticulocyte lysate, APF II

The preparation of reticulocytes, reticulocyte lysate and reticulocyte APF II (ATP-dependent proteolysis fraction II) is described in [7,8].

2.1.2. Ubiquitin

Ubiquitin was purchased from Sigma (Munich). No difference in biological activity to our ubiquitin prepared from pig blood according to [7] was detected as tested with APF II [7]. The ubiquitin concentration necessary for half-maximal activation. (7-10-fold) was ca. 1.5-2.0 μM.

^{125}I-CT-ubiquitin and ^{125}I-CT-ubiquitin-m ($5-6 \times 10^8$ cpm/mg) were synthesized with native and methylated ubiquitin (see below), respectively, according to the chloramine-T procedure [7,9]. ^{125}I-BH-ubiquitin (3.2×10^8 cpm/mg) was prepared according to a modified Bolton-Hunter procedure [10,11]. The incubation mixture contained 2 μg ubiquitin with a two-fold molar excess of ^{125}I-Bolton-Hunter reagent (Amersham). After derivatization of ubiquitin with ^{125}I-

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Bolton-Hunter reagent 1 mg of cold-labelled BH-ubiquitin was added and instead of the gel filtration step the ^{125}I-BH-ubiquitin was precipitated with 5% trichloroacetic acid. The pellet obtained by centrifugation was twice resuspended and washed with 5% trichloroacetic acid. The final pellet was solubilized by addition of 500 μl urea (0.5 mg/ml) and 10 μl 2 M Tris. The pH was adjusted to pH 7–8 with HCl. The solubilized ^{125}I-BH-ubiquitin was dialyzed against 5 l 20 mM Tris/HCl (pH 7.4) overnight to yield the final product. The labelled ubiquitin contained ca. 0.6 mol Bolton-Hunter reagent/mol.

Native ubiquitin was methylated (ubiquitin-m) according to [2]. The degree of methylation was determined according to [12] (see also section 3). Poly-Asp (5–15 kDa) was obtained from Sigma (Taufkirchen, FRG).

Calmodulin. Bovine testis calmodulin was isolated according to [13] and purified further by affinity chromatography according to [14]. The biological activity of calmodulin was tested [15] with phosphorylase kinase in the AutoAnalyzer test [16,17]. The concentration of calmodulin necessary for half maximal activation of phosphorylase kinase (maximal activation 6–7-fold) was 30–60 nM; the corresponding value for BH-calmodulin was ca. 120 nM (J. Kaiser and H.P. Jennissen, unpublished results).

^{125}I-BH-calmodulin (4.5×10^8 cpm/mg) was labelled with ^{125}I according to a modified Bolton-Hunter procedure [10,11]. The major change in the procedure was that the gel filtration buffer did not contain gelatine (to reduce non-specific adsorption) but 1 mg/sample cold-labelled BH-calmodulin. After modification and gel filtration the labelled protein was precipitated with 5% trichloroacetic acid and taken up in 100 μl 1 M NaOH, 2 ml 10 mM sodium β-glycerophosphate, 0.1 mM $CaCl_2$. After adjustment of the pH to 7–8 with 1 M HCl the protein was dialyzed against 5 l 10 mM sodium β-glycerophosphate, 0.1 mM Ca^{2+} (pH 7.0). Under the above conditions BH-calmodulin contained ca. 0.9 mol Bolton-Hunter reagent/mol.

2.1.3. Preparative isolation of ubiquityl-calmodulin conjugate

120 ubiquitination mixtures (0.1 ml) containing 1 mg/ml calmodulin, 6.4 mg/ml reticulocyte lysate, 0.17 mg/ml ^{125}I-CT-ubiquitin (40 000 cpm/μg) were prepared according to [5] and purified on fluphenazine Sepharose [5]. The EGTA eluates were precipitated with 5% trichloroacetic acid taken up in 100 μl sample buffer [18] containing 10 mM EGTA and were separated electrophoretically on 12.5% polyacrylamide gels in the presence of SDS [18]. The unstained and unfixed gels (ca. 112 cm^2) were blotted on nitrocellulose membranes (0.2 μm pore diameter, Schleicher and Schüll, Dassel, FRG) in a semi-dry procedure (SatoroBlott, Sartorius, Göttingen, FRG) for 2 h in 50 mM sodium borate buffer, 20% methanol, pH 9.0, at constant voltage of 4.5 V and 0.5 mA/cm^2, with the polyacrylamide gel and the nitrocellulose sandwiched between three layers of Whatman No. 3 filter paper (Whatman, Maidstone, UK) on each side. The nitrocellulose membranes were stained with amido black (0.1% w/v amido black, 25% v/v methanol, 10% v/v acetic acid) and were destained with 25% v/v methanol, 10% v/v acetic acid for 10 min. The radioactive conjugate bands (i.e. mono-ubiquitination product) were determined autoradiographically [3,4,6] (also see below) and were then cut out. The conjugate containing membrane pieces were first washed with blotting buffer (50 mM sodium borate buffer, 20% methanol, pH 9.0) on a Büchner funnel and then incubated in a volume of 0.25 ml/cm^2 membrane for 30 min of 100% trifluoroacetic acid (TFA). The TFA eluates were concentrated and dried in an exicator. The dried samples (slightly brown color) were taken up in water and the protein was precipitated with 5% trichloroacetic acid yielding the final product (ca. 560 pmol conjugate). In a similar fashion 60 control ubiquitination mixtures (see above) were run in the absence of calmodulin under identical electrophoresis and blotting conditions. Portions of the nitrocellulose membranes corresponding to the molecular mass range of 26–30 kDa were cut out and the proteins were eluted with TFA and dried as described above.

2.2. *Analytical methods*

The enzyme *uCaM-Synthetase* was quantitated with the standard FP-test [5]. All other information is given in the text or figure legends.

In general the specific activity of uCaM-synthetase in APF II was 110–120 fkat/mg.

Incubation mixtures for analytical *conjugate formation* with ubiquitin and calmodulin derivatives were essentially prepared according to [5]. All other information is given in the text or figure legends.

Electrophoresis on polyacrylamide gels (15% gels) in the presence of SDS was performed according to [18]. Autoradiographic analysis of electrophoresis gels in the presence of enhancer (film exposure 40 h at −80°C, processing at room temperature) was performed as described in [4,5]. All other information is given in the text or figure legends.

Protein [19] was determined on an AutoAnalyzer II (Technicon) employing bovine serum albumin as standard.

For *amino acid analysis* the isolated mono-ubiquitination product of calmodulin and contaminating control proteins (see above) were hydrolyzed in vacuum for 72 h in 6 N HCl, 0.1% w/v phenol at 110°C. The hydrolysate was dried in an exicator and taken up in 0.5–1.5 ml 16 mM Na_2HPO_4/TFA (pH 6.95). Amino acid analysis according to the orthophthaldialdehyde (OPA) method was performed on a Spherisorb ODS II column (3 μm beads, column length 12.5 cm, 4.6 mm i.d., Grom, Herrenburg, FRG) as described by [13]. The column was calibrated with external amino acid standards (calibration kit, Sigma, Taufkirchen, FRG). N^6-trimethyllysine was obtained from Calbiochem (Frankfurt am Main, FRG). In the described system trimethyllysine eluted between Ala and Arg.

3. RESULTS AND DISCUSSION

3.1. *Methylation of ubiquitin*

In order to block the free primary amino groups of ubiquitin and thus inhibit a selfpolymerization reaction the protein was methylated with formaldehyde and subsequently reduced [2]. As seen in Table I the molar ratio of primary amino groups/mol ubiquitin (N-terminal Met, 7 Lys) was reduced from 8.1 to 0.23, indicating that one primary amino group per five molecules of ubiquitin remained intact. A similar residual value (0.24) was obtained with the control polypeptide poly-Asp (Table I). Attempts to obtain higher methylation ratios by increasing the formaldehyde concentration were unsuccessful (not shown).

3.2. *Formation of ubiquityl-calmodulin from derivatives of ubiquitin and calmodulin*

In Fig. 1 (lane 6) three conjugates are formed by uCaM-synthetase (APF II) between calmodulin and the

Table I

Methylation of ubiquitin

	Amino groups	
	titrated values mol/mol	expected values mol/mol
Ubiquitin	8.1	8.0
Ubiquitin-m (methylated)	0.23	0
Poly-Asp	1.0	1.0
Poly-Asp-m (methylated)	0.24	0

The primary amino groups were titrated according to [12]. The number of titratable groups for ubiquitin were derived from the amino acid sequence [21]. For further details see section 2 and the text

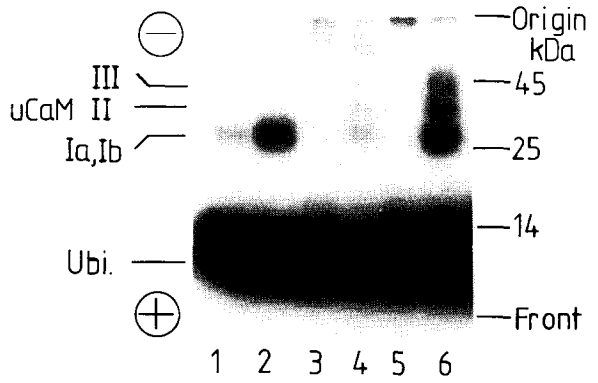

Fig. 1. Ca^{2+}-dependent ubiquitination of bovine testis calmodulin with three different ^{125}I-ubiquitin derivatives. The incubation mixtures contained 50 mM Tris/HCl, 1 mM DTE, 5 mM $MgCl_2$, 1 mM ATP, 10 mM phosphocreatine, 0.1 mg/ml creatine kinase, 250 μg/ml calmodulin (15 μM), 1.35 mg/ml ^{125}I-CT-ubiquitin or ^{125}I-CT-ubiquitin-m or ^{125}I-BH-ubiquitin, 3.6 mg/ml APF II. All mixtures (0.1 ml) contained 6.6×10^5 cpm (i.e. identical specific radioactivities). Mixtures with calcium contained 1 mM $CaCl_2$, mixtures without calcium 1 mM EGTA. After incubation for 2 h the mixtures were boiled for 5 min, centrifuged and the supernatants precipitated with 5% trichloroacetic acid. The pellets were resuspended in 80 μl sample buffer [18] which contained 10 mM EGTA [4] and analyzed on 15% polyacrylamide gels according to [18]. For autoradiography the X-ray film was exposed for 24 h and developed at room temperature [5]. Unconjugated ^{125}I-ubiquitin runs at the buffer front. For further details and the molecular mass standards employed (not shown) see section 2 and the text. The incubation mixtures from which the samples were derived contained 250 μg/ml calmodulin and: lane 1: 1.35 mg/ml ^{125}I-CT-ubiquitin-m (methylated + EGTA; lane 2: 1.35 mg/ml ^{125}I-CT-ubiquitin-m (methylated) + Ca^{2+}; lane 3: 1.35 mg/ml ^{125}I-BH-ubiquitin + Ca^{2+}; lane 4: 1.35 m/ml ^{125}I-BH-ubiquitin + EGTA; lane 5: 1.35 mg/ml ^{125}I-CT-ubiquitin + EGTA; lane 6: 1.35 mg/ml ^{125}I-CT-ubiquitin + Ca^{2+}.

chloramine-T derivative ^{125}I-CT-ubiquitin: the first-order (Ia, Ib; 27–30 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 [5]). Because of overexposure Ia and Ib cannot be differentiated on the autoradiogram [5].

Lane 4 in Fig. 1 shows that the incorporation of the Bolton-Hunter derivative ^{125}I-BH-ubiquitin (of identical specific radioactivity as ^{125}I-CT-ubiquitin) into calmodulin again leads to the same three conjugates. From lanes 4 and 6 (Fig. 1) it can be estimated that the amount of ^{125}I-BH-ubiquitin incorporated into calmodulin is 4–5-fold lower than that of ^{25}I-CT-ubiquitin. This might be due to different affinities of the two derivatives for uCaM-synthetase. The detection of the higher order conjugates (II and III) in the ^{125}I-BH-ubiquitin conjugates (lane 4) shows that the lysine in ubiquitin necessary for selfconjugation is not blocked by the Bolton-Hunter reagent.

However, if ^{125}I-CT-ubiquitin-m is substituted for the non-methylated species (compare to lane 6) only a single band (see lane 2) corresponding to the mono-ubiquitination product of calmodulin ($M_r \approx 28\,000$) is formed. This demonstrates that only one lysine on calmodulin is modified with ubiquitin. On longer exposures a minor radioactive band (maximally 1–2% of the main band) corresponding to a second-order conjugate is sometimes observed (not shown). This observation can be explained on the basis of the residual primary amino groups of ubiquitin (see Table II) which should allow some degree of selfpolymerization. In all cases practically no conjugates are formed in the absence of Ca^{2+} (lanes 1,3,5, + EGTA).

In the experiments of Fig. 1 derivatives of ubiquitin were conjugated to native calmodulin. The calcium dependence of this reaction indicates the biological significance for intact cellular systems. However, it could be hypothesized that the derivatization of ubiquitin leads to an altered conformation of ubiquitin allowing the artifactual formation of the conjugates with calmodulin. Therefore calmodulin was labelled by the Bolton-Hunter method (^{125}I-BH-calmodulin) so that native ubiquitin could be employed in the conjugation mixture. Since the Bolton-Hunter reagent also leads to a modification of lysine residues in calmodulin

Table II

Amino acid analysis of ubiquityl-calmodulin prepared by preparative blotting on nitrocellulose followed by TFA elution

Amino acid	Calmodulin		^{125}I-ubiquityl-calmodulin	
	I mol/mol	II composition from sequence [22]	III mol/mol	IV composition from sequences [21,22]
Asx	22.1	23	28.3	30
Glx	26.9	27	38.8	39
Serine	3.7	4	8.8	7
Glycine	12.9	11	26.4	17
Threonine	11.0	12	14.3	19
Histidine	1.0	1	3.9	2
Alanine	11.0	11	17.2	13
Tm-Lysine	0.8	1	0.7	1
Arginine	5.8	6	9.8	10
Tyrosine	0	2	0.57	3
Methionine	7.1	9	5.6	10
Valine	7.1	7	14.1	11
Phenylalanine	7.2	8	10.2	10
Isoleucine	8.4	8	12.4	15
Leucine	9.1	9	19.0	18
Lysine	7.0	7	9.0	14
Proline	ND	2	ND	5
Cysteine	ND	0	ND	0
Tryptophan	ND	0	ND	0

In column I (calmodulin) the data correspond to mean values calculated from hydrolyses at 24, 48 and 72 h. The data in column III (conjugate) were obtained after 72 h of hydrolysis; the amino acids of the contaminating proteins have been subtracted. The amino acid composition of ubiquitin can be calculated from the difference between column IV and column II. For further details see section 2, Fig. 3 and the text. ND, not determined: Asx, aspargine and aspartic acid; Glx, glutamine and glutamic acid; Tm-Lysine, trimethyllysine

the lysine which is substrate of uCaM-synthetase could be modified. That this is not the case is seen in Fig. 2 (lanes 1,3,5) at different ubiquitin concentrations. Three conjugates are formed with ^{125}I-BH-calmodulin as with native calmodulin (compare Fig. 1) excluding the above hypothesis. The Bolton-Hunter labelling of calmodulin neither significantly decreases its ability to be conjugated with ubiquitin, nor the Ca^{2+}-dependence of this reaction (not shown). The labelling of calmodulin also unequivocally proves that the conjugates detected with labelled ubiquitin (compare Fig. 1) contain calmodulin (Fig. 2, lanes 1,3,5). The experiments with unlabelled methylated ubiquitin (Fig. 2, lanes 2,4,6) again demonstrate under these different conditions that there is only one conjugation site on calmodulin.

3.3. *Blotting and amino acid composition (Tm-lys) of uCaM*

A major question remaining is to which single lysine residue of calmodulin ubiquitin is conjugated and whether lysine 115 plays a role. In order to answer the latter question the mono-ubiquitination product of calmodulin was prepared in preparative amounts by electrophoresis and blotting (see section 2). The acid hydrolysates were subjected to amino acid analysis on a reverse phase column employing the precolumn OPA derivatization method [13]. Since other proteins of 25–30 kDa seem to contaminate the mono-ubiquitination product of calmodulin the amino acid profile found (Fig. 3A) was at variance with the expected composition of the conjugate based on a molar ratio of 1:1 ubiquitin/calmodulin. In order to correct for these contaminating proteins control preparations without added calmodulin were run (see section 2) in equivalent amounts. The amino acid profile of this control preparation is shown in Fig. 3B. A subtraction of these amino acids from the uncorrected conjugate preparation (see Fig. 3A) lead to the composition shown in Fig. 3C, and Table II. Since trimethyllysine is a rare amino acid it can be expected to be a good marker for differentiation of calmodulin in the conjugate (Fig. 3A) from contaminating proteins. This was indeed the case since no trimethyllysine could be detected in the contaminating proteins (see Fig. 3B). Quantification of the data of Fig. 3C in Table II demonstrates that 0.7 mol trimethyllysine per mol ubiquityl calmodulin conjugate is detected. The yield of trimethyllysine is reduced through the elution step with trifluoroacetic acid. This is also seen for control blotted and TFA-treated purified calmodulin (Table II) in which only 0.8 mol trimethyllysine/mol is detected. Other amino acids which are partially or fully destroyed by TFA are tyrosine and methionine. The high amount of the amino acid glycine in the analysis is due to contamination with glycine buffer of the electrophoresis system. In spite of these corrections only ca. 50% of the amino acids including trimethyllysine are in agreement with the expected composition indicating additional contaminating proteins not corrected for by the controls. Thus the major result is the finding of trimethyllysine in a molar ratio of ca. 1 mol/mol conjugate (Table II).

Further evidence for the correctness of the analysis is obtained from the following calculation. Based on the specific radioactivity of the isolated first-order ubiquityl-calmodulin conjugate 137 pmol conjugate was hydrolyzed and applied to the column (see Fig. 3). The amount of trimethyllysine detected by the OPA method corresponded to a nearly equimolar amount of 110 pmol. Since no trimethyllysine is found in the controls (Fig. 3B) this quantitative result is in excellent agreement with the molar ratio obtained from the total amino acid composition (see above and Table II). The detection of molar amounts of trimethyllysine in the conjugate conclusively demonstrates that this amino acid is not involved in the ubiquitination reaction and in addition does not protect calmodulin from ubiquitination as was supposed [20].

In agreement with the results of this paper previous work has strongly indicated that the ubiquitination of

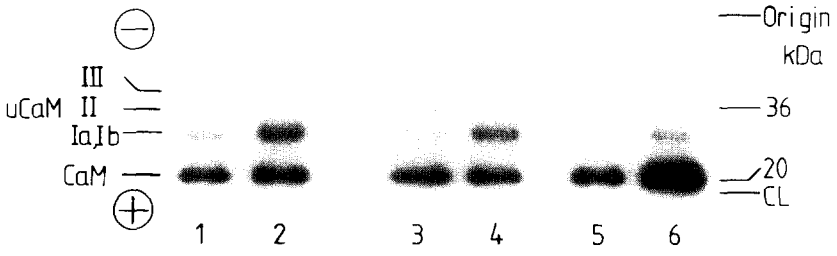

Fig. 2. Ubiquitination of bovine testis ^{125}I-BH-calmodulin with native and methylated non-radioactive ubiquitin. The incubation mixtures contained 50 mM Tris/HCl, 1 mM DTE, 5 mM $MgCl_2$, 1 mM ATP, 0.1 mM $CaCl_2$, 10 mM phosphocreatine, 0.1 mg/ml creatine kinase, 105 μg/ml ^{125}I-BH-calmodulin (6.3 μM), 11–100 μg/ml ubiquitin (1.3–11.8 μM), 6.3 mg/ml reticulocyte lysate. All mixtures (0.1 ml) contained 4.5×10^5 cpm. After incubation for 1 h 30 μl of the mixtures were added to 50 μl sample buffer [18] which contained 10 mM EGTA [4] and analyzed on 15% polyacrylamide gels according to [18]. For further details see legend to Fig. 1, section 2 and the text. The incubation mixtures from which the samples were derived contained 105 μg/ml ^{125}-BH-calmodulin and: lane 1: 150 μg/ml ubiquitin; lane 2: 150 μg/ml ubiquitin-m (methylated); lane 3: 50 μg/ml ubiquitin; lane 4: 50 μg/ml ubiquitin-m (methylated); lane 5: 11 μg/ml ubiquitin; lane 6: 11 μg/ml ubiquitin-m (methylated).

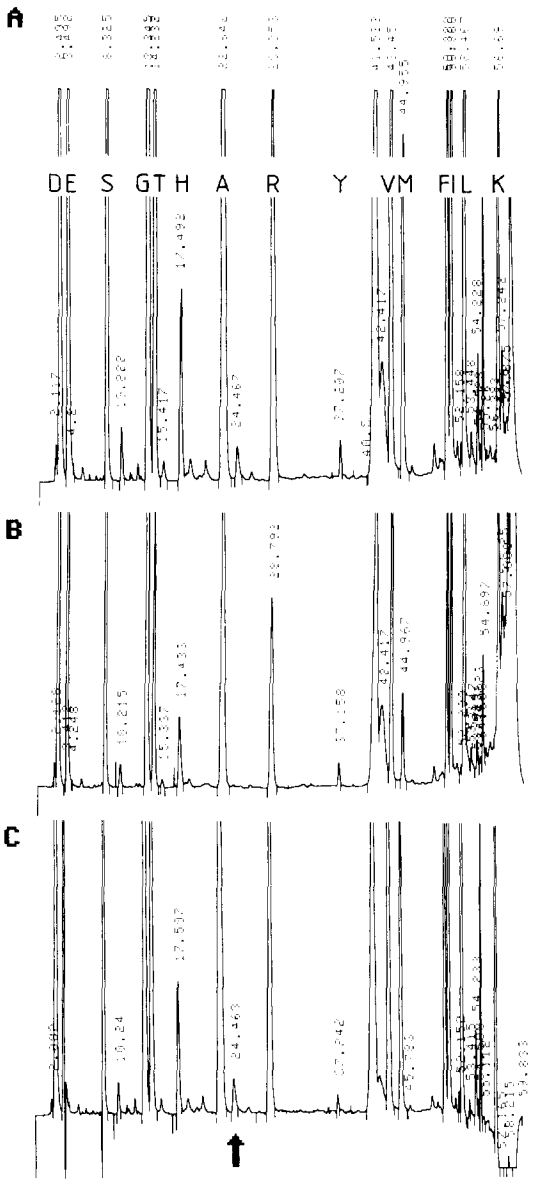

Fig. 3. Demonstration of trimethyllysine in ubiquityl-calmodulin (mono-ubiquitination product). Ubiquityl-calmodulin conjugate (ca. 140 pmol/100 μl) prepared as described in section 2 was subjected to amino acid analysis on a reverse phase column by the OPA method. The chromatograms (optical density vs time, min) are depicted to demonstrate trimethyllysine (see arrow). The recordings were made with a Shimadzu fluorescence detector (350 nm/450 nm) (Shimadzu, Düsseldorf) linked to a Chromatopac C-R3A integrator/computer fitted with a printer and a diskette drive for storage and retrieval of data. For further details see section 2, the text and Table II. (A) Uncorrected analysis of preparatively isolated ubiquityl-calmodulin conjugate. (B) Analysis of contaminating proteins prepared from control runs. (C) Corrected analysis of conjugate (difference between A and B).

mammalian calmodulin is of biological importance, since the specificity and uniqueness of important regulatory properties of the system exclude an artifactual behavior with high probability: (a) the conjugation reaction depends on the presence of micromolar concentrations of Ca^{2+} [4], (b) only the free form of calmodulin can be conjugated, the bound form being protected (e.g. in phosphorylase kinase) [5], (c) all rabbit tissues studied so far contain uCaM-synthetase [3], (d) addition of ATP/Mg^{2+} and ^{125}I-ubiquitin to crude tissue extracts containing endogenous calmodulin leads to the formation of one major conjugate, namely uCaM [3]. Whether the main function of calmodulin ubiquitination is to target calmodulin towards proteolysis [6], a function which is supported by the results of this paper (poly-ubiquitin chain linked to specific lysine; see also [2]), or whether ubiquityl-calmodulin itself possesses intrinsic biological activity has to be resolved by further work.

Acknowledgments: We thank Mrs G. Botzet and Mrs A. Wensing for excellent technical help. This work was supported by a grant (Je 84/7-2) from the Deutsche Forschungsgemeinschaft and the Fonds der Chemie.

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