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Calcium Affects the Spontaneous Degradation of Aspartyl/Asparaginyl Residues in Calmodulin†

Irene M. Ota and Steven Clarke*

Department of Chemistry and Biochemistry and the Molecular Biology Institute, University of California, Los Angeles, California 90024

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ABSTRACT: We have previously shown that the D-aspartyl/L-isoaspartyl protein carboxyl methyltransferase recognizes two major sites in affinity-purified preparations of bovine brain calmodulin that arise from spontaneous degradation reactions. These sites are derived from aspartyl residues at positions 2 and 78, which are located in apparently flexible regions of calmodulin. We postulated that this flexibility was an important factor in the nonenzymatic formation and enzymatic recognition of D-aspartyl and/or L-isoaspartyl residues. Because removal of Ca^{2+} ions from this protein may also lead to increased flexibility in the four Ca^{2+} binding regions, we have now characterized the sites of methylation that occur when calmodulin is incubated in buffers with or without the calcium chelator ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA). Calmodulin was treated at pH 7.4 for 13 days at 37 °C under these conditions and was then methylated with erythrocyte D-aspartyl/L-isoaspartyl methyltransferase isozyme I and *S*-adenosyl-L-[*methyl*-^{3}H]methionine. The ^{3}H-methylated calmodulin product was purified by reverse-phase HPLC and digested with various proteases including trypsin, chymotrypsin, endoproteinase Lys-C, clostripain, and *Staphylococcus aureus* V8 protease, and the resulting peptides were separated by reverse-phase HPLC. Peptides containing Asp-2 and Asp-78, as well as calcium binding sites II, III, and IV, were found to be associated with radiolabel under these conditions. When calmodulin was incubated under the same conditions in the presence of calcium, methylation at residues in the Ca^{2+} binding regions was not observed. These results suggest that there may be a correlation between the flexibility of polypeptide segments and the potential of their aspartyl and asparaginyl residues to undergo degradation via succinimide-linked reactions.

Protein carboxyl methyltransferase type II catalyzes the transfer of methyl groups from *S*-adenosylmethionine into ester linkages in peptides and proteins at altered aspartyl residues, including D-aspartate and L-isoaspartate. These residues appear to originate from the spontaneous decomposition of proteins via succinimide intermediates, and this methylation reaction may play a role in the repair or degradation of these damaged proteins (McFadden & Clarke, 1982; Clarke, 1985; Johnson et al., 1987; Galletti et al., 1988; O'Connor & Yutzey, 1988). To understand the factors involved in the formation of L-isoaspartyl and D-aspartyl residues, we mapped the sites of methylation in bovine brain calmodulin, an M_r 17 000 protein that mediates the effects of Ca^{2+} on cellular metabolism [for reviews, see Manalan and Klee (1984) and Wang et al. (1985)]. We found that L-isoaspartyl residues originating from aspartyl residues at the 2- and 78-positions were major sites of methylation in affinity-purified calmodulin (Ota & Clarke, 1989a). These residues are located in two apparently flexible regions of the protein; one near the N-terminus (Babu et al., 1988) and one at a seven-turn α-helical region that connects the two globular domains (Babu et al., 1988; Bayley et al., 1988; Persechini & Kretsinger, 1988). We suggested that the segmental flexibility of the polypeptide chain might allow succinimide formation.

Recently, it was found that incubation of calmodulin under physiological conditions of temperature and pH in the absence of calcium resulted in an increase in its methyl-accepting capacity (Johnson et al., 1987, 1989). We have now mapped the locations of methylation sites after calmodulin was incubated under similar conditions in the presence or absence of calcium. Incubation under either condition results in an increase in the methyl acceptor capacity of sites originating from Asp-2 and Asp-78 relative to unincubated material. Significantly, incubation in the absence of calcium produces new

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* Address correspondence to this author at the Department of Chemistry and Biochemistry, University of California.

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sites of methylation that appear to be associated with calcium binding sites II, III, and IV. These results suggest that the removal of calcium from the calcium binding sites might induce enough flexibility in these regions to permit the formation of methylatable L-isoaspartyl and D-aspartyl residues.

Experimental Procedures

Preparation of Calmodulin Incubated in the Presence of Calcium or EGTA. Calmodulin [Sigma, bovine brain, purified by affinity chromatography (Gopalakrishna & Anderson, 1982)] at 150 μM was incubated in the absence of calcium in 50 mM potassium *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (K-HEPES), 2 mM ethylene glycol bis-(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA),[1] pH 7.4, and 0.2 mg/mL sodium azide in a final volume of 20 μL in a 0.5-mL polypropylene microfuge tube for 13 days at 37 °C. Calmodulin was incubated in the presence of calcium under the same conditions except that 1 mM calcium chloride was substituted for 2 mM EGTA.

Methylation and Purification of Calmodulin. D-Aspartyl/L-isoaspartyl protein methyltransferase was used to incorporate radiolabeled methyl groups into the calmodulin preparations described above. Calcium chloride was added to the EGTA-preincubated calmodulin, and EGTA was added to the calcium-preincubated calmodulin in order to equalize EGTA and calcium concentrations in these samples. Specifically, 5 μL of 50 mM K-HEPES and 8 mM EGTA, pH 7.4, or 5 μL of 50 mM K-HEPES and 4 mM calcium chloride, pH 7.4, were added to 20 μL of the calcium-preincubated and EGTA-preincubated samples, respectively. To enzymatically methylate these samples, 12 μL of partially purified human erythrocyte protein D-aspartyl/L-isoaspartyl carboxyl methyltransferase isozyme I [prepared by ammonium sulfate precipitation and DEAE-cellulose chromatography and concentrated by Amicon ultrafiltration to an activity of approximately 15 pmol of methyl groups transferred to saturating ovalbumin per minute per microliter of enzyme at 37 °C (Ota et al., 1988)] and 8 μL of *S*-adenosyl-L-[*methyl*-^{3}H]methionine [Amersham Corp., 15 Ci/mmol, 66.7 μM in dilute H_2SO_4 (pH 2.5–3.5)/ethanol, 9:1 (v/v)] were added, and the mixture was incubated for 1 h at 37 °C. Unpreincubated calmodulin was methylated similarly in HEPES/calcium chloride buffer with EGTA added as above. The methylated calmodulin samples were purified by reverse-phase HPLC as described previously (Ota & Clarke, 1989a).

Digestion of ^{3}H-Methylated Calmodulin with Proteases and Separation of the Fragments by Reverse-Phase HPLC. A portion of the radiolabeled ^{3}H-methylated calmodulin was combined with approximately 50 μg of unlabeled calmodulin and digested with either trypsin, endoproteinase Lys-C, clostripain, chymotrypsin, or *Staphylococcus aureus* V8 protease as described previously (Ota & Clarke, 1989a). The fragments were separated by reverse-phase HPLC and identified by amino acid analysis (Ota et al., 1987).

Redigestion of Large Fragments. Cyanogen bromide cleavage of the endoproteinase Lys-C generated fragment from ^{3}H-methylated calmodulin, 1–94, was done as described previously (Ota & Clarke, 1989a). *Staphylococcus aureus* V8 protease digestion of the endoproteinase Lys-C generated fragment, 95–148, from EGTA-preincubated ^{3}H-methylated calmodulin was done as follows. HPLC fractions containing radiolabeled fragment 95–148 were lyophilized and resuspended in a total volume of 21 μL of 0.2 M sodium citrate, pH 6.0, and digested with 5 μL, 0.37 unit, of *Staphylococcus aureus* V8 protease (Sigma, 4.5 units/mg of solid) for 40 min at 37 °C. A similar procedure was used to digest the chymotrypsin-generated fragment, 110–138. Fractions 43–45 from an HPLC separation of a chymotrypsin digest of EGTA-preincubated, ^{3}H-methylated calmodulin were lyophilized and resuspended in a total of 20 μL of 0.2 M sodium citrate, pH 6.0, and digested with 2.5 μL, 0.19 unit, of *S. aureus* V8 protease for 20 min at 37 °C. The fragments were separated by reverse-phase HPLC. The reverse-phase column (Vydac, C_4, 300-Å pore, 0.46 × 25 cm) was equilibrated in buffer A, 0.1% TFA (trifluoroacetic acid), and the fragments eluted at a flow rate of 1 mL/min with a gradient of buffer B (90% acetonitrile, 9.9% H_2O, and 0.1% trifluoroacetic acid) that increased at 0.5%/min.

Trypsin Digestion of Fragment 1–36 from Calcium-Preincubated and EGTA-Preincubated Calmodulin Samples. Fragment 1–36 was isolated from cyanogen bromide cleavage of fragment 1–94 which was generated by endoproteinase Lys-C digestion of calcium-preincubated and EGTA-preincubated ^{3}H-methylated calmodulin samples. HPLC fractions 47–49 from the cyanogen bromide cleavages of both the EGTA-preincubated and calcium-preincubated calmodulin fragment 1–94 were lyophilized, and each sample was resuspended in 0.2 M sodium citrate, pH 6.0, in a total volume of 20 μL; 2.4 μL of 1 mg/mL trypsin [Sigma, bovine pancreas, treated with diphenylcarbamyl chloride, 7500–9000 N^{α}-benzoyl-L-arginine ethyl ester (BAEE) units/mg of protein] in 1 mM hydrochloric acid and 2.6 μL of 5 mg/mL calcium chloride were added, and the peptides were digested for 20 min at 37 °C. The fragments were applied to a C_4 reverse-phase HPLC column that was equilibrated at 20% buffer B (as above) and 80% buffer A (as above) and the peptides eluted isocratically.

Results

Calcium Binding Sites Become Methyl Acceptors after Extended Preincubation of Calmodulin in the Presence of EGTA. Bovine brain calmodulin was incubated at pH 7.4 at 37 °C for 13 days in the presence of 2 mM EGTA or 1 mM calcium chloride [cf. Johnson et al. (1987, 1989)]. To assess the formation of methylatable D-aspartyl and/or L-isoaspartyl residues, this preparation was incubated with protein carboxyl methyltransferase and *S*-adenosyl-L-[*methyl*-^{3}H]methionine, and the reaction products were purified by C_4 reverse-phase high-performance liquid chromatography as described previously (Ota & Clarke, 1989a). The methylatability of calmodulin increased significantly during the 13-day incubation in the presence and absence of calcium. Incorporation of radiolabel into the EGTA-incubated calmodulin was approximately 5-fold greater compared to nonincubated calmodulin. There was a 4.5-fold increase in the methyl label incorporated into the calcium-incubated samples.

The methylation sites of the calcium-incubated and EGTA-incubated calmodulin samples were compared with those of unincubated calmodulin by identifying radioactivity in specific proteolytic fragments of ^{3}H-methylated calmodulin (Figure 1). Calmodulin samples were digested with trypsin, chymotrypsin, clostripain, endoproteinase Lys-C, and *Staphylococcus aureus* V8 protease and the resulting peptides separated by reverse-phase HPLC. A 10-fold excess of unmethylated bovine calmodulin was added prior to digestion to aid in the identification of the individual peptides. In this approach, the use of multiple proteolytic digestions minimizes

[1] Abbreviations: [^{3}H]AdoMet, *S*-adenosyl-L-[*methyl*-^{3}H]methionine; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; TFA, trifluoroacetic acid; HPLC, high-performance liquid chromatography.

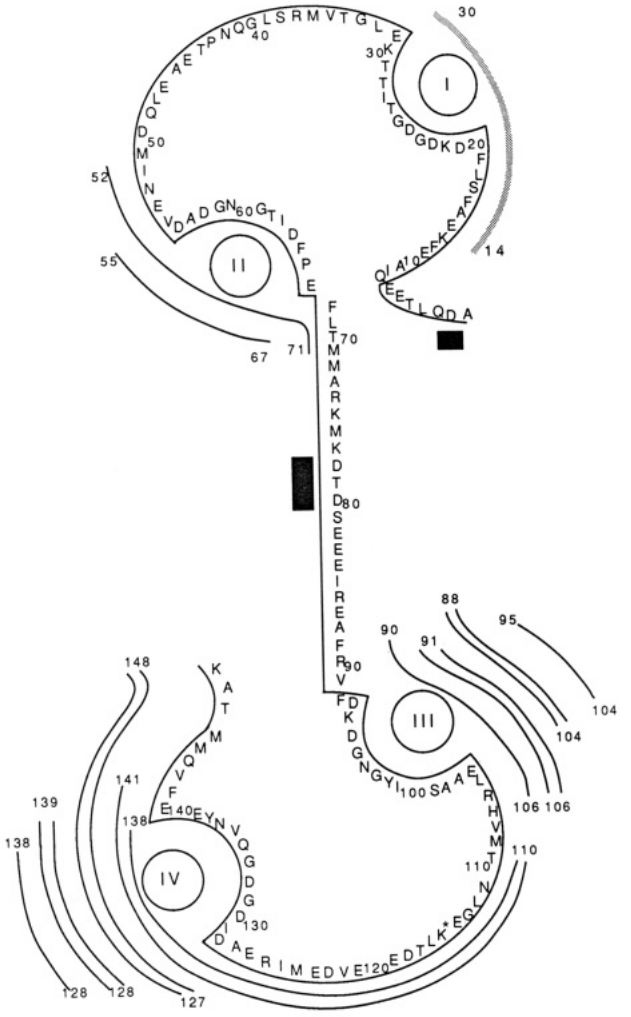

FIGURE 1: Sequence of bovine brain calmodulin indicating calcium binding sites I–IV. Dark boxes indicate major methylation sites in affinity-purified calmodulin (Ota & Clarke, 1989a). Peptide fragments shown as single lines are associated with methyl esters in EGTA-preincubated calmodulin, while fragment 14–30 shown as a hatched bar is not associated with radiolabel (see text).

problems in the identification of the D-aspartyl and L-iso-aspartyl methyl ester derivatives of the calmodulin peptide fragments. In general, the methylated derivatives of the larger fragments coelute on HPLC with the unmodified peptides; for smaller peptides, the methylated species usually elute 1–3 min later than the unmodified species (Ota et al., 1987; Ota & Clarke, 1989a).

In this study, we found that the distribution of [^{3}H]methyl radioactivity in the peptides derived from calmodulin incubated in calcium was similar to that seen with native preparations (Figures 2–6); these results suggest that spontaneous isomerization reactions at Asp-2 and Asp-78 can account for the bulk of methylation sites in liganded calmodulin. On the other hand, the radiolabel profiles of the EGTA-incubated samples were quite different (Figures 2–6). These results indicate that in the absence of calcium new sites become methyl acceptors. The identification of these new sites is described below.

Calcium Binding Site II ($D_{56}AD_{58}GN_{60}GTID_{64}FPE$) Becomes a Methyl Acceptor after Incubation with EGTA. Digestion of ^{3}H-methylated calmodulin samples with endoproteinase Lys-C produces radiolabeled fragment 1–94, which contains calcium binding sites I and II, as well as other aspartyl and asparaginyl residues (Figures 1 and 2). Cyanogen bromide cleavage of the unincubated calmodulin fragment 1–94 shows that the methyl acceptor sites are present in

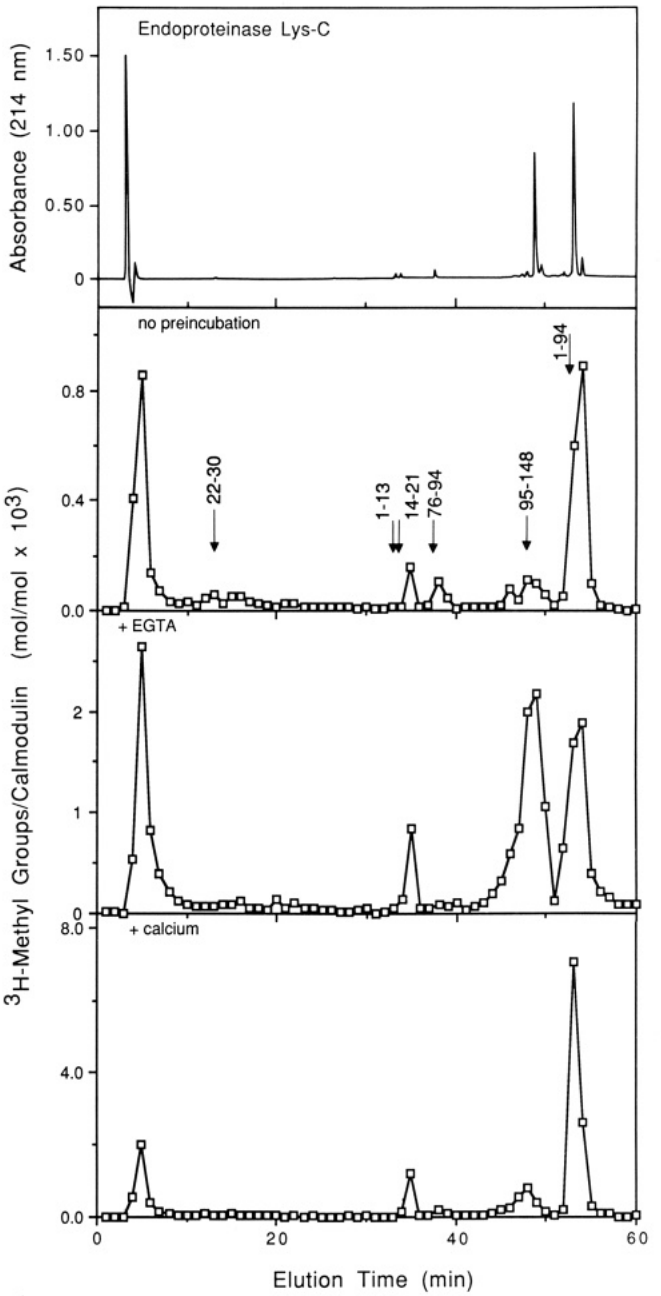

FIGURE 2: Endoproteinase Lys-C digestions of ^{3}H-methylated calmodulin samples. Calmodulin was incubated in the presence of EGTA or in the presence of calcium for 13 days at pH 7.4 at 37 °C. The calcium and EGTA concentrations were then normalized, and these samples, as well as unincubated affinity-purified calmodulin, were methylated with protein carboxyl methyltransferase and [^{3}H]AdoMet for 1 h at 37 °C. The methylated calmodulin molecules were purified by reverse-phase HPLC and digested with endoproteinase Lys-C, and the resulting fragments were separated by reverse-phase HPLC and identified by amino acid analysis as described previously (Ota & Clarke, 1989a). The peptides were detected by their absorbance at 214 nm, and the radioactivity was determined by counting a portion of each fraction in fluor. The elution positions of the peptides are designated by arrows. The large peak of radiolabel at 4–7 min was not volatile either before or after base treatment and may be [^{3}H]-AdoMet which was not fully removed when ^{3}H-methylated calmodulin was purified from [^{3}H]AdoMet by HPLC (Ota & Clarke, 1989a). The absorbance profile is shown for the calcium preincubated sample; similar profiles were obtained for the other samples.

fragments 1–36 and 77–94 (Figure 3); these peptides were demonstrated previously to contain the highly methylated Asp-2 and Asp-78 sites (Ota & Clarke, 1989a). Cleavage of the calcium-incubated sample shows that methylation is limited to these fragments as well but is enhanced 8-fold and 3.3-fold

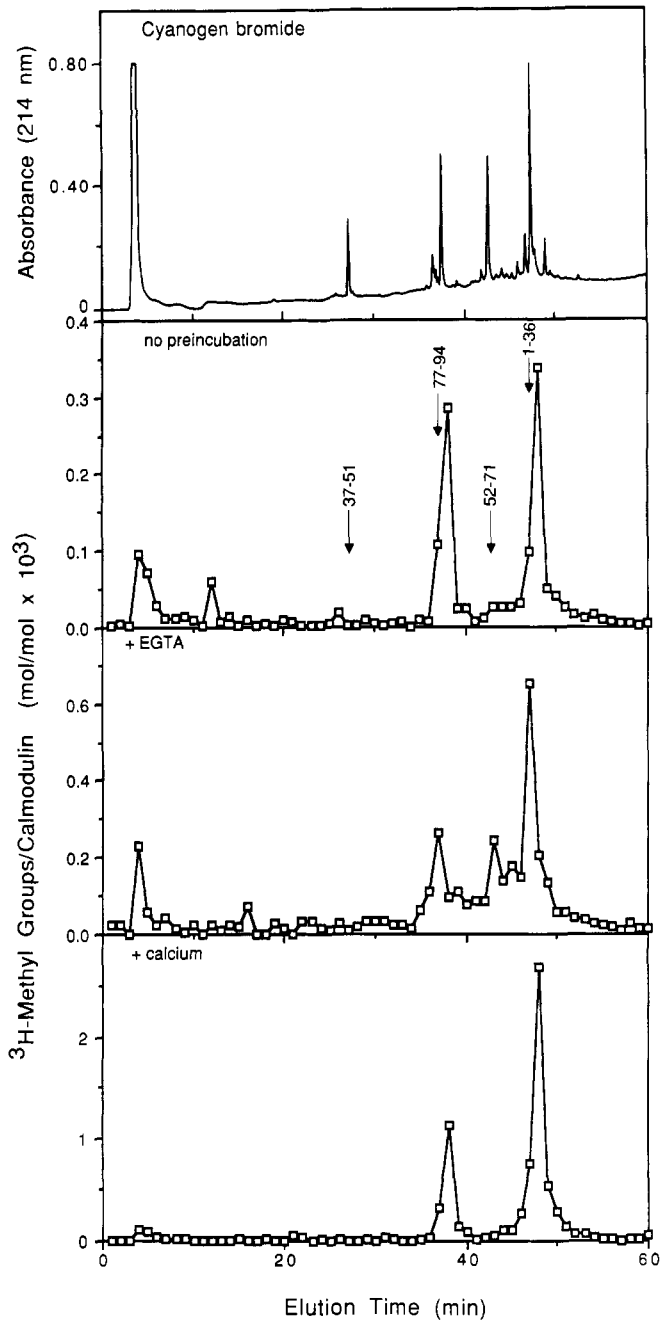

FIGURE 3: Cyanogen bromide cleavage of fragment 1–94 generated by endoproteinase Lys-C digestion of ^{3}H-methylated calmodulin samples. The fractions containing fragment 1–94 from endoproteinase Lys-C digestion of the enzymatically methylated unincubated, EGTA-preincubated, and calcium-preincubated calmodulin samples (Figure 2) were lyophilized, resuspended in 0.1% TFA, cleaved with cyanogen bromide, and separated by reverse-phase HPLC as described previously (Ota & Clarke, 1989a). The absorbance profile is shown for the calcium preincubated sample; similar profiles were obtained for the other samples.

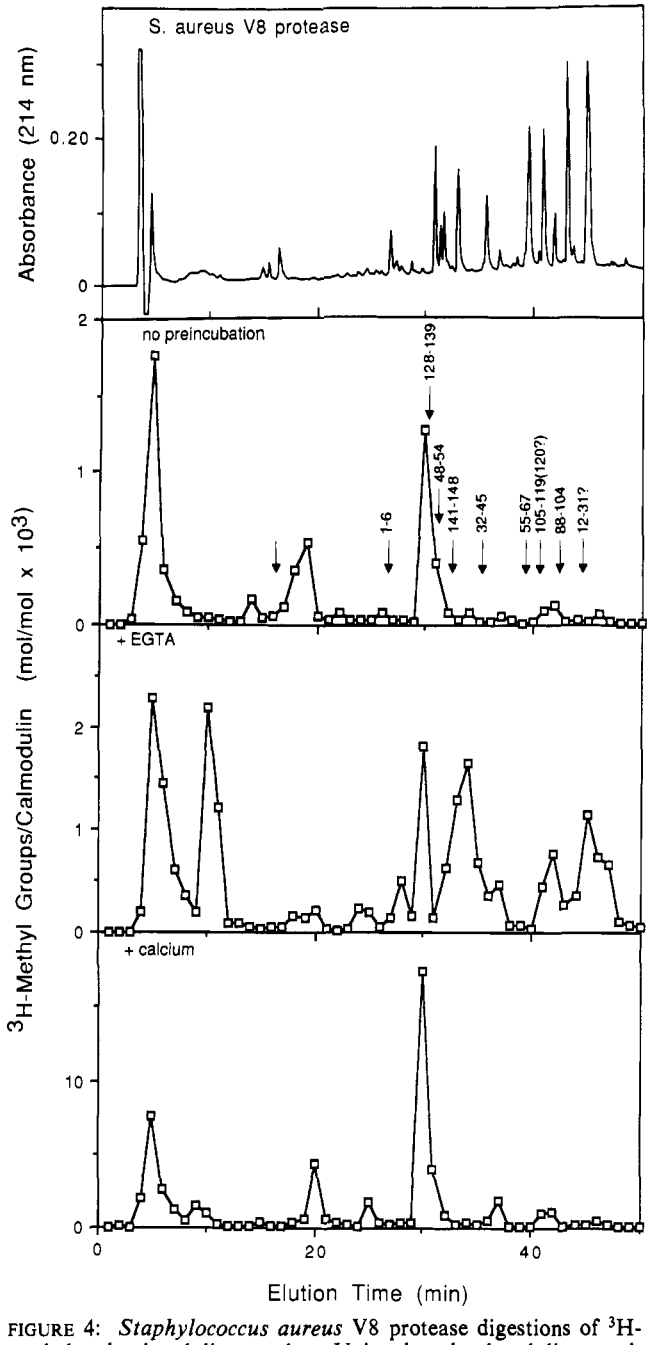

FIGURE 4: *Staphylococcus aureus* V8 protease digestions of ^{3}H-methylated calmodulin samples. Unincubated calmodulin or calmodulin incubated in the presence of EGTA or in the presence of calcium was methylated, purified, and digested with *S. aureus* V8 protease as described under Experimental Procedures, and the fragments were separated by reverse-phase HPLC as described previously (Ota & Clarke, 1989a). The absorbance profile is shown for the sample that was not preincubated; similar profiles were obtained for the other samples.

at each site, respectively. However, cyanogen bromide cleavage of the EGTA-preincubated sample reveals a new peak of radioactivity that appears to be associated with fragment 52–71, containing calcium binding site II, as well as Asn-53 (Figure 3).

A separate digestion of ^{3}H-methylated calmodulin samples with *S. aureus* V8 protease produced fragment 55–67, that contains Asx residues only at the calcium binding site. Very little radiolabel appears to be associated with this peptide for the native and calcium-preincubated samples, but the digest of the EGTA-preincubated sample contained a new radioactive peak at 41–43 min that may be associated with this fragment eluting at 39.2 min (Figure 4). This result was complicated by the fact that two other Asx-containing peptides, 105–119 at 40.7 min and 88–104 at 42.4 min, elute in this region. Fragment 105–119 appeared to contribute some of the radiolabel seen in these fractions since radiolabel was still found at 40 and 42 min when endoproteinase Lys-C fragment 95–148, which does not contain residues 55–67, was digested with *S. aureus* V8 protease (data not shown). Fragment 88–104 did not appear to contribute to the 41–43-min radioactive peak but appears to be associated mostly with the radioactive peak at fractions 44–48 min.

Calcium Binding Site III ($D_{93}KD_{95}GN_{97}GYISAAE$) Becomes a Methyl Acceptor Site after Preincubation with

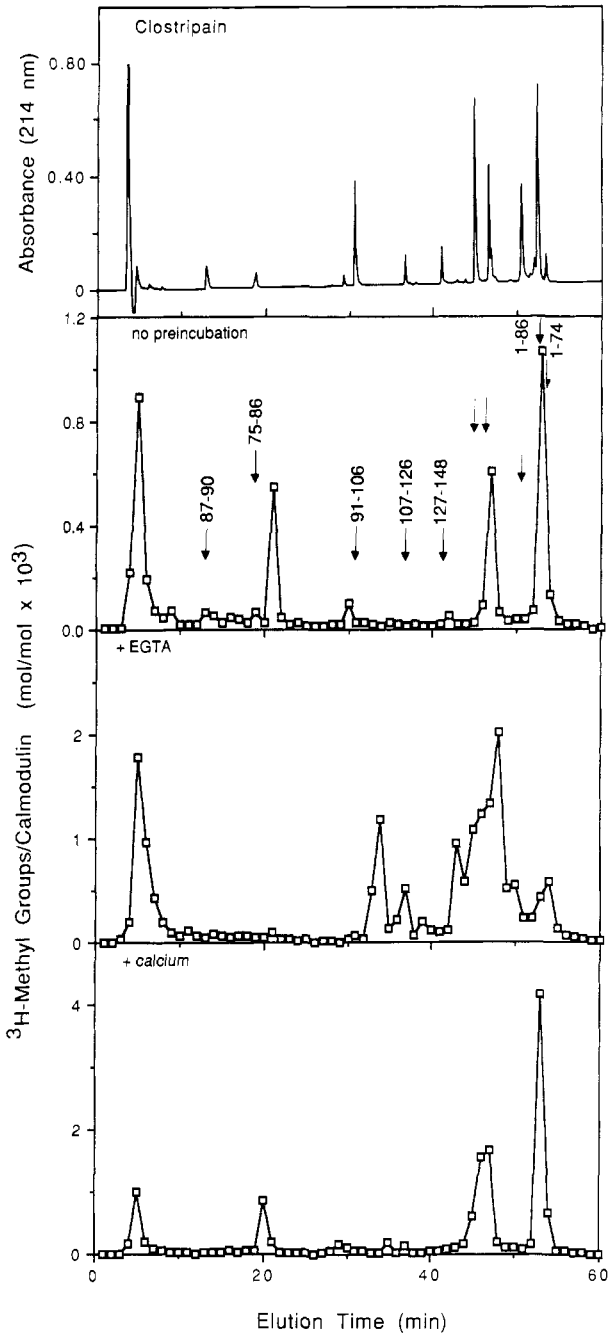

FIGURE 5: Clostripain digestions of ^{3}H-methylated calmodulin preparations preincubated in the presence or absence of calcium. Unincubated calmodulin or calmodulin preincubated in the presence of EGTA or in the presence of calcium was methylated, purified, and digested with clostripain as described under Experimental Procedures. The absorbance profile is shown for the sample that was not preincubated; similar profiles were obtained for the other samples.

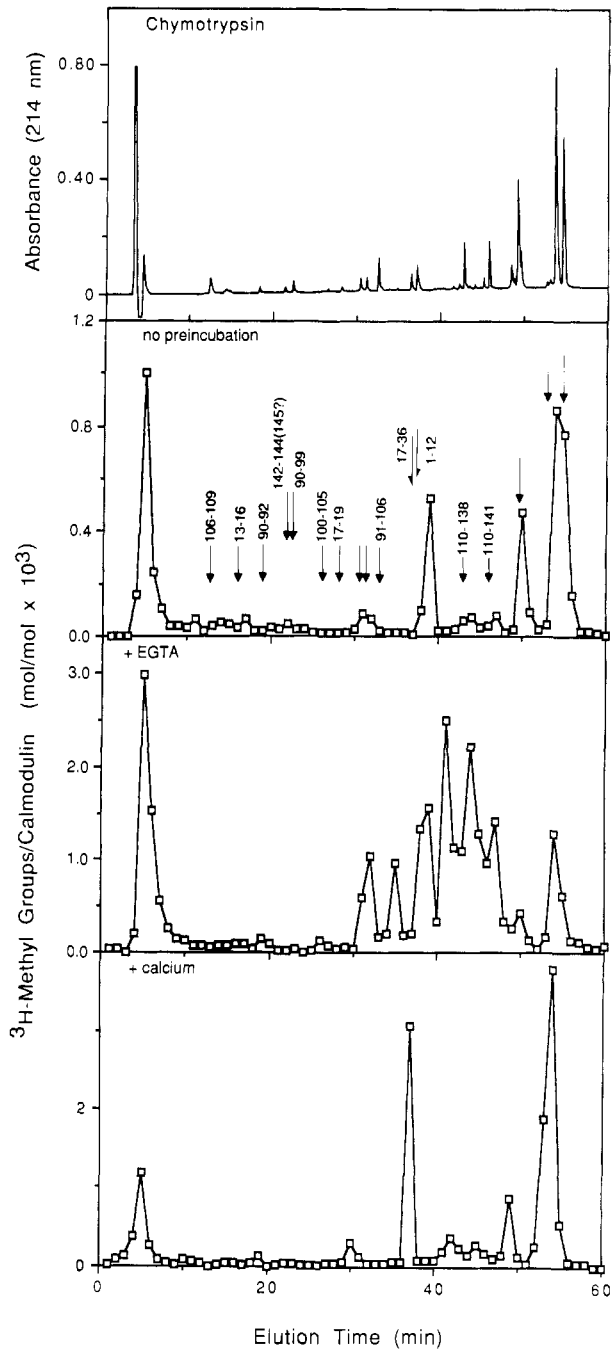

FIGURE 6: Chymotrypsin digestions of ^{3}H-methylated calmodulin samples. Unincubated calmodulin or calmodulin incubated in the presence of EGTA or in the presence of calcium was methylated, purified, and digested with chymotrypsin, and the fragments were separated by reverse-phase HPLC as described under Experimental Procedures. The absorbance profile is shown for the EGTA preincubated sample; similar profiles were obtained for the other samples.

EGTA. Endoproteinase Lys-C digests calmodulin into two large fragments, 1–94 and 95–148, as well as some smaller fragments (Figure 2). Since a new large radiolabeled peak appears at 47–50 min where only a very small peak is present in the unincubated or calcium-incubated samples, it is clear that EGTA incubation can enhance methylation in residues 95–148 that contain calcium binding sites III and IV. Both calcium binding sites III and IV appear to become major methyl acceptors after incubation with EGTA. The best evidence that calcium binding site III (residues 93–104) becomes a methyl acceptor comes from clostripain digestion of intact ^{3}H-methylated calmodulin. If the radiolabel profiles are compared between the unincubated, EGTA-incubated, and calcium-incubated samples, a new radiolabeled peak is found at 33–34 min just after fragment 91–106 at 31.5 min (Figure 5). This fragment includes all of the Asx residues from calcium binding site III. Trypsin digestion produces a similar peptide, 90–106, eluting at 31.5 min. A new radioactive peak, probably representing the methylated 90–106 peptide, appears in the EGTA-incubated sample at 34 min, but not in the unpreincubated or calcium-preincubated samples (data not shown). Finally, chymotrypsin digestion produces peptide 91–106 eluting at 32.5 min which appears to be associated with radiolabel at 35 min (Figure 6).

Calcium Binding Site IV ($D_{129}ID_{131}GD_{133}GQVN_{137}YEE$) Appears To Become a Methyl Acceptor after EGTA Preincubation. Methylation can be localized to calcium binding

site IV, at residues 129–140, by analyzing the results of several digestions. Clostripain digestion produces fragment 127–148 eluting at 41.9 min, and it appears to be associated with radiolabel in the same region at 43 and 44 min (Figure 5). Trypsin digestion produces the same peptide eluting at 41.65 min that is associated with radiolabel at 42–44 min which is not prominent in the unincubated and calcium-preincubated samples (data not shown). *S. aureus* V8 protease digestion of intact calmodulin produces fragment 128–139 eluting at 30.8 min, and this may be associated with radioactivity at 32–35 min (Figure 4). A peak of radiolabel also appears in this region when fragment 95–148 is digested with *S. aureus* V8 protease, indicating that peptides 48–54 and 32–45 which also contain Asx residues do not account for all of the radioactivity present in these fractions (data not shown). Chymotrypsin digestion produces two related peptides, 110–138 eluting at 42.7 min and 110–141 eluting at 45.7 min. These fragments appear to be associated with radiolabel at 43–45 and 46–48 min, respectively (Figure 6). Since these chymotryptic peptides contain several other Asx residues (Asn-111, Asp-118, and Asp-122) as well as calcium binding site IV, *S. aureus* V8 protease was used to redigest fragment 110–138, and these digestions show that at least part of the radiolabel can be localized to calcium binding site IV (data not shown).

Calcium Binding Site I at Residues 20–31 ($D_{20}KD_{22}GD_{24}GTITTKE$) May Not Become a Methyl Acceptor Site by Preincubation with EGTA. Although calcium binding domains II, III, and IV appear to become significant methyl acceptor sites after extended preincubation of calmodulin in the calcium-free form, calcium binding site I does not become a major methyl acceptor site. Since peptides containing the highly methylated Asp-2 site always elute within a minute of peptides containing calcium binding site I in trypsin, chymotrypsin, and clostripain digestions, it is difficult to determine whether there is methylation at this site. To increase the separation between these peptides on reverse-phase HPLC columns, an isocratic gradient was used. To further eliminate the complexities that arise from the presence of other peptides that may elute near the peptides of interest, fragment 1–37, derived from cyanogen bromide cleavage of the endoproteinase Lys-C produced peptide, 1–94, was used (Figure 3). This fragment from EGTA-incubated methylated calmodulin was digested with trypsin, and on the isocratic system, fragment 14–30 eluted at 15.95 min and fragment 1–13 eluted at 19.51 min with the radiolabel appearing at 23–24 min. Trypsin digestion of the calcium-incubated sample gave the same results (data not shown). These results suggest that only one of these peptides, 1–13, identified above as a major methyl acceptor, is a site of methylation and that calcium binding site I is not a methyl acceptor.

Methylation of Calmodulin Preincubated in the Presence or Absence of Calcium Is Enhanced at Asp-2 and the Asp-78 Region. The major sites of methylation were found to originate from Asp-2 and Asp-78 in affinity-purified calmodulin (Ota & Clarke, 1989a). The methylation at these sites in calcium-preincubated calmodulin was enhanced approximately 8-fold at Asp-2 and 3.3-fold at Asp-78. This is based on a complete digestion of calmodulin that was achieved by endoproteinase Lys-C digestion of intact ^{3}H-methylated calmodulin followed by cyanogen bromide cleavage of the endoproteinase Lys-C generated 1–94 fragment. In EGTA-preincubated calmodulin, methylation was enhanced 3.1-fold at Asp-2 and 1.4-fold at Asp-78. Methylation of minor sites that were not identified in the native calmodulin studies above also appears to be somewhat enhanced. These sites were not identified.

Discussion

A small fraction of calmodulin purified from bovine brain was previously found to be a substrate for the D-aspartyl/L-isoaspartyl protein carboxyl methyltransferase and to be methylated at two major sites. Both of these sites originated from aspartyl residues, one at position 2 and one at position 78 (Ota & Clarke, 1989a). These results were somewhat unexpected because asparaginyl residues have been shown to spontaneously form methylatable L-isoaspartyl and D-aspartyl residues more readily than aspartyl residues in model peptides under physiological conditions (Geiger & Clarke, 1987; Stephenson & Clarke, 1989). In fact, two of the six Asn residues in calmodulin are present in Asn-Gly sequences, and these have been shown to form methylatable residues especially rapidly in denatured polypeptides (Bornstein & Balian, 1977; Blodgett et al., 1985; Meinwald et al., 1986; Geiger & Clarke, 1987; Stephenson & Clarke, 1989). Based partially on these studies, it had been suggested that the two Asn-Gly sequences in calmodulin would be likely candidates for the major sites of methylation (Johnson et al., 1985, 1987, 1989) although other evidence did favor aspartyl sites (Brunauer & Clarke, 1986). We found, however, no evidence for significant methylation at sites derived from any of the Asn residues in affinity-purified preparations of calmodulin (Ota & Clarke, 1989a).

This distribution of methylation sites appears to be a function of the three-dimensional structure of calmodulin. The formation of L-isoaspartyl residues via a succinimide route requires that the peptide nitrogen atom be in a position to attack the side-chain carbonyl of the Asx residue (Clarke, 1987). Although the crystal structure of the calcium form of bovine brain calmodulin shows that the peptide bond nitrogen of Thr-79 is not in a position to attack the side-chain carbonyl carbon of Asp-78 to form a succinimide (Babu et al., 1988), evidence from both crystal studies (Babu et al., 1988) and solution studies (Persechini & Kretsinger, 1988; Bayley et al., 1988) indicates that these residues are present in an apparently flexible region of calmodulin. This flexibility thus might allow these residues to move into positions that would permit succinimide formation. Asp-2 is also located in another apparently flexible region at the exposed N-terminus of the protein (Babu et al., 1988). The failure to detect methylation sites at other Asp or at Asn residues, particularly those in the 4 calcium binding sites (11 Asp and 3 Asn), may reflect both the poor positioning of the peptide bond nitrogen and carboxyl carbon atoms for succinimide formation as well as the relative inflexibility of these sites (Babu et al., 1988). Examination of the crystal structure of the calcium form of bovine brain calmodulin shows that none of the 14 Asx residues in the calcium binding domains have their side-chain carbonyl carbons arranged within 3 Å of the nitrogen atom of the succeeding residue (Babu et al., 1988).

In this work, we found that the distribution of methyl acceptor sites in calmodulin can be significantly altered by preincubating this protein for extended periods of time in the presence or absence of calcium [cf. Johnson et al. (1987, 1989)]. For calmodulin preincubated in the presence of calcium, we showed enhanced methylation at the Asp-2 and Asp-78 sites. However, extended preincubation of calmodulin in the absence of calcium resulted in a new methylation pattern with major sites of methylation now detectable at calcium binding sites II, III, and IV. Each of these sites contains several Asx residues in the sequences *Asp*-Ala-*Asp*-Gly-*Asn*-Gly-Thr-Ile-*Asp*-Phe-Pro-Glu, *Asp*-Lys-*Asp*-Gly-*Asn*-Gly-

Tyr-Ile-Ser-Ala-Ala-Glu, and *Asp*-Ile-*Asp*-Gly-*Asp*-Gly-Gln-Val-*Asn*-Tyr-Glu-Glu, respectively. We were unable to identify which particular Asx residues were methylated in these binding sites. It is interesting to note that even under these conditions, the formation of methylatable residues does not appear to be restricted to Asn-Gly sequences since domain IV, which does not contain any Asn-Gly sequences, becomes a methyl acceptor site. Under these preincubation conditions, methylation was also still found to occur at Asp-2 and Asp-78, and methylation at these sites was enhanced by incubation for the 13-day period.

Why does the removal of calcium from calmodulin prior to extended preincubation allow the calcium binding sites to become methyl acceptor sites? One possibility is that the ligand-free binding sites are less constrained and allow Asx residues to undergo the degradation reactions that result in the formation of methylatable L-isoaspartyl and possibly D-aspartyl residues. It may also be possible that when calcium is removed, new contacts between the Asx residues and neighboring residues are made that could catalyze succinimide formation [cf. Ota et al. (1987) and Kossiakoff (1988)].

Why does calcium binding site I not become a methyl acceptor in analogy to the other calcium binding sites when calmodulin is preincubated for extended periods in the absence of calcium? This site contains three aspartyl residues that could potentially form methylatable residues. One difference between calcium binding site I and the other calcium binding sites is that there are no asparaginyl residues at site I. Since asparaginyl residues have been demonstrated to be more susceptible to succinimide formation than aspartyl residues in short peptides (Geiger & Clarke, 1987; Stephenson & Clarke, 1989), it may be that methylatable residues do not form readily from the aspartyl residues at site I. Alternatively, methylatable residues may form in site I but may not be recognized by the methyltransferase. While most L-isoaspartyl-containing peptidyl methyl acceptors have been found to have K_m's for this enzyme that range from 0.4 to 6.3 μM (Ota & Clarke, 1989b), a peptide, Tyr-Val-Ser-L-isoAsp-Gly-Asp-Gly, has been found to display a much higher K_m of 469 μM (J. Lowenson and S. Clarke, unpublished results). Calcium binding site I contains aspartyl residues in the sequence Asp-Lys-Asp-Gly-Asp-Gly, and if an L-isoaspartyl residue were to form at the second Asp residue in this site, its affinity for the methyltransferase may be so low that it would not be readily detected as a methyl acceptor in our experiments.

Calmodulin is a calcium-dependent regulatory molecule and activates target enzymes only when calcium is bound (Wang et al., 1985). The concentration of calcium in cells is very low, usually in the submicromolar range, and with the concentration of calmodulin at approximately 10 μM in bovine brain, calmodulin would not be expected to have calcium bound at all four sites at all times (Huang et al., 1985). Thus, calmodulin isolated from most tissues might be predicted to have unliganded calcium binding sites that would be potential methylation sites. If this is the case, it is not immediately apparent why the sites of methylation in purified bovine brain calmodulin appear to be limited to Asp-2 and Asp-78 sites. However, this preparation of calmodulin is prepared by a hydrophobic interaction chromatography procedure that depends on the differing affinities of calmodulin for the resin in the presence and absence of calcium (Jamieson & Vanaman, 1979; Gopalakrishna & Anderson, 1982). Calmodulin that is denatured by oxidation or heat treatment binds only two calcium ions and does not bind effectively to these types of affinity columns (Gopalakrishna & Anderson, 1985). Thus, this affinity purification may select against calmodulin molecules which have altered aspartyl and asparaginyl residues in the calcium binding sites.

In preliminary experiments, we have incubated intact red cells with [*methyl*-^{3}H]methionine to incorporate radiolabeled methyl groups into cell components. We purified calmodulin from these cells without using affinity chromatography and analyzed the distribution of methyl groups on the polypeptide chain. We find that the pattern of methylated peptides on HPLC is similar to that observed here with calmodulin incubated in the absence of calcium. We also detect additional methylated peptides. It is possible that other types of damage occur in erythrocytes that we have not reproduced in our in vitro incubations, and these might expose new sites of methylation in calmodulin. For instance, oxidative damage has been shown to increase the methylation of hemoglobin (O'-Connor & Yutzey, 1988).

Protein carboxyl methyltransferases have been postulated to participate in the repair of altered aspartyl residues in age-damaged proteins (McFadden & Clarke, 1982). Johnson et al. (1987) have recently shown that incubation of calmodulin for 28 days at 37 °C results in a loss of its ability to activate the calcium–calmodulin-dependent protein kinase. Further incubation with purified methyltransferase and *S*-adenosylmethionine was found to effect the partial reactivation of this activity. Since the damaged calmodulin in these experiments was formed by preincubating in the presence of EGTA, methylation at altered aspartyl and asparaginyl residues in the calcium binding sites as well as at Asp-2 and Asp-78 may lead to reactivation. An alternative pathway for the metabolism of altered L-isoaspartyl- and D-aspartyl-containing proteins may be their removal from the cell by proteolytic systems that recognize damaged aspartyl residues. Although specific L-isoaspartyl- and D-aspartyl-recognizing proteases have not been found, there is at least one peptidase which appears to preferentially cleave bonds adjacent to isoaspartyl and succinimide residues (Momand & Clarke, 1987).

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Registry No. D-Asp, 1783-96-6; L-Asp, 56-84-8; L-Asn, 70-47-3; Ca, 7440-70-2; protein carboxyl methyltransferase type II, 9055-09-8.

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Characterization and Differential Expression of Human Vascular Smooth Muscle Myosin Light Chain 2 Isoform in Nonmuscle Cells†

C. Chandra Kumar,*,‡ Sheela R. Mohan,‡ Paul J. Zavodny,§ Satwant K. Narula,§ and Paul J. Leibowitz‡
Departments of Tumor Biology and Molecular Biology, Schering Corporation, 60 Orange Street, Bloomfield, New Jersey 07003
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ABSTRACT: The 20-kDa regulatory myosin light chain (MLC), also known as MLC-2, plays an important role in the regulation of both smooth muscle and nonmuscle cell contractile activity. Phosphorylation of MLC-2 by the enzyme MLC kinase increases the actin-activated myosin ATPase activity and thereby regulates the contractile activity. We have isolated and characterized an MLC-2 cDNA corresponding to the human vascular smooth muscle MLC-2 isoform from a cDNA library derived from umbilical artery RNA. The translation of the in vitro synthesized mRNA, corresponding to the cDNA insert, in a rabbit reticulocyte lysate results in the synthesis of a 20 000-dalton protein that is immunoreactive with antibodies raised against purified chicken gizzard MLC-2. The derived amino acid sequence of the putative human smooth muscle MLC-2 shows only three amino acid differences when compared to chicken gizzard MLC-2. However, comparison with the human cardiac isoform reveals only 48% homology. Blot hybridizations and S1 nuclease analysis indicate that the human smooth muscle MLC-2 isoform is expressed restrictively in smooth muscle tissues such as colon and uterus and in some, but not all, nonmuscle cell lines. Previously reported MLC-2 cDNA from rat aortic smooth muscle cells in culture is ubiquitously expressed in all muscle and nonmuscle cells, and it was suggested that both smooth muscle and nonmuscle MLC-2 proteins are identical and are probably encoded by the same gene. In contrast, the human smooth muscle MLC-2 cDNA that we have characterized from an intact smooth muscle tissue is not expressed in skeletal and cardiac muscles and also in a number of nonmuscle cells. Nevertheless, MLC-2 protein species is readily detectable in all the nonmuscle cell lines using antibodies to smooth muscle MLC-2 protein. Two-dimensional gel analysis of the ^{35}S-labeled proteins from a nonmuscle cell line indicates three protein species that are immunoprecipitated with MLC-2 antibodies. Comparison of the two-dimensional gel pattern indicates the absence of one MLC-2 protein species in the cell lines that do not express the smooth muscle MLC-2 mRNA. Together, these results suggest that the smooth muscle and nonmuscle MLC-2 isoforms are separate and are possibly encoded by separate genes. Hence, the MLC-2 cDNA sequence reported in this paper corresponds to a novel and distinct smooth muscle isoform.

The two major contractile proteins actin and myosin are present in almost all eukaryotic cells. Myosin is a hexameric protein which forms the core of the thick filaments of muscle. All myosin filaments share the same architecture of two myosin heavy chains (MHCs) and two pairs of light chains (MLCs). Two of these light chains are classified as phosphorylatable regulatory chains (MLC-2), and the other two are nonphosphorylatable, alkali light chains (MLC-1 or MLC-3) (Harrington & Rodgers, 1984). Myosin light chain 2 (MLC-2) has a molecular weight of 20K and plays an important role in the regulation of smooth muscle contraction

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* To whom correspondence should be addressed.
‡ Department of Tumor Biology.
§ Department of Molecular Biology.

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