Characterization of the autophosphorylation of chicken gizzard caldesmon

Gisele C. SCOTT-WOO and Michael P. WALSH

Department of Medical Biochemistry, Faculty of Medicine, University of Calgary, Calgary, Alberta, Canada T2N 4N1

Caldesmon, an actin- and calmodulin-binding protein of smooth muscle, is a protein serine/threonine kinase capable of Ca^{2+} /calmodulin-dependent autophosphorylation [Scott-Woo & Walsh (1988) *Biochem. J.* **252**, 463–472]. Phosphorylation nullifies the inhibitory effect of caldesmon on the actin-activated Mg^{2+} -ATPase activity of smooth-muscle myosin [Ngai & Walsh (1987) *Biochem. J.* **244**, 417–425]. We have characterized the kinase activity of caldesmon of chicken gizzard smooth muscle. Autophosphorylation requires Ca^{2+} /calmodulin, but is unaffected by other second messengers (Ca^{2+} /phospholipid/diacylglycerol, cyclic AMP or cyclic GMP), and is inhibited by the calmodulin antagonists chlorpromazine and compound 48/80, with 50% inhibition at 39.8 μM and 12.0 ng/ml respectively. Half-maximal activation of autophosphorylation occurs at 60–80 nM- Ca^{2+} and 0.14 μM -calmodulin, and maximal activity at 0.14–0.18 μM - Ca^{2+} and 1 μM -calmodulin; activation is gradually lost at higher Ca^{2+} and calmodulin concentrations. Autophosphorylation is pH-dependent, with maximal activity over the range pH 7–9, and requires free Mg^{2+} in addition to the MgATP^{2-} substrate. The K_m for ATP is $15.6 \pm 4.1 \mu\text{M}$ (mean $\pm$ S.D., $n = 4$), and kinase activity is inhibited by increasing ionic strength [half-maximal inhibition at $I = 0.094 \pm 0.009 \text{ M}$ (mean $\pm$ S.D., $n = 4$)]. Autophosphorylation does not affect the rate of hydrolysis of caldesmon (free or bound to calmodulin) by α -chymotrypsin. However, a slight difference in peptides generated from phospho- and dephospho-forms of caldesmon is observed. The binding of phospho- or dephospho-caldesmon to F-actin protects the protein against chymotryptic digestion, but does not alter the pattern of peptide generation. Characterization of proteolytic fragments of caldesmon generated by α -chymotrypsin and *Staphylococcus aureus* V8 protease enables localization of the phosphorylation sites and the kinase active site within the caldesmon molecule.

INTRODUCTION

The primary mechanism whereby Ca^{2+} ions regulate the contractile state of a smooth-muscle cell involves phosphorylation of myosin by Ca^{2+} /calmodulin-dependent myosin light-chain kinase (Hartshorne, 1987). Substantial evidence suggests the existence of secondary Ca^{2+} -dependent regulatory systems in smooth muscle (Kamm & Stull, 1985). One such secondary control mechanism may involve the protein caldesmon (Walsh, 1987).

Caldesmon is the major calmodulin-binding protein of smooth muscle, the caldesmon–calmodulin interaction being Ca^{2+} -dependent. It is also capable of interacting with actin in a Ca^{2+} -independent manner (Sobue *et al.*, 1981).

These observations suggested the possible involvement of caldesmon in Ca^{2+} -mediated regulation of smooth-muscle contraction. Such a physiological function is supported by the observations that isolated caldesmon inhibits the actin-activated Mg^{2+} -ATPase activity of phosphorylated smooth-muscle myosin, without affecting the phosphorylation state of the myosin (Ngai & Walsh, 1984), and that caldesmon is located *in situ* on actin filaments (Marston & Lehman, 1985). We demonstrated that caldesmon can be phosphorylated by a Ca^{2+} /calmodulin-dependent protein kinase (Ngai & Walsh, 1984) and that phosphorylation blocks its inhibitory effect on the actomyosin ATPase (Ngai & Walsh, 1987). Subsequent studies indicated that caldesmon itself is the

protein kinase and this is an intermolecular autophosphorylation (Scott-Woo & Walsh, 1988). We report here the kinetic and regulatory properties of caldesmon autophosphorylation and proteolytic-digestion studies which lead to a tentative map of the domain structure of caldesmon.

MATERIALS AND METHODS

Materials

[γ - ^{32}P]ATP (10–40 Ci/mmol) was purchased from Amersham Corp. (Oakville, Ontario, Canada). Dithiothreitol, CNBr-activated Sepharose 4B, cyclic AMP, cyclic GMP, ATP, L- α -phosphatidyl-L-serine, 1,3-diolein, chlorpromazine and compound 48/80 were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Chymotrypsin A₄ was purchased from Boehringer-Mannheim (Dorval, Quebec, Canada). Materials for SDS/PAGE were purchased from Bio-Rad Laboratories (Mississauga, Ontario, Canada). General laboratory reagents used were of analytical grade or better, and were purchased from Fisher Scientific (Calgary, Alberta, Canada).

Protein purifications

The following proteins were purified by previously described methods: bovine brain calmodulin (Walsh *et al.*, 1984), chicken gizzard actin (Ngai *et al.*, 1986) and caldesmon (Scott-Woo & Walsh, 1988).

Enzymic assays

[32 P] P_i incorporation into caldesmon was measured as described for myosin phosphorylation (Walsh *et al.*, 1983) under the conditions described in individual Figure legends. Conditions for determination of Mg^{2+} -dependence, pH-dependence, ionic-strength-dependence and K_m for ATP were as follows. For Mg^{2+} -dependence: 20 mM-Tris/HCl (pH 7.5)/0–10 mM- $MgCl_2$ /0.1 mM- $CaCl_2$ /2.5 μ M-calmodulin/0.7 μ M-caldesmon/0.5 mM- $[\gamma\text{-}^{32}P]$ -ATP (31 c.p.m./pmol). For pH-dependence: 40 mM-buffer (sodium acetate/acetic acid for pH 4.0–5.5; Pipes for pH 6.0–7.0; Mops for pH 7.0–8.0; Tris for pH 8.5–9.0)/5 mM- $MgCl_2$ /0.1 mM- $CaCl_2$ /2.5 μ M-calmodulin/0.7 μ M-caldesmon/0.5 mM- $[\gamma\text{-}^{32}P]$ -ATP (31 c.p.m./pmol). For ionic-strength-dependence: 20 mM-Tris/HCl (pH 7.5)/5 mM- $MgCl_2$ /0.1 mM- $CaCl_2$ /NaCl to give $I =$

0–0.45 M/2.5 μ M-calmodulin/0.35 μ M-caldesmon/0.5 mM- $[\gamma\text{-}^{32}P]$ -ATP (100 c.p.m./pmol). For K_m (ATP): 20 mM-Tris/HCl (pH 7.5)/5 mM- $MgCl_2$ /0.1 mM- $CaCl_2$ /2.5 μ M-calmodulin/0.35 μ M-caldesmon/1–1000 μ M- $[\gamma\text{-}^{32}P]$ -ATP (600 c.p.m./pmol).

Other methods

Protein concentrations were determined by the Coomassie Brilliant Blue dye-binding assay (Spector, 1978), with dye reagent purchased from Pierce Chemical Co. (Rockford, IL, U.S.A.), or by spectrophotometric measurements for calmodulin [$A_{277}^{1\%} = 1.9$ (Klee, 1977)]. Calmodulin was coupled to CNBr-activated Sepharose 4B as previously described (Walsh *et al.*, 1982). Other methods were as previously described (Clark *et al.*, 1986).

Fig. 1. Effects of calmodulin antagonists on caldesmon autophosphorylation

Caldesmon (0.7 μ M) was incubated at 30 °C in 20 mM-Tris/HCl (pH 7.5)/5 mM- $MgCl_2$ /0.1 mM $CaCl_2$ (○) or 1 mM-EGTA (●)/0.1 μ M-calmodulin/0.5 mM- $[\gamma\text{-}^{32}P]$ -ATP (12 c.p.m./pmol) in the presence of the indicated concentrations of calmodulin antagonists [chlorpromazine in (a) or compound 48/80 in (b)]. Samples (0.2 ml) of reaction mixtures (total volume 1.75 ml) were withdrawn at selected times for quantification of protein-bound [^{32}P] P_i . An equal volume of SDS-gel sample buffer was added to the remainder of each reaction mixture and boiled before SDS/PAGE and autoradiography. Data are plotted as the maximum caldesmon phosphorylation achieved (mol of P_i /mol of caldesmon) as a function of the concentration of calmodulin antagonist. The insets show autoradiographs of the caldesmon band (5 μ g of caldesmon/lane) at the end of the phosphorylation reactions; from left to right: caldesmon + Ca^{2+} , caldesmon – Ca^{2+} , caldesmon + Ca^{2+} + increasing concentrations of calmodulin antagonist.

Table 1. Regulation of caldesmon kinase activity by second messengers

The rate of caldesmon phosphorylation was determined under the following conditions, with additions as indicated in the Table: 20 mM-Tris/HCl (pH 7.5)/5 mM-MgCl₂/0.5 mM-[γ -³²P]ATP (44 c.p.m./pmol)/1 μ M-caldesmon. Additions: EGTA (1 mM); CaM (calmodulin; 1 μ M) Ca²⁺ (0.1 mM); phospholipid (40 μ g of phosphatidylserine/ml); diacylglycerol (DAG; 0.8 μ g of 1,3-diolein/ml). These results represent a typical experiment.

Conditions	Kinase activity (pmol P _i /min mg)
+ EGTA	20.4
+ EGTA + CaM	52.9
+ Ca ²⁺	18.3
+ Ca ²⁺ + CaM	509.6
+ EGTA + phospholipid/DAG	126.1
+ Ca ²⁺ + phospholipid/DAG	103.6
+ 10 μ M-Cyclic AMP	4.7
+ 1 mM-Cyclic AMP	7.1
+ 10 μ M-Cyclic GMP	5.1
+ 1 mM-Cyclic GMP	8.5

Fig. 2. Ca²⁺-dependence of caldesmon autophosphorylation

Caldesmon (2.5 μ M) was incubated at 30 °C in 10 mM-Mops (pH 7.1)/2 mM-MgCl₂/1 mM-EGTA/2.5 μ M-calmodulin/0.3 mM-[γ -³²P]ATP (21 c.p.m./pmol) with various amounts of CaCl₂ to give the free Ca²⁺ concentrations indicated (Fabiato, 1981). Values represent the means of triplicate determinations (s.d. < 0.75 %).

RESULTS

Regulation by second messengers of caldesmon autophosphorylation

Table 1 indicates that caldesmon autophosphorylation requires both Ca²⁺ and calmodulin. Partial activation by phosphatidylserine/1,3-diolein was observed, but was unaffected by Ca²⁺. Neither cyclic AMP nor cyclic GMP was capable of activating caldesmon. Ca²⁺/calmodulin-dependent autophosphorylation is inhibited by the calmodulin antagonists chlorpromazine and compound

Fig. 3. Calmodulin-dependence of caldesmon autophosphorylation

Caldesmon (1.42 μ M) was incubated at 30 °C in 10 mM-Mops (pH 7.1)/2 mM-MgCl₂/0.218 μ M-Ca²⁺ (free)/0.3 mM-[γ -³²P]ATP (50 c.p.m./pmol), with various amounts of calmodulin as indicated. Values represent the means of triplicate determinations (S.D. < 2.0 %). Similar results were obtained in five independent experiments using caldesmon concentrations of 1.42 μ M (as shown) or 0.3 μ M.

48/80, with 50 % inhibition at 39.8 μ M and 12.0 ng/ml respectively (Fig. 1). The Ca²⁺-dependence of autophosphorylation (Fig. 2) is biphasic, with activation at low [Ca²⁺] (half-maximal activation occurred at 79 nM-Ca²⁺ and maximal activity at 0.18 μ M-Ca²⁺); activation was decreased at higher [Ca²⁺]. In separate experiments using different protein preparations, half-maximal activation was observed at 60 nM-Ca²⁺ and maximal activity at 0.14 μ M-Ca²⁺. Again, activation was gradually decreased at higher [Ca²⁺]. The calmodulin-dependence of autophosphorylation is also biphasic (Fig. 3): half-maximal activation occurred at 0.14 μ M-calmodulin and maximal activity at 1.0 μ M-calmodulin. Activation was decreased at higher calmodulin concentrations.

Kinetic parameters of caldesmon autophosphorylation

Caldesmon has a *K_m* for MgATP²⁻ of 15.6 $\pm$ 4.1 μ M (mean $\pm$ s.d., *n* = 4) and requires free Mg²⁺ in addition to MgATP²⁻; maximal activity occurred at 2 mM-Mg²⁺ in the presence of 0.5 mM-ATP and did not change significantly over the range 2–10 mM. The pH-dependence of autophosphorylation was studied over the range pH 4–9. Maximal activity occurred over the range pH 7–9; activity fell dramatically below pH 7. Autophosphorylation was found to be sensitive to ionic strength: half-maximal inhibition occurred at *I* = 0.094 $\pm$ 0.009 M (mean $\pm$ s.d., *n* = 4) (results not shown).

Proteolytic digestion of phospho- and dephospho-caldesmon

Fig. 4 depicts the time course of digestion of

Fig. 4. Time course of digestion of caldesmon with α -chymotrypsin

Caldesmon ($3.5 \mu\text{M}$) was incubated at 30°C in 20 mM-Tris/HCl ($\text{pH } 7.5$)/ 50 mM-KCl / 1 mM-EGTA / $0.5 \text{ mM-dithiothreitol}$ with $0.5 \mu\text{g}$ of α -chymotrypsin/ml [proteinase/substrate = $1:1000$ (w/w)]. Samples ($20 \mu\text{l}$) of reaction mixtures were withdrawn at selected time intervals, added to an equal volume of SDS-gel sample buffer and boiled for 2 min before SDS-PAGE ($10 \mu\text{g}$ sample/lane). From left to right: marker proteins (phosphorylase *b*, 97.4 kDa ; bovine serum albumin, 66.2 kDa ; ovalbumin, 45 kDa ; carbonic anhydrase, 29 kDa ; soya-bean trypsin inhibitor, 20.1 kDa ; lysozyme, 14.4 kDa); undigested caldesmon; digestion for 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 40, 50 and 60 min respectively. The major fragments of caldesmon are numbered 1–9.

dephospho-caldesmon by α -chymotrypsin to identify the major proteolytic fragments. The rate of digestion of caldesmon, determined by laser densitometry of Coomassie Blue-stained gels of digestion time courses, was not significantly affected by phosphorylation and/or the presence of bound Ca^{2+} /calmodulin (results not shown). However, a slight difference between the peptides generated from phospho- and dephospho-caldesmons was observed (Fig. 5). A prominent peptide of 26 kDa (band 7a) is generated from dephospho-caldesmon (Fig. 5a, lanes 4 and 5) but not from the phospho-caldesmon (Fig. 5a, lanes 2 and 3). Rather, the latter contains a $29/28 \text{ kDa}$ doublet (Fig. 5a, lanes 2 and 3). The prominent 11 kDa peptide observed in lanes 3 and 5 is a breakdown product of calmodulin, which is much more susceptible to proteolysis in the absence than in the presence of Ca^{2+} (Walsh *et al.*, 1977). This conclusion was verified in separate experiments in which calmodulin alone was digested under identical conditions in the presence and the absence of Ca^{2+} . No digestion occurred in the presence of Ca^{2+} , and an 11 kDa peptide was generated in the absence of Ca^{2+} . Other than the differences just defined, the digestion patterns in Fig. 5 are identical. The Ca^{2+} -dependent mobility shift exhibited by calmodulin (Grab *et al.*, 1979) is clearly apparent. The autoradiogram (Fig. 5b) shows that the pattern of phosphopeptides generated is unaffected by the presence of bound calmodulin. The two major phosphopeptides (26 and 18 kDa ; Fig. 5b) do not correspond to major stained peptides in Fig. 5a. In five independent experiments, caldesmon was phosphorylated to $2.04 \pm 0.18 \text{ mol of P/mol}$. No dephosphorylation occurred during the course of chymotryptic digestion, and 98% of the incorporated

radioactivity was recovered in the 26 kDa and 18 kDa phosphopeptides, confirming our previous report (Scott-Woo & Walsh, 1988). We conclude that the 26 kDa and 18 kDa peptides are major fragments of caldesmon which stain poorly with Coomassie Brilliant Blue.

In contrast with calmodulin binding, actin binding does alter the rate of degradation of phospho-caldesmon (Fig. 6). Fig. 6(a) depicts the disappearance of native caldesmon (141 kDa), which is slowed down by the presence of actin. The same conclusion is demonstrated more clearly in Fig. 6(b), which shows the appearance and disappearance of band 3 (95 kDa), both of which are significantly slowed down by actin. This effect is specific for actin, since ovalbumin, a protein of similar M_r , at the same concentration did not affect the rate of proteolysis of caldesmon (results not shown). The peptides and phosphopeptides generated in the presence and absence of actin were indistinguishable, as shown by SDS/PAGE and autoradiography. Dephospho-caldesmon was similarly affected by actin (results not shown). Quantitative binding of caldesmon to actin under the conditions used in the experiment of Fig. 6 was verified by a sedimentation method (Ngai *et al.*, 1984). Results for caldesmon digestion identical with those obtained with actin were obtained with actin/tropomyosin ($3:1$ molar ratio) (results not shown).

When non-phosphorylated caldesmon was digested with *S. aureus* V8 protease and the digest subjected to affinity chromatography on a column of calmodulin-Sepharose 4B, the unbound fraction, which contained several fragments of caldesmon, exhibited a small amount of Ca^{2+} -independent histone kinase activity. On the other hand, a peak of Ca^{2+} -dependent kinase activity

Fig. 5. Effect of phosphorylation and calmodulin binding on the digestion of caldesmon by chymotrypsin

Caldesmon (7.0 μM) was incubated at 30 $^{\circ}\text{C}$ in 20 mM-Tris/HCl (pH 7.5)/5 mM-MgCl₂/0.1 mM-CaCl₂ or 1 mM-EGTA/0.5 mM-dithiothreitol/2.5 μM -calmodulin/0.5 mM- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (42 c.p.m./pmol). In the presence of Ca²⁺, phosphorylation to the extent of 1.9 mol of P_i/mol of caldesmon was determined, whereas in the absence of Ca²⁺ no phosphorylation was detected. Conditions were adjusted to 20 mM-Tris/HCl (pH 7.5)/50 mM-KCl/3.5 mM-MgCl₂/1.75 μM -calmodulin/0.35 mM-ATP/0.1 mM-CaCl₂ or 1 mM-EGTA/0.5 mM-dithiothreitol/3.5 μM -caldesmon, and digestion was initiated by addition of α -chymotrypsin [proteinase/caldesmon = 1:1000 (w/w)]. Digestions were arrested at 60 min by addition of an equal volume of SDS-gel sample buffer and boiling for 2 min before SDS/PAGE and autoradiography. Panel (a) shows the Coomassie Blue-stained gel and panel (b) the corresponding autoradiogram. Key to lanes: 1, molecular-mass marker proteins (numbers at the left indicate their sizes in kDa); 2, phosphorylated caldesmon + Ca²⁺; 3, phosphorylated caldesmon - Ca²⁺; 4, non-phosphorylated caldesmon + Ca²⁺; 5, non-phosphorylated caldesmon - Ca²⁺; 6, undigested calmodulin + Ca²⁺; 7, undigested calmodulin - Ca²⁺. Arrows indicate major phosphopeptides of 26 and 18 kDa.

was eluted with EGTA (Fig. 7). The latter activity peak contained a 65 kDa peptide. No autophosphorylation was detected in this peak, suggesting that the phosphorylation sites are not retained in this 65 kDa peptide (results not shown). Consistent with this observation, when phosphorylated caldesmon was digested with *S. aureus* proteinase, the 65 kDa peptide was not phosphorylated (results not shown).

Fig. 7 shows that the histone kinase activity in the unbound fraction (first peak) is actually higher in the absence than in the presence of Ca²⁺. This was verified in two independent experiments in which the initial rates of histone phosphorylation were determined from phosphorylation time-course assays: 44.6 pmol of P_i/min per mg in the absence of Ca²⁺ and 21.9 pmol/min per mg in its presence. The Ca²⁺-dependent activity (peak II) was determined to be 1.48 nmol of P_i/min per mg in the presence of Ca²⁺ and 0.12 nmol/min per mg in its absence.

DISCUSSION

Five calmodulin-dependent protein kinases have been characterized to date: myosin light-chain kinase (Stull

et al., 1986), glycogen phosphorylase *b* kinase (Cohen, 1980), and calmodulin-dependent kinases I (Nairn & Greengard, 1987), II (Nairn *et al.*, 1985a) and III (Nairn *et al.*, 1985b). Caldesmon was identified as a Ca²⁺/calmodulin-dependent protein kinase capable of autophosphorylation (Scott-Woo & Walsh, 1988). It can be distinguished from the above five kinases on the basis of molecular mass and substrate specificity. Caldesmon most closely resembles calmodulin-dependent kinase II. Both have a high affinity for MgATP²⁻: ~16 μM for caldesmon and 3–114 μM for calmodulin-dependent kinase II (Stull *et al.*, 1986). Both phosphorylate brain synapsin I, but at distinct sites (G. C. Scott-Woo & M. P. Walsh, unpublished work). Caldesmon has subunits of ~140 kDa, whereas the calmodulin-dependent kinase II has subunits of 50 and 60 kDa and a native molecular mass of ~500 kDa (Stull *et al.*, 1986).

Caldesmon exhibits an interesting dependence on Ca²⁺ and calmodulin. Both dependence curves are biphasic, with activation over the range of 25–180 nM-Ca²⁺ and 100 nM–1 μM -calmodulin and decreased activation at higher concentrations. The cytosolic free Ca²⁺ concentration in resting smooth-muscle cells has been estimated at ~130 nM with the use of Ca²⁺ indicator dyes (quin-2

Fig. 6. Effect of actin on digestion of phosphorylated caldesmon

Caldesmon (7.0 μM) was phosphorylated to the extent of 1.9 mol of P_i /mol by incubation at 30 °C in 20 mM-Tris/HCl (pH 7.5)/5 mM-MgCl₂/0.1 mM-CaCl₂/0.5 mM-dithiothreitol/2.5 μM-calmodulin/0.5 mM-[γ -³²P]ATP (42 c.p.m./pmol). Conditions were adjusted to 20 mM-Tris/HCl (pH 7.5)/50 mM-KCl/3.5 mM-MgCl₂/1.75 μM-calmodulin/0.35 mM-ATP/1 mM-EGTA/0.5 mM-dithiothreitol/3.5 μM-caldesmon in the presence (□) or the absence (○) of actin (35 μM). Digestion was initiated by addition of α -chymotrypsin [proteinase/caldesmon = 1:1000 (w/w)]. Samples were withdrawn at the indicated times for SDS/PAGE. Panel (a) shows the disappearance of intact caldesmon (141 kDa; band 1 in Fig. 4), and panel (b) shows the appearance of a major 95 kDa band (band 3 in Fig. 4) quantified by laser densitometry of the Coomassie Blue-stained gels. Values represent the means ($\pm$ S.D.) of four separate experiments.

Fig. 7. Calmodulin-Sepharose affinity chromatography of caldesmon digested with *S. aureus* V8 protease

Caldesmon (7 μM) was incubated at 30 °C for 3 min with *S. aureus* V8 protease [enzyme/substrate = 1:2000 (w/w)] in 20 mM-Tris/HCl (pH 7.5)/1 mM-EGTA/5 mM-dithiothreitol. Digestion was stopped by addition of di-isopropyl fluorophosphate (final concn. 2 mM). Remaining procedures were carried out at 4 °C or on ice. CaCl₂ was added to a final concentration of 1.2 mM (0.2 mM excess over EGTA), and the digest was loaded on to a column (1 cm × 10 cm) of calmodulin-Sepharose previously equilibrated with 20 mM-Tris/HCl (pH 7.5)/0.2 mM-CaCl₂/5 mM-dithiothreitol. Flow rate = 10 ml/h; fraction size = 1.0 ml. The column was washed with equilibration buffer until A_{280} returned to baseline, and bound proteins were eluted (indicated by the arrow) with 20 mM-Tris/HCl (pH 7.5)/1 mM-EGTA/5 mM-dithiothreitol. Samples of column fractions were examined by SDS/PAGE and assayed for histone kinase activity in the presence (○) and the absence (●) of Ca²⁺. Key to gel lanes: 1, untreated caldesmon (20 μg); 2–8 = fractions 8, 10, 12, 27, 28, 29 and 30 respectively (20 μl/lane). The positions of molecular-mass marker proteins are indicated (a–h): myosin heavy chain (205 kDa), β -galactosidase (116), phosphorylase *b* (97.4), bovine serum albumin (66.2), ovalbumin (45), carbonic anhydrase (29), soya-bean trypsin inhibitor (20.1) and lysozyme (14.4).

Fig. 8. Tentative domain map of caldesmon

On the basis of earlier work and the digestion studies described herein, a tentative domain map of caldesmon depicting the calmodulin (CaM)- and actin-binding sites, the kinase active site and the sites of autophosphorylation (Phos.) was constructed (see the Discussion section). Major chymotryptic peptides are numbered as in Fig. 4. Asterisks (*) denote phosphopeptides in digests of ^{32}P -caldesmon. The N- and C-termini are also shown.

and fura-2) (Williams & Fay, 1986; Williams *et al.*, 1987). Cytosolic free $[\text{Ca}^{2+}]$ rises to $\sim 0.5\text{--}0.7\ \mu\text{M}$ in response to contractile agents (such as carbachol) or depolarizing stimuli (Williams *et al.*, 1987). The calmodulin concentration in smooth muscle is $\sim 20\text{--}50\ \mu\text{M}$ (Hartshorne, 1987). Caldesmon is $11\ \mu\text{M}$ (Ngai & Walsh, 1985a) and myosin light-chain kinase is $4.6\ \mu\text{M}$ (Ngai & Walsh, 1985a); these are the major calmodulin-binding proteins in smooth muscle. The Ca^{2+} -dependence of caldesmon autophosphorylation described here therefore suggests that caldesmon may be at least partially phosphorylated in the resting cell.

Proteolytic-digestion studies indicate that calmodulin binding and phosphorylation have no effect on the rate of digestion, and only phosphorylation has a slight effect on the pattern of peptides generated. Calmodulin binding and phosphorylation therefore do not induce major conformational changes in caldesmon, although they do have important effects on its functional properties. Binding of caldesmon to actin has more pronounced effects on chymotryptic digestion: although the same peptides are generated from caldesmon, whether free or bound to actin or actin/tropomyosin, the rate of digestion is significantly decreased when caldesmon is bound. Interpretation of the results of these digestion studies is greatly facilitated by the fact that caldesmon is highly susceptible to proteolytic degradation (Ngai & Walsh, 1985b; Szpacenko & Dabrowska, 1986); under the conditions used (i.e. a very high caldesmon:proteinase

ratio), neither actin nor tropomyosin was degraded to any extent.

Limited proteolysis has also been very useful in elucidating the domain structure of caldesmon. Calmodulin- and actin-binding and ability to inhibit the actin-activated myosin Mg^{2+} -ATPase were all retained in an M_r -18000 peptide (Szpacenko & Dabrowska, 1986), which Fujii *et al.* (1987) have placed at the C-terminus. Chymotryptic digestion of phosphorylated caldesmon generated labelled peptides of 115, 95 and 26 kDa, the 26 kDa phosphopeptide retaining essentially all the incorporated phosphate (Scott-Woo & Walsh, 1988). None of these peptides retained the calmodulin-binding site, since they were not bound to calmodulin-Sepharose in the presence of Ca^{2+} (results not shown). Analysis of time courses of digestion of caldesmon by SDS/PAGE, autoradiography and kinase activity measurements (see Figs. 4, 5 and 7), coupled with the earlier work of Szpacenko & Dabrowska (1986), Fujii *et al.* (1987) and Scott-Woo & Walsh (1988), enables construction of a tentative map depicting the locations of the important functional domains of caldesmon (Fig. 8). Chymotrypsin cleaves caldesmon initially at two alternative sites to generate 115 and 24 kDa peptides or 95 and 42 kDa peptides. The 115 and 95 kDa peptides retain the phosphorylation sites, whereas the 24 and 42 kDa peptides retain the actin- and calmodulin-binding sites and the capacity to inhibit the actin-activated myosin Mg^{2+} -ATPase (Szpacenko & Dabrowska, 1986; Fujii *et al.*,

1987). The 95 kDa chymotryptic peptide is further degraded to 63 and 26 kDa peptides, the latter retaining the phosphorylation sites. This 26 kDa phosphopeptide could correspond to either the *N*-terminus or the *C*-terminus of the 95 kDa parent peptide. We have shown previously that a 93 kDa fragment of caldesmon can be generated during purification, perhaps by the action of calpain (Scott-Woo & Walsh, 1988). This peptide retains calmodulin-binding, kinase activity and phosphorylation sites, and therefore must correspond to the *C*-terminus of caldesmon, assuming that the assignment by Fujii *et al.* (1987) of the calmodulin-binding site is correct. Therefore the 26 kDa phosphopeptide corresponds to the *C*-terminus of the 95 kDa parent peptide and hence is located in the middle of the molecule. Finally, a 65 kDa peptide generated by *S. aureus* V8 protease binds to immobilized calmodulin in a Ca^{2+} -dependent manner, and exhibits histone kinase activity which requires Ca^{2+} and calmodulin; however, this peptide does not contain the phosphorylation sites, since it does not exhibit autophosphorylation, and digestion of phosphorylated caldesmon by *S. aureus* V8 protease generates the same peptide which is not phosphorylated.

An important area for future study will be the elucidation of the function of the *N*-terminal half of caldesmon. Caldesmon is known to exist in two major forms, of 140–150 kDa, as exemplified by chicken gizzard, and 70–80 kDa, as exemplified by human platelets. The lower-molecular-mass form binds to calmodulin and actin and inhibits the actin-activated myosin Mg^{2+} -ATPase. It will be interesting to determine whether or not this species of caldesmon is capable of autophosphorylation.

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