Autophosphorylation of smooth-muscle caldesmon

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Caldesmon, a major actin- and calmodulin-binding protein of smooth muscle, has been implicated in regulation of the contractile state of smooth muscle. The isolated protein can be phosphorylated by a co-purifying Ca^{2+} /calmodulin-dependent protein kinase, and phosphorylation blocks inhibition of the actomyosin ATPase by caldesmon [Ngai & Walsh (1987) *Biochem. J.* **244**, 417–425]. We have examined the phosphorylation of caldesmon in more detail. Several lines of evidence indicate that caldesmon itself is a kinase and the reaction is an intermolecular autophosphorylation: (1) caldesmon (141 kDa) and a 93 kDa proteolytic fragment of caldesmon can be separated by ion-exchange chromatography: both retain caldesmon kinase activity, which is Ca^{2+} /calmodulin-dependent; (2) chymotryptic digestion of caldesmon generates a Ca^{2+} /calmodulin-independent form of caldesmon kinase; (3) caldesmon purified to electrophoretic homogeneity retains caldesmon kinase activity, and elution of enzymic activity from a fast-performance-liquid-chromatography ion-exchange column correlates with caldesmon of M_r 141 000; (4) caldesmon is photoaffinity-labelled with 8-azido- $[\alpha\text{-}^{32}\text{P}]\text{ATP}$; labelling is inhibited by ATP, GTP and CTP, indicating a lack of nucleotide specificity; (5) caldesmon binds tightly to Affi-Gel Blue resin, which recognizes proteins having a dinucleotide fold. Autophosphorylation of caldesmon occurs predominantly on serine residues (83.3%), with some threonine (16.7%) and no tyrosine phosphorylation. Autophosphorylation is site-specific: 98% of the phosphate incorporated is recovered in a 26 kDa chymotryptic peptide. Complete tryptic/chymotryptic digestion of this phosphopeptide followed by h.p.l.c. indicates three major phosphorylation sites. Caldesmon exhibits a high degree of substrate specificity: apart from autophosphorylation, brain synapsin I is the only good substrate among many potential substrates examined. These observations indicate that caldesmon may regulate its own function (inhibition of the actomyosin ATPase) by Ca^{2+} /calmodulin-dependent autophosphorylation. Furthermore, caldesmon may regulate other cellular processes, e.g. neurotransmitter release, through the Ca^{2+} /calmodulin-dependent phosphorylation of other proteins such as synapsin I.

INTRODUCTION

Caldesmon was discovered as a major calmodulin-binding protein of smooth muscle, which is also capable of interacting with F-actin (Sobue *et al.*, 1981). Caldesmon inhibits the actin-activated myosin Mg^{2+} -ATPase in a contractile system reconstituted *in vitro* from purified actin, myosin, tropomyosin, calmodulin and myosin light-chain kinase, without affecting myosin phosphorylation (Ngai & Walsh, 1984), suggesting that caldesmon may play a role in the regulation of smooth-muscle contraction. These observations have been confirmed by several investigators using caldesmon and myosin from a variety of sources (Dabrowska *et al.*, 1985; Marston & Lehman, 1985; Sobue *et al.*, 1985; Clark *et al.*, 1986; Lash *et al.*, 1986; Lim & Walsh, 1986; Chalovich *et al.*, 1987).

Caldesmon can be phosphorylated by a co-purifying Ca^{2+} /calmodulin-dependent protein kinase and dephosphorylated by a protein phosphatase (Ngai & Walsh, 1984, 1985a). Phosphorylation of caldesmon blocks its inhibitory effect on the smooth-muscle actin-activated myosin Mg^{2+} -ATPase (Ngai & Walsh, 1984, 1987).

A possible physiological role for caldesmon in modulating actin–myosin interactions and therefore the contractile state of smooth muscle has been proposed, and this function may in turn be regulated by Ca^{2+} -dependent reversible phosphorylation of caldesmon (Walsh, 1987). Caldesmon kinase is distinct from two other Ca^{2+} /calmodulin-dependent protein kinases, i.e. myosin light-chain kinase and glycogen phosphorylase *b* kinase (Ngai & Walsh, 1985a). In the present paper we provide several lines of evidence indicating that caldesmon itself is a kinase and that caldesmon phosphorylation is actually an intermolecular autophosphorylation reaction. Furthermore, autophosphorylation is site-specific and occurs predominantly on serine residues. Caldesmon exhibits a high degree of substrate specificity, caldesmon itself and brain synapsin being the best substrates.

MATERIALS AND METHODS

Materials

$[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (10–40 Ci/mmol) was purchased from Amersham Corp. (Oakville, Ontario, Canada) and 8-

azido- $[\alpha\text{-}^{32}\text{P}]\text{ATP}$ (3.8 Ci/mmol) from ICN Biomedicals (Montreal, Que., Canada). CNBr-activated Sepharose 4B, ninhydrin spray reagent, dithiothreitol, calf thymus histones II-S, III-S and V-S, glycogen synthase, α -casein, phosphorylase *b*, protamine, phosphovitin, M_r markers for SDS/PAGE, anti-(mouse IgG)-alkaline phosphatase conjugate and 5-bromo-4-chloroindol-3-yl phosphate were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Chymotrypsin A_4 and subtilisin were purchased from Boehringer Mannheim (Dorval, Quebec, Canada). DEAE-Sephacel and the Mono Q HR 5/5 prepacked column were purchased from Pharmacia (Dorval, Quebec, 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) and synapsin I (Ueda & Greengard, 1977), chicken gizzard myosin light-chain kinase (Ngai *et al.*, 1984), myosin (Persechini & Hartshorne, 1981), the 20 kDa light chain of myosin (Hathaway & Haeberle, 1983), actin (Ngai *et al.*, 1986), vinculin, filamin and α -actinin (Feramisco & Burridge, 1980), tropomyosin (Bretscher, 1984), rabbit skeletal-muscle actin (Pardee & Spudich, 1982; Zot & Potter, 1981) and troponin (I+T+C) (Potter, 1982), bovine cardiac C-protein (Starr & Offer, 1982) and bovine aortic caldesmon (Clark *et al.*, 1986). Fodrin (bovine brain) was generously provided by C. Y. Wang and Dr. J. H. Wang (Department of Medical Biochemistry, University of Calgary).

Caldesmon with caldesmon kinase activity (CaD/CaDK) was purified by a modification of the procedure of Ngai *et al.* (1984). The initial stages of purification (DEAE-Sephacel and calmodulin-Sepharose chromatography) were performed as described previously (Ngai *et al.*, 1984). Fractions from calmodulin-Sepharose chromatography with the highest activity were pooled and loaded directly on to a DEAE-Sephacel column (1 cm $\times$ 20 cm) previously equilibrated with 20 mM-Tris/HCl (pH 7.5)/0.1 M-NaCl/0.1 mM-dithiothreitol/1 mM-EGTA. The column was washed with equilibration buffer, and CaD/CaDK was eluted with a linear salt gradient (0.1–0.15 M-NaCl, total volume 50 ml) in buffer. Alternatively, dithiothreitol was added to the CaD/CaDK to a final concentration of 5 mM, and the sample was loaded on to a prepacked anion-exchange f.p.l.c. column (Mono-Q HR 5/5). CaD/CaDK was eluted as described in the legend to Fig. 6.

Electrophoretic and immunoblotting procedures

Electrophoresis was performed as previously described (Clark *et al.*, 1986). Isoelectric focusing was carried out in tube gels as described by O'Farrell (1975), and gels were stained with Fast Green (Allen *et al.*, 1980). Proteins were transblotted as described by Ngai & Walsh (1985b) and detected by using, as the primary antibody, a monoclonal antibody raised against human platelet caldesmon (Dingus *et al.*, 1986), which was generously provided by Dr. J. Bryan (Baylor College of Medicine, Houston, TX, U.S.A.). Anti-(mouse IgG)-alkaline phosphatase conjugate was used as the secondary

antibody. Detection was performed with 5-bromo-4-chloroindol-3-yl phosphate as previously described (McDonald *et al.*, 1987).

Photoaffinity labelling

Proteins were covalently labelled with 8-azido- $[\alpha\text{-}^{32}\text{P}]\text{ATP}$ essentially as described by Potter & Haley (1983).

'Cleveland' peptide mapping

Caldesmon (40 μg) and the 93 kDa polypeptide (48 μg) were electrophoresed and the 141 kDa and 93 kDa bands cut out of the gel after brief staining and destaining for digestion by the *Staphylococcus aureus* V8 protease (1 μg) in a second gel as described by Cleveland *et al.* (1977).

Phosphoamino acid analysis

Caldesmon was phosphorylated under standard reaction conditions (see below) for 1 h to ensure maximal phosphorylation (2.95 mol of P_i /mol). The phosphorylated protein was dialysed overnight against two changes (10 litres each) of 10 mM- NH_4HCO_3 , freeze-dried and hydrolysed in 6 M-HCl (125 μl) for 2 h at 110 $^\circ\text{C}$. The hydrolysate was dried with a Speed Vac Concentrator (Savant model RH 20-12) for 2 h and redissolved in 30 μl of distilled deionized water. Standards of phosphoserine, phosphothreonine and phosphotyrosine were added, and half of the sample was spotted on to a cellulose thin-layer plate (Eastman-Kodak) for two-dimensional thin-layer electrophoresis (Hunter & Sefton, 1980). Standards were detected with ninhydrin spray reagent, and radiolabelled phosphoamino acids were identified by autoradiography. $[\text{P}_i]$ incorporation was quantified by scraping the phosphoamino acids from the plate and liquid-scintillation counting.

Enzymic assays

$[\text{P}_i]$ incorporation into caldesmon or exogenously added substrates was measured as described for myosin phosphorylation (Walsh *et al.*, 1983). Standard reaction mixture consisted of 20 mM-Tris/HCl (pH 7.5), 5 mM- MgCl_2 , 0.1 mM- CaCl_2 , 2.5 μM -calmodulin and 0.5 mM- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (~ 10000 c.p.m./nmol). To monitor enzymic activity during purification, 50 μl samples of column fractions were added per ml of reaction mixture in the presence or the absence of exogenously added substrate (histone III-S; 0.1 mg/ml).

To determine the substrate specificity of caldesmon kinase, the enzyme was preincubated for 1 h under standard reaction conditions in the presence of unlabelled ATP to ensure maximal phosphorylation. The putative substrate and radiolabelled ATP were then added to final concentrations of 0.1 or 0.2 mg/ml and 0.5 mM respectively. At the end of a second hour, a sample of the reaction mixture was added to an equal volume of SDS-gel sample buffer and boiled. The remainder of the reaction mixture was used to determine $[\text{P}_i]$ incorporation as previously described (Walsh *et al.*, 1983). Radiolabelled samples were subjected to SDS/PAGE and autoradiography. $[\text{P}_i]$ incorporation into the putative substrates was quantified by densitometric scanning of the autoradiographs. Controls were included

Fig. 1. Separation of caldesmon kinase activity into two peaks by ion-exchange chromatography

Caldesmon containing endogenous caldesmon kinase activity was further purified by ion-exchange chromatography (DEAE-Sephacel) as described in the Materials and methods section. Protein elution (A_{280} , —) and histone kinase activity in the presence of Ca^{2+} and calmodulin ($\bigcirc$ --- $\bigcirc$) were monitored. Bound protein eluted with a shallow $[\text{NaCl}]$ gradient (----). Flow rate = 12 ml/h; fraction size = 1 ml. Fractions were pooled as indicated (A and B) and analysed by SDS/PAGE. Key to gel lanes: 1, DEAE-Sephacel column load (10 μg); 2, pool A (1 μg); 3, pool B (10 μg). These gels were stained with Coomassie Brilliant Blue. Caldesmon ($\cdot$) of 141 kDa and the 93 kDa polypeptide ($\bigcirc$) are indicated.

for each substrate to determine substrate phosphorylation in the absence of caldesmon kinase. Net phosphate incorporation owing to caldesmon kinase is reported. In other experiments designed to determine initial rates of protein phosphorylation (see Table 1), reactions were initiated by the addition of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and samples were withdrawn at regular time intervals for quantification of total $^{32}\text{P}_i$ incorporation and for SDS/PAGE. Stained bands were cut out of the gel for scintillation counting to quantify $^{32}\text{P}_i$ incorporation into both caldesmon itself and exogenous substrate.

Other methods

Protein concentrations were determined by the Coomassie Blue dye-binding assay (Spector, 1978) by using 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) and myosin ($A_{280}^{1\%} = 5.6$; Greene *et al.*, 1983). Calmodulin was coupled to CNBr-activated Sepharose 4B as previously described (Walsh *et al.*, 1982).

RESULTS

Autophosphorylation of caldesmon

Caldesmon containing endogenous Ca^{2+} /calmodulin-dependent caldesmon kinase activity consists predominantly of the 141 kDa caldesmon polypeptide (Fig. 1, lane 1). Minor contaminants of lower M_r , including a 93 kDa polypeptide, are apparent in this preparation. When subjected to ion-exchange chromatography on DEAE-Sephacel, the 93 kDa polypeptide was recovered in the flow-through fractions, whereas the 141 kDa caldesmon was eluted in the salt gradient at ~ 0.12 M-NaCl (Fig. 1).

Measurements of histone kinase activity in the presence of Ca^{2+} and calmodulin revealed that enzymic activity was associated with both peaks A and B. Previously (Ngai & Walsh, 1985a), we suggested that the 93 kDa polypeptide may be Ca^{2+} /calmodulin-dependent caldesmon kinase. However, the data in Fig. 1 suggest that kinase activity is also associated with caldesmon itself. This conclusion was supported by the finding that the 93 kDa polypeptide is actually a proteolytic fragment of caldesmon which is presumably generated during purification, probably by the action of calpain (Ca^{2+} -activated neutral proteinase). 'Cleveland' peptide mapping of caldesmon and the 93 kDa polypeptide with *S. aureus* V8 proteinase generated seven peptides, six of which were common to the two proteins, and one of which was unique to the caldesmon digest (Fig. 2). The 93 kDa polypeptide was also capable of Ca^{2+} /calmodulin-dependent autophosphorylation (Fig. 3), indicating that this proteolytic fragment retains the calmodulin-binding site, the kinase active site and the site(s) of phosphorylation. As would be expected, the 93 kDa polypeptide was retained on a calmodulin affinity column in the presence of Ca^{2+} and was eluted with EGTA (results not shown).

These observations raised the possibility that caldesmon is a kinase which autophosphorylates in a Ca^{2+} /calmodulin-dependent manner. The following experimental results support this conclusion. Partial proteolysis of caldesmon with α -chymotrypsin (proteinase: substrate = 1:1000, w/w) generated a series of peptides, the major ones of which were of 115, 95, 65 and 42 kDa, plus several in the region of 18–30 kDa. The 42 kDa fragment and several smaller fragments, together with undigested caldesmon, bound to a calmodulin affinity column in the presence of Ca^{2+} and could be eluted with EGTA. This fraction exhibited Ca^{2+} /calmodulin-dependent histone kinase activity (Fig. 4a)

Fig. 2. 'Cleveland' peptide mapping of caldesmon and the 93 kDa polypeptide

The patterns of digestion of caldesmon (CaD) and the 93 kDa polypeptide were compared by treating the two proteins with *S. aureus* V protease as described by Cleveland *et al.* (1977). Key to gel lanes: 1, M_r marker proteins (a = phosphorylase b, 97.4 kDa; b = bovine serum albumin, 66.2 kDa; c = ovalbumin, 45 kDa; d = carbonic anhydrase, 29 kDa; e = soya-bean trypsin inhibitor, 20.1 kDa; f = lysozyme, 14.4 kDa); 2 and 4 = caldesmon + 0 and 1 μ g, respectively, of *S. aureus* V8 protease; 3 and 5 = 93 kDa polypeptide + 0 and 1 μ g, respectively, of *S. aureus* V8 protease. Six common peptides [33, 30 (doublet), 18, 13 and 12 kDa] and one peptide (14 kDa) unique to the caldesmon digest are apparent: the common peptides are denoted by horizontal lines at the right, and the unique peptide is shown by the arrow. The *S. aureus* V8 protease (27 kDa; Houmard & Drapeau, 1972) did not co-migrate with any of these peptides and was not visible on the stained gel at the loading level used.

and autophosphorylation (Fig. 4b). The other peptides were recovered in the flow-through fractions and exhibited Ca^{2+} /calmodulin-independent histone kinase activity (Fig. 4a) and autophosphorylation (Fig. 4b). In fact, the activity expressed in the absence of Ca^{2+} was consistently higher than that in the presence of Ca^{2+} . Similar results were obtained with two different preparations of CaD/CaDK.

Fig. 5 shows that only undigested caldesmon (lanes 2, 5 and 6) and the 115 and 95 kDa chymotryptic fragments (lanes 1 and 2) are cross-reactive with a monoclonal antibody to human platelet caldesmon. The 93 kDa fragment (lane 4), which overlaps with the 95 kDa chymotryptic fragment, but retains the calmodulin-binding site, does not contain the antigenic site.

Fig. 3. Autophosphorylation of the 93 kDa polypeptide

Incorporation into the 93 kDa polypeptide was determined, under conditions described in the Materials and methods section, in the presence of 0.1 mM- CaCl_2 (O) or 1 mM-EGTA (□). Protein concentration was 0.1 mg/ml and samples (0.1 ml) were withdrawn at the indicated times for determination of $[\text{P}^{32}]\text{P}_i$ incorporation. Phosphorylation of the 93 kDa polypeptide in the presence of Ca^{2+} was verified by SDS/PAGE and autoradiography. The inset shows the autoradiograms in the presence (lane 1) and the absence (lane 2) of Ca^{2+} .

Ion-exchange f.p.l.c. of calmodulin-Sepharose-purified caldesmon enabled purification of the 141 kDa protein to > 96% purity (based on ten preparations) as determined by scanning laser densitometry of Coomassie Blue-stained gels (Fig. 6). Immunoblotting with a monoclonal antibody to human platelet caldesmon revealed minor contamination of the purified caldesmon (Fig. 6, lane b) with cross-reactive polypeptides, which are presumably proteolytic fragments of caldesmon (Fig. 6, lane c). These could account for the < 3% of contaminating proteins detected by densitometric scanning of Coomassie Blue-stained gels of the preparation. Fig. 6 also depicts the protein elution profile from this f.p.l.c. column, the gel pattern of selected fractions and the profile of Ca^{2+} /calmodulin-dependent histone kinase activity. It is clear that the enzymic activity correlates with the 141 kDa caldesmon polypeptide. Measurements of caldesmon autophosphorylation gave a profile identical with that shown for histone kinase activity (results not shown). The appearance of multiple peaks and trailing apparent in the elution profile is probably due to multiple charge variants of caldesmon (Bretscher, 1984). The presence of charge variants of caldesmon is shown by isoelectric focusing of the Mono Q-purified protein (Fig. 6, lane a): seven major bands are apparent, with pI

Fig. 4. Generation of Ca^{2+} /calmodulin-independent caldesmon kinase by partial proteolysis with α -chymotrypsin

Caldesmon (1.0 mg/ml) was digested at 30 °C with α -chymotrypsin (1.0 $\mu\text{g}/\text{ml}$) in 20 mM-Tris/HCl (pH 7.5)/50 mM-KCl/1 mM-EGTA/0.5 mM-dithiothreitol in a reaction volume of 5 ml for 2 min. Digestion was stopped by the addition of di-isopropyl fluorophosphate (0.1 M in propan-2-ol) to a final concentration of 2 mM. CaCl_2 (2 mM) and additional dithiothreitol (5 mM) were added to the digest, which was loaded on a column (1 cm $\times$ 10 cm) of calmodulin-Sepharose previously equilibrated with 20 mM-Tris/HCl (pH 7.5)/5 mM-dithiothreitol/0.2 mM- CaCl_2 . Flow rate = 10 ml/h; fraction size = 1 ml. After all unbound fragments were washed off with equilibration buffer, bound peptides were eluted with 20 mM-Tris/HCl (pH 7.5)/5 mM-dithiothreitol/1 mM-EGTA (applied at the arrow). Selected fractions were assayed for histone kinase activity (a) and autophosphorylation (b). The inset in (a) shows Coomassie Blue-stained SDS/polyacrylamide gels of the total digest (left lane) and the pooled flow-through fractions (right lane).

values in the range 5.78–5.9, plus additional minor bands. Two-dimensional isoelectric-focusing-SDS/PAGE and Coomassie Blue staining revealed that all these bands had $M_r = 141\,000$ and all cross-reacted with the monoclonal anti-(human platelet caldesmon) antibody in immunoblots of two-dimensional gels (results not shown).

Photoaffinity labelling of caldesmon purified by f.p.l.c. indicated that caldesmon contains a nucleotide-binding site (Fig. 7). Caldemon was incubated with 5 mM- MgCl_2 and 10 μM -8-azido- $[\alpha\text{-}^{32}\text{P}]$ -ATP alone (Fig. 7, lane 1) or with 0.8 mM-ATP (lane 2), 0.8 mM-GTP (lane 3) or 0.8 mM-CTP (lane 4), and photoactivated as described in the Materials and methods section. Azido-ATP labelling

Fig. 5. Immunoblotting of caldesmon and its fragments

Key to lanes: 1, chymotryptic peptides of caldesmon which are not retained on calmodulin-Sepharose; 2, total chymotryptic digest of caldesmon; 3, brain synapsin I; 4, 93 kDa polypeptide; 5, 6, untreated caldesmon; 7, M_r marker proteins (a = myosin heavy chain, 205 kDa; b = β -galactosidase, 116 kDa; c = 97.4 kDa; d = 66.2 kDa; e = 45 kDa).

was observed in the absence of unlabelled nucleotides (lane 1), and was decreased by ATP, GTP and CTP to 44.7, 48.4 and 58.8% respectively of the labelling observed in the presence of 8-azido- $[\alpha\text{-}^{32}\text{P}]$ ATP alone (lanes 2–4). No photoaffinity labelling occurred in the absence of photoactivation (lane 5). Quantitative data on the extents of labelling were obtained by laser densitometry of the autoradiograms shown in Fig. 7 and by gel slicing and scintillation counting of radioactivity. The extent of azido-ATP labelling of caldesmon was also decreased by the non-hydrolysable ATP analogue adenosine 5'- $[\beta\gamma\text{-imido}]$ triphosphate (to 44.5%) and by NAD (to 67.5%).

Controls for these photoaffinity-labelling experiments were carried out with actin, which is known to bind ATP with high affinity, and tropomyosin, which does not bind ATP. The results demonstrated, as expected, very low tropomyosin labelling (< 100 c.p.m./nmol). On the other hand, actin was strongly labelled with azido-ATP (1790 c.p.m./nmol). This labelling was inhibited by ATP (to 490 c.p.m./nmol), but not by GTP (1740 c.p.m./nmol) or CTP (1680 c.p.m./nmol). The extent of labelling of caldesmon (4310 c.p.m./nmol) was actually greater than that observed with the positive control, actin. The photoaffinity-labelling experiments indicate that caldesmon possesses a high-affinity ATP-binding site(s), consistent with it being a kinase. The ATP-binding site in caldesmon, unlike that in actin, lacks nucleotide specificity.

Additional evidence that caldesmon is a kinase is provided by the observation that caldesmon binds tightly to an Affi-Gel Blue column: a high concentration (1.7 M) of NaCl was required to elute the protein (results not shown). This resin has a high affinity for proteins which contain a dinucleotide fold (Thompson *et al.*, 1975); however, it is not absolutely specific for such proteins.

Fig. 6. F.p.l.c. purification of caldesmon

After calmodulin-Sepharose affinity chromatography, caldesmon was further purified by f.p.l.c. on a Mono Q HR 5/5 column. Flow rate = 1 ml/min; fraction size = 1 ml. The column was pre-equilibrated with 20 mM-Tris/HCl (pH 7.5)/1 mM-EGTA/5 mM-dithiothreitol/0.1 M-NaCl (solution A) and the protein was applied in the same buffer. Bound proteins were eluted by application of the indicated [NaCl] gradients (---), where solution B was 20 mM-Tris/HCl (pH 7.5)/1 mM-EGTA/5 mM-dithiothreitol/0.5 M-NaCl. Protein (A_{280} , —) and histone kinase activity (O---O) were measured, and selected fractions were analysed by SDS/PAGE and Coomassie Blue staining. Key to gel lanes: 1–28 = column load, fractions 2, 6, 9, 19–36, 38, 42, 46, 51, 52 and 53 respectively. The gel insets depict isoelectric focusing (pH gradient = 4.5–8.25) of Mono Q-purified caldesmon (100 µg) stained with Fast Green (a), SDS/PAGE of Mono Q-purified caldesmon (15 µg) stained with Coomassie Blue (b) and the corresponding immunoblot (2 µg) (c). The arrow indicates caldesmon (141 kDa).

Characterization of caldesmon autophosphorylation

Phosphoamino acid analysis by two-dimensional thin-layer electrophoresis revealed that most (83.3%) of the P_i incorporated into caldesmon was on serine residues, with some (16.7%) on threonine and none on tyrosine residues. Site-specificity of autophosphorylation was probed initially by partial chymotryptic digestion of the phosphorylated protein. Digestion of phosphorylated caldesmon generated major phosphopeptides of 115, 95 and 26 kDa (Fig. 8). Laser densitometry of autoradiograms of digests of phosphorylated caldesmon revealed that 98.0% of the radioactivity in phosphorylated caldesmon was recovered in the 26 kDa peptide. This peptide was cut out of the gel and completely digested with trypsin and chymotrypsin. The resultant peptides were separated by h.p.l.c. on a reverse-phase C_{18} column. Quantification of [^{32}P] P_i in the eluted fractions revealed three major phosphopeptides (Fig. 9), indicating a high degree of site specificity.

Fig. 10 demonstrates that caldesmon autophosphorylation is an intermolecular reaction, since the specific enzymic activity is dependent on the protein concentration. If it were an intramolecular reaction, the specific activity would be completely independent of protein concentration.

Substrate specificity

A wide variety of putative substrates of caldesmon kinase was investigated. Apart from chicken gizzard caldesmon itself, brain synapsin I was the best substrate of chicken gizzard caldesmon kinase (Table 1). Heat-treated chicken gizzard caldesmon, which lacks kinase activity, was not as good a substrate (0.41 mol of P_i /mol of protein) as the non-heated protein. Similarly, heat-treated bovine aortic caldesmon (0.33 mol of P_i /mol of protein) and bovine cardiac caldesmon (0.16 mol of P_i /mol of protein) were phosphorylated by the chicken gizzard enzyme. Various histones (calf thymus types

Fig. 7. Photoaffinity labelling of caldesmon with 8-azido- $[\alpha\text{-}^{32}\text{P}]\text{ATP}$

Photoaffinity labelling of f.p.l.c.-purified caldesmon (0.2 mg/ml) was carried out for 5 min at 22 °C in 20 mM-Tris/HCl (pH 7.5)/5 mM-MgCl₂/10 μM -8-azido- $[\alpha\text{-}^{32}\text{P}]\text{ATP}$. Reactions were quenched by addition of SDS-gel sample buffer and boiling before SDS/PAGE and autoradiography. The Figure shows the resultant autoradiogram. Key to lanes: 1, caldesmon + 8-azido- $[\alpha\text{-}^{32}\text{P}]\text{ATP}$; 2–4, as for 1, but with 0.8 mM-ATP, -GTP or -CTP respectively; 5, as for 1, but without photoactivation. Loading = 5 μg of caldesmon (CaD)/lane. The following M_r marker proteins are indicated: a = 205 kDa; b = 116 kDa; c = 97.4 kDa; d = 66 kDa; e = 45 kDa; f = 29 kDa; g = 20.1 kDa.

II-S, III-S and V-S) and casein were phosphorylated by caldesmon, albeit to low stoichiometry (~ 0.1 mol of P_i /mol of protein) (Table 1). Nevertheless, histones provide a useful substrate *in vitro*, since they are available cheaply in relatively large amounts. The isolated 20 kDa light chain of smooth-muscle myosin was phosphorylated to 0.42 mol of P_i /mol, but intact myosin was not a substrate of caldesmon kinase. Several other proteins were shown not to be substrates of caldesmon kinase: smooth-muscle myosin light-chain kinase, bovine cardiac C-protein, bovine brain fodrin, rabbit skeletal-muscle glycogen synthase, phosphorylase *b*, troponin (I + T + C), actin and tropomyosin, smooth-muscle actin, filamin, vinculin and α -actinin, protamine and phosvitin. As shown in Table 1, the presence of an exogenous substrate inhibited the rate of autophosphorylation; casein was, however, an exception.

DISCUSSION

Although the physiological role of caldesmon has not been clearly established, a significant body of evidence indicates that the isolated protein can regulate the actin-activated Mg^{2+} -ATPase activity of smooth-muscle myosin. When reconstituted with purified actin, myosin,

Fig. 8. Partial chymotryptic digestion of phosphorylated caldesmon

Caldesmon (0.5 mg/ml) was phosphorylated to 1.2 mol of P_i /mol by incubation with 20 mM-Tris/HCl (pH 7.5)/5 mM-MgCl₂/1 mM-dithiothreitol/0.1 mM-CaCl₂/calmodulin (42 μg /ml)/0.2 mM- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (200 c.p.m./pmol) at 30 °C for 5 min in a reaction volume of 0.4 ml. Phosphorylation was arrested by addition of EGTA to a final concentration of 1 mM, and caldesmon was immediately digested with α -chymotrypsin [proteinase:caldesmon = 1:2000 (w/w)] at 30 °C. Samples (20 μl) of the reaction mixture were withdrawn at selected times and added to an equal volume of boiling SDS-gel sample buffer. Digests (20 μg /lane) were analysed by SDS/PAGE and autoradiography. The autoradiogram is shown in this Figure. Key to lanes: 1, 1 min digestion; 2, 10 min digestion; 3, 60 min digestion. Numbers at the left indicate molecular masses (kDa) of the major phosphopeptides.

tropomyosin, calmodulin and myosin light-chain kinase, caldesmon inhibits the actin-activated myosin Mg^{2+} -ATPase without affecting myosin phosphorylation (Ngai & Walsh, 1984). Two alternative mechanisms have been considered which could regulate this inhibitory effect of caldesmon. Firstly, the Ca^{2+} -dependent binding of calmodulin to caldesmon removes the caldesmon from F-actin and thereby relieves the caldesmon-induced inhibition of actin-myosin interaction (Sobue *et al.*, 1982). However, the high calmodulin concentration required to dissociate caldesmon from F-actin suggests that this mechanism may be unphysiological (Marston & Smith, 1985; Lehman, 1986). Nevertheless, the possibility remains that Ca^{2+} /calmodulin binding to caldesmon

Fig. 9. Separation of tryptic/chymotryptic fragments of the 26 kDa phosphopeptide of caldesmon by reverse-phase h.p.l.c.

The ^{32}P -labelled 26 kDa peptide derived by partial chymotryptic digestion of caldesmon (phosphorylated for 5 min to 1.2 mol of P_i /mol) was cut out of the gel, swollen for 1 h in acetic acid/methanol/water (1:5:4, by vol.), washed with 50 % (v/v) methanol and freeze-dried. The dried slices were re-swollen in 50 mM- NH_4HCO_3 (pH 8.0)/1 mM-dithiothreitol containing trypsin (75 $\mu\text{g}/\text{ml}$) and α -chymotrypsin (75 $\mu\text{g}/\text{ml}$) and incubated for 24 h at 30 °C. The eluate was collected and the gel slices were further digested with 50 mM- NH_4HCO_3 (25 $\mu\text{g}/\text{ml}$) (pH 8.0)/1 mM-dithiothreitol containing trypsin (25 $\mu\text{g}/\text{ml}$) and α -chymotrypsin (25 $\mu\text{g}/\text{ml}$) for 6 h at 30 °C. The eluates (which contained all the radioactivity) were combined, freeze-dried and dissolved in 250 μl of aq. 0.1 % (v/v) trifluoroacetic acid. The digest (200 μl) was applied to a Vydac reverse-phase C_{18} column at a flow rate of 1 ml/min and 30 °C. Fractions (0.5 ml) were collected for determination of $^{32}\text{P}\text{P}_i$ by Čerenkov counting. Peptides were eluted by using the following linear gradient elution protocol, where solution A is 0.1 % trifluoroacetic acid and solution B is 0.1 % trifluoroacetic acid in acetonitrile: time 0, 0 % B; time 50 min, 25 % B; time 65 min, 65 % B; time 72 min, 0 % B. Peptide elution was monitored by recording A_{214} : 80 discrete peptide peaks were observed. As shown in the Figure, which depicts the elution of peptide-bound $^{32}\text{P}\text{P}_i$, only three phosphopeptides were detected. The recovery of radioactivity from the column was 77.7 %.

Fig. 10. Caldesmon phosphorylation is an intermolecular reaction

The specific activity of a caldesmon kinase was determined at the indicated caldesmon concentrations under the following conditions: 20 mM-Tris/HCl (pH 7.5)/5 mM- MgCl_2 /0.1 mM- CaCl_2 /2.5 μM -calmodulin/0.5 mM- $[\gamma\text{-}^{32}\text{P}]\text{-ATP}$ (31 000 c.p.m./nmol) at 30 °C in a reaction volume of 1.5 ml. Samples (0.2 ml) were withdrawn at 2, 4, 6, 8, 10, 12 and 14 min of incubation for determination of protein-bound $^{32}\text{P}\text{P}_i$. Specific activities were calculated by linear regression analysis of the linear time-course data obtained at each caldesmon concentration; $r = 0.966$.

Table 1. Substrate specificity of caldesmon kinase

Exogenous substrate	Specific activity (nmol of P_i /min per mg)		Maximal stoichiometry (mol of P_i /mol)
	Substrate phosphorylation	Autophosphorylation	
None	—	0.73	1.16
Synapsin I	0.63	0.09	1.55
Aorta caldesmon	0.54	0.09	0.33
Histone III-S	0.27	0.32	0.11
Casein	0.54	0.77	0.16
Phosphorylase b	0.06	0.59	0.07

could relieve inhibition of the actomyosin ATPase without dissociation of caldesmon from the thin filament (Smith *et al.*, 1987). Secondly, phosphorylation of caldesmon by a Ca^{2+} /calmodulin-dependent protein kinase blocks its inhibition of the actin-activated myosin

Mg^{2+} -ATPase (Ngai & Walsh, 1984, 1987). Consistent with a potential physiological role of caldesmon phosphorylation, we have demonstrated the existence of caldesmon phosphatase activity in smooth muscle (Ngai & Walsh, 1984, 1985a) and have demonstrated that stoichiometric phosphorylation of caldesmon can occur in a reconstituted actomyosin system on a similar time scale as relief of inhibition of the actin-activated myosin Mg^{2+} -ATPase (Ngai & Walsh, 1987). Lash *et al.* (1986) have shown that caldesmon enhances the affinity of phosphorylated and non-phosphorylated forms of smooth-muscle heavy meromyosin for actin by approx. 40-fold, suggesting that caldesmon could enhance the affinity of the myosin cross-bridge for actin while inhibiting the actomyosin ATPase. Our prediction then

would be that Ca^{2+} -dependent phosphorylation of caldesmon would reverse these effects.

As part of a continuing study of the caldesmon phosphorylation/dephosphorylation system, we have investigated the Ca^{2+} /calmodulin-dependent caldesmon kinase in more detail. We showed previously that the kinase co-purified with caldesmon through calmodulin-Sepharose affinity chromatography (Ngai & Walsh, 1984, 1985a). This preparation consists predominantly of caldesmon (141 kDa) (Fig. 1). Several observations led to the conclusion that caldesmon itself is the kinase and that caldesmon phosphorylation is therefore an autophosphorylation. (1) Ion-exchange chromatography of the affinity-purified caldesmon/caldesmon kinase preparation separated two peaks of Ca^{2+} /calmodulin-dependent caldesmon kinase activity, one associated with caldesmon itself and the other with a 93 kDa polypeptide shown to be a proteolytic fragment of caldesmon. Caldesmon is known to be extremely sensitive to proteolysis (Ngai & Walsh, 1985b). (2) Partial proteolysis of caldesmon with α -chymotrypsin generated a Ca^{2+} /calmodulin-independent form of caldesmon kinase. (3) Caldesmon could be further purified (to > 97% homogeneity) by f.p.l.c. Caldesmon kinase activity elution correlated very well with elution of the 141 kDa caldesmon polypeptide, and the < 3% of contaminating proteins could be accounted for by proteolytic fragments of caldesmon as revealed by immunoblotting using monoclonal antibodies against human platelet caldesmon. (4) Caldesmon was photoaffinity labelled with 8-azido- $[\alpha\text{-}^{32}\text{P}]\text{ATP}$. Labelling could be inhibited by ATP, GTP, CTP, adenosine 5'- $[\beta\gamma\text{-imido}]\text{triphosphate}$ and NAD, indicating a lack of nucleotide specificity similar to that shown by other protein kinases (Flockhart *et al.*, 1984). The reliability of these results was substantiated by positive (actin) and negative (tropomyosin) controls and quantification of the photoaffinity-labelling data. (5) Caldesmon bound tightly to an Affi-Gel Blue column, which recognizes proteins with a dinucleotide fold or similar structure (Thompson *et al.*, 1975). Taken together, these observations are strongly supportive of the conclusion that caldesmon is a Ca^{2+} /calmodulin-dependent protein kinase capable of autophosphorylation.

We also examined some characteristics of the phosphorylation of caldesmon. Autophosphorylation was found to be an intermolecular reaction and occurred predominantly on serine residues, with some threonine and no tyrosine phosphorylation. A high degree of site-specificity of autophosphorylation was demonstrated by proteolysis of phosphorylated caldesmon with trypsin and chymotrypsin. Three phosphorylation sites, all in a 26 kDa peptide, were indicated by these studies. This is consistent with the maximum stoichiometry of phosphorylation observed on prolonged incubation of caldesmon with MgATP^{2-} (2.95 mol of P_i /mol of caldesmon). However, in order to be of functional significance, phosphorylation extents achieved at much shorter times must correlate with functional changes. We have previously shown that a phosphorylation extent of ~ 1.0 mol of P_i /mol is achieved in 5 min, i.e. on the same time scale as loss of actomyosin Mg^{2+} -ATPase inhibitory activity (Ngai & Walsh, 1987). We have repeatedly observed phosphorylation of 0.9–1.2 mol of P_i /mol of caldesmon within 5 min. The phosphopeptide studies reported herein suggest that there are three

phosphorylation sites located close together (within 26 kDa) and that all three are partially phosphorylated during the first 5 min. Prolonged incubation results in additional phosphate incorporation, primarily into the same three sites (results not shown). Perhaps phosphorylation of any one of the three phosphorylation sites is sufficient to block caldesmon's inhibitory action on the actomyosin Mg^{2+} -ATPase.

Finally, caldesmon kinase exhibits a high degree of substrate specificity: of a wide variety of potential protein substrates examined, only brain synapsin I was identified as a good substrate. This protein has been implicated in the regulation of neurotransmitter release from presynaptic nerve terminals (Böhler & Greengard, 1987). Nerve depolarization results in synapsin I phosphorylation and neurotransmitter release. Isolated synapsin I is phosphorylated by cyclic-AMP-dependent protein kinase, protein kinase C and calmodulin-dependent protein kinases I and II (Nester & Greengard, 1986); the latter are clearly distinguishable from caldesmon. The possibility arises therefore that caldesmon may regulate its own function (inhibition of the actomyosin ATPase) by Ca^{2+} /calmodulin-dependent autophosphorylation, but also other cellular processes, e.g. neurotransmitter release, through the Ca^{2+} /calmodulin-dependent phosphorylation of proteins such as synapsin I. In this context, we have demonstrated by immunoblotting techniques the presence of caldesmon in chicken and bovine brain (Ngai & Walsh, 1985b; Clark *et al.*, 1986).

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