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Identification of mitogen-activated protein kinase phosphorylation sequences in mammalian h-Caldesmon

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h-Caldesmon in vascular smooth muscle is phosphorylated in response to pharmacologic stimulation. Although many kinases phosphorylate h-caldesmon, in vitro, the responsible kinase in intact tissue is unknown. The sites of phosphorylation in caldesmon from intact canine aortas have recently been identified and are consensus sequences for a proline-directed protein kinase. In this study, we investigated the phosphorylation of h-caldesmon by mitogen-activated protein kinase (MAPK). Purified, recombinant MAPK phosphorylated porcine stomach h-caldesmon to a stoichiometry approaching 2 mol phosphate/mol protein. Phosphorylated h-caldesmon was subjected to proteolysis and the phosphopeptides were purified by high performance liquid chromatography. Two major phosphopeptides were identified and sequenced. These two peptides, VTS * PTKV and S * PAPK, were identical to the sequences of the sites phosphorylated in intact tissue. Antibodies to several enzymes implicated in the cascade of activation of MAPK were used to evaluate vascular smooth muscle by Western blotting. All components were found to be present. These data suggest that MAPK can function as a 'caldesmon kinase' in vascular smooth muscle.

Caldesmon; Mitogen-activated protein kinase; Vascular smooth muscle

1. INTRODUCTION

Mammalian h-caldesmon is a 93 kDa actin-binding protein present in both vascular and non-vascular smooth muscles [1,2]. h-Caldesmon can cross-link actin to myosin via binding of the N-terminus of caldesmon to the S2 region of myosin heavy chain, and binding of the C-terminus to the amino-terminal domain of actin [3]. In arterial smooth muscle h-caldesmon can be phosphorylated in response to pharmacologic stimulation [4,5]. In particular, agonists which stimulate sustained or tonic contraction of the muscle also increased the level of phosphate incorporated into h-caldesmon. Several kinases have been reported to phosphorylate h-caldesmon in vitro, including protein kinase C [6–8] and Ca^{2+}/calmodulin-dependent protein kinase II [9]. However, the kinase responsible for h-caldesmon phosphorylation in the intact smooth muscle, has not been identified with certainty. Recently, the sequences of the sites phosphorylated in intact canine aortas were determined and found to be: VTS * PTKV and S * PAPK [8]. These sequences implicated a proline-directed protein kinase as the endogenous caldesmon kinase.

Two main families of proline-directed protein kinases have been described: $p34^{cdc2}$ and mitogen-activated protein kinase (MAPK). $p34^{cdc2}$ is the protein kinase component of maturation promoting factor (MPF) which is involved in cell cycle regulation in proliferating cells [10]. MPF has not been identified in the contractile phenotype of vascular smooth muscle. MAPK, also known as extracellular signal-regulated kinase (or ERK) is present in some muscles and can phosphorylate gizzard caldesmon, in vitro [11]. There are at least three isoenzymes of MAPK [12] and, as the name would imply, these enzymes have been implicated in growth factor-mediated events such as proliferation and hypertrophy. In other tissues, MAPK is believed to activate ribosomal S6 kinase via a cascade that can be initiated as a result of protein kinase C stimulation by phorbol esters [13,14].

In this paper, we present evidence that h-caldesmon is phosphorylated by MAPK on two sites that are identical to the sites phosphorylated in intact tissue. These data suggest that MAPK is a caldesmon kinase and that caldesmon phosphorylation in intact vascular smooth muscle can be modulated through a cascade of enzymes that lead to activation of MAPK.

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Abbreviations: MAPK, mitogen-activated protein kinase; MOPS, 4-morpholinepropanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; HPLC, high performance liquid chromatography; TFA, trifluoroacetic acid; SDS, sodium dodecyl sulfate; PDBu, phorbol-12,13-dibutyrate.

2. EXPERIMENTAL

2.1. *Materials*

Most chemicals and reagents were purchased from Sigma. [^{32}P]ATP and [^{32}P]orthophosphate were obtained from New England Nuclear.

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Antibodies specific for MAPK and p32^{cdc2} were purchased from Biodesign; antibodies specific for *ras* were from Oncogene Science; and antibodies specific for *raf* and MAPK-kinase (anti-STE7-VIII) were from Upstate Biotechnology Inc.

2.2. *Proteins*

h-Caldesmon was purified from porcine stomachs as previously described [15]. p34^{cdc2} was purified from colcemid-treated HeLa cells using p13-Sepharose chromatography [16]. Purified, recombinant, rat skeletal muscle MAPK was a gift from Dr. Tim Haystead (University of Virginia).

2.3. *Phosphorylation reactions*

For sequencing analysis, h-caldesmon (1 mg) was phosphorylated by MAPK in 1 ml of a buffer consisting of 12.5 mM MOPS, pH 7.2, 0.25 mM [^{32}P]ATP, 12.5 mM β-glycerophosphate, 7.5 mM $MgCl_2$, 0.5 mM EGTA, 50 μM sodium fluoride, 0.5 mM sodium orthovanadate and 2 mM dithiothreitol. After termination of the reaction by boiling, phosphorylated h-caldesmon was separated from [^{32}P]ATP on a column of Sephacryl S-200 developed with 50 mM NH_4HCO_3 and digested with *S. aureus* protease. For time-course experiments, h-caldesmon (0.125 mg/ml) was phosphorylated in the same buffer for varying lengths of time. The reactions were initiated by the addition of ATP, terminated by the addition of SDS-sample buffer, and h-caldesmon was separated from ATP by polyacrylamide gel electrophoresis.

2.4. *High performance liquid chromatography and peptide sequencing*

Phosphopeptides were purified by HPLC using a combination of C18 reverse-phase chromatography and anion-exchange chromatography utilizing an MA7P column from Bio-Rad. Phosphopeptides were loaded on the C18 column in H_2O/0.1% TFA and eluted by increasing concentrations of CH_3CN/0.1% TFA. Phosphopeptides were eluted from the MA7P column with a gradient of NaCl from 0 to 500 mM in 10 mM Tris, pH 8.5. Purified phosphopeptides were sequenced using an Applied Biosystems model 477 sequencer [8]. Phosphoamino acids were prepared from purified phosphopeptides by acid hydrolysis at 110°C for 3 h, in vaccuo. Phosphoamino acids were separated on cellulose thin layer plates (Kodak) in a buffer consisting of acetic acid/formic acid/water (15:5:80) at 10°C.

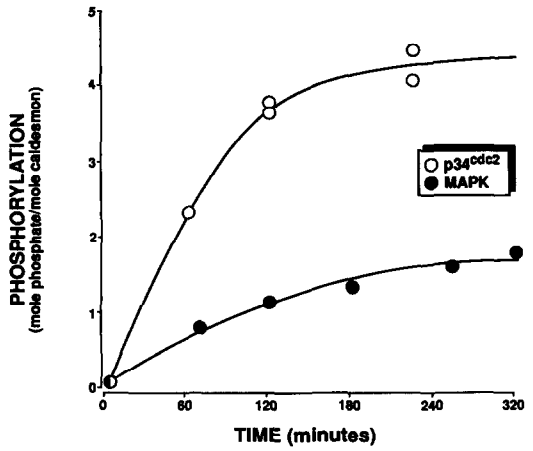

Fig. 1. Time-course of porcine stomach h-caldesmon phosphorylation by p34^{cdc2} and MAPK. h-Caldesmon was phosphorylated as described in section 2. Reactions were initiated by the addition of ATP and terminated by the addition of SDS. The stoichiometry of phosphorylation was determined by gel assay after accurate determination of ATP specific activity. Open and filled circles show phosphorylation by p34^{cdc2} and MAPK, respectively.

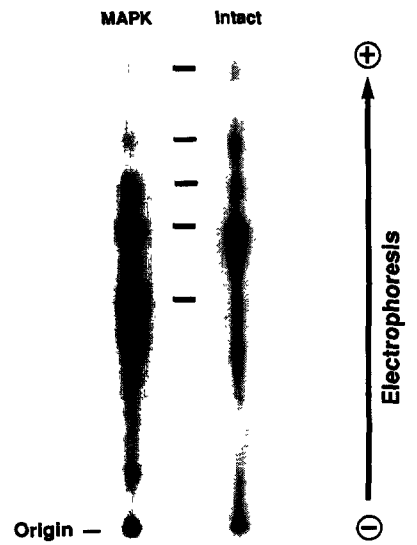

Fig. 2. Phosphopeptide maps of h-caldesmon phosphorylated in intact tissue and by MAPK in vitro. Reactions were performed as described in section 2. Thin layer electrophoresis of both samples was performed concurrently to minimize experimental variability. The mobility of the major spots in the intact tissue phosphopeptide map (Intact) corresponded to the mobility of the sites in the map of h-caldesmon phosphorylated by MAPK (MAPK).

2.5. *Phosphopeptide mapping*

Porcine carotid arteries were loaded for 90 min with ^{32}P as previously described [4], and then stimulated with 1 μM phorbol-12,13-dibutyrate (PDBu) for 60 min. The muscles were then freeze-clamped and ground to a fine powder under liquid N_2. h-Caldesmon was purified by immunoprecipitation according to procedures we have previously described [4]. Phosphorylated h-caldesmon was electrophoretically transferred to nitrocellulose and digested with a combination of α-chymotrypsin and then TPCK-treated trypsin in a buffer of 50 mM NH_4HCO_3. The phosphopeptides were separated on silica gel-60 thin layer plates (EM Science) in a buffer consisting of acetic acid/formic acid/water (15:5:80) and subjected to autoradiography. Phosphate content in the various caldesmon preparations was determined by the method of Buss and Stull [17].

2.6. *Immuno-blotting techniques*

Proteins were extracted from cultured cells by scraping the cells in the presence of SDS-sample buffer containing 1 mg/ml each of TPCK and TLCK. Proteins were extracted from tissues that were ground to a fine powder under liquid N_2 using the same buffer. Extracted proteins were separated by SDS-PAGE, transferred to nitrocellulose and then probed with the various protein-specific antibodies. SDS-PAGE was performed using the buffer system of Porzio and Pearson [18].

3. RESULTS

3.1. *Phosphorylation of caldesmon by MAPK and p34^{cdc2}*

When porcine stomach h-caldesmon was incubated with purified p34^{cdc2}, phosphate was incorporated to levels greater than 4 mol phosphate/mol protein (4.4 mol/mol maximal, Fig. 1). On the other hand, purified MAPK incorporated less than 2 mol phosphate/mol h-caldesmon (maximal value, 1.7 mol/mol, Fig. 1). Be-

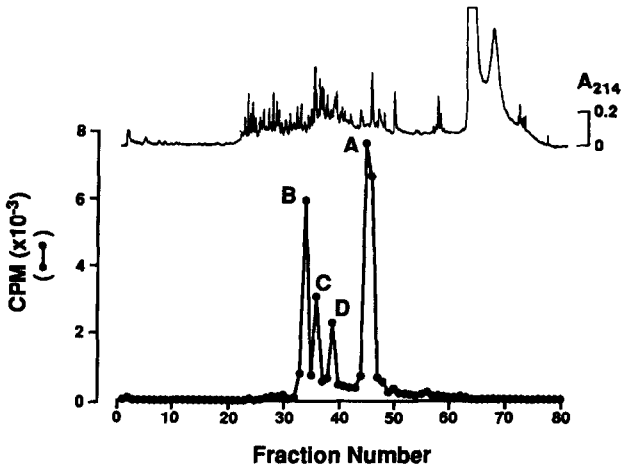

Fig. 3. Initial purification of h-caldesmon phosphopeptides. Porcine stomach h-caldesmon was phosphorylated by MAPK, digested with *S. aureus* protease and purified by HPLC as described in section 2 and shown here. These data show the absorbance and radioactivity profiles for elution of the initial digest from a C18 column.

cause of the similarity of stoichiometry of MAPK phosphorylation in vitro to caldesmon phosphorylated in intact tissues, we suspected that MAPK might also phosphorylate the same sites, in vitro. To test this, we compared phosphopeptide maps of caldesmon phosphorylated in intact porcine carotid arteries with the maps of caldesmon phosphorylated by MAPK, in vitro (Fig. 2). The relative mobilities of the phosphopeptides were similar in the two circumstances. The main difference appeared to be in the relative ratio of radioactivity in the various spots. This raised the possibility that purified caldesmon might contain endogenous phosphate. As shown in Table I, an assay of 4 preparations confirmed this suspicion. Purified caldesmon contained variable amounts of phosphate ranging from 0.34 to 0.62 mol/mol of protein. Thus, comparison of relative ratios of phosphate in caldesmon, as opposed to mobility, was judged to be unreliable, resulting from the likely attenuation of phosphorylation in vitro resulting from the presence of significant amounts of unlabeled phosphate.

Table I

Determination of the amount of phosphate in purified h-caldesmon

Preparation (ID#)	Phosphate (mol PO_4/mol caldesmon)
HSCD-2	0.61 ± 0.05 (4)
HSCD-3	0.51 ± 0.06 (4)
HSCD-4	0.34 ± 0.02 (5)
HSCD-5	0.62 ± 0.05 (4)

h-Caldesmon was purified from hog stomachs (HSCD preparations). The amount of phosphate covalently bound to h-caldesmon in these purified preparations was then determined by the method of Buss and Stull [17].

3.2. *Sequence of MAPK phosphorylation sites in h-caldesmon*

We sequenced the sites in caldesmon phosphorylated by MAPK in vitro. Fig. 3 summarizes the HPLC profiles of the phosphopeptides generated by digestion of MAPK-phosphorylated h-caldesmon with *S. aureus* protease. Four radioactive peaks were identified on the initial elution from a C18 HPLC column (Fig. 3). The two major peaks accounted for 83% of the total phosphate incorporated into h-caldesmon. The remaining two peaks accounted for 10% and 7% of total incorporated phosphate, respectively. These two minor peaks were subfractionated into several smaller peaks following anion-exchange chromatography and subsequent isocratic elution from a C18 column. However, no reliable sequence could be obtained.

The two major peaks were successfully sequenced and found to contain only one phosphopeptide each. Peak A, containing 61% of the total phosphate incorporated, yielded the sequence beginning with WLTKTPDGNKSPAPKPSDL-. In order to unambiguously determine the identity of the phosphorylated amino acid, this peptide was further digested with endoproteinase Lys-C and the resulting phosphopeptide purified by C18 HPLC. The sequence of the resulting phosphopeptide was S * PAPK (Table II). Peak B, containing 22% of total incorporated phosphate, yielded the sequence KQSVDKVTSPTKV. This peptide was further digested with TPCK-trypsin and the phosphopeptide purified by C18 HPLC to yield VTS * PTKV (Table II). Serine was identified as the phosphorylated residue in both peptides A and B by phosphoamino acid analysis. In addition, the ratio of the dithiothreitol (DTT) adduct of PTH-serine to the amount of PTH-serine (i.e. DTT-serine/PTH-serine) was increased above control levels in the cycles that were identified as phosphoserine.

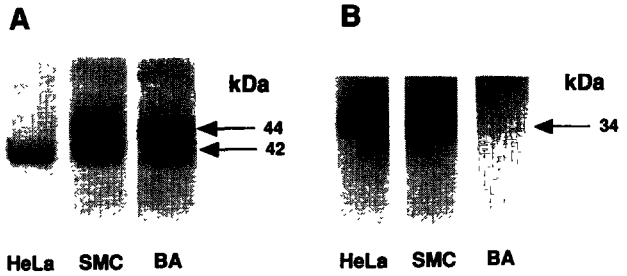

Fig. 4. MAPK vs. $p34^{cdc2}$ in smooth muscle. Proteins from cultured cells and frozen-ground tissues were extracted in 3% SDS gel sample buffer in the presence of protease inhibitors. Following SDS gel electrophoresis, the samples were transferred to nitrocellulose and evaluated for MAPK (A) or $p34^{cdc2}$ (B). HeLa, HeLa cells; SMC, cultured bovine aortic smooth muscle cells; BA, bovine aortic extract.

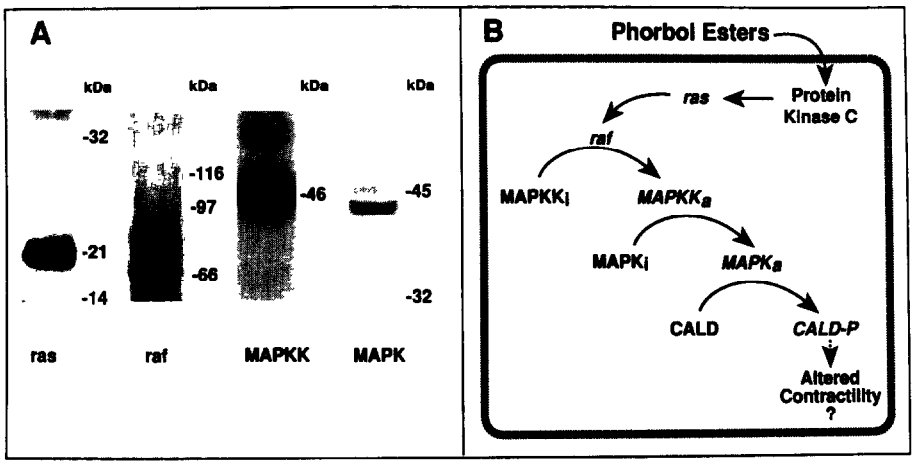

Fig. 5. Identification of MAPK cascade proteins in bovine aortic smooth muscle. Proteins were extracted from bovine aortic muscle and subjected to SDS-PAGE (7.5% gel) followed by Western blotting. Lanes were incubated with antibodies specific for *ras*, *raf*, MAPK-kinase and MAPK (A). MAPK-kinase was partially purified by chromatography on DEAE-Sephacel prior to immunoblotting. A proposed scheme for the mechanism of caldesmon phosphorylation in response to phorbol ester treatment of vascular muscle is shown in B.

3.3. *Identification of other enzymes implicated in MAPK activation*

Although MAPK phosphorylation sites of h-caldesmon in vitro are identical to those phosphorylated in intact vascular smooth muscle, it has not been previously established whether all of the putative enzymes involved in the MAPK activation cascade are also present in vascular muscle. In agreement with the data of Childs et al. [11], who identified MAPK in rat aorta and chicken gizzard, we found MAPK in vascular tissue (Fig. 4A). In vascular as well as other types of smooth muscle that we have surveyed, antibody reactivity towards two protein bands of molecular weights 42 and 44 kDa is routinely observed, although the relative amounts of p42 and p44 vary among the different tissues (data not shown). In addition, *ras*, *raf* and MAPK-kinase were readily identified by immunoblot of bovine aortic smooth muscle (Fig. 5A). While $p34^{cdc2}$ is a potential candidate for caldesmon kinase based on its ability to phosphorylate proline-directed sites in h-caldesmon, we found no evidence for this enzyme in differentiated smooth muscle (Fig. 4B). Thus, the cascade shown in Fig. 5B could summarize the sequence of steps involved in caldesmon phosphorylation as it has been proposed for phosphorylation of ribosomal S6 kinase [14,30].

Table II

Sequences of the sites on h-caldesmon phosphorylated by MAPK

Peptide	Sequence	PAA analysis	Amount of total phosphate incorporated into h-caldesmon
A	S * PAPK	Serine	61%
B	VTS * PTKV	Serine	22%
C	ND[a]	Serine and threonine	10%
D	ND[a]	Serine	7%

Peptides corresponding to peaks A–D of Fig. 3 were sequenced to give the above results. Phosphoamino acid analysis of peak C showed approximately equal amounts of phosphoserine and phosphothreonine.

[a] ND, the sequences of the phosphopeptides in these peaks could not be determined.

4. DISCUSSION

The data presented in this paper provide additional evidence that MAPK is one of the protein kinases that can phosphorylate h-caldesmon in smooth muscle. In an earlier study, we determined that h-caldesmon isolated from phorbol ester-stimulated canine aortic muscle is phosphorylated at two sites: VTS * PTKV and S * PAPK [8]. This provided conclusive evidence for the involvement of a proline-directed protein kinase in the phosphorylation of caldesmon in the contractile phenotype of smooth muscle. In the present study, we find that 83% of the phosphate incorporated into h-caldesmon by MAPK is identified in the same two sites: VTS * PTKV and S * PAPK. The remaining 17% is incorporated into sites that could not be sequenced. These minor site(s) may have resulted from either partial digestion of the protein by *S. aureus* protease or from phosphorylation of h-caldesmon by MAPK on alternative sites, albeit at low stoichiometry.

MAPK is not the only proline-directed protein kinase that has been reported to phosphorylate caldesmon. Yamashiro et al. have shown that l-caldesmon in REF 2A cells is phosphorylated during the M phase of the cell cycle, resulting in dissociation of this caldesmon isoform from actin [19,20]. Recently, Childs et al. have phosphorylated h-caldesmon in vitro [11]. Although

phosphopeptide sequences were not reported, they obtained a stoichiometry of approximately 2 mol PO_4/mol caldesmon. Through peptide mapping they concluded that the MAPK phosphorylation sites and those previously reported for $p34^{cdc2}$ must be different [21]. In the contractile phenotype of vascular smooth muscle there is no detectable $p34^{cdc2}$ by Western blotting, although both MAPK and $p34^{cdc2}$ can be detected in the proliferating phenotype. Thus, MAPK is likely to serve as caldesmon kinase in contracting vascular muscle.

We have also identified other components of the MAPK cascade in vascular smooth muscle. Previous work by others has established that vascular muscle contains several members of the *ras* family [22,23]. In addition, we find that the protein kinases *raf* and MAPK-kinase (MEK) [24] are also present. Thus, caldesmon phosphorylation and ribosomal S6 kinase activation may occur in parallel since they share the same cascade [14]. This cascade is especially interesting since several growth factors and peptide hormone receptors are coupled to it [14,25–28].

In conclusion, there appear to be three members of the caldesmon kinase family. 1-Caldesmon is phosphorylated in intact platelets by cAMP-dependent protein kinase [29] and in REF 2A cells by $p34^{cdc2}$ [19,20]. Our work [8] and the work of Childs et al. [11] provides good evidence that MAPK is an h-caldesmon kinase.

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