Phosphorylation of caldesmon by smooth-muscle casein kinase II

CINDY SUTHERLAND¹,², BERNARD S. RENAX,² DON J. MCKAY² and MICHAEL P. WALSH¹,²*

¹MRC Group in Signal Transduction and ²Department of Medical Biochemistry, University of Calgary, 3330 Hospital Dr. N.W., Calgary, Alberta, Canada T2N 4N1

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Summary

A caldesmon kinase activity was partially purified from an extract of chicken gizzard smooth muscle by sequential chromatography on columns of DEAE-Sephacel, MonoQ and Superose 12. This kinase was identified as casein kinase II by Western blotting using peptide-directed antibodies raised against the α, α' and β subunits of human casein kinase II; the smooth muscle enzyme consisted of similar subunits of M, 43 000 (α), 39 000 (α'), and 27 000 (β). Phosphorylation of caldesmon and casein by smooth muscle casein kinase II was optimal at ~0.1 M NaCl, did not require second messengers, and was inhibited by heparin. The kinase utilized either GTP or ATP as a substrate. Caldesmon was phosphorylated to ~1 mol P₁ mol⁻¹ caldesmon by smooth muscle casein kinase II with a Kₘ for caldesmon of 4.9 μM. Two-dimensional thin-layer electrophoresis indicated phosphate incorporation into both serine and threonine. All the incorporated phosphate was recovered in the N-terminal peptide (residues 1-152) generated by cleavage at cysteine 153 with 2-nitro-5-thiocyanobenzoic acid. Purification of tryptic phosphopeptides and N-terminal sequencing revealed two principal sites of phosphorylation: serine 73 and threonine 83. The following four synthetic peptides corresponding to this domain of caldesmon were examined as substrates of casein kinase II: A = RRREVNAQNSVAEE; B = AQNSVAEE; C = RSTDDEAA; D = SVAEEETKRSTDDE. Interestingly, only peptides C and D were phosphorylated and both only at threonine. Phosphorylation of intact caldesmon did not affect the pattern of chymotryptic digestion suggesting that it does not induce a significant conformational change in the protein substrate. Phosphorylation also had no effect on the binding of caldesmon to actin or on the caldesmon-mediated inhibition of actomyosin MgATPase activity. However, phosphorylation completely abolished the interaction of caldesmon with immobilized smooth muscle myosin. These results are consistent with the localization of the myosin-binding domain near the N-terminus of caldesmon and of the actin-binding domain near the opposite end of the elongated molecule. Casein kinase II may therefore play a role in regulating caldesmon-myosin interaction and the ability of caldesmon to cross-link actin and myosin filaments in smooth muscle.

Introduction

Smooth-muscle caldesmon (Walsh, 1990; Marston & Redwood, 1991; Sobue & Sellers, 1991) is an elongated protein molecule (74 nm × 1.9 nm) (Graceffa et al., 1988) which is associated with the thin filaments of the contractile apparatus (Breitarder & Lynch, 1985; Furst et al., 1986; Lehman et al., 1989). Caldesmon contains a myosin-binding site near the N-terminus (Sutherland & Walsh, 1989) and an actin-binding site near the C-terminus (Sobue & Sellers, 1991). Two potential physiological functions have been proposed for caldesmon: (1) regulation of actin-myosin interaction, and therefore the contractile state of the muscle, through its interaction with actin and inhibition of actin-activated myosin MgATPase activity (Ngai & Walsh, 1984); and (2) organization of the contractile filaments, via the cross-linking of actin and myosin filaments, so that they maintain the proper orientation and spatial relationship for efficient contraction in response to nervous or hormonal stimulation (Hemrich & Chalovich, 1988; Ikebe & Reardon, 1988; Walsh, 1990; Marston et al., 1992).

One way in which the interactions of caldesmon with actin and myosin may be regulated is via reversible phosphorylation, and caldesmon has been shown to be phosphorylated in intact smooth muscle strips upon...
stimulation by a variety of contractile agonists (Park & Rasmussen, 1986; Adam et al., 1990, 1992; Bárany et al., 1992a, b). Furthermore, several protein serine/threonine kinases have been shown to phosphorylate caldesmon in vitro: Ca\(^{2+}\)/calmodulin-dependent protein kinase II (CaM kinase II) (Abougou et al., 1989; Ikebe et al., 1990; Scott-Woo et al., 1990), protein kinase C (Tanaka et al., 1990), casein kinase II (Bogatcheva et al., 1993), cdc2 kinase (Mak et al., 1991a, b) and mitogen-activated protein (MAP) kinase (Childs et al., 1992; Adam & Hathaway, 1993). Adam and coworkers have shown, in intact canine aortic smooth muscle treated with phorbol ester, that caldesmon is phosphorylated at the same sites as are phosphorylated in vitro by MAP kinase (Adam et al., 1992; Adam & Hathaway, 1993). The possibility that other kinases phosphorylate caldesmon in vivo in response to other extracellular signals remains unexplored.

In this report, we describe the phosphorylation of caldesmon by smooth-muscle casein kinase II and the effects of this phosphorylation on the structural and functional properties of caldesmon. We conclude that casein kinase II may play a role in regulation of contractile filament organization in smooth muscle through its ability to phosphorylate caldesmon resulting in disruption of the caldesmon-myosin interaction and thereby of the cross-linking of actin and myosin filaments by caldesmon.

Materials and methods

**Materials**

\(\gamma^3P\)ATP (\(> 5000 \text{ Ci mmol}^{-1}\)) and \(\gamma^3P\)GTP (\(> 5000 \text{ Ci mmol}^{-1}\)) were purchased from Amersham (Oakville, Ontario, Canada), DEAE-Sephacel, monoQ and Superose 12 from Pharmacia (Mississauga, Ontario, Canada), casein from Sigma (St Louis, MO, USA) and heparin (sodium salt) from Calbiochem (La Jolla, CA, USA). Electrophoresis reagents were purchased from Bio-Rad (Mississauga, Ontario, Canada). General laboratory reagents used were of analytical grade or better and were purchased from CanLab (Edmonton, Alberta, Canada). Polyclonal antibodies raised in rabbits against synthetic peptides corresponding to caldesmon (67-78) with the addition of three arginine residues to the N-terminus to allow the peptide to bind to P81 phosphocellulose paper in phosphorylation assays; peptide B = AQNSVAEEE, corresponding to caldesmon (70-78); peptide C = RSTDDEAA, corresponding to caldesmon (81-88); and peptide D = SVAEEETKRRSTDE, corresponding to caldesmon (73-86). Peptides were shown to be > 95% pure by analytical methods described below.

**Methods**

The following proteins were purified by previously-described methods: chicken gizzard caldesmon (Sutherland & Walsh, 1989), actin (Ngai et al., 1986), myosin (Persechini & Hartshorne, 1981), tropomyosin (Smillie, 1982) and myosin light chain kinase (Ngai et al., 1984), and bovine brain calmodulin (Walsh et al., 1984). Chicken gizzard myosin was coupled to CNBr-activated Sepharose 4B (Pharmacia) by standard procedures described by the manufacturer, but eliminating exposure to low pH. Myosin-Sepharose was stored at 4°C in 50 mM Tris-HCl (pH 7.5), 50 mM KC1, 1 mM dithiothreitol, 0.02% (w/v) NaN3.

**Partial purification of caldesmon kinase activity**

Frozen chicken gizzards (25 g) were minced, homogenized in 4 vols (100 ml) of Buffer A (20 mM Tris-HCl (pH 7.5), 5 mM EDTA, 5 mM dithiothreitol) containing leupeptin (10 \(\mu\)g ml\(^{-1}\)) and pepstatin (10 \(\mu\)g ml\(^{-1}\)) with a Brinkmann Polytron at setting 7 for 2 × 10 s, and centrifuged at 30 000 g for 30 min. The supernatant was applied to a column (1 × 20 cm) of DEAE-Sepharose previously equilibrated with Buffer A. The column was washed with Buffer A and bound proteins were eluted with a linear 0-0.4 M NaCl gradient (100 ml). Fractions (1.5 ml) were collected at a flow rate of 25 ml h\(^{-1}\). Samples of column fractions were assayed for caldesmon kinase activity as described below. The column elution profile and kinase assay results are shown in Fig. 1. Comparison of the autoradiograms in panel B of Fig. 1 indicates a major peak of caldesmon kinase activity which peaks at fractions 58 and 59. Other regions of the profile exhibit caldesmon kinase activity (e.g., fractions 45 and 48-51), but these are quantitatively significantly lower than the major peak. Consequently, fractions 58-60 were pooled, dialysed vs Buffer A containing 0.2 M NaCl (2 × 5 l) and centrifuged at 100 000 g for 1 h. The supernatant was passed through a 0.45 \(\mu\)m filter and applied to a monoQ HR5/5 column previously equilibrated with Buffer A containing 0.2 M NaCl. The column was washed with equilibration buffer and bound proteins were eluted with a linear 0.2-0.6 M NaCl gradient followed by a step to 0.8 M NaCl in Buffer A. Fractions (3 ml) were collected at a flow rate of 5 ml min\(^{-1}\). Samples of column fractions were assayed for caldesmon kinase activity as described below. The column elution profile and kinase assay results are shown in Fig. 2. A single peak of caldesmon kinase activity was detected. Peak fractions (39-41) were pooled from three monoQ columns and subjected to that shown in Fig. 2 and concentrated on a DEAE-Sephaloc column: the combined monoQ pools were dialysed vs 20 mM Tris-HCl (pH 7.5), 5 mM EDTA, 1 mM dithiothreitol, applied to a DEAE-Sephaloc column (1 × 10 cm) previously equilibrated with the same buffer, and the caldesmon kinase activity was eluted in a small volume of equilibration buffer containing 0.5 M NaCl. The kinase preparation (in 1-ml batches) was then applied to a Superose 12 HR16/30 column previously equilibrated with 20 mM Tris-HCl (pH 7.5), 0.5 M NaCl, 5 mM EDTA, 1 mM dithiothreitol. Fractions (1 ml) were collected at a flow rate of 0.5 ml min\(^{-1}\). The column elution profile and kinase assay and immunoblotting results are shown in Fig. 3. Caldesmon kinase-containing fractions were pooled as shown, dialysed vs 20 mM Tris-HCl (pH 7.5), 0.3 M NaCl, 5 mM EDTA, 1 mM dithiothreitol and concentrated on a 1-ml monoQ column. Bound protein was eluted with a linear 0.3-0.55 M NaCl gradient and a step to 0.6 M NaCl collecting 0.5-ml fractions at a flow rate of 1 ml min\(^{-1}\). Caldesmon kinase-containing fractions were pooled, NaN3 added to 0.02% (w/v) and stored at −80°C.

**Synthetic peptides**

The following four peptides were synthesized using a Beckman model 990B automated peptide synthesizer and purified by preparative reverse-phase HPLC as previously described (Litwin et al., 1991): peptide A = RRREVNAQNSVAAEE, corresponding to caldesmon (67-78) with the addition of three arginine residues to the N-terminus to allow the peptide to bind to P81 phosphocellulose paper in phosphorylation assays; peptide B = AQNSVAAEE, corresponding to caldesmon (70-78); peptide C = RSTDDEAA, corresponding to caldesmon (81-88); and peptide D = SVAEEETKRRSTDE, corresponding to caldesmon (73-86). Peptides were shown to be > 95% pure by analytical