Phosphorylation of Caldesmon by p21-activated Kinase

IMPLICATIONS FOR THE Ca$^{2+}$ SENSITIVITY OF SMOOTH MUSCLE CONTRACTION*

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We have previously shown that p21-activated kinase, PAK, induces Ca$^{2+}$-independent contraction of Triton-skinned smooth muscle with concomitant increase in phosphorylation of caldesmon and desmin but not myosin-regulatory light chain (Van Eyk, J. E., Arrell, D. K., Foster, D. B., Strauss, J. D., Heinonen, T. Y., Furmaniak-Kazmierczak, E., Cote, G. P., and Mak, A. S. (1998) J. Biol. Chem. 273, 23433–23439). In this study, we provide biochemical evidence implicating a role for PAK in Ca$^{2+}$-independent contraction of smooth muscle via phosphorylation of caldesmon. Mass spectroscopy data show that stoichiometric phosphorylation occurs at Ser$^{657}$ and Ser$^{687}$ abutting the calmodulin-binding sites A and B of chicken gizzard caldesmon, respectively. Phosphorylation of Ser$^{657}$ and Ser$^{687}$ has an important functional impact on caldesmon. PAK-phosphorylation reduces binding of caldesmon to calmodulin by about 10-fold whereas binding of calmodulin to caldesmon partially inhibits PAK phosphorylation. Phosphorylated caldesmon displays a modest reduction in affinity for actin-tropomyosin but is significantly less effective in inhibiting actin-activated S1 ATPase activity in the presence of tropomyosin. We conclude that PAK-phosphorylation of caldesmon at the calmodulin-binding sites modulates caldesmon inhibition of actin-myosin ATPase activity and may, in concert with the actions of Rho-kinase, contribute to the regulation of Ca$^{2+}$ sensitivity of smooth muscle contraction.

Recent data strongly implicate the monomeric Rho family GTPases in modulating Ca$^{2+}$ sensitivity of smooth muscle contraction (1, 2). RhoA-activated kinase, Rho kinase, enhances activity (3) and/or by phosphorylating Ser19 of MLC directly (4). Our finding that PAK induces Ca$^{2+}$-independent contraction in skinned smooth muscle fibers (8) and the recent demonstration that an unknown kinase besides MAPK phosphorylates gizzard caldesmon in vivo (20) suggest that PAK phosphorylation of caldesmon may be involved in the regulation of Ca$^{2+}$ sensitivity of smooth muscle contraction. Here, we report biochemical evidence supporting a role of PAK in the Ca$^{2+}$ sensitivity of smooth muscle contraction via phosphorylation of caldesmon. Specifically, we have identified the sites of phosphorylation may be involved in the regulation of Ca$^{2+}$ sensitivity of smooth muscle contraction via phosphorylation of caldesmon. Phosphorylation of Caldesmon and Identification of Phosphorylation Sites—Caldesmon (1–2 mg/ml) was phosphorylated by GST-mPAK3 (−5 μg/ml), at 37°C for 60 min, at 0 mM Tris, pH 7.5, 100 mM NaCl, 5 mM MgCl$_2$, 1 mM [γ-$^{32}$P]ATP (1–5 × 10$^6$ cpm/nmol), 0.5 mM DTT. Quantification of phosphorylation and analysis of phosphorylated amino acids were performed as described before (25).

Approximately 200 μg of caldesmon was dissolved in 200 μl of 100 mM NH$_4$HCO$_3$, pH 7.9, containing 10 μg of endopeptidase Glu-C. The digestion was carried out overnight at room temperature. The digest solutions were evaporated to dryness and redissolved in 5% acetic acid

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The abbreviations used are: SMPP1, smooth muscle myosin light chain phosphatase; MLCK, myosin light chain kinase; PAK, p21-activated kinase; MLC, 20-kDa regulatory myosin light chain; MAPK, mitogen-activated protein kinase; DTT, dithiothreitol; MS/MS, tandem mass spectrometry; GST, glutathione S-transferase.

MATERIALS AND METHODS

Protein Preparation—b-Caldesmon and α β tropomyosin were purified from chicken gizzards essentially as described by Bretcher (21). Skeletal muscle actin was purified from rabbit muscle as outlined in (22). Smooth muscle myosin S1 was prepared by papain cleavage of gizzard myosin (23). Rabbit skeletal myosin S1(A1) was prepared by cleavage with chymotrypsin as described in (24). Recombinant murine PAK3, was expressed from the plasmid pGST-mPAK3 in Escherichia coli JM101 and/or JM110 cells as described before (8).

Phosphorylation of Caldesmon and Identification of Phosphorylation Sites—Caldesmon (1–2 mg/ml) was phosphorylated by GST-mPAK3 (−5 μg/ml), at 37°C for 60 min, in 20 mM Tris, pH 7.5, 100 mM NaCl, 5 mM MgCl$_2$, 1 mM [γ-$^{32}$P]ATP (1–5 × 10$^6$ cpm/nmol), 0.5 mM DTT. Quantification of phosphorylation and analysis of phosphorylated amino acids were performed as described before (25).

Approximately 200 μg of caldesmon was dissolved in 200 μl of 100 mM NH$_4$HCO$_3$, pH 7.9, containing 10 μg of endopeptidase Glu-C. The digestion was carried out overnight at room temperature. The digest solutions were evaporated to dryness and redissolved in 5% acetic acid
(200 μl). For nanoelectrospray mass spectrometry analysis, 20-μl aliquots of the sample solutions were desalted using ZipTip™ C18 (Millipore, Bedford, MA). Approximately 1–2 μl of the desalted solutions were used for both precursor ion scanning and tandem mass spectrometry (MS/MS) analyses.

Phosphopeptides were detected by precursor ion scanning (precursors of m/z 79) in negative ion mode on a API 3000 triple quadrupole mass spectrometer (Perkin Elmer/SCIEX Concord, ON, Canada). Precursor ion spectra were acquired in multiple channel acquisition mode, typically over a period of 3 min (m/z 400–2000, 0.5 mass units step size, 5 msec dwell time). Argon was used as the collision gas, and the collision offset voltage was 80 V. Phosphopeptide sequencing was achieved by MS/MS using a prototype quadrupole time-of-flight mass spectrometer (QqTOFMS, Perkin Elmer/SCIEX) equipped with a nanoelectrospray ionization source. Product ion spectra were carried out in positive ion mode using argon as the collision gas and a collision energy of 60 eV (laboratory frame of reference). MS/MS spectra were typically acquired every 2 s over a period of 3 min.

**Caldesmon-Calmodulin Interaction**—Interaction between calmodulin and phosphorylated and nonphosphorylated caldesmon was studied using intrinsic Trp fluorescence as described previously (15). The binding buffer was 20 mM Tris-HCl, pH 7.2, 0.5 mM CaCl₂, 100 mM NaCl, 1 mM DTT. The excitation wavelength was 285 nm with a slit width of 10 nm. Intensity measurement was made with a 290-nm filter at 330 nm (laboratory frame of reference). MS/MS spectra were typically acquired every 2 s over a period of 3 min.

**RESULTS**

Chicken gizzard h-caldesmon was phosphorylated in vitro using a constitutively active murine GST-PAK3 to a maximum of 2 mol of phosphate per mol of caldesmon as shown in Fig. 1. Only phosphorylated Ser was recovered from a hydrolysate of 32P-labeled caldesmon (Fig. 1, inset).

To locate the phosphorylation sites, caldesmon phosphorylated by GST-PAK to 2 mol of phosphate/mol of protein was subjected to digestion by endoprotease Glu-C, which cleaves peptide bonds on the COOH-side of Glu residues. The resulting digest was analyzed for phosphorylated peptides by precursor ion scanning as described in “Materials and Methods.” Two major doubly deprotonated ions were detected (Fig. 2A, peaks a and b) together with a number of minor peaks representing minor sites of phosphorylation. MS/MS sequence analysis determined that peak a (m/z 648.0) and peak b (m/z 754.0) correspond to two singly phosphorylated peptides, Gly651-Val-Arg-Asn-Ile-Lys-p-Ser-Met-Trp-Glu660 and Thr678-Ala-Gly-Leu-Lys-Val-Gly-Val-p-Ser-Arg-Ile-Asn-Lys-Glu691, A and B, respectively (data not shown). Ser687, being the only Ser in peptide A, can be unambiguously assigned as the site of phosphorylation in this peptide. There are adjacent Ser residues in peptide B however. MS/MS sequence analysis indicated that Ser687 is most likely the site of phosphorylation (Fig. 2C) because a b-type fragment ion at m/z 813.5 corresponding to the unphosphorylated peptide, Thr-Ala-Gly-Leu-Lys-Val-Gly-Val-Ser686, was detected (data not shown). The nonphosphorylated counterparts of peptides A and B were not detected, indicating that Ser687 and Ser686 were fully phosphorylated, largely accounting for the observed stoichiometry of 2 mol of phosphate/mol of protein (Fig. 1). The precursor ion scan of the endoprotease Glu-C digest of unphosphorylated caldesmon shows no trace of peak a (m/z 648.0) or peak b (m/z 754.0), indicating that Ser687 and Ser686 are genuine PAK-target sites (Fig. 2B). Background peaks at m/z 529, 543, and 558, which amount to less than 12% of peak b, were detected in the unphosphorylated...
caldesmon sample but were not analyzed further.

According to the model of Marston and Redwood (Ref. 26, and Fig. 2), Ser657 and Ser687 are located at the amino-terminal ends of calmodulin-binding sites A and B (15, 16) in subdomain 4. We therefore examined whether Ser 657 and Ser 687 were accessible to PAK when caldesmon formed a complex with Ca\(^{2+}\)/calmodulin. GST-PAK3-phosphorylated caldesmon-calmodulin complex at a similar initial rate but reach a stoichiometry of 1.2 mol of phosphate/mol of protein (Fig. 1). Calmodulin was not phosphorylated by PAK, and Ca\(^{2+}\) did not affect PAK activity under the same conditions (data not shown). This result suggests that binding of calmodulin to sites A and B of caldesmon renders Ser657 and/or Ser687 less accessible to PAK.

To determine whether introduction of phosphate groups to Ser657 and Ser687 at the calmodulin-binding sites A and B can affect calmodulin-binding, we compared binding of phosphorylated and nonphosphorylated caldesmon to calmodulin using intrinsic Trp fluorescence measurements (Fig. 3). Phosphorylated and nonphosphorylated caldesmon have similar fluorescence spectra, each exhibiting a similar emission maximum at 350 nm, which suggests that the phosphate groups do not cause significant changes in the environments surrounding the Trp residues which are major determinants for calmodulin-binding (15). Binding of Ca\(^{2+}\)-calmodulin, which contains no Trp, increased the intrinsic Trp fluorescence of caldesmon by a maximum of about 70% (Fig. 3B) and caused a blue shift of the emission maximum from 350 to 340 nm (Fig. 3A). On the other hand, calmodulin increases the fluorescence intensity by less than 40% at saturation accompanied by a smaller shift in emission maximum from 350 to 345 nm (Fig. 3B). As shown in
Fig. 3. Effect of phosphorylation on binding of caldesmon to calmodulin. A, Trp fluorescence emission spectra of phosphorylated caldesmon with calmodulin (- - -) and unphosphorylated caldesmon with calmodulin (—). In the absence of calmodulin, the emission spectra of phosphorylated and nonphosphorylated caldesmon are virtually identical as indicated by the solid triangles (▲). Excitation was at 295 nm. B, binding curves of phosphorylated (○) and nonphosphorylated (▲) caldesmon to calmodulin using intrinsic Trp fluorescence measurements. \( \Delta F \) is the change in fluorescence in caldesmon at 330 nm induced by calmodulin; \( I_o \) is fluorescence of caldesmon in the absence of calmodulin. \( \lambda = \) wavelength. Inset, 12% SDS-polyacrylamide gel electrophoresis of the phosphorylated and unphosphorylated caldesmon and calmodulin at the end of the binding studies.

Fig. 3B, phosphorylation reduces the affinity of caldesmon for Ca\(^{2+}\)-calmodulin by about 10-fold and increases the \( K_d \) from 0.1 to 0.9 \( \mu M \).

We compared the ability of caldesmon and its phosphorylated counterpart to interact with actin-tropomyosin and to inhibit actin-activated myosin ATPase activity because the calmodulin-binding sites A and B have been shown to bind actin (27), and site B is in the middle of the tropomyosin-linked actin-binding and inhibitory region in subdomain 4 (28). As shown in Fig. 4, caldesmon phosphorylated to 2 mol of phosphate/mol of protein has a modest reduction in affinity for actin-tropomyosin; \( K_d \) was increased by less than 2-fold from 1.0 to 1.7 \( \mu M \). However, phosphorylation of caldesmon induces a significant release of inhibition of actin-S1 ATPase (Fig. 5) in the presence or absence of tropomyosin. At 0.2 mol/mol of caldesmon/actin, nonphosphorylated caldesmon inhibits actin-activated skeletal myosin S1 ATPase activity by 80% in the presence of tropomyosin, whereas about 40% inhibition was observed by the same amount of phosphorylated caldesmon (Fig. 5B). Similar results were obtained using smooth muscle S1 (data not shown). In the absence of tropomyosin (Fig. 5A), caldesmon is much less effective in inhibition as reported by others (28, 29); 0.4–0.5 mol/mol of nonphosphorylated caldesmon/actin is required to cause a 40% inhibition whereas similar amounts of phosphorylated caldesmon inhibit by 20%.

**DISCUSSION**

This study provides biochemical evidence to support the hypothesis that phosphorylation of caldesmon by PAK may play a role in inducing Ca\(^{2+}\)-independent contraction in smooth muscle. The strategic location of Ser\(^{657}\) and Ser\(^{687}\) in the calmodulin-binding sites A and B provides a crucial clue to our under-
standing of how phosphorylation of these sites may affect the function of caldesmon. The sequences around Ser657 and Ser687 are conserved in chicken, mouse, and human caldesmon, and these regions also form parts of the extended actin-binding regions in subdomain 4 (28), further underscoring their importance. Ser657 and Ser687 are not recognized by MAPK (25, 30), casein kinase II (31), Ca^{2+}-dependent calmodulin kinase II (32), and protein kinase C (33), all of which have been shown to

**FIG. 4.** Effect of phosphorylation on binding of caldesmon to actin-tropomyosin. Binding curves of unphosphorylated caldesmon (●) and phosphorylated caldesmon (○) to actin-tropomyosin was analyzed by sedimentation.

**FIG. 5.** Inhibition of actin-activated skeletal S1-ATPase activity by caldesmon and phosphorylated caldesmon. A, inhibition of actin-activated S1-ATPase activity by unphosphorylated (●) and phosphorylated caldesmon (○) in the absence of tropomyosin. B, same as panel A except that tropomyosin was present.
phosphorylate caldesmon in vitro. The sequences surrounding Ser\textsuperscript{657} (Arg-Asn-Ile-Lys-Ser\textsuperscript{657}-Met-Trp-Glu) and Ser\textsuperscript{687} (Lys-Val-Gly-Val-Ser\textsuperscript{687}-Arg-Ile-Asn) in caldesmon, and Ser\textsuperscript{19} (Glu-Arg-Ala-Thr-Ser\textsuperscript{19}-Asn-Val-Phe) in \(\alpha\)-calmodulin have a hydrophobic residue in the +2 position which agrees with Brzeska \textit{et al.} (39) who showed that a Tyr at the +2 position is strongly preferred by PKA1. As well, seven of the eight autophosphorylation sites in PKA1 have a hydrophobic residue in the +2 position (40). However, Tuazon \textit{et al.} (34), using a series of synthetic peptide substrates, identified the signature determinants for PKA1 phosphorylation as KRES, which bears little resemblance to the caldesmon and MLC phosphorylation sites except for the presence of a basic residue between positions -1 and -5. It appears, therefore, secondary structures and a hydrophobic amino acid at the +2 position are equally important determinants for PKA recognition.

Not unexpected, we found that phosphorylation of Ser\textsuperscript{657} and Ser\textsuperscript{687} interfered with interaction between calmodulin and caldesmon. We have shown previously that although Trp\textsuperscript{959} and Trp\textsuperscript{962} in sites A and B, respectively, are major determinants for caldesmon-calmodulin interaction, though amino acid residues surrounding the Trp residues also contribute to optimal binding (15). NMR data showed that sites A and B in synthetic peptides simultaneously bind to the two hydrophobic regions of calmodulin affecting all eight Met residues in the “Met puddles” (35) and become \(\beta\)-helical upon binding to calmodulin (36, 37). Furthermore, the helix formed by site A is amphiphilic such that Ser\textsuperscript{657} is located on the polar surface (36). Introduction of phosphate groups at these sites likely interferes with the contacts between the polar surface of site A and calmodulin but should have a minor impact on hydrophobic interactions, as would be suggested by fluorescence data (Fig. 3A), which indicate that phosphorylation of caldesmon alone does not affect the environment surrounding Trp residues in sites A and B. This is also consistent with our finding that binding of calmodulin to sites A and B ablates subsequent phosphorylation of caldesmon by PKA, indicating that Ser\textsuperscript{657} and/or Ser\textsuperscript{687} become less accessible to PKA (Fig. 1).

The actin-binding sites span an extended region in subdomain 4 of caldesmon (28). Introduction of two phosphates at Ser\textsuperscript{657} and Ser\textsuperscript{687} is unlikely to induce extensive disruption in the actin-binding regions, thus abrogating interaction. This may account for the modest reduction in affinity of phosphorylated caldesmon for Ca\textsuperscript{2+}-calmodulin, thus abrogating interaction between calmodulin and MLC phosphorylation sites except for the presence of a basic residue between positions -1 and -5. It appears, therefore, secondary structures and a hydrophobic amino acid at the +2 position are equally important determinants for PKA recognition.

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