THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 275, No. 3, Issue of January 21, pp. 1959–1965, 2000

Printed in U.S.A.

Phosphorylation of Caldesmon by p21-activated Kinase

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

(Received for publication, October 27, 1999)

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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 the Ca^{2+} sensitivity of contraction by phosphorylating the myosin-binding subunit of SMPP1[1] resulting in inhibition of its activity (3) and/or by phosphorylating Ser^{19} of MLC directly (4). Rac1 and cdc42 have been implicated in the cytoskeletal remodeling processes that accompany lamellapodia and filopodia formation in many types of non-muscle cells (6). One of the key downstream effectors of Rac1 and cdc42 is the Ser/Thr kinase, PAK (7). We have shown previously that infusion of Triton-skinned guinea pig *Taenia coli* smooth muscle fibers with constitutively active PAK3 induces Ca^{2+}-independent contraction to about 60% of the force obtained in the presence of Ca^{2+}/calmodulin (8). PAK-induced contraction is accompanied by an increase in the phosphorylation of caldesmon and desmin but not MLC even though PAK is able to phosphorylate MLC at Ser^{19} *in vitro* (8, 9). These results suggest that Rac and cdc42, in contrast to Rho, induce smooth muscle contraction by altering the properties of actin and/or intermediate filaments.

Smooth muscle caldesmon is speculated to be a thin filament regulatory protein by virtue of its ability to inhibit actin-tropomyosin activated ATPase activity of myosin (10–12). It is an 89-kDa protein that binds in an extended conformation along filaments of actin-tropomyosin. It houses binding sites for myosin (13), tropomyosin (14), calmodulin (15, 41), and actin (16). *In vitro,* caldesmon-mediated inhibition of actomyosin ATPase activity can be regulated by calcium binding proteins, including calmodulin (17, 18) or caltropin (19).

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 and have studied phosphorylated caldesmon with respect to its affinity for actin-tropomyosin and calmodulin and its ability to inhibit actomyosin ATPase activity.

MATERIALS AND METHODS

Protein Preparation—h-Caldesmon and $\alpha\ \beta$ tropomyosin were purified from chicken gizzards essentially as described by Bretscher (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 $\times$ 10^5 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_4HCO_3, pH 7.9, containing 10 μg of endoproteinase Glu-C. The digestion was carried out overnight at room temperature. The digest solutions were evaporated to dryness and redissolved in 5% acetic acid

* This work was funded by the Medical Research Council of Canada Grants MT-14303 (to A. S. M.) and MT-14375 (to J. V. E.) and Ontario Heart and Stroke Foundation Grants T-3458 (to A. S. M.) and T-3759 (to J. V. E.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] 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.

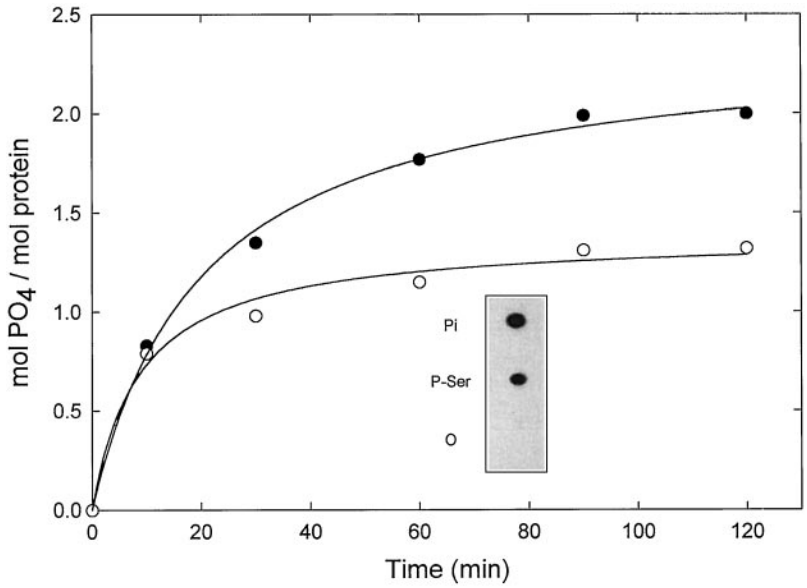

FIG. 1. **Phosphorylation of caldesmon by mPAK3 in the presence and absence of calmodulin.** Caldesmon (1 mg/ml) was phosphorylated by recombinant constitutively active GST-mPAK3 in the presence (○) or absence (●) of Ca^{2+}/calmodulin (0.4 mg/ml) as outlined under "Materials and Methods." *Inset*, autoradiogram of ^{32}P-labeled amino acid recovered from partial acid hydrolysis of phosphorylated caldesmon; P_i, inorganic phosphate; P-Ser, phosphorylated Ser; O, origin.

(200 μl). For nanoelectrospray mass spectrometry analysis, 20-μl aliquots of the sample solutions were desalted using ZipTip™ C_{18} (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 quadruple 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.

Calmodulin-Caldesmon 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 $CaCl_2$, 100 mM NaCl, 1 mM DTT. The excitation wavelength was 295 nm with a slit width of 10 nm. Intensity measurement was made with a 290-nm filter at 330 nm and a slit width of 10 nm. Binding curves were fitted to a binding equation to obtain dissociation constants as described before (15) except that binding stoichiometry was set to 1 mol of caldesmon per mol of calmodulin as previously determined (15).

Actin-binding Assays—Two nmol of actin was mixed with 0.4 nmol of tropomyosin and 0 to 1.5 nmol of phosphorylated or nonphosphorylated caldesmon in 200 μl of binding buffer (40 mM Tris, pH 7.5, 100 mM NaCl, 5 mM $MgCl_2$, and 1 mM DTT). Protein mixtures were allowed to equilibrate for 30 min prior to centrifugation at 100,000 × *g* in a Beckman TL-100 ultracentrifuge. Pellets were rinsed with actin-binding buffer once and dissolved in 100 μl of 0.05% (v/v) TFA in water. Relative amounts of caldesmon, tropomyosin, and actin were determined by high performance liquid chromatography using a Zorbax SB300-C8 HPLC column (5).

Actin-activated S1-ATPase Assays—Actin-activated myosin S1 ATPase assays in the presence and absence of tropomyosin were conducted in a 96-well ELISA plate (100 μl assay volume). Inorganic phosphate was determined colorimetrically as described in (38). ATPase buffer contained 40 mM Tris, pH 7.5, 50 mM NaCl, 5 mM $MgCl_2$, and 1 mM DTT. Myosin S1 ATPase activity was determined for reaction mixtures containing 0–4 μM caldesmon, 10 μM actin, and 0.5 μM S1 with or without 2 μM tropomyosin for 10 min at 37 °C. ATPase reaction was initiated with 4 mM ATP and terminated by adding 100 μl of a solution containing 3% ascorbic acid, 0.5 M HCl, 4% SDS, and 0.5% (w/v) ammonium molybdate. Color was allowed to develop for 6 min prior to the addition of 2% sodium citrate and 2% sodium *m*-arsenite followed by 10-min incubation before absorbance at 650 nm was measured in a Molecular Dynamics E-max plate reader. Phosphate content was determined by comparison to a potassium phosphate standard curve. Less than 10% of the ATP was hydrolyzed over the course of the reaction. Phosphate release was linear within the 10-min reaction.

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 ^{32}P-labeled caledesmon (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 endoproteinase 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. 2*A, 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, Gly^{651}-Val-Arg-Asn-Ile-Lys-p-Ser-Met-Trp-Glu^{660} and Thr^{678}-Ala-Gly-Leu-Lys-Val-Gly-Val-Ser-p-Ser-Arg-Ile-Asn-Lys-Glu^{691}, A and B, respectively (data not shown). Ser^{657}, being the only Ser in peptide A, can be unambiguously assigned as the site of phosphorylation in this peptide. There are two adjacent Ser residues in peptide B however. MS/MS sequence analysis indicated that Ser^{687} is most likely the site of phosphorylation (Fig. 2*C*) because a b-type fragment ion at *m/z* 813.5 corresponding to the unphosphorylated peptide, Thr-Ala-Gly-Leu-Lys-Val-Gly-Val-Ser^{686}, was detected (data not shown). The nonphosphorylated counterparts of peptides A and B were not detected, indicating that Ser^{657} and Ser^{687} 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 endoproteinase 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 Ser^{657} and Ser^{687} are genuine PAK-target sites (Fig. 2*B*). Background peaks at *m/z* 529, 543, and 558, which amount to less than 12% of peak **b**, were detected in the unphosphorylated

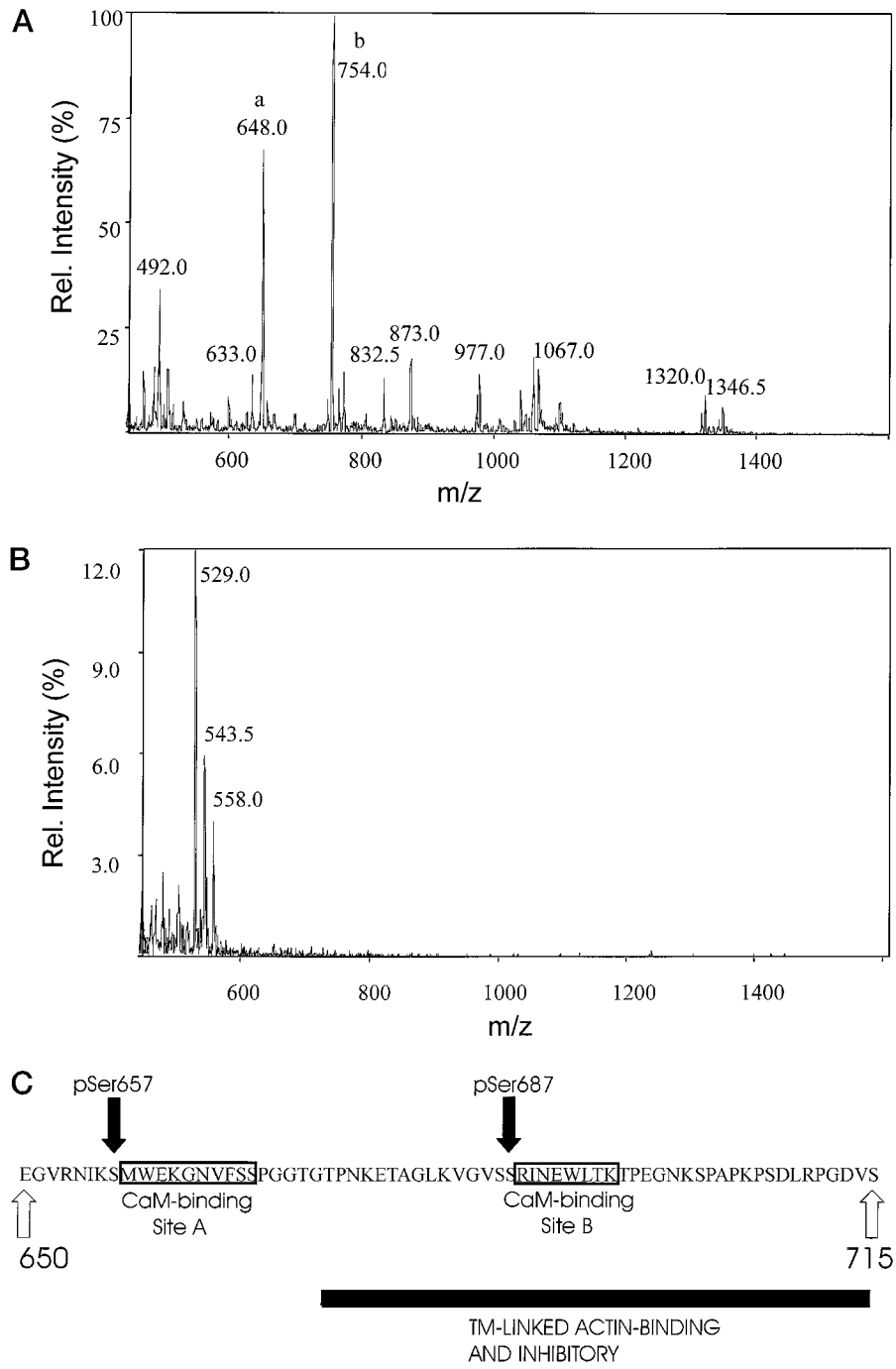

FIG. 2. **Identification of phosphorylation sites in caldesmon incubated with mPAK3.** *A*, precursor ion scan of *m/z* 79 (-ve ion mode) of the endoproteinase Glu-C digest of phosphorylated chicken gizzard caldesmon. Peaks **a** (*m/z* = 648.0) and **b** (*m/z* = 754.0) represent the two major doubly-deprotonated ions. Sequence analyses by tandem mass spectrometry determined the sequences of peaks **a** and **b** to be Gly^{651}-Val-Arg-Asn-Ile-Lys-p-Ser-Met-Trp-Glu^{660} and Thr^{678}-Ala-Gly-Leu-Lys-Val-Gly-Val-Ser-p-Ser-Arg-Ile-Asn-Lys-Glu^{691}, respectively. *B*, precursor ion scan of *m/z* 79 of the endoproteinase Glu-C digest of unphosphorylated caldesmon. *C*, the functional domains in subdomain 4 of caldesmon; the calmodulin-binding sites A and B are *boxed*, and the phosphorylation sites by PAK at Ser^{657} and Ser^{687} are indicated.

caldesmon sample but were not analyzed further.

According to the model of Marston and Redwood (Ref. 26, and Fig. 2*C*), Ser^{657} and Ser^{687} 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 Ser^{657} and/or Ser^{687} less accessible to PAK.

To determine whether introduction of phosphate groups to Ser^{657} and Ser^{687} 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. 3*B*) and caused a blue shift of the emission maximum from 350 to 340 nm (Fig. 3*A*). 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. 3*B*). As shown in

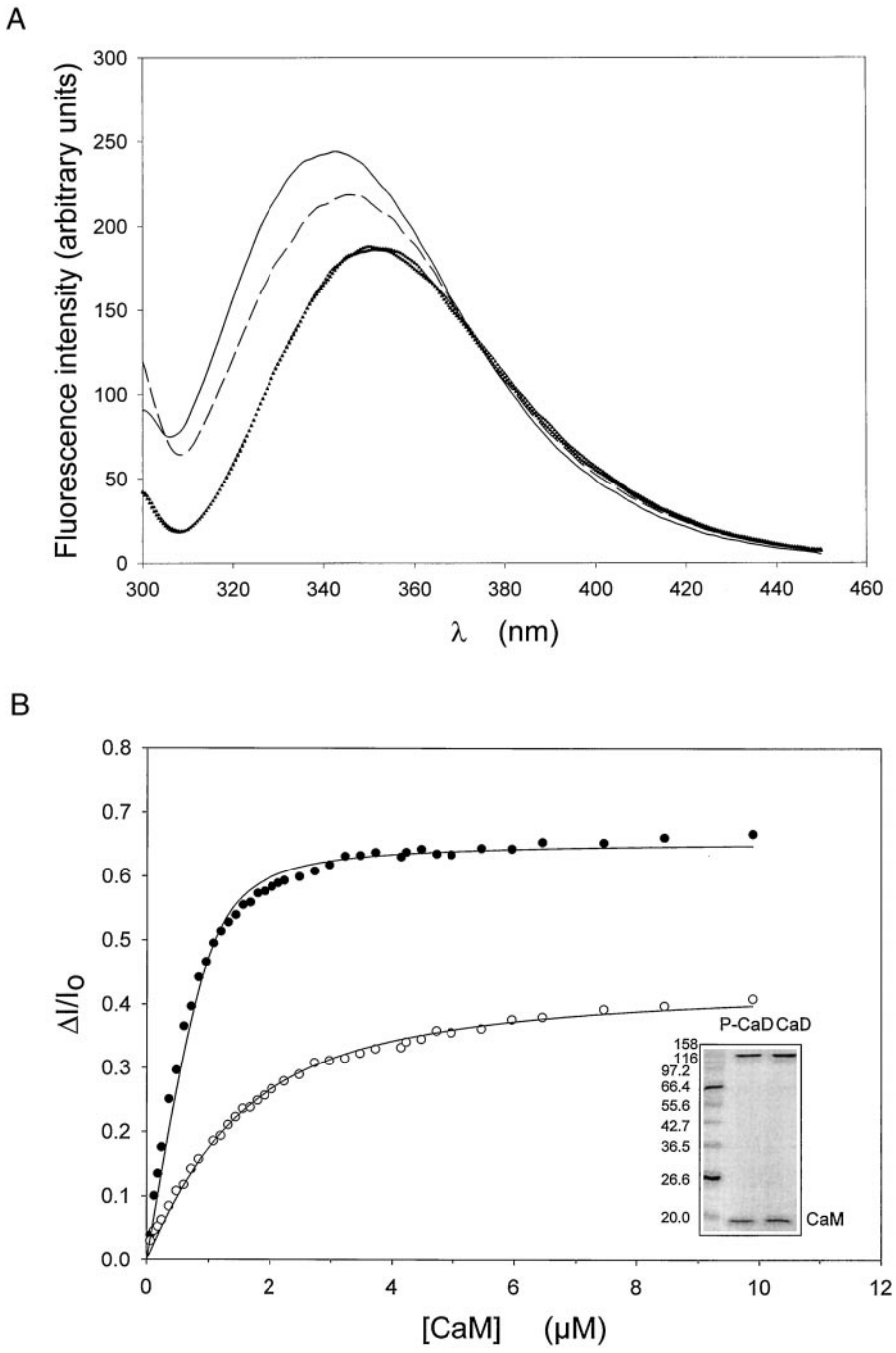

FIG. 3. **Effect of phosphorylation on binding of caldesmon to calmodulin.** *A*, Trp fluorescence emission spectra of phosphorylated caldesmon with calmodulin (- - -) and unphosphorylated caldesmon with calmodulin (—). In the absence of calmodulin, the emission spectra of phosphorylated and nonphosphorylated 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. ΔI is the change in fluorescence in caldesmon at 330 nm induced by calmodulin; I_o is fluorescence of caldesmon in the absence of calmodulin. λ = wavelength. *Inset*, 12% SDS-polyacrylamide gel electrophoresis of the phosphorylated and unphosphorylated caldesmon and calmodulin at the end of the binding studies.

Fig. 3*B*, 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 μM.

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 site A has 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 μM. 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. 5*B*). Similar results were obtained using smooth muscle S1 (data not shown). In the absence of tropomyosin (Fig. 5*A*), 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-

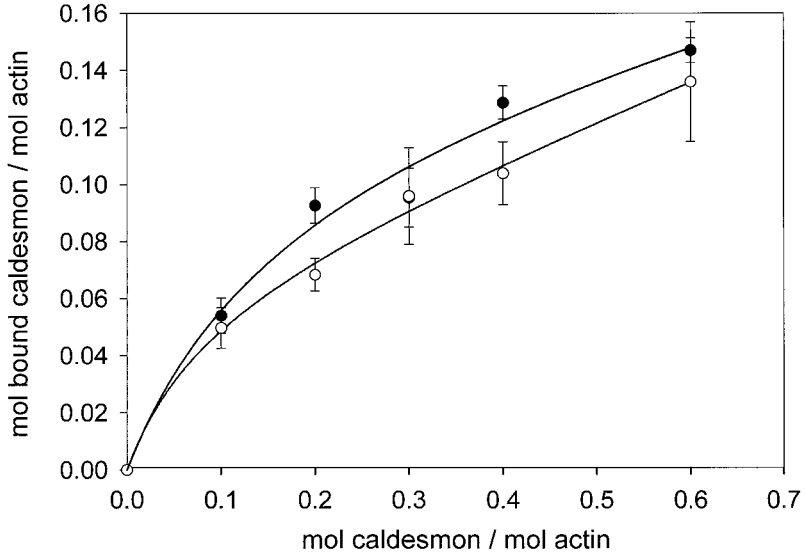

FIG. 4. **Effect of phosphorylation on binding of caldesmon to actin-tropomyosin.** Binding curves of unphosphorylated caldesmon (●) and phosphorylated caldesmon (○) to actin-tropomyosin was analyzed by sedimentation.

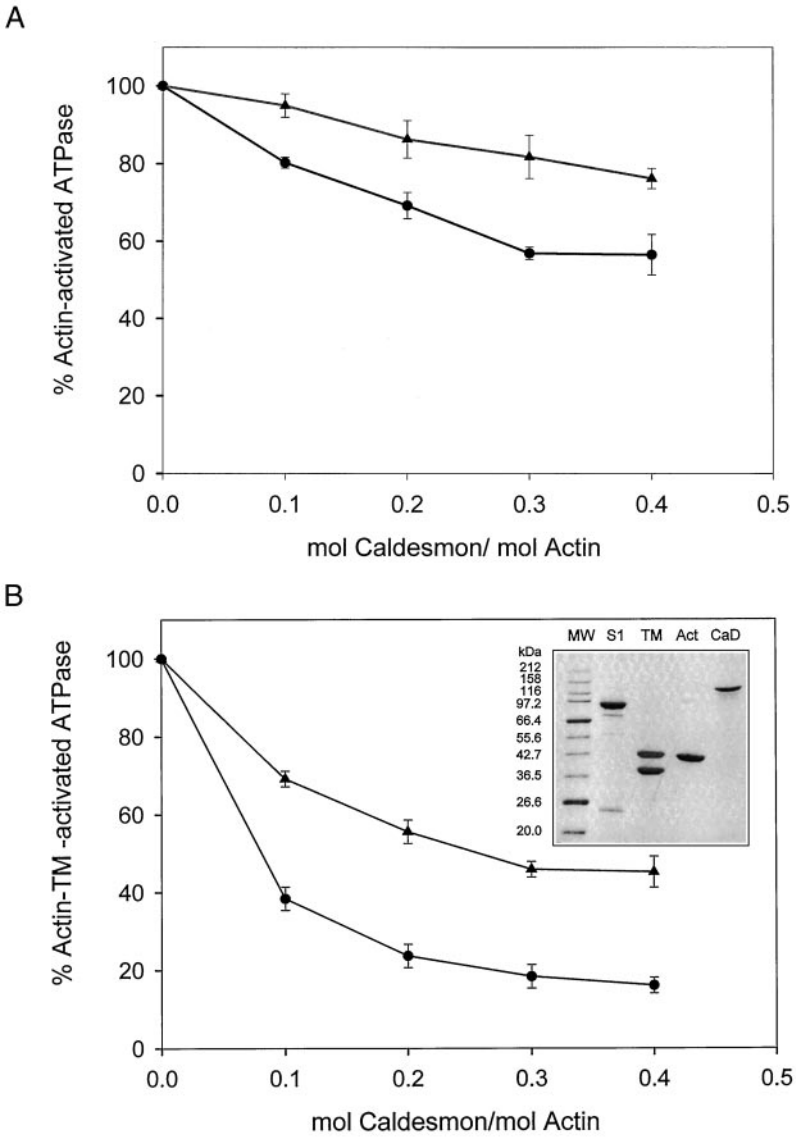

FIG. 5. **Inhibition of actin-activated skeletal S1-ATPase activity by caldesmon and phosphorylated caldesmon.** *A*, inhibition of actin-activated S1-ATPase activity by unphosphorylated (●) and phosphorylated caldesmon (▲) in the absence of tropomyosin. *B*, same as *panel A* except that tropomyosin was present.

standing of how phosphorylation of these sites may affect the function of caldesmon. The sequences around Ser^{657} and Ser^{687} 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. Ser^{657} and Ser^{687} 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

phosphorylate caldesmon *in vitro*. The sequences surrounding Ser^{657} (Arg-Asn-Ile-Lys-Ser^{657}-Met-Trp-Glu) and Ser^{687} (Lys-Val-Gly-Val-Ser-Ser^{687}-Arg-Ile-Asn) in caldesmon, and Ser^{19} (Gln-Arg-Ala-Thr-Ser^{19}-Asn-Val-Phe) in MLC have a hydrophobic residue in the +2 position which agrees with Brzeska *et al.* (39) who showed that a Tyr at the +2 position is strongly preferred by PAK1. As well, seven of the eight autophosphorylation sites in PAK1 have a hydrophobic residue in position +2 (40). However, Tuazon *et al.* (34), using a series of synthetic peptide substrates, identified the signature determinants for PAK1 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 PAK recognition.

Not unexpected, we found that phosphorylation of Ser^{657} and Ser^{687} interfered with interaction between calmodulin and caldesmon. We have shown previously that although Trp^{659} and Trp^{692} 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 α-helical upon binding to calmodulin (36, 37). Furthermore, the helix formed by site A is amphiphilic such that Ser^{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. 3*A*), 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 attenuates subsequent phosphorylation of caldesmon by PAK, indicating that Ser^{657} and/or Ser^{687} become less accessible to PAK (Fig. 1).

The actin-binding sites span an extended region in subdomain 4 of caldesmon (28). Introduction of two phosphates at Ser^{657} and Ser^{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 actin-tropomyosin (Fig. 4), which might perhaps be because of a reorientation of the caldesmon molecule along the actin-tropomyosin filament toward forming a noninhibitory state. This interpretation is consistent with our finding that phosphorylation significantly reduces the ability of caldesmon to inhibit myosin S1-ATPase. A synthetic peptide spanning from Gly^{651} to Ser^{667} containing site A has been shown to bind actin and enhance contraction in saponin-treated single hyper-permeable ferret aorta smooth muscle cells (27). Ser^{687} and site B are situated in the midst of a tropomyosin-dependent actin-binding region, residues 669–710, which is believed to be involved in tropomyosin-linked caldesmon inhibition of actomyosin ATPase activity (28). It is conceivable that phosphorylation of Ser^{687} and Ser^{657} is responsible for altering the tropomyosin-dependent and tropomyosin-independent inhibition, respectively.

As shown in Fig. 5*B*, PAK-phosphorylation attenuates (~50%), but does not abolish, caldesmon inhibition of actin-TM-activated myosin S1 ATPase activity invoking a phosphorylation mechanism by which caldesmon function can be modulated independently of Ca^{2+}/calmodulin. It appears that caldesmon can exist in a number of states endowed with different inhibitory activities depending on its phosphorylation status and binding to Ca^{2+}/calmodulin. One of these states, which is generated by Ca^{2+}-independent phosphorylation of Ser^{657} and Ser^{687} with PAK, possesses intermediate inhibitory activity compared with the fully inhibitory nonphosphorylated caldesmon and the noninhibitory Ca^{2+}/calmodulin-caldesmon complex. It is possible that introduction of phosphate groups to the calmodulin-binding region may engender a simulacrum of the Ca^{2+}-calmodulin-bound state of caldesmon, thus accounting for the partial reversion of inhibition. Formation of a complex between phosphorylated caldesmon and calmodulin, however, appears unfavorable in view of results showing that the affinity of phosphorylated caldesmon for Ca^{2+}/calmodulin is reduced 10-fold and that Ser^{657} and/or Ser^{687} in the caldesmon-calmodulin complex are less accessible to PAK (Fig. 3). Taken together, data from this study and others suggest that Rac1cdc42 and Rho GTPase may act in concert to target thin- and thick-filament, respectively, modulating Ca^{2+} sensitivity of smooth muscle contraction.

Acknowledgments—We thank S. Bagrodia and R. A. Cerione for the gifts of GST-mPAK3. We extend thanks to Nina Buscemi and Lenny Organ for technical assistance and to Dr. Irena Neverova for helpful suggestions.

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J. Biol. Chem. 2000, 275:1959-1965.
doi: 10.1074/jbc.275.3.1959
