Modulation of smooth muscle contractility by CHASM, a novel member of the smoothelin family of proteins

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Abstract **Cyclic nucleotides acting through their associated protein kinases, the cGMP- and cAMP-dependent protein kinases, can relax smooth muscles without a change in free intracellular calcium concentration ($[Ca^{2+}]_i$), a phenomenon referred to as Ca^{2+} desensitization. The molecular mechanisms by which these kinases bring about Ca^{2+} desensitization are unknown and an understanding of this phenomenon may lead to better therapies for treating diseases involving defects in the contractile response of smooth muscles such as hypertension, bronchospasm, sexual dysfunction, gastrointestinal disorders and glaucoma. Utilizing a combination of real-time proteomics and smooth muscle physiology, we characterized a distinct subset of protein targets for cGMP-dependent protein kinase in smooth muscle. Among those phosphoproteins identified was calponin homology-associated smooth muscle (CHASM), a novel protein that contains a calponin homology domain and shares sequence similarity with the smoothelin family of smooth muscle specific proteins. Recombinant CHASM was found to evoke relaxation in a concentration dependent manner when added to permeabilized smooth muscle. A co-sedimentation assay with actin demonstrated that CHASM does not possess actin binding activity. Our findings indicate that CHASM is a novel member of the smoothelin protein family that elicits Ca^{2+} desensitization in smooth muscle.**

1. Introduction

Smooth muscle relaxation occurs through one of the two mechanisms: passive relaxation following the removal of the contractile stimulus or active relaxation induced by the activation of cyclic nucleotide-dependent protein kinases in the lasting presence of the contractile stimulus [1–3]. Nitric oxide (NO), which has been implicated in the regulation of smooth muscle tone, signals through multiple mechanisms including stimulation of guanosine 3′,5′-cyclic monophosphate (cGMP) synthesis. Increases in intracellular [cGMP] (or [cAMP]) by cyclic-nucleotide elevating agents lead to the relaxation of intact smooth muscle [1].

One potential mechanism of cGMP-mediated smooth muscle relaxation is through a decrease in intracellular calcium concentration ($[Ca^{2+}]_i$) [4,5]. cGMP-dependent protein kinase (PKG) is an important effector for transducing cGMP signals into biological responses [6,7]. Several possible cyclic nucleotide-dependent protein kinase substrates have been identified including: the inositol 1,4,5-triphosphate (IP_3) receptor, IP_3-associated cGMP kinase substrate (IRAG), phospholamban, the large conductance calcium-activated potassium channels (BK_{Ca}), and the plasma membrane Ca^{2+}-pump. Phosphorylation of the IP_3 receptor is believed to inhibit IP_3-induced Ca^{2+} release [8]. Similarly, phosphorylation of IRAG is associated with an inhibition of IP_3-dependent Ca^{2+} release [9]. Phosphorylation of phospholamban causes activation of the Ca^{2+}-ATPase in the sarcoplasmic reticulum thereby decreasing $[Ca^{2+}]_i$ [10]. Stimulation of PKG produces membrane polarization and subsequent vasodilation through BK_{Ca} channel activation by channel phosphorylation [11]. Phosphorylation of the plasma membrane Ca^{2+} pump increases the affinity of the pump for Ca^{2+} [12] and promotes extrusion of Ca^{2+} into the extracellular space. These cyclic-nucleotide-dependent protein kinase substrates all mediate smooth muscle relaxation by decreasing $[Ca^{2+}]_i$. However, cyclic nucleotides acting through their associated kinases can relax permeabilized smooth muscle without a comparable change in $[Ca^{2+}]_i$ [5,13–17]. This phenomenon, whereby a decrease in myosin regulatory light chain (RLC) phosphorylation and force occurs without a proportionate decline in $[Ca^{2+}]_i$, is referred to as Ca^{2+} desensitization [18–20].

Inhibition of MLCK activity has been proposed as one mechanism of Ca^{2+} desensitization as cyclic nucleotide-induced phosphorylation of MLCK reduces the activity of MLCK in vitro. However, inhibitory phosphorylation of MLCK in vivo appears to be primarily due to CaMKII. An additional mechanism of Ca^{2+} desensitization is thought to involve the activation of smooth muscle myosin phosphatase (SMPP-1M). Reports have demonstrated that cGMP administration can accelerate dephosphorylation of RLC when MLCK activity is inhibited [16,17]. The mechanism of cyclic nucleotide-dependent activation of SMPP-1M may involve

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Abbreviations: CaMKII, calcium/calmodulin-dependent protein kinase II; CHASM, calponin homology-associated smooth muscle protein; NO, nitric oxide; cGMP, guanosine 3′,5′-cyclic monophosphate; PKG, cGMP-dependent protein kinase; 8Br-cGMP, 8-bromoguanosine 3′,5′-cyclic monophosphate; SMPP-1M, myosin phosphatase; $[Ca^{2+}]_i$, intracellular calcium concentration

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direct phosphorylation of the myosin-binding (MYPT1) subunit of SMPP-1M. Evidence for a direct interaction between SMPP1-1M and PKG has been presented [21,22]. In these studies, PKG-Iα was targeted to SMPP-1M through a leucine zipper interaction with MYPT1, and disruption of the PKG-Iα/SMPP-1M interaction inhibited cGMP-mediated dephosphorylation of myosin RLC. However, other investigations have demonstrated that direct phosphorylation of MYPT1 by PKG does not appear to alter the enzyme's activity in vitro [23,24]. The observation that cGMP causes Ca^{2+} desensitization in smooth muscle through activation of myosin phosphatase activity [16,17] therefore suggests an indirect mechanism involving a mediator protein(s). Previous studies have identified multiple proteins that are phosphorylated during cGMP-induced Ca^{2+} desensitization, including telokin [16], and the 20 kDa heat shock-related protein (HSP20) [25]; however, a direct effect on myosin phosphatase activity has yet to be demonstrated for these proteins. The objectives of the present study were to identify any additional smooth muscle proteins that were phosphorylated during cGMP-dependent relaxation and to evaluate their role in smooth muscle Ca^{2+} desensitization.

2. Materials and methods

2.1. Materials

CaMKII was provided by Dr. Anthony Means (Duke University). Smooth muscle MLCK was purified using a previously established protocol [26]. Sequencing grade trypsin was from Promega (Madison, WI). *Staphylococcus aureus* α-toxin was from List Biological Laboratories (Campbell, CA). Precission Protease™ was from Amersham Biosciences (Piscataway, NJ). PKA catalytic subunit, PKG-Iα, and 8-Br-cGMP were from Calbiochem (San Diego, CA). Microcystin was obtained from Alexis Biochemicals (San Diego, CA). β-escin was from Sigma (St. Louis, MO). Recombinant $p42^{MAPK}$ was purified from *Escherichia coli* BL21(DE3)[pET-MK] [27]. Constitutively active S218D/S222D MEK1 was a gift from Dr. Andrew Catling (University of Virginia).

2.2. Two-dimensional gel electrophoresis

For two-dimensional gel electrophoresis, radio-labeled ileum samples (see below) were homogenized in glass on glass homogenizers in sample buffer containing 5 M urea, 4% CHAPS, 1 mM DTT, and 10 nM microcystin and centrifuged at $15\,000 \times g$ for 15 min. The supernatant was removed for two-dimensional gel analysis. Proteins were visualized with silver staining and gels were dried and subjected to autoradiography. Spots of interest were excised from the gels and processed for mass spectrometric analysis.

2.3. Real-time identification of phosphoproteins in smooth muscle by mass spectrometric analysis

Rabbit ileum longitudinal smooth muscle was removed from rabbits anesthetized by halothane and exsanguinated according to approved animal protocols. Sheets of ileum smooth muscle (~1 cm × 0.5 cm) were permeabilized with *S. aureus* α-toxin (20 μg/ml) for 40 min at room temperature in calcium-free solution containing 1 mM EGTA (G1). Following permeabilization, muscle sheets were washed in Ca^{2+}-free solution and contracted with submaximal calcium (pCa6.3). After 10 min, muscle sheets were washed in 10 mM EGTA solution containing 0.5 mM ATP (G10′) and then incubated in the same solution with [γ-^{32}P]ATP (1 mCi/sheet) for 5 min. Muscles were then stimulated with either vehicle (water) or 8-Br-cGMP (100 μM). Muscles were snap frozen in liquid nitrogen and prepared for two-dimensional gel electrophoresis, as above. Proteins exhibiting increased phosphorylation in response to 8-Br-cGMP stimulation were cut from the gels and in-gel digested with trypsin [28]. Extracted tryptic peptides were purified with Poros R2 (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions (http://protana.com). The peptides were concentrated in a nano-electrospray capillary and placed in the source head of a QSTAR Pulsar hybrid mass spectrometer (Applied Biosystems) to derive de novo peptide sequences. Peptide sequences were searched against protein and DNA non-redundant databases using the FASTS algorithm [29].

2.4. Expression and purification of recombinant CHASM

Recombinant CHASM was produced from I.M.A.G.E. cDNA clone 3593616 (Research Genetics). The open reading frame was amplified by polymerase chain reaction (PCR) using the 5′ primer, CGCGGATCCATGGAGCAGACAG-3′ and the 3′ primer, GTGCTCGAGTGCGGCCGCCC-3′. The resultant PCR product was in-frame inserted into vector pGEX-6P-1 (Amersham Pharmacia Biotech). *E. coli* cells were cultured in Luria broth containing 50 μg/ml ampicillin overnight at 37 °C. Cells were induced with 400 μM IPTG for 2 h at 37 °C and GST-CHASM was isolated using glutathione-Sepharose 4B beads. For in situ experiments, the CHASM recombinant protein was cleaved by treatment with PreScission Protease™ as described by the manufacturer (Amersham Pharmacia Biotech). A phosphorylation site mutant CHASM protein (S301A) was generated with the QuickChange Site Directed mutagenesis kit (Stratagene, La Jolla, CA). The CHASM S301A mutant protein was expressed and purified as described above.

2.5. In vitro phosphorylation of recombinant CHASM by PKG, PKA, CaMKII, MAPK, rMYPT1K, and MLCK

Phosphorylation of CHASM (0.55 mg/ml) was performed at 25 °C in 25 mM HEPES, pH 7.2, 1 mM $MgCl_2$, and 0.1 mM DTT, with 0.2 mM [γ-^{32}P]ATP and 30 ng/ml of either PKG-Iα, PKA catalytic subunit, activated $p42^{MAPK}$, CaMKII, rMYPT1K or MLCK. MAPK was activated by phosphorylation with recombinant S218D/S222D MEK1 as previously described [27]. Assays with MLCK and CaMKII were carried out in a buffer that also contained 10 μM $CaCl_2$ and 2.5 μM calmodulin. Reactions were terminated at the indicated time points by addition of Laemmli sample buffer. Proteins were resolved by SDS–PAGE and phosphorylated CHASM was visualized by autoradiography.

2.6. Muscle tension measurements

For in situ force measurements, sheets of longitudinal muscle were peeled and cut into small strips (3 mm × 250 μM). Muscle strips were attached to a force transducer (SensorOne AE801, Sausalito, CA) in a "bubble" chamber and stretched to 1.3 times resting length. Muscle strips were permeabilized by incubation for 30 min with 50 μM β-escin in an intracellular solution containing 1 mM EGTA and no added Ca^{2+} (G1) with 10 μM A23187 added for the final 10 min to deplete intracellular calcium stores. All experiments were carried out at room temperature.

2.7. Actin co-sedimentation assay

An actin co-sedimentation assay was performed to investigate the ability of CHASM to bind actin. Precission Protease™-cleaved, recombinant CHASM protein (1 μM) was incubated with F-actin (9 μM) in F-actin buffer (25mM Tris–HCl, pH 7.5, 130 mM KCl, 0.1 mM $CaCl_2$, 8.6 mM $MgCl_2$, 1 mM ATP, and 1 mM dithiothreitol) for 1 h at 25 °C. In some experiments, tropomyosin (1 μM) was added as a postive-control for actin binding. After the incubation, the protein mixtures were ultracentrifuged at $150{,}000 \times g$ for 1 h at 4 °C. The supernatant was separated from the pellet and the proteins present in each fraction were resolved by SDS–PAGE with Coomassie or silver staining.

3. Results

3.1. Identification of a novel smooth muscle protein phosphorylated in response to 8-Br-cGMP administration

In order to identify possible candidate proteins that contribute to cyclic nucleotide-induced Ca^{2+} desensitization, we permeabilized sheets of rabbit ileum smooth muscle and treated them with either vehicle (water) or 8-Br-cGMP, a non-hydrolyzable analogue of cGMP, in the presence of exogenously added [γ-^{32}P] ATP. Addition of [γ-^{32}P] ATP with

8-Br-cGMP followed by flash freezing with liquid nitrogen ensured that only those phosphorylation events directly mediated by PKG would be identified, thereby greatly reducing the complexity of phosphoproteins that one would normally observe following steady state ^{32}P-labelling of intact muscles. Acute 8-Br-cGMP-stimulation of ileum smooth muscle was associated with an increase in phosphorylation of a number of proteins (Fig. 1A). Proteins exhibiting an increase in ^{32}P incorporation (phosphorylation) in response to 8-Br-cGMP were identified in 2D gels by mass spectrometry. Consistent with previously published results, we found increased phosphorylation of telokin [16] (Fig. 1A, labeled 5), and the 20 kDa heat shock-related protein, HSP20 [30–32] (labeled 2), in response to 8-Br-cGMP stimulation (mass spectrometry sequence data not shown). An increase in phosphorylation in response to 8-Br-cGMP stimulation was also detected for actin depolymerizing factor (labeled 4). Phosphorylation of a ubiquitin conjugating enzyme (labeled 3) was detected. The roles of these proteins in cyclic nucleotide-dependent smooth muscle relaxation remain undefined. Some proteins that demonstrated increases in phosphorylation with 8-Br-cGMP-induced relaxation were not identified due to insufficient protein quantity.

Most interestingly, we observed a fourteen fold increase in the phosphorylation of an acidic protein of approximately 60 kDa (Fig. 1A, labeled 7) when the vehicle treated control was compared with 8-Br-cGMP stimulation, 1.0 ± 0.3 versus 14.2 ± 1.4 (mean $\pm$ S.E.M. for $n = 4$ separate experiments). Two peptides obtained from the trypsin digestion of this protein were sequenced by nanospray mass spectrometry and aligned within the sequence of a RIKEN cDNA putative gene product (Fig. 1B). The sequence of the RIKEN putative gene product revealed the presence of a calponin homology (CH) domain at its carboxyl terminus (Fig. 2). We have designated this protein CHASM for Calponin Homology-Associated Smooth Muscle protein. The next highest scoring sequence was smoothelin (expectation score of 0.18), a smooth muscle-specific protein also characterized by the presence of a CH-domain at its carboxyl terminus [33].

A FASTA search of the public databases with the full length CHASM sequence showed a high degree of similarity in the C terminus (residues 347–451) with the CH2 domains found in the smoothelins (Fig. 2). Smoothelin proteins are found exclusively in differentiated smooth muscle tissue [34]. Although the entire N-terminal domain of CHASM shares minimal similarity with the smoothelins, close inspection of the primary sequence alignment reveals a number of additional amino acids that are conserved in all four proteins (Fig. 2). Many of these residues are basic or acidic amino acids that are distributed throughout the entire protein sequence. Notably, and in contrast to the smoothelins, the CHASM protein possesses a single, consensus site for phosphorylation by cyclic nucleotide-dependent protein kinases (Ser 301) (Fig. 2).

3.2. CHASM is phosphorylated by cyclic nucleotide-dependent kinases

Based on the amino acid sequence of CHASM, a single consensus phosphorylation sequence for PKG/PKA was predicted by PhosphoBase analysis [35]. Accordingly, PKG and

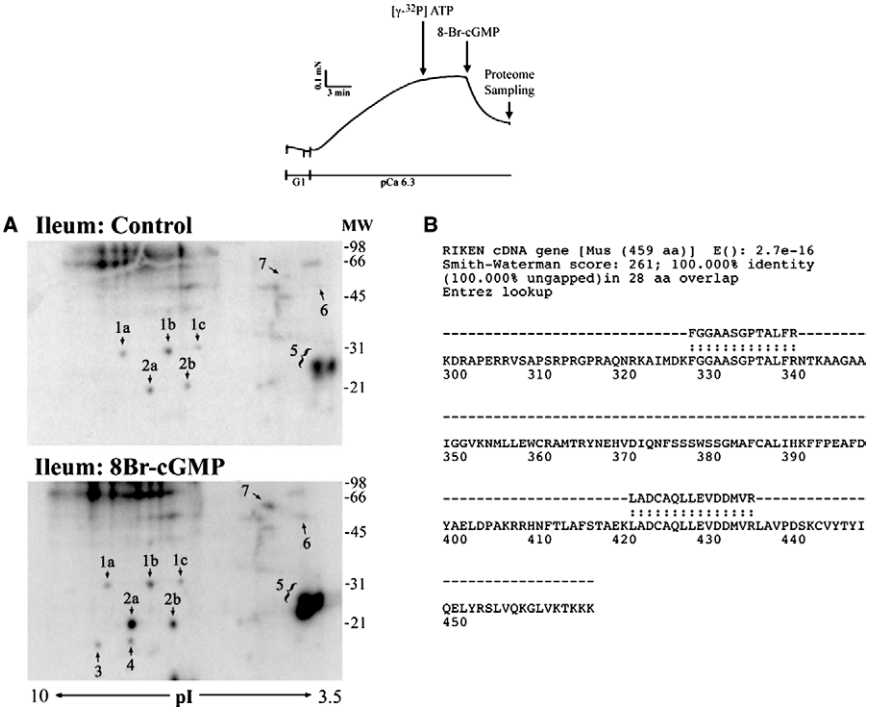

Fig. 1. Identification of CHASM as a novel target for cGMP-dependent protein kinase. (A) Autoradiograms of control and 8-Br-cGMP-stimulated rabbit ileum smooth muscle showing acute phosphorylation events. Sheets of α-toxin permeabilized smooth muscle were washed in G1 and contracted with pCa 6.3. Following a 5 min incubation with [γ-^{32}P] ATP, vehicle (water) or 50 μM 8-Br-cGMP was added. After 5 min, muscle sheets were rapidly frozen in liquid nitrogen and processed for 2D-gel electrophoresis. Phosphorylated proteins were identified by tandem mass spectrometry: (1a,b,c) HSP27, (2a,b) p20, (3) ubiquitin conjugating enzyme, (4) actin depolymerizing factor (5) telokin, and (7) CHASM. The protein labeled (6) was below detection limits. Autoradiograms are representatives of at least 6 independent experiments. (B) Alignment of tryptic peptides from protein 7, sequenced by nanospray tandem mass spectrometry, using the FASTS algorithm.

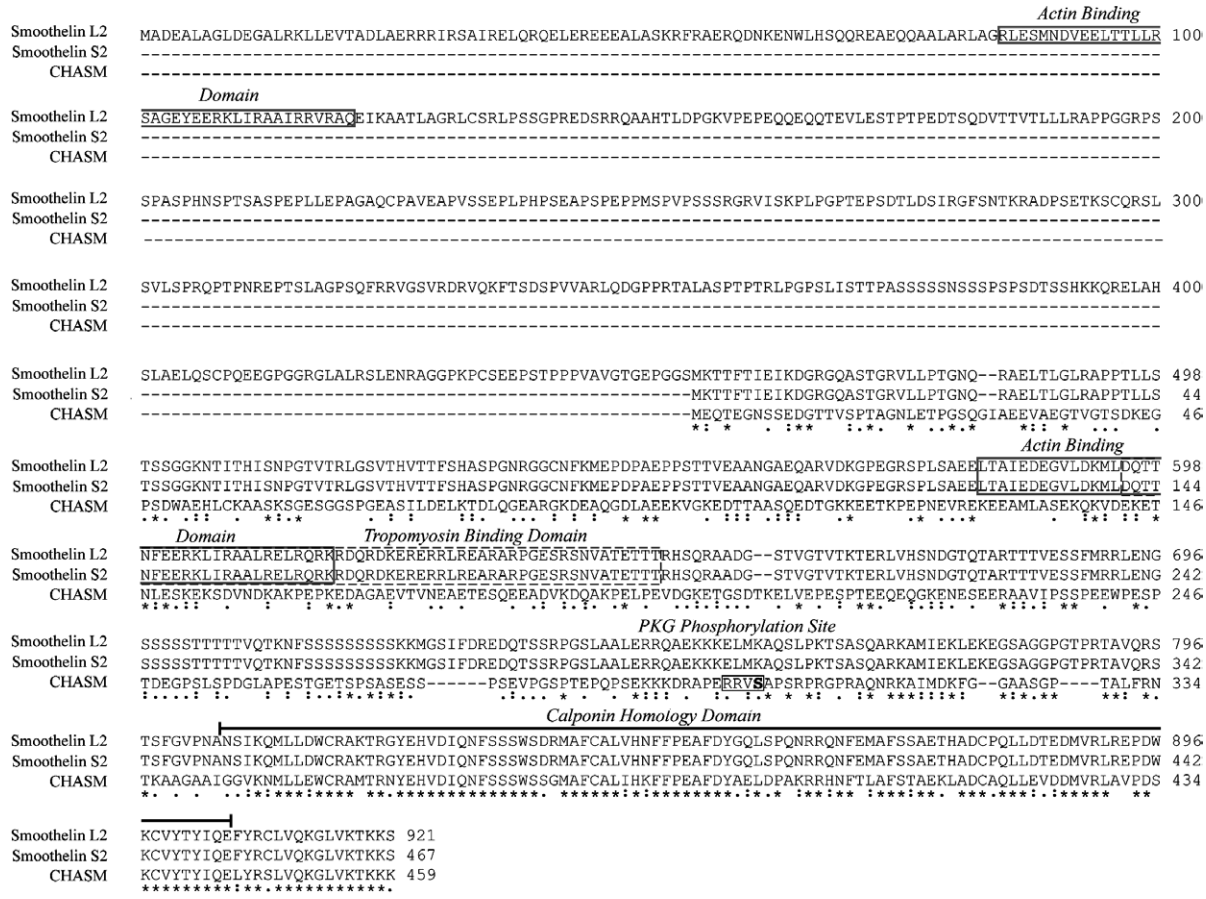

Fig. 2. Sequence alignment of mouse CHASM with the smoothelin isoforms. The sequence analysis of CHASM (NM_024230), smoothelin S2 (AAF25580), and smoothelin L2 (AAF01480) was completed using the ClustalW multiple sequence alignment program (www.ebi.ac.uk http://www.ebi.ac.uk). The locations of the calponin homology domain, the single PKG phosphorylation site, the tropomyosin binding domain, and the actin binding domains are indicated. Amino acid residues identical in all proteins are indicated with (*), conserved substitutions are denoted by (:), and semi-conserved substitutions are denoted by (.).

PKA phosphorylated recombinant CHASM equally well in vitro (Fig. 3A). CHASM was phosphorylated to a stoichiometry of 1 mol of phosphate/mol of protein and phosphoamino acid analysis indicated phosphorylation of serine alone (Fig. 3C). Mutation of Ser 301 to Ala completely abolished the in vitro phosphorylation of CHASM by PKG (Fig. 3B), confirming PKG phosphorylation at a single residue. These findings suggest that cGMP- and cAMP-activated kinase(s) could mediate CHASM phosphorylation in smooth muscle. Although Ser 301 is also a predicted Ca^{2+}/calmodulin-dependent kinase II (CaMKII) consensus sequence, CHASM was a poor substrate for CaMKII (Fig. 3A). Similarly, CHASM was not significantly phosphorylated by mitogen-activated protein kinase (MAPK), recombinant myosin phosphatase-associated kinase (rMYPT1K) or myosin light chain kinase (MLCK), suggesting that CHASM is phosphorylated only by cyclic nucleotide-dependent protein kinases and that no significant phosphorylation occurs in the presence of Ca^{2+}-dependent kinases in vitro.

3.3. CHASM elicits Ca^{2+} desensitization in β-escin permeabilized smooth muscle strips

Next, we examined the physiological effects of CHASM on β-escin permeabilized strips of rabbit ileum longitudinal smooth muscle. In these preparations, it is possible to strictly control $[Ca^{2+}]_i$ thereby providing a method of evaluating the effects of recombinant proteins in the absence of changes in calcium levels [14,16]. Contraction was induced by incubation in submaximal calcium prior to the addition of recombinant CHASM. CHASM relaxed submaximally contracted smooth muscle at constant $[Ca^{2+}]_i$ in a concentration-dependent manner (Fig. 4A). A maximum relaxation of $42.5 \pm 4.3\%$ ($n = 5$) was observed with 20 μM CHASM (Fig. 4B). A buffer control did not elicit any significant relaxation in the permeabilized muscle (data not presented).

3.4. Actin co-sedimentation assay

We examined the ability of full-length CHASM protein to co-sediment with actin in vitro. The CHASM protein did not co-sediment with actin (Fig. 5). Binding studies using actin (3 μM) with increasing amounts of CHASM (1, 3, 6, and 9 μM) were also unable to reveal any co-sedimentation of CHASM with actin (results not shown). While tropomyosin was found to be associated with actin following the co-sedimentation procedure, the addition of tropomyosin did not enable CHASM co-sedimentation with actin. These results suggest that the single CH-domain present in CHASM is insufficient for actin binding. Outside of the single CH-domain, CHASM does not contain any additional actin-binding or tropomyosin-binding domains.

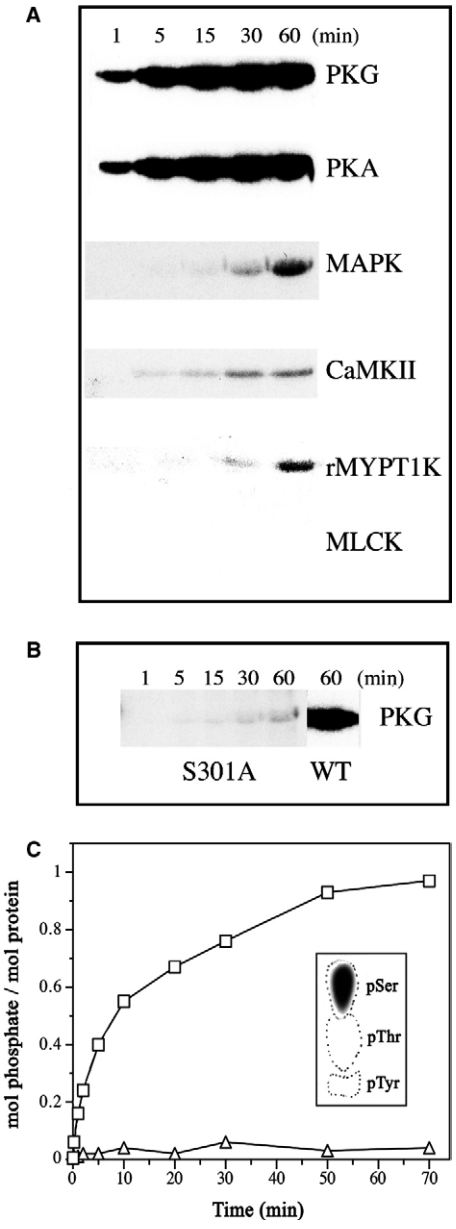

Fig. 3. Analysis of CHASM phosphorylation. (A) Autoradiograms showing the time course of phosphorylation of recombinant CHASM by the indicated protein kinases. (B) Autoradiograms showing the time course of phosphorylation of recombinant Ser301 → Ala CHASM. (C) Stoichiometry of phosphorylation by PKG of wild-type (●) and S301A (▲) CHASM proteins. Inset: phospho-amino acid analysis of PKG-phosphorylated CHASM.

4. Discussion

The function of the smoothelin family of proteins is unknown; however, the smoothelins are found only in contractile smooth muscle cells and are often used as markers for the differentiated phenotype of smooth muscle [33]. Two isoforms of smoothelin have been identified: a 59 kDa isoform (smoothelin A) that is expressed in visceral smooth muscle such as intestine [33] and a 100 kDa isoform (smoothelin B) that is expressed in vascular smooth muscle [34]. More recently, Leonhart and colleagues [36] have defined two alternate splice variants that contain spectrin family similarity. In this report, we present an additional member of the smoothelin family of proteins. The protein, termed CHASM, is unique among the smoothelin family in that it possesses a site for phosphorylation by cyclic nucleotide kinases.

In the present study, protein phosphorylation during cGMP-induced relaxation in smooth muscle was evaluated using a combination of real-time proteomics and smooth muscle physiology. Increased phosphorylation of the CHASM protein was identified as an early event in cGMP-induced smooth muscle relaxation. Both smoothelin isoforms (and CHASM) are characterized by the presence of a calponin homology domain at their carboxyl-termini. There is no evidence that the smoothelins play a role in smooth muscle Ca^{2+} desensitization or are phosphorylated in response to 8-Br-cGMP stimulation. Notably, there are no predicted PKG phosphorylation sites within the smoothelin sequences (based on a PhosphoBase analysis) [35]. Furthermore, the amino terminal domain of CHASM (residues 1–346), which contains the predicted phosphorylation site, is quite divergent from the smoothelins.

Calponin is an actin-binding protein that is almost exclusively expressed in smooth muscle [37]. The calponin homology domains were identified in this protein as regions that contributed to its actin binding and have since been identified in a number of actin-binding proteins and signaling molecules [38]. Calponin and other CH-domain containing proteins may regulate smooth muscle contractility via the thin filament regulatory system [37]. Upon association with actin filaments, calponin inhibits the actin-activated Mg^{2+}-ATPase activity of myosin [39]. We have demonstrated that CHASM is unable to associate with actin filaments in vitro. Previous reports [40] have demonstrated that the single CH-domain of calponin is neither necessary nor sufficient for its actin binding [40] and support our finding that the single CH-domain found in CHASM is not sufficient for actin binding. It has been suggested that two CH-domains in tandem are required for actin-binding (reviewed in [41]). The smoothelins possess novel actin binding domains that are responsible for association with actin containing filaments [36]. The large vascular specific smoothelin isoform contains two actin binding domains, whereas the small visceral specific isoform contains only one actin binding domain (Fig. 2). Both smoothelin isoforms also contain a tropomyosin binding domain [36]. CHASM lacks the novel actin binding domains found in the smoothelin proteins. Importantly, we have found that the addition of the full length CHASM protein to microcystin-treated smooth muscles (to block dephosphorylation of myosin by endogenous myosin phosphatase activity) did not cause relaxation (preliminary data not presented). This finding supports a hypothesis that CHASM possesses a regulatory role in the signaling process, potentially through modulation of myosin phosphatase activity, rather than a disruption of myosin ATPase activity and cross-bridge cycling.

The NO-cGMP pathway contributes to relaxation by decreasing the Ca^{2+} sensitivity of cross-bridge phosphorylation [42]. The evidence suggests that this phenomenon is modulated through myosin phosphatase regulation [16,17,42]. The association between increases in CHASM phosphorylation and the cGMP-dependent inhibition of contraction suggests that CHASM may be important in modulating this process. CHASM's ability to elicit relaxation without a change in $[Ca^{2+}]_i$ indicates that CHASM functions by activating the Ca^{2+} desensitization pathway. Based on the significant in-

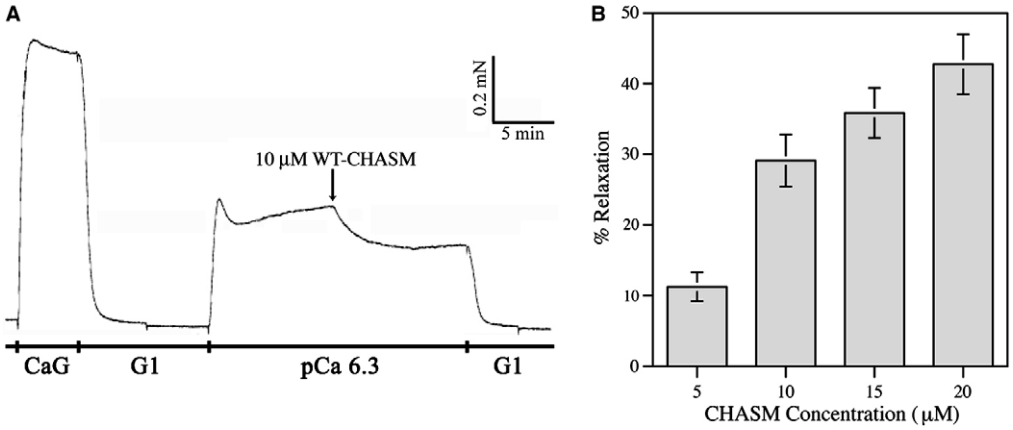

Fig. 4. CHASM-induced smooth muscle calcium desensitization. (A) β-escin permeabilized rabbit ileum smooth muscle strips were contracted with submaximal calcium (pCa 6.3), and at the plateau of contraction wild-type, Precission Protease™-cleaved CHASM was added. (B) The concentration-dependence of relaxation induced by CHASM. Percent relaxations are calculated from the plateau of the pCa 6.3 contraction. Data are representatives of at least $n = 4$ experiments.

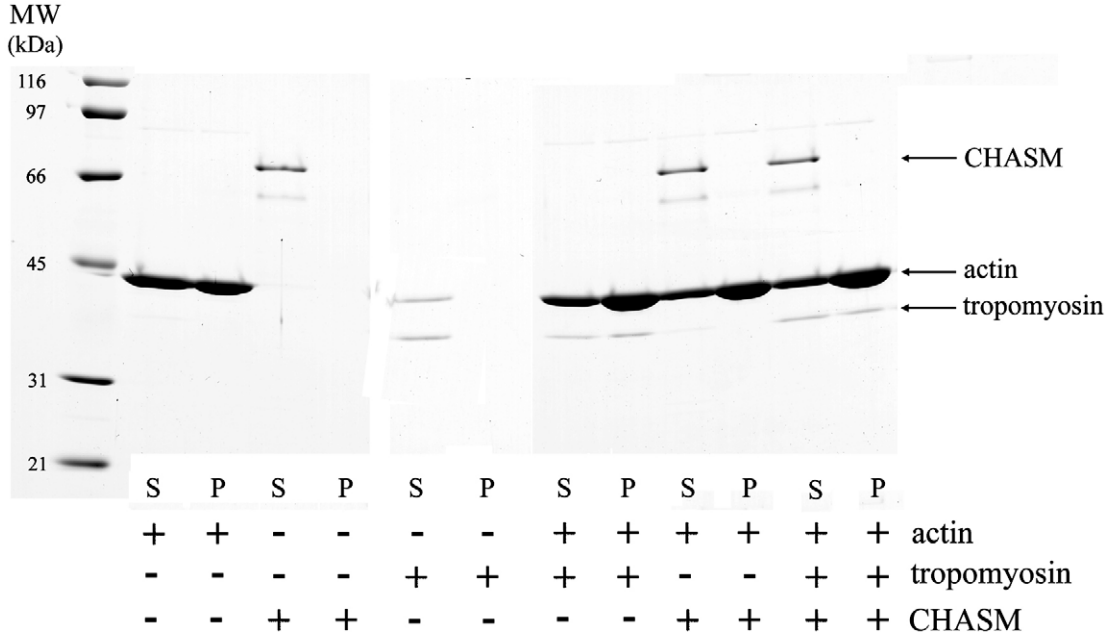

Fig. 5. Co-sedimentation assay of CHASM with F-actin and tropomyosin. F-actin was sedimented by ultracentrifugation and associated proteins were identified by SDS–polyacrylamide electrophoresis: S, ultracentrifuged supernatant; P, ultracentrifuged precipitate. Precission Protease™-cleaved CHASM (1 μM) was incubated with (+) or without (−) F-actin (9 μM) and tropomyosin (1 μM) in F-actin buffer for co-sedimentation assay.

crease in CHASM phosphorylation observed during the early stages of smooth muscle relaxation, a role for cGMP-induced CHASM phosphorylation seems likely. Further evaluation is necessary to elucidate the precise mechanism of CHASM regulation and the role of phosphorylation therein.

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