Mutagenesis analysis of human SM22: characterization of actin binding

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SM22 is a 201-amino acid actin-binding protein expressed at high levels in smooth muscle cells. It has structural homology to calponin, but how SM22 binds to actin remains unknown. We performed site-directed mutagenesis to generate a series of NH2-terminal histidine (His)-tagged mutants of human SM22 in Escherichia coli and used these to analyze the functional importance of potential actin binding domains. Purified full-length recombinant SM22 bound to actin in vitro, as demonstrated by cosedimentation assay. Binding did not vary with calcium concentration. The COOH-terminal domain of SM22 is required for actin affinity, because COOH terminally truncated mutants [SM22-(1–186) and SM22-(1–166)] exhibited markedly reduced cosedimentation with actin, and no actin binding of SM22-(1–151) could be detected. Internal deletion of a putative actin binding site (154-KKAQEHKR-161) partially prevented actin binding, as did point mutation to neutralize either or both pairs of positively charged residues at the ends of this region (KK154LL and/or KR160LL). Internal deletion of amino acids 170–180 or 170–186 also partially or almost completely inhibited actin cosedimentation, respectively. Of the three consensus protein kinase C or casein kinase II phosphorylation sites in SM22, only Ser-181 was readily phosphorylated by protein kinase C in vitro, and such phosphorylation greatly decreased actin binding. Substitution of phosphorylated by protein kinase C in vitro, and such phosphorylation sites in SM22, only Ser-181 was readily phosphorylated by protein kinase C (PKC) and two more potential targets for casein kinase II (CKII), although no SM22 phosphorylation has been demonstrated (5, 7). It remains unknown whether calcium binding or phosphorylation alters SM22-actin association.

Since its first discovery in chicken gizzard smooth muscle by Lees-Miller and colleagues (12, 14), SM22 has been identified in many different species (3, 9, 10, 13, 14, 17, 18, 20) and has been given different names, including transgelin (11), WS3-10 (21), and mouse p27 (1). In adult vertebrates, accumulation of SM22 protein is restricted to smooth muscle, where it is one of the most abundant proteins. The DNA and protein sequences of SM22 are highly conserved across species, and homologs have been found in invertebrate species as distant as Caenorhabditis elegans (unc-87) (6) and Drosophila melanogaster (mp20) (2). Despite its apparent evolutionary importance and its abundance within smooth muscle, the function of SM22 is still poorly characterized. We previously showed that SM22 decorates contractile filament bundles within cultured tracheal smooth muscle cells that exhibit a differentiated phenotype (5). A number of other investigators also found that SM22/transgelin can bind to and/or gel actin (10, 15, 16), possibly through a predicted actin binding site that contains four positively charged amino acids. However, no prior study has experimentally evaluated whether this putative actin binding site is required for SM22-actin binding.

In a recent study of calponin-actin interaction, Gimon and Mital (4) also assessed SM22-actin interactions but found no SM22-actin binding, either by cosedimentation assay in vitro or immunolocalization of transfected SM22 in vivo. These investigators did, however, demonstrate that COOH-terminal tandem repeats within calponin are important for its actin binding, and one such sequence occurs within the COOH-terminal domain of SM22. These observations suggest the possibility that this region of high homology with calponin might participate in SM22-actin interactions.

Finally, SM22 possesses a potential EF-hand calcium binding domain, but there is only indirect evidence that SM22 binds calcium (17), and the functional significance of calcium interaction is unclear. Furthermore, the amino acid sequence of human SM22 includes one potential site for phosphorylation by protein kinase C (PKC) and two more potential targets for casein kinase II (CKII), although no SM22 phosphorylation has been demonstrated (5, 7). It remains unknown whether calcium binding or phosphorylation alters SM22-actin association.
We undertook the present study to clarify further how SM22 binds to actin. To test the hypotheses that portions of the COOH-terminal domain of SM22 determine its binding to actin and that calcium concentration and/or phosphorylation by PKC or CKII modulate this binding, we generated a series of recombinant wild-type and mutant human SM22 proteins and used these to evaluate 1) the functional importance of regions within the COOH-terminal domain of SM22 for actin binding in vitro and in vivo and 2) the potential influences of calcium binding and phosphorylation on SM22-actin association.

MATERIALS AND METHODS

Cloning and protein expression and purification. Human total RNA was isolated from uterine tissue with use of a Qiagen RNA extraction kit, and a cDNA containing the full-length SM22 coding region was generated by RT-PCR. The first-strand cDNA was generated by RT by use of random hexamers and oligo(dT) primer under standard conditions, and the coding region was amplified using gene-specific primers flanking the coding region. A BamHI site was added to the sense primer and an HindIII site to the antisense primer. The wild-type cDNA and all cDNA mutants, produced by PCR, were ligated in frame into BamHI- and HindIII-digested pQE-30 bacterial expression vector (Qiagen) containing an NH2-terminal MRGHHHHHGS tag (hereafter designated “His tag”). A stop codon was introduced at the 3’ end of each clone, and the cDNA sequence of each clone was confirmed by the dideoxy-NTP method. Figure 1A shows the structure of the 5’-His-tagged recombinant wild-type and mutant SM22 proteins used in this study. Mammalian expression vectors encoding 5’-FLAG-tagged SM22 variants were constructed by subcloning wild-type or mutant cDNAs into HindIII- and KpnI-digested pFLAG-CMV2 expression vector (Sigma Chemical).

Recombinant His-tagged wild-type and mutant SM22 variants were expressed in Escherichia coli and purified by affinity chromatography on nickel [Ni-nitrilotriacetic acid (NTA)] columns (Qiagen) as follows. Transformed E. coli were grown to optical density at 600 nm of 0.6–1.0, and the recombinant protein expression was induced for 4 h after addition of 1 mM isopropyl-β-D-thiogalactopyranoside. Bacteria with recombinant protein expression were harvested and resuspended in 50 mM sodium phosphate, pH 8.0, 500 mM NaCl, 50 mM imidazole, and 1 mg/ml lysozyme. Lysate was centrifuged to remove cell debris, then the supernatant was passed through the Ni-NTA column. Retained proteins were eluted and stored in buffer containing 50 mM sodium phosphate, pH 8.0, 300 mM NaCl, and 250 mM imidazole and were >90% pure. The identity of wild-type NH2-terminal His-SM22 protein was confirmed by NH2-terminal amino acid sequencing.

A polyclonal antibody to His-SM22 was generated in New Zealand White rabbits (Pocono Rabbit Farms), with this recombinant protein used as immunogen. The identity of recombinant proteins used in this study was confirmed by Western blot with anti-RGS-His antibody (Qiagen) and our own anti-SM22 antibody, with enhanced chemiluminescence used to visualize immunoreactive bands.

In vitro phosphorylation. PKC was purchased from Sigma Chemical. CKII was purchased from New England Biolabs. Two micrograms of each recombinant SM22 variant were incubated at 30°C for 1 h in kinase buffer [20 mM Tris-HCl (pH 7.5), 50 mM KCl, 10 mM MgCl2] containing 5 μCi of [γ-32P]ATP. The reaction mixtures were analyzed by SDS-PAGE or purified by passage over Ni-NTA columns for actin binding assay (see below).

Cosedimentation assay. Rabbit skeletal muscle actin was purchased from Sigma Chemical. Recombinant His-SM22 proteins were precentrifuged at 120,000 g for 15 min at 20°C to remove protein aggregates. Cosedimentation was assessed in buffer containing 12 mM potassium phosphate (pH 6.8), 20 mM imidazole, 24 mM NaCl, 2 mM MgCl2, 1 mM ATP, and 1 mM EGTA. Each reaction contained 10 μM actin with 3 μM His-SM22 variant in a final volume of 100 μl and was incubated at room temperature for 1 h and then centrifuged at 120,000 g for 30 min at 20°C. Pellets were washed once, then resuspended in 50 μl of cosedimentation buffer. Proteins within pellets and corresponding supernatants were then size fractionated by 15% SDS-PAGE. Equal volumes were
loaded in each lane. To evaluate how calcium concentration affects binding of recombinant SM22 proteins to actin, 1 mM EGTA was replaced with 25–400 μM CaCl₂.

**In vivo binding of SM22 to actin.** To evaluate the in vivo physiological relevance of the COOH-terminal domain of SM22 for actin binding suggested by cosedimentation assays in vitro (see below), we transfected cultured canine tracheal smooth muscle cells at 70% confluence with expression plasmids encoding NH₂-terminal FLAG-tagged wild-type human SM22, FLAG-SM22-(1–151), or FLAG-SM22-(S181D) using Lipofectamine (GIBCO) reagent. Two days later, cells were fixed and dually stained for smooth muscle α-actin and FLAG epitope with use of anti-smooth muscle α-actin primary antibody (Sigma Chemical) with rhodamine-conjugated secondary antibody and anti-FLAG M1 monoclonal antibody (Sigma Chemical) with FITC-conjugated secondary antibody. Nuclei were stained using Hoechst-33342, and cells were imaged on a Nikon immunofluorescence microscope equipped with a Senys digital camera.

**RESULTS**

**Expression of recombinant SM22.** Figure 1A shows the structure of the NH₂-terminal His-tagged wild-type and mutant SM22 proteins used in this study. As shown in Fig. 1B, recombinant protein in isopropyl-β-D-thiogalactopyranoside-treated *E. coli* represented the most abundant species in crude lysate, and a single affinity purification step, using an Ni-NTA column, resulted in high purity (Fig. 1B). Each recombinant protein migrated at its expected size during SDS-PAGE, and each was recognized by mouse anti-RGS-His monoclonal antibody and rabbit anti-SM22 polyclonal antibody (data not shown).

**Recombinant SM22 cosediments with actin.** Wild-type His-SM22 readily cosedimented with actin, as shown in Fig. 2. Truncation of the 15 COOH-terminal amino acids, yielding SM22-(1–186), decreased the binding substantially. Further COOH-terminal deletion to SM22-(1–166) reduced binding to actin even further, whereas essentially no SM22-(1–151) could be found in the actin pellet. Thus amino acids within the COOH-terminal quarter of SM22 are required for full actin binding activity.

Figure 2 also illustrates a previously reported phenomenon, that SM22 proteins with intact COOH terminus (wild-type SM22 and internal deletion or point mutants) can spontaneously degrade in a discreet fashion, such that a minor, shorter protein is formed through cleavage of the COOH terminus (7); we have observed that the spontaneously shortened form of wild-type SM22 is still recognized by the antibody directed against the NH₂-terminal His tag (data not shown), confirming the COOH-terminal site of cleavage. Note that these spontaneously COOH-terminally shortened SM22 derivatives, such as those generated through site-directed mutagenesis, also demonstrate diminished binding to actin.

Previously, Prinjha and co-workers (15) proposed residue 154–161 of wild-type SM22/transgelin as a potential actin binding site. To evaluate whether this region participates in actin binding, we generated an SM22 mutant from which amino acids 154–161 were internally deleted [SM22-(Δ154–161)] and found that its binding to actin was reduced markedly (Fig. 2). This putative actin binding region (154-KKAKQEHKR-161) contains four positively charged residues. To explore their role in the SM22-actin interaction, we substituted the positively charged amino acids KK at 154/155 and/or KR at 160/161 with uncharged LL. Neutralization of the positive charges at either end of the putative actin binding site [i.e., SM22-(KK154LL) or SM22-(KR160LL)] decreased cosedimentation with actin, whereas substitution of all four positively charged residues with leucine [SM22-(LLLL)] decreased actin binding even further but not completely in our assay. These data show that the putative actin binding site predicted by Prinjha and co-workers does play a role in actin binding, probably through electrostatic interaction. However, because deletion of the entire amino acid 154–161 domain did not fully inhibit actin binding, other regions within the COOH-terminal domain of SM22 must also be important.

Gimona and Mital (4) previously demonstrated that COOH-terminal tandem repeats in calponin h1 and h2 are required for calponin-actin binding activity. The amino acid sequence of these repeats shares high homology with amino acids 175–195 of SM22. Furthermore, as shown in our serial deletion experiments, truncation of part or all of these amino acid residues significantly reduced SM22-actin binding. To further evaluate the importance of these residues in actin binding, we generated two additional internal deletion

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![Fig. 2](http://jap.physiology.org/DownloadedFrom/10.22033.3.onOctober30,2017)
mutants lacking amino acids 170–180 or 170–186. As shown in Fig. 2, SM22-(Δ170–180) exhibited reduced actin binding, whereas SM22-(Δ170–186) displayed nearly complete inhibition of actin cosedimentation. These data confirm that residues in the COOH-terminal domain of SM22 that share homology with the calponin tandem repeats also, in part, determine binding of SM22 to actin.

Calcium concentration does not influence SM22-actin binding. Sequence analysis reveals an EF-hand potential calcium binding site located in SM22 at amino acids 108–119 (108-KTDMFQTVDLFE-119), and a previous report suggested that membrane binding of SM22 can be influenced by calcium concentration (17). To evaluate whether calcium concentration affects the binding of SM22 to actin in vitro, we repeated cosedimentation assays using full-length, wild-type His-SM22 and actin in the presence of 25–400 μM CaCl₂. As shown in Fig. 3, replacing EGTA with increasing concentrations of CaCl₂ had no influence on the cosedimentation of SM22 with actin. Thus calcium concentration did not alter SM22-actin association under the conditions of our study.

Phosphorylation of SM22 at Ser-181 by PKC inhibits SM22-actin binding in vitro. Sequence analysis reveals three potential phosphorylation sites in SM22. A PKC consensus target motif (S/T-X-R/K) appears at amino acids 181–183, and two CKII consensus target motifs (S/T-X-X-D/E) are located at amino acids 16–19 and 139–142 (Fig. 4A). We tested whether any of these sites could be phosphorylated by either kinase in vitro by incubating full-length wild-type His-SM22 with enzyme in the presence of [γ-32P]ATP. PKC readily phosphorylated full-length SM22 (Fig. 4B, lanes 1 and 3) but barely labeled the COOH-terminal truncated SM22-(1–151) mutant (Fig. 4B, lane 4). Furthermore, substitution of Ser-181 with aspartic acid in full-length SM22 also markedly reduced PKC phosphorylation (Fig. 4B, lane 2). Thus Ser-181 is the predominant PKC phosphorylation site in vitro. In marked contrast, CKII did not phosphorylate SM22 in our experiments (Fig. 4B, lanes 5 and 6).

Because Ser-181 occurs within the COOH-terminal region shown above to be important in determining actin binding, we tested whether PKC-phosphorylated full-length SM22 binds actin with altered affinity. To do so, we included ~10,000 dpm PKC-phosphorylated SM22 in the cosedimentation experiments performed to evaluate calcium binding (see above). As shown in Fig. 3, PKC-phosphorylated SM22 bound to actin in a much smaller quantity than did nonphosphorylated SM22 (the vast majority of total SM22 present), and this reduced binding occurred independent of calcium concentration. Incubation of full-length recombinant SM22 with PKC buffer without enzyme did not alter SM22-actin binding (data not shown). Thus PKC phosphorylation on Ser-181 reduces SM22 binding to actin in vitro. This result is further strengthened by our finding that replacing Ser-181 with aspartic acid reduces binding of SM22-(181D) to actin partially but not completely (Fig. 2).

SM22 colocalizes with smooth muscle α-actin in smooth muscle in vivo. To test the physiological relevance of our findings in living smooth muscle cells, we transfected subconfluent canine tracheal smooth muscle cells with expression vectors encoding NH₄-terminal FLAG-tagged wild-type SM22 or with FLAG-SM22-(1–151) mutant, which did not bind to actin in vitro. Consistent with our findings in vitro, wild-type SM22 colocalized with filaments containing smooth muscle α-actin in cultured airway smooth muscle (Fig. 5, top), but the COOH-terminal truncation mutant was widely dispersed throughout the cytoplasm, reflecting apparent lack of actin binding (Fig. 5, middle). We also evaluated the binding of FLAG-SM22-(181D) in vivo; as shown in Fig. 5 (bottom), this mutant does colocalize with actin filaments. Thus, as in our cosedimentation assay, substitution of Ser-181 with aspartic acid (to

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**Fig. 4.** A: consensus phosphorylation targets for casein kinase II (CKII) and PKC in full-length recombinant SM22. B: phosphorylation of wild-type (wt) or mutant (as indicated) SM22 by PKC but not CKII.
mimic PKC phosphorylation) is not sufficient to block actin binding completely.

**DISCUSSION**

The purpose of this study was to clarify how SM22 binds to actin. To do that, we expressed and purified human SM22 with an NH₂-terminal His tag and used a cosedimentation assay to demonstrate that this recombinant protein binds actin in vitro (Fig. 2). Deletion of amino acids 152–201 completely ablated actin binding. Sequence analysis had previously suggested that amino acids 154–161 may be an actin binding site (15). Using COOH-terminal truncation, internal deletion, and point mutation, we generated a series of SM22 variants (Fig. 1) and used them to evaluate the functional importance of this region. Internal deletion of amino acids 154–161 markedly reduced but did not completely ablate SM22-actin cosedimentation; a similar result was obtained on neutralization of the four positively charged residues at either end of this domain (Fig. 2). Importantly, COOH-terminal SM22 truncation mutants that retained amino acids 154–161 also exhibited reduced actin affinity, as did internal deletion mutants lacking amino acids 170–180 or 170–186 (Fig. 2). Together, these results indicate that amino acids 154–161 partially, but not completely, determine actin binding by SM22. It is noteworthy that amino acids 175–195 exhibit high homology with tandem repeat domains in the COOH-terminal portion of calponin h1 and h2 and that, in calponin, these tandem repeat sequences are important for full actin affinity (4). Our results demonstrate that, as in calponin, residues in this region determine binding of SM22 to actin, possibly by serving as a second actin binding site or by participating in a larger actin binding domain that spans residues within amino acids 152–201. These results represent the first experimental demonstration of the importance of the COOH-terminal domain of SM22 for actin binding and of important sequences within this larger region.

We sought to test the physiological relevance of these in vitro findings in living cells by evaluating the distribution of SM22 and smooth muscle α-actin in canine tracheal myocytes transfected with plasmids encoding FLAG-tagged SM22 variants. Wild-type FLAG-SM22 appeared in bundles that also contained smooth muscle α-actin (Fig. 5, top), demonstrating the colocalization of these proteins in airway myocytes. In contrast, FLAG-SM22-(1–151) was widely dispersed throughout the cytoplasm (Fig. 5, middle). Thus the COOH-terminal domain that determines actin binding in vitro is also required for actin association in vivo.

Although several earlier studies have suggested that SM22 binds to and/or gels actin filaments (10, 15, 16), a recent report from Gimona and Mital (4) demonstrated no SM22-actin binding by cosedimentation analysis. Furthermore, these authors did not find colocalization of SM22 with actin in transfected fibroblasts but, rather, demonstrated HA-tagged SM22 in vacuoles. We are uncertain as to the cause of the discrepancy between their findings and ours. Perhaps subtle differences in experimental conditions during cosedimentation assays or our analysis of SM22 distribution...
in transfected smooth muscle cells, rather than fibroblasts, accounts for our divergent findings.

It is well documented that kinases and calcium mobilization are involved in regulation of smooth muscle contraction (19). Sequence analysis reveals that human SM22 contains consensus phosphorylation targets for CKII (16-SKIE-19 and 139-TKND-142) and PKC (181-SNR-183). Although both CKII sites are conserved in vertebrate SM22s, we failed to demonstrate any phosphorylation of our recombinant SM22 by this enzyme in vitro. In contrast, the PKC target at Ser-181 was efficiently phosphorylated by PKC in vitro (Fig. 4), and this phosphorylation partially inhibited actin cosedimentation (Fig. 3), as did substitution of Ser-181 with aspartic acid to simulate PKC phosphorylation (Fig. 2). However, when transfected into airway myocytes, FLAG-tagged SM22-(181D) still colocalized with actin filaments (Fig. 5, bottom). There are two potential explanations for this finding: 1) binding of this mutant was partially inhibited, but this inhibition went undetected because of the limitations of our immunocytochemical localization method, or 2) addition of a negative charge at Ser-181 was insufficient to alter actin binding in vivo. Furthermore, it remains unclear whether substitution of Ser-181 with aspartic acid fully mimics the important physiological effects of phosphorylation by PKC, if such phosphorylation actually occurs in vivo. Interestingly, no phosphorylation of endogenous SM22 has been demonstrated (5). In contrast to the still uncertain role of PKC phosphorylation in controlling SM22-actin interaction, our results suggest that direct calcium binding by SM22 does not regulate this process (Fig. 3), even though SM22 contains an EF-hand sequence. Of course, this finding does not exclude the possibility that in vivo intracellular free calcium might indirectly affect SM22-actin binding, for example, through interaction of SM22 with calcium-calmodulin complex or other calcium binding contractile myofilament components.

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