Regulation of Ca^{2+}/calmodulin kinase II by a small C-terminal domain phosphatase

Samudra S. GANGOPADHYAY*†, Cynthia GALLANT*†, Eric J. SUNDBERG†, William S. LANE‡ and Kathleen G. MORGAN*†[1]

*Department of Health Sciences, Sargent College, Boston University, 635 Commonwealth Avenue, Boston, MA 02215, U.S.A., †Boston Biomedical Research Institute, 64 Grove Street, Watertown, MA 02472, U.S.A., and ‡Mass Spectrometry and Proteomics Resource Laboratory, Harvard University, 16 Divinity Avenue, Cambridge, MA 02138, U.S.A.

We present here the identification and characterization of an SCP3 (small C-terminal domain phosphatase-3) homologue in smooth muscle and show, for the first time, that it dephosphorylates CaMKII [Ca^{2+}/CaM (calmodulin)-dependent protein kinase II]. SCP3 is a PP2C (protein phosphatase 2C)-type phosphatase that is primarily expressed in vascular smooth muscle tissues and specifically binds to the association domain of the CaMKIIγ G-2 variant. The dephosphorylation is site-specific, excluding the Thr^{287} associated with Ca^{2+}/CaM-independent activation of the kinase. As a result, the autonomous activity of CaMKIIγ G-2 is not affected by the phosphatase activity of SCP3. SCP3 co-localizes with CaMKIIγ G-2 on cytoskeletal filaments, but is excluded from the nucleus in differentiated vascular smooth muscle cells. Upon depolarization-induced Ca^{2+} influx, CaMKIIγ G-2 is activated and dissociates from SCP3. Subsequently, CaMKIIγ G-2 is targeted to cortical adhesion plaques. We show here that SCP3 regulates phosphorylation sites in the catalytic domain, but not those involved in regulation of kinase activation. This selective dephosphorylation by SCP3 creates a constitutively active kinase that can then be differentially regulated by other phosphorylation-dependent regulatory mechanisms.

Key words: autophosphorylation, Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), protein phosphatase 2C (PP2C), small C-terminal domain (CTD) phosphatase, vascular smooth muscle.

INTRODUCTION

CaMKII [Ca^{2+}/CaM (calmodulin)-dependent protein kinase II] is a ubiquitous protein that has numerous functions in fundamental cellular processes including metabolism, cell cycle control [1], cell shape variation, gene transcription [2] and the regulation of ion channel and cytoskeletal function [3–5]. CaMKII is activated by Ca^{2+}-bound CaM. There are four isoforms: α, β, δ and γ. *In vitro* studies have shown that CaMKII can autophosphorylate at several sites [6,7]. Thr^{286} (numbering according to that of the α isoform, Thr^{287} for β, δ and γ) is the best explored site and has been linked to the generation of a Ca^{2+}/CaM-independent persistence of kinase activity. There are other known autophosphorylation sites (e.g. Thr^{253}, Ser^{279}, $Thr^{305/306}$ and Ser^{314}; numbering according to the α isoform), but the *in vivo* functions of these sites are less well understood.

Previously, we reported the identification of six variants of CaMKIIγ in dVSM (differentiated vascular smooth muscle) [8]. CaMKIIγ G-2 has a novel association domain that is distinct from the common association domain found in all other CaMKIIγ variants. We have previously shown that CaMKIIγ G-2 translocates to cortical adhesion plaques upon activation and regulates contractility of smooth muscle through an ERK (extracellular-signal-regulated kinase)-mediated pathway [9].

Phosphorylated CaMKII is believed to be regenerated to its unphosphorylated state by the action of phosphatases in the cell. Accordingly, dephosphorylation of Thr^{286} by phosphatases in neurons has been reported [10]. A PP1 (protein phosphatase 1) is reported to dephosphorylate CaMKII associated with postsynaptic densities, but the soluble form of CaMKII is reported to be dephosphorylated by a PP2A [11,12]. PP1, PP2A [11,12] and PP2C decrease autonomous activity of CaMKII, but PP2B does not inhibit autonomous activity [10]. A PP2C has also been reported to dephosphorylate CaMKII *in vitro* [13], but its *in vivo* function has not been determined. The nature of the relevant CaMKII phosphatase in other cell types is not known.

CTD (C-terminal domain) phosphatases are known to be involved in the dephosphorylation of the CTD of RNA polymerase II. CTD phosphatases consist of a phosphatase catalytic domain and a BRCT (breast cancer 1 C-terminal) domain. However, SCPs (small CTD phosphatases) lack the BRCT domain (Figure 1A). To date, three isoforms (SCP1–3) have been identified in humans. The only known functions of SCPs are that human SCP1 can dephosphorylate the CTD of the largest RNA polymerase II subunit *in vitro* [14] and that some human SCP isoforms are suggested to function as global silencers of neuronal genes [15].

The present study was initiated to identify new CaMKII binding proteins. We have used the novel association domain of CaMKIIγ G-2 as a bait in a yeast two-hybrid assay and here present the identification and characterization of a SCP homologue in smooth muscle that binds to this novel association domain. SCP3 is a PP2C-type phosphatase that is primarily expressed in vascular smooth muscle tissues. This phosphatase dephosphorylates CaMKIIγ G-2 but, as we show here, the dephosphorylation is site-specific in that Thr^{287} is protected from dephosphorylation. We show that SCP3 regulates phosphorylation of CaMKIIγ G-2 at sites that are not involved in the autonomous activation of the kinase, but have been shown to regulate other functions, such as targeting of the kinase.

Abbreviations used: BRCT domain, breast cancer 1 C-terminal domain; CaM, calmodulin; CaMKII, Ca^{2+}/CaM-dependent protein kinase II; CBD, chitin-binding domain; CHO cell, Chinese hamster ovary cell; CTD, C-terminal domain; DTT, dithiothreitol; dVSM, differentiated vascular smooth muscle; MS/MS, tandem MS; PNPP; *p*-nitrophenyl phosphate; PP, protein phosphatase; SCP, small CTD phosphatase; SPR, surface plasmon resonance.

[1] To whom correspondence should be addressed (email kmorgan@bu.edu).

The nucleotide sequence data reported for ferret (*Mustela putorius furo*) SCP3 will appear in the DDBJ, EMBL, GenBank® and GSDB Nucleotide Sequence Databases under the accession number DQ465034.

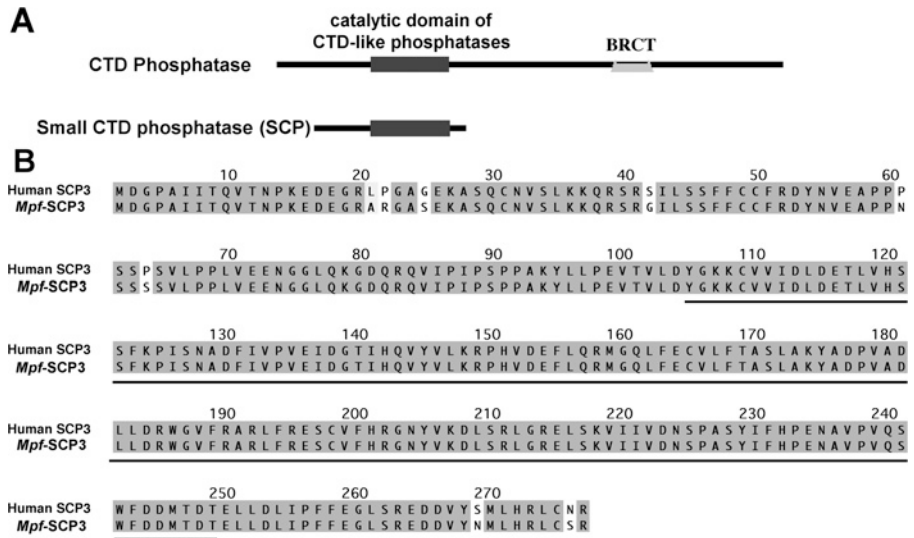

Figure 1 SCP3 sequence

(**A**) Diagrammatic presentation of the domains (as indicated) of CTD phosphatase and small CTD phosphatase. (**B**) Alignment of ferret (*Mustela putorius furo, Mpf*) SCP3 with human (*Homo sapiens*) SCP3. The identical residues are shaded dark grey, similar residues are shaded light grey and unmatched residues are not shaded. The catalytic domain of CTD phosphatase is underlined. The GenBank® accession number of Ferret SCP3 is DQ465034.

EXPERIMENTAL

Yeast two-hybrid assay

The cDNA corresponding to the novel 99-amino-acid residue sequence was cloned into the bait vector pBD-GAL4 Cam (Stratagene). A HybriZap 2.1 aortic cDNA two-hybrid library was screened [8]. The method of screening was according to the instructions of the manufacturer (Stratagene) [16].

Protein expression

The cDNA of SCP3, that of the novel G-2 association domain (corresponding to the 99 amino acid residues) and that of the association domain of CaMKIIγ C-1 (residues 331–495) were expressed and purified by IMPACT-CN system (intein-mediated purification with an affinity chitin-binding tag; New England Biolabs). The procedures used were according to the manufacturers' instructions.

Antibodies

For the SCP3 antibody, *Escherichia coli*-expressed pure protein was injected into rabbits and a polyclonal antibody was raised. The antibody was purified from the serum using an affinity column (AminoLink Kit, Pierce Biotechnology). The purified antibody was tested against recombinant SCP3 and aorta whole-tissue homogenate. It recognizes the recombinant protein and also recognizes a single protein of similar molecular mass in aorta tissue homogenate. This antibody was used for immunoblot and imaging studies.

The CaMKIIγ G-2 antibody is the same as that used in our previous study [9] and pan-CaMKIIγ antibody is the same as that used in [8]. The phospho-Thr^{287}-specific antibody was from Upstate/Millipore. This is a mouse monoclonal antibody raised against a peptide corresponding to residues 281–294 of the rat CaMKIIα subunit. This antibody has been extensively used and has been shown to not only be specific for activated CaMKIIα phosphorylated at Thr^{286}, but also for the analogous Thr^{287} in the γ isoforms [8]. Anti-CBD (chitin-binding domain) mouse monoclonal antibody was from New England Biolabs (Ipswich, MA, U.S.A.). Thr^{306} antibody was from PhosphoSolutions (Aurora, CO, U.S.A.).

Affinity labelling of antibody

The fluorescent labelling of antibodies was performed using kits from Molecular Probes. The purified antibody was dialysed against PBS to remove any ammonia or amines, and the procedure of labelling was according to the instructions of the manufacturer.

Surface plasmon resonance analysis (Biacore)

The affinities and kinetics of the molecular interactions between SCP3 and the association domains of CaMKIIγ G-2 and C-1 were measured by SPR (surface plasmon resonance) analysis using a Biacore 300 instrument (Biacore). Purified SCP3 protein was immobilized to a CM-5 sensor chip (Biacore) using the standard amine coupling method. Approx. 500 resonance units of SCP3 were immobilized. A negative control sensor chip surface was prepared by activation and blocking with ethanolamine. All binding experiments were performed at 25 °C in PBS, pH 7.4. To test for binding of CaMKIIγ G-2 and C-1 association domains to SCP3, a 250 nM concentration of each association domain was injected over both the SCP3-immobilized and negative control surface and the differential response was measured. For affinity and kinetic analysis of CaMKIIγ G-2 association domain binding to SCP3, serial dilutions from 250 to 0.3 nM of the former were injected over the SCP3-immobilized and control surfaces. Differential response curves were analysed using the BIAevaluation 4.1 software (Biacore). The on- (k_a) and off-rates (k_d) were fitted simultaneously using a 1:1 Langmuir binding model, and K_d values were determined by the ratio k_d/k_a.

Pull-down assay

Purified SCP3 was coupled to a resin by AminoLink kit from Pierce Biotechnology. Cleaned aorta tissue (see below) was attached to a force recording device to confirm viability and quick-frozen with a solid CO_2/acetone slurry containing DTT (dithiothreitol). The tissue was homogenized in a homogenization

buffer {20 mM Tris/HCl, pH 8.0, 50 mM NaCl, 10 % (v/v) glycerol, 0.1 % (v/v) Triton X-100, 5 mM $MgCl_2$, 1 mM ATP and protease inhibitor cocktail [8]}. The coupled resin was removed from the column and incubated with the tissue homogenate with rotation at 4 °C overnight. The beads were washed with the washing buffer, which was identical to homogenization buffer, except that the NaCl concentration was 150 mM. Finally, the proteins were extracted from the beads with SDS/PAGE loading sample buffer containing SDS. The extracted proteins were electrophoresed and Western blots were performed with a CaMKIIγ G-2 specific antibody. The same blot was stripped and labelled with anti-CBD antibody.

CaMKIIγ expression and purification

COS-7 (African green monkey simian-virus-40-transfected kidney fibroblast cell line)/CHO (Chinese hamster ovary) cells were routinely maintained in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10 % (v/v) fetal bovine serum and penicillin/streptomycin, at 37 °C in the presence of 5 % (v/v) CO_2. CaMKIIγ variant cDNAs, as described previously [8], were cloned into a pcDNA4 His-Max TOPO vector. For transfection, cells were grown in 100-mm-diameter Petri dishes to approx. 90 % confluence. Each plate was transfected with 30–40 μg of plasmid DNA and 50 μl of Lipofectamine™ 2000 transfection reagent (Invitrogen). After 48 h of incubation, cells were harvested by scrubbing with a rubber policeman and cell pellets were either stored at −80 °C or used immediately for purification.

Cell pellets from ten plates were resuspended in 2 ml of lysis buffer [50 mM phosphate buffer (1.6:1 K_2HPO_4/KH_2PO_4), pH 7.0, 0.5 M NaCl, 10 % (v/v) glycerol, 0.1 % (v/v) Tween-20 and protease inhibitor cocktail] and mixed by pipetting. After centrifugation at 15000 ***g*** for 10 min, the supernatant was added to the Talon spin column (BD Biosciences). After rocking for 30 min at room temperature (25 °C), the column was washed three times with lysis buffer, followed by washing (four times) with wash buffer [50 mM phosphate buffer pH 7.0, 1.0 M NaCl, 10 % (v/v) glycerol, 0.1 % Tween-20, 75 mM imidazole and protease inhibitor cocktail]. Finally, the protein was eluted from the column with elution buffer [50 mM phosphate buffer pH 7.0, 50 mM NaCl, 10 % (v/v) glycerol, 200 mM imidazole and protease inhibitor cocktail] and concentrated by filter centrifugation. The detailed method of purification was according to the manufacturer's instructions for the Talon column. The purified proteins were further purified by passing the material through a Superdex 75 size-exclusion column (FPLC) with a buffer containing 50 mM Pipes, pH 7.0, 20 mM NaCl, 0.5 mM EDTA and 10 % (v/v) glycerol. The CaMKII fractions were tested by activity measurements, and pooled fractions were stored at −80 °C in small aliquots.

Phosphatase assay

The phosphatase activity of SCP3 was measured with PNPP (*p*-nitrophenyl phosphate) as the substrate. The phosphatase assay buffer contained 50 mM Tris/acetate, 10 mM $MgCl_2$, 0.5 mM DTT, 10 % (v/v) glycerol and 20 mM PNPP. Purified SCP3 (0.5 μg) was added to 200 μl of the assay mixture and incubated at 30 °C for 30 min. The reaction was stopped by the addition of 800 μl of 250 mM NaOH and the absorbance of *p*-nitrophenol was measured at 410 nm. For detection of dephosphorylation, 5 μg of CaMKIIγ G-2 was incubated with 1 μg of SCP3 (0.5 ml total volume) in the same assay buffer as indicated above in the presence or absence of reagents described in the Figures. The released phosphate was detected by incubation with 0.5 ml of Biomol Green Reagent (Biomol International) at 30 °C for 30 min. Developed colour was detected by absorbance measurement at 620 nm.

Autophosphorylation of CaMKII, autonomous activity and dephosphorylation

Purified CaMKIIγ (1 μg) proteins were autophosphorylated in the presence of Ca^{2+}/CaM and ATP (or [γ-^{32}P]ATP) for 30 s in a 50 μl reaction mixture as described in [8]. The reaction mixture was passed through a Micro Bio-Spin P-30 column (BioRad), pre-equilibrated with the phosphatase assay buffer, to remove the excess ATP. The eluted protein was subjected to dephosphorylation with PP1 (NE Biolabs), PP2A (Upstate/Millipore) and purified SCP3 (equimolar to CaMKII protein) in the buffer as recommended by the suppliers (for PP1 and PP2A) and phosphatase assay buffer (for SCP3) at 30 °C. At the time intervals indicated in the Figures, aliquots of 10 μl were withdrawn and the reaction stopped by mixing with sample loading buffer containing SDS. Thr^{287} dephosphorylation was measured by Western blot with a phospho-Thr^{287}-specific antibody and total dephosphorylation was tested by autoradiography of the electrophoresed samples. For the detection of Thr^{306} phosphorylation, CaMKIIγ G-2 protein was autophosphorylated for 15 s at 30 °C, and EGTA was added to a final concentration of 3.3 mM. After incubation for 30 s at 30 °C, the reaction mixture was passed through the spin column as described above and dephosphorylated by SCP3. Phosphorylation was detected by an immunoblot developed with anti-phospho-Thr^{306} antibody.

For autonomous activity measurements in the presence of SCP3, the eluted protein from the spin column was subjected to dephosphorylation with purified SCP3 (equimolar to CaMKII protein) in phosphatase assay buffer at 30 °C for 10 min. Autonomous activity [(Ca^{2+}-independent activity/total activity) × 100] of SCP3 treated and untreated CaMKIIγ G-2 was measured as described in [7].

MS

The phosphorylation site determination by MS was performed by the Harvard Microchemistry and Proteomics Analysis Facility. The results were obtained by a single microcapillary reverse-phase HPLC run, directly coupled to the nano-electrospray ionization source on a Finnigan LCQ DECA XP Plus quadrupole ion-trap mass spectrometer. This instrument configuration is capable of acquiring individual sequence [MS/MS (tandem MS)] spectra on-line at high sensitivity ($\ll$1 fmole) for multiple peptides in the chromatographic run. These MS/MS spectra were then correlated with known sequences using the algorithm SEQUEST, developed at the University of Washington, and programs developed in the facility [17]. MS/MS peptide sequences were then reviewed for consensus with known proteins and the results were manually confirmed for fidelity.

Cell isolation from aorta tissue, staining and imaging

Ferrets (Marshall Farms, North Rose, NY, U.S.A.) were anaesthetized by an overdose of chloroform following procedures approved by the Institutional Animal Care and Use Committee of Boston University and the abdominal aorta was excised quickly to a dish containing oxygenated PSS (physiological saline solution; 120 mM NaCl, 5.9 mM KCl, 25 mM $NaHCO_3$, 11.5 mM dextrose, 2.5 mM $CaCl_2$ 1.2 mM $MgCl_2$ and 1.2 mM NaH_2PO_4, pH 7.4). The tissue was cut into small strips and used for cell isolation. Single cells from the aorta tissue were isolated as previously described [18]. For each 100 mg of aorta (wet weight), the digestion medium A consisted of 4.2 mg of CLS 2 collagenase

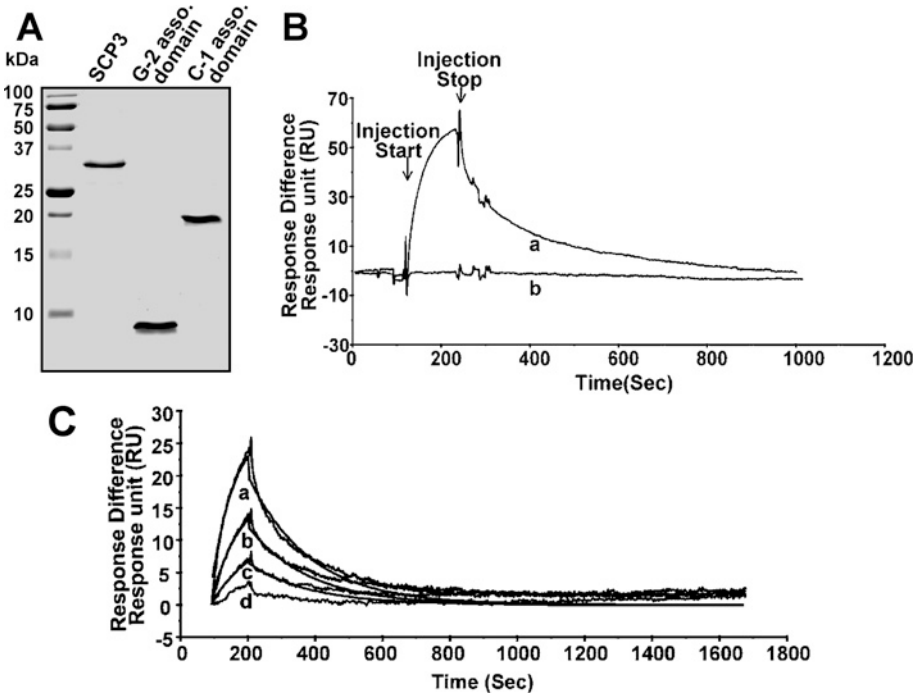

Figure 2 Protein expression and purification and SPR analysis of *in vitro* interactions

(**A**) Coomassie Brilliant Blue-stained protein gel of purified proteins (1 μg each) as indicated. All three proteins were expressed in *E. coli* (see the Experimental section). Molecular-mass markers in kDa are shown in the first lane. (**B**) An SPR sensorgram showing relative responses of injections of CaMKIIγ G-2 (a) and C-1(b) association domain, both at 250 nM concentration, over an SCP3-immobilized surface. (**C**) Kinetic analysis of serial dilutions (250–0.3 nM) of CaMKIIγ G-2 association domain binding to SCP3. The lines depict the relative responses of each injection (a, 125 nM; b, 62 nM; c, 31 nM and d, 15 nM of G-2 association domain) with the superimposed fitted lines showing the result of global curve-fitting of both k_a and k_d for three of the concentrations (a, 125 nM; b, 62 nM and c, 31 nM) using a 1:1 Langmuir binding model with the BIAevaluation 4.1 software.

(type II, 390 units/mg) dissolved in 4 ml of Hanks saline solution, 4.8 mg of elastase (3.63 units/mg) dissolved in 4 ml of Hanks saline solution and 5000 units of soybean trypsin inhibitor in a final total volume of 7.5 ml Ca^{+2}/Mg^{+2}-free Hanks balanced saline solution. For all experiments, isolated cells on one coverslip were first tested to confirm that they shortened in response to phenylephrine.

Confocal microscopy

Cells were fixed with 2 % (w/v) paraformaldehyde, permeabilized with 0.1 % (v/v) Triton X-100, blocked with 10 % (v/v) goat serum and incubated with the appropriate concentration of fluorescently labelled primary and secondary antibodies. Images were obtained with a Kr/Ar laser (Radiance 2000) scanning confocal microscope equipped with Nikon X-60 (numerical aperture 1.4)/40× magnification oil-immersion objectives.

Fluorescence microscopy

CHO cells were grown on coverslips and transfected with SCP3 cDNA. Cells were fixed 48 h after transfection with 4 % (w/v) paraformaldehyde, labelled with SCP3 antibody and Alexa Fluor® 488-conjugated goat anti-rabbit secondary antibody. Images were obtained using a Nikon Eclipse TE2000-E microscope.

RESULTS

SCP3 interacts with the novel association domain of CaMKIIγ G-2

To identify the binding partners of the novel association domain of CaMKIIγ G-2, a yeast two-hybrid assay was performed. The cDNA corresponding to the novel sequence of the CaMKIIγ G-2 association domain (residues 463–561) was used as a bait in screening a ferret aorta cDNA yeast two-hybrid library [19]. A single specific clone exhibited positive interactions and was isolated. Analysis of the cDNA sequence by a BLAST search revealed that the interacting protein is a homologue of a human SCP3, exhibiting a 97 % sequence identity. The SCP3 homologue derived from the aorta cDNA library is a 31 kDa protein with 276 amino acid residues. A catalytic domain with CTD-like phosphatase activity comprises residues 104–248. The amino acid sequence of the SCP3 homologue aligned with that of the human SCP3 isoform is shown in Figure 1(B).

CaMKIIγ G-2 association domain and SCP3 interact with high affinity

The *in vitro* interaction of SCP3 and CaMKIIγ G-2 was measured by SPR analysis. Purified full-length recombinant SCP3, the novel 99-amino-acid sequence of G-2 and the association domains of CaMKIIγ C-1 (residues 332–495) were used for these experiments (Figure 2A). Injection of the G-2 association domain fragment through a flow cell to which SCP3 was immobilized produced a reproducible and reversible increase in the SPR response with the non-specific response to a blank control surface subtracted (Figure 2B). Previously we have shown that G-2 forms a multimer in spite of having novel sequence in its association domain [8]. If the association between the CaMKIIγ G-2 association domain and SCP3 is due specifically to the novel sequence present in this variant, then the association domains of other CaMKIIγ variants will not bind. Indeed, this is what was observed. When a recombinant protein fragment corresponding to the association domain of CaMKIIγ C-1 (and identical with the association domains from all CaMKIIγ variants other than

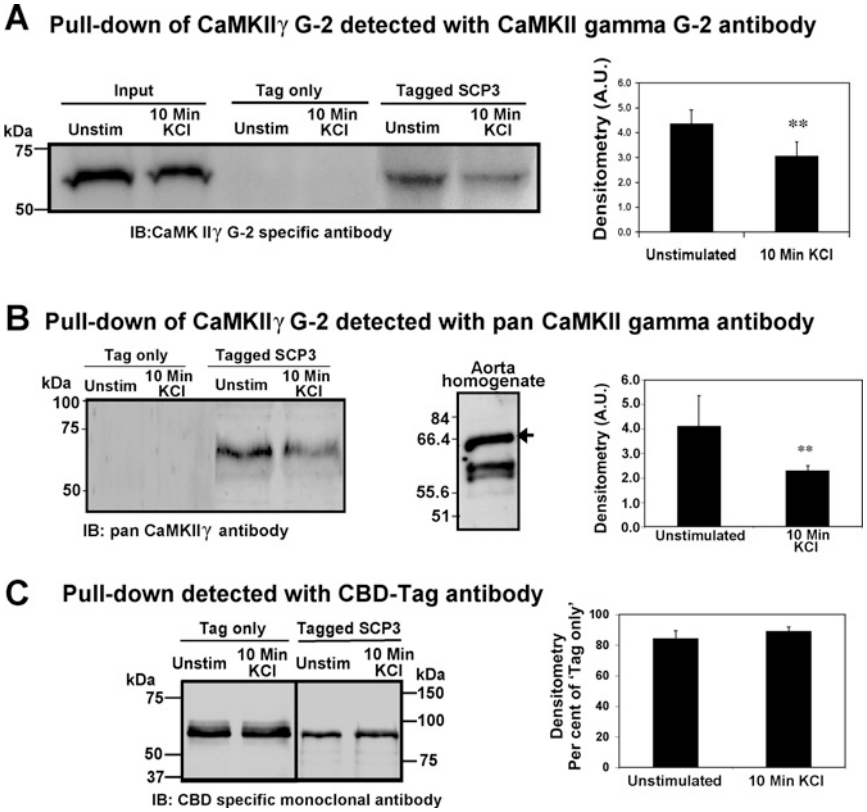

Figure 3 Pull-down assay: detection of CaMKIIγ G-2 bound to SCP3 in tissue homogenate

(**A**) Homogenates from unstimulated and KCl-stimulated tissues were tested as indicated. IB, immunoblot; unstim, unstimulated. Input: tissue homogenate, Tag only: pull-down with the CBD protein tag as negative control, Tagged SCP3: pull-down with the CBD-tagged SCP3. Right panel, the densitometry of the tagged SCP3 immunoblots probed with CaMKIIγ G-2 specific antibody. Densitometry data are normalized by dividing by the respective input signal ($n = 4$). (**B**) The signal as obtained from the pull-down blot by probing with a pan-CaMKIIγ antibody (left panel). Middle panel: Western blot of aorta whole cell homogenate probed with pan-CaMKIIγ antibody. The arrow corresponds to the G-2 variant. Right panel: the densitometry of the tagged SCP3 immunoblots probed with a pan-CaMKIIγ antibody and the data are the average absolute densitometric values of the digital signals ($n = 3$). (**C**) The signal as obtained from pull-downs probed with anti-CBD antibody. Right panel: the densitometry of tagged SCP3 immunoblots probed with an anti-CBD antibody. Densitometry data are normalized by dividing by the respective tag-only signal ($n = 3$). The two-tailed *t*-test *P* values < 0.01 is denoted by **. Approximate molecular masses in kDa are indicated at the side of the blot. A.U., absorbance units.

γ G-2) was injected over the same flow cell, there was no detectable increase in the SPR response (Figure 2B). In order to measure the affinity of the SCP3/CaMKIIγ G-2 association domain interaction, various concentrations of the CaMKIIγ G-2 association domain were injected and the kinetics of the specific association and dissociation with SCP3 were determined, with the resulting ratio of these rates corresponding to an affinity of 36 nM (Figure 2C). Thus, the interaction between CaMKII association domains and SCP3 is specific to the G-2 variant of CaMKII and is of relatively high affinity.

Full-length endogenous CaMKIIγ G-2 binds to SCP3

To test the interaction of full-length endogenous CaMKIIγ G-2 and SCP3, a pull-down assay was performed. SCP3 was expressed with an N-terminal CBD tag and an affinity resin was made by immobilizing the tagged protein on chitin beads. The interactions were tested against ferret aorta tissue whole-cell homogenates flash-frozen under two conditions: (i) unstimulated tissue; and (ii) tissue depolarized with physiological saline solution containing 51 mM KCl. The pulled-down proteins were subjected to SDS/PAGE and immunoblotted with a CaMKIIγ G-2-specific antibody [9]. To eliminate the possibility of interaction between CaMKIIγ G-2 and the tag sequence, a negative control experiment was performed with the tag sequence alone. Immunoblot results (Figure 3A) show that CaMKIIγ G-2 is pulled down with SCP3-labelled beads under both the conditions tested, but that there is no signal detected for the tag-only control. Quantitative densitometric measurements of bands ($n = 4$) indicate that the interaction is significantly decreased (approx. 50 %) in samples from depolarized muscle (right panel, Figure 3A). The pulled-down samples were further tested with a pan-CaMKIIγ antibody (left panel, Figure 3B). We have used this antibody against aorta whole-cell homogenate, and multiple bands are detected corresponding to the multiple CaMKIIγ variants in this tissue (middle panel, Figure 3B). In contrast, in the pull-downs, only a single band was detected with the pan-CaMKII antibody. We found that the intensity of signal in 10 min KCl-treated tissue homogenate is significantly less (approx. 50 %) than that from unstimulated tissue sample (right panel, Figure 3B). The results from two antibodies describes a similarity in the decrease of signals in 10 min KCl-stimulated samples and suggest that hetero-oligomerization of G-2 with the other variants known to be present in this tissue does not occur. To test the linearity of loading and whether there was equally efficient CBD sedimentation in both the sets of tissue homogenates, the same blot was stripped and labelled with anti-CBD monoclonal antibody. As seen in Figure 3(C), the CBD signal levels remain unchanged in both negative controls (tag-only) and in pull-down samples (left panel, Figure 3C). CaM kinases are autophosphorylated and activated upon depolarization

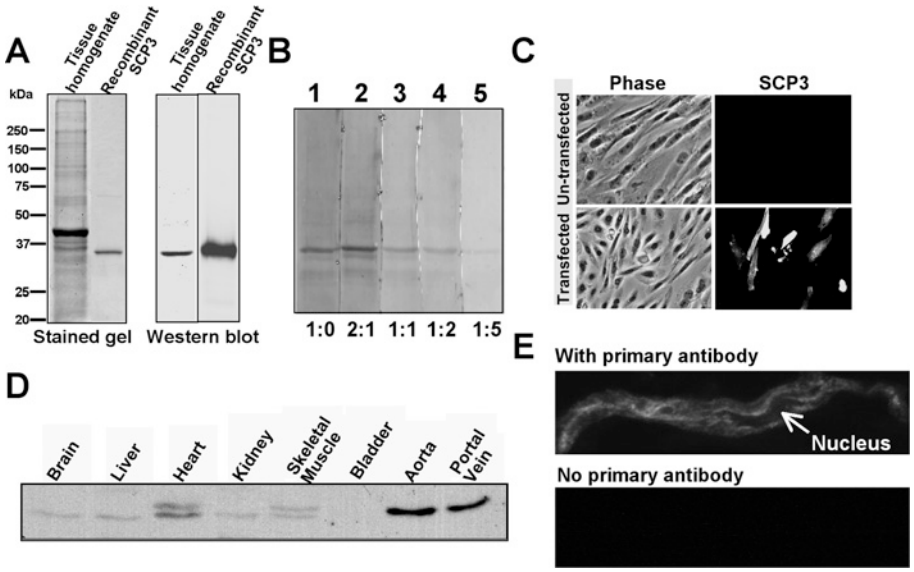

Figure 4 Tissue/cellular distribution of SCP3

(**A**) Left panel: the Coomassie Brilliant Blue-stained SDS/PAGE gel of aorta tissue whole cell homogenate and recombinant *E. coli* expressed SCP3. Right panel: Western blot of the corresponding proteins detected with SCP3 antibody as indicated. Approximate molecular masses in kDa are indicated at the side of the blot. (**B**) Immunoblot of aorta tissue whole cell homogenate probed with SCP3 antibody in the presence of antigen (SCP3 protein) with the antibody/antigen ratio indicated at the bottom. (**C**) Fluorescence microscopic images of CHO cells at identical microscope settings of both transfected and untransfected cells as indicated, stained with SCP3 antibody and Alexa Fluor® 488-conjugated goat anti-rabbit secondary antibody. Fluorescence image (SCP3) and phase-contrast image (Phase) are shown. (**D**) Immunoblot of protein matched (20 μg) whole cell homogenates of tissues as indicated detected by SCP-specific antibody. (**E**) Confocal image of freshly enzymatically isolated smooth muscle cell immunostained with SCP3 antibody (top) or stained with secondary antibody (bottom) and visualized at identical settings.

of smooth muscle tissues [19], thus the interaction of CaMKIIγ G-2 and SCP3 is diminished during depolarization.

SCP3 is a cytosolic protein whose expression is vascular-specific

An anti-SCP3 antibody was raised to assess differential protein expression across tissues and at the subcellular level. This antibody recognizes a single band at a molecular mass appropriate for SCP3 (31 kDa) on a Western blot of aorta whole cell homogenate (Figure 4A). Binding of the antibody to the antigen on the blot is diminished by increasing amounts of the antigen in the antibody incubation buffer, demonstrating specificity (Figure 4B). To test the specificity in a cellular environment, we stained CHO cells, both untransfected and transfected with the expression construct of SCP3 cDNA, with the SCP3 antibody (Figure 4C). The results show that the cells transfected with the cDNA of SCP3 and expressing the protein are stained with the antibody (lower right panel, Figure 4C) and untransfected cells are not detectably stained (upper panel of Figure 4C).

An immunoblot of a protein-matched tissue homogenate (Figure 4D) shows that the expression of SCP3 is most pronounced in aorta and portal vein and that no signal is detected in bladder. Only faint signals are detected in brain, heart, liver, kidney and skeletal muscle, and the faint signals could represent the vasculature of the tissues. Thus the expression of SCP3 appears to be largely restricted to the vasculature. It is of note that doublets are visible in the samples from heart and skeletal muscle tissue homogenates. Our prediction is that this could be due to alternatively spliced isoforms; however, at the present time there is no report of alternative splicing of SCP3.

Previously described SCP family members have been associated with the functions of RNA polymerase dephosphorylation and gene silencing and, as a result, nuclear distribution of SCP3 was expected. Imaging of freshly dissociated differentiated vascular smooth muscle cells with the anti-SCP3 antibody shows that SCP3 is localized on filamentous structures, but is not present in the nucleus (upper panel, Figure 4E). We were unable to detect any signal from cells not treated with the primary (anti-SCP3) antibody (lower panel, Figure 4E). These results imply a non-nuclear function of this small CTD phosphatase homologue.

CaMKIIγ G-2 and SCP3 co-localize in unstimulated, but not depolarized, dVSM cells

To evaluate whether CaMKIIγ G-2 and SCP3 interact in the cellular environment, we performed confocal imaging to assess their cellular localization. Since both the CaMKIIγ G-2 and SCP3 antibodies were raised in rabbit, they had to be directly coupled to fluorophores, resulting in somewhat diminished signals. Imaging of dissociated smooth muscle cells indicates that SCP3 partially co-localizes (arrowheads in red, green and merge images, Figure 5) with CaMKIIγ G-2 under unstimulated conditions. However, upon depolarization, CaMKII translocates to the cell cortex, as previously described [9], but SCP3 does not, and the two proteins no longer co-localize (Figure 5). Thus cellular SCP3 dissociates from CaMKIIγ G-2 upon depolarization, consistent with the pull-down experiments.

CaMKIIγ G-2 is dephosphorylated by SCP3

Multiple sites of CaMKII are known to be autophosphorylated in the presence of Ca^{2+}/CaM and ATP [5,10]. CaMKIIγ G-2 was autophosphorylated in the presence of Ca^{2+}/CaM and [γ-^{32}P]ATP for 30 s so that the phosphorylation sites would be labelled with ^{32}P. The labelled phosphorylated CaMKIIγ G-2 was treated with recombinant SCP3. Under these conditions, CaMKIIγ G-2 was largely dephosphorylated within 10 min, leaving only a faint signal. This faint signal, although close to baseline, was maintained at up to 30 min of incubation (upper panel, Figure 6A). A parallel control reaction of dephosphorylation in the absence of SCP3 (results not shown) did not detect any change in the

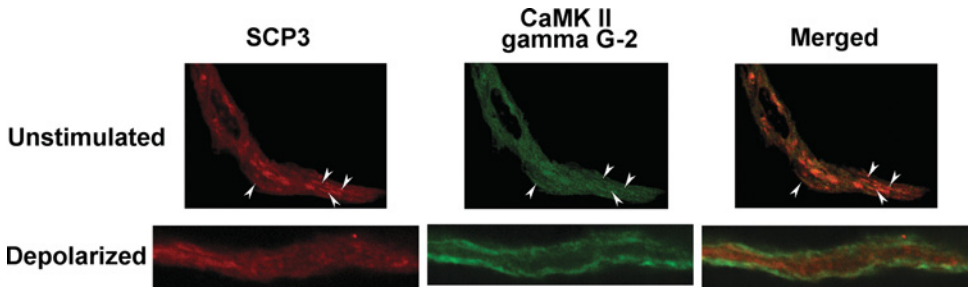

Figure 5 Co-staining of freshly dissociated vascular smooth muscle cells

Cells were fixed under unstimulated and depolarized conditions and co-stained with Alexa Fluor® 555-labelled SCP3 antibody (red) and Alexa Fluor® 465-labelled CaMKIIγ G-2 antibody (green). The arrowheads indicate co-localization of the two proteins.

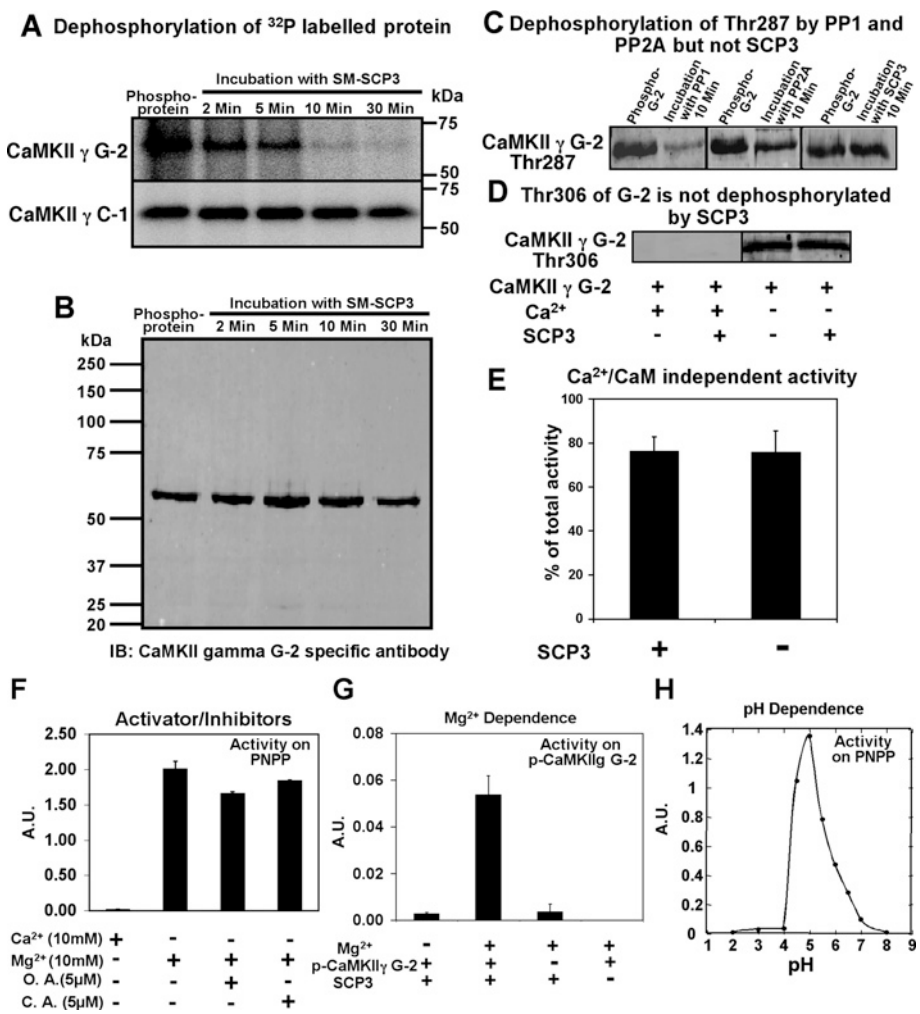

Figure 6 Dephosphorylation of CaMKIIγ by SCP3 and function of SCP3 as a PP2C phosphatase

(**A**) Autoradiogram of autophosphorylated CaMKIIγ G-2 and C-1 and dephosphorylation by SCP3 (0.1 μg). Time of incubation in the presence of SCP3 is indicated above. (**B**) A full length blot of autophosphorylated CaMKIIγ G-2, subsequently dephosphorylated by SCP3, was probed with CaMKIIγ G-2 specific antibody. (**C**) Immunoblots of phospho-CaMKIIγ G-2 detected by phospho-Thr^{287} specific antibody under the conditions indicated above blots. (**D**) Detection of dephosphorylation at Thr^{306} of G-2 in the presence and absence of SCP3. Autophosphorylation at Thr^{306} is seen only in the absence of Ca^{2+}. (**E**) Ca^{2+}/CaM-independent activity (% total activity) of CaMKIIγ G-2 in the presence or absence of SCP3. (**F**) Activity and inhibition of SCP3 using PNPP as a substrate in the presence or absence of ions. Activity was measured (absorption unit, A.U.) as the absorption of *p*-nitrophenol, the product of phosphatase reaction. C.A., cyclosporin A; O.A., okadaic acid. (**G**) Activity of SCP3 on phospho-CaMKIIγ G-2 in the presence or absence of Mg^{2+} as indicated. The released phosphate was detected by Biomol Green Reagent at 620 nm. (**H**) Plot of activity versus pH of SCP3. Activity was measured (absorption units, A.U.) as the absorption of *p*-nitrophenol, the product of phosphatase reaction. Approximate molecular masses in kDa are indicated at the side of the blot.

^{32}P signal of CaMKIIγ G-2 for up to 30 min of observation. Some residual signal would be expected, due to the lack of dephosphorylation of Thr^{287} discussed below, with phospho-antibodies. An identical experiment using SCP3 and CaMKIIγ C-1 showed no change in phosphorylation with up to 30 min of incubation (lower panel, Figure 6A), consistent with the lack of binding between SCP3 and CaMKIIγ C-1 (Figure 2B). A blot detected with the CaMKIIγ G-2-specific antibody from a parallel reaction of G-2 dephosphorylation shows the similarity of the lane loading and also ruled out the possibility of any degradation/proteolysis of the protein (Figure 6B). Thus it appears that the anchoring association domain of G-2 is necessary for dephosphorylation by SCP3.

Residue Thr^{287} of CaMKII is known to be involved in the generation of Ca^{2+}/CaM-independent (e.g. autonomous) activity. Therefore, we tested whether the Thr^{287} of CaMKIIγ G-2 was dephosphorylated by SCP3 using a phospho-Thr^{287}-specific antibody. CaMKIIγ G-2 was autophosphorylated in the presence of Ca^{2+}/CaM and ATP for 30 s and was subsequently subjected to dephosphorylation for 10 min in the presence of PP1 (NE Biolabs), PP2A (Millipore) and SCP3. As seen in Figure 6(C), upper panel, there is dephosphorylation at Thr^{287} of G-2 by PP1 and also to a lesser extent by PP2A, but there was no change in the signal of CaMKIIγ G-2 phosphorylated at Thr^{287}, whether in the presence of SCP3 or not. Thus, SCP3 must decrease ^{32}P-labelled CaMKIIγ G-2 by dephosphorylation of residues other than Thr^{287}.

We have also tested for possible dephosphorylation of the Thr^{306} site of G-2 by SCP3. The Thr^{306} site is known to be inhibitory with respect to kinase activity [3–5]. This site is phosphorylated only when Ca^{2+} is removed from the reaction mixture by the addition of EGTA after prior activation of CaMKII [3–5]. As shown in Figure 6(D), there is no detectable decrease in the phosphorylation of this site after incubation with SCP3. Thus, although SCP3 does not globally dephosphorylate all sites other than Thr^{287}, it must dephosphorylate several sites, given the large decrease in the ^{32}P signal.

Since the generation of Ca^{2+}/CaM-independent activity of CaMKII is associated with its autophosphorylation at Thr^{287}, we also measured Ca^{2+}/CaM-independent activity of activated CaMKIIγ G-2 in the presence or absence of SCP3. Purified CaMKIIγ G-2 was autophosphorylated in the presence of Ca^{2+}/CaM and ATP, subjected to dephosphorylation in the presence or absence of SCP3 for 10 min and autonomous activity was measured (Figure 6E). The results show that the Ca^{2+}/CaM-independent activity of CaMKIIγ G-2 is not affected by SCP3 treatment, consistent with the negative phospho-Thr^{287} immunoblot and confirming that CaMKIIγ G-2 dephosphorylated by SCP3 retains autonomous activity. These results also indicate that the sites dephosphorylated by SCP3 are not involved in the generation of autonomous activity and suggests that SCP3 instead may regulate other functions of CaMKIIγ G-2, which has a direct role in smooth muscle contractility [9].

The CaMKII-binding SCP3 is a PP2C type phosphatase

Phosphatases are classified according to their dependence on metal ions and their sensitivity to specific inhibitors. PP1 as well as PP2A phosphatases are Ca^{2+}-independent, whereas PP2B phosphatases are Ca^{2+}-dependent. PP2C phosphatases are known to be Mg^{2+}-dependent. The activity of SCP3 on a generic phosphatase substrate, PNPP, is dependent on Mg^{2+} ions, and Ca^{2+} cannot substitute for Mg^{2+} (Figure 6F). However, since anomalous results have been reported in some cases for the Mg^{2+}-dependence of phosphatase activity on PNPP [20,21], phosphatase activity was tested on phospho-CaMKIIγ G-2 as a substrate. SCP3 was found to be Mg^{2+}-dependent with respect to G-2 dephosphorylation as well (Figure 6G). Okadaic acid (an inhibitor of PP1 and PP2A type Ser/Thr phosphatases) [22] and cyclosporin A (an inhibitor of PP2B type Ser/Thr phosphatases) [23] did not inhibit the activity of SCP3 (Figure 6F). The dependence on pH was tested over the range 2–8 and optimum activity for SCP3 was obtained at pH 5.0 (Figure 6H). This profile indicates that SCP3 functions as a PP2C type phosphatase.

CaMKIIγ is autophosphorylated at multiple sites

In an effort to investigate the sites of autophosphorylation on CaMKIIγ, we analysed its phosphorylation state by MS. We were unable to express large enough quantities of CaMKIIγ G-2 for phosphorylation site determination by MS, but since the sequence of CaMKIIγ G-2 and that of the more easily expressed CaMKIIγ C-1 are identical in the catalytic/regulatory domain and the first one third of the association domain, we used the CaMKIIγ C-1 protein. Purified CaMKIIγ C-1 was autophosphorylated in the presence of Ca^{2+}/CaM and ATP for 10 min, separated by SDS/PAGE, subjected to in-gel tryptic digestion and analysed by microcapillary reverse-phase HPLC nano-electrospray MS/MS. The CaMKIIγ phosphorylation sites identified are illustrated in detail in Supplementary Figures 1 and 2 (at http://www.BiochemJ.org/bj/412/bj4120507add.htm). Some of these sites have been previously reported for the α and β isoforms (Thr^{287}, Thr^{254} and Ser^{280}; numbering according to the γ isoform), however, we also found some unique phosphorylation sites (e.g. Ser^{26}, Thr^{262}, Ser^{319} and Ser^{350}) not previously reported. These phosphorylation sites are found predominantly in the catalytic/regulatory domain of CaMKIIγ and at least one (Thr^{254}) has been shown to regulate CaMKII targeting [24].

DISCUSSION

We show here that SCP3 binds specifically to the CaMKIIγ G-2 association domain, a novel function for SCP phosphatases. SCP3 is primarily expressed in vascular tissues. Although only nuclear functions have previously been attributed to the SCP family of phosphatases, we show here that SCP3 is excluded from the nucleus in differentiated cells. Thus, despite containing a CTD phosphatase domain, it is unlikely that SCP3 dephosphorylates the CTD of RNA polymerase II, as has been described for SCP1. On the basis of the specific binding to the CaMKIIγ G-2 association domain, selective dephosphorylation of sites on CaMKIIγ G-2, as well as cellular co-localization and co-pull-down from homogenates, it appears that this SCP isoform regulates non-nuclear CaMKII.

The structurally and functionally diverse family of phosphatases have been grouped into three distinct gene families, namely PPP, PPM and PTP [25]. PP1, PP2A and PP2B belong to the PPP family and PP2C belongs to the PPM family. Besides the dependence on Mg^{2+} of PP2C phosphatases, they are also characterized by the insensitivity to the inhibitors okadaic acid and cyclosporin A [22,23]. Thus SCP3 has the functional properties of a PP2C-type phosphatase. PP2C family proteins contain 11 conserved domains [26], however, and SCP3 has little sequence homology with these domains.

On the basis of *in vitro* evidence, a presumed CaMKII phosphatase [27] has been reported that is Mn^{2+}-dependent, but is a PP2C type phosphatase distinct from any class of phosphatases. A comparison of sequences between SCP3 and this phosphatase reveals negligible similarity. Thus, SCP3 is a unique CaMKII phosphatase.

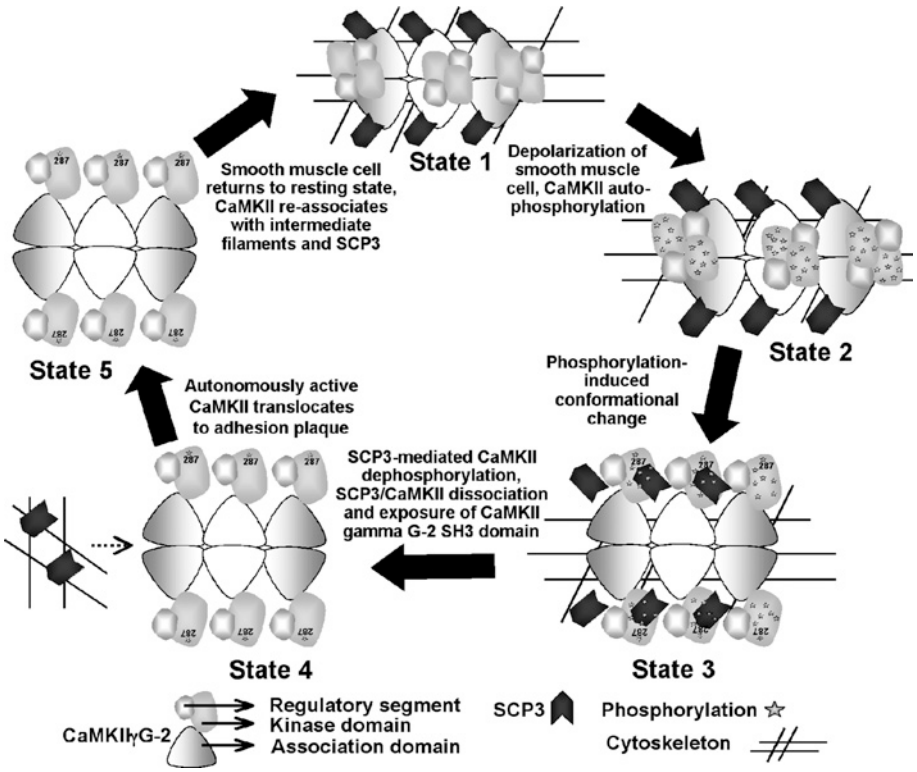

Figure 7 Proposed model for SCP3 interactions with CaMKIIγ G-2 in the smooth muscle cell

The G-2 oligomer is drawn based on results shown in Rosenberg et al. [30]. The holoenzyme is composed of two stacked hexameric rings. A side view of the molecule is shown. The dodecameric holoenzyme is modelled as a 6-mer for simplicity. State 1: under unstimulated conditions, there is maximal association of CaMKIIγ G-2 with SCP3. State 2: in depolarized tissues, CaMKIIγ G-2 undergoes autophosphorylation. State 3: after the activation-induced conformational changes and phosphorylation of the kinase, SCP3 is associated with phosphorylation sites on CaMKIIγ G-2. State 4: dephosphorylation of CaMKII by SCP3 leads to the completion of dissociation of SCP3 from CaMKII. State 5: the dissociated CaMKIIγ G-2 is targeted to the adhesion plaques. See the text for details.

In the present work we have tested the activity of SCP3 on phospho-CaMKIIγ. Multiple sites have been reported to be phosphorylated in the α and β isoforms. MS data confirm that the γ isoform is autophosphorylated at Thr^{287}, Thr^{254} and Ser^{280} positions, as previously reported [6,28]. In addition, we report four novel sites, including Ser^{26}, Thr^{262}, Ser^{319} and Ser^{350}. Autophosphorylation of Thr^{253} on the α isoform is known to be involved in the targeting of CaMKII to specific subcellular sites (postsynaptic densities) [24] and, taken together with our previous findings for CaMKIIγ G-2, our results may suggest that the dephosphorylation by SCP3 may perform a regulatory function, such as targeting of the γ G-2 variant of CaMKII.

When SCP3 dephosphorylates CaMKIIγ G-2, Thr^{287} is not dephosphorylated. As a result, the Ca^{2+}/CaM-independent activity (commonly called 'autonomous activity' or 'memory') [5] of the G-2 variant is maintained in the presence of SCP3. This makes a regulatory mechanism possible, where the fully active kinase can be potentially regulated by the action of SCP3 on other phosphorylation sites of CaMKIIγ G-2. We speculate that protection of Thr^{287} from dephosphorylation is related to the conformational changes known to be caused by the autophosphorylation of the molecule in concert with its binding of potential substrates or docking proteins subsequent to the conformational shift. This could protect this site from binding to SCP3 or could remove it from the reach of SCP3 as it is bound to the association domain.

Binding between SCP3 and the novel association domain of CaMKIIγ G-2, as obtained by SPR analysis, reveals a relatively high affinity interaction. However, the amount of full-length CaMKIIγ G-2 associated with the phosphatase in cells is regulated, since the amount of kinase pulled down with SCP3 is decreased in homogenates from depolarized tissue compared with homogenates from unstimulated tissue. The association domain of CaMKIIγ G-2 is unique among CaMKIIγ variants in containing polyproline, which bind to SH3 domains, which we have shown are involved in the targeting of CaMKIIγ G-2 [9]. Our finding indicates that SCP3 does not bind to the C-1 association domain and the C-1 variant is not dephosphorylated by SCP3. These results imply that binding is indeed required for dephosphorylation.

The data from our previous work involving CaMKIIγ G-2 [9], and the results presented here, allow us to propose a possible mechanistic model by which SCP3 regulates CaMKII (Figure 7). However, this is only one possible interpretation of the available data. We have previously shown that CaMKIIγ G-2 is associated with the cellular cytoskeleton in unstimulated cells [9]. Under these same unstimulated conditions, we now know that there is maximal association of CaMKII G-2 with SCP3 (state 1). However, when CaMKIIγ G-2 is activated in depolarized tissues [9] and consequently undergoes autophosphorylation (state 2), we have previously shown that CaMKII G-2 subsequently translocates from the cytoskeleton to cortical adhesion plaques. This translocation can be prevented by a polyproline decoy peptide. In the present study we show that, after activation of the kinase (state 2), the association between SCP3 and CaMKII G-2 is diminished (state 3). Activation of CaMKII is known to

cause conformational changes in the molecule. We speculate that either the conformational changes or the appearance of phosphorylation sites on the activated CaMKII catalytic domain or both, attract the phosphatase, and consequently weaken the association of SCP3 with the G-2 association domain (state 3, Figure 7). Dephosphorylation of CaMKII at sites other than Thr^{287} and Thr^{306} by SCP3 are then expected to lead to the completion of dissociation of the phosphatase (state 4), exposing the polyproline regions in the G-2 association domain required for targeting of CaMKIIγ G-2 to cortical adhesion plaques [9] (state 5, Figure 7). Adhesion plaque proteins such as p130 Cas are known to contain SH3 (Src homology 3) domains that may attract CaMKIIγ G-2 to those sites [29].

We have previously reported that CaMKIIγ G-2 directly binds to the cytoskeletal proteins vimentin and α-actinin and co-localizes with them in unstimulated cells [9]. When G-2 translocates to the cortical adhesion plaques, one of two mechanisms could explain its dissociation from the cytoskeleton: (i) CaMKIIγ G-2 is not directly bound to the cytoskeleton, but it is bound via SCP3 (thus dissociation of G-2 from SCP3 releases G-2 from the cytoskeleton) or (ii) CaMKIIγ G-2 phosphorylates the cytoskeleton upon activation, causing a disruption of the association of CaMKII and the cytoskeleton.

In conclusion, the present study identifies an SCP3 homologue (SCP3) predominantly expressed in smooth muscle that is non-nuclear-located in differentiated cells and interacts with a novel variant of CaMKIIγ (variant G-2) through its association domain. After activation of CaMKIIγ G-2, SCP3 dephosphorylates CaMKIIγ G-2 at sites implicated in the regulation of the kinase, but in a manner that we have shown maintains the autonomous activity of the kinase.

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