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# Phosphorylation at Ser<sup>26</sup> in the ATP-binding site of Ca<sup>2+</sup>/calmodulin-dependent kinase II as a mechanism for switching off the kinase activity

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## Synopsis

CaMKII (Ca<sup>2+</sup>/calmodulin-dependent kinase II) is a serine/threonine phosphotransferase that is capable of long-term retention of activity due to autophosphorylation at a specific threonine residue within each subunit of its oligomeric structure. The  $\gamma$  isoform of CaMKII is a significant regulator of vascular contractility. Here, we show that phosphorylation of CaMKII  $\gamma$  at Ser<sup>26</sup>, a residue located within the ATP-binding site, terminates the sustained activity of the enzyme. To test the physiological importance of phosphorylation at Ser<sup>26</sup>, we generated a phosphospecific Ser<sup>26</sup> antibody and demonstrated an increase in Ser<sup>26</sup> phosphorylation upon depolarization and contraction of blood vessels. To determine if the phosphorylation of Ser<sup>26</sup> affects the kinase activity, we mutated Ser<sup>26</sup> to alanine or aspartic acid. The S26D mutation mimicking the phosphorylated state of CaMKII causes a dramatic decrease in Thr<sup>287</sup> autophosphorylation levels and greatly reduces the catalytic activity towards an exogenous substrate (autocamtide-3), whereas the S26A mutation has no effect. These data combined with molecular modelling indicate that a negative charge at Ser<sup>26</sup> of CaMKII  $\gamma$  inhibits the catalytic activity of the enzyme towards its autophosphorylation site at Thr<sup>287</sup> most probably by blocking ATP binding. We propose that Ser<sup>26</sup> phosphorylation constitutes an important mechanism for switching off CaMKII activity.

**Key words:** activity switch, Ca<sup>2+</sup>/calmodulin-dependent kinase II (CaMKII), CaMKII  $\gamma$ , phosphorylation, serine/threonine protein kinase

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## INTRODUCTION

CaMKII (Ca<sup>2+</sup>/calmodulin-dependent kinase II) is a ubiquitously expressed serine/threonine protein kinase, which functions as a multimeric holoenzyme consisting of 12 monomers [1,2]. The holoenzyme is organized into a ring-like structure and the catalytic/autoregulatory component of each subunit is attached to the hexameric ring by a stalk-like extension. Two such hexameric rings stacked on top of each other form the dodecameric holoenzyme. The association domain forms the core of the holoenzyme, whereas the catalytic activity resides in the peripheral 'foot'-like structures [1,3].

Thr<sup>287</sup> is the primary site responsible for regulation of the autonomous activity of CaMKII [4]. Activation of the kinase by Ca<sup>2+</sup>/CaM (calmodulin) causes autophosphorylation of Thr<sup>287</sup>, which generates the fully active form of CaMKII. Each monomer is phosphorylated at Thr<sup>287</sup> by adjacent CaMKII monomers [5]. Thr<sup>287</sup> phosphorylation results in Ca<sup>2+</sup>-independent activity of CaMKII that persists upon the removal of Ca<sup>2+</sup>. Several other autophosphorylation sites have been described for CaMKII (Ser<sup>26</sup>, Thr<sup>254</sup>, Thr<sup>262</sup>, Ser<sup>280</sup>, Thr<sup>305</sup>, Thr<sup>306</sup>, Ser<sup>319</sup> and Ser<sup>350</sup>) ([6], but see [6a], [7–9]); however, only for some of them (Thr<sup>305</sup>, Thr<sup>306</sup> and Thr<sup>254</sup>) are the functional consequences known [8,10,11]. The most curious of these additional sites is Ser<sup>26</sup> due to its location in the ATP-binding

**Abbreviations used:** 2D, two-dimensional; CaM, calmodulin; CaMKII, Ca<sup>2+</sup>/calmodulin-dependent kinase II; DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; FBS, fetal bovine serum; LC-MS/MS, liquid chromatography tandem MS; PKA, protein kinase A; PSS, physiological saline solution; SCP3, small C-terminal domain phosphatase 3; TCA, trichloroacetic acid; VSMC, vascular smooth muscle cell; wt, wild-type.

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site. This phosphorylation site is the main focus of the present work.

Four isoforms of CaMKII are known:  $\alpha$ ,  $\beta$ ,  $\gamma$  and  $\delta$  [12]. Alternative splicing of these gene products result in multiple variants [13]. The  $\alpha$  and  $\beta$  isoforms have been extensively studied and are expressed primarily in neuronal tissues and brain [12,14]. They are reported to play a crucial role in behaviour and memory through their influence on LTP (long-term potentiation) at PSDs (postsynaptic densities) [15].

Unlike  $\alpha$  and  $\beta$  isoforms,  $\delta$  and  $\gamma$  isoforms are, in general, ubiquitously expressed [16]. The *CAMK2D* (encoding  $\delta$  CaMKII) gene products have been extensively studied in cardiac muscle [15–18]. The  $\gamma$  isoform has been studied in the vasculature [11,19–21] and in many other tissue types [12,16,22]. The  $\delta$  isoform has been linked with proliferative VSMCs (vascular smooth muscle cells), whereas the  $\gamma$  isoform appears to be more closely linked with non-proliferative contractile VSMCs [20,23]. Vascular tone is maintained by the contractility of differentiated VSMCs. CaMKII has been shown to be a significant regulator of vascular tone as shown by experiments with antisense knockdown of CaMKII and small molecule inhibitors [20,24].

Our group has isolated six CaMKII  $\gamma$  variants, C-1, C-2, G-1, G-2, B and J, from an aorta smooth muscle cDNA library [19]. The main differences between these variants occur in the segment that connects the catalytic/regulatory domain with the association domain. The C-1 and C-2 variants lack variable region 1 (V1) and variable region 2 (V2). Furthermore, C-2 lacks an eight amino acid sequence (residues 23–30) that forms part of the ATP-binding motif of the catalytic domain. Interestingly, this variant displays defective autophosphorylation at Thr<sup>287</sup>, but surprisingly is still able to phosphorylate an exogenous substrate [19]. Our laboratory has previously reported that this deleted sequence contains a serine residue (Ser<sup>26</sup>), as determined by LC–MS/MS (liquid chromatography tandem MS) of the recombinant C-1 variant, is autophosphorylated when the kinase is activated by Ca<sup>2+</sup>/CaM [7].

In the present paper, we provide evidence that phosphorylation of CaMKII  $\gamma$  at this novel autophosphorylation site, Ser<sup>26</sup>, occurs in vascular tissue and is important for the termination of the sustained activity of CaMKII  $\gamma$ .

## EXPERIMENTAL

### Tissue preparation

Freshly isolated aorta from the ferret was prepared as previously described [11]. Stimulated or unstimulated aorta rings were quick-frozen in 5 mM DTT (dithiothreitol) with 10% (w/v) TCA (trichloroacetic acid) in solid CO<sub>2</sub>/acetone and stored at –80 °C for later use. All procedures have been approved by the Boston University Institutional Animal Care and Use Committee. The animals were maintained in a manner consistent with the NIH Guide for the Care and Use of Laboratory Animals and were obtained and used in compliance with federal, state

and local laws. Physiological experiments were performed using a PSS (physiological saline solution) as described previously [20]; 51 mM PSS with 51 mM NaCl replaced by KCl was used as depolarizing stimulus.

### 2D (two-dimensional) gels and immunoblotting

Samples were quick frozen in a solid CO<sub>2</sub>/acetone slurry containing DTT and TCA, and washed in acetone with 5 mM DTT. IEF (isoelectric focusing) tube gels and PAGE gels were prepared as previously described [19]. Ampholytes used were 3–10 and 5–6. Gels were transferred on to PVDF membranes and processed using a standard immunoblotting protocol. Blots were probed with a total CaMKII  $\gamma$  antibody, a G-2 CaMKII  $\gamma$  antibody and a vimentin antibody (see the Antibodies and reagents section). A two-colour LiCor Odyssey (LI-COR Biosciences) instrument was used to visualize bands quantify densitometric results. Images shown in Figures were adjusted identically for each complete dataset for better visualization ( $n = 3–15$ ) but this did not alter quantitative values.

### Colorimetric dephosphorylation assay

A mixture containing 50 mM Tris acetate (pH 5.0), 10 mM MgCl<sub>2</sub>, 0.5 mM DTT, 10% (v/v) glycerol, 0.1 mM phosphopeptides (pSer<sup>26</sup>, pThr<sup>254</sup>, pThr<sup>262</sup>, pSer<sup>280</sup>, pThr<sup>287</sup>, pSer<sup>319</sup> and pSer<sup>350</sup>) was used for the 500  $\mu$ l reaction with or without purified SCP3 (small C-terminal domain phosphatase 3). The peptide sequences used for the experiment are as follows: pSer<sup>26</sup>, H<sub>2</sub>N-GKGAFS(p)VVRRC-COOH; pThr<sup>254</sup>, H<sub>2</sub>N-INQMLT(p)INPAK-COOH; pThr<sup>262</sup>, H<sub>2</sub>N-PAKRIT(p)ADQAL-COOH; pSer<sup>280</sup>, H<sub>2</sub>N-RSTVAS(p)MMHRQ-COOH; pThr<sup>287</sup>, H<sub>2</sub>N-MHRQET(p)VECLR-COOH; pSer<sup>319</sup>, H<sub>2</sub>N-FSAAKS(p)-LLNKK-COOH; pSer<sup>350</sup>, H<sub>2</sub>N-KGSTES(p)CNTTT-COOH. The reaction mixture was incubated at 30 °C for 30 min, then 500  $\mu$ l of Biomol Green reagent (see the Antibodies and reagents section) was added and the samples were incubated for another 30 min at 30 °C. The extent of the reaction was calculated from the absorbance at 620 nm according to the Biomol Green reagent protocol.

### Antibody generation

Polyclonal antibodies for phosphorylated Ser<sup>280</sup> and Ser<sup>26</sup> were generated by immunization of rabbits with the phosphopeptides CaMKII pSer<sup>280</sup> [H<sub>2</sub>N-RSTVAS(p)MMHRQ-COOH] and CaMKII pSer<sup>26</sup> [H<sub>2</sub>N-LGKGAFS(p)VVRRCVKTSTQE-COOH]. These peptides were synthesized as octavalent MAPs (multiple antigenic peptides) using MAP resin from EMD Chemicals. The peptides were synthesized at BBRI (Boston Biomedical Research Institute) using an Applied Biosystems Model 433A Peptide Synthesizer and Fmoc (fluoren-9-ylmethoxycarbonyl) to protect  $\alpha$ -amino groups. Rabbits were immunized with the peptides following standard procedures at Caprologics.

### Cell culture

COS-7 cells were maintained in DMEM (Dulbecco's modified Eagle's medium) culture medium supplemented with 4.5 g/l

D-glucose, L-glutamine, 110 mg/l sodium pyruvate, 50 units/ml penicillin, 50  $\mu$ g/ml streptomycin and 10% (v/v) FBS (fetal bovine serum). His-tagged CaMKII  $\gamma$  C-1 and mutant cDNAs in pcDNA4/HisMax TOPO vector were transfected into COS-7 cells by using Lipofectamine<sup>TM</sup> reagent (Invitrogen). The amount of Lipofectamine<sup>TM</sup> and the plasmid were titrated for the experiment according to the Lipofectamine<sup>TM</sup> protocol using OPTI-MEM to increase the transfection efficiency. Plasmids were expressed for 24 h. Cells were lysed on ice with phosphate lysis buffer (20 mM sodium phosphate, 140 mM NaCl<sub>2</sub>, 3 mM MgCl<sub>2</sub>, 0.5% Nonidet P40, 1 mM DTT, 1 $\times$  protease inhibitor mixture and a standard immunoblotting technique was followed.

### Site-directed mutagenesis

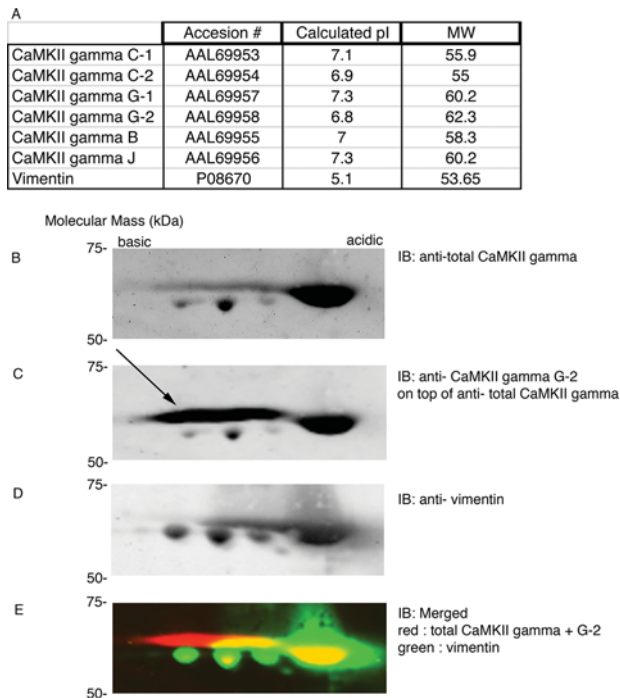
C-1 cDNA in pcDNA4/HisMax TOPO was used to mutate Ser<sup>26</sup> to alanine (A) or aspartic acid (D) using the primers S26A-sense 5'-GCAAGGGTGCTTTCGCTGTGGTCCGCAGG-3', S26A-antisense 5'-CCTGCGGACCACAGCGAAAGCACCCCTTGC-3', S26D-sense 5'-GGCAAGGGTGCTTTCGATGTGGTCCGCAGGTG-3' and S26D-antisense 5'-CACCTGCGGACCACATCGAAAGCACCCCTTGCC-3'. QuikChange II XL site-directed mutagenesis kit was used according to the manufacturer's protocol. Mutations were confirmed later with sequencing by Eurofins MWG Operon.

### Dot-blot kinase assay

A solution containing 50 mM Pipes (pH 7.0), 10 mM MgCl<sub>2</sub>, 10 mM CaCl<sub>2</sub>, 0.5  $\mu$ g/ml CaM, 20  $\mu$ M autocamtide-3 (H<sub>2</sub>N-Lys-Lys-Ala-Leu-His-Arg-Gln-Glu-Thr-Val-Asp-Ala-Leu-COOH), 0.225 mM ATP, 0.1 mg/ml BSA, 1 $\times$  protease inhibitor and 5  $\mu$ l of CaMKII was used in a 50  $\mu$ l kinase-assay reaction. The reaction was stopped using 50 mM EDTA. Next, the peptide was separated from the kinase using 300 kDa cut-off spin filtration columns. Then 2  $\mu$ l of the autocamtide-3 fraction of the assay were spotted onto a nitrocellulose membrane. Dot blots were blocked and analysed with anti-total phosphothreonine antibody (see the Antibodies and reagents section). In parallel experiments under identical conditions, negative controls, consisting each of no CaM, no CaM and no ATP, and BSA only, were tested with CaMKII wt (wild-type) and resulted in no signal.

### Molecular modelling

For modelling the catalytic domain of CaMKII  $\gamma$  C-1 isoform, we have used the Swiss Model server (<http://swissmodel.expasy.org/>) [25–27]. The model was built using the structure of PKA (protein kinase) (PDB entry 1ATP) as a template. Residues 12–261 of C-1 were aligned with residues 42–282 of PKA. Rebuilding of the missing loops was done by the Swiss Model server. Co-ordinates for ATP and two Mn<sup>2+</sup> ions present in 1ATP were introduced into the C-1 model and the structure was optimized with Phenix [28]. Ser<sup>26</sup> was either phosphorylated or mutated to aspartic acid using COOT [29] and the structures were energy optimized without ATP using Phenix.



**Figure 1** Multiple post-translational modifications of CaMKII are suggested by 2D gel analysis of vascular tissue homogenates

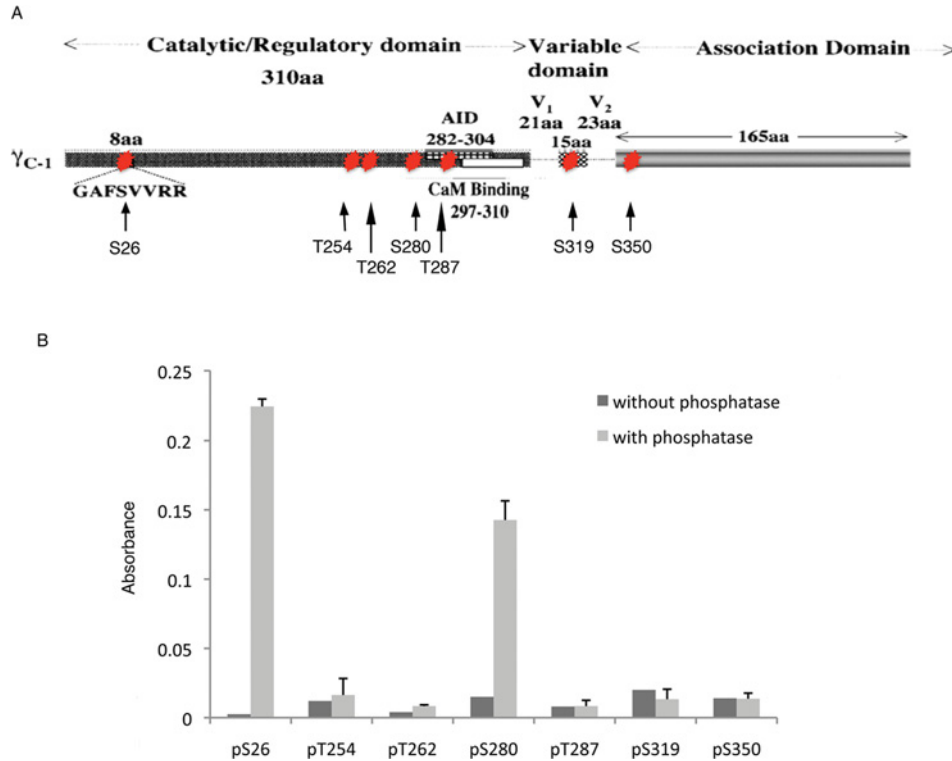
(A) Calculated molecular mass (Mw) and pIs of CaMKII  $\gamma$  variants. Vimentin (P08670) was used for comparison as a marker. (B) Representative CaMKII  $\gamma$  immunoblot (rabbit antibody) of a 2D gel of a whole cell homogenate of aorta tissue. (C) Subsequent probing of blot shown in (B) with a second rabbit antibody specific for the CaMKII  $\gamma$  G-2 variant (shown with arrow) (D) Probe of same blot for vimentin (mouse antibody) as a reference protein. (E) Merged view of CaMKII  $\gamma$  variants and G-2 variant (red) and vimentin (green). The brightness of the entire blot has been uniformly altered for visual display, digital values are unchanged.

### Antibodies and reagents

The antibodies used in present study were rabbit anti-total CaMKII  $\gamma$  (1:250 dilution; Upstate/Millipore); rabbit anti-CaMKII  $\gamma$  G-2 [21] (1:500 dilution); mouse anti-vimentin antibody (1:400 dilution; Sigma); mouse anti-tubulin (1:1000 Cell Signaling); mouse anti-His-tag antibody (1:5000 dilution; Cell Signaling); rabbit anti-phosphothreonine antibody (1:500 dilution; Cell Signaling); mouse anti-pThr<sup>287</sup> antibody (Millipore); rabbit anti-CaMKII G-2 (1:500 dilution) [21]; fluorescently labelled secondary antibodies (1:1000 dilution; LI-COR Biosciences). DMEM (Gibco), FBS (Gibco), OPTI-MEM (Invitrogen), protease inhibitor (Roche), QuikChange II XL (Stratagene), Lipofectamine<sup>TM</sup> (Invitrogen), Biomol Green reagent (Biomol International). cDNA sequences are from ferret.

### Statistics

Unless indicated otherwise, significance of difference between two individual datasets was taken at  $P < 0.05$  by a two-tailed paired Student's  $t$  test. Data are presented as means  $\pm$  S.E.M.



**Figure 2** Ser<sup>280</sup> and Ser<sup>26</sup> autophosphorylation sites are selectively dephosphorylated by SCP3

(A) CaMKII  $\gamma$  C-1 domain structure. Red stars mark the residues previously shown to be autophosphorylated. aa, amino acid(s). (B) Dephosphorylation of phosphopeptides with and without SCP3 measured by a colorimetric assay for free phosphate.

## RESULTS

### 2D gels indicate multiple post-translational modifications of CaMKII $\gamma$ in vascular tissue

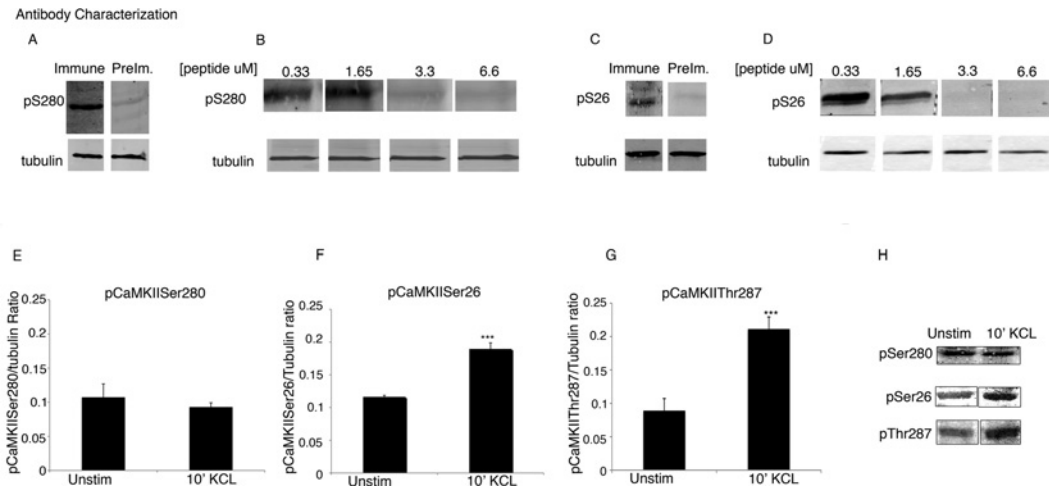
To determine whether CaMKII  $\gamma$  variants are phosphorylated at multiple sites in vascular tissue, we analysed vascular homogenates by 2D gel electrophoresis followed by immunoblotting, using vimentin as a reference protein. Vimentin has a calculated pI of 5.1 and post-translationally modified forms of vimentin from human arterial smooth muscle cells are known to run within the range 3.8–4.9 (Figure 1A) in 2D maps [30]. As shown in Figure 1(A), the calculated pIs of CaMKII  $\gamma$  variants cover the range 6.8–7.3. Figure 1(B) shows a Western blot of all CaMKII  $\gamma$  variants separated by 2D gel electrophoresis and stained with an anti-total CaMKII  $\gamma$  antibody. In addition to probing with the total CaMKII  $\gamma$  antibody (rabbit polyclonal, red channel), we subsequently co-labelled the blot with a CaMKII  $\gamma$  G-2 variant-specific antibody (also a rabbit polyclonal, red channel) to confirm the identity of CaMKII spots on the 2D gel. The CaMKII  $\gamma$  G-2-specific antibody typically stains an oval apparently containing several forms of the G-2 variant on the blot (Figure 1C, arrow). The same membrane was probed with an anti-vimentin antibody (monoclonal mouse, green channel) as a reference protein (Figures 1A and 1D). Figure 1(E) shows a merged image

of total CaMKII and G-2 signals (red channel) and the vimentin signal (green channel). Despite their expected pI of around 7.0, CaMKII  $\gamma$  variants overlap with vimentin in a pI range below 5.0. Since the observed pIs for CaMKII  $\gamma$  variants vary from the calculated pI by roughly 2.0, these findings suggest that CaMKII variants are phosphorylated in aorta tissue. A single-site phosphorylation shifts the pI approximately 0.5 unit in the acidic direction [31]. Thus, comparison of the calculated and observed pI values suggests the presence of up to four to five phosphate groups or other post-translational modifications on CaMKII in vascular tissue.

### CaMKII Ser<sup>26</sup> and Ser<sup>280</sup> peptides are selectively dephosphorylated by SCP3

Previous *in vitro* analysis of autophosphorylation sites in the presence of Ca<sup>2+</sup>/CaM on recombinant CaMKII  $\gamma$  by LC-MS/MS of recombinant CaMKII  $\gamma$  identified new phosphorylation sites (Ser<sup>26</sup>, Thr<sup>262</sup>, Ser<sup>319</sup> and Ser<sup>350</sup>) and confirmed other phosphorylation sites (Thr<sup>287</sup>, Thr<sup>254</sup> and Ser<sup>280</sup>) [7]. We have also previously described SCP3 that binds CaMKII  $\gamma$  and decreases incorporated <sup>32</sup>P, but does not dephosphorylate Thr<sup>287</sup> [7]. However, it is not known whether any of the other identified phosphorylated site(s) are dephosphorylated by this phosphatase. Thus, we synthesized phosphopeptides containing all the





**Figure 3** Phosphorylation of Ser<sup>26</sup> but not Ser<sup>280</sup> is regulated by vascular tissue depolarization

(A–D) Antibody controls, tubulin used as a loading control. (A) Whole cell aorta homogenate immunoblotted with pCaMKII Ser<sup>280</sup> immune or preimmune serum as indicated. (B) Whole cell aorta homogenate probed with pCaMKII Ser<sup>280</sup>-specific antibody in the presence of increasing amounts of a competing pCaMKII Ser<sup>280</sup> peptide used as the immunogen to raise the antibody. (C) Whole cell aorta homogenate immunoblotted with pCaMKII Ser<sup>26</sup> immune or preimmune serum. (D) Whole cell aorta homogenate probed with pCaMKII Ser<sup>26</sup>-specific antibody in the presence of increasing amounts of the pCaMKII Ser<sup>26</sup> peptide used as the immunogen to raise the antibody. (E) Average densitometry for pCaMKII Ser<sup>280</sup> staining of unstimulated tissue or that depolarized with 51 mM PSS. (F) Average densitometry for pCaMKII Ser<sup>26</sup> staining of unstimulated tissue or that depolarized with 51 mM PSS,  $P < 0.01$ . (G) Average densitometry for pCaMKII Thr<sup>287</sup> staining of unstimulated tissue or that depolarized with 51 mM PSS,  $P < 0.01$ . (H) Typical blots stained with anti-pCaMKII Ser<sup>280</sup>, pCaMKII Ser<sup>26</sup> and pCaMKII Thr<sup>287</sup> antibodies;  $n = 3–15$ . The brightness of entire blot has been uniformly altered for visual display; digital values are unchanged.

previously identified [7,19] autophosphorylation sites: Ser<sup>26</sup>, Thr<sup>254</sup>, Thr<sup>262</sup>, Ser<sup>280</sup>, Thr<sup>287</sup>, Ser<sup>319</sup> and Ser<sup>350</sup> (Figure 2A). Dephosphorylation of these peptides by SCP3, was analysed using a colorimetric phosphate release assay. The results (Figure 2B) demonstrate selective dephosphorylation of only two sites, Ser<sup>26</sup> and Ser<sup>280</sup> by SCP3. In contrast, Thr<sup>254</sup>, Thr<sup>262</sup>, Thr<sup>287</sup>, Ser<sup>319</sup> and Ser<sup>350</sup> were not significantly dephosphorylated. Thus, these findings raise the question of whether and how the phosphorylation/dephosphorylation of these sites is regulated *in vivo*. Thus, we focused specifically on Ser<sup>26</sup> and Ser<sup>280</sup>.

### Phosphorylation of CaMKII at Ser<sup>280</sup> is not regulated by depolarization in vascular tissue

Ser<sup>280</sup> is located within a conserved stretch of 39 residues that constitutes the autoinhibitory and the Ca<sup>2+</sup>/CaM-binding domain in the canonical kinase fold. It is located only seven residues away from Thr<sup>287</sup>, the site responsible for the autonomous activity of CaMKII. It appears, however, to have a different function from that of Thr<sup>287</sup> because as shown above it is dephosphorylated by SCP3, while Thr<sup>287</sup> is not. To test if phosphorylation of Ser<sup>280</sup> is regulated *in vivo*, a phosphospecific Ser<sup>280</sup> antibody was generated using the sequence H<sub>2</sub>N-RSTVAS(p)MMHRQ-COOH. Specificity of the antibody was confirmed by comparison with preimmune serum and by probing aorta homogenate with the pCaMKII Ser<sup>280</sup>-specific antibody in the presence of competing antigen (Figures 3A and 3B).

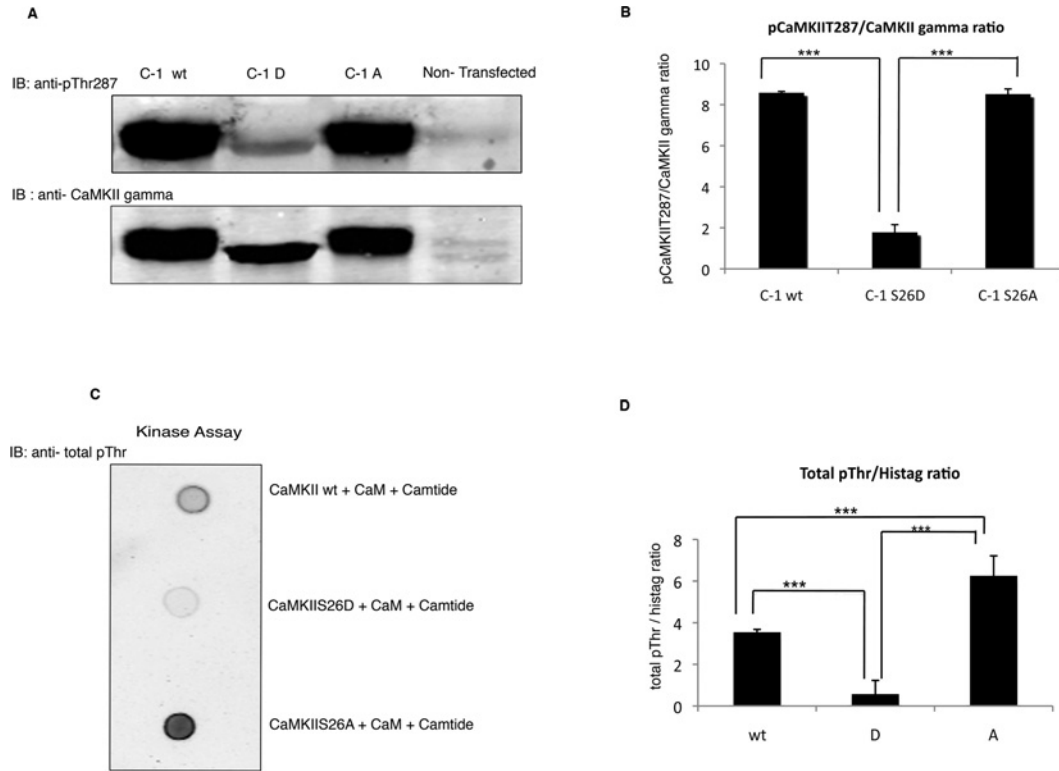
Vascular tissues were quick-frozen in the presence or absence of the depolarizing stimulus (51 mM KCl PSS). Depolarization

is known to open voltage-dependent Ca<sup>2+</sup> channels in this tissue, raising intracellular Ca<sup>2+</sup> levels and activating CaMKII. Tissue homogenates were probed by immunoblot with the anti-pCaMKII Ser<sup>280</sup> antibody. Mean densitometry results show no significant difference between the unstimulated and stimulated vascular samples (Figure 3E), suggesting that phosphorylation of Ser<sup>280</sup> is not regulated under these physiological conditions.

As a positive control, a pThr<sup>287</sup>-specific antibody was used to confirm CaMKII activation in these tissues (Figure 3G).

### Phosphorylation of Ser<sup>26</sup> is regulated by depolarization in vascular tissue

Ser<sup>26</sup> is located within a conserved stretch of nine residues (LGKGAFSVV) that constitute the upper lid of the ATP-binding site in the canonical kinase fold (bold letters indicate conserved residues). To test the physiological importance of CaMKII phosphorylation at Ser<sup>26</sup> during vascular depolarization, a phosphospecific antibody against Ser<sup>26</sup> was raised using the phosphopeptide sequence H<sub>2</sub>N-LGKGAFS(p)-VVRRCVKKTSTQE-COOH. Antibody specificity was tested by comparing preimmune and immune serum staining of aorta homogenates. A signal at the expected molecular mass was detected only with immune serum (Figure 3C). The identity of the band was confirmed with a pCaMKII Thr<sup>287</sup>-specific antibody (results not shown). Next, the phosphopeptide used to raise the antibody was mixed with the antibody in a competition assay. As shown in Figure 3(D), the competing peptide decreased the antibody signal in a concentration-dependent manner.



**Figure 4** Phosphomimic mutation of Ser<sup>26</sup> decreases Thr<sup>287</sup> autophosphorylation and leads to an impaired catalytic activity toward exogenous Thr<sup>287</sup> substrate peptide (A) Lysates from His-tagged C-1 wt, C-1 S26D or C-1 S26A or mock-transfected (NT) COS-7 cells probed with the indicated antibodies. (B) Densitometric quantification of the results of three similar experiments. \*\*\**P* ≤ 0.0001. (C) CaMKII wt, CaMKII S26D and CaMKII S26A recombinant proteins mixed with autocamtide-3 in a kinase activating buffer and dotted on membrane and probed for phosphothreonine. Peptide and kinase were separated using a sizing spin column and the peptide fraction used for dot blot. (D) Quantification of the results from five similar experiments. The results are normalized by the amount of kinase used in each assay. \*\*\**P* ≤ 0.001. The brightness of entire blot has been uniformly altered for visual display; digital values are unchanged.

Next, we tested CaMKII phosphorylation at Ser<sup>26</sup> during vascular depolarization. The level of Ser<sup>26</sup> phosphorylation on CaMKII was compared between unstimulated versus depolarized quick-frozen vascular tissue homogenates. As is shown in Figure 3(F), densitometric analysis of the phospho-Ser<sup>26</sup> immunoblots normalized to tubulin co-staining shows a significant increase (~50%) with depolarization for 10 min with KCl PSS. Thus, phosphorylation of Ser<sup>26</sup> on CaMKII is regulated during contraction in vascular tissue.

### A phosphomimic S26D mutation inhibits Thr<sup>287</sup> autophosphorylation of CaMKII

We postulated that Ser<sup>26</sup> phosphorylation of CaMKII might alter kinase activity. To test this hypothesis, we determined the effect of mutation of Ser<sup>26</sup> to alanine (A) or aspartic acid (D) in recombinant CaMKII. Kinase activity was assayed by monitoring Thr<sup>287</sup> autophosphorylation. COS-7 cells were transfected with CaMKII wt, the S26D mutant or the S26A mutant. The COS-7 cell line used has no endogenous detectable

CaMKII protein expression (Figure 4A). Therefore the CaMKII holoenzyme in the transfected COS-7 cells is a homo-oligomer. The Thr<sup>287</sup> phosphorylation level was measured by probing the transfected COS-7 cell lysates in immunoblots. The results were normalized for transfection efficiency by probing co-stained immunoblots with an anti-total CaMKII  $\gamma$  antibody (Figure 4A). Densitometric analysis of the normalized Thr<sup>287</sup> phosphorylation levels of the S26D and S26A mutants and wt CaMKII indicates that the level of Thr<sup>287</sup> phosphorylation is markedly reduced in the S26D mutant (~80%) (Figure 4B). Thr<sup>287</sup> phosphorylation of wt and S26A were not significantly different. These results indicate that phosphorylation of Ser<sup>26</sup> inhibits Thr<sup>287</sup> phosphorylation in the CaMKII  $\gamma$  homo-oligomer while the S26A mutation has no effect.

### The phosphomimic CaMKII S26D mutant cannot phosphorylate exogenous CaMKII Thr<sup>287</sup> substrate

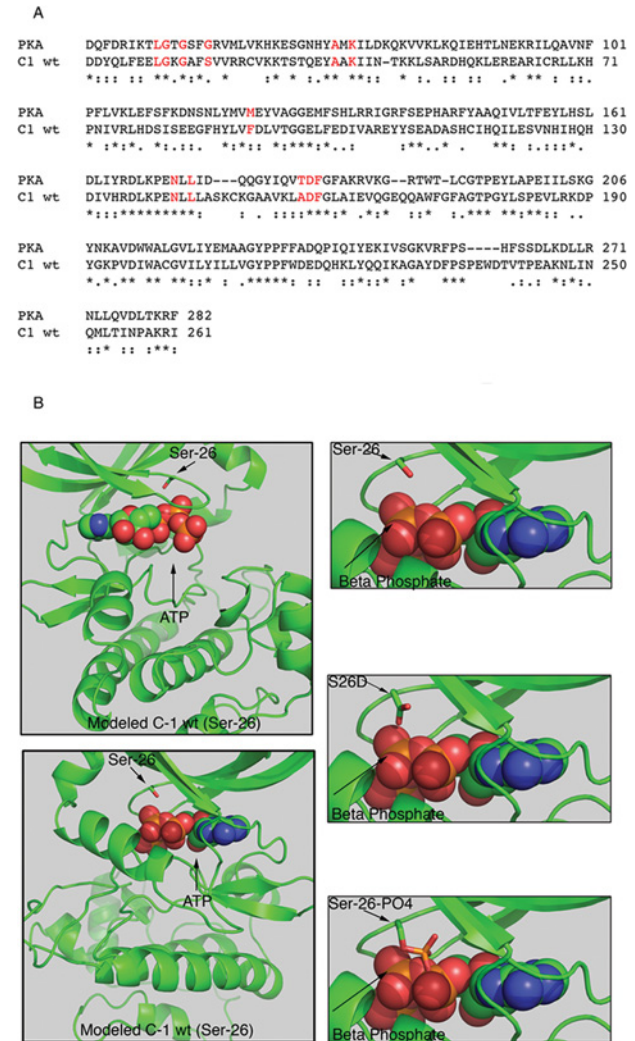
To investigate whether the observed inhibition of Thr<sup>287</sup> phosphorylation in the S26D mutant is due to a direct inhibition

of kinase activity, an *in vitro* kinase assay was performed using a 13-amino-acid peptide containing Thr<sup>287</sup> (autocamtide-3) as a substrate. His-tagged purified recombinant CaMKII wt, the S26D mutant and the S26A mutant were used *in vitro* dot-blot kinase assays containing 20  $\mu$ M autocamtide-3, 5  $\mu$ g/ml CaM and 10 mM CaCl<sub>2</sub> to activate the kinase. The phosphorylated peptide was separated from the holoenzyme (~680 kDa) using a sizing spin column. As shown in Figure 4(C) and is quantified in Figure 4(D), the S26D mutant caused negligible threonine phosphorylation of the exogenous peptide even though significant amounts of peptide phosphorylation were seen with the S26A mutant and wt kinase. The S26A mutant showed significantly more activity in phosphorylating the substrate peptide than did wt kinase, suggesting that autophosphorylation of the wt kinase leads to less than the maximal possible kinase activity.

Taken together, these results indicate that the negative charge on Ser<sup>26</sup> of CaMKII  $\gamma$  introduced by the mutation or by autophosphorylation of wt kinase inhibits the catalytic activity of the enzyme towards its autophosphorylation site at Thr<sup>287</sup>.

### Structural interpretation of the CaMKII inhibition by Ser<sup>26</sup> phosphorylation

In the absence of a three-dimensional structure of CaMKII phosphorylated on Ser<sup>26</sup> we used homology modelling to explain a plausible mechanism for the kinase inhibition by Ser<sup>26</sup> phosphorylation or S26D mutation. Ser<sup>26</sup> is located in a stretch of residues that form the first two  $\beta$ -strands and the connecting loop of the  $\beta$  domain in the canonical kinase fold. We refer to this segment as the ‘upper lid’ of the ATP-binding site because these residues make extensive interactions with the ATP molecule, specifically with the triphosphate moiety, as observed, for example, in the structure of the PKA–ATP complex [32]. Glycine is the most commonly found amino acid at the position corresponding to CaMKII Ser<sup>26</sup>. This semi-conserved glycine residue plays an important role in positioning the triphosphate moiety for catalysis by making a strong hydrogen bond with one of the  $\beta$ -phosphate oxygen atoms. Besides the serine residue found in all CaMKII isoforms there are also alanine and histidine found among the very few substitutions at this position in the kinase superfamily. Thus, there must be a strong evolutionary selection at this position and it is probable that the glycine  $\rightarrow$  serine substitution plays an important function specific for CaMKII. To evaluate the possible consequences of introducing an acidic group at this position, we have modelled the wt CaMKII  $\gamma$  C-1 isoform, its phosphorylated form and the S26D mutant based on the structure of PKA (PDB entry 1ATP). We found the PKA structure to be a better template for the modelling than the recently published structure of CaMKII  $\gamma$  isoform (PDB entry 1V7O), because in the latter the loop containing Ser<sup>26</sup> is disordered due to the absence of the triphosphate moiety in the bound inhibitor. In the modelled structure of C-1, all the interactions between the protein and ATP are well preserved including a correct geometry and bond lengths for the bound metal (Mn<sup>2+</sup>), which positions the  $\gamma$  phosphate for catalysis. Therefore we are confident that our model has all the essential features of



**Figure 5 Atomic model depicting a role for Ser<sup>26</sup> phosphorylation** (A) Alignment with the protein whose structure was used for modelling and marking the residues that were used for structural homology alignment residues involved in ATP binding are shown in red (B) On the left, the front and the back view (rotated 180° on the y-axis) of the modelled C-1 wt structure with ATP. On the right, close view of atomic structure of unphosphorylated Ser<sup>26</sup>, S26D and phosphorylated Ser<sup>26</sup> (Ser<sup>26</sup>-PO<sub>4</sub>) on modelled CaMKII C-1 structure with ATP. Atomic colour code: Phosphate (orange), oxygen (red), carbon (green), nitrogen (blue). Structures drawn using the PyMOL Molecular Graphics System, version 1.2r3pre, Schrödinger.

the CaMKII structure. The modelled structures suggest that the negatively charged side chain carboxy group of the aspartic acid residue or the phosphate attached to the serine side chain oxygen would clash with the  $\beta$ -phosphate group of ATP, thus either sterically interfering with the catalysis, i.e. with the transfer of the  $\gamma$  phosphate to a substrate such as Thr<sup>287</sup> or inhibiting the ATP binding (Figure 5). Furthermore, owing to the orientation of the Ser<sup>26</sup> side chain towards the catalytic cleft, and its position close to the  $\beta$ -phosphate of the bound ATP, this side chain is not readily accessible to an external kinase molecule. It appears





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#### AUTHOR CONTRIBUTION

Mehtap Yilmaz, Kathleen Morgan and Samudra Gangopadhyay conceived and designed the experiments; Mehtap Yilmaz and Samudra Gangopadhyay performed the experiments; Paul Leavis designed and synthesized the peptides; Mehtap Yilmaz and Zenon Grabarek modelled the three-dimensional protein structure; Mehtap Yilmaz prepared the Figures and drafted the paper; Mehtap Yilmaz, Kathleen Morgan and Zenon Grabarek analysed the data, interpreted the results and revised and edited the paper before acceptance.

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