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CaMKII Kinase Activity, Targeting and Control of Cellular Functions: Effect of Single and Double Phosphorylation of CaMKIIα

A'qilah Banu binte Abdul Majeed‡, Elizabeth Pearsall‡, Helen Carpenter, Joshua Brzozowski, Phillip W. Dickson, *PhD*, John A. P. Rostas, *PhD*, and Kathryn A. Skelding, *PhD**

***Abstract*— Calcium/calmodulin-stimulated protein kinase II (CaMKII) is a ubiquitously expressed multifunctional kinase, which regulates many cellular processes, including synaptic plasticity and proliferation. CaMKII is activated by binding calmodulin (CaM), triggered by an increase in intracellular Ca^{2+}. CaMKII can autophosphorylate at several residues, including T253, T286, and T305/306, which alters CaMKII activity and targeting in different ways. Phosphorylation at T286 induces autonomous activity, whereas phosphorylation at T305/306 prevents CaMKII activation. Phosphorylation at T253 has no effect on CaMKII activity *in vitro*. Phosphorylation at each of these sites changes the subcellular location of CaMKII, and alters protein interactions. To investigate the mechanisms by which dual phosphorylation of CaMKII might regulate cellular functions we examined the effects of double phosphomimic mutation (at T253D/T286D or T286D/T305D) on kinase activity and targeting. We showed that both double phosphomimic mutations altered targeting whereas only T286D/T305D altered kinase activity *in vitro*. We also showed that overexpressing either T253D/T286D or T286D/T305D altered cell proliferation rates, and that this effect was different from the effects observed with the relevant single phosphomimic mutation. These results indicate the importance of targeting as a regulatory mechanism in CaMKII control of cell function.**

***Keywords* — calcium/calmodulin-stimulated protein kinase II, CaMKII, cell proliferation, kinase activity, protein phosphorylation, protein targeting**

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‡ AB Abdul Majeed and E Pearsall contributed equally to this work.

School of Biomedical Sciences and Pharmacy, and the Hunter Medical Research Institute, Faculty of Health, The University of Newcastle, Callaghan, New South Wales, Australia.

*Correspondence to Kathryn A Skelding (e-mail: Kathryn.skelding@newcastle.edu.au).

I. INTRODUCTION

Calcium/calmodulin stimulated protein kinase II (CaMKII) is a multifunctional serine/threonine protein kinase, that is one of the major Ca^{2+} sensors in cells. CaMKII is normally activated by binding calmodulin (CaM), triggered by a rise in intracellular Ca^{2+}. CaMKII is encoded by four highly homologous genes in mammals (α, β, γ, and δ), and one or more isoforms are expressed in virtually every tissue. CaMKII is involved in regulating many aspects of neuronal function, including long-term plasticity, learning, and memory consolidation (1-6). CaMKII has also been implicated in the regulation of other non-neuronal biological processes, such as cell growth and proliferation (7-9), and fertilisation (10).

The biological properties of CaMKII are controlled by multi-site autophosphorylation, (11, 12). CaMKII can autophosphorylate at several sites, including T253, T286, and T305/306. Phosphorylation at the well-characterised site, T286, increases the affinity for CaM (13), and causes CaMKII to become autonomously active (14). Once CaM has dissociated from the CaM binding site, T305/306 autophosphorylation can occur (15). Following phosphorylation at T305/306, CaM can no longer bind so CaMKII cannot be activated by Ca^{2+}/CaM, resulting in an enzyme that is insensitive to changes in Ca^{2+}/CaM (16). Phosphorylation at another site, T253, has no direct effect on CaMKII activity *in vitro*, but alters the proteins with which CaMKII associates (8, 17).

The properties of CaMKII can also be controlled by

targeting to specific cellular locations through interactions with binding proteins (8, 11, 12). For example, CaMKII translocation to the post-synaptic density (PSD) is dependent on the phosphorylation state of CaMKII. Autophosphorylation of CaMKII at T286 or T253 causes CaMKII to bind to the PSD (17, 18). By contrast, phosphorylation of T305/306 causes CaMKII to translocate from the PSD to the cytosol (19).

Most studies examining the regulation and subsequent functions of CaMKII have examined CaMKII that is phosphorylated at a single site, however, it is likely that phosphorylation at more than one site will occur *in vivo*. Recent evidence has emerged that suggests that the various phosphorylation sites influence the capacity of each other to regulate CaMKII (17, 20, 21). For example, long-term potentiation (LTP) induction requires CaMKII activation and phosphorylation of T286 (22, 23). Pharmacological and transgenic experiments involving mice demonstrated that LTP induction was dependent on the activation of CaMKII (1, 24). Furthermore, overexpression of an active CaMKII fragment in neurons prevented saturated synaptic potentiation and LTP following synaptic stimulation (25, 26). It was therefore expected that transgenic mice bearing a phosphomimic mutation of CaMKIIα at T286 (T286D) should exhibit saturated potentiation, however, at high-frequency stimulation normal LTP was induced, and at low-frequency stimulation long-term depression (LTD) was favored (27). This suggests that some other factor is modifying CaMKII function *in vivo*. Pi et al (21) showed that the functional outcomes mediated by CaMKII following T286 autophosphorylation could be modified by additional phosphorylation at T305/306. Overexpression of a T286D/T305D/T306D phosphomimic mutant form of CaMKIIα in hippocampal neurons, caused depression that occluded LTD, whereas overexpression of the T286D/T305A/T306A 305/306 phosphonull mutant form of αCaMKII prevented LTD and produced LTP (21). Hence, whether LTP or LTD occurs is dependent on whether CaMKII is phosphorylated at T286, T305/306, or a combination of the two. Furthermore, Pi et al (20) also showed that synaptic strength and spine size can be altered depending on the phosphorylation state of T286 and T305/306. Triple phosphomimic mutation (T286D/T305D/T306D) of CaMKIIα causes an increase in spine size, but a decrease in synaptic strength. By contrast, overexpression of the T286D phosphomimic mutant form of CaMKIIα increased spine size and enhanced synaptic strength. This shows that the T305/306 phosphorylation sites act as control points in which the effect of T286 autophosphorylation on synaptic strength can be altered. However, the mechanism responsible for these interactions is not known. It may be due to kinase activity, targeting, or a combination of both. Other CaMKII phosphorylation sites have also been shown to potentially influence each other, namely T253 and T286. Phosphorylation of CaMKIIα at both T253 and T286 produces additive binding to the PSD when compared to that of either site alone (17).

To investigate the mechanisms by which dual phosphorylation of CaMKII might regulate cellular functions we examined the effects of double phosphomimic mutation (at T253D/T286D or T286D/T305D) on kinase activity, CaM binding and interaction with protein binding partners *in vitro* and the effects on cell proliferation of overexpressing these mutants. Our results indicate the importance of targeting as a regulatory mechanism in CaMKII control of cell function.

II. Methods

A. Generation of FLAG-CaMKII mutants

Fusion proteins containing the FLAG tag at the N-terminus were generated by inserting CaMKIIα cDNA from rat brain (a gift from H Schulman) into the FLAG CMV-4 expression vector (Stratagene, Chatswood, NSW, Australia). The point mutations of CaMKIIα (T253D, T286D, T305D, T253D/T286D, T286D/T305D) in the CMV-4 vector were created using the QuickChange Site Directed Mutagenesis Kit (Stratagene). The following oligonucleotides were used (Geneworks, Hindmarsh, SA, Australia): 5′-CAATAAGATGCTG**GA**CATCAACCCGTCC-3′ for T253D, 5′-GCATGCACAGACAGGAG**GA**CGTGGACTGCCTGA AG-3′ for T286D, and 5’–TGAAGGGAGCCATCCTC**GA**CACTATGCTGG-3’ for T305D. The presence of the mutation and the absence of other changes was confirmed by sequencing.

Recombinant CaMKIIα was expressed in COS-7 cells after transfection with Lipofectamine 2000 (Life Technologies, Mulgrave, VIC, Australia) according to the manufacturer’s instructions. CaMKII (wild-type, T253D, T286D, and T253D/T286D) was purified as described previously (28). Mutants containing the T305D mutation were purified via size exclusion chromatography, using the AKTA design FPLC (Fast protein liquid chromatography; GE Healthcare, Castle Hill, NSW, Australia) and a Superose^{TM} 12 column (10 – 300mm GL, GE Healthcare). The Superose^{TM} 12 column was pre-equilibrated at a flow rate of 0.5 ml/min using 50 mM Tris-HCl, 150 mM NaCl, pH 7.4 which had been degassed and filtered. The GE Healthcare calibration low molecular and high molecular weight kits were used to calibrate the column. By comparing the elution volume parameter (V_e) of the CaMKII protein with the values of several calibration standards, the molecular weight could be determined, and

proteins could be extracted based on their size. The samples were eluted at 0.5 ml/min in 50 mM Tris-HCl, 150 mM NaCl, pH 7.4. The eluates were monitored by UV spectrometry at 280 nm. Glycerol (final concentration: 20%), PMSF (final concentration: 0.1 mM), DTT (final concentration: 1 mM), and NaN_3 (final concentration: 0.02%) were added to the fractions that contained CaMKII. The relative concentration of purified CaMKIIα was determined by quantification of western blot using antibodies to FLAG.

B. Binding to calmodulin (CaM) sepharose

The ability of the various wild-type and phosphomimic mutant forms of CaMKII to bind to CaM was determined by using CaM sepharose, as described previously (28).

C. Phosphorylation of tyrosine hydroxylase 1 (TH1)

Human tyrosine hydroxylase isoform 1 (TH1) was used as an exogenous substrate to determine the kinase activity of the various phosphomimic mutants. TH1 was made as described previously (29). TH was phosphorylated using the various wild-type and phosphomimic mutant forms of CaMKII (29). The reaction mixture contained 50 mM Tris-HCl (pH 7.4), 2.5 µg CaM, 2 mM $CaCl_2$, 12.5 mM $MgCl_2$, 100 µM ATP, and 8 µg TH1. The reaction was started by adding 0.86 µg of wild-type or phosphomimic mutant CaMKIIα, the reaction was incubated at 30°C for 3 min, and the reaction was stopped by adding 6 x electrophoresis sample buffer and boiling for 5 min.

D. Phosphorylation of Syntide 2.

Phosphorylation of syntide-2 was quantitated using the CycLex CaM kinase II activity ELISA Kit (MBL International Corporation, Woburn, MA, USA), as per the manufacturer's instructions.

E. PSD Binding Assay

The binding assay was based on the method of Yoshimura and Yamuchi (30), using the PSD-enriched detergent insoluble (P3x) subcellular fraction (which was obtained as previously described, (17)). Wild-type or phosphomimic mutant forms of CaMKII (20µl) were mixed with PSD-enriched subcellular fraction (20 µg), and incubated for 10 min at 4°C. After this time, the samples were centrifuged at 100,000 g for 30 min at 4°C. The pellet was resuspended in 40 mM Tris-HCl, pH 7.6, containing 1 mM DTT, 50 mM NaCl, 10% ethyleneglycol, 0.05% Tween-20 and centrifuged at 100,000g for 30 min. The relative amount of FLAG-CaMKIIα bound to the PSD fraction was determined by western blot.

F. Western Blotting

CaMKII autophosphorylation at T253 and T286, TH phosphorylation at S19 by CaMKII, and PSD binding were measured by western blotting. Reaction mixtures were separated using 10% SDS-polyacrylamide gel electrophoresis (PAGE), and then transferred to nitrocellulose membranes, using standard techniques (31). The primary antibodies used were as follows: phospho-T253-CaMKII (1:20,000 (17)), anti-FLAG (M2; 1:1,000; Sigma-Aldrich, Castle Hill, NSW, Australia), phospho-T286-CaMKII (1:1,000; Abcam, Cambridge, MA, USA), total CaMKIIα (6G9, 1: 5000, Millipore North Ryde, NSW, Australia), actin (AC-15; 1:50,000; Sigma-Aldrich), total TH1 (1:2,000; (32)), and phospho-S19-TH (1:1000; (29)). Primary antibody binding was detected by incubation with donkey anti-rabbit or sheep anti-mouse horseradish peroxidase-linked secondary antibody, and the ECL Plus Immunoblotting Detection System (GE Healthcare). Blots were scanned with a Fujifilm LAS-4000 Imaging System (Brookvale, NSW, Australia) and analysed with MultiGauge Software (Fujifilm).

G. Overlay Binding Assay

The overlay binding assay was performed as described previously (8).

H. Cell Lines

COS-7 (ATCC CRL-1651) and MDA-MB-231 (ATCC HTB-26; human breast cancer cells) were purchased from the ATCC (Manassas, VA, United States of America) and were maintained in DMEM (Life Technologies), supplemented with 15 mM HEPES (Life Technologies), 2 mM glutamine (Life Technologies), and 10% heat-inactivated fetal calf serum (FCS; Sigma-Aldrich).

I. Cell Proliferation

MDA-MB-231 cells were transiently transfected with wild-type and phosphomimic mutant forms of CaMKIIα using Lipofectamine 2000 (Life Technologies). Proliferation was assessed 48 h post-transfection using the CellTiter-Blue Cell Viability Assay (Promega, Alexandria, NSW, Australia). Transfections were performed in triplicate, and three independent experiments were performed.

J. Data Analysis

All statistical analyzes were conducted using GraphPad

Prism software V6.0 (GraphPad, San Diego, CA, USA). Comparisons between lines were made using one-way analysis of variance (ANOVA), with a Tukey post-test. All data is presented as the mean ± standard error of the mean (SEM) for the number of replicates (n).

III. Results

A. Generation of recombinant wild-type and phosphomimic mutant CaMKII proteins

Single and double phosphomimic mutant forms of CaMKIIα (as well as a wild-type [WT] form) were generated, expressed in COS-7 cells and purified using binding to CaM sepharose beads and size exclusion chromatography or, for mutants containing the T305D mutation, just size exclusion chromatography). All constructs were able to be expressed at a high enough concentration for further examination (Supplementary Figure 1).

B. The effects of phosphomimic mutations on CaMKII binding to CaM sepharose

To investigate the effect of phosphomimic mutations on the interaction between CaMKII and CaM, we measured the binding of the single and double phosphomimic CaMKII mutants to CaM sepharose. Under the conditions of this assay where CaM is immobilized on sepharose beads, WT CaMKIIα bound weakly to CaM; the majority of WT CaMKIIα was recovered in the flow through fraction and all of the CaMKIIα that bound to the beads was eluted with a single application of EGTA (Figure 1). The T253D phosphomimic mutation did not alter the binding of CaMKIIα to CaM sepharose (Figure 1). As expected from the known effects of phosphorylation at T286 (13), the T286D phosphomimic mutation strongly increased the affinity of CaMKIIα for CaM; almost all the applied CaMKIIα bound to the beads and a small amount remained bound after a single elution with EGTA (Figure 1). Consistent with previously published results (16)), the T305D phosphomimic mutation, which is within the CaM binding site itself, prevented CaMKIIα from binding to CaM (Figure 1).

The behaviour of the T253D/T286D double phosphomimic mutant was very similar to that observed with the single T286D phosphomimic mutant (Figure 1). Additionally, the double phosphomimic mutation (T253D/T286D) increased the amount (~2-fold) of CaMKIIα that remained bound to the CaM sepharose following EGTA elution, when compared to the single T286D mutant (Figure 1) showing that the T253D mutation which, on its own, had no effect on CaM binding, was able to modify CaM binding when combined with the T286D mutation. The behaviour of the T286D/T305D double phosphomimic mutant was very similar to that of the single T305D phosphomimic mutant (Figure 1). This shows that, the presence of a phosphomimic mutation within the CaM binding site can negate the ability of the T286D mutation to greatly enhance CaMKIIα binding to CaM under the conditions of this assay.

C. The effects of phosphomimic mutations on CaMKII activity

The effects of phosphomimic mutations on the kinase activity of CaMKIIα were measured using two substrates: a low molecular weight peptide substrate (Syntide-2) and a macromolecular protein substrate CaMKII (human tyrosine hydroxylase, TH) with which CaMKIIα is known to interact in a way that is modified by phosphorylation (8).

Syntide-2 phosphorylation activity of the T253D mutant was indistinguishable from that of WT CaMKIIα (Figure 2), which is consistent with our previous findings that T253 phosphorylation has no direct effect on CaMKII activity (17). The presence of the T286D mutation did not change the rate of phosphorylation in the presence of Ca^{2+}/CaM despite its enhancement of the affinity of CaM binding (Figure 1). In the absence of Ca^{2+}/CaM, the T286D mutation induced ~25% maximal kinase activity (Figure 2) which is consistent with the level of autonomous activity induced by T286 phosphorylation reported by others (33). As expected, the T305D mutation blocked CaM stimulated activity and induced no autonomous activity (Figure 2).

The activity of the T253D/T286D double phosphomimic mutant was not significantly different from that of the T286D mutant (Figure 2), showing that the T253D mutation which, on its own, had no effect on CaMKII activity under the conditions of this assay, also did not alter the effects of the T286D mutation on CaMKII activity. By contrast, the activity of the T286D/T305D double mutant showed a clear interaction between the effects of the phosphomimic mutations. The enhancement in the affinity of CaMKIIα binding to CaM produced by the T286D mutation was strong enough to partially overcome the inhibition of CaM binding produced by the T305D mutation and produced increased CaMKII activity in the presence of Ca^{2+}/CaM (Figure 2). Furthermore, in the presence of the T305D mutation the T286D mutation was no longer able to induced autonomous CaMKII activity (Figure 2).

Tyrosine hydroxylase is phosphorylated by CaMKII at S19 (34). TH is also a CaMKII binding protein whose interaction with CaMKII is greatly enhanced by phosphorylation of CaMKII at T253 and phosphorylation of TH at S19 (8). To investigate the potential effects of such allosteric interactions between CaMKII and a protein substrate on the effects of phosphomimic mutations in CaMKII we measured S19 phosphorylation activity of CaMKII using human TH1 which shows the same phosphorylation sensitive interaction with CaMKII as TH2

(results not shown). Overall, the results were the same as those obtained using Syntide-2 as the substrate (Figure 3). This shows that, despite the dramatic and selective effects of T253D phosphomimic mutation on binding to both phosphorylated and non-phosphorylated TH (8), such allosteric interactions had no direct effects on the kinase activity of CaMKII.

D. Effects of phosphomimic mutations on CaMKII binding to proteins

Phosphorylation of CaMKII can alter the targeting of CaMKII (8, 11, 12, 35, 36). Perhaps the best-studied example of phosphorylation state altering CaMKII targeting is the translocation of CaMKII to the postsynaptic density (PSD). Autophosphorylation of CaMKII at T286 or T253 causes CaMKII to bind to the PSD (17, 18). By contrast, phosphorylation of T305/306 causes CaMKII to translocate away from the PSD (19). Additionally, dual phosphorylation of CaMKII at T253 and T286 causes an additive increase in the amount of CaMKII bound to the PSD (17). Herein, we demonstrate that single phosphomimic mutation of T286D or T253D significantly increased ($p < 0.01$ for both) the amount of CaMKIIα bound to the PSD (Figure 4), when compared to WT-CaMKIIα, whereas single phosphomimic mutation ($p < 0.001$) of T305D significantly decreased the amount of CaMKIIα bound to the PSD (Figure 4). Furthermore, double phosphomimic mutation of T253D/T286D further increased the amount of CaMKIIα bound to the PSD ($p < 0.0001$), when compared to single phosphomimic mutation of either site alone (Figure 4). Double phosphomimic mutation of T286D/T305D significantly decreased the amount of CaMKIIα bound to the PSD, compared to WT-CaMKIIα ($p < 0.01$) and T286D-CaMKIIα ($p<0.001$) the amount bound was not significantly different from that of T305D-CaMKIIα (Figure 4).

The results in Figure 4 show that phosphorylation of CaMKII at multiple sites can significantly alter CaMKII targeting to isolated PSDs. To investigate the protein interactions involved in this targeting we used a CaMKII overlay binding assay (8) on immobilised proteins from a PSD fraction prepared from rat brain cortex. Single and double phosphomimic mutation of CaMKIIα produced a variety of changes in the binding of CaMKII to other proteins (Figure 5). The binding of CaMKIIα to some proteins was unaffected by any of the phosphomimic mutations. By contrast, CaMKIIα binding to other proteins was enhanced by single phosphomimic mutant, when compared to WT-CaMKIIα (Figure 5; arrow), whereas in other cases, single phosphomimic mutation decreased binding (Figure 5; *). The double phosphomimic mutants showed a pattern of protein binding that could not be predicted from the combination of the behaviour of the corresponding single phosphomimics (Figure 5). For instance, the T286D/T305D mutant bound to a protein band of ~75kDa that did not bind either single phosphomimic or WT CaMKIIα (Figure 5, #) whereas it no longer bound to other proteins (e.g. 10kDa, Figure 5, *) that did bind the T286D mutant. Similarly, the T253D/T286D mutant bound to some proteins (Figure 5, #) that did not bind either single phosphomimic or WT CaMKIIα and the presence of the T286D mutation in the double phosphomimic negated the ability of the T253D mutation to inhibit the binding of CaMKIIα to the 10kDa band (Figure 5, *).

E. Effects of overexpression of CaMKIIα phosphomimic mutants on cell proliferation.

Recent evidence has shown that functional outcomes mediated by CaMKIIα following T286 autophosphorylation could be modified by additional phosphorylation at T305/306 (21). To determine whether CaMKII that is phosphorylated at both T286 and T253 could also modify functional outcomes, we transiently transfected MDA-MB-231 breast cancer cells with single and double phosphomimic mutant forms of CaMKIIα. We have previously shown that CaMKII that is phosphorylated at different sites affects cell proliferation differently (8, 37). Overexpression of a WT or T286D phosphomimic mutant form of CaMKIIα in MDA-MB-231 cells significantly increases cell proliferation, whereas overexpression of a T253D phosphomimic mutant form of CaMKIIα causes cells to stop dividing (8, 37).

Following transient transfection of MDA-MB-231 cells with WT, T253D, T286D, T305D, T253D/T286D, and T286D/T305D-CaMKIIα, cells expressed detectable levels of CaMKIIα at 48 h post-transfection, whereas empty vector (EV) transfected cells exhibited no detectable level of CaMKIIα (Figure 6A). Proliferation of MDA-MB-231 cells was measured at 48 h post-transfection (Figure 6B). Transfection of the various CaMKIIα phosphomimic mutants had varied effects on cell proliferation. Overexpression of WT, T286D or T305D phosphomimic mutant forms of CaMKIIα significantly increased cell proliferation when compared to the EV control cells (Figure 6B; $p < 0.01$ for WT and T286D; $p<0.05$ for T305D), whereas overexpression of the T253D phosphomimic mutant form of CaMKIIα significantly decreased cell proliferation (Figure 6B; $p < 0.001$).

Cells overexpressing the double phosphomimic mutant T253D/T286D exhibited a significantly faster rate of proliferation when compared to cells expressing the corresponding single phosphomimic mutants (Figure 6B;

T253D: $p < 0.0001$; T286D: $p < 0.001$). The comparison of the effects of T253D and T253D/T286D is particularly striking as the presence of the T286D mutation reversed the effects of the T253D mutation. Additionally, cells overexpressing the T286D/T305D double phosphomimic mutant exhibited a significantly faster rate of proliferation when compared to cells expressing the corresponding T286D or T305D phosphomimic mutants (Figure 6B; $p < 0.05$ for both). This indicates that the double phosphomimic mutation of T253D and T286D, or T286D and T305D produce a different functional effect when compared to mutation of any of these sites on their own.

IV. Discussion

CaMKII is an abundantly expressed multifunctional protein kinase, that is important for many cellular functions, including long-term plasticity, learning, (1, 2), and cell proliferation (7-9). The properties of CaMKII are controlled by multi-site autophosphorylation and by targeting to specific subcellular microdomains through interactions with particular proteins. To date, most studies examining the regulation of CaMKII and its functional consequences have examined CaMKII that is phosphorylated at a single site. However, it is likely that CaMKII will be phosphorylated at more than one site *in vivo*. This could occur in several ways with potentially different outcomes. If the multimeric CaMKII holoenzyme contains a mixture of subunits that are non-phosphorylated or phosphorylated at only a single site each, the functional consequences of the phosphorylations may be simply the sum of the effects of the single sites. However, if some subunits become phosphorylated at more than one site whose combined allosteric effects modify the function of the subunit in a new way, the functional consequences may be different from that expected based on the effects of the single phosphorylation changes.

Phosphorylation of T286 and T305 may individually cause allosteric changes to multiple areas of the CaMKII molecule but they are both known to have an effect on the CaM binding site (amino acids 293-310) (12). Phosphorylation of T305, which is within the CaM binding site, directly disrupts the conformation of the binding site and blocks CaM binding (16). Phosphorylation of T286 greatly enhances the affinity of CaM binding to CaMKII (13) partly, or wholly, by indirect allosteric modification of the CaM binding site. This allosteric interaction between phosphorylation at T286 and T305 can explain the CaM binding (Figure 1) and kinase activity (Figures 2 and 3) of the T286D/T305D double mutant: under conditions that favour CaM binding (but not under limiting conditions): the allosteric enhancement of CaM binding by the T286D mutation can partially overcome the disruption of the binding site by the T305D mutation. Whether the same effect can occur with phosphorylation, as opposed to phosphomimic mutation, remains to be established.

The interaction between the effects of phosphorylation at T286 and T305 can also have functional consequences through modifying CaMKII targeting. Figure 5 shows that the pattern of protein binding by the double mutant was different from that of either of the single mutants suggesting that protein binding regions outside the CaM binding site are also modified by phosphorylation at T286 and/or T305. Using electrophysiological and morphological measures of synaptic function in cultured neurons following the expression of various phosphomimic mutants of CaMKII, Pi et al (20, 21) have shown that phosphorylation of CaMKII at T305/306 can modify and even reverse the functional consequences of CaMKII phosphorylation at T286. Based on our results (Figures 1-5) the mechanisms responsible for the interaction between the effects of T286 and T305/T306 phosphorylation reported by Pi et al could have been due to effects on kinase activity or targeting or both.

Using cell proliferation as another cellular function that is known to be regulated by CaMKII (7-9), we have shown that expressing the T286D and T305D phosphomimic mutants each stimulated cell proliferation to the same extent as WT. Since WT, T286D and T305D CaMKIIα were expressed at the same level (Figure 6A) but had very different kinase activities (Figures 2 and 3), the enhancement of proliferation is likely to be due to the increased level of expression of CaMKIIα and is likely mediated via targeting of CaMKII. Similarly, the further enhancement of proliferation produced by overexpression of the T286D/T305D double mutant cannot be explained by differences in expression level or activity of CaMKII and also is most likely to represent the consequences of altered targeting of CaMKII.

Phosphorylation of T253 has no effect on CaMKII activity either by itself ((17), Figures 2 and 3) or in combination with T286D (Figures 2 and 3) but it is known to alter targeting of CaMKII (8, 12, 17). One of the protein binding regions on CaMKII that is allosterically regulated by phosphorylation at T253 is amino acid 310-320 that is immediately adjacent to the CaM binding site (12). Although the T253D mutation by itself had no effect on CaM binding, when combined with the T286D mutation, the combined allosteric effects produced a greater enhancement of CaM binding than observed with T286D alone (Figure 1). Interactions between the allosteric effects of T253D and T286D mutations can also be seen in CaMKII binding to other proteins (Figure 5). For example, the T253D mutation inhibited CaMKII binding to a 10kDa band (Figure 5) but, in the T253D/T286D double mutant, the allosteric effects of the T286D mutation negated the inhibition by the T253D mutation (Figure 5). The double mutant also bound to protein(s) which bound neither the WT CaMKII nor the single mutants (Figure 5). We have previously shown that overexpression of T253D CaMKII inhibits cell proliferation

by blocking cell division whereas overexpressing T286D CaMKII has no effect on cell proliferation, when compared to overexpression of WT CaMKII (8, 37). In this study we have shown that overexpressing the T253D/T286D double mutant at the same level as the single mutants reversed the inhibition by the T253D mutation and produced an even greater stimulation of proliferation than by the T286D mutation alone (Figure 6). Taken together these results suggest that the mechanisms by which phosphorylation at T253 alters CaMKII dependent functions is likely to involve changes in CaMKII targeting.

V. Conclusions

Our results illustrate the importance of CaMKII targeting through specific protein-protein interactions in regulating a variety of cellular functions. It is through specific, phosphorylation-sensitive interactions with proteins, whose expression and/or subcellular location can vary between cell types, that a ubiquitously expressed kinase such as CaMKII can regulate very different cellular functions in different tissues (11, 12).

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Figure 1. Effect of single and double phosphomimic mutation of CaMKIIα on binding to calmodulin (CaM). COS-7 cells were transiently transfected (using Lipofectamine 2000) with various FLAG-tagged CaMKIIα constructs (wild-type [WT], T253D, T286D, T305D, T253D/T286D, T286D/T305D). Twenty-four hours post-transfection, cells were lysed, insoluble material was removed by centrifugation, and lysates were added to CaM Sepharose columns. Following incubation for 1 hour, the columns were opened, and the flow through was collected (*flowthrough*). The columns were washed, and then bound proteins were eluted in 8 steps using EGTA (*eluted fractions*). Proteins that remained bound to beads following these elution steps (*remained bound*) were removed by boiling in SDS Sample Buffer for 15 mins. Proteins (10µl) were separated via 4-12% SDS-PAGE, transferred to nitrocellulose membranes, and CaMKIIα expression was detected using an anti-FLAG antibody. Blots are representative of 3 independent experiments.

Figure 2. Effect of single and double phosphomimic mutation of CaMKIIα on ability to phosphorylate syntide-2. The ability of purified recombinant CaMKIIα (wild-type [WT], T253D, T286D, T305D, T253D/T286D, T286D/T305D) to phosphorylate syntide-2 *in vitro* in the presence (black bars) or the absence (grey bars) of calcium and calmodulin was measured using the CycLex CaM kinase II activity ELISA Kit. Measurements were performed in duplicate, n=3.

Figure 3. Effect of single and double phosphomimic mutation of CaMKIIα on ability to phosphorylate tyrosine hydroxylase 2 (TH1) at S19. Purified recombinant TH1 was phosphorylated at S19 by CaMKIIα (wild-type [WT], T253D, T286D, T305D, T253D/T286D, T286D/T305D) *in vitro* under various conditions. (A) Following incubation at 30◦C for 3 min, TH1 was separated via 10% SDS-PAGE, transferred to nitrocellulose, and probed with antibodies to TH1 (Total TH) and phospho-S19-TH (pS19-TH). Blots are representative of 3 independent experiments. (B) Densitometric quantitation of band signal intensity of samples phosphorylated in the presence (black bars) or the absence (grey bars) of calcium and calmodulin. n=3.

Figure 4. Effect of single and double phosphomimic mutation of CaMKIIα on ability to bind to the post-synaptic density (PSD). The ability of purified recombinant FLAG-tagged CaMKIIα (wild-type [WT], T253D, T286D, T305D, T253D/T286D, T286D/T305D) to bind to the PSD-enriched P3x subcellular fraction *in vitro* was measured in solution via a PSD binding assay. Purified CaMKII (1 µg) was incubated with 20 µg of PSD fraction for 10 min at 4◦C. Following this, samples were centrifuged at 100,000g for 30 min at 4◦C. The pellet was resuspended in 40 mM Tris, pH 7.6, containing 1 mM DTT, 50 mM NaCl, 10% ethyleneglycol, and 0.05% Tween and centrifuged again at 100000g for 30 min. The amount of FLAG-tagged CaMKII bound to the PSD fraction was determined by quantification of Western blot, and was normalised to the amount of WT-CaMKII bound to the PSD fraction. n=3. * denotes statistical significance ($p < 0.05$).

Figure 5. CaMKII binding patterns vary with single and double phosphomimic mutation. Detergent insoluble (P3x) membrane proteins (30µg) from the forebrain were examined for their ability to bind CaMKII in the overlay binding assay. Arrow: binding increased by single phosphomimic mutation compared to WT-CaMKIIα; *: binding of single phosphomimic mutation decreased compared to WT-CaMKIIα; #: binding was only observed following double phosphomimic mutation. Blots are representative of 3 independent experiments.

Figure 6. Functional consequences of multiple phosphorylation of CaMKII. MDA-MB-231 breast cancer cells were transiently transfected with either empty vector (EV), wild-type (WT), T253D, T286D, T305D, T253D/T286D, or

T286D/T305D CaMKIIα. Forty-eight hours later, (A) FLAG-tagged CaMKII expression was determined by western blotting (anti-FLAG antibody; actin was used as a loading control), and (B) cell proliferation was measured using the CellTiter Blue Assay. Results are presented as percentage of 0 h post-transfection. Experiments were performed in triplicate, n=3. * denotes statistical significance ($p < 0.05$) from EV control, unless otherwise stated.

Supplementary Figure 1. Expression and purification of CaMKII wild-type and phosphomimic mutant constructs in COS-7 cells. COS-7 cells were transiently transfected (using Lipofectamine 2000) with various FLAG-tagged CaMKIIα constructs (wild-type [WT], T253D, T286D, T305D, T253D/T286D, T286D/T305D). Twenty-four hours post-transfection, cells were lysed, and CaMKIIα was purified using Calmodulin Sepharose beads (WT, T253D, T286D, T253D/T286D) or via FPLC (T305D, T286D/T305D). Whole cell lysates (20µl; *Lysate*) or CaM sepharose purified CaMKII (10µl; *Purified*) were separated via 4-12% SDS-PAGE, transferred to nitrocellulose membranes, and CaMKIIα expression was detected using an anti-FLAG antibody (indicated by arrow).

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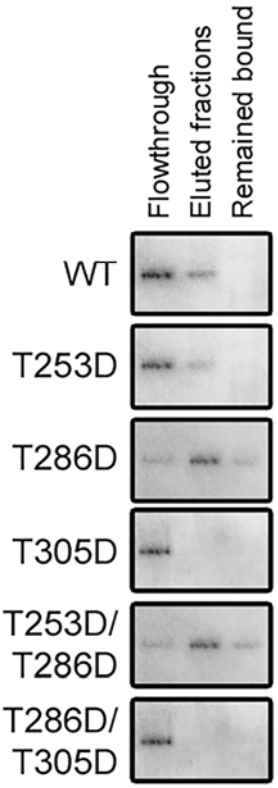

Figure 1.

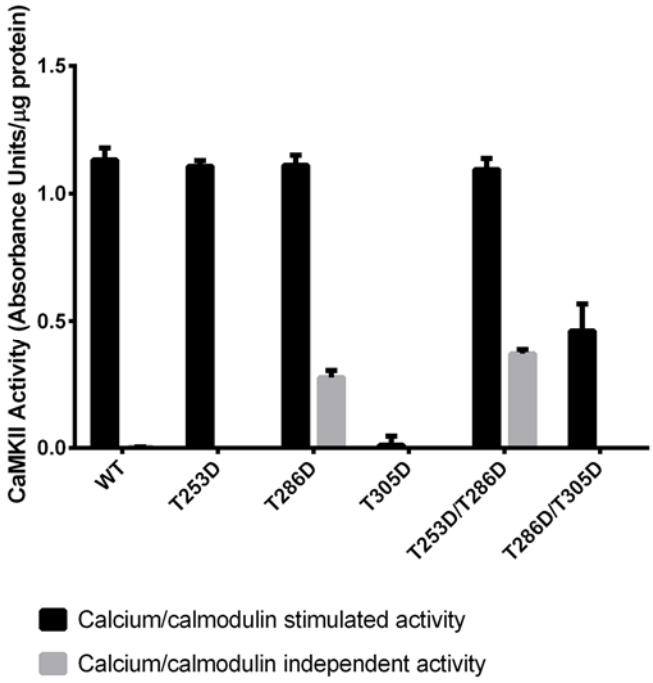
CaMKII Activity (Absorbance Units/µg protein)
1.5
1.0
0.5
0.0
WT
T253D
T286D
T305D
T253D/T286D
T286D/T305D
Calcium/calmodulin stimulated activity
Calcium/calmodulin independent activity

Figure 2.

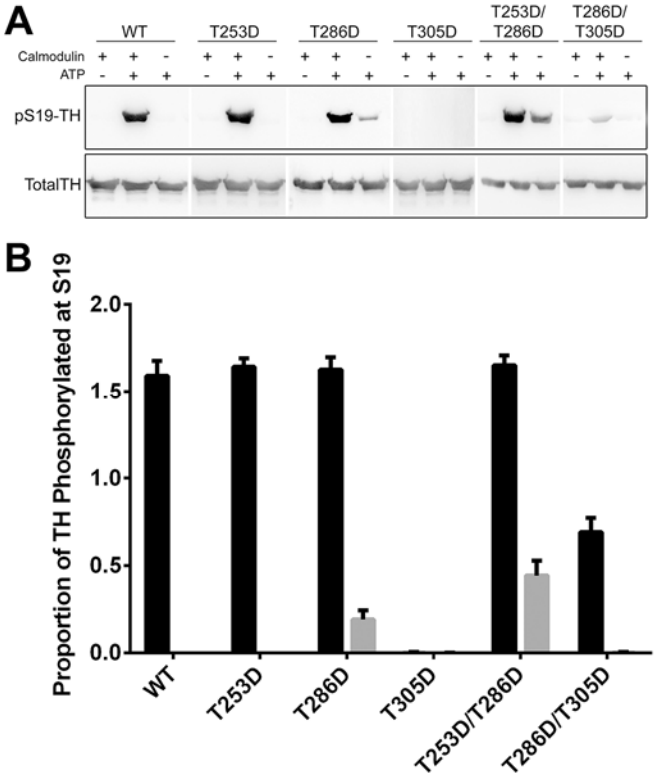
A
WT
T253D
T286D
T305D
T253D/ T286D
T286D/ T305D
Calmodulin
ATP
pS19-TH
TotalTH
B
Proportion of TH Phosphorylated at S19
2.0
1.5
1.0
0.5
0.0
WT
T253D
T286D
T305D
T253D/T286D
T286D/T305D

Figure 3.

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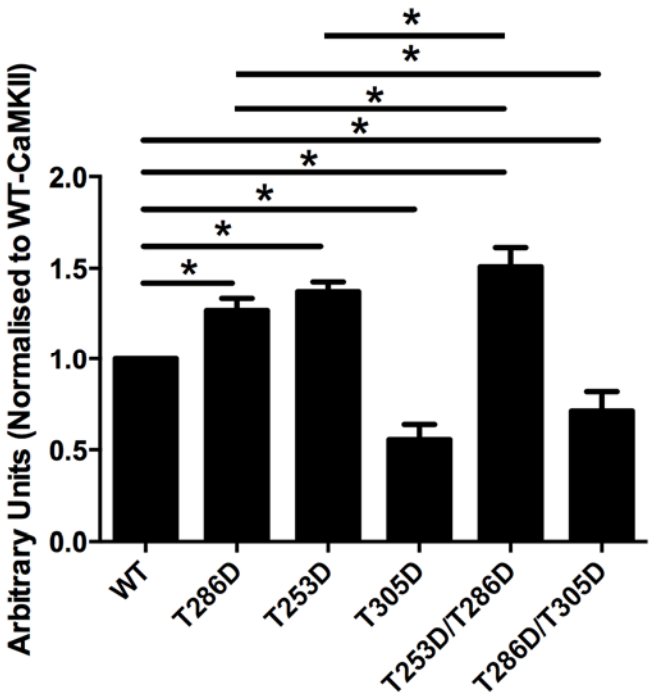


Figure 4.

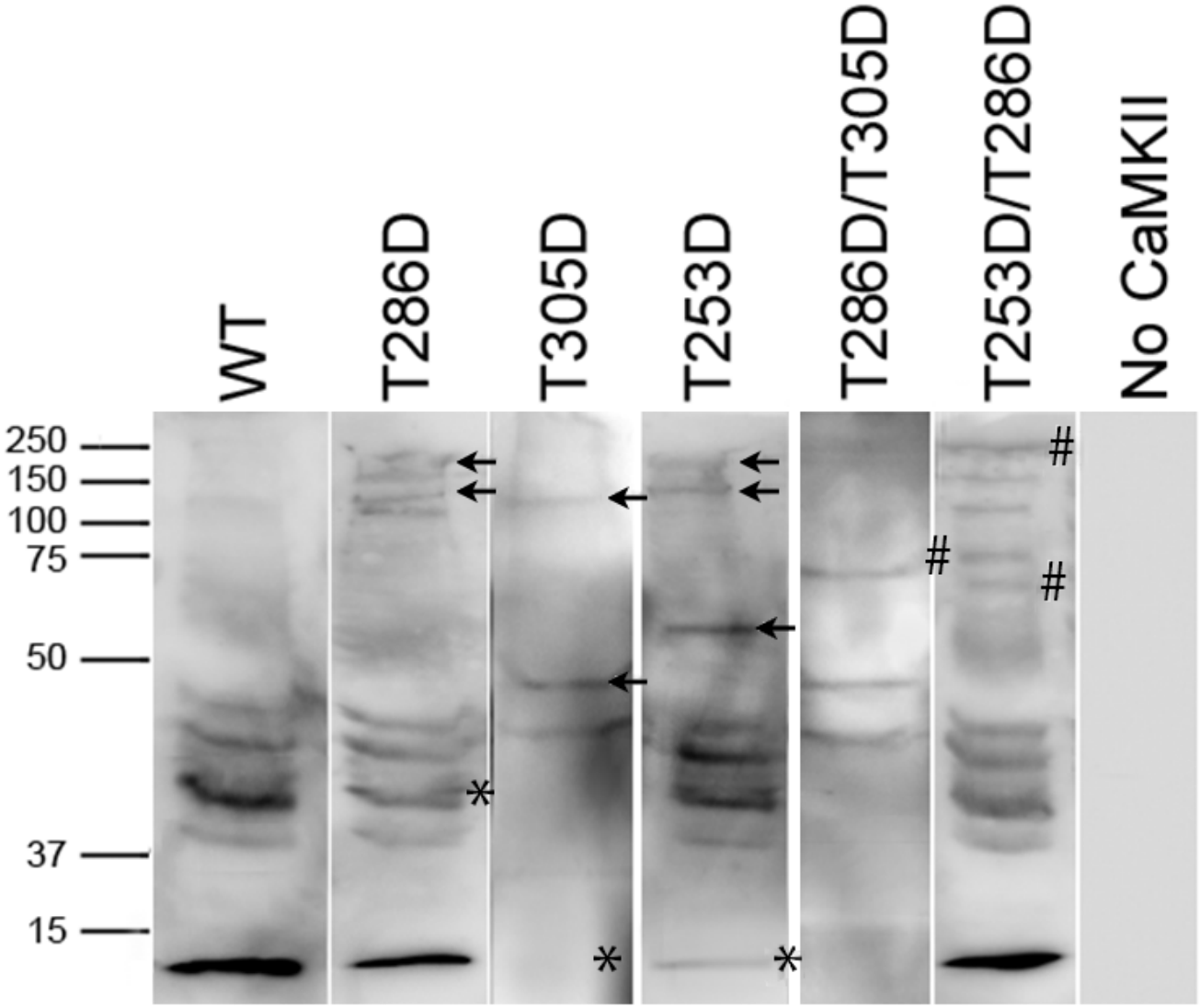

WT
T286D
T305D
T253D
T286D/T305D
T253D/T286D
No CaMKII
250
150
100
75
50
37
15
*
#

Figure 5.

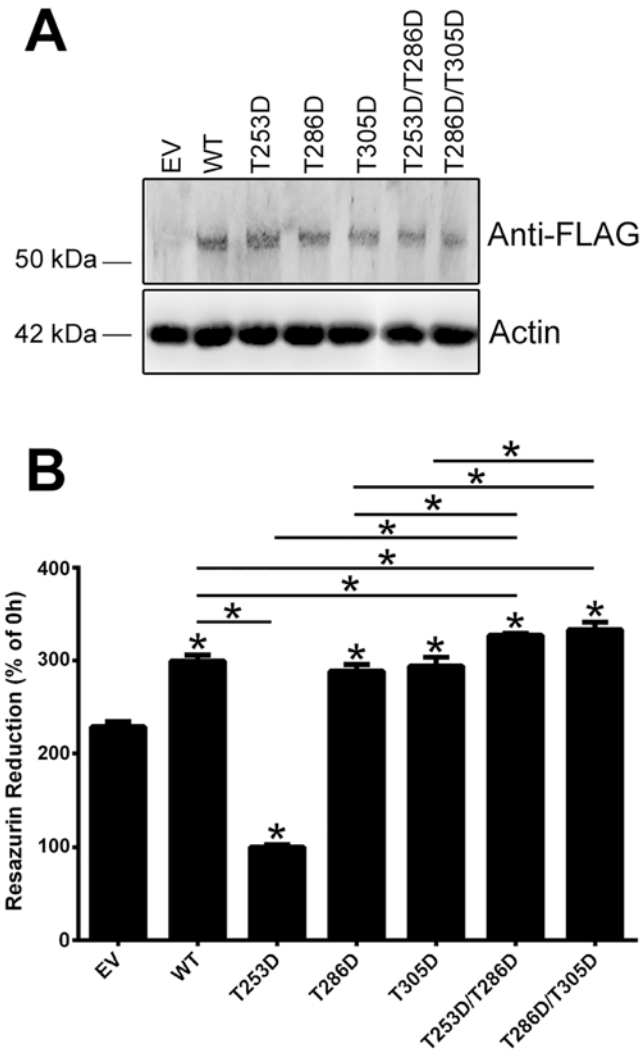
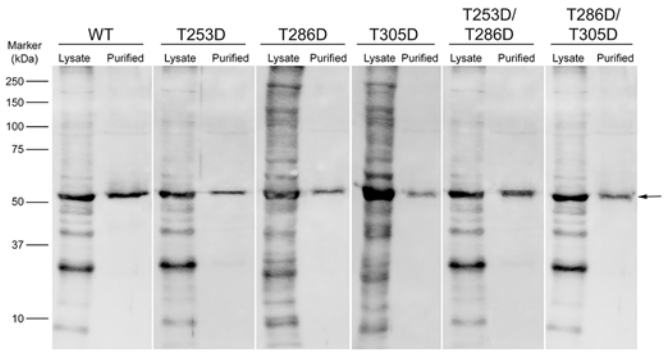

A
EV
WT
T253D
T286D
T305D
T253D/T286D
T286D/T305D
50 kDa
Anti-FLAG
42 kDa
Actin
B
Resazurin Reduction (% of 0h)
400
300
200
100
0
EV
WT
T253D
T286D
T305D
T253D/T286D
T286D/T305D

Figure 6.


Marker (kDa)
WT
T253D
T286D
T305D
T253D/ T286D
T286D/ T305D
Lysate
Purified
250
150
100
75
50
37
10

Supp Fig 1.