Regulation of CaMKII by Phospho-Thr253 or Phospho-Thr286 Sensitive Targeting Alters Cellular Function

Kathryn A. Skelding[1], Tatsuo Suzuki[2], Sarah Gordon[1], Jing Xue[3], Nicole M. Verrills[1], Phillip W. Dickson[1], John A.P. Rostas[1]*

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

[2]Department of Neuroplasticity, Shinshu University Graduate School of Medicine, 3-1-1 Asahi, Matsumoto, Japan

[3]Cell Signalling Unit, Children's Medical Research Institute, Wentworthville, New South Wales, Australia

* Address for Correspondence:

John A.P. Rostas

Faculty of Health, Bowman Building, The University of Newcastle, University Drive, Callaghan, New South Wales 2308, Australia

Email: John.Rostas@newcastle.edu.au

Telephone: +61 2 4921 5615

Fax: + 61 2 4921 5669

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Abbreviations: Brain and Acute Leukaemia, Cytoplasmic protein 1-6-8 (BAALC 1-6-8); Calcium/calmodulin-stimulated protein kinase II (CaMKII); fetal calf serum (FCS); microtubule associated protein fraction (MAPF); myelin basic protein (MBP); neurogranin (NRG); polyvinylpyrrolidone-40 (PVP-40); post-synaptic density (PSD); protein kinase C (PKC); protein phosphatase $2A_1$ ($PP2A_1$); tris-buffered saline (TBS); tyrosine hydroxylase isoform 2 (TH2); wild-type (WT)

ABSTRACT

Calcium/calmodulin-stimulated protein kinase II (CaMKII) is an important mediator of synaptic function that is regulated by multisite phosphorylation and targeting through interactions with proteins. A new phosphorylation site at Thr253 has been identified *in vivo*, that does not alter CaMKII activity, but does alter CaMKII function through interactions with binding proteins. To identify these proteins, as well as to examine the specific effects following Thr253 or Thr286 phosphorylation on these interactions, we developed an *in vitro* overlay binding assay. We demonstrated that the interaction between CaMKII and its binding proteins was altered by the phosphorylation state of both the CaMKII and the partner, and identified a CaMKII-specific sequence that was responsible for the interaction between CaMKII and two interacting proteins. By comparing CaMKII binding profiles in tissue and cell extracts, we demonstrated that the CaMKII binding profiles varied with cell type, and also showed that overexpression of a CaMKII Thr253 phospho-mimic mutant in human neuroblastoma and breast cancer cells dramatically altered the morphology and growth rates when compared to overexpression of non-phosphorylated CaMKII. This data highlights the importance of the microenvironment in regulating CaMKII function, and describes a potentially new mechanism by which the functions of CaMKII can be regulated.

1. INTRODUCTION

Calcium/calmodulin-stimulated protein kinase II (CaMKII) is a family of multifunctional serine (Ser)/threonine (Thr) protein kinases encoded by four genes (α, β, γ, *∂*) [1]. One or more members of this family are found in virtually every tissue, and mediate a diverse range of physiological responses. CaMKII is expressed most abundantly in neurons, and is involved in regulating many aspects of neuronal function, including neurotransmitter synthesis and release, cellular morphology and neurite extension, long-term plasticity, and learning [2-4]. Non-neuronal CaMKII has been implicated in the regulation of other biological processes, such as fertilisation [5], osteogenic differentiation [6], and the maintenance of vascular tone [7].

The biological properties of CaMKII are regulated by multi-site phosphorylation and targeting to specific subcellular locations through interactions with specific target proteins [8]. The roles of two phosphorylation sites, namely Thr286 and Thr305/6, have been well characterised. Phosphorylation at either of these sites has a profound impact on the activity of CaMKII, as well as targeting to various subcellular locations [9, 10]. Recently, the consequences of phosphorylation at Thr253 have begun to be characterised. This phosphorylation site, which is present in every isoform and conserved across species, has been shown to be phosphorylated *in vivo* [11, 12]. Unlike phosphorylation at Thr286 or Thr305/306, phosphorylation at Thr253 has no direct effect on the activity of CaMKII *in vitro*, however it has marked effects on CaMKII targeting [11]. In addition, phosphorylation of Thr253 occurs only in a small proportion of cellular CaMKII, much of which is associated with the post-synaptic density (PSD) [11,

12]. This suggests that the functional consequences of Thr253 phosphorylation may be concentrated at this and other specialised cellular locations.

It has been well established that the appropriate targeting of signalling molecules plays an important role in establishing the cellular responses to extracellular stimuli [13, 14]. Changes in the autophosphorylation of CaMKII can regulate translocation and CaMKII binding to specific subcellular locations, such as the PSD. Phosphorylation of Thr305/6 decreases the amount of CaMKII bound to the PSD, stimulating translocation from the PSD to the cytosol [15]. In contrast, phosphorylation of either Thr286 or Thr253 enhances binding to the PSD, with phosphorylation at both Thr286 and Thr253 exhibiting at least an additive increase in binding [11]. Once located in the PSD, CaMKII can phosphorylate many different substrates, including subunits of the NMDA receptor, the AMPA receptor, and ion channels [16-18].

There is evidence that suggests that the micro-environment in which CaMKII can be found plays a major role in regulating CaMKII function [8]. It is well established that CaMKII located in different subcellular locations behaves differently [19]. In addition, there is indirect evidence that suggests that allosteric changes caused by the microenvironment can bias CaMKII phosphorylation towards different sites. For example, *in vitro* rates of CaMKII phosphorylation at either Thr286 or Thr253 can be dramatically influenced by different concentrations of ATP, and the rate of Thr286 phosphorylation is more rapid than that observed for Thr253 phosphorylation [11, 20]. In contrast, Thr253 phosphorylation occurs at a relatively rapid rate on CaMKII that is

associated with the PSD, even in the absence of added calmodulin [21]. In addition, the reciprocal interaction between CaMKII phosphorylation and CaMKII targeting can be illustrated by studying the interaction of two binding proteins that are present at the PSD (NMDA receptor and CASK). CaMKII that is phosphorylated at Thr286 binds to the NR2B subunit of the NMDA receptor [22, 23]. This binding changes the conformation of CaMKII, keeping it autonomously active. Additionally, this interaction inhibits the phosphorylation of CaMKII at Thr305/306 [23]. In contrast, CaMKII binding to the scaffold protein CASK results in a very different outcome. Whilst CASK binding also changes the conformation of CaMKII, keeping it autonomously active, the conformation is changed in a way that favours autophosphorylation of Thr305/306 [24]. Once the CASK-bound CaMKII becomes phosphorylated on Thr305/306, it dissociates from CASK and releases a pool of CaMKII insensitive to changes in calcium levels. These scenarios highlight the complexity of CaMKII regulation, and demonstrate the importance of molecular environment and the interaction with binding partners in controlling CaMKII function.

Most of the binding sequences on CaMKII responsible for interacting with binding proteins are unknown. Except for the CaMKIIβm splice insert, which contains a putative SH3-domain binding region [25, 26], CaMKII does not contain the well known consensus-binding motifs. Multiple parts of CaMKII can be involved in the binding interactions, with most of the identified sites being located in the C-terminal half of the molecule [8]. For example, BAALC-1-6-8 (Brain and Acute Leukaemia, Cytoplasmic protein) interacts with the C-terminal

end of the regulatory domain of α-CaMKII (aa 310 – 320) [27], and F-actin binds to β-CaMKII in the V1 insert region of the association domain [28].

To identify the proteins responsible for CaMKII targeting, as well as to elucidate the roles of Thr253 and Thr286 phosphorylation in this targeting, we have developed and optimised an *in vitro* overlay binding assay, based on the method described by McNeill and Colbran [29]. We have examined the interactions between binding proteins and CaMKII, and have investigated the effects of phosphorylation of both the CaMKII and the binding partner on this interaction. In addition, we have identified a major functional consequence following the overexpression of a CaMKII Thr253 phospho-mimic mutant in human neuroblastoma and breast cancer cells, which is potentially mediated by the expression/absence of particular binding proteins.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Primary Antibodies: Rabbit antibodies to phospho-Thr253-α-CaMKII were made as described previously [11]. Mouse antibodies to phospho-Thr286-CaMKII, total α-CaMKII, and the FLAG epitope were purchased from Affinity BioReagents (Redfern, NSW, Australia), Chemicon (now Millipore, North Ryde, NSW, Australia), and Sigma (M2, Castle Hill, NSW, Australia), respectively.

2.1.2 Cell Lines: COS-7 (ATCC CRL-165; monkey kidney cells), SHSY5Y (ATCC CRL-2266; human neuroblastoma cells), 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 (Invitrogen, Mount Waverley, VIC, Australia), supplemented with 15 mM HEPES (Invitrogen), 2 mM glutamine (Invitrogen), and 10% heat-inactivated fetal calf serum (FCS; Invitrogen).

2.1.3 Proteins: Purified human tyrosine hydroxylase isoform 2 (TH2) was made and phosphorylated to high stoichiometry at Ser19 and Ser40 as described previously [30]. BAALC 1-6-8 was produced as previously described [27]. Recombinant human desmin and α-synuclein were obtained from RayBiotech (Norcross, GA, USA). Histone, myelin basic protein (MBP), microtubule associated protein fraction (MAPF), and protein phosphatase $2A_1$ ($PP2A_1$) were purchased from Worthington Biochemical Corporation (Lakewood, NJ, USA), Sigma, Cytoskeleton (Denver, CO, USA), and Calbiochem (now Merck, Melbourne, VIC, Australia), respectively. Neurogranin was obtained from Abnova (Taipei, Taiwan), and was phosphorylated to high stoichiometry as described previously [31].

2.1.4 Peptides: Synthetic peptides 310-320 (TRNFSGGKSGG; corresponding to the α-CaMKII specific sequence from aa 310 – 320), T253 (NKMLTINPSK; corresponding to the α-CaMKII sequence from aa 249 - 258) and 253D (NKMLDINPSK; corresponding to the Thr253 α-CaMKII phospho-mimic sequence from aa 249 - 258), were synthesised, purified to ≥ 94% purity by HPLC, and analysed by mass spectroscopy by Auspep (Parkville, VIC, Australia).

2.2 Generation of CaMKII Mutants and FLAG-CaMKII

FLAG-CaMKII mutants (T286D – α-CaMKII with a phospho-mimic mutation at Thr286; T253D – α-CaMKII with a phospho-mimic mutation at Thr253) and wild-type (WT) FLAG-α-CaMKII were generated [11] and purified [32] as previously described. The relative concentration was determined by quantification of Western blots using antibodies to α-CaMKII or FLAG.

2.3 Preparation of Brain Tissue

All procedures were performed in accordance with the guidelines of the University of Newcastle Animal Ethics Committee. Six to eight week old Sprague-Dawley rats (Animal Resource Centre, Perth, WA, Australia) were sacrificed by decapitation, the brain was removed rapidly and chilled in ice cold 0.32 M sucrose for 1 min. Subcellular fractions from whole forebrain and cerebellum were prepared as previously described [11].

2.4 Western Blotting

Subcellular fractions (30 µg), recombinant proteins (500 ng unless otherwise state), or cell lysates (5 – 30 µg) were separated using 10% SDS-PAGE, and then transferred to nitrocellulose membranes as described previously [33]. Cell lysates were made as follows: cells were pelleted and then resuspended in lysis buffer (1% NP-40/tris-buffered saline, 2 mM EDTA, 20 µM sodium orthovanadate, 50 mM sodium fluoride, complete protease inhibitor cocktail) for 20 min at 4°C. SDS sample buffer was added, and the lysate was boiled for 10 min. 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, Castle Hill, NSW, Australia). Blots were scanned with a Fujifilm LAS-3000 Imaging System (Brookvale, NSW, Australia) and analysed with Multi Gauge Software (Fujifilm).

2.5 Overlay Binding Assay

The overlay binding assay was based on the assay described in McNeil and Colbran [29], and was modified as follows. Proteins (30 µg for subcellular fractions and cell lysates; 500 ng for purified proteins unless otherwise stipulated) were separated using 10% SDS-PAGE, and were transferred to PVDF membranes. For all incubations with CaMKII or antibodies, membranes were sealed in plastic containers in the volumes specified; all other incubations and washes were in larger volumes (> 30 ml) in large containers. Membranes were blocked with tris-buffered saline/0.05% Tween-20 (TBS/T; 50 mM Tris, 150 mM sodium chloride, pH 7.4, with 0.05% Tween-20) containing 1% polyvinylpyrrolidone-40 (PVP-40; Sigma) for 3 h at room temperature, and then overlayed with 0.5 – 4 ml of 0.1 – 1 µg/ml (2 – 20 µM) of wild-type (WT)-α-CaMKII, T286D-α-CaMKII, or T253D-α-CaMKII for 18 h at 4°C, with gentle

agitation. Blots were washed three times (5 min each) with TBS/T to remove unbound CaMKII, and the remaining CaMKII was cross-linked to the PVDF by incubating the blots in 20 ml 5% glutaraldehyde (Sigma) for 40 min at room temperature. Blots were washed twice (5 min each) in TBS/T, and the glutaraldehyde was blocked with 20 ml 0.15M ethanolamine (Sigma) for 10 min at room temperature, with shaking. Blots were washed three times (5 min each) with TBS/T, and were then incubated in 5 ml anti-FLAG antibody (M2 clone, 1:1,000 dilution) for 1 h at room temperature. Blots were washed three times (5 min each) with TBS/T, and incubated in 20 ml sheep anti-mouse horseradish peroxidase-linked secondary antibody (1:5,000) for 1 h at room temperature. Blots were washed three times (5 min each) with TBS/T, and were then visualised using the ECL Plus Immunoblotting Detection System. Blots were scanned using a Fujifilm LAS-3000 Imaging System, and analysed with Multi Gauge Software.

2.6 Peptide Inhibition Assay

The peptide inhibition assay was performed in a similar fashion as the overlay binding assay, with the following modification. Peptide (either 310-320, T253, or 253D) was added to the overlay mixture (0.25 µg/ml of CaMKII in TBS/T) to a final concentration of 10 mg/ml (10 mM), and mixed for 30 min at 4°C. The CaMKII-peptide mixture was then added to the blots, and incubated for 18 h at 4°C.

2.7 Pull Down Assay

The interaction between native TH2 and CaMKII was examined in solution via a pull down assay. Affinity gel to which the anti-FLAG M2 antibody had been conjugated (anti-FLAG beads; Sigma) was washed twice in TBS, and once in TBS/1% Triton X-

100/1% ovalbumin. Either WT- or T253D-α-CaMKII (0.5 µg; 0.8 *p*mol) was captured by the anti-FLAG beads by incubation at 4°C for 2 h, with continuous mixing. Beads were washed three times (5 min each) with TBS/1% Triton X-100/1 % ovalbumin, to remove any unbound CaMKII. TH2 (1 µg; 20 *p*mol) was added to the CaMKII-FLAG beads and incubated for 20 min at 4°C, with mixing. Beads were washed three times (5 min each) with TBS/1% Triton X-100/1% ovalbumin, and then boiled for 5 min in SDS sample buffer, to remove any TH2/CaMKII that had been captured.

2.8 Creation of Stable CaMKII Expressing Cell Lines

Stable CaMKII expressing SHSY5Y and MDA-MB-231 cell lines were created by Lipofectamine 2000 transfection. Briefly, 5 x 10^5 cells were cultured in 10 cm dishes overnight at 37°C/5% CO_2. WT-, T286D-, or T253D-α-CaMKII DNA (1.6 µg) was mixed with Lipofectamine 2000 (Invitrogen) for 20 min at room temperature, and cells were incubated overnight at 37°C in the DNA/Lipofectamine mixture. The medium was removed, and fresh DMEM containing 10% FCS was added to the cells. Twenty-four hours later, the cells were placed under Geneticin (G418; Invitrogen) selection (500 µg/ml for the MDA-MB-231 cells; 600 µg/ml for the SHSY5Y cells). Cells were cultured for up to three months post-transfection.

2.9 Growth of Stable CaMKII Expressing Cell Lines

The growth rates of SHSY5Y and MDA-MB-231 cell lines stably overexpressing CaMKII (either WT, T286D, or T253D) were examined by the CellTitre-Blue™ Cell Viability Assay (Promega, Alexandria, NSW, Australia) according to the manufacturer's instructions. Briefly, cells were seeded at 1 x 10^4/well in a 96-well

plate in DMEM containing 10% FCS. At various time points (0, 6, 16, 24, 48, 72 h post-plating), 20 µl CellTitre-Blue™ reagent was added to each well, and the plates were incubated at 37°C for an additional 4 h. Fluorescence was measured at $560_{Ex}/590_{Em}$ on a FLUOstar OPTIMA plate reader (BMG Labtechnologies, Offenburg, Germany), and growth rates for each cell line were calculated. Assays were plated in triplicate, and repeated at least three times.

As there were inadequate SHSY5Y-T253D and MDA-MB-231-T253D cell numbers to perform the CellTitre-Blue™ Cell Viability Assay, cells were counted every second day over a three-month period, and the number of cell divisions over a 100 day period was calculated. After three-months, cells were stained with trypan blue and assessed for viability.

2.10 Data Analysis

All statistical analyses were conducted using one-way analysis of variance (ANOVA) with a Bonferroni post-test. All data is presented as the mean ± standard error of the mean (SEM) for the number of replicates (n).

3. RESULTS

3.1 Characterisation of an Overlay Binding Assay

We have developed an overlay binding assay in order to examine the interactions of both phosphorylated and non-phosphorylated CaMKII with various proteins. Previous studies that have attempted this have used radio-labelled CaMKII [29, 34, 35], autophosphorylated with ^{32}P-ATP under conditions that result in phosphorylation primarily at Thr286. As we wanted to examine the binding of both phosphorylated and non-phosphorylated CaMKII, we developed a new binding assay that did not involve radio-labelled CaMKII. Phospho-mimic mutants of CaMKII (T286D – CaMKII with a phospho-mimic mutation at Thr286; T253D – CaMKII with a phospho-mimic mutation at Thr253) were used in this study for their stability (phospho-mimic mutants are unaffected by phosphatase activity) and specificity (phospho-mimic mutants exhibit stoichiometric changes at individual sites without changes at other sites).

We examined the ability of non-phosphorylated α-CaMKII (wild-type; WT), and CaMKII with a phospho-mimic mutation at Thr286 (T286D) or Thr253 (T253D) to bind to a panel of proteins that are known to interact with CaMKII, either as substrates (MAP-2 [36], TH2 [30], desmin [37], MBP, histones [38] (Figure 1), α-synuclein [39]), or by direct interaction (BAALC 1-6-8 [27], $PP2A_1$ [40]). Using this panel of proteins, we demonstrated that phospho-mimic mutations at Thr253 or Thr286 had variable and independent effects on CaMKII binding. Different CaMKII binding patterns were observed for each protein. The binding for some proteins was significantly enhanced following Thr253 phospho-mimic mutation (Figure 1B and 1D) (BAALC 1-6-8, TH2; $p = 0.006$ and $p = 0.02$, respectively), whilst others were

significantly decreased (Figure 1E) (MBP; $p = 0.0004$), when compared to WT or Thr286 mutation. Other proteins exhibited significantly increased binding following Thr286 phospho-mimic mutation (Figure 1A) (MAP-2; $p = 0.0004$). In addition, some proteins that bound WT-α-CaMKII exhibited no significant change in binding following either Thr253 or Thr286 phospho-mimic mutation (Figure 1C) (desmin). These findings imply that phosphorylation of CaMKII at Thr253 or Thr286 independently alters binding to specific proteins. However, for all of the proteins examined, phospho-mimic mutation of Thr286 or Thr253 always altered CaMKII binding (i.e. increased or decreased) in the same direction, or did not change binding relative to WT-α-CaMKII.

INSERT FIGURE 1

Not all proteins were found to bind using this overlay binding assay. Histones (Figure 1F), α-synuclein, and $PP2A_1$ (data not shown) did not bind at any concentration tested (500 ng – 2 µg). This demonstrates that this overlay binding assay does not result in a blanket binding to all immobilised proteins, but that specific binding interactions can be detected.

Following optimisation, this assay could detect small differences in binding, and was shown to be quantitative under appropriate conditions (Figure 2A and 2B). The amount of CaMKII bound was found to be proportional to the amount of binding protein on the membrane, as well as the concentration of CaMKII in the overlay solution (Figure 2A and 2B). In addition, the ratio of WT to phospho-mimic mutant CaMKII binding remained the same for a constant concentration of CaMKII (1 µg/ml;

20 μM), across a wide range of concentrations of the target protein immobilised on the blot (0.13 – 1 μg MBP [Figure 2A], and 500 ng and 62ng TH2 [Figure 1B and Figure 3A, respectively]).

INSERT FIGURE 2

Furthermore, the effects of phospho-mimic mutation on binding to proteins in the overlay binding assay correlated with the effects seen on the interactions between native proteins in solution (Figure 2C). The enhancement of T253D-α-CaMKII binding to TH2 relative to WT-α-CaMKII binding was similar whether the binding occurred to native soluble TH2 (as determined by pull down assay; black bars of Figure 2C) or to immobilised TH2 on PVDF membranes (as determined by overlay assay; white bars of Figure 2C). This suggests that this overlay assay may be useful for predicting CaMKII binding interactions *in vivo*.

3.2 CaMKII Binding is Sensitive to the Phosphorylation State of the Target Protein

In Figure 1, we demonstrated that phospho-mimic mutation of CaMKII could alter the binding of CaMKII. To determine whether the phosphorylation state of the target protein can also have an effect on binding, we phosphorylated TH2 and neurogranin, two proteins that exhibited varied CaMKII binding effects following phospho-mimic mutation of CaMKII. The binding of CaMKII to TH2 was significantly enhanced (2-fold increase; $p = 0.02$) by Thr253 phospho-mimic mutation, and this binding was further enhanced by the phosphorylation of TH2 at Ser19 and Ser40 by CaMKII (8-fold increase; T253D binding for TH2 vs. pTH2 $p = 1 \times 10^{-10}$) (Figure 3A). Despite

this enhanced overall CaMKII binding following phosphorylation of TH2, the same relative WT and phospho-mimic mutant binding pattern was observed.

INSERT FIGURE 3

In contrast, the binding of CaMKII to neurogranin (NRG) was completely abrogated by phospho-mimic mutation at Thr286, and unaffected by Thr253 mutation (Figure 3B; WT/T253D vs. T286D $p = 0.0001$). In addition, phosphorylation of neurogranin by protein kinase C (PKC) prevented the binding of neurogranin to all three forms of α-CaMKII. These results demonstrate the CaMKII binding is sensitive to the phosphorylation state of the target protein, and that the effects of this phosphorylation are varied between different target proteins and different CaMKII phospho-mimic mutants.

3.3 Identification of Regions of CaMKII Responsible for Binding to Specific Proteins

We next investigated the regions of CaMKII that are responsible for binding by testing whether short peptides corresponding to specific sequences in α-CaMKII could inhibit the binding between α-CaMKII and binding proteins. Previously, aa 310 – 320 (an α-CaMKII specific sequence) was shown to be able to block the interaction between WT-α-CaMKII and BAALC 1-6-8 [27] using a pull-down assay. Thus, we examined the ability of this 310-320 peptide to inhibit both WT- and T253D-α-CaMKII via the overlay binding assay. As a control, we also examined a peptide corresponding to aa 249 – 258 (T253; which includes the Thr253 site), as this peptide has physical properties similar to the 310-320 peptide (pI 11.5 for 310-320

peptide, 10.6 for T253 peptide; net charge 2 for both peptides; and average hydrophilicty 0.4 for 310-320 peptide, 0.1 for T253 peptide). This peptide also serves to investigate whether the region containing Thr253 is involved in direct binding to target proteins. To determine whether the Thr253 phospho-mimic site was involved in these interactions, a peptide corresponding to aa 249 – 258, with the Thr at 253 being substituted for an Asp (253D) was also examined for its capacity to interfere with WT- and T253D-α-CaMKII binding via the overlay binding assay.

We found that the 310-320 peptide inhibited WT-α-CaMKII binding to BAALC -1-6-8 (Figure 4A). This demonstrates that WT-α-CaMKII binding to BAALC 1-6-8 in the overlay assay is via the same site as in the native protein. Interestingly, 310-320 peptide did not inhibit the binding of T253D-α-CaMKII to BAALC -1-6-8. The 310-320 peptide did not interfere with CaMKII binding to TH2 (Figure 4B) or neurogranin (Figure 4C) but, unexpectedly, the peptide inhibited WT-α-CaMKII binding to MBP (Figure 4D), a protein with which BAALC 1-6-8 shares no sequence homology. This demonstrates that a variety of proteins can interact through a single site on CaMKII, and that there are multiple sites on CaMKII that are involved in protein binding. Furthermore, the control T253 peptide with similar physicochemical properties but different sequence did not affect WT-α-CaMKII binding to BAALC 1-6-8 (Figure 4A) or MBP (Figure 4D). These results demonstrate the specificity of inhibition by the 310-320 peptide.

INSERT FIGURE 4

As the interaction between T253D-α-CaMKII and BAALC 1-6-8 is much stronger than the binding of WT-α-CaMKII to BAALC 1-6-8, it was not clear whether the inability of the 310-320 peptide to inhibit the binding of T253D-α-CaMKII to BAALC 1-6-8 was due to the fact that the strength of binding relative to WT was too high, or whether a new site was involved. As such, we performed the same peptide inhibition assay where the peptide concentration was kept constant (10 mM), but the CaMKII concentration was sequentially reduced so that the peptide:CaMKII ratio varied from 1:1 to 32:1 (data not shown). Even at these reduced concentrations of CaMKII, no effect on binding was observed in the presence of the 310-320 peptide (data not shown). Despite the fact that the WT-α-CaMKII binding to MBP is approximately three-fold stronger than the T253D-α-CaMKII binding to BAALC 1-6-8 (Figure 4E), the 310-320 peptide still inhibits WT-α-CaMKII binding to MBP. Taken together, these results suggest that the inability of the 310-320 peptide to inhibit the binding of T253D-α-CaMKII to BAALC 1-6-8 is due to the interaction taking place via a different binding site.

The 253D peptide did not inhibit WT- or T253D-α-CaMKII binding to any of the proteins (Figure 4), which suggests that the Thr253 phosphorylation site is not involved in the direct binding of CaMKII to these proteins, but rather phosphorylation of Thr253 alters binding via allosteric modulation of a distant site.

3.4 CaMKII Binding Varies with Subcellular Location and Cell Type

As we have previously shown that CaMKII phosphorylated at Thr253 is enriched in specific subcellular fractions [11], we examined the CaMKII binding profiles in subcellular fractions from different brain regions (Figure 5). The CaMKII binding

profiles differ between forebrain and cerebellum, and also between detergent soluble (non-junctional membrane-enriched; S3x) and detergent insoluble (PSD-enriched; P3x) fractions of a crude membrane preparation. Not all protein bands, as observed by Coomassie staining, contained proteins capable of binding in this overlay binding assay (Figure 5). We have noted over twenty proteins/bands whose binding to CaMKII is phospho-mimic mutation sensitive. In addition, we have identified several of these proteins, including MBP (in the cerebellum P3x sample) by mass spectrometry (MALDI-TOF), and confirmed these identifications by comparing the binding profile of the recombinant proteins to that observed for the band that was examined. It is interesting to note that MBP is also present in the forebrain P3x fraction (indicated by the bottom arrow on the forebrain P3x diagram in Figure 5), but that the binding profile of the band that contains it is different from that observed in the cerebellum P3x fraction, suggesting the presence of more than one CaMKII binding protein in the band in the forebrain fraction. Indeed, MALDI-TOF analysis of the ~10 kDa band isolated from the forebrain P3x was found to contain a mixture of proteins (of which MBP was only a minor protein), whereas the ~10 kDa band isolated from the cerebellum P3x consisted predominantly of MBP (data not shown). In addition, a T253D-α-CaMKII enhanced binding protein was identified in the S3x fraction from forebrain that was of the same molecular weight as BAALC 1-6-8, and exhibited the same binding pattern as that observed for purified BAALC 1-6-8 (Figure 1D). Western blotting with an antibody to BAALC 1-6-8 [27] demonstrated that BAALC 1-6-8 was primarily expressed in this S3x fraction, and co-migrated with the CaMKII binding protein indicated in Figure 5 (data not shown).

INSERT FIGURE 5

These results indicate that the expression of CaMKII binding proteins varies with both subcellular location, and with cell type, as forebrain and cerebellum contain different populations of cells. Therefore, variations in the expression and intracellular location of binding proteins could give rise to cell-specific functional responses to CaMKII activation.

3.5 Functional Consequences of Thr253 Phosphorylation

We also examined the binding profiles in two established cultured cell lines. SHSY5Y is a very slow growing, non-invasive, human neuroblastoma cell line. MDA-MB-231 is a very rapidly dividing, highly invasive human breast cancer cell line. CaMKII binding profiles were found to be different for these two cell lines (Figure 6A), suggesting that CaMKII expression may have some different functional outcomes in these cells, due to the presence/absence of various binding partners.

INSERT FIGURE 6

To investigate this, WT-, T286D-, or T253D-α-CaMKII DNA were transfected into these cell lines, and overexpression of α-CaMKII was confirmed by western blotting (Figure 6B). WT- and T286D-α-CaMKII overexpression did not affect morphology of either the SHSY5Y (Figure 6B) or MDA-MB-231 (Figure 6C) cells. Remarkably, both SHSY5Y and MDA-MB-231 cells overexpressing T253D-α-CaMKII exhibited dramatic alterations in morphology when compared to parental cells, with cells rounding and losing their processes, and a dramatic reduction in cell division was

observed. However, after a three month period, these cells still excluded trypan blue, and were therefore still viable (data not shown).

WT- and T286D-α-CaMKII overexpression in SHSY5Y cells, whilst not affecting morphology, significantly increased (2-fold; $p = 0.001$) the growth rate of these cells. In contrast, WT- and T286D-α-CaMKII overexpression in MDA-MB-231 cells did not alter the growth rate.

4. DISCUSSION

CaMKII is regulated by multi-site phosphorylation, which can alter enzyme activity, and targeting to cellular microdomains through interactions with binding proteins [8]. These proteins integrate CaMKII into multiple signalling pathways, which may lead to varied functional outcomes following CaMKII phosphorylation, depending on the identity and location of the binding partner. A new phosphorylation site on CaMKII (Thr253) has been identified *in vivo* [11]. Thr253 phosphorylation does not affect enzyme activity, and appears to control CaMKII purely by targeting. However, how phosphorylation of CaMKII affects interactions with binding partners is largely unknown. In this study we describe a new assay that enables the identification of binding partners that interact with both phosphorylated and non-phosphorylated forms of CaMKII, and demonstrate that these interactions are dependent on the phosphorylation state of CaMKII as well as the relevant binding protein. Furthermore, we identify the CaMKII region responsible for binding to two proteins (aa 310 – 320). In addition, we have identified a major functional consequence following the overexpression of a CaMKII Thr253 phospho-mimic mutant in human neuroblastoma and breast cancer cells, which is potentially mediated by the expression/absence of a particular binding protein.

We have described a non-radioactive overlay binding assay that allows the investigation and identification of interactions between CaMKII and its binding partners. Following optimisation, this assay was found to be quantitative under appropriate conditions, and highly sensitive, as small differences in binding could be determined (Figure 2). In addition, the amount of CaMKII bound was proportional to

the concentration of target protein immobilised on the membrane, as well as the concentration of CaMKII in the overlay solution (Figure 2), and the ratio of WT to phospho-mimic mutant CaMKII binding remained constant, regardless of the concentration of the target protein (MBP Figure 2A; TH2 Figures 1B and 3A). These findings indicate that this assay may be useful for detecting interactions *in vivo*, where interactions between CaMKII and binding partners can be detected irrespective of the abundance of the target protein, and that we can adjust the sensitivity of the assay so that a comparison can be made between high and low affinity interactions.

Using phospho-mimic mutants to simulate phosphorylation and binding studies with CaMKII, purified proteins and tissue extracts, we have shown that site-specific phosphorylation at Thr253 or Thr286 can enhance, inhibit, or have no effect on the binding of CaMKII to particular proteins (Figure 1). The effects of phospho-mimic mutation at these sites on binding to proteins are independent of each other but, interestingly, where effects are observed with both sites, the effects were never in the opposite direction for any of the proteins examined (e.g. if the binding is increased following Thr253 phospho-mimic mutation when compared to WT, it is also increased, or remains unchanged following Thr286 phospho-mimic mutation). Even in tissue extracts, we have not identified any individual protein bands for which Thr253 and Thr286 phosphorylation have opposite effects on binding (Figure 5 and data not shown).

We have identified an α-CaMKII sequence (aa 310 – 320) that is responsible for the binding to MBP, and confirmed previous findings that this sequence was responsible for the interaction between CaMKII and BAALC 1-6-8 [27] (Figure 4). Interestingly,

peptides corresponding to the non-phosphorylated and phospho-mimic aa 249 – 258 sequence (which incorporates Thr253) did not inhibit binding to any protein examined (Figure 4). This suggests that most interactions between CaMKII and its specific binding partners are via a site distant to Thr253, whose conformation is altered by allosteric interactions caused by phosphorylation at Thr253.

The interaction between WT-α-CaMKII and MBP that was identified using the overlay binding assay (Figure 1E), and the subsequent inhibition of this interaction with the 310-320 peptide (Figure 4D) is interesting. Since oligodendrocytes are not known to express α-CaMKII, this interaction either does not occur *in vivo*, or occurs via a region of CaMKII that is conserved among other CaMKII isoforms as MBP also binds γ-CaMKII [38]. However, the 310-320 peptide corresponds to a CaMKII region that is α-specific. This suggests that there may be multiple sites on MBP that can interact with the various isoforms of CaMKII. In addition, even though MBP and α-CaMKII do not appear to be co-expressed in the same cell type, Golli proteins (which contain MBP sequences in addition to unique peptide sequences), are expressed in axonal and dendritic processes throughout various brain regions [41]. Whether these proteins bind to α-CaMKII through sequences similar to MBP remains to be established.

Both TH2 and MAP-2 are known substrates of CaMKII [30, 42]. Our findings in Figures 1A and 1B confirm this interaction, and also further demonstrate that this binding is increased following Thr286 or Thr253 phospho-mimic mutation. MAP-2 is known to play a role in microtubule assembly and disassembly, as well as in the interaction between microtubules and actin filaments [43], and tyrosine

hydroxylase is the rate-limiting enzyme in catecholamine synthesis [44]. Therefore, autophosphorylation-modulated interactions between CaMKII and MAP-2 and TH2 may play a role in regulating cell division and catecholamine synthesis, respectively.

BAALC 1-6-8 is widely expressed in the frontal part of the brain, and is localised to synaptic sites [27]. Myristoylation and palmitoylation of BAALC 1-6-8 results in its targeting to lipid rafts [27]. Previously, α-CaMKII has been shown to associate with lipid rafts [45], but whether this association was a direct interaction or occurred via binding to another protein was not known. Previously, non-phosphorylated α-CaMKII was shown to bind directly to BAALC 1-6-8 [27]. Our findings (Figure 1D) confirm this interaction using the overlay binding assay, and also demonstrate that this interaction is significantly increased following Thr253 phospho-mimic mutation. These results provide a potential mechanism by which BAALC 1-6-8 may target CaMKII to lipid rafts, and perhaps accounts for the association of CaMKII with lipid rafts.

Previously, neurogranin and CaMKII have been shown to regulate the ability of calmodulin to bind calcium. However, when CaMKII was autophosphorylated under conditions that favoured Thr286 phosphorylation, neurogranin no longer had any effect on calmodulin [46]. Our results (Figure 3B) provide an explanation for this phenomenon by showing that neurogranin can interact directly with CaMKII and that the Thr286 phospho-mimic mutation of CaMKII abrogates this interaction. This direct interaction may possess physiological relevance in two models of neuropathology. In animal models of

hypothyroidism, a decreased expression of neurogranin is observed [47], and CaMKII translocation from the cytosol to the PSD is inhibited [48]. In light of our findings, the decreased expression of neurogranin in hypothyroidism may account for the lack of CaMKII translocation that is observed. In addition, in rodent models of hypoxia, there is an increase in the level of phosphorylated neurogranin [49], and CaMKII is depleted from the cytosol and translocated to a Triton-insoluble fraction in neurons [50, 51]. Our findings that phosphorylation of neurogranin completely prevents the interaction with CaMKII (Figure 3B) may explain this translocation of CaMKII.

The phosphorylation of target protein can also modify binding of CaMKII, and the effects of phosphorylation are varied. When TH2 is phosphorylated at Ser19 and Ser40 by CaMKII (Figure 3A), the interaction between CaMKII and phospho-TH2 is enhanced 8-fold ($p = 1 \times 10^{-10}$). In contrast, when neurogranin is phosphorylated by PKC (Figure 3B), the interaction between CaMKII and phospho-neurogranin is completely abolished. When subcellular fractions isolated from cerebellum and forebrain were treated with λ phosphatase, altered binding to specific protein bands was observed (data not shown). This suggests that the interaction between CaMKII and particular target proteins can involve phosphorylation-effects induced by other kinases (or, as in the case of TH2, by CaMKII itself). Hence, this provides a mechanism whereby the functional effects of different signalling pathways can interact (as in the case of PKC phosphorylated neurogranin), and the interaction of CaMKII with a substrate can be modified in itself by the activity of CaMKII (e.g. WT- and T253D-α-CaMKII both interact with neurogranin, whereas T286D-α-CaMKII does not). This may provide a molecular mechanism for cross-talk between

CaMKII-dependent and other pathways, which can result in different functional outcomes depending on the signalling pathway in which CaMKII has interacted. In the case of the interaction between CaMKII and TH2, a 'feed-forward loop' may be created – CaMKII phosphorylates TH2, which subsequently increases the affinity for CaMKII, leading to CaMKII phosphorylating TH2, and so forth. However, the physiological function of such a feed-forward mechanism remains to be determined.

It has been well established that CaMKII located in different subcellular compartments behaves differently [19], and hence the association of CaMKII with various binding partners can alter the phosphorylation and function of CaMKII. We confirmed that the expression of CaMKII binding proteins varies with subcellular location and cell type (Figures 5 and 6A). Variations in expression and intracellular location of binding proteins can give rise to cell specific functional responses to CaMKII activation. We demonstrated that overexpression of T253D-α-CaMKII in human neuroblastoma (SHSY5Y) and breast cancer (MDA-MB-231) cell lines dramatically altered cell morphology and growth rates (Figure 6B and 6C), whereas WT and T286D overexpression had no effect on morphology. However, WT- and T286D-α-CaMKII overexpression in SHSY5Y cells significantly increased (2-fold; $p = 0.001$) the growth rate of these cells (Figure 6B). In contrast, WT- and T286D-α-CaMKII overexpression in MDA-MB-231 cells did not alter the growth rate (Figure 6C). This suggests that there are two separate effects occurring in these cell lines: a T253D-dependent effect that is common to both cell lines, and a second WT-dependent effect that increases the growth rate of the SHSY5Y cells but has no effect on the MDA-MB-231 cells. These

effects may be due to the presence/absence of one or more binding proteins that are differentially expressed in the two cell lines.

Current evidence suggests that the micro-environment in which CaMKII can be found plays a dramatic role in regulating CaMKII function [8]. There is indirect evidence suggesting that allosteric changes caused by the microenvironment can bias CaMKII phosphorylation either towards Thr286 or Thr253 [11, 20, 21]. We hypothesise, that while the bulk of cellular CaMKII may undergo autophosphorylation at both Thr253 and Thr286, there are specific pools of CaMKII that can selectively autophosphorylate at only Thr253 or Thr286 due to the influence of local binding proteins. As a consequence of the altered binding interactions caused by these autophosphorylation events demonstrated by our results, these differentially phosphorylated pools of CaMKII may then exchange and/or recruit new binding protein partners, which results in differences in the targeting of CaMKII between these subsets, and hence, different functional outcomes. Thus, the complement of CaMKII interacting proteins expressed by a cell, as well as their subcellular location, may determine the functional consequences of CaMKII activation in that cell. As CaMKII is a ubiquitously expressed multifunctional kinase, a mechanism must be available to selectively regulate its wide and varied cellular functions. The interaction between CaMKII and its binding partners provides such a cell and stimulus-specific functional selectivity, and is an additional way in which the function of CaMKII is regulated.

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Figure 1. CaMKII binding to recombinant and purified proteins. (A) Microtubule associated protein-2 (MAP-2), (B) human tyrosine hydroxylase isoform 2 (TH2), (C) desmin, (D) brain and acute leukaemia cytoplasmic protein 1-6-8 (BAALC 1-6-8), (E) myelin basic protein (MBP), and (F) histones, were examined for their ability to bind CaMKII using the overlay binding assay. Except for MAP-2 (which was 200 ng), 500 ng of protein was examined for their ability to bind CaMKII in the overlay binding assay. Representative blots for each interaction are shown, and the exposure times for each blot were as follows: 5 min (MAP-2), 2 min (TH2), 5 min (desmin), 2 min (BAALC 1-6-8), 30 sec (MBP), 5 min (histones). Three independent binding experiments were performed for each protein, and the average binding relative to WT-α-CaMKII binding was calculated, and is represented as the mean ± SEM. * denotes statistical significance ($p < 0.05$; see text for actual values) relative to WT binding.

Figure 2. Characterisation of the overlay binding assay. (A) Various amounts of MBP (0.13 µg – 1 µg) were separated using 10% SDS-PAGE, transferred to PVDF, and then probed with a constant concentration of wild-type (WT-), T286D-, or T253D-α-CaMKII. Representative blots for each interaction are shown. Data is presented as mean arbitrary units ± SEM (n = 3). (B) MBP (500 ng) was transferred to PVDF, and then probed with varying amounts of WT-α-CaMKII (0.002 ng/ml – 250 ng/ml). A representative blot is shown. Two independent experiments were performed, and the average binding relative to the 250 ng/ml binding was calculated, and is represented as the mean ± SEM. (C) The amount of binding detected by the overlay binding assay is of a similar level as that observed following a pull down assay. Four independent experiments were performed, and the average binding

relative to WT-α-CaMKII binding was calculated, and is represented as the mean ± SEM.

Figure 3. CaMKII binding is sensitive to the phosphorylation state of the target protein. (A) Non-phosphorylated (500 ng and 62 ng) and phosphorylated TH2 (62 ng) and (B) non-phosphorylated and phosphorylated neurogranin (NRG; 500 ng) were examined for their ability to bind CaMKII in the overlay binding assay. Representative blots for each interaction are shown, and the exposure times for each blot were 2 min for TH2 and phospho-TH2, and 5 min for neurogranin and phospho-neurogranin. Data is presented as the average binding relative to WT-α-CaMKII binding was calculated, and is represented as the mean ± SEM (n = 6 for non-phosphorylated TH2, n = 3 for phosphorylated TH2, and phosphorylated and non-phosphorylated neurogranin). * denotes statistical significance ($p < 0.05$; see text for actual values) relative to WT binding.

Figure 4. Determination of regions of CaMKII responsible for binding to specific proteins. The ability of peptides corresponding to various regions of CaMKII (310-320, 249-258 wild-type [T253], 249-258 phosphomimic T253D [253D]) to inhibit binding to a panel of proteins was examined in the overlay binding assay. (A) BAALC 1-6-8 (500 ng), (B) TH2 (500 ng), (C) neurogranin (500 ng), and (D) MBP (500 ng) were separated using 10% SDS-PAGE, transferred to PVDF, and then probed with wild-type (WT)-α-CaMKII (0.25 µg/ml CaMKII), WT + 310-320 peptide (10 mg/ml peptide), WT + T253 peptide (10 mg/ml peptide), WT + 253D peptide (10 mg/ml peptide), T253D-α-CaMKII 0.25 µg/ml CaMKII), T253D-α-CaMKII + 310-320 peptide (10 mg/ml peptide), T253D-α-CaMKII + T253 peptide

(10 mg/ml peptide), or T253D-α-CaMKII + 253D peptide (10 mg/ml peptide) (as described in *Materials and Methods*). Representative blots for each interaction are shown, and the exposure time for each blot were as follows: 60 sec (BAALC 1-6-8 WT), 30 sec (BAALC 1-6-8 T253D), 3 min (TH2 WT), 2 min (TH2 WT), 5 min (neurogranin WT and T253D), 30 s (MBP WT and T253D). Three independent binding experiments were performed for each protein, and the average binding relative to the no peptide control, is represented as the mean ± SEM. (E) CaMKII binding to BAALC 1-6-8 and MBP expressed as arbitrary units, normalised for exposure times.

Figure 5. CaMKII binding patterns vary with cell type and subcellular fraction. Detergent soluble (S3x) and insoluble (P3X) membrane proteins (30 µg) from the cerebellum and forebrain were examined for their ability to bind CaMKII in the overlay binding assay; Coomassie blue stained lanes are also included. Representative blots for each subcellular fraction are shown. Arrows indicate bands that are consistently altered with phospho-mimic mutation at either Thr253 or Thr286.

Figure 6. Functional consequences of Thr253 phosphorylation. (A) Cell lysates (30 µg) from two different cell types (SHSY5Y human neuroblastoma; MDA-MB-231 breast cancer) were examined for their ability to bind CaMKII in the overlay binding assay. Representative blots for each cell line are shown. Arrows indicate bands that are consistently altered with phospho-mimic mutation at either Thr253 or Thr286. The functional outcomes following transfection of WT, T286D, or T253D in (B) SHSY5Y and (C) MDA-MB-231 were also examined. CaMKII DNA was transfected into cell lines using Lipofectamine 2000, and cells stably expressing

CaMKII were obtained by Geneticin selection. Morphology was examined, and photomicrographs were obtained approximately two months post-transfection. Growth rates were measured by CellTitre-Blue™ assay or cell count, and are expressed as cell divisions per 100 days (mean ± SEM; n = 3). CaMKII expression was also examined in the SHSY5Y cell line by western blotting (30 µg for WT- and T286D-α-CaMKII expressing cells; 5 µg for T253D-α-CaMKII expressing cells). * denotes statistical significance ($p < 0.05$; see text for actual values) relative to WT.

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