Published in final edited form as:

Biochimie. 2017 November ; 142: 158–167. doi:10.1016/j.biochi.2017.09.002.

Peptides derived from transcription factor EB bind to calcineurin at a similar region as the NFAT-type motif

Ruiwen Song^{a,1}, Jing Li^{a,2,1}, Jin Zhang^{a,1}, Lu Wang^a, Li Tong^a, Ping Wang^a, Huan Yang^a, Qun Wei^a, Huaibin Cai^b, and Jing Luo^{a,*}

^aDepartment of Biochemistry and Molecular Biology, College of Life Sciences, Beijing Normal University, Beijing, 100875, China

^bTransgenics Section, Laboratory of Neurogenetics, National Institute on Aging, National Institutes of Health, Bethesda, MD, 20892, USA

Abstract

Calcineurin (CN) is involved in many physiological processes and interacts with multiple substrates. Most of the substrates contain similar motifs recognized by CN. Recent studies revealed a new CN substrate, transcription factor EB (TFEB), which is involved in autophagy. We showed that a 15-mer QSYLENPTS YHLQQS peptide from TFEB (TFEB-YLENP) bound to CN. When the TFEB-YLENP peptide was changed to YLAVP, its affinity for CN increased and it had stronger CN inhibitory activity. Molecular dynamics simulations revealed that the TFEB-YLENP peptide has the same docking sites in CN as the 15-mer DQYLAVPQHPYQWAK motif of the nuclear factor of activated T cells, cytoplasmic 1 (NFATc1-YLAVP). Moreover expression of the NFATc1-YLAVP peptide suppressed the TFEB activation in starved Hela cells. Our studies first identified a CN binding site in TFEB and compared the inhibitory capability of various peptides derived from CN substrates. The data uncovered a diversity in recognition sequences that underlies the CN signaling within the cell. Studies of CN-substrate interactions should lay the groundwork for developing selective CN peptide inhibitors that target CN-substrate interaction *in vitro* experiments.

Keywords

Calcineurin; Transcription factor EB; Nuclear factor of activated T cells; LxVP-type motif; TFEB-YLENP peptide

*Corresponding author. Department of Biochemistry and Molecular Biology, Gene Engineering and Biotechnology, Beijing Key Laboratory, No. 19, Xijiekouwaidajie, Beijing Normal University, Beijing, 100875, China. luojing@bnu.edu.cn (J. Luo).

¹The authors should be regarded as joint first authors.

Present address: Department of Biochemistry and Molecular Biology, University of Chicago, Chicago, IL 60635, USA.

Declaration of conflict of interest

We declare that we have no conflict of interest. The paper is original paper. Neither the entire paper nor any part of its content has been accepted elsewhere.

1. Introduction

Calcineurin (CN) is a serine/threonine phosphatase that is activated by calcium/calmodulin ($\text{Ca}^{2+}/\text{CaM}$). Its structure is conserved from yeast to humans, and consists of a regulatory subunit (CNB; 19 kDa) and a catalytic subunit (CNA; 61 kDa). CNA has four regions: a catalytic domain, a CNB-binding helix (BBH), a CaM-binding domain (CBD), and a C-terminal autoinhibitory domain (AI) [1].

CN plays critical roles in many biological processes, including the immune response, cardiac hypertrophy, and neurodegenerative diseases. To carry out its diverse functions, it interacts with multiple substrates and targeting proteins such as the nuclear factor of activated T cells (NFAT), A-kinase anchoring protein 79 (AKAP79), and endogenous regulator of calcineurin 1 (RCAN1) [2–5]. After decades of investigation, we have some understanding of how CN interacts with its substrates and dephosphorylates specific phosphorylated residues [6]. For example, CN dephosphorylates four members of the NFAT family, including NFATc1, NFATc2, NFATc3, and NFATc4. The NFAT protein has two binding sites for CN: the PxIxIT motif that is located near the N-terminus of the regulatory region, and the LxVP motif that is located near the C-terminus of the regulatory domain and that we named as the NFAT-YLAVP motif [7–10]. It is reported that the 15-mer DQYLAVPQHPYQWAK motif in NFATc1 (NFATc1-YLAVP) binds to a hydrophobic pocket at the interface of the CNA and CNB subunits. It has been shown that three residues, namely Y, L, and V, in the sequence YLAVP form the NFAT motif that interacts with CN. The Leu and Val residues are embedded into a hydrophobic groove on the surface joining CNA and CNB, and both side chains fit in the available space. In addition, the Tyr occupies the gap between the two EF-hand domains of CNB [10,11]. Binding of the NFATc1-YLAVP motif requires the presence of Ca^{2+} and CaM, indicating that the interaction between CN and the NFATc1-YLAVP motif requires the activated form of CN. Furthermore, A238L, a viral protein inhibitor of CN, occupies a critical substrate recognition site on CN and competitively inhibits CN activity. A crystal structure of the A238L-CN complex showed that CN interacts with the NSNFLCVKKLNKYGK motif in A238L that are analogous to the LxVP motifs of CN substrates [11]. Recently, we found that the GSSHLAPPNPDQFL motif derived from RCAN1 (RCAN1-HLAPP) interacts with CN and that the association between CN and its LxVP-type substrates, including NFAT and RCAN1, can be disrupted by quercetin [12]. The last Pro is conserved and very important in the RCAN1-HLAPP motif and the NFATc1-YLAVP motif.

Autophagy is a cellular process that is crucial for maintaining cellular homeostasis by transporting targeted cytosolic proteins and impaired organelles to lysosomes for degradation. Recent studies have highlighted the significance of the transcription machinery, notably transcription factor EB (TFEB), in autophagy-related gene expression. Medina et al. have reported that TFEB, a master regulator of autophagic and lysosomal biogenesis, is a substrate of CN [13,14]. Autophagy and lysosomal biogenesis can be induced by lysosomal Ca^{2+} signaling via CN-mediated activation of TFEB. Under normal conditions, TFEB is phosphorylated and present in the cytoplasm. When CN is activated by lysosomal Ca^{2+} release through mucolipin 1 (MCOLN1), it dephosphorylates TFEB at Ser211 and Ser142 and TFEB translocates to the nucleus. This process of TFEB translocation is similar to that

of NFAT. TFEB, as a transcription factor, regulates the expression of target genes such as *MAPLC3*, *SQSTM1*, and *UVRAG*.

CN physically interacts with TFEB. This led us to study the interaction between CN and TFEB. The sequence of ⁷³QSYLENPTSYHLQQS⁸⁷ encoded by residues 73–87 of TFEB then attracted our attention, as it contains a close match to the NFATc1-YLAVP motif and the RCAN1-HLAPP motif. Our previous studies have focused on screening small molecule inhibitors of CN and blocking the CN/NFAT signaling pathway in immune response and neurodegenerative diseases [15–17]. The present study on CN-substrate interactions is helpful for understanding and developing selective CN peptide inhibitors for impaired CN-dependent signaling *in vitro* experiments.

2. Materials and methods

2.1. Reagents

GFP-tagged TFEB plasmid was purchased from OriGene Technologies (Beijing, China). The RII phosphopeptide (derived from cAMP-dependent protein kinase regulatory subunit, Type II), was purchased from Biomol Research Laboratories, Inc. (PA, USA) [18]. Other peptides used in the experiments were synthesized by SciLight-Peptide Co. (Beijing, China) and are shown in Table 1. All of the other reagents were of standard laboratory grade and the highest quality available from commercial suppliers.

2.2. Preparation of mouse brain lysates

Male Kunming mice (weight 16 ± 2 g, 4 weeks of age) were supplied by the Vital River Corp (a joint venture with Charles River Laboratories). The animals were housed in groups under the following laboratory conditions: temperature 20 ± 1 °C, humidity 40–60%, and 12 h light/dark cycle with food and water ad libitum. The mice were killed under sodium pentobarbital anesthesia, and we did our best to minimize any suffering. The brains were removed and homogenized at 4 °C by passage through a syringe into a solution of 50 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, 0.1 mM EGTA, 1.0 mM dithiothreitol, 0.2% NP-40, 1.0 mM phenylmethylsulfonyl fluoride, 5 µg/ml leupeptin, 5 µg/ml aprotinin, and 2 µg/ml pepstatin. After sonication and centrifugation for 60 min at $16,000 \times g$ and 4 °C, the supernatant was used as a source of CN in subsequent GST pull-down assays.

2.3. Expression of GST fusion protein, pull-down assays, and CN phosphatase assay

Plasmids encoding peptides fused to GST were produced by direct cloning of oligonucleotides into *EcoRI* + *XhoI*-digested pGEX-4T-1 plasmids. The sequences of the oligonucleotides used are shown in Table 1S (Supplemental Information). The GST fusion proteins were expressed in *Escherichia coli* and quantified by the Bradford procedure. CNA was detected in mouse brain lysates by western blotting. Unless otherwise specified, all pull-down experiments were performed in 50 mM Tris-HCl, 1.5 mM CaCl₂ (pH 7.5), 1.0 mM dithiothreitol, 2 µM CaM, and 0.5 mM MnCl₂. Glutathione-agarose beads coated with GST or GST peptide were incubated with 500-µl aliquots of brain lysates for 1 h at 4 °C with end-over shaking. The beads were recovered by centrifugation, washed five times with 50 mM Tris-HCl, 50 mM NaCl, 1 mM CaCl₂, 0.1% β-mercaptoethanol, and 0.2 mM

phenylmethylsulfonyl fluoride (pH 7.4), mixed with 20 μ l of SDS-PAGE sample buffer, boiled, centrifuged, and immunoblotted with anti-CNA antibody (pan-calcineurin A antibody, 1:1000, CST) or anti-GST antibody.

The cDNAs for the CNA and CNB were isolated from rat brain cDNA libraries. CNA and CNB were expressed in *E. coli* and purified in our lab. The purification scheme of CNB involved sequential hydrophobic chromatography, DEAE chromatography, and gel filtration. CaM from the bovine brain was purified by DEAE-cellulose 52 and Phenyl-Sepharose column in our lab [19]. CaM-Sepharose was prepared by coupling to CNBr-activated Sepharose. The CNA subunit was purified by CaM-Sepharose 4B affinity column. The enzyme activities of the reconstituted CN complex were found to be comparable to that of the bovine brain enzyme [20].

The purified proteins were concentrated with an Amicon Ultra Filter Unit, diluted in 0.5 mM dithiothreitol, 50 mM Tris-HCl, 0.1 mg/ml BSA, and 50% glycerol, and analyzed by SDS-PAGE. A colorimetric assay was used to determine the activity of CN with RII phosphopeptide as a substrate and using the Calcineurin Colorimetric Drug Discovery Kit (AK-804, Enzo Life Sciences) [21]. The amount of PO_4 released was determined calorimetrically with the classic malachite green reagent. The reaction was terminated after incubation at 37 °C for 30 min. The CN activity of each sample was determined in triplicate. Phosphatase activities are presented as percentages of the control.

2.4. Cell culture and transfection

Plasmids encoding LxVP peptides were fused to FLAG through direct cloning of overhang-double-stranded annealed oligonucleotides into *EcoRI* + *XhoI*-digested pEGFP-C1 plasmids. HEK293T cells were maintained in Dulbecco's modified Eagle's medium supplemented with 5 μ M L-glutamine, 10% fetal calf serum, and sodium pyruvate. The cells were transfected with FLAG-LxVP cDNA for 12 h, cultured for an additional 24 h, harvested, and lysed in lysis buffer (20 mM Tris-HCl, 10 mM NaCl, 1 mM EDTA, and 0.5% NP-40; pH 8.0). The lysates were used in subsequent experiments.

2.5. Microscale thermophoresis (MST)

The MST method has been described in detail elsewhere [22,23]. The K_d values for the binding of FAM-labeled peptides to CN were measured using a Monolith NT.115 from NanoTemper Technologies. FAM-labeled TFEB-YLENP or TFEB-YLAVP peptide (10 μ l) was added to serial dilutions of CN (CNA:CNB:CaM, 1:1:2) in PBS (10 μ l). Samples were incubated at 25 °C for 1 h, loaded into silica capillaries (Polymicro Technologies), and measurements were performed at 20 °C with 20% LED power and 80% IR-laser power. The data were analyzed with NanoTemper Analysis software, v.1.2.101.

2.6. Fluorescence polarization binding assay (FP)

The interaction between CN and the FAM-labeled TFEB-YLAVP peptide was studied in black 96-well flat-bottom plates, and measured using a SPECTROstar Omega (BMG, Germany) [24]. The program parameter settings were 495 nm excitation wavelength and 520 nm observed emission wavelength. To calculate CN-binding affinities, 300 nM FAM-labeled

TFEB-YLAVP peptide in 100 μ l of Tris-HCl (pH 7.4) containing 0.2 mg/ml BSA was titrated with increasing concentrations of purified recombinant CN. Competitive binding assays were performed by mixing peptide-CN complex with FAM-labeled TFEB-YLAVP peptide (300 nM) and CN (10 μ M). Unlabeled competitor peptide (NFATc1-YLAVP) was preincubated with increasing concentrations of CN for 15 min before addition of fluorescently labeled peptides. All binding and competition assays were performed for 15 min at 25 °C.

2.7. Model building and simulation

The simulations were based on the published crystal structure of the A238L-CN complex obtained from the RCSB Protein Data Bank (PDB ID: 4F0Z) [11]. For CN, we built the following three peptide/CN systems: CN binding to the “mutant” TFEB-YLAVP peptide, CN binding to the TFEB-YLENP motif, and CN binding to the FLCVK motif from A238L. Two additional residues were included with the neutralized terminals at the N and C terminals of the original structures. We constructed the initial structures by mutating the corresponding residues in the A238L-CN crystal structure. We assigned the titration states of the ionizable residues (aspartate, glutamate, lysine, arginine, histidine, and tyrosine) based on pKa predictions performed with PROPKA [25]. We found that almost all of the residues were in their default titration states, with only Asp121 having to be changed to a protonation state. Using the SOLVATE program, we dissolved the system in a box of water, with at least 10 Å between the protein and the border of the box. After that, we used 150 mM NaCl to neutralize the net charge of the system with the AUTOIONIZE plugin from Visual Molecular Dynamics [26].

We used NAMD2 to perform the simulations [27], the CHARMM36 force field for proteins and ions [28,29], and the TIP3P model for explicit water [30], using periodic boundary conditions to perform all simulations with a time step of 2 fs. We used the SHAKE algorithm to fix the bond distances involving hydrogen atoms in the simulations [31].

After an initial 10,000 steps of energy minimization with all C α atoms fixed, the system was equilibrated in an NVT ensemble (constant number, volume, and temperature) at 310 K for 500 ps. During the process, all protein C α atoms were restricted ($k = 1$ kcal/mol/Å⁻²) to allow mitigation of the side chains and water. All of the following equilibrium simulations were conducted in an NVT ensemble for 100 ns, restricting the active site to its configuration in the crystal structure.

For all simulations, we used Langevin dynamics to hold a constant temperature with damping coefficient γ of 0.5 ps⁻¹. We employed a cutoff distance of 12 Å to calculate short-range, nonbonded interactions, and long-range electrostatic forces were described by the particle mesh Ewald (PME) method [32]. The similar simulation protocol has been applied to our previous studies for CN [12,33–35].

2.8. Statistical analysis

To quantitatively assess marker protein expression for imaging analyses, we used identical settings and ImageJ (NIH). This method involves selecting the areas using freehand selection tools and then measuring their mean optical intensities or area fractions. The

intensity of the background was subtracted from the intensity of selected area to determine the net mean intensity. The data were analyzed with GraphPad Prism 5. All results are reported as mean $\pm$ SEM.

3. Results

3.1. Peptides derived from transcription factor EB bind to CN in mouse brain lysates

We tested whether the TFEB-YLENP peptide interacted with CN; GST pull-down assays and anti-CNA immunoblotting confirmed that GST-TFEB-YLENP bound to CNA. The interaction between GST-TFEB-YLENP and CNA was greatly inhibited by the addition of synthetic TFEB-YLENP peptides (Fig. 1A). Western blotting with anti-GST antibody showed that equivalent amounts of GST and GST-TFEB-YLENP fusion proteins were used in each reaction, and a GST control did not interact with CNA.

As we expected, the interaction of CN with each GST-peptide fusion protein was weakened by the point “mutations” TFEB-YLENP^{Y75A}, TFEB-YLENP^{L76A}, and TFEB-YLENP^{P79A} (Fig. 1B). Replacement of the Tyr-Leu-Pro tripeptide in the YLENP peptide with Ala (Y75A/L76A/P79A) abolished CN binding (Fig. 1B). In contrast, Glu-77 seems to be a negative binding determinant, since substituting Glu-77 with Ala (E77A) enhanced CN binding. Substitution of both Glu-77 and Asn-78 with Ala and Val, respectively, to increase the similarity to the NFATc1-YLAVP motif led to a further increase in polypeptide-CN complex formation (Fig. 1C and D; E77A/N78V = YLAVP “mutant”).

3.2. Peptides derived from transcription factor EB bind to purified recombinant CN

To quantitatively define the interaction between CN and TFEB-YLENP peptide, we resorted to MST. We mixed 40 nM of fluorescently labeled TFEB-YLENP peptide with serial dilutions of CN (22 μ M–1 nM). The experiment was conducted in PBS. The result of the MST is shown in Fig. 2A. The FAM-labeled TFEB-YLENP peptide bound to CN with a dissociation constant (K_d) of 4.5 ± 0.6 μ M. A concentration of 20 nM of fluorescently labeled TFEB-YLAVP peptide was mixed with the CN dilutions. Fig. 2B demonstrates that the “mutant” TFEB-YLAVP peptide bound to CN with a dissociation constant (K_d) of 2.4 ± 0.4 μ M. The MST data show that the binding affinity of the mutant TFEB-YLAVP peptide for CN is higher than that of the wild-type TFEB-YLENP peptide (Fig. 2C). The result accords with the findings of the GST pull-down experiments.

We set up an FP experiment to define the binding affinities between CN and the TFEB-YLAVP peptide. We found that CN bound to the FAM-labeled TFEB-YLAVP peptide (Fig. 2D). This result is consistent with the outcome of the MST experiments. In the subsequent competition binding assay, the concentration of CN was fixed at 10 μ M. We found that the complex formation between CN and the FAM-labeled TFEB-YLAVP peptide could be almost completely displaced by unlabeled NFATc1-YLAVP peptide at a concentration above 20 μ M. The IC_{50} calculated by the competitive binding curve was about 5.0 μ M. Owing to the extensive amino acid conservation in the two peptides, cross-competition experiments revealed competition for CN binding (Fig. 2E), which suggests that both peptides interact with the same CN region.

3.3. A similar binding pattern for both TFEB-YLENP and LxVP-type peptides to CN

We conducted molecular dynamics simulations to study the binding of CN to the TFEB-YLENP peptide and its YLAVP derivative. Based on the crystal structure of the A238L-CN complex (PDB ID: 4F0Z), we constructed the initial structures by altering equivalent residues, and each peptide-CN complex was simulated for 100 ns.

According to the crystal structure of the A238L-CN complex and simulations of the binding of FLCVK to CN, three residues, namely Y, L, and N in the TFEB-YLENP peptide facilitated binding to CN (Fig. 3A). Similarly, the three residues, Y, L, and V in the mutant TFEB-YLAVP facilitated binding to CN (Fig. 3B). In the TFEB-YLAVP motif, Leu and Val are embedded in a hydrophobic groove on the interface of CNA and CNB, and both side chains fitted the available space. In addition, we found that the Tyr fits into the gap between the two EF-hand domains of CNB (Fig. 3C). However, in the TFEB-YLENP motif, Glu faced outward instead of involvement with the binding. Gln didn't insert into the CNA:CNB interface as deep as the equivalent residue Val in YLAVP.

The interaction between CN and the TFEB-YLENP peptide is weaker than that between CN and the TFEB-YLAVP peptide, as shown by the average interaction energies based on the whole trajectories (Fig. 3D). This is in agreement with our binding assay and measurement of K_d , which also suggest that the binding between TFEB-YLENP peptide and CN is weaker than that between TFEB-YLAVP and CN. Albeit the different binding affinities, it demonstrates that the “mutant” TFEB-YLAVP peptide is able to bound to the hydrophobic LxVP-binding pocket at the CNA:CNB interface in CN, similar to the NFATc1-YLAVP motif.

3.4. CsA bound to cyclophilin could compete with activated CN for the mutant TFEB

Rodriguez et al. [10] observed that NFAT resembles the immunosuppressant-immunophilin complex, in that LxVP-type site such as cyclosporin A (CsA)-cyclophilin (CyP) inhibit CN by blocking substrate recognition. We examined the effect of the CsA-CyP complex on the interaction of mutant TFEB-YLAVP with CN in GST pull-down assays. When lysates were incubated with the classic immunosuppressant CsA (20 μ M) in the absence of exogenous recombinant CyP, the amount of CNA that was pulled down by the GST fusion protein of TFEB-YLAVP was reduced. Further of CyP (200 nM) almost completely blocked the interaction between CN and the TFEB-YLAVP derivative (Fig. 4A). These observations demonstrate that the interaction between the TFEB-YLAVP peptide and CN is similar to that of the NFATc1-YLAVP motif.

Our data are in agreement with the results of Medina et al. [13]. They found that the interaction between TFEB and CN plus CaM was stronger than that between TFEB and CN alone. Moreover, CsA or FK506 could reduce TFEB nuclear translocation during starvation.

CNA (1-511) consists of the catalytic domain and three regulatory domains: a CNB-binding domain (BBH, 350-370), a CaM-binding domain (CBD, 389-413), and an autoinhibitory domain (AID, 457-482). We compared the effects of GST fusion CNA expression vectors and two domain deletion derivatives: GST-CNAabc (1-456, deletion of the AID) and GST-CNAab (1-388, deletion of the AID and CBD). When a GFP-TFEB plasmid was transfected

into HeLa cells, we found that the absence of the CaM-binding domain of CN reduced the interaction between GFP-TFEB and CN in the GST pull-down assay. This demonstrates that the state of activation of CN affects its binding to TFEB (Fig. 4B and C). This conclusion is consistent with what is known about the interaction between the NFATc1-YLAVP motif and CN. In our previous work, we showed that quercetin, a CN inhibitor, bound to a similar region of CN as CsA, namely at the interface between CNA and CNB [15]. Moreover, quercetin significantly inhibited interactions between CN and LxVP-type substrates. In the present work, we showed that quercetin blocks the interaction between GST-CNA and GFP-TFEB (Fig. 4D and E).

3.5. Expression of NFATc1-YLAVP peptide blocks TFEB activation in starved HeLa cells

CN was found to catalyze the rapid dephosphorylation of Ser95 of the cAMP-dependent protein kinase type II regulatory subunit (RII). The 19-residue phosphopeptide (DLDVPIPGRFDRRVpSVAAE) was dephosphorylated with kinetics comparable to those of intact RII ($K_m = 26 \mu\text{M}$; $V_{\max} = 1.7 \mu\text{mol min}^{-1} \text{mg}^{-1}$) and used to assay CN activity [15]. We determined the dose-response profiles and IC_{50} values of other peptides derived from CN substrates using RII phosphopeptide as the substrate (Table 2). The result showed that other NFATc1-like motifs were present in CN substrates such as NFATc2 and RCAN1.

Rodriguez et al. reported the importance of individual residues in the NFATc1-YLAVP in CN inhibition. The NFATc2-ILLVP motif binds less strongly to CN than do the homologous peptides from other NFAT family proteins that lack the aromatic residues that precede the conserved Leu residues. Our enzymatic data show that compared to the NFATc1-YLAVP peptide, NFATc2-ILLVP has a lower inhibitory ability. Similarly, His and Pro residues in the RCAN1-HLAPP peptide could not fit in the local regions like Tyr and Val in the NFATc1-YLAVP motif. Our results for CN activity assay are consistent with the finding that the binding between CN and the RCAN1-HLAPP motif was weaker than that between CN and the NFATc1-YLAVP motif in our GST pull-down experiments [12]. Rodriguez et al. also reported the residues in the C-terminal portion of NFATc1-YLAVP ($^1\text{DQYLAVPQH}^{10}\text{PYQ}^{13}\text{WAK}$), such as ^{10}P and ^{13}W also contribute to the inhibitory capacity. Therefore, we speculated that TFEB-YLAVP has a lower inhibitory capacity probably due to the influence of other residues.

In general, dephosphorylation of proteins by CN requires docking via an LxVP motif. We investigated the inhibitory effects of synthetic peptides corresponding to the LxVP motif in NFATc1 and TFEB on CN-dependent dephosphorylation and nuclear translocation of TFEB from cytoplasm to nucleus, including TFEB-YLENP, TFEB-YLAVP, and NFATc1-YLAVP peptides. We transiently transfected HEK293T cells with plasmids expressing GFP-TFEB and FLAG-LxVP. Expression of NFATc1-YLAVP, but not TFEB-YLENP or “mutant” TFEB-YLAVP, significantly inhibited nuclear translocation in response to starvation (Fig. 5A and B), and TFEB dephosphorylation (Fig. 5C).

4. Discussion

NFAT has been the focus in studies of CN-substrate interactions. The highly conserved PxIxIT motif in NFATc1-c4 was the first identified. Garcia-Cozar et al. found that the

NFATc2-PRIET peptides spanning the NFATc2 docking site for CN inhibit the binding of activated CN to NFATc2 [36]. The measured K_d value for the binding of NFATc2-PRIET peptide to CN is about 25 μ M [6]. Subsequently, a high-affinity PVIVIT peptide (MAGPHPVIVITGPHEE) was selected from combinatorial peptide libraries based on the PxIxIT docking motif [7]. The K_d value for the binding of the artificially designed peptide to CN is 0.5 μ M [6]. This peptide potently inhibited NFAT activation (NFATc1-c3) and NFAT-dependent gene expression in T cells, without affecting the CN activity. Aramburu et al. also replaced the wild-type NFATc2 protein with the high-affinity PVIVIT sequence [7]. They found that a mutant NFATc2-PVIVIT was significantly dephosphorylated even in resting cells and required lower concentrations of Ca^{2+} stimuli to be fully dephosphorylated.

Several years later, the second CN binding site on NFAT, named the LxVP motif, was identified. Rodriguez et al. reported that CN binds the NFATc1-YLAVP motif with high affinity [10]. The NFATc1-YLAVP peptide at a concentration of 10 μ M significantly inhibits CN activity to 23.4% of the control level. Individual Y3A substitutions in NFATc1-¹DQ³YLAVPQHPYQWAK¹⁵ impaired the inhibitory activity dramatically ($\text{IC}_{50} > 100 \mu\text{M}$). The NFATc1-YLAVP peptide inhibits NFAT-dependent transcription and downstream gene expression. Further investigation found the binding ability of the LxVP-type motif among all isoforms of NFAT is different. The NFATc2-ILLVP motif binds less strongly to CN than do the homologous peptides from other NFAT family proteins that lack the aromatic residues that precede the conserved Leu residues.

Martinez-Martinez and Rodriguez et al. have reported there is cross-competition between the NFATc2-PxIxIT and the NFATc1-LxVP motif [37]. Their data suggest that the CN sequences that interact with the two binding motifs might be somehow interdependent. Similarly, Gal et al. found that the two binding motifs in NFATc1 bind simultaneously to overlapping epitopes in the catalytic CNA domain of CN. Their findings suggest that it is possible to inhibit CN/NFAT interaction with a single molecule [38]. Therefore, there is certainly more to be learned about the two CN/NFAT docking interactions in CN signaling.

In our present study, we first confirmed that the TFEB-YLENP peptide binds to CN. Changing the TFEB-YLENP sequence to TFEB-YLAVP increased the affinity of the polypeptide for CN. In the molecular dynamics simulations of the interaction between CN and the TFEB-YLENP peptide, the Phe (Y), Leu (L), and Pro (P) residues each contributed to binding. In contrast, the side chain of Glu (E) always points to the outside and has no effect on CN binding to the TFEB-YLENP peptide. N231 may contribute to the formation of an unstable hydrogen bond with Y341 of CN. Hence, according to the average interaction energies based on the complete trajectories, binding between CN and the TFEB-YLAVP peptide is stronger than that between CN and the TFEB-YLENP peptide. Thus, the Ala and Val substitutions enhanced CN binding compared with the wild-type TFEB-YLENP peptide.

Although we have identified that TFEB-derived peptides could bind to CN and inhibit its activity, they did not inhibit TFEB dephosphorylation and nuclear translocation effectively in response to starvation. One possible reason is that the affinity of TFEB-derived peptides binding to CN and the inhibitory capacity of CN are too low to block the downstream signaling. We have shown above that even TFEB-YLAVP inhibits CN less strongly than the

NFATc1-YLAVP motif. The IC_{50} of the NFATc1-YLAVP peptide was found to be 9 μM , compared with 190 μM for the IC_{50} of the TFEB-YLAVP “mutant” peptide. Our study found that the NFATc1-YLAVP motif was able to block phosphorylation of TFEB and its subsequent nuclear translocation. Martinez-Martinez and Rodriguez et al. have reported that expression of NFATc1-YLAVP peptide *in vivo* blocks NFATc2 dephosphorylation, not NFATc1 itself [38]. Compared with NFATc2-ILLVP, the NFATc1-YLAVP peptide interacts with CN more strongly and is sufficient to block CN/NFATc2 interaction and inhibit CN/NFATc2 downstream signaling.

Therefore, it is necessary to develop high-affinity selective peptides that can affect the CN downstream signaling. For example, Aramburu et al. have developed the high-affinity PVIVIT peptide ($K_d = 0.5 \mu M$) in place of the wild-type PRIET sequence ($K_d = 25 \mu M$) in order to inhibit CN downstream signaling in response to physiological stimuli. They speculated that the many protein-protein interactions, especially those involving enzymesubstrate interactions or transient docking interactions are low to moderate affinity in order to facilitate information transfer from one intracellular location to another, to ensure reversibility, and to prevent inappropriate activation [7]. Beyond that, the inhibitors that target CN-LxVP interactions and CN downstream signaling should show efficient inhibition of CN activity, just like the NFATc1-YLAVP peptide, CsA/CyP, and FK506/FKBP.

In summary, we first show that the TFEB-YLENP peptide binds to CN at a similar region as the LxVP-type motifs, the known CN docking sites present in the transcription factors NFAT, AKAP79, and RCAN1. By modifying the TFEB-YLENP peptide, we enhanced its interaction with CN. The expression of the NFATc1-YLAVP peptide blocked the dephosphorylation of TFEB in starved Hela cells. The results increase our understanding of how CN interacts with the LxVP-type motifs of substrates. Moreover, this study will be potentially useful for developing selective CN peptide inhibitors that target CN-substrate interactions and inhibit CN-dependent signaling *in vitro* experiments.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The present work was supported by the National Natural Science Foundation of China (Project 81373389 and 31540011).

Appendix A. Supplementary data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.biochi.2017.09.002>.

References

1. Rusnak F, Mertz P. Calcineurin: form and function. *Physiol Rev.* 2000; 80:1483–1521. [PubMed: 11015619]

2. Rao A. Signaling to gene expression: calcium, calcineurin and NFAT. *Nat Immunol.* 2009; 10:3–5. [PubMed: 19088731]
3. Müller MR, Rao A. NFAT, immunity and cancer: a transcription factor comes of age. *Nat Rev Immunol.* 2010; 10:645–656. [PubMed: 20725108]
4. Oliveria SF, Dell'Acqua ML, Sather WA. AKAP79/150 anchoring of calcineurin controls neuronal L-type Ca^{2+} channel activity and nuclear signaling. *Neuron.* 2007; 55:261–275. [PubMed: 17640527]
5. Mehta S, Li H, Hogan PG, Cunningham KW. Domain architecture of the regulators of calcineurin (RCANs) and identification of a divergent RCAN in yeast. *Mol Cell Biol.* 2009; 29:2777–2793. [PubMed: 19273587]
6. Li H, Rao A, Hogan PG. Interaction of calcineurin with substrates and targeting proteins. *Trends Cell Biol.* 2011; 21:91–103. [PubMed: 21115349]
7. Aramburu J, Yaffe MB, López-Rodríguez C, Cantley LC, Hogan PG, Rao A. Affinity-driven peptide selection of an NFAT inhibitor more selective than cyclosporin A. *Science.* 1999; 285:2129–2133. [PubMed: 10497131]
8. Martínez-Martínez S, Rodríguez A, Lopez-Maderuelo MD, Ortega-Perez I, Vazquez J, Redondo JM. Blockade of NFAT activation by the second calcineurin binding site. *J Biol Chem.* 2006; 281:6227–6235. [PubMed: 16407284]
9. Liu J, Arai K, Arai N. Inhibition of NFATx activation by an oligopeptide: disrupting the interaction of NFATx with calcineurin. *J Immunol.* 2001; 167:2677–2687. [PubMed: 11509611]
10. Rodríguez A, Roy J, Martínez-Martínez S, Lopez-Maderuelo D, Nino-Moreno P, Orti L, Pantoja-Uceda D, Pineda-Lucena A, Cyert MS, Redondo JM. A conserved docking surface on calcineurin mediates interaction with substrates and immunosuppressants. *Mol Cell.* 2009; 33:616–626. [PubMed: 19285944]
11. Grigoriu S, Bond R, Cossio P, Chen JA, Ly N, Hummer G, Page Cyert M, Peti W. The molecular mechanism of substrate engagement and immunosuppressant inhibition of calcineurin. *Plos Biol.* 2013; 11:e1001492. [PubMed: 23468591]
12. Zhao Y, Zhang J, Shi X, Li J, Wang R, Song R, Wei Q, Cai H, Luo J. Quercetin targets the interaction of calcineurin with LxVP-type motifs in immunosuppression. *Biochimie.* 2016; 127:50–58. [PubMed: 27109380]
13. Medina DL, Di Paola S, Peluso I, Armani A, De Stefani D, Venditti R, Montefusco S, Scotto-Rosato A, Prezioso C, Forrester A, Settembre C, Wang W, Gao Q, Xu H, Sandri M, Rizzuto R, De Matteis MA, Ballabio A. Lysosomal calcium signalling regulates autophagy through calcineurin and TFEB. *Nat Cell Biol.* 2015; 17:288–299. [PubMed: 25720963]
14. Medina DL, Fraldi A, Bouche V, Annunziata F, Mansueto G, Spanpanato C, Puri C, Pignata A, Martina JA, Sardiello M, Palmieri M, Polishchuk R, Puertollano R, Ballabio A. Transcriptional activation of lysosomal exocytosis promotes cellular clearance. *Dev Cell.* 2011; 21:421–430. [PubMed: 21889421]
15. Lei H, Luo J, Tong L, Peng LQ, Qi Y, Jia ZG, Wei Q. Quercetin binds to a similar region with FKBP-FK506 and CyPA-CsA on calcineurin. *Food Chem.* 2011; 127:1169–1174. [PubMed: 25214110]
16. Luo J, Sun L, Lin X, Liu G, Yu J, Parisiadou L, Xie C, Ding J, Cai H. A calcineurin- and NFAT-dependent pathway is involved in α -synuclein-induced degeneration of midbrain dopaminergic neurons. *Hum Mol Genet.* 2014; 23:6567–6574. [PubMed: 25051958]
17. Ma B, Yu J, Xie C, Sun L, Lin S, Ding J, Luo J, Cai H. Toll-Like receptors promote mitochondrial translocation of nuclear transcription factor of activated T-Cells in prolonged microglial activation. *J Neurosci.* 2015; 35:10799–10814. [PubMed: 26224862]
18. Blumenthal D, Takio K, Hansen RS, Krebs EG. Dephosphorylation of cAMP-dependent protein kinase regulatory subunit (Type II) by calmodulin-dependent protein phosphatase. *J Biol Chem.* 1986; 262:8140–8145.
19. Wei Q, Pervaiz S, Jackson AJ, Puett D, Lee EYC, Brew K. Effects of interaction with calcineurin on the reactivities of calmodulin lysines. *J Biol Chem.* 1988; 263:19541–19544. [PubMed: 2848832]

20. Wei Q, Lee EY. Expression and reconstitution of calcineurin A and B subunits. *Biochem Mol Biol Int.* 1997; 41:169–177. [PubMed: 9043646]
21. Wang H, Zhou CL, Lei H, Wei Q. Inhibition of calcineurin by quercetin in vitro and in Jurkat cells. *J Biochem.* 2010; 147:185–190. [PubMed: 19880376]
22. Jerabek-Willemsen M, Wienken CJ, Braun D, Baaske P, Duhr S. Molecular interaction studies using microscale thermophoresis. *Assay Drug Dev Technol.* 2011; 9:342–353. [PubMed: 21812660]
23. Lin CC, Melo FA, Ghosh R, Suen KM, Stagg LJ, Kirkpatrick J, Arold ST, Ahmed Z, Ladbury JE. Inhibition of basal FGF receptor signaling by dimeric Grb2. *Cell.* 2012; 149:1514–1524. [PubMed: 22726438]
24. Carne Mulero M, Orzáez M, Messeguer J, Messeguer A, Pérez-Payá E, Pérez-Riba M. A fluorescent polarization-based assay for the identification of disruptors of the RCAN1-calcineurin protein complex. *Anal Biochem.* 2010; 398:99–103. [PubMed: 19891949]
25. Olsson MH, Søndergaard CR, Rostkowski M, Jensen JH. PROPKA3: consistent treatment of internal and surface residues in empirical pKa predictions. *J Chem Theory Comput.* 2011; 7:525–537. [PubMed: 26596171]
26. Humphrey W, Dalke A, Schulten K. VMD: visual molecular dynamics. *J Mol Graph.* 1996; 14:33–38. 27–28. [PubMed: 8744570]
27. Phillips JC, Braun R, Wang W, Gumbart J, Tajkhorshid E, Villa E, Chipot C, Skeel RD, Kalé L, Schulten K. Scalable molecular dynamics with NAMD. *J Comput Chem.* 2005; 26:1781–1802. [PubMed: 16222654]
28. Best RB, Zhu X, Shim J, Lopes PE, Mittal J, Feig M, Mackerell AD Jr. Optimization of the additive CHARMM all-atom protein force field targeting improved sampling of the backbone ϕ , ψ and side-chain $\chi(1)$ and $\chi(2)$ dihedral angles. *J Chem Theory Comput.* 2012; 8:3257–3273. [PubMed: 23341755]
29. Klauda JB, Venable RM, Freites JA, O'Connor JW, Tobias DJ, Mondragon-Ramirez C, Vorobyov I, Mackerell AD Jr, Pastor RW. Update of the CHARMM all-atom additive force field for lipids: validation on six lipid types. *J Phys Chem B.* 2010; 114:7830–7843. [PubMed: 20496934]
30. Jorgensen WL, Chandrasekhar J, Madura JD, Impey RW, Klein ML. Comparison of simple potential functions for simulating liquid water. *J Chem Phys.* 1983; 79:926–935.
31. Ryckaert JP, Ciccotti G, Berendsen HJ. Numerical integration of the Cartesian equations of motion of a system with constraints: molecular dynamics of n-alkanes. *J Comp Phys.* 1977; 23:327–341.
32. Darden T, York D, Pedersen LG. Particle mesh Ewald: an Nlog(N) method for Ewald sums in large systems. *J Chem Phys.* 1993; 98:10089–10092.
33. Li J, Jia Z, Zhou W, Wei Q. Calcineurin regulatory subunit B is a unique calcium sensor that regulates calcineurin in both calcium-dependent and calcium-independent manner. *Proteins.* 2009; 77:612–623. [PubMed: 19536897]
34. Chen Q, Wu W, Li J, Wei Q. The polarity of the amino acid residue 118 of calcineurin B is closely linked to calcineurin enzyme activity. *IUBMB Life.* 2010; 62:561–567. [PubMed: 20552635]
35. Feng YD, Li J, Zhou WC, Jia ZG, Wei Q. Identification of the critical structural determinants of the EF-hand domain arrangements in calcium binding proteins. *Biochim Biophys Acta.* 2012; 1824:608–619. [PubMed: 22285364]
36. Garcia-Cozar FJ, Okamura H, Aramburu JF, Shaw KT, Pelletier L, Showalter R, Villafranca E, Rao A. Two-site interaction of nuclear factor of activated T cells with activated calcineurin. *J Biol Chem.* 1998; 273:23877–23883. [PubMed: 9727000]
37. Martínez-Martínez S, Rodríguez A, López-Maderuelo MD, Ortega-Pérez I, Vázquez J, Redondo JM. Blockade of NFAT activation by the second calcineurin binding site. *J Biol Chem.* 2006; 281:6227–6235. [PubMed: 16407284]
38. Gal M, Li S, Luna RE, Takeuchi K, Wagner G. The LxVP and PxIxIT NFAT motifs bind jointly to overlapping epitopes on calcineurin's catalytic domain distant to the regulatory domain. *Structure.* 2014; 22:1016–1027. [PubMed: 24954618]

Fig. 1. Peptides derived from transcription factor EB bind to CN in mouse brain lysates
 (A) The TFEB-YLENP peptide binds CNA in mouse brain lysates in pull-down assays. Bound CNA was visualized by western blot with monoclonal anti-CNA antibody, shown in the upper blot; the input is shown in the middle panel. The bottom blot confirms that equal amounts of GST fusion proteins were used in the reactions. (B) Alterations of the TFEB-YLENP peptide decrease the interaction between peptides and CNA in mouse brain lysates assayed by GST pull-down. (C) The TFEB-YLENP derivatives TFEB-YLENP^{N78V} and TFEB-YLENP^{E77A, N78V} interact more strongly with CNA. (D) Densitometric quantification of CNA bound by TFEB-YLENP^{E77A} and TFEB-YLENP^{E77A, N78V} and histograms showing the relative intensity units of bound CN. Data were presented as mean $\pm$ SEM (n = 3), * p < 0.05; ** p < 0.01 compared with the TFEB-YLENP group.

Fig. 2. Peptides derived from transcription factor EB bind to purified recombinant CN
 (A) The affinity of FAM-labeled TFEB-YLENP peptide for CN. (B) The affinity of FAM-labeled TFEB-YLAVP peptide for CN. CN was titrated against fixed concentrations of labeled TFEB-YLENP (40 nM) and TFEB-YLAVP (20 nM). The top panel shows the isotherm derived from the raw data, fitted to a sigmoidal dose-response curve. The bottom panel displays the raw data for thermophoresis recorded at 20 °C using 20% LED power and 80% MST power. (C) Comparison of K_d values between TFEB-YLENP/CN and TFEB-YLAVP/CN from three individual experiments. The individual experimental values were

showed. (D) The interaction between CN and 300 nM FAM-labeled TFEB-YLAVP measured by fluorescence polarization. (E) Complex formation between CN and 300 nM FAM-labeled TFEB-YLAVP in the presence of unlabeled NFATc1-YLAVP peptide by fluorescence polarization.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Fig. 3. A similar binding pattern for both TFEB-YLENP and LxVP-type peptides to CN (A and B) The distance (upper) between C-alpha atoms of the peptide and CN, and their interaction energy (lower) during the simulations. (C) The YLENP (shiny) and YLAVP (brushed) peptides bind to CN in a similar manner, as shown in the last snapshots of the simulation. Both CNA (ice blue) and CNB (orange) are shown as transparent surfaces and cartoons, whereas the LxxP peptides are drawn as sticks. (D) Binding between CN and the TFEB-YLENP peptide is weaker than that between CN and the TFEB-YLAVP peptide, as shown by the average interaction energies based on the complete trajectories.

Fig. 4. CsA bound to cyclophilin competes with activated CN for the mutant TFEB

(A) Competition for binding of CN to TFEB-YLAVP by CsA alone and by the CsA-CyP complex (20 μM and 200 nM, respectively). (B) Binding of GST-CNA and its deletion mutants to GFP-TFEB in HeLa cells. (C) TFEB bound by CNA and its mutants was measured densitometrically, and the histograms show the relative intensity units of bound TFEB. TFEB bound by CNA is set at 100%. Data were presented as mean ± SEM (n = 3), * $p < 0.05$ compared with CNA group. (D) Quercetin (50 μM and 100 μM) competes with binding of GFP-TFEB to GST-CNA. (E) The bar graph depicts the ratios of bound TFEB and GST-CN in the presence of quercetin as percentages of the control binding (100%). Data were presented as mean ± SEM (n = 3), ** $p < 0.01$; *** $p < 0.001$ compared with the control group.

Fig. 5. Expression of NFATc1-YLAVP peptide blocks TFEB activation in starved HeLa cells
 (A) Starvation induces nuclear translocation of GFP-TFEB. (B) NFATc1-YLAVP peptide inhibits TFEB nuclear translocation. The graph shows the percentages of TFEB translocation in starved and peptide-treated cells. Data were presented as mean $\pm$ SEM ($n = 3$), $*p < 0.05$; $**p < 0.01$ compared with the starved group. (C) Transfection of NFATc1-YLAVP cDNA induces TFEB phosphorylation in starved HeLa cells. Empty vector cDNA and NFATc1-YLAVP cDNA were transfected into HeLa cells, and the cells were starved. The blot shows the expression of GFP-TFEB in total lysates and indicates the positions of phosphorylated and dephosphorylated proteins.

Table 1

Abbreviations and sequences of the peptides used.

Abbreviations	Sequences
TFEB-YLENP	QSYLENPTSYHLQQS
TFEB-YLAVP	QSYLAVPTSYHLQQS (mutant)
NFATc1-YLAVP	DQYLAVPQHPYQWAK
NFATc2-ILLVP	ESILLVPPTWPKPLV
RCAN1-HLAPP	GSSHLAPPNPDKQFL
RII	DLDVPIPGRFDRRVSVAAE
RII phosphopeptide	DLDVPIPGRFDRRVpSVAAE
FAM-labeled TFEB-YLENP	5(6)-carboxyfluorescein-labeled QSYLENPTSYHLQQS
FAM-labeled TFEB-YLAVP	5(6)-carboxyfluorescein-labeled QSYLAVPTSYHLQQS (mutant)

Table 2

Inhibitory effect of various peptides derived from CN's substrates on RII dephosphorylation.

Abbreviations of the peptides	IC ₅₀
NFATc1-YLAVP	9 μ M
NFATc2-ILLVP	130 μ M
RCAN1-HLAPP	131 μ M
TFEB-YLAVP	190 μ M