Published in final edited form as:

Trends Cell Biol. 2011 February ; 21(2): 91–103. doi:10.1016/j.tcb.2010.09.011.

Interaction of calcineurin with substrates and targeting proteins

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Abstract

Calcineurin is a calcium-activated protein phosphatase with a major role in calcium signaling in diverse cells and organs, and importance in clinical practice as the target of the immunosuppressive drugs cyclosporin A and FK506. Cell biological studies have focused mainly on the role of calcineurin in transcriptional signaling. Calcium entry in response to extracellular stimuli results in calcineurin activation and signal transmission from the cytosol into the nucleus through dephosphorylation and nuclear translocation of the transcription factor NFAT. This initiates a cascade of transcriptional events involved in physiological and developmental processes. Molecular analyses of the calcineurin/NFAT interaction have been extended recently to encompass the interaction of calcineurin with other substrates, targeting proteins and regulators of calcineurin activity. These studies have increased our understanding of how this essential calcium-activated enzyme orchestrates intracellular events in cooperation with other signaling pathways, and suggested a link between altered calcineurin signaling and the developmental anomalies of Down syndrome.

Introduction

Calcineurin, a unique calcium/calmodulin activated protein serine/threonine phosphatase 1⁴, is one of the key players directing the flow of information from local or global calcium signals to effectors that control immediate cellular responses and alter gene transcription. To carry out its diverse biological functions (Table 1, Supplementary material), calcineurin communicates with a large number of substrates and other proteins, in some cases at restricted intracellular locations. In this review, we focus on molecular interactions of calcineurin with partner proteins that shape downstream cellular responses. We take as examples calcineurin-substrate interactions that are obligatory for signaling (NFAT, Crz1, TRESK), or that are optional and serve to channel input from calcineurin to one specific isoform of a substrate (KSR2), a calcineurin-scaffold protein interaction that is essential for communication with certain substrates in hippocampal neurons (AKAP79), and a calcineurin-modulatory protein interaction that regulates calcineurin enzymatic activity (Rcn1, RCAN1).

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Overview of the enzymatic activity of calcineurin

Calcineurin is a heterodimer of a catalytic A subunit (calcineurin A) of ~60kDa and a regulatory B subunit (calcineurin B) of ~19kDa. Calcineurin A possesses an N-terminal catalytic domain, a helical calcineurin B-binding segment, a calmodulin-binding segment and an autoinhibitory peptide (Figure 1A). The latter three regions together comprise the regulatory region of the A subunit. Activation of calcineurin involves the participation of two distinct EF-hand containing subunits, calcineurin B and calmodulin. Calcineurin B, the regulatory component of calcineurin, is tightly associated with calcineurin A, whereas calmodulin binding to calcineurin A is Ca^{2+} -dependent. Based on crystal structures 5· 6, the catalytic domain of calcineurin A has a typical β -sandwich fold with two β -sheets located centrally and a mixture of α -helices and β -strands on the outside. Calcineurin B has four EF-hands that bind four Ca^{2+} ions (Figure 1A). Ca^{2+} -binding sites 1 and 2 in the N-terminal lobe are of lower affinity, with K_{d} s (dissociation constants, or the Ca^{2+} concentrations needed for half occupancy) in the micromolar range, whereas Ca^{2+} -binding sites 3 and 4 in the C-terminal lobe bind Ca^{2+} with nanomolar K_{d} s and cannot readily be decalcified 7· 8. It is believed that Ca^{2+} binding at the high-affinity sites serves to stabilize the heterodimeric structure of calcineurin, and these sites are therefore often referred to as structural sites. The low affinity sites serve as Ca^{2+} sensors and play a regulatory role in that their occupancy is dependent on elevation of intracellular Ca^{2+} . Occupancy of these sites results in partial stimulation of calcineurin activity, but more importantly, allows calmodulin binding to its target site in the regulatory domain in a Ca^{2+} -dependent manner 9· 11.

A proposed mechanism for calcineurin activation, involving sequential changes in conformation, is depicted in Figure 1B 10· 11. Full activation is thought to require a conformational change, initiated by binding of calcium ions to the low affinity sites on calcineurin B (Form I to Form II), that causes dissociation of the calmodulin-binding region from the calcineurin B-binding helix of calcineurin A, binding of calmodulin (Form III), and displacement of the autoinhibitory peptide from the active site (Form IV). In the crystal structure of a partially-activated calcineurin A/calcineurin B heterodimer, where all four Ca^{2+} binding sites of calcineurin B are loaded with Ca^{2+} but calmodulin is not present (Form II in Figure 1B), the autoinhibitory peptide of calcineurin A α , residues 469-486, occludes the active site of the enzyme 6. Recent work indicates that displacement of the autoinhibitory peptide from the catalytic site depends on interaction of calmodulin with other parts of the calcineurin A regulatory region in addition to the recognized calmodulin target sequence 12· 13.

In vitro enzymatic assays conducted at different concentrations of Ca^{2+} [10] have helped to place this scheme for calcineurin activation in a physiological setting. There is little phosphatase activity at Ca^{2+} concentrations of 100 nM or less, corresponding to cytoplasmic Ca^{2+} in a resting cell, but considerable activity at concentrations in the range of a few hundred nanomolar to a few micromolar, corresponding to those achieved during physiological stimulation. Only a rough quantitative correlation can be drawn, since calcineurin activity depends, for example, on the concentrations of free Mg^{2+} and calmodulin in the cell, which may differ from those chosen for a particular enzymatic assay. Other parameters affecting calcineurin signaling in cells that are not accounted for in the assays *in vitro* are the rates of activation and deactivation of calcineurin in response to oscillating global Ca^{2+} signals and to large excursions of local Ca^{2+} concentration that occur in some cellular microdomains, and the rates of rephosphorylation of substrates by opposing kinases.

Calcineurin, immunosuppressive drugs, and NFAT transcription factors

A major impetus to the study of calcineurin was its recognition as the target of the immunosuppressive drugs cyclosporin A and FK506, which are widely used in transplant medicine. Cyclosporin A and FK506 form complexes, respectively, with the immunophilins cyclophilin A and FKBP12 5' 6' 14' 16 and inhibit calcineurin phosphatase activity against peptide and protein substrates 16. The immunosuppressive action of these compounds is due to preventing dephosphorylation of NFAT-family transcription factors in T cells and other immune cells 17.

The Ca^{2+} signaling pathway in T lymphocytes that leads to immune effector responses is well understood. Signals initiated at the T cell antigen receptor trigger the release of calcium from endoplasmic reticulum (ER) stores. Depletion of ER calcium stores is sensed by an N-terminal EF-hand of the single-pass ER membrane protein STIM1 (stromal interaction molecule 1) and relayed to the STIM1 cytoplasmic domain, which activates ORAI1 calcium release-activated calcium (CRAC) channels located in the plasma membrane 18' 19. Sustained calcium influx through CRAC channels activates calmodulin and calcineurin. Calcineurin activation initiates a linear sequence of signaling events consisting of dephosphorylation of conserved phosphoserine residues in the regulatory domain of NFAT (Figure 2), a conformational change in NFAT to expose its nuclear localization sequence, translocation of NFAT into the nucleus, and transcriptional activation of NFAT target genes (Figure 5A). The reverse process of NFAT rephosphorylation and nuclear export is controlled by a number of kinases including CK1, GSK3 and DYRK 20' 22. CK1, the kinase that targets phosphorylation sites in the SRR-1 region of NFAT that are central for nuclear import, docks at an FxxxF motif in NFAT, an interaction that has been exploited experimentally to construct a hyperactivable NFAT protein discussed in a later section 22 (Figure 2).

Although effector immune responses figured prominently in early studies of calcineurin-NFAT signaling, they form just one part of a larger picture. The calcineurin/NFAT pathway translates calcium signals into gene expression and affects diverse biological processes depending on the cell type. Dysregulation of calcineurin-NFAT signaling can contribute to pathologies including cardiac hypertrophy 23' 24, autoimmune disease 25' 26, osteoporosis 27, and potentially Alzheimer's disease 28' 29, Down syndrome 21 and cancer 30' 32.

Direct interaction between calcineurin and NFAT

NFAT proteins have served as a model for the role of docking in signal transmission, and to define sites of calcineurin-substrate interaction. Two binding sites for calcineurin on NFAT have been recognized: the PxIxIT motif located near the N terminus of the regulatory region and an LxVP motif located near its C terminus (Figure 2).

The PxIxIT motif

The calcineurin docking site of NFAT proteins was originally identified by examining a series of engineered NFAT1 proteins with alanine substitutions across conserved motifs in the NFAT1 regulatory domain. Only two substitutions, in the calcineurin docking sequence and the nuclear localization sequence, interfered with NFAT1 nuclear localization in response to stimulation 33. A synthetic peptide including the native NFAT1 docking sequence, PRIEIT, was shown to compete with NFAT for binding to calcineurin and to prevent NFAT dephosphorylation 33' 34. An optimized PxIxIT peptide obtained by screening a randomized peptide library, PVIVIT, had 50-fold higher affinity for calcineurin than PRIEIT, measured as K_d 35. When the native PRIEIT sequence in NFAT1 was replaced by PVIVIT, NFAT1 was dephosphorylated and partially localized to the nucleus

even under resting conditions 35. Presumably the enhanced association of substrate with calcineurin allowed it to compete effectively with autoinhibitory peptide for access to the calcineurin catalytic site, even in the absence of a Ca^{2+} signal. Conversely, when a Thr>Ala substitution was introduced at the last position of the PRIET motif, resulting in a reduction in calcineurin binding affinity by about 10-fold, NFAT1 became unresponsive to calcium stimulation and remained in the cytoplasm 33. These findings with altered NFAT docking sites provide insight into the logic of calcineurin-substrate signaling that is further explored below.

Structural basis for calcineurin substrate interaction through the PxIxIT motif

The crystal structure of calcineurin in complex with PVIVIT peptide has been determined and provides a structural basis for calcineurin-substrate recognition 36 (Figure 3). In the crystal structure, PVIVIT is juxtaposed along the outside edge of β -strand 14 of the catalytic domain of calcineurin in an extended conformation, adding an additional strand to one of the existing β -sheets in the central β -sandwich (Figure 3A). Each residue at conserved positions of the PVIVIT peptide shows complementarity with the surface of calcineurin (Figure 3B). A string of surface hydrophobic pockets provides anchor points for the peptide: the proline is buried in a hydrophobic pocket created by residues from loops connecting β -strands 11/12 and 13/14. The side chains of the two conserved isoleucines probe the hydrophobic recesses formed by the joining of the two central β -sheets, and the threonine is part of a hydrogen bond network that also includes Asn330 of calcineurin, to which the threonine is linked directly 36, 37. The PxIxIT motif is not only highly conserved among the four members of calcium-activated NFAT family of transcription factors (Figure 2), but it is also shared in modified form by many other calcineurin substrates and regulatory proteins from yeast to human (Figure 4A, discussed below). The calcineurin-PVIVIT structure thus illustrates the essential features of calcineurin binding to many of its substrates.

The observation that PVIVIT peptide is in contact with a second calcineurin A molecule in the crystal structure has generated some discussion as to whether two copies of calcineurin are indeed part of the physiologically relevant functional enzyme-substrate complex 38. We believe that the second binding mode is best interpreted as a crystal contact in this case, though the possibility remains that it might be used in assembling some larger calcineurin complexes in cells. Similarly, calmodulin and a calmodulin-binding peptide from calcineurin A were crystallized as a domain-swapped dimer, but actually formed a 1:1 complex in solution 39.

The LxVP motif

While the PxIxIT site is considered the primary site for calcineurin binding for NFAT and is used by all members of the NFAT family of transcription factors, one or more additional sites may coordinate with the PxIxIT site to mediate the interaction of NFAT with calcineurin. Recently, the LxVP motif, first characterized a decade ago as a calcineurin-interacting peptide of NFAT4 and NFAT2 40–42, has attracted new attention 43, 44. The LxVP motif is located near the C-terminal end of the NFAT regulatory region after the third SPxx repeat (Figures 2, 4B). The LxVP peptide of NFAT1 binds less tightly to calcineurin than the corresponding peptides from other NFAT-family proteins 42, 45, a difference that has been ascribed to the absence of an aromatic residue preceding the conserved Leu, and to the NFAT1 sequence C-terminal to the LxVP consensus (44; see Figures 2, 4B). The difference in binding strengths could be a source of differential regulation of NFAT activity by calcineurin at the level of protein-protein interaction inside the cell. Rodríguez et al. 44 argue that the LxVP motif makes a major contribution to calcineurin-NFAT2 signaling, a view that needs to be confirmed by mutating LxVP motif residues in the context of full-

length NFAT or an NFAT regulatory domain-green fluorescent protein fusion, and examining calcineurin sensitivity as judged by nuclear translocation of NFAT.

The LxVP peptides potentially block calcineurin phosphatase activity against the phosphoR11 peptide, a preferred calcineurin peptide substrate derived from the protein substrate PKA regulatory subunit R11 α . Based on this finding, on LxVP competition with immunosuppressive/immunophilin complexes, and on *in silico* docking, it was proposed that LxVP peptides bind to a site located near the calcineurin B-binding helix and the active site of calcineurin 44. Consistent with the fact that the proposed site overlaps the binding site for immunosuppressant/ immunophilin complexes, mutation of certain calcineurin residues, identified as contact residues by *in silico* docking, diminished calcineurin binding to LxVP peptides as well as to immunosuppressant/ immunophilin complexes 44. Introducing the corresponding mutations into yeast calcineurin diminished the ability of calcineurin to bind several of its substrates in yeast 44, although among the substrates tested, only Rcn1 was shown to contain an LxVP motif. In summary, the studies with synthetic peptides clearly demonstrate an interaction of LxVP peptides from NFAT and Rcn1 with calcineurin, but additional work is needed to understand how the LxVP site functions together with the PxIxIT site in the context of an intact NFAT regulatory domain.

Biological variation in the PxIxIT motif and its role in signaling

Sequence variations in the PxIxIT motif fine-tune interaction affinities

Natural PxIxIT motif-containing calcineurin recognition sites show pronounced sequence variations at both conserved and non-conserved positions, giving rise to a graded variation in calcineurin binding affinity over a wide range of K_d 36–46–47 (Figure 4A). The crystal structure (Figure 3) sheds light on how side-chains of different shapes and sizes can be accommodated at the docking surface of calcineurin, and single residue substitutions help in understanding how affinity tuning is achieved by placing different amino acid residues along the core PxIxIT motif 36. Single-residue replacements at both conserved and non-conserved positions affect calcineurin binding, and each position seems to contribute independently to affinity, with β -strand-promoting residues being favored after Pro, and polar residues with short side-chains -- such as Thr and Ser -- favored at the threonine position for high affinity 35–36.

The observation that each residue makes an independent contribution to PxIxIT peptide binding marks the calcineurin-PxIxIT interaction as a special case, since the free energy of binding interactions is a global thermodynamic property, rather than a local property. The explanation in this case is that it is likely that the PxIxIT peptide undergoes a random-to-order transition upon binding to calcineurin, and the peptide is placed in a stereotyped register with respect to the calcineurin surface, with the result that the contributions of individual residues to the free energy of binding become uncoupled and mostly reflect the properties of the individual residues and their local environment. Independent energetic contributions from individual residues have also been observed in the interaction between PDZ domain proteins and their target peptides, which adopt a similar “ β -augmentation” binding mode 48.

Some intriguing questions arise when individual calcineurin-PxIxIT affinities are considered in the context of the estimated calcineurin concentrations in different types of cells. For example, it is likely that there will be low occupancy by calcineurin of NFAT docking sites in T cells, but high occupancy of docking sites on the K^+ channel TRESK in neuronal cells, based both on the measured affinities and on the sharply higher levels of calcineurin present in neurons than in T cells. Why have the natural docking sites of NFAT proteins not increased in affinity during the course of evolution? A partial answer was provided by the

experiment mentioned above, in which introducing the high-affinity PVIVIT site into NFAT1 led to NFAT activation in the absence of a calcium signal. The next two sections describe more extensive studies of NFAT and of the yeast protein Crz1, a transcription factor activated when yeast are subjected to physiological stresses, and underline the conclusion that inappropriate increases in the affinity of the calcineurin-substrate interaction can have deleterious biological consequences.

Tinkering with evolution by stabilizing calcineurin-substrate docking: NFAT

Altering NFAT activity *in vivo* by manipulating calcineurin-NFAT docking can have striking biological consequences. The point was examined in transgenic mice expressing an NFAT1 variant in which the native PRIET docking site was replaced by PVIVIT (VIVIT-NFAT1), and in a second strain of mice where a weakened CK1 docking site was introduced in addition to the PVIVIT mutation to further compromise NFAT phosphorylation (ASILA-VIVIT NFAT1). Both VIVIT- and ASILA-VIVIT NFAT1 were hyperactivable in T cells, displaying more persistent nuclear localization upon stimulation, and more readily triggering production of the NFAT-dependent cytokines IL-2, TNF α , and IFN- γ at low levels of stimulation 49. An unanticipated observation was that mice expressing ASILA-VIVIT NFAT1 at low levels in all organs were less likely to survive embryonic development than their wildtype littermates. In surviving mice, the expression of hyperactivable NFAT variants was mosaic in several tissues, presumably because tissues were preferentially populated by rare precursor cells that did not express the hyperactivable NFAT, again emphasizing the disadvantage imposed by increased NFAT signaling. Even the less hyperactivable version, VIVIT-NFAT1, was completely excluded from expression in the heart and brain, where the function of NFAT is known to be essential 49.

Tinkering with evolution by stabilizing calcineurin-substrate docking: Crz1

In the yeast *Saccharomyces cerevisiae*, calcineurin activity is required for growth under physiological stresses such as high salt, alkaline pH and cell wall stress 50. Certain stress responses in yeast are mediated by the transcription factor Crz1, a protein unrelated to NFAT that is also recognized by calcineurin via a PxIxIT docking sequence (Figure 4). When the high affinity PVIVIT sequence was substituted for the native Crz1 sequence, yeast showed better survival under high salt conditions, consistent with the demonstrated increase in calcineurin-Crz1 signaling. However, these cells became more susceptible to alkaline pH stress, possibly due to a titration effect by which the high affinity Crz1 diverted calcineurin from its substrate(s) in the independent alkaline pH stress pathway 47. These and other observations indicate that the docking sequences selected by evolution offer calcineurin binding affinities that tune the engagement and dephosphorylation of individual substrates to the signaling needs of the cells and tissues.

Interaction of calcineurin with other substrates and adapters

Recent work has extended the study of calcineurin-protein interactions beyond NFAT 33, 35-37, 40-42, 44, 45 and yeast calcineurin substrates 47, 50-53. It has long been recognized that calcineurin differs from the related phosphatase PP1 54, 55 in its lack of specific tightly-associated targeting subunits. What is now evident from the examples discussed below is that reversible docking with both substrates and scaffold proteins is a recurring theme in directing cellular signaling downstream of calcineurin. The K⁺ channel TRESK is the second mammalian substrate with a thoroughly documented PxIxIT docking site. The MAP kinase scaffold KSR2 is a calcineurin substrate with the primary docking interaction at an LxVP site. The calcineurin-AKAP79 interaction, which is critical for calcineurin signaling in neurons and perhaps in cardiac and vascular smooth muscle cells, shows that PxIxIT docking is utilized by a calcineurin scaffold protein as well as by substrates.

TRESK

TRESK (TWIK-related spinal cord K⁺ channel; KCNK18), a member of the two-pore domain potassium channel family (2PK), was first cloned from human spinal cord 56 and mouse cerebellum 57. TRESK may be linked to nociception because of its abundance in dorsal root ganglion neurons 58, 59 and its sensitivity to volatile anesthetics 60. As a class, 2PK channels provide background potassium currents that maintain the negative membrane potential and regulate the firing rate of neurons. The currents through 2PK channels can be modulated by a variety of inputs, but TRESK is unique in its strong response to elevation of intracellular calcium. TRESK expressed in *Xenopus* oocytes is strikingly activated by calcineurin in response to calcium mobilizing stimulation 57, 61, and it is assumed that native TRESK in neuronal cells is subject to similar regulation (Figure 5C). The intracellular loop between transmembrane segments 2 and 3 of TRESK contains a PxIxIT calcineurin-docking motif (PQIIS in human and PQIVID in mouse; Figure 4A) that interacts strongly with calcineurin 36. Docking at the PxIxIT-binding site is necessary for channel activation by elevated cytoplasmic calcium, as evidenced by the findings that mutations predicted to reduce the calcineurin affinity of the PQIVID motif in murine TRESK reduced the activation of TRESK in response to calcium, and that saturating the calcineurin docking site by injecting excess PVIVIT peptide into oocytes eliminated the response of TRESK channels to calcium signaling 61. A cluster of serine residues — Ser 274, Ser276, and Ser279 in murine TRESK — has been identified as the likely locus at which dephosphorylation controls channel activation 57.

KSR2

KSR (Kinase Suppressor of Ras) is a protein scaffold that interacts with the core kinase components of the ERK signaling cascade to facilitate signaling from Ras to ERK 62, 63 (Figure 5D). There are two isoforms of mammalian KSR, KSR1 and KSR2. Receptor tyrosine kinases induce dephosphorylation of both isoforms via protein phosphatase 2A (PP2A), at Ser392 in KSR1 and at Ser469 in KSR2, releasing the KSR proteins from a complex with 14-3-3 proteins that inhibits their scaffolding role 43, 64. However, only KSR2 binds calcineurin, is dephosphorylated by calcineurin, and contributes to calcium-mediated ERK activation 43. In response to elevated cytoplasmic calcium, calcineurin dephosphorylates KSR2 at Ser198, Thr287, and Ser310, a 14-3-3 binding site distinct from the Ser469 site dephosphorylated by PP2A. Efficient dephosphorylation of these residues by calcineurin requires calcineurin docking via an LxVP motif (LSVP) present only in KSR2, since substitution of alanine residues in the LSVP docking site interferes with the calcineurin-KSR2 interaction, with KSR2 dephosphorylation by calcineurin, and with calcium-dependent ERK signaling 43. The effect of mutating the docking site is limited to the calcium-calcineurin pathway, since activation of the mutant KSR2 through the EGFR-PP2A pathway common to both KSR isoforms is preserved. This example of primary docking of calcineurin through an LxVP site suggests other substrates will be found to use this alternative docking mode, and raises the possibility that additional docking sequences remain to be identified.

AKAP79/150

Human AKAP79 (AKAP150 in rodents) is a multivalent anchoring molecule that targets PKA, PKC and calcineurin to specific subcellular locations, in some cases adjacent to sites of calcium entry such as the L-type Ca²⁺ channel and the NMDA receptor channel.

Calcineurin-AKAP79 anchoring in hippocampal neurons regulates the L-type Ca²⁺ channel itself, with channel dephosphorylation by calcineurin attenuating Ca²⁺ current and opposing channel phosphorylation and enhancement of current by PKA 65. Despite the abundance of calcineurin in neurons, unanchored calcineurin is ineffective in regulating channel activity.

PVIVIT competes with AKAP79 for binding to calcineurin, indicating that the AKAP79 calcineurin-anchoring sequence, PIAIIT, occupies the PxIxIT-binding site on calcineurin 65. At first glance, it might appear that AKAP79 is serving as a targeting subunit, directing calcineurin to the L-type Ca^{2+} channel, in the way that targeting subunits associate with PP1 catalytic subunit to direct its activity to specific locations and substrates 54, 55. However, this simple interpretation conflicts with the accepted view that calcineurin anchored to AKAP79 is enzymatically inactive.

It is worth reviewing the conclusion that AKAP79 inhibits calcineurin. Overexpression of AKAP79 fragments that included the PIAIIT calcineurin-anchoring peptide blocked calcineurin-dependent hypertrophic growth of cardiac myocytes and blunted calcineurin-dependent rundown of recombinant GluR1 receptor currents 66, 67. The inhibition can be interpreted as reflecting competition between PIAIIT and the PxIxIT peptide of NFAT for the same docking surface on calcineurin in the first case, and between the AKAP79 fragment and full-length AKAP79 in the second case, and does not necessarily imply that calcineurin was catalytically inactive. Peptide fragments corresponding to several distinct regions of AKAP79 inhibited calcineurin *in vitro* 67–69, but the structural basis for inhibition has not been established and the inhibitory fragments included segments rich in charged residues, leaving it open to question whether the inhibition was specific. A cautious view is that calcineurin-AKAP79 complex formation has not been definitively shown to prevent the activation of calcineurin by Ca^{2+} .

Anchoring of calcineurin by AKAP79 is required to couple L-type Ca^{2+} channel activity in hippocampal neurons to NFAT3 nuclear import 65. The fact that AKAP79 positions calcineurin in a microdomain subject to large Ca^{2+} transients and in proximity to calmodulin bound to the cytoplasmic tail of the L-type Ca^{2+} channel may facilitate phosphatase activation 65, but the privileged role of scaffolded calcineurin in communicating to NFAT has not been fully explained. A second intriguing question, in view of the dual use of the PxIxIT-binding site by AKAP79 and NFAT, is how a balance between calcineurin-AKAP anchoring and calcineurin-NFAT recognition is maintained to allow signaling to proceed.

In the central nervous system, AKAP79/150 also participates in synaptic plasticity by targeting PKA, PKC and calcineurin to the postsynaptic densities of excitatory synapses, in protein complexes that include the NMDA and AMPA receptor channels 70–73 (Figure 5E). Together these enzymes regulate AMPA receptor trafficking by reversible protein phosphorylation during long-term potentiation (LTP) and long-term depression (LTD), two major cellular mechanisms widely believed to underlie learning and memory 74, 75. Calcineurin and calcineurin-AKAP79 docking have been most clearly implicated in LTD in hippocampal pyramidal neurons 73, 76.

Interaction of calcineurin with its RCAN regulators

RCAN (regulator of calcineurin) proteins comprise a family of endogenous calcineurin regulators that are conserved from yeast to human and that are essential for normal calcineurin signaling. They have complex effects in cells, including both positive and negative contributions to calcineurin signaling, whose precise mechanisms are still unresolved. RCAN proteins have appeared under many names in the literature. Here we will follow the updated nomenclature 77 in referring to the proteins as a class and to the human proteins as RCANs, but we will refer specifically to the protein of *S cerevisiae* as Rcn1.

RCANs were first identified in fungi in screens for calcineurin-binding proteins 78 or calcineurin inhibitors 79. Mammalian RCANs were discovered by database searches of the human genome for genes homologous to the yeast counterparts 80. Human RCAN1, one of the mammalian homologues, is encoded within the Down syndrome critical region on

chromosome 21. RCAN1 is abundantly expressed in heart, brain, and muscle, and is over-expressed in the brains of individuals with Down syndrome and Alzheimer's disease 81, 82. RCAN1 has drawn much attention recently because of reports linking the function of this protein to vertebrate embryonic development and tumor progression 21, 30, 32.

The detailed role of RCAN1 in calcineurin signalling is a topic of ongoing debate. RCAN1 physically interacts with calcineurin, inhibits calcineurin phosphatase activity *in vitro* 78, 79, 83, 84, and blocks the activation of NFAT in cultured mammalian cell lines when over-expressed 82, 84, 85. Transgenic over-expression of RCAN1 in the heart diminished the cardiac hypertrophy triggered by expression of constitutively active calcineurin, by β -adrenergic receptor stimulation with isoproterenol, or by exercise, in each case presumably by depressing calcineurin-NFAT signaling 86. T cells from RCAN1-deficient mice showed a lowered threshold for calcineurin-dependent gene transcription, again indicating that a physiological role of RCAN1 is to limit cellular calcineurin activity 87. In this context, the induction of the RCAN1 exon 4 variant by calcineurin-NFAT signaling 88 has been interpreted as part of a negative feedback loop in which elevated calcineurin activity leads to accumulation of RCAN1 inhibitor 79, 88-90 (Figure 5F).

In contrast to the evidence that over-expressed RCANs negatively regulate calcineurin signaling, genetic studies in yeast and mice indicate that RCAN proteins expressed at endogenous levels serve to facilitate calcineurin activity 79, 89, 91-93. Thus yeast *rcn1* null mutants displayed diminished calcineurin signaling 79. RCAN1/RCAN2-deficient mice exhibited reduced NFAT transcriptional activity and decreased cardiac hypertrophic responses to pressure overload, similar to calcineurin A β -deficient mice 93. The mice also lacked the normal exercise-induced switch toward oxidative-type fibers in skeletal muscle 93, another calcineurin-dependent transcriptional response.

In yeast the stimulatory effect of Rcn1 on calcineurin requires phosphorylation of Rcn1 by Mck1, a GSK3 protein kinase, at Ser 113 within the conserved FLISPP motif that is a signature of RCAN proteins 89. The corresponding serine residue, Ser108, in mammalian RCAN1 can be phosphorylated by GSK3 94 and is required for activation of calcineurin by mammalian RCAN1 expressed in yeast 89. Phosphorylation by GSK3 is contingent in each case on a priming phosphorylation at a nearby site (Ser 117 in yeast Rcn1, Ser112 in human RCAN1) by a MAP kinase 89, 94, 95.

It is the steps following phosphorylation that are debated. Phosphorylated yeast Rcn1 is targeted for proteasomal degradation by the ubiquitin ligase SCF^{cdc4} 46, 96, prompting the suggestion that there is no stimulatory effect of RCAN proteins, only a relief of tonic calcineurin inhibition following RCAN phosphorylation 96. A second proposed mechanism that depends on relief of tonic inhibition is that phosphorylated human RCAN is sequestered in a complex with 14-3-3 protein 95. It has been argued, however, that these models are not compatible with observations that certain deletion mutants of Rcn1 exhibit apparently normal phosphorylation and degradation in yeast cells, and yet do not stimulate calcineurin activity 46. Recently, a distinct mechanism was reported in which TAK1 (TGF- β activated kinase 1) phosphorylates RCAN1 at two positions, Ser94 and Ser136, converting RCAN1 from an inhibitor to a facilitator of calcineurin-NFAT signaling, but again the basis of the facilitation remains undefined 92 (Figure 5F). Phosphorylated Rcn1 and RCAN1 are themselves substrates of calcineurin 89, 94 (Figure 5F), adding another layer of control to an already complex regulatory network.

The mechanisms of inhibition of calcineurin are less controversial. Systematic studies of truncation mutants of RCAN have mapped several regions that inhibit calcineurin 46, 79, 83, 94, 97. A PSVVVH sequence near the C terminus of RCAN competes with PVIVIT

peptide for binding to calcineurin, and, despite the substitution for three consensus residues of the PxIxIT motif, binds calcineurin with a K_d nearly comparable to that of the PRIEIT peptide 46 (Figure 4A). The PSVVVH motif is thought to suppress calcineurin signaling in cells by blocking the access of substrates to the PxIxIT-docking surface 46· 97· 98, and in this respect the inhibition by RCAN proteins parallels one mode of inhibition by AKAP79. However, there is as yet no structural explanation of how other noncontiguous inhibitory regions in RCAN interfere with calcineurin enzymatic activity *in vitro* and calcineurin signaling in cells. Some recent findings underscore the critical need to understand positive and negative regulation of calcineurin by RCAN proteins. It has been proposed that RCAN1 and the NFAT kinase DYRK1A — both encoded on chromosome 21 and increased in gene dosage in individuals with Down syndrome — together largely account for developmental anomalies observed in Down syndrome through altered NFAT signaling 21. In this case an elevated RCAN1 level is proposed to inhibit dephosphorylation of NFAT. Elevated levels of DYRK1A would act in the same direction, countering calcineurin phosphatase activity by directly phosphorylating serine residues in the conserved SPxx repeat 3 of the NFAT1 regulatory domain and priming NFAT for subsequent phosphorylation by GSK3 β and CK1 20. Indeed, in cells, DYRK1A over-expression expedites the export of NFAT from the nucleus and reduces NFAT transcriptional activity 20· 21. The effects of mild over-expression of RCAN1 and DYRK1A in Down syndrome may be amplified when diminished NFAT activation leads to diminished positive feedback of NFAT2 and NFAT4 on their own promoters 21. The hypothesis relating developmental anomalies to a gene dosage effect finds concrete support in the impaired cardiac valve development of murine embryos engineered to express modestly increased amounts of RCAN1 and DYRK1A 21. Further, the low incidence of solid tumors in individuals with Down syndrome has been attributed to attenuated VEGF-mediated angiogenic signaling via calcineurin and NFAT, and inhibition of tumor growth in mice trisomic for a large segment of murine chromosome 16 — including the region corresponding to the Down syndrome critical region on human chromosome 21 — has been traced in part to an *Rcan1* gene dosage effect 32.

Perspectives

Calcineurin was purified from brain more than three decades ago 99· 100, and was recognized almost two decades ago as the clinical target of immunosuppressive agents in transplant medicine 16· 101· 102. The downstream effector of calcineurin in T lymphocyte activation is NFAT 103· 104, and NFAT was the focus in early studies of calcineurin-substrate interactions, leading to the definition of two substrate-recognition motifs. Extension of the studies to other substrates uncovered an almost playful diversity in recognition sequences that underlies a serious strategy for directing signaling within the cell. Interestingly, it now appears that calcineurin binding to key scaffold proteins and regulators builds on the same basic interactions, expanding them with additional interactions or additional partner proteins as needed.

Several fundamental questions remain to be addressed. The pioneering work of Klee and colleagues 9·11 sketched out the steps in activation of calcineurin, yet a concrete structural picture of these steps is still elusive. Given the fundamental role of Ca²⁺ oscillations and Ca²⁺ microdomains in cellular signaling, rapid assays of local calcineurin activity are needed to follow the timing and localization of calcineurin activation in cells with high resolution. There is certainly more to be learned about calcineurin-substrate docking interactions, and about the mechanics of signaling by calcineurin anchored by AKAP79 or other targeting proteins. The pivotal role of RCAN proteins in integrating converging cellular pathways that modulate calcineurin activity remains an active area of research. It is from these disparate pieces that a coherent picture of calcineurin signaling will be built.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was funded by NIH grants AI84167 and AI40127 (to A.R. and P.G.H.).

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Figure 1.

Calcineurin domain organization and proposed mechanism of activation. **(a)** Regional organization of calcineurin. Here and in Figure 1b, calcineurin A and calcineurin B are color-coded in shades of red and green, respectively. **(b)** The proposed mechanism of activation of calcineurin. In this widely accepted model of calcineurin activation [10–11], Ca^{2+} occupancy of the low affinity sites on calcineurin B causes dissociation of the calmodulin-binding region of calcineurin A from the calcineurin B-binding region and causes the transition from Form I to Form II, facilitating the subsequent binding of calmodulin (Form III), which leads to displacement of the autoinhibitory peptide and full calcineurin activation (Form IV). The structure of the calcineurin A regulatory region between the calcineurin B-binding helix and the autoinhibitory peptide in resting calcineurin (Form I) remains to be determined. In Form IV, the portions of calcineurin A between the calcineurin B-binding helix and the calmodulin binding site and C-terminal to the calmodulin binding site are depicted as random coil, but may in fact be structured. Recent

evidence indicates that calmodulin contacts with calcineurin A may be more extensive than those illustrated here for a typical calmodulin partner 12, 13. The depictions of calcineurin are modified from PDB entry 1AUI 6, and the depiction of calmodulin is based on its complex with a Dap kinase peptide, PDB entry 1YR5. The figure was generated with Chimera 105.

Figure 2.

Functional organization of the N-terminal regulatory region of NFAT. The casein kinase 1 docking site (FxxxF) and the calcineurin docking site (PxIxIT) are highlighted. The red circles denote phosphoserine residues. TAD: transactivation domain; SRR: serine rich region; SP: SPxx repeat; NLS: nuclear localization signal.

Figure 3 (a)

Figure 3.

Crystal structure of calcineurin in complex with PVIVIT peptide. **(a)** An extended PVIVIT peptide (cyan) is aligned parallel to β -strand 14 as a continuation of a core β -sheet (red) of calcineurin A. Calcineurin A (CNA) is colored yellow, and calcineurin B (CNB), green. **(b)** A close-up view of the docking interaction between PVIVIT peptide and calcineurin A. Residues at the conserved P_xI_xI_T positions make specific contacts with calcineurin A, which include close van der Waals packing against nonpolar recesses on the surface of calcineurin A and the indicated hydrogen bond between Thr11 of PVIVIT and N330 of calcineurin A. For clarity, the PVIVIT peptide residues are labeled in a separate depiction at right. The figures were generated with PyMol 106 from coordinates in PDB entry 2P6B 36.

Consensus	P x I x I T						
VIVIT	P	V	I	V	I	T	0.5 μM
NFAT1	P	R	I	E	I	T	25 μM
TRESK (human)	P	Q	I	I	I	S	5 μM
TRESK (mouse)	P	Q	I	V	I	D	10 μM
Crz1	P	I	I	S	I	Q	15 μM
Slm1	P	N	I	Y	I	Q	40 μM
Slm2	P	E	F	Y	I	E	20 μM
Hph1	P	V	I	A	V	N	250 μM
RCAN1 (human)	P	S	V	V	V	H	45μM
Rcn2	P	S	I	T	V	N	100μM
Rcn1	G	A	I	T	I	D	~1mM
AKAP79 (human)	P	I	A	I	I	T	0.5μM
AKAP5 (lizard)	G	I	M	I	M	T	ND

Consensus	L x V P					
NFAT1	I	L	L	V	P	P
NFAT2	Y	L	A	V	P	Q
NFAT3	Y	L	A	V	P	S
NFAT4	F	L	S	V	P	S
KSR2	T	L	S	V	P	R
Rcn1	Y	L	K	V	P	E

Figure 4.

Conserved calcineurin docking sequences identified among calcineurin substrates and interactors. (a) PxIxIT motif sequences. In addition to PVIVIT and the NFAT1 docking site, the sequences shown are from the K⁺ channel TRESK; the *S cerevisiae* transcription factor Crz1 and other yeast proteins involved in adaptation to physiological stress; the calcineurin

regulatory protein RCAN1 and its *S cerevisiae* homologues; and the scaffold protein AKAP79, also termed AKAP5, and its *A carolinensis* homologue encoded by Ensembl transcript ENSACAT00000015823. The measured K_{ds} for PxIxIT peptide binding to calcineurin are shown next to the corresponding peptide sequences 36, 37, 46, 47. ND, not determined. **(b)** LxVP motif sequences. The rank order of calcineurin binding to the NFAT LxVP motifs was estimated indirectly to be NFAT3 ~ NFAT4 > NFAT2 >> NFAT1 42.

Figure 5 - (a)

Figure 5 - (b)

Figure 5 - (c)

Figure 5 - (d)

Figure 5 - (e)

Figure 5 - (f)

Figure 5.

Cartoon representations of the signaling pathways discussed in this review. **(a)** The calcineurin-NFAT pathway in T cells. Ligand binding to the T cell receptor triggers an initial cytoplasmic Ca^{2+} signal via phospholipase $\text{C}\gamma$, inositol 1,4,5-trisphosphate (IP3), and release of Ca^{2+} from ER stores; and a sustained Ca^{2+} signal via the ER Ca^{2+} sensor STIM1 and plasma membrane ORAI1 calcium release-activated calcium (CRAC) channels. The channels providing a sustained or repetitive calcium signal can be different in other types of cells. Elevated cytoplasmic Ca^{2+} activates calmodulin and calcineurin, and calcineurin dephosphorylates the transcription factor NFAT, causing NFAT to translocate into the nucleus and initiate gene transcription. **(b)** The calcineurin-Crz1 pathway in yeast. When

yeast grow under conditions of physiological stress, Ca^{2+} is released into the cytoplasm from the vacuole or other sources, and calcineurin is activated. The transcription factor Crz1 is dephosphorylated, translocates into the nucleus, and activates genes involved in the stress response. **(c)** Background K^+ conductance in neurons. In response to elevated cytoplasmic Ca^{2+} , calcineurin dephosphorylates the 2PK channel TRESK, resulting in increased plasma membrane K^+ conductance, and lowering neuronal excitability or firing rate. The cartoon depicts only one of the two TRESK monomers that form the channel. **(d)** Crosstalk between calcium signaling and MAP kinase signaling. In response to calcium signals, calcineurin dephosphorylates the scaffold protein KSR2 at phosphoserine residues including a 14-3-3 protein target site. KSR2 translocates to the plasma membrane and facilitates the activation of ERK by scaffolding Raf-MEK-ERK signaling complexes. **(e)** Long-term depression (LTD) in hippocampal neurons. AKAP79 positions calcineurin at the postsynaptic density through an association with other scaffold proteins such as PSD-95 and SAP-97. Calcineurin, thus positioned, responds to NMDA receptor activation and the resulting Ca^{2+} influx by dephosphorylating Ser845 of GluR1 and triggering the internalization of AMPA receptors from the postsynaptic membrane. **(f)** Negative and positive regulation of calcineurin by RCAN1. The expression of RCAN1 is induced by calcineurin-NFAT signaling, and unphosphorylated RCAN1 functions in a feedback loop to inhibit calcineurin. Phosphorylation by the kinases GSK3 (glycogen synthase kinase 3) or TAK1 (TGF- β activated kinase 1) converts RCAN1 into a facilitator of calcineurin signaling. GSK3 phosphorylates RCAN1 at Ser108 after priming phosphorylation by a MAP kinase at Ser 112. TAK1 phosphorylates RCAN1 at Ser94 and Ser136. Calcineurin dephosphorylates the GSK3 target site, presumably damping the action of the kinase and the phosphorylated RCAN1 (i). Calcineurin is not known to dephosphorylate the TAK1 target sites, but activated calcineurin dephosphorylates and inhibits the TAK1/TAB1/TAB2 kinase complex, thereby opposing its action on RCAN1 (ii).

Table 1

Examples of calcineurin substrates and binding partners*

Transcriptional regulation	REFS
NFAT	S1
TORC2/CRTC2	S2
Elk-1	S3
Retinoblastoma protein (Rb)	S4
MEF2A	S5, S6
Receptors and ion channels	
TRESK	S7, S8
TRPV1	S9, S10
AMPA receptor	S11-S13
NMDA receptor	S14-S16
GABA(A) receptor	S17, S18
IP3R	S19, S20
Kv2.1	S21
KATP	S22
Scaffold and regulatory proteins	
PKA RII subunit	S23
DARPP-32	S24
Inhibitor-I	S25
RCAN	S26
Cabin/Cain	S27, S28
KSR2	S29
RACK1	S30
CIB1	S31
Membrane trafficking	
Dephosphins	S32, S33
Cytoskeleton	
MAP2	S34, S35
Tubulin	S34
Tau	S34
Slingshot1L	S36
Cell cycle and apoptosis	
Fizzy/Cdc20	S37, S38
BAD	S39
Drp1	S40, S41
Unknown function	
CRHSP-24	S42
Yeast stress responses	
Crz1	S43, S44
Hph1	S45

Slm1 and Slm2	S46
Targeting proteins	
AKAP79/150	S47
Calsarcin-1, -2 and -3	S48
Muscle LIM protein (MLP)	S49

* Table reproduced in Supplementary materials with full reference list.