## Targeting the Calcineurin-NFAT Interaction by Solution NMR Spectroscopy

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Targeting the Calcineurin-NFAT Interaction
by Solution NMR Spectroscopy

A dissertation presented
by
Shuai Li
to
The Department of Chemistry and Chemical Biology
in partial fulfillment of the requirements
for the degree of
Doctor of Philosophy
in the subject of
Chemistry

Harvard University
Cambridge, Massachusetts
January, 2015
Targeting the Calcineurin-NFAT Interaction by Solution NMR Spectroscopy

Abstract

The serine/threonine phosphatase calcineurin (Cn) targets the nuclear factors of activated T cells (NFATs) that activate cytokine genes. Calcium influx activates Cn to dephosphorylate multiple serine residues within the ~200 residue NFAT regulatory domain, which triggers joint nuclear translocation of NFAT and Cn. The dephosphorylation process relies on the interaction between Cn and the conserved motifs PxIxIT and LxVP, which are located N- and C-terminal to the phosphorylation sites in NFAT’s regulatory domain. Here, we show that an NFATc1-derived 15-residue peptide segment containing the conserved LxVP motif binds to an epitope on Cn’s catalytic domain (CnA), which overlaps with the previously established PxIxIT binding site on CnA and is distant to the regulatory domain (CnB). Both NFAT motifs partially compete for binding but do not fully displace each other on the CnA epitope, revealing that both segments bind simultaneously to the same epitope on the catalytic domain.

The Cn-NFAT signaling pathway has been well credentialed as a potential target in the treatment of graft transplant rejection, autoimmune diseases and cardiovascular disorders, and is a common target of immunosuppressive drugs cyclosporin A (CsA) and FK506. Although effective in the disruption of Cn phosphatase activity, CsA and FK506 also result in undesired side effects and toxicity, promoting the discovery of alternative inhibitors which can selectively inhibit the Cn-NFAT interaction without altering the functioning of other Cn substrates. Several peptides directly inhibiting NFAT binding to Cn, as well as several small molecule inhibitors of
NFAT-Cn association targeting an allosteric site on Cn have been developed previously. However, to-date there have been no reported small molecule inhibitors directed against the PxIxIT-binding site on Cn. Here, we report the fragment-based discovery of several direct-acting small molecule inhibitors targeting the Cn-NFAT interaction, and show that they selectively inhibit NFAT-dephosphorylation and NFAT-mediated gene expression without affecting Cn phosphatase activity against other substrates. We further demonstrate that the binding site for these inhibitors coincides with the core PxIxIT-binding site on Cn. The development of these inhibitors provides a new tool for probing Cn-NFAT signaling, further optimization of which may provide an alternative strategy for immunosuppressive therapy.
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<td>activator protein 1</td>
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<tr>
<td>βME</td>
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<td>calmodulin</td>
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<td>DTT</td>
<td>dithiothreitol</td>
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<td>DYRK</td>
<td>dual-specificity tyrosine-phosphorylation regulated kinase</td>
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<td>1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide</td>
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<td>FBDD</td>
<td>fragment-based drug design</td>
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<td>Acronym</td>
<td>Definition</td>
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<td>IPTG</td>
<td>isopropylthio-E-D-galactoside</td>
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<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
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<td>MAP3K</td>
<td>mitogen-activated protein kinase kinase kinase</td>
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<td>nuclear factor of activated T cells</td>
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<td>NHR</td>
<td>NFAT homology region</td>
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<td>nuclear localization region</td>
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<td>NOE</td>
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<td>PLCγ</td>
<td>phospholipase Cγ</td>
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<td>PMA</td>
<td>phorbol 12-myristate 13-acetate</td>
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<tr>
<td>PMSF</td>
<td>phenylmethylsulfonylfuranelide</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>pNPP</td>
<td>p-nitrophenyl phosphate</td>
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<tr>
<td>PP1</td>
<td>protein phosphatase 1</td>
</tr>
<tr>
<td>PP2A</td>
<td>protein phosphatase 2A</td>
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<tr>
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<td>protein phosphatase 2B</td>
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<td>PP3</td>
<td>protein phosphatase 3</td>
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<td>PPlase</td>
<td>peptidyl-prolyl isomerases</td>
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<td>PRE</td>
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<tr>
<td>PVIVIT</td>
<td>14-mer VIVIT peptide</td>
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<tr>
<td>RCAN</td>
<td>regulator of calcineurin</td>
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<tr>
<td>RHR</td>
<td>Rel-homology region</td>
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<tr>
<td>RII</td>
<td>RII regulatory subunit of cAMP-dependent protein kinase</td>
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<td>RTK</td>
<td>receptor tyrosine kinases</td>
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<tr>
<td>SAR</td>
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<td>SP</td>
<td>serine-proline-rich repeats</td>
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<td>SPR</td>
<td>surface plasmon resonance</td>
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<td>SRR</td>
<td>serine-rich regions</td>
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<td>STD</td>
<td>saturation transfer difference</td>
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<td>STIM</td>
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<td>TAD</td>
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<td>TCR</td>
<td>T-cell receptors</td>
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<td>tonicity element binding protein</td>
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CHAPTER 1: Structural Delineation of the Calcineurin-NFAT Interaction by NMR

1.1 Introduction

1.1.1 The biological background of calcineurin

Calcineurin (Cn), also known as protein phosphatase 2B (PP2B) or protein phosphatase 3 (PP3) is a Ca^{2+}- and calmodulin-dependent serine/threonine protein phosphatase implicated in a number of biological contexts, such as regulation and development of the immune, nervous, cardiovascular and musculoskeletal systems, as well as in apoptosis and necrosis\textsuperscript{1-6}. Cn is a heterodimeric protein consisting of a catalytic subunit A (CnA, 58-64kDa) that contains an active site dinuclear metal center (Fe^{3+} and Zn^{2+}), and a tightly associated, myristoylated, Ca^{2+}-binding subunit B (CnB, 19kDa). In addition to the phosphatase catalytic domain, CnA contains a CnB-binding domain, a calmodulin-binding domain (Caε) as well as a carboxy-terminal autoinhibitory domain (AI) (Figure 1.1A)\textsuperscript{4,7,8}. CnB contains four EF-hand structural motifs that bind four Ca^{2+} ions. EF-hands 3 and 4 in the C-terminal lobe of CnB bind Ca^{2+} with higher affinity, and are often referred to as structural sites that serve to stabilize the heterodimeric structure of Cn. Ca^{2+} binding sites 1 and 2 in the N-terminal lobe are of lower affinity, and play a regulatory role in Cn activation (Figure 1.1B)\textsuperscript{9}. The primary sequence of both subunits and the heterodimeric quaternary structure is highly conserved from yeast to mammals.

A mechanism for Cn activation, involving sequential changes in its conformation, has been proposed based on the cumulative biochemical and structural data available (Figure 1.2)\textsuperscript{9}. Briefly, at low Ca^{2+} levels, only the two higher affinity “structural” Ca^{2+} binding sites are occupied in CnB. The CaM-binding region of CnA forms intramolecular interactions with the...
CnB binding helix of CnA, and the AI domain occupies the active site, locking Cn in an “inactive” conformation. As the intracellular Ca$^{2+}$ concentration rises, Ca$^{2+}$ populates also the two lower affinity EF-hands on CnB, which causes dissociation of the CaM binding region from the CnB binding helix of CnA, and subsequent binding of CaM. This binding in turn causes displacement of the AI peptide from the active site and results in the transition of Cn from “inactive” conformation to a “fully active” confirmation.

Figure 1.1 Functional architecture of calcineurin. (A) Primary sequence and domain structure of CnA. The amino acid sequence represents the rat CnAĮ. Adapted from Rusnak et al (2000). (B) Regional organization of CnB. Adapted from Li et al (2011).
Figure 1.2 Proposed mechanism of activation of calcineurin. Transition from Form I to Form II: binding of Ca^{2+} to low affinity sites on CnB (green) frees the CaM binding domain of CnA (red). Form III: CaM binds to Cn. Form IV: The autoinhibitory peptide is released from the active site causing full activation of Cn for substrate binding. Adapted from Li et al (2011)^9.

Unlike the related protein phosphatases PP1 and PP2A, which can form up to hundreds of holoenzymes by swapping their respective regulatory domains, Cn has one invariable regulatory domain (CnB) and can only form one holoenzyme. Therefore, Cn has a relatively narrow substrate specificity as compared to other serine/threonine protein phosphatases (Table 1.1)^8. Phosphoproteins listed below, such as inhibitor 1^10, the RII subunit of cAMP-dependent protein kinase^11, are preferentially dephosphorylated by Cn, whereas others such as casein, synapsin 1, and calmodulin kinase II are dephosphorylated at much slower rates or not at all^10. The substrate
specificity of Cn is not due only to a specific sequence but rather is determined by a variety of primary and higher-order structural features conferring to it an overall selectivity that is different from those of other protein phosphatase.

Table 1.1 Substrate specificity of calcineurin

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<th>$k_{cat}$</th>
<th>$k_m$</th>
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<td>Inhibitor 1$^a$</td>
<td>2.8</td>
<td>0.003</td>
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<tr>
<td>RII subunit</td>
<td>2.7</td>
<td>0.02</td>
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<tr>
<td>Neurogranin</td>
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<td>0.013</td>
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<td>Phosphorylase kinase (α subunit)$^a$</td>
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<td>0.006</td>
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<td>Neuromodulin</td>
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<td>Microtubule-associated proteins</td>
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<td>Tau factor</td>
<td>0.6–0.8</td>
<td>0.002</td>
</tr>
<tr>
<td>DARPP-32$^a$</td>
<td>0.2</td>
<td>0.014</td>
</tr>
<tr>
<td>DLDVPIPGRFDRRVSVAE$^b$</td>
<td>2.2</td>
<td>0.026</td>
</tr>
<tr>
<td></td>
<td>5.9$^c$</td>
<td>0.023</td>
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<tr>
<td>DLDVPIPGRFDRRVYVAE</td>
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<tr>
<td></td>
<td>0.3$^c$</td>
<td>0.003</td>
</tr>
<tr>
<td>$p$-Nitrophenyl phosphate</td>
<td>26</td>
<td>23.0</td>
</tr>
</tbody>
</table>

$^a$ Values of $k_{cat}$ measured in the absence of added metal are underestimated.

$^b$ Synthetic peptide routinely used for calcineurin assays. Values of 12 s$^{-1}$ and 0.1 mM have been reported recently (62, 95). For comparison at pH 7.5 in the absence of added metal the $k_{cat}$ value for the dephosphorylation of the peptide substrate by crude calcineurin is 40 s$^{-1}$.

$^c$ Assayed in the presence of 1 mM Ni$^{2+}$.

A model for the catalytic mechanism of Cn has been proposed based on the available biochemical and chemical data (Figure 1.3). At the active site of Cn, the two metal ions, Fe$^{3+}$ and Zn$^{2+}$, are coordinated by several catalytic residues: Fe$^{3+}$ bridges Asp90, His92 and Asp118 of CnA, and Zn$^{2+}$ is coordinated by Asp118, Asn150, His199, and His 281 of CnA. In this
dephosphorylation reaction, His-151 serves both as a general base and as a general acid. Briefly, in the first step of this mechanism, His-151 functions as a general base to remove the proton from the Fe$^{3+}$-activated water molecule. In the transition state, the Fe$^{3+}$ functions as a Lewis acid to lower the pKa of the water molecule and activates it for nucleophilic attack of the phosphate ester. Following P-O bond scission, the negatively charged leaving group is neutralized by protonation and leaves the active site. In the product-inhibited state, the orthophosphate molecule bridges the two metal ions of the active center, which is then regenerated as the bound phosphate is replaced by another water molecule.

**Figure 1.3 Proposed catalytic mechanism for calcineurin.** (A) His-151 deprotonates a Fe$^{3+}$-bound water molecule to form hydroxide. (B) In the transition state, the leaving group is protonated by His-151 and leaves the active site. (C) In the product-inhibited state, the phosphate molecule bridges the two metal ions. (D) The enzyme is regenerated as the phosphate exchanges with a water molecule. Adapted from Rusnak et al (2000).
1.1.2 The biological background of NFAT

The nuclear factors of activated T cells (NFATs) are transcription factors that regulate numerous genes upon T cell activation\textsuperscript{13-15}. The NFAT proteins upregulate cytokines required for T cell proliferation and stimulate cell growth and differentiation during the T cell response\textsuperscript{16}. The NFAT family encompasses five proteins evolutionarily related to the Rel/NFκB family, namely, NFAT1 (also known as NFATc2 or NFATp), NFAT2 (NFATc1 or NFATc), NFAT3 (NFATc4), NFAT4 (NFATc3 or NFATx) and NFAT5/TonEBP (tonicity element binding protein, also known as OREBP). Four of the five NFAT proteins, NFAT1-NFAT4, are part of the T cell response mechanism (Table 1.2)\textsuperscript{17}, but the NFAT proteins also take part in different regulatory mechanisms of other immune cells in the vertebrate immune system, such as dendritic cells, B cells, NKT cells, megakaryocytes, as well as other systems of cell differentiation outside the immune system, such as fiber-type specification in differentiated skeletal muscle, cardiac valve development, and osteoclast differentiation\textsuperscript{13,18-21}.

Table 1.2 NFAT family of transcription factors\textsuperscript{17}

<table>
<thead>
<tr>
<th>NFAT family member</th>
<th>Alternative names</th>
<th>Regulation</th>
<th>Expression in the immune system</th>
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<tr>
<td>NFAT1</td>
<td>NFATc2 and NFATp</td>
<td>Calcium–calcineurin</td>
<td>Yes</td>
</tr>
<tr>
<td>NFAT2</td>
<td>NFATc1 and NFATc</td>
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<td>Yes</td>
</tr>
<tr>
<td>NFAT3</td>
<td>NFATc4</td>
<td>Calcium–calcineurin</td>
<td>No</td>
</tr>
<tr>
<td>NFAT4</td>
<td>NFATc3 and NFATx</td>
<td>Calcium–calcineurin</td>
<td>Yes</td>
</tr>
<tr>
<td>NFAT5</td>
<td>TonEBP and OREBP</td>
<td>Osmotic stress</td>
<td>Yes</td>
</tr>
</tbody>
</table>
The highly conserved C-terminal half of the 930-residue NFAT contains two domains, the N-terminal, and C-terminal domains of NFAT Rel homology regions (RHR), which are involved in binding DNA and activator protein 1 (AP1), respectively, and have been characterized structurally (Figure 1.4)\textsuperscript{22-25}. Dimers of Fos and Jun form quaternary complexes with NFAT and DNA, which have an extensive network of protein-protein contacts, contribution to their stability and cooperative nature. AP1 proteins are the main transcriptional partners of NFAT during T-cell activation. Other than Fos and Jun proteins, there are several other families of NFAT transcriptional partners, including MAF and GATA-binding proteins\textsuperscript{17}. Binding to different transcription factors and coactivators allows NFAT to integrate different signaling pathways and activate specific sets gene expressions in response to various stimuli.

Figure 1.4 The NFAT-AP1-DNA complex. (A) X-ray crystal structure of the NFAT-AP1-DNA complex. The N-terminal domain (RHR-N) of the NFAT RHR is shown in yellow; RHR-C is in green; Fos is red; Jun is blue (PDB entry: 1A02). Adapted from Chen et al (1998)\textsuperscript{22}. (B) Solution NMR structure of the core NFATc1/DNA complex. Core NFATc1 is shown in yellow and the 12-mer DNA sequence containing murine ARRE2 site is shown in black (PDB entry: 1A66)\textsuperscript{25}.
The N-terminal half known as the NFAT homology region (NHR) contains a transactivation domain (TAD) and a regulatory domain (Figure 1.5). The regulatory domain of approximately 200 residues is moderately conserved among NFAT proteins and contains a nuclear localization region (NLS), three serine-proline-rich repeats (SP) and two serine-rich regions (SRR), as well as the docking sites for Cn and NFAT kinases. Phosphorylation status of the SP and SRR segments, which reflects the intensity of Ca\(^{2+}\)/Cn signaling and the activities of NFAT kinases, controls the subcellular distribution, DNA binding affinity, as well as transcriptional activity of NFAT by yet unknown mechanisms.

Figure 1.5 Location of functional segments in the NFAT homology region. (A) Schematic representation of NFAT N-terminal sequence (amino acid residues 1-400). The following abbreviations are used to represent the functional segments: Transactivation Domain, TAD; the conserved binding motifs of Cn PxIxIT and LxVP, CN-1 and CN-2; Serine rich regions, SRR-1 and SRR-2; Serine-Proline rich repetition segments, SP1, SP2 and SP3; Nuclear Localization Sequence, NLS. (B) NFAT conserved Cn-binding motifs, PxIxIT and LxVP, primary amino acid sequences throughout the different human NFAT1-4 isoforms. Note that other than the conserved PxIxIT and LxVP motifs, the flanking WxK motif is also moderately conserved.
1.1.3 The Ca\(^{2+}\)-calcineurin-NFAT signaling pathway

In resting T cells, the regulatory domain of NFAT is heavily phosphorylated, preventing NFAT from entering into the nucleus. Four kinases are known to phosphorylate NFAT, which
include, casein kinase 1 (CK1, also known as CSK1A1), glycogen synthase kinase 3 (GSK3), protein kinase A (PKA), and the dual specificity tyrosine phosphorylation kinase (DYRK)\textsuperscript{21,27-30}. Among these four kinases, GSK3 is an export kinase, responsible for the rephosphorylation of NFAT inside the nucleus and its relocation to the cytoplasm. By contrast, a maintenance kinase keeps NFAT proteins in a fully phosphorylated state and prevents their translocation to the nucleus. The kinase type, substrate specificity and phosphorylation sites of several NFAT kinases are listed in Table 1.3\textsuperscript{21}. Selective regulation of each NFAT protein by multiple kinases allows continuous modulation of NFAT activities.

**Table 1.3 NFAT kinases\textsuperscript{21}**

<table>
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<th>NFAT kinase</th>
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<th>Substrate</th>
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<td>GSK3</td>
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<td>NFAT1</td>
<td>SP2</td>
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<td></td>
<td>NFAT2</td>
<td>SP2 and SP3</td>
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<tr>
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<td>Export and maintenance</td>
<td>NFAT1</td>
<td>SRR1</td>
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<tr>
<td>DYRK1</td>
<td>Export</td>
<td>NFAT1 and NFAT2</td>
<td>SP3</td>
</tr>
<tr>
<td>DYRK2</td>
<td>Maintenance</td>
<td>NFAT1 and NFAT2</td>
<td>SP3</td>
</tr>
</tbody>
</table>

The distinguishing feature of NFAT is its activation by Ca\textsuperscript{2+} and the Ca\textsuperscript{2+}-dependent phosphatase Cn. In the cytoplasm, NFAT is activated by cell surface receptors that are coupled to “store-operated” Ca\textsuperscript{2+} entry via phospholipase C\textgreek{y} (PLC\textgreek{y}) (Figure 1.7)\textsuperscript{13,21,38}. Briefly, activation of PLC\textgreek{y} by ligand binding to cell surface receptors, such as, T-cell receptors (TCR) and receptor tyrosine kinases (RTK), leads to the production of inositol-1,4,5-trisphosphate (InsP3), which in turn results in the release of Ca\textsuperscript{2+} from endoplasmic reticulum (ER) Ca\textsuperscript{2+} stores. This Ca\textsuperscript{2+} depletion from ER is sensed by stromal interaction molecules (STIM1 and STIM2) on the ER
membrane, triggering influx of extracellular Ca\(^{2+}\) into the cytosol. Rising Ca\(^{2+}\) levels, such as those caused by mitogens, activate calcineurin, which then dephosphorylates multiple phosphoserines on NFAT and initiates a joint nuclear translocation event of both NFAT and calcineurin (Figure 1.6). The nuclear entry event of NFAT also involves other scaffold proteins, such as importin-β and tubulin-α, which function as adaptors. In the nucleus, NFAT assembles with other transcription factors and coactivators, such as AP1, to initiate gene expressions and the immune response. NFAT proteins are rephosphorylated and inactivated by NFAT kinases.

Figure 1.7 The calcineurin-NFAT signaling pathway. Activation of PLCγ caused by ligand binding to the TCRs and RTKs leads to the production of InsP3, further resulting in the release of Ca\(^{2+}\) from ER Ca\(^{2+}\) stores. This Ca\(^{2+}\) depletion in turn activates STIM1 or STIM2 and causes influx of extracellular Ca\(^{2+}\) into the cytosol and activation of the Ca\(^{2+}\)-Cn-NFAT signaling pathway. Adapted from Müller et al (2010).
1.1.4 The calcineurin-NFAT PxIxIT interaction

Efficient dephosphorylation of NFAT by Cn requires a docking interaction between the two. Two distinct Cn-binding segments have been identified in the NHR of all NFAT isoforms involved in T cell response (NFAT1-NFAT4), which contain the conserved PxIxIT and LxVP motifs and are labeled CN-1 and CN-2, respectively, as shown in Figure 1.5. The NHR of NFAT was found unstructured, both in its active and inactive states\textsuperscript{39}.

The major docking site for Cn, CN-1, which is located in the N-terminal regulatory domain of NFAT, was discovered first and is highly conserved among NFAT isoforms\textsuperscript{40}. Peptides containing the endogenous PxIxIT sequence (PRIEIT in NFAT1 and NFAT2) bind Cn with low-to mid-micromolar affinities\textsuperscript{40}. The nonconserved amino acids (x) in positions 2 and 4 of the PxIxIT motif modulate the Cn binding affinities, and these residues are thought to cause fine-tuning of the Cn-NFAT interaction under different physiological conditions, as well as prevent constitutive activation of NFA\textsuperscript{43}. Affinity-driven selection of peptide inhibitors resulted in an optimized PxIxIT peptide that binds Cn with a 200 nM dissociation constant (K\textsubscript{d}). In this more potent NFAT inhibitors, residues in positions x were optimized and selected as valines yielding the PVIVIT sequence\textsuperscript{9,41}.

Structures of heterodimeric CnAB and catalytic subunit CnA in complex with a 14-mer NFAT peptide containing the PVIVIT sequence have been determined with X-ray crystallography\textsuperscript{42} and solution nuclear magnetic resonance (NMR) spectroscopy (Figure 1.8)\textsuperscript{43}, respectively. The PxIxIT binding site is well-established, which is located on the catalytic subunit of Cn, away from the metal ions in the active center and far from CnB. Specifically, PVIVIT augments the 4-3-2-13-14 β-sheet of CnA by backbone hydrogen bond\textsuperscript{42}.
1.1.5 The calcineurin-NFAT LxVP interaction

More recently, a second Cn-binding segment was discovered that contains the LxVP sequence. This sequence is only moderately conserved among the NFATs family (Figure 1.5B) and is less understood than the NFAT-PxIxIT motif. Located C-terminal to NFAT regulatory domain (Figure 1.5A), the LxVP peptide binds Cn with a similar affinity as that of the PxIxIT segment\(^\text{26}\). It has been reported that LxVP peptide in NFATc1 (NFAT2) inhibits NFAT-dependent transcription and modifies the enzymatic activity of calcineurin\(^\text{45}\). Furthermore, it was observed that blocking the interaction of Cn with the PxIxIT motif alone does not prevent NFAT nuclear translocation. Thus, it was concluded that the interaction of Cn with the LxVP motif is necessary for an efficient NFAT dephosphorylation process\(^\text{39,44}\).

![Figure 1.8 Interaction between calcineurin A and VIVIT peptide.](image)

(A) The solution NMR structure of CnA in complex with VIVIT peptide (red). (B) Residues of VIVIT peptide (right) forms hydrogen bonds with residues Met329 and Ile331 in the β14 strand of CnA. (C) PVIVIT is accommodated in the hydrophobic pockets of CnA separated by residues Leu275, Tyr288, Met290, and Phe299. Adapted from Takeuchi et al (2007)\(^\text{43}\).
Figure 1.9 Proposed LxVP binding site on calcineurin A/B interface. (A) Overview of the crystal structure of calcineurin in complex with A238L. The PxIxIT and LxVP binding pockets are highlighted in yellow and green, respectively. (B) Cartoon representation of the Cn-A238L complex. Adapted from Grigoriu et al (2013)\textsuperscript{46}. (C) Cn residues putatively involved in the interaction with NFAT2-LxVP peptide as identified by in silico docking. The in silico modeling locates the NFAT2-LxVP peptide (sticks) in a hydrophobic groove (blue) comprising amino acids from both Cn subunits. Adapted from Rodriguez et al (2009)\textsuperscript{45}.
Recently, it was suggested based on mutation studies and docking calculations that a peptide containing the NFAT-LxVP motif interacts with Cn at a region near the interface of CnA and CnB (Figure 1.9C)\(^5\). An LxVP binding site was also suggested based on X-ray co-crystal structure of CnAB with the viral inhibitor protein A238L. In this complex, A238L binds to the CnA and CnB interface with an FLCVK motif (Figure 1.9A and B)\(^6\). However, as we can see, besides the P to K replacement, the FLCVK segment also lacks the flanking residues, WxK, found in NFAT’s LxVP segments (Figure 1.5), which were recently shown to be important for NFAT activity\(^5\). Therefore, it is not clear where the LxVP binding site is located on Cn, and how NFAT interacts with Cn using both PxIxIT and LxVP motifs. A significant part of this dissertation is devoted to the structural delineation of the Cn-NFAT interaction, in particular, the Cn-LxVP interaction.

1.2 Structure of calcineurin A subunit

1.2.1 NMR resonance assignments of calcineurin A

To obtain a detailed picture of the Cn-NFAT interaction, we have carried out NMR mainchain assignment of CnA (2-347) in unligated form, as well as, in complex with the 14-amino-acid-residue peptide containing the PVIVIT sequence\(^4\). Although a complete assignment was not possible mainly due to the paramagnetic line broadening induced by the Fe\(^{3+}\) in the CnA catalytic center, the assignment was extensively verified by amino-acid selective labeling of Arg, Leu, Lys, and Val, which cover more than one third of all the CnA residues. Nevertheless, the core PVIVT binding site was successfully assigned and the assignments were used to successfully determine the solution NMR structure of the CnA-PVIVIT peptide complex\(^4\) and provide the basis for investigation of the interactions of CnA with physiological interaction partners, such as
the LxVP motif of NFAT, and small molecules that disrupt the Cn-NFAT interaction. The materials and experiments are as follows:

CnAα, comprising residues 2-347 with substitutions Y341S, L343A, and M347D, was produced as a cleavable GST fusion protein in Rosetta (DE3) cells. Cleavage of the fusion protein with PreScission protease produces CnA (2-347) with an additional GPLG sequence at its N-terminus. The CnA obtained is enzymatically active as described previously. The [U-2H, 13C, 15N] CnA was obtained by growing E. coli in modified M9 Celtone medium, which consists of 1 kg/L 99.8% D2O, 8.5 g/L Na2HPO4, 3 g/L KH2PO4, 0.5 g/L NaCl, 40 mg/L carbenicillin, 15 mg/L chloramphenicol, 1 g/L 15NH4Cl (99.9% enriched), 2 g/L 2H6 13C-glucose (97% enriched), 1 g/L 2H (>97% enriched), 13C (>97% enriched), 15N (>98% enriched) Celtone® Base Powder, supplemented with 2 mM MgSO4, 0.1 mM CaCl2, 10 mg/L ZnSO4, and 10 mg/L FeCl3. To obtain selectively labeled CnA, E. coli was grown in modified M9 Celtone medium, and desired amino acids are added after induction of protein expression at 7-10 times the amount present in the Celtone media (Spectra Stable Isotopes).

After reaching an OD600 = 0.6, protein expression was induced by the addition of 1 mM IPTG at 37 °C. The E. coli cells were harvested after 36-48 hr of IPTG induction for uniformly labeled samples, and after 24 hr for selectively labeled samples. The harvested cells were resuspended in 40 ml of PBS with 2mM dithiothreitol (DTT), and 0.2 mg mL−1 phenylmethylsulfonylfluorolide (PMSF) at 4 °C. The cells were disrupted by sonication and the insoluble fraction was removed by centrifugation at 15000 g for 20 min. CnA was purified from the supernatant by Glutathione Sepharose 4 Fast Flow. CnA was eluted with 40 ml of PBS containing 2 mM DTT and 25 mM of reduced glutathione. PreScission protease was added to the concentrated elution fraction, and the solution was dialyzed against 1L of PBS with 2 mM
DTT for 15 hr at 4 °C. The digested solution was passed through a PD-10 desalting column and Glutathione Sepharose 4 Fast Flow to remove PreScission™ protease and the cleaved GST tag. The elution protein was further purified with Superdex75 size-exclusion column. A typical final yield of CnA was 2 mg/L culture.

After obtaining the isotope-labeled CnA, NMR experiments were performed to achieve the mainchain assignment of CnA. All NMR experiments were performed on a Bruker Avance 750 MHz spectrometer equipped with a cryogenic probe at 298 K. All spectra were collected using 0.4-0.6 mM protein in 10 mM sodium phosphate buffer (pH 6.8) containing 150 mM NaCl, 2mM DTT and 90% H2O / 10% D2O with or without 1.2 molar excess of the non-labeled PVIVIT peptide. The backbone assignment of CnA in unligated and in complex with the PVIVIT peptide was accomplished by using standard TROSY triple resonance experiments49. The following experiments were performed, 2D $^1$H$^{15}$N-TROSY-HSQC50, 3D TROSY-HNCA51, 3D TROSY-HNCOCA, 3D TROSY-HNCOCA, 3D TROSY-HNCCACB, 3D TROSY-hNcaNH52, and 3D $^1$H$^1$H-NOESY-15N-TROSY (mixing time: 200ms) experiments. To confirm the sequence-specific assignments, 4 types of amino-acid-specifically labeled CnA were prepared; $[^{U-2}$H$^{15}$N, $^1$H$^{14}$N Arg], $[^{U-2}$H$^{15}$N, $^1$H$^{14}$N Lys], $[^{U-2}$H$^{15}$N, 1-13C Val] and $[^{U-2}$H$^{15}$N, 1-13C Leu]. Spectra were processed using XWINNMR and analyzed with Sparky53.

As shown in Figure 1.10A, the 40 kDa CnAα (2-347) exhibits a well dispersed 2D $^1$H$^{15}$N-TROSY-HSQC spectrum, reflecting the α-β mixed structure of the protein54-56. About 210 backbone resonances out of 326 expected resonances are identified in the $^1$H$^{15}$N TROSY-HSQC spectrum of $[^{U-2}$H$^{13}$C$^{15}$N] CnA. Absence of the other CnA amide resonances is primarily due to the paramagnetic broadening caused by the Fe$^{3+}$ ion in the CnA catalytic center. Insufficient amide proton D to H back exchange after expression of the protein in D$_2$O is another
reason for incomplete assignment. In an attempt to overcome this back-exchange problem we prepared partially deuterated sample of CnA using deuterated expression media but H₂O instead of D₂O as solvent. This allowed for the detection of ~20 additional resonances out of the ~110 missing CnA signals. Indeed, none of the assigned resonances are from nuclei within a 10 Å distance from the Fe³⁺ ion (Figure 1.11A). Despite several attempts, we were not able to replace the Fe³⁺ ion in the catalytic center with a diamagnetic metal without affecting the CnA catalytic function, protein folding, and 3D structure. Upon titration of the non-labeled PVIVIT peptide to CnA, CnA resonances exhibited slow exchange chemical shift perturbation⁴³ and the signal intensity of CnA is rather uniform in the PVIVIT bound state, thus the residue-specific assignments were carried out first for the CnA-PVIVIT complex and then independently for ligand-free CnA.

The chemical shifts were externally referenced relative to DSS for ¹H and indirectly for ¹³C and ¹⁵N. The sequence-specific resonance assignment of the Hᴺ and Nᴴ atoms are deposited in BMRB data base in the absence and presence of the PVIVIT peptide with the accession numbers 19980 and 19981, respectively. The coordinates of the CnA-PVIVIT complex were deposited to the PDB with the accession number 2JOG⁴³.

Note that other than backbone resonance assignments, several sidechain resonance assignments of the CnA residues in the PVIVIT binding site were also established while pursuing the structure determination of the CnA-PVIVIT complex. These sidechain resonance assignments are, Tyr-288 Hδ: 6.90 ppm; Hɛ: 6.55 ppm, Met-290 Hɛ: 1.65 ppm, Phe-299 Hɛ: 6.55 ppm; Hζ: 7.36 ppm, Met-329 Hɛ: 1.90 ppm, Ile-331 Hβ: 1.93 ppm, Hγ: 0.95/1.40 ppm, Hδ: 1.91 ppm (Figure 1.10B).
Figure 1.10 NMR resonance assignment of CnA. (A) Amide resonance assignment of CnA. 2D $^1\text{H}^\text{15}\text{N}-\text{TROSY-HSQC}$ spectrum of [U-$^2\text{H}^\text{15}\text{N}^\text{13}\text{C}$] CnA recorded on Bruker Avance 750MHz spectrometer at 298K. Sequence specific assignments were indicated. The spectral region that is boxed in the left panel is enlarged. Several residues exhibited two cross peaks, reflecting the presence of multiple conformers. Adapted from Takeuchi et al (2014)\textsuperscript{47}. (B) Sidechain resonance assignment of CnA residues in the PVIVIT binding site. A strip view of the $^{13}\text{C}$-NOESY spectrum of [U-$^2\text{H}$/FIMY-$^1\text{H}$] CnA and [U-$^2\text{H}^\text{15}\text{N}$/ILV-$^1\text{H}^\text{13}\text{C}_\text{metyl}$] VIVIT complex.
Figure 1.11 Mapping of assigned residues on CnA surface. (A) The CnA surface was colored according to the availability of sequence specific assignment for the closest amide proton. The surface with and without the assignment is colored blue and gray, respectively. The iron and zinc ions in the catalytic center were drawn as orange and purple spheres. (B) Mapping of assigned residues in a ribbon representation of CnA. All residues with available assignments are indicated with spheres. The residues for which the assignments were confirmed by Arg-negative, Lys-negative, 1-$^{13}$C Leu, and 1-$^{13}$C Val selective labeling were colored yellow, green, red, and purple, respectively. Adapted from Takeuchi et al (2014)\textsuperscript{47}.

The impact of paramagnetic broadening is even more severe in triple resonance 3D NMR experiments, which require longer pulse sequences. Thus, in addition to the TROSY-type triple-resonance experiments, we extensively used $^{15}$N and 1-$^{13}$C selectively labeled samples\textsuperscript{57}, which enable verification of the sequence-specific assignment obtained by triple-resonance NMR experiments. One third of the residues were selectively labeled to confirm the sequence specific assignments based on 3D triple resonance NMR experiments (Figure 1.11B). Nevertheless, the combination of TROSY-type triple-resonance NMR experiments and selective labeling provides reliable assignments of most residues outside of the paramagnetic center. As a result, $\sim$60\% of the observable HN cross peaks are assigned in the unligated form and in the complex bound to PVIVIT. This corresponds to $\sim$40\% of non-proline residues in the CnA construct used. Residues
that exhibited substantial chemical shift differences between the unligated and PVIVIT-bound state are located near the exposed β14 strand of the central CnA β sheet. This formed the basis for the NOE-based structure determination of the CnA-PVIVIT complex and is consistent with the X-ray structure of the complex. The NMR resonance assignments of CnA in unligated and in PVIVIT-peptide-bound states serve as the basis for investigating the CnA-NFAT interaction by solution NMR spectroscopy.

1.2.2 X-ray crystal structure of calcineurin A

To understand the structural basis underlying the activation and substrate interactions of Cn, as well as to obtain a free CnA crystal form that would permit CnA/small-molecule-inhibitor or CnA-peptide complex formation by ligand-soaking or co-crystallization techniques, we also solved the X-ray crystal structure of the free calcineurin catalytic domain (CnA 19-347). The first 18 amino acids of CnA were removed through cloning, as the flexible nature of this loop might affect the crystal formation of CnA.

In this crystallographic study, an *E. coli* expressed His-GB1 fusion protein of the CnA, consisting of residues 19-347 with Y341S, L343A and M347D substitutions, was first purified by Ni²⁺-affinity chromatography (Qiagen). After overnight cleavage by TEV protease, the His-GB1 was removed by a second round of Ni²⁺-affinity chromatography, and CnA (19-347) was further purified using anion-exchange and S75 size-exclusion chromatography. Crystallization of this CnA (19-347) construct was achieved by the sitting-drop vapor-diffusion method at pH 7.25 using 1.0 M succinic acid as both solvent and precipitant. The diffraction results show that the CnA crystal belong to the orthorhombic space group P2₁2₂₁, with unit-cell parameters \(a=86.83\ \text{Å}, b=111.1\ \text{Å}, c=161.5\ \text{Å}, \alpha=90^\circ, \beta=90^\circ, \gamma=90^\circ\) and a resolution of 2.17Å (Table 1.4).
Table 1.4 X-ray data-collection statistics

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</tr>
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<tr>
<td>Rwork / Rfree (%)</td>
<td>26.9 / 27.0</td>
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*Values in parentheses are for the highest resolution shell

Following purification, CnA was concentrated to 7.0 mg ml$^{-1}$ in 10 mM Tris-HCl pH 7.4, 50 mM NaCl, and 1 mM CaCl$_2$. Initial crystallization conditions were screened with the Index and Crystal Screen kits from Hampton Research (Aliso Viejo, California, USA) using the sitting-drop vapor-diffusion method. Briefly, sitting drops consisting of 1 µl of the 7.0 mg ml$^{-1}$ CnA protein solution and 1 µl crystallization solution were mixed and equilibrated against a reservoir containing 0.5 ml crystallization solution. Two sets of conditions were identified with succinic acid or Tacsimate (a pH titrated mixture of organic acids containing 1.8305 M malonic acid, 0.25 M ammonium citrate tribasic, 0.12 M succinic acid, 0.3 M DL-malic acid, 0.4 M sodium acetate trihydrate, 0.5 M sodium formate, 0.16 M ammonium tartrate dibasic) as precipitant, respectively. After optimization of the initial crystallization conditions, 2 µl of the 7.0 mg ml$^{-1}$ CnA solution and 1 µl crystallization solution (1.0 M succinic acid, pH 7.25) were mixed in a
sitting drop, equilibrated against 0.5 ml crystallization solution and kept at 294 K. CnA crystals appeared within 1 hr as needles and grew to a maximum size of 0.2×0.01×0.01 mm in 24 hr.

The CnA crystal was cryoprotected using reservoir solution supplemented with 15% (v/v) glycerol and flash-cooled in liquid N₂. X-ray diffraction data were collected at 100 K on beamline 24-ID-E at the Advanced Photon Source, Argonne National Laboratory. Diffraction patterns were recorded on a CCD detector with a crystal-to-detector distance of 349.6 mm. The oscillation angle was 1.0° and the exposure time was 30 s per frame. A total of 90 frames were collected to a 2.17 Å resolution. The diffraction data collected were used to solve the crystal structure of CnA by molecular replacement. The catalytic domain from the 2.3 Å resolution structure of calcineurin in complex with PVIVIT peptide (PDB entry 2P6B) was used as the search model. Refinement of the initial model lowered the $R_{\text{work}}$ and $R_{\text{free}}$ 26.9% and 27.0%, respectively, in the resolution range of 161.45-2.17 Å.

As shown in Figure 1.12, there are four CnA molecules in the asymmetric unit of the crystal structure. Notably, the active site of CnA is open to the solvent channels in the crystal packing. Thus, by employing ligand-soaking techniques, this CnA crystal form would allow complex formation of CnA with small molecule inhibitors targeting the Cn active site. However, the β14 strand of CnA, which is the core binding pocket for the VIVIT peptide, is buried in the crystal packing. For a direct inhibitor of the CnA-NFAT interaction, which competes directly with VIVIT for the β14 stand on CnA surface, we expect that the crystal packing would prevent its binding to CnA. Therefore, unless the small molecule inhibitor being studied binds allosterically and away from the VIVIT binding site, the usefulness of this crystal form for CnA-inhibitor co-crystal formation by ligand-soaking is limited.
Figure 1.12 X-ray crystal structure of free CnA. Overview of the four CnA molecules in the asymmetric unit. The core-VIVIT binding site is tightly packed between two CnA molecules, whereas the active site of CnA (boxed) is solvent accessible. The dimetals at the active sites are shown as blue (Zn$^{2+}$) and orange (Fe$^{3+}$) spheres.

1.3 Structural and biophysical characterization of calcineurin-NFAT interaction

1.3.1 The NFATc1-derived LxVP peptide interacts with calcineurin A

To characterize Cn binding of NFAT’s homology domain we selected two different NFAT peptides containing the PxIxIT and LxVP motifs, respectively. The location of the peptides in the 400-residue NFAT Homology Region (NHR) is depicted in Figure 1.5A and is labeled CN-1
and CN-2, respectively. The sequence alignments for both segments among human isoforms of NFATs are provided in Figure 1.5B. Note that there is a WxK motif C-terminal to the LxVP sequence, which motivated us to use the longer 15-residue segment of NFATc1 for our binding studies. Indeed, the WxK motif seems to be important for binding of the LxVP motif (see below), which is consistent with previous studies\textsuperscript{45}.

The NFAT-LxVP peptide containing the sequence 384-DQY\textsubscript{AVA}PQHPYQWAK-398 (NFAT2) was produced as a GB1 fusion protein in an \textit{E. coli} strain, BL21 (DE3). The GB1 fusion protein has the PreScission\textsuperscript{TM} protease recognition site between GB1 and NFAT-LxVP peptide, which added a GP dipeptide at its N-terminus. For purification purposes, the construct was expressed with the LEHXXXXXXX tag at its C terminus. For the expression of uniformly $^2$H$^{15}$N$^{13}$C labeled NFAT-LxVP peptide, \textit{E. coli} harboring the GB1- NFAT-LxVP fusion vector were grown in M9 medium, which consists of 1 kg/L 99.8% D$_2$O, 6 g/L Na$_2$HPO$_4$, 3 g/L KH$_2$PO$_4$, 0.5 g/L NaCl, 50 mg/L Ampicillin, 1 g/L $^{15}$NH$_4$Cl (99.9% enriched), 2 g/L $^2$H$_6$, $^{13}$C$_6$-glucose (97% enriched), 2mM MgSO$_4$, and 0.1 mM CaCl$_2$. The cells were incubated at 37 °C, and after reaching an OD (600nm) = 0.7, protein expression was induced by the addition of 1 mM IPTG for overnight in 25 °C. The cells were harvested and resuspended in 40 ml of buffer A (50 mM Tris-HCl [pH 8.0], 300 mM NaCl, and 20 mM imidazole) with 0.2 mg/ml PMSF at 4 °C. The suspended cells were disrupted by sonication, and the insoluble fraction was removed by centrifugation at 15,000g for 20 min. The supernatant was applied to a 5 ml column of Ni-NTA agarose. After washing the resin with 40 ml of buffer A, the GB1-NFAT-LxVP peptide was then eluted with 40 ml of buffer consisting of 50 mM Tris-HCl (pH 7.5), 300 mM NaCl, and 300 mM imidazole. The elution fraction was concentrated to about 2 ml by 3000-MWC-membrane ultrafiltration. PreScission\textsuperscript{TM} protease and 1 mM DTT were added to the concentrated elution
fraction to allow for overnight digestion. The digested solution was diluted with buffer A to reduce imidazole concentration to less than 30mM and passed through Ni-NTA column. The peptide was eluted with PBS buffer at pH = 3.4.

Similarly, the NFAT-PRIEIT peptide containing the following NFAT1 sequence: WAAKPAGASGLSPRIEITPSHELQAVGPLRM, was produced as a GB1 fusion protein in an *E. coli* strain, BL21 (DE3). The GB1 fusion protein has the TEV recognition site between GB1 and NFAT-PRIEIT peptide. For purification purposes, the construct was expressed with the LEHRRRHHH tag at its N-terminus. For the expression of uniformly $^2$H$^{15}$N$^{13}$C labeled NFAT-PRIEIT peptide, *E. coli* harboring the correct plasmid was grown in M9 medium, which consists of 1 kg/L 99.8% D$_2$O, 6 g/L Na$_2$HPO$_4$, 3 g/L KH$_2$PO$_4$, 0.5 g/L NaCl, 50 mg/L Ampicilin, 1 g/L $^{15}$NH$_4$Cl (99.9% enriched), 2 g/L $^{2}$H$_6, ^{13}$C$_6$-glucose (97% enriched), 2mM MgSO$_4$, and 0.1 mM CaCl$_2$. The cells were incubated at 37 °C, and after reaching an OD (600nm) = 0.7, protein expression was induced by the addition of 1 mM IPTG for O/N in 25°C. The cells were harvested and resuspended in 40 ml of buffer A (50 mM Tris-HCl pH 8.0, 300 mM NaCl, and 20 mM imidazole) with 0.2 mg/ml PMSF at 4°C. The suspended cells were disrupted by sonication, and the insoluble fraction was removed by centrifugation at 15,000g for 20 min. The supernatant was applied to a 5 ml column of Ni-NTA agarose. After washing the resin with 40 ml of buffer A, the GB1-PxIxIT peptide was eluted with 40 ml of buffer consisting of 50 mM Tris-HCl pH 7.5, 300 mM NaCl, and 300 mM imidazole. The elution fraction was incubated with TEV in the presence of 1mM DTT and 0.5mM EDTA overnight to allow for cleavage. The digested solution was diluted with buffer A (without Imidazole) to reduce imidazole concentration to less than 30mM and passes through the Ni-NTA column. The flow-through containing the peptide was collected followed by S75 size-exclusion column chromatography.
The peptide containing the well-established, 15-residue PVIVIT sequence and a C-terminal His\textsubscript{6} tag (GPHPVIVITGPHEELEHHHHHH) was expressed with similar procedures as described previously\textsuperscript{43}.

The CnA construct used in this study is the same as that described in previous section and has been proven to be biologically relevant. It comprises residues 2–347 of the A chain with Y341S, L343A and M347D substitutions to increase the local hydrophilicity for improved protein solubility to perform NMR experiments. These residues are located at a turn connecting the main catalytic domain to the CnB binding helix and are >20 Å away from the active site. The mutations do not affect the enzymatic activity of CnA. The heterodimeric CnAB construct used in this study does not have such mutations in its CnA domain but exhibits equivalent LxVP binding characteristics, consistent with the binding data on CnA alone. The plasmid for expressing the heterodimeric CnAB was obtained from Addgene (Plasmid ID 11787) and was expressed as a tandem expression system by \textit{E. coli} as was previously described\textsuperscript{42,58}. The bacteria expressing His-tagged CnAB were harvested and the heterodimeric CnAB protein complex was further purified using a Ni-NTA gravity column (Qiagen). After the His-tag was cleaved by overnight digestion with thrombin at 4°C, the protein solution was dialyzed to remove imidazole and passed again through a Ni-NTA column to remove the His-tag, followed by a S200 size-exclusion column. The CnAB complex consists of full length CnA\textsubscript{δ} and CnB. As the size of the heterodimeric CnAB approaches the limit of solution NMR detection, we were not able to perform NMR assignment for this protein complex.

We set out to determine whether the NFAT-LxVP peptide binds CnA subunit, CnB subunit or both. Using NMR and fluorescence polarization (FP) experiments, we found that the LxVP peptide containing a 15-residue conserved segment binds to CnA with an affinity that is on the
same order of magnitude as to CnAB (Table 1.5, Figure 1.13). Related to our efforts are recent reports of molecular biology experiments and in silico docking, which suggested that the NFAT fragment containing the LxVP motif binds to a hydrophobic pocket at the interface between the two calcineurin subunits\textsuperscript{45}. This is inconsistent with our experimental results as the CnA/B interface is not presented in the CnA protein used here. And thus, it would be difficult to explain with the nearly identical LxVP affinities to CnA and CnAB, with $K_d$ values of 3.1 and 3.2 $\mu$M, respectively (Table 1.5).

Table 1.5 Equilibrium dissociation constants for the NFAT-LxVP and PxIxIT peptides interactions with CnA and CnAB\textsuperscript{26}

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<th>CnA</th>
<th>CnAB</th>
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<td>LxVP</td>
<td>$K_d = 3.1 \pm 0.44 \mu$M</td>
<td>$K_d = 3.2 \pm 0.70 \mu$M</td>
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<tr>
<td>PRIEIT</td>
<td>$K_d = 2.5 \pm 0.76 \mu$M</td>
<td>$K_d = 4.2 \pm 1.1 \mu$M</td>
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<tr>
<td>PVIVIT</td>
<td>$K_d = 0.53 \pm 0.11 \mu$M</td>
<td>$K_d = 0.51 \pm 0.13 \mu$M</td>
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* The equilibrium dissociation constants were measured using the FP assay from Figure 1.13D

All NMR experiments were performed on either Bruker Avance 500, 600, 750, or 800 MHz spectrometers equipped with cryogenic probes. All NMR spectra were collected with proteins dissolved in 30 mM sodium phosphate buffer (pH 6.8) containing 150 mM NaCl, 1 mM DTT (in the experiments with spin labeled-peptides, no DTT was used), and 90% H$_2$O/10% D$_2$O at 298 K. Spectra were processed using nmrPipe\textsuperscript{59} and analyzed with SPARKY\textsuperscript{53}. The partial backbone resonance assignment of CnA\textsuperscript{47} and the backbone resonance assignment of the LxVP and PxIxIT peptides were accomplished using standard TROSY triple-resonance experiments as described previously\textsuperscript{43}. 

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Figure 1.13 The NFAT-LxVP peptide interacts with CnA of Cn. (A) 2D NMR HSQC spectrum of free 62 μM $^{15}$N-labeled NFAT-LxVP peptide. The signal marked with an asterisk is an extra residue of the C-terminal His tag and is not relevant in this context. (B) Inset of A, which contains the relevant peaks and serves as a reference for the panels below. (C) 2D NMR HSQC spectra of $^{15}$N-NFAT-LxVP peptide in the presence of non-labeled CnA (left) and non-labeled CnAB (right), which was acquired at an LxVP:Cn molar ratio of 1:1.2. In all 2D spectra, 10 contour plot levels are drawn starting at 1.5e+05 and increasing by a factor of 1.40 for each additional level. Peak heights for complexes of $^{15}$N-NFAT-LxVP peptides with CnA and CnAB are documented in the bar graphs of Figure 1.15 and Figure 1.18. (D) Semi-logarithmic plots of fluorescence polarization experiments for determining $K_d$ of NFAT-LxVP (red), NFAT-PRIEIT (blue) and PVIVIT (black) peptides. Each FITC labeled peptide (30 nM) was incubated with increasing amounts of GST-CnA (left) and CnAB (right). The binding affinity was determined using Graphpad® Prism6 by fitting the data to a single-site binding model. Note that a dimeric GST fusion construct was used for CnA to increase the molecular mass of the complex and increase the fluorescence anisotropic difference between bound and free peptide. Thus, the bound FP of GST-CnA is larger than that of CnAB. Adapted from Gal et al (2014)[26].
**Figure 1.13A** shows an HSQC spectrum of the unbound $^{15}$N-labeled NFAT-LxVP peptide. An expansion displaying the spectral region that contains all the peptide mainchain resonance peaks, excluding a C-terminal histidine, is shown in **Figure 1.13B**. To gain a better understanding of the binding properties of the NFAT-LxVP peptide to calcineurin, we then added either non-labeled CnA (**Figure 1.13C**- left) or non-labeled CnAB (**Figure 1.13C**- right) to $^{15}$N-labeled NFAT-LxVP. A comparison of the HSQC spectra in **Figure 1.13C** shows that in both cases several peaks are broadened, and thus reduced peak intensity, upon the addition of either the non-labeled CnA alone or the heterodimer CnAB. Most affected residues are L387 and A388 of the LAVP motif, and Y394 and W396 of the C-terminal flanking sequence (**Figure 1.5**). A short polypeptide such as the NFAT-LxVP peptide used here exhibits long $^1$H transverse relaxation times due to its fast tumbling in solution$^{60}$. If binding to a larger protein such as Cn, occurs, the bound residues would exhibit slower tumbling and faster signal decay due to enhanced dipolar interaction, which is directly translated to line broadening and lower peaks heights. This is particularly effective if the protein is not deuterated. Indeed, the broadening of resonances observed in **Figure 1.13C** identifies those residues that get immobilized upon contacting Cn. The broadening may also be in part due to slow chemical exchange-type motion in the bound state with distinct chemical shifts. In this context, it is important to consider that chemical shift changes arise from a combination of direct contacts with the binding partner and induced effects on the conformational distribution, especially in the case of flexible peptides with a limited lifetime in the bound state.

Importantly, inspection of **Figure 1.13C** indicates that the same set of resonance signals at the core of the binding motif in the NFAT-LxVP sequence is broadened upon binding to either CnA or CnAB. This indicates a similar binding mode of NFAT-LxVP to both CnA and CnAB.
suggesting for the role CnA plays in this interaction. Some residues exhibit slightly different broadening behavior when CnA or CnAB were added into the NFAT-LxVP sample. For example, the intensity reduction of the signal originating from Q391 is more pronounced when bound to CnAB (Figure 1.13C-right). This may be the result of the higher molecular weight of the CnAB-LxVP complex, as compared to the CnA-LxVP complex, and the rather dynamic nature of the NFAT-LxVP in complex with Cn. It may also be the result of exchange between slightly different bound conformations of NFAT-LxVP in the presence of the heterodimeric CnAB.

In order to better understand the binding mechanism of LxVP, we then proceeded to compare the strength of the interactions between NFAT-LxVP and CnA or CnAB. Thus, we employed a FP assay to measure the dissociation constants ($K_d$) for the interactions between a fluorescein isothiocyanate (FITC)-tagged NFAT-LxVP peptide and CnA (GST-tagged to increase molecular weight of the bound form for better FP signals; GST-CnA) or CnAB. The FP assay is a well-established method for analyzing macromolecular interactions in which one of the polypeptide pair is labeled with a fluorophore for signal detection. In this assay, the complex formation can be deduced from the changes in the FP (anisotropy) at the specific wavelength corresponding to the emission peak of the fluorophore. Here, FITC is used as the fluorophore, which has an excitation wavelength and emission wavelength of 494 nm and 518 nm, respectively. Fluorescence measurements were made on 30 µl samples arrayed in 384-well plates. An EnVision Multilabel Reader was used to measure the polarization and monitor the interaction between Cn and FITC-labeled peptides. All measurements were done in triplicate. Experimental polarization data from simple and competitive binding experiments were fitted using the GraphPad Prism6, with error bars representing SD. The concentration dependence of the polarization is then used to extract the dissociation constant of the proteins pair of interest.
**Figure 1.13D** shows the FP curves corresponding to a titration of an increasing concentration of GST-CnA (left) and CnAB (right) into samples of native FITC-tagged NFAT-LxVP (red line) and FITC-tagged NFAT-PRIEIT peptides (blue line), as well as the well-established, affinity-enhanced, FITC-tagged PVIVIT (black line) peptides. All FP curves were fitted using a single-site binding model by Prism, which yields the dissociation constants (K\textsubscript{d}) shown in Table 1.5. Our results are consistent with the K\textsubscript{d} value of 2.5 µM measured previously with isothermal titration calorimetry (ITC) for the binding of LxVP peptide to calcineurin\textsuperscript{39}. The structure of the complex between the affinity-optimized PVIVIT peptide and calcineurin\textsuperscript{42,43}, and the PVIVIT binding affinity to CnA and CnAB are well established and serve as positive controls in our experimental scheme. Indeed, the obtained K\textsubscript{d} values for the binding of the well-established PVIVIT to calcineurin suggest that our experimental conditions are congruent with previously reported data indicating a dissociation constant of about 0.5 µM between the PVIVIT peptide and Cn\textsuperscript{48,61}. These results clearly show that the NFAT-LxVP peptide can bind to either CnA alone or CnAB with similar affinities, highlighting the role that CnA plays in this specific interaction.

1.3.2 LxVP and PVIVIT peptides bind to overlapping epitopes on calcineurin A

Having established that NFAT-LxVP binds to the catalytic domain of calcineurin (CnA), we then turned to identify the exact binding epitope of LxVP on CnA surface at a residue level using NMR-chemical shift perturbation (CSP) experiments. Briefly, we titrated increasing amounts of non-labeled NFAT-LxVP peptide into a solution NMR sample of \textsuperscript{15}N, \textsuperscript{2}H-CnA containing residues 2-347 with substitutions Y341S, L343A, and M347D, and recorded the 2D HSQC spectrum of CnA at each state.
Figure 1.14 Characterization of the CnA:NFAT-LxVP binding epitope by NMR spectroscopy. (A) 2D $^1$H-$^1$H TROSY-HSQC NMR spectrum of [U-2$^15$N] CnA. The spectrum was acquired on a 600MHz spectrometer with 2048 (10 ppm) × 128 (30 ppm) complex points (SW) in the direct and indirect dimensions, respectively. (B) Insets of representative regions from the series of the 2D HSQC TROSY spectra acquired during the titration of unlabeled NFAT-LxVP peptide (residues 384-400 of NFATc1) into [U-2$^15$N] CnA (residues 2-347). The spectra show CnA peaks in the absence of NFAT-LxVP (red) and in the presence of 1:0.25 (cyan), 1:1 (green), 1:3 (purple) and 1:5.5 (yellow) CnA:NFAT-LxVP molar ratios as depicted in the color legend on top of the panels. A complete record of the chemical shift perturbations for the assigned signals of CnA together with paramagnetic relaxation enhancement experiments are shown in Figure 1.15. (C) Solution structure of the complex of CnA (grey) with a 15-residue PVIVIT peptide. The active site metals (Zn$^{2+}$/Fe$^{3+}$) are shown as solid spheres. (D) Residues of CnA that exhibit chemical shift changes upon binding the high-affinity binder PVIVIT. (E) Residues of CnA that exhibit chemical shift changes upon binding to the 15-residues NFAT-LxVP peptide. Large and small spheres represent chemical shift differences larger than 0.1 ppm and 0.05 ppm, respectively. Adapted from Gal et al (2014).
**Figure 1.14A** shows a 2D $^1$H$^{15}$N TROSY-HSQC spectrum of CnA alone in NMR buffer. We then titrated non-labeled NFAT-LxVP peptide into the $^{15}$N, $^2$H-CnA sample. Upon each addition of the NFAT-LxVP peptide, we monitored the chemical shifts perturbation of CnA residues by recording 2D TROSY-HSQC spectra of the newly formed complex. **Figure 1.14B** shows three insets from different regions of the CnA HSQC spectra (boxed in **Figure 1.14A**) wherein both types of shifted and non-shifted residues can be observed. The resonance assignments of these residues are adapted from our previous work$^{47}$. As shown in **Figure 1.14B**, the shifted residues of CnA upon LxVP binding showed fast-to-intermediate exchange, which would be reasonable for the low micro-molar affinity interactions between the NFAT-LxVP peptide and CnA.

To identify the LxVP binding site on Cn, we measured the chemical shift changes of CnA upon addition of the LxVP peptide. We then compared the chemical shift perturbation (CSP) of CnA forming complex with PVIVIT (**Figure 1.15A**) or with NFAT-LxVP (**Figure 1.15B**). CnA residues with the largest chemical shift difference upon binding to the peptide containing the NFAT-LxVP motif are in decreasing order: 331, 327, 324, 326, 299, 332, 323, 102, 298, 270, 328, 277, 193, 294, 242, 269 and 83. This comparison clearly shows that the LxVP and PVIVIT peptides interact with the same region of CnA. Note that the NMR spectrum of CnA is not fully assigned due to line-broadening caused by the paramagnetic metal (Fe$^{3+}$) in the enzyme’s active site, and the unassigned residues appear as blank regions in the histograms. In addition to the chemical shift changes documented in **Figures 1.15A** and **B**, some residues experience severe line broadening during the addition of the NFAT-LxVP peptide as they appear to have reached the limit of intermediate exchange. The CnA residues that experience intermediate exchange are 73, 79, 104, 192, 194, 273, 294, 296, 323, 329, 330, and 333. These residues fall within the areas that also show significant chemical shift changes following the CnA-peptide complex formation.
Figure 1.15 PVIVIT and LxVP motifs bind to overlapping surfaces on CnA. (A) Chemical shift differences of CnA residues when bound with the high-affinity binder PVIVIT peptide. The structure of the optimized PVIVIT peptide and CnA is known and thus the experiment acts as positive control. (B) Chemical shift differences of CnA residues when bound with the NFATLxVP peptide. Note that the LxVP peptide binds an order of magnitude weaker than the PVIVIT peptide which has been optimized for binding. Thus the chemical shift changes for the LxVP peptide are significantly smaller. (C) Utilization of paramagnetic relaxation enhancement (PRE) experiment to identify the region of CnA that binds to the LxVP peptide. The histogram represents the peak intensity ratio of calcineurin residues bound to an LxVP peptide covalently attached to a nitrooxide spin-label (methanethiosulphonate, MTSL). The spin label was attached to the single cysteine on the NFAT-LxVP peptide at position Q385C. The data depicted the peak intensity ratio of two identical sequentially acquired HSQC spectra, before and after the addition of 5 molar excess of ascorbic acid. The ratio is calculated by directly dividing the peak intensity of the latter spectrum by the first one. Adapted from Gal et al (2014)26.
Note that we only have incomplete resonance assignments for CnA$^{57}$. We miss assignments around the paramagnetic center of CnA due to paramagnetic line broadening. Moreover, we lack assignments for many residues in the hydrophobic core of the CnA protein since we have to deuterate the protein for NMR spectroscopy. This deuteration leads to loss of amide signals in the center of the CnA protein. Since the CnA (residues 2-347) protein was expressed in a folded state, back-exchange of internal amide protons from D to H does not happen easily and could not be achieved despite many attempts. However, as we can see in Figure 1.11, most surface residues outside the area of the paramagnetic center are assigned, and the identification of the binding epitope is safe. Moreover, the assignments of the cross peaks of the binding epitope have been validated by structure determination of the Cn/VIVIT peptide complex$^{42,43}$.

The chemical shift perturbation assay reports on both direct, and indirect or allosteric effects occurring due to the complex formation. As an orthogonal assay that reports only direct spatial-related interaction, we used spin labeling to map the LxVP binding site on CnA. The methanethiosulfonate (MTSL) spin-label was attached to the thiol of mutated Qγ85C in the second position of the NFAT-LxVP peptide comprising residues 384-400. In this experiment, a five molar excess of the MTSL spin label [(1-oxyl-2,2,5,5-tetramethylpyrroline-3-methyl)-methanesulfonothioate] was incubated with a Q385C mutant of the GB1-NFAT-LxVP protein sample for 4 hr at room temperature. Excess spin label was removed by PD-10 desalting column and size-exclusion chromatography using Superdex75 column. Further purification was done in a similar way to the native LxVP peptide in order to remove the GB1 tag and purify the resulting spin labeled-LxVP peptide.

The MTSL molecule serves as a spatial probe capable of delivering information regarding the relative position of the NFAT-LxVP peptide with respect to CnA. We compared the intensities of
CnA backbone NH cross peaks when bound to NFAT-LxVP-MTSL before and after reducing the spin label with 5 fold excess of ascorbic acid (Figure 1.15C). The same binding region on CnA impacted by the PVIVIT peptide also exhibits the largest reduction in intensity upon binding of the NFAT-LxVP-MTSL peptide. This clearly reveals CnA residues that are in close proximity to the spin label at the NFAT-LxVP peptide.

Our two NMR assays provide congruent results that the strongest spin label effects in the paramagnetic relaxation enhancement (PRE) experiment are observed at the amino acids that also exhibit the largest chemical shift differences (Figure 1.15B and C), and the effects are located approximately at the same binding site as the well-established PVIVIT peptide. Based on these experiments, we concluded that the same stretch of residues on the surface of CnA indeed is contacted by both the PVIVIT and LxVP motifs of NFAT. Note that the PRE broadening effects depend directly on the inverse sixth power of the distance to the spin label and not on indirect (allosteric) effects and can report distances up to 20 Å. However, the accuracy is limited due to the mobility of the spin label itself. On the other hand, the CSPs, which measure both direct and indirect (allosteric) contacts, are not related to distances to this single site spin label. Therefore, the PRE data are not directly correlated to the CSPs and should not be identical.

It is informative to visualize the CSP on CnA of the two NFAT segments containing the PxIxIT and LxVP motifs. Figure 1.14C shows a cartoon of CnA in complex with the PVIVIT peptide (orange). The Cn residues with the largest chemical shift changes upon binding the PVIVIT and NFAT-LxVP peptides are indicated with red and blue spheres in Figures 1.14D and 1.14E, respectively. As can be seen, the similarity of the sets of CnA residues that interact with PVIVIT or NFAT-LxVP peptides reveals that the two peptides interact with approximately the same epitope on CnA. However, the NFAT-LxVP peptide affects more strongly a helical region
adjacent to the PVIVIT binding site (top left of Figure 1.14E). Thus, the residues that are perturbed upon binding by both peptides on CnA are overlapped but not identical.

1.3.3 LxVP and PVIVIT motifs compete for binding to calcineurin partially

Having shown that both NFAT-LxVP and PVIVIT containing peptides bind to an overlapping surface of CnA, we asked whether the two motifs bind in a competitive fashion. First, we recorded $^1$H-$^{15}$N correlated spectra of $^{15}$N-labeled NFAT-LxVP in the free form and upon addition of unlabeled CnAB in a similar way as described in Figure 1.13C. In a second step, we added the unlabeled high affinity binder PVIVIT peptide to the CnAB-LxVP complex to see whether we can compete out the $^{15}$N-labeled NFAT-LxVP peptide for CnAB binding. The PVIVIT peptide was expressed with a His-GB1 tag. The GB1 tag is a small globular and soluble protein that enhances expression levels and was not removed in this experiment. In order to ensure the competition specificity of PVIVIT and rule out the effects of GB1, we also tested GB1 alone as a negative control. Figure 1.16A shows the $^1$H-$^{15}$N HSQC NMR spectrum of NFAT-LxVP peptide in the free form. Figure 1.16B depicts the same spectrum following the addition of unlabeled CnAB. It exhibits a subset of the NFAT-LxVP $^1$H-$^{15}$N cross-peaks with decreased intensity as shown in Figure 1.13 for addition of CnA. Four peaks that display the strongest intensity changes (L387, A388, V389 and Y394) are labeled in the spectra. In order to document and compare the size of the intensity losses, cross sections are drawn for L387 as a representative case. Addition of unlabeled GB1-PVIVIT to this sample (Figure 1.16C, left panel) leads to the reappearance of some of the affected signals (L387, A388, V389, the residues of the LxVP segment), while others remain suppressed (Y394). As a negative control, addition of GB1 only without the PVIVIT peptide does not produce this effect (Figure 1.16C, right panel). In addition, GB1 did not interact with NFAT-LxVP (Figure 1.17).
Figure 1.16 PVIVIT competes out NFAT-LxVP from CnAB. 2D HSQC spectra of the following: (A) 62 μM $^{15}$N-labeled NFAT-LxVP alone. (B) $^{15}$N-labeled NFAT-LxVP in complex with CnAB in 1:1.2 molar ratio. Peaks of the residues contacting CnAB are strongly reduced and also shifted. (C) Addition of unlabeled His$_6$-GB1-PVIVIT (left) in a 1:1.2:5 molar ratio restores intensity of some peaks (L387, A388, V389; the LxVP motif) but not others (Y294, W396, A397, K398; the WxK motif). Addition of unlabeled His$_6$-GB1 as a control (right) does not restore the intensity changes. The spectra were acquired on a 600 MHz spectrometer with 2048 (10 ppm) × 128 (30 ppm) complex points in the direct and indirect dimensions, respectively. In order to document the intensity changes, cross sections are drawn through the peak of L387 in all four panels. (D) Bar graph indicating the relative peak intensity of NFAT-LxVP peaks corresponding to each of the four spectra of A-C with peak colors similar to the representative bars. The error bars indicate the noise levels in the respective spectra as extracted from SPARKY using median of 10,000 calculated points. Adapted from Gal et al (2014).
Figure 1.17 Titration of non-labeled NFAT-LxVP peptide into $^{15}$N-His-GB1 protein. (A) 2D HSQC NMR spectrum of free $^{15}$N His-GB1. (B) 2D HSQC NMR spectrum of $^{15}$N His-GB1 protein bound to NFAT-LxVP peptide in a 1:1.2 molar ratio. (C) Overlay of the spectrum in B onto spectrum in A. This shows that no chemical shift perturbation of the 15N His-GB1 resonances occurs upon LxVP addition. Adapted from Gal et al (2014).
A complete summary of the peak intensity changes of δxVP in this competitive NMR experiment is given in the bar graph of Figure 1.16D. As typical in such NMR experiments, loss of peak heights and intensities indicates residues that bind to the much larger protein partner, while unaffected peaks are thought to not directly touch the larger protein and remains flexible. Detailed inspection of Figure 1.16D suggests that the segment containing residues D384 to Q391 of the NFAT-LxVP peptide is tightly bound when added to CnAB, but it is also displaced almost completely from CnAB when GB1-PVIVIT was added. In contrast, the segment containing Y394 to L399 although clearly binds CnAB to a similar extent, but is not displaced by the GB1-PVIVIT peptide.

Interestingly, the segment that did not recover upon PVIVIT addition, contains the conserved WxK sequence, which thus seems to be important for the NFAT-LxVP peptide binding and responsible for the fact that PVIVIT, or more generally, the PxIxIT peptides cannot fully displace the NFAT-LxVP peptide. Based on the $K_d$ listed in Table 1.5, the addition of 75 µM CnAB to the sample of 62 µM NFAT-LxVP causes 90% of the peptide to be bound to CnAB. This agrees with the peak intensity changes for residues L387-V389. The addition of four molar excess of the high affinity PVIVIT peptide should have resulted in complete displacement. Yet, some of the NFAT-LxVP residues remain bound. These observations suggest that both NFAT segments, namely, the 15-residue NFAT-LxVP and the 15-residue PVIVIT only partially compete for the same binding site on Cn and that the PVIVIT does not fully displace the NFAT-LxVP peptide. In addition, spectra of CnAB-bound $^{15}$N-labeled NFAT-LxVP with and without unlabeled PVIVIT peptide are not identical. Thus, both peptides must bind simultaneously on the surface of CnAB. In order to test whether the same effects occur if only using CnA alone, we performed similar experiments with only CnA and obtained equivalent results as shown in Figure 1.18.
Figure 1.18 PVIVIT partially competes with the NFAT-LxVP motif for binding with CnA. Competition assay based on 2D HSQC NMR spectra indicating that the $^{15}$N-labeled NFAT-LxVP peptide is partially displaced from CnA by addition of the PVIVIT peptide. (A) 2D HSQC spectrum of free $^{15}$N labeled LxVP. A series of 2D HSQC NMR spectra (right) showing the $^{15}$N labeled NFAT-LxVP peptide in free form (left), bound to CnA in 1:3 molar ratio (middle) and following addition of the PVIVIT peptide in 1:3:10 molar ratio (right). (B) Histograms indicating the relative peak intensity of NFAT-LxVP peaks in the different spectra of panel A with peaks color similar to the representative bars. Obviously, similar effects are found for binding of LxVP segment to the catalytic domain CnA as to heterodimeric CnAB, and similar competition/displacement effects are seen. Thus, inspection of the histogram suggests that binding in the context of both peptides is entirely driven by the Q591-E400 segment. However, this is not the case when the PxIxIT peptide is bound in the absence of LxVP (Figure 1.20). This supports our conclusion that the two peptides compete partially but do not fully displace each other. Adapted from Gal et al (2014)\textsuperscript{26}.
In addition to partially displacing the NFAT-LxVP segment by the well-established PVIVIT high affinity-binder, we also checked if a peptide with the endogenous NFAT-PRIEIT sequence (GEFWAASKASGLSPRIEITPSHELIEQAVGPLRM) would be partially displaced from calcineurin by the addition of a 15-residue peptide containing the LxVP motif in NMR experiments. As shown in Figure 1.13D as well as Table 1.5, the NFAT-LxVP and NFAT-PRIEIT peptides bind with comparable affinities to CnA and CnAB. To verify the latter result by an orthogonal experiment, we also measured direct binding of the His-GB1-NFAT-PRIEIT peptide and the His-GB1-NFAT-LxVP peptide to GST-CnA using the BLItz® technology, which gives association and dissociation rates, as well as the equilibrium dissociation constants of protein-protein interactions (PPI). Using the BLItz® biophysical instrument, we found that the association and dissociation rates are ten-fold faster for the NFAT-PRIEIT peptide as compared to the NFAT-LxVP peptide. However, the $K_d$ values measured using the BLItz® technology are almost identical for both NFAT-PxIxIT and NFAT-LxVP peptides, at about 0.64 μM and 0.46 μM, respectively (Figure 1.19 and Table 1.6). This is consistent with previous reports\(^9,44\) as well as our FP assay results (Table 1.5), which further confirms that the NFAT- PRIEIT and NFAT-LxVP peptides bind CnA with almost equal affinities.

Table 1.6 Global fitting data for binding of GST-calcineurin A to His-GB1-NFAT-PRIEIT and His-GB1-NFAT-LxVP based on BLItz® measurement\(^26\)

<table>
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<tr>
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<th>$k_a$ (Ms(^{-1}))</th>
<th>$k_d$ (s(^{-1}))</th>
<th>$K_d$ (μM)</th>
<th>$K_d$ Error (μM)</th>
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</thead>
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<td>His-GB1-NFAT-PRIEIT</td>
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<td>0.07458</td>
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<tr>
<td>His-GB1-NFAT-LxVP</td>
<td>1884</td>
<td>8.8E-04</td>
<td>0.467</td>
<td>0.0394</td>
</tr>
</tbody>
</table>
Figure 1.19 Measurement of binding affinity and kinetics of LxVP and PxIxIT using the BLItz® technology. (A) Concentration dependent curve for binding of GST-CnA to His-GB1-NFAT-PRIEIT. Measured kinetic constants are reported: $k_a$: 10680 1/Ms; $k_d$: 6.881E-03 1/s; $K_D$: 0.6446 µM. (B) Concentration dependent curve for binding of GST-CnA to His-GB1-NFAT-LxVP. Measured kinetic constants are reported: $k_a$: 1884 1/Ms; $k_d$: 8.8E-04 1/s; $K_D$: 0.467 µM. The kinetic analysis of His-GB1-NFAT-PRIEIT and His-GB1-NFAT-LxVP peptides with GST-CnA was carried out on the BLItz® system using the Advanced Kinetics module, where the His-tagged peptide was first loaded on Ni-NTA biosensors followed by monitoring the association and dissociation of the GST-CnA protein to the peptide. The same experiment was repeated for different concentrations of GST-CnA with final sample concentrations being 0.2 µM, 1 µM, and 10 µM, respectively. The data was analyzed and fitted globally using the BLItz® system. Adapted from Gal et al (2014)²⁶.

In a complementary experiment we checked whether the peptide containing the LxVP motif can displace the PRIEIT peptide, where we added non-labeled CnAB to a sample of $^{15}$N-labeled NFAT-PRIEIT peptide. Similar to the previous experiment, LxVP peptide, was expressed and measured with a GB1-tag. The GB1 tag alone was also used as negative control. Figure 1.20 (top panel) shows the 2D HSQC NMR spectrum of the free $^{15}$N- NFAT-PRIEIT peptide.
Addition of non-labeled CnAB causes a subset of peaks to shift or even disappear (middle panel). The cumulative loss of peak intensity indicates the complex formation between the NFAT-PRIEIT peptide and heterodimeric CnAB. We then added the non-labeled NFAT-LxVP peptide to examine whether it also partially competes out the NFAT-PRIEIT peptide for CnAB binding (lower-left panel). Unlike the previous experiment depicted in Figure 1.16, where the tighter binder, GB1-VIVIT peptide, was used, the two peptides with endogenous sequences examined here now have comparable affinities towards calcineurin (Table 1.5 and 1.6). Yet, if competing with four-fold excess of GB1-LxVP peptide on exactly the same binding epitope of Cn, a full displacement of $^{15}$N-NFAT-PRIEIT should occur according to the law a mass action. However, again, only a subset of the attenuated peaks reappeared upon addition of the GB1-LxVP peptide. This confirms partial but not full competition between the NFAT-LxVP and NFAT-PRIEIT peptides.

Two representative peaks are labeled in the spectra, residue E19 and T21. The reappearance of residue E19 located in the PRIEIT motif suggests that a displacement of it from the binding epitope on Cn took place following the addition of the NFAT-LxVP peptide. Thus, the data provide additional support that the NFAT-PRIEIT and NFAT-LxVP peptides bind to an overlapping region on Cn competitively. The histogram in Figure 1.20B details the relative intensities of all NFAT-PRIEIT residues in each mode: free (black bars), bound to Cn (red bars) after the addition of GB1-LxVP (blue bars) and after the addition of GB1 as a negative control (green bars). It can be seen that the residues in the core of the binding motif exhibit the most pronounced intensity decrease upon CnAB binding as well as intensity recovery after LxVP addition. Note that some residues do not fully recover after LxVP addition indicates simultaneous binding of both peptides.
Figure 1.20 GB1-LxVP partially displaces $^{15}$N-PxIxIT from CnAB. 2D HSQC spectra of the following: (A) 75 μM $^{15}$N-labeled NFAT-PRIEIT, (B) $^{15}$N-labeled NFAT-PRIEIT peptide in complex with CnAB in 1:1.2 molar ratio, (C) addition of GB1-LxVP-His$_6$ (C-left) and unlabeled His$_6$-GB1 (C-right) in a 1:1.2:5 molar ratio. Spectra were acquired on a 600MHz spectrometer with 2048 (10 ppm) × 128 (30 ppm) complex points in the direct and indirect dimensions, respectively. In order to document the intensity changes, cross sections are drawn through the peaks of T21 and E19 in all panels. (E) Bar graph reporting the intensities of NFAT-PRIEIT peaks corresponding to each of the spectra of A-C with peak colors similar to the representative bars. The error bars indicate the noise levels in the respective spectra as extracted from SPARKY using median of 10,000 calculated points. Adapted from Gal et al (2014).
1.3.4 FP assay confirms competitive binding of LxVP and PxIxIT to calcineurin

To further validate the competitive binding of the two NFAT derived peptides containing the PxIxIT and LxVP motifs, respectively, on both CnA and CnAB, we used a FP competitive displacement assay. As shown in Figure 1.21, we labeled the NFAT-LxVP peptide with a fluorescein isothiocyanate (FITC) tag at its C terminus and incubated a 30 nM solution of the peptide with 1.2 μM of GST-CnA (left panel) or 3.0 μM heterodimeric CnAB (right panel) in a sample volume of 30 μl. To displace the FITC-labeled NFAT-LxVP peptide, we then added increasing amounts of unlabeled NFAT-PRIEIT peptide. The polarization change of the sample is recorded on an EnVision Multilabel Reader.

In Figure 1.21A, the red and blue lines represent the constant amount of FITC-tagged NFAT-LxVP peptide with and without the corresponding Cn protein, respectively, and in the absence of NFAT-PRIEIT peptide. The green line represents the sample wells wherein increasing amounts of NFAT-PRIEIT peptide were added. It can be seen, that the FITC-labeled 30 nM NFAT-LxVP peptide can be fully displaced at high molar excess of the NFAT-PRIEIT peptide with final concentrations in the mid to high μM range.

Figure 1.21B exhibit the results of a complementary experiment, wherein 30 nM FITC-tagged NFAT-PRIEIT peptide is incubated with 1.2 μM GST-CnA (left panel) or 3.0 μM of CnAB (right panel). Increasing concentrations of NFAT-LxVP peptide displaces the FITC-tagged NFAT-PRIEIT peptide from both Cn proteins. These data support the notion that both NFAT-PxIxIT and NFAT-LxVP peptides bind to adjacent or overlapping epitopes on CnA. Note that, in this FP assay, higher concentrations of CnAB (3.0 μM) were used as compared to GST-CnA (1.2 μM), while the concentration of the FITC-labeled peptide was the same. Thus, more titrant
peptide is needed to displace bound peptide in the case of CnAB, which shifts the FP displacement curves for CnAB to the right as compared to those for GST-CnA. The fact that a large excess of one peptide is required to displace the other directly indicates that the binding sites for both peptides are not identical, and the overlap in the epitopes contribute only a modest amount to the overall affinity.

Figure 1.21 Monitoring the displacement of NFAT-PRIEIT and NFAT-LxVP peptides using FP assay. (A) Displacement of FITC-labeled NFAT-LxVP peptide by the NFAT-PRIEIT peptide. Increasing concentrations of NFAT-PRIEIT peptide were titrated into a mixture of 30 nM FITC-NFAT-LxVP peptide and 1.2 μM GST-CnA (left) and 3.0 μM CnAB (right). (B) Displacement of FITC-labeled PRIEIT by the LxVP peptide. Increasing concentrations of LxVP peptide were titrated into a mixture of 30 nM FITC-PRIEIT peptide with 1.2 μM GST-CnA (left) and 3.0 μM CnAB (right). Note that the NFAT-derived PxIxIT and LxVP peptides will always be presented at equimolar concentrations as part of the same NFAT molecule. The experiment results shown here document that the two NFAT segments target the same epitope on CnA but does not displace each other as part of full-length NFAT. Adapted from Gal et al (2014).26
The high excess of NFAT-PRIEIT or NFAT-LxVP peptide compared to the FITC-labeled peptide used here is different from the NMR competition experiment (Figures 1.20). In the NMR experiment, we used approximately equimolar amounts of both peptides at high μM concentrations. In vivo, the PRIEIT and LxVP segments will always be present at equimolar concentrations, since they are on the same NFAT molecule. Thus, the NMR competition experiments that showed simultaneous binding to the overlapping epitopes approximate this situation better. This is confirmed by the results of Figure 1.21. At the lowest titrant concentrations, we have equimolar concentrations of NFAT-LxVP and NFAT-PxIxIT, and the two NFAT-derived peptides do not displace each other, which is consistent with the equimolar NMR competition experiments described above. The fact that the separate peptides containing either the LxVP or the PxIxIT peptide can displace the other peptide at high molar excess supports the finding that the two NFAT peptides compete for the same binding site on Cn. That displacement can happen at all at high excess of one peptide over the other indicates that the PxIxIT-Cn or the LxVP-Cn complex alone adapts a bound structure that is incompatible with the other peptide binding. Only at approximately equal peptide concentrations, a bound trimeric complex of Cn-LxVP-PxIxIT can form where the two peptides interact with Cn as well as with each other.

1.4 Conclusion

The importance of the Cn-NFAT signaling pathway is self-explanatory. NFAT-specific inhibition of Cn in T-cells has long been an objective for obtaining novel safe immune-suppressing agents (see Chapter 2). Inhibitors broadly blocking the phosphatase activity of calcineurin, such as cyclosporin A (CsA) or FK506 in complex with their cytoplasmic receptors, cyclophilin and FKBP12, respectively, have been tremendously successful in clinic and are now
routinely used to prevent rejection in organ transplants. However, the general inhibition of Cn’s enzymatic activity also affects all other functions of calcineurin outside the immune system and has led to severe side effects in the long-term treatment of transplant patients. Thus, there is a great need for specific inhibitors targeting Cn’s catalytic activity against NFAT only, and not blocking its function in other contexts outside the immune system. Indeed, initial inhibitors with such specificity have been found already that prevent NFAT binding to Cn, its dephosphorylation, nuclear translocation, binding to DNA and upregulation of cytokines, but do not inhibit Cn’s general phosphatase activity against other targets. Given the importance of this binding event, it is imperative to achieve a better structural understanding of the calcineurin-NFAT interaction at a molecular level, which would facilitate the search for novel NFAT-specific Cn inhibitors for safe immunosuppression.

The discovery of two distinct Cn binding motifs, PxxIT and LxVP, seemed to make it unlikely that the regulatory domain of NFAT could be displaced from Cn with a single molecule. This is in contrast to a previous study where single compounds could inhibit NFAT dephosphorylation and nuclear translocation. Thus, a more detailed investigation of the interaction sites of the two binding peptides was desirable.

In this study, we embarked on defining the binding site of an NFAT-derived peptide containing the LxVP motif of NFATc1 on the surface of calcineurin. Using NMR spectroscopy we have discovered that the conserved NFAT-LxVP motif binds to the same CnA surface region as the high affinity PVIVIT peptide. This finding was unexpected and is inconsistent with recent suggestions based on mutagenesis and modeling that locate the LxVP binding at the CnA/B interface. Our findings are also inconsistent with the claim that the binding site of an LxVK motif of the viral A238L peptide represents NFAT’s LxVP binding. Besides the L to K
difference, the viral peptide also lacks the flanking sequences native to NFATs, including the WxK motif (Figure 1) that anchors the peptide onto CnA. Indeed, it has previously been shown that W to A mutation in the WxK motif impaired the inhibition activity of the mutated LxVP peptide\(^{45}\). Moreover, the binding affinity of the NFAT-LxVP peptide to calcineurin is similar with both the heterodimeric CnAB and the CnA subunit alone (Table 1.5). This binding affinity also resembles that of the peptide containing the NFAT-PxIxIT motif, which is known to bind explicitly to CnA. The similarity of the binding affinities indicates that the LxVP binding event manifests equal importance as the PxIxIT interaction. This result is consistent with previous reports that peptides containing the LxVP sequence derived from NFATc1 (i) disrupt the NFAT-Cn interaction, (ii) displace the PxIxIT peptide of NFATc2 from Cn, (iii) prevent NFAT dephosphorylation, and (iv) inhibit NFAT nuclear translocation\(^{66}\). Therefore, the LxVP binding motif of NFAT is of equal importance to the NFAT-PxIxIT motif in understanding, as well as inhibiting the Cn-NFAT interaction.

The data presented here reveal that both peptides bind to epitopes on CnA that overlap but are not identical. The peptides containing the LxVP and PxIxIT motifs bind simultaneously when presented at equimolar concentrations as shown in Figures 1.16, 1.20 and 1.21. Equimolar concentrations resemble the binding event of full-length NFAT where both motifs are on the same polypeptide chain. Simultaneous binding at equimolar concentrations is also consistent with the FP displacement experiments of Figure 1.21, which show that the peptides cannot displace each other at the starting concentrations of the titration. For example, as shown in Figure 1.21A, 30 nM FITC tagged NFAT-LxVP peptide could not be displaced from 1.2 \(\mu\)M CnA by the PRIEIT peptide of up to 10 \(\mu\)M in concentration, and similar effects are observed in complementary titrations (Figure 1.21B).
Figure 1.22 LxVP and PxIxIT peptides bind to an overlapping epitope on the CnAB heterodimer. (A) Model for the conformational changes upon Cn interaction with different concentrations of LxVP and PxIxIT containing NFAT peptides. As suggested by the experimental data, LxVP (I) or PxIxIT (III) peptides alone form bound conformations incompatible with binding the other peptide. Equimolar concentration of the binding peptides leads to a trimeric bound structure of a different conformation (II). The trimetric condition resembles the binding event of full-length NFAT where both motifs are of equimolar concentration on the same polypeptide chain (IV). (B) Location of the NεR-derived LxVP binding site on the surface representation of CnAB (PDB ID: 1AUI). The residues of the NMR-derived LxVP and PxIxIT binding sites are colored blue and red, respectively, with the overlapping site colored in magenta. The groove connecting the LxVP binding site to Cn’s catalytic center was shadowed in blue. The site in the CnA/CnB interface suggested in a previous study is marked in orange. (C) Model of NFAT regulatory domain in complex with CnAB depicting simultaneous PxIxIT and LxVP binding to an overlapping epitope. The lowest energy model out of 10 independent calculations using CYANA is shown. Phosphorylation sites are depicted with red spheres. The CnAB structures are shown in the same color as in panel B. Adapted from Gal et al (2014).

The fact that one peptide can displace the other at high excess while binding to a common but not entirely identical epitope suggests the following binding model, which is visualized with the cartoon of Figure 1.22A. Briefly, one peptide alone adopts a bound conformation with Cn
that does not allow the other peptide to bind without changing its conformation (I and III). Thus, high excess of one peptide displaces the other. When both peptides are present at equal concentrations they adopt a different trimeric bound conformation with Cn and do not displace each other (II). If one peptide is in excess, the equilibrium shifts to the conformation that is incompatible with the binding of the other peptide (transition from II to I or III).

The three states described in this cartoon of Figure 1.22A are visible in the NMR experiments: the free NFAT-LxVP and NFAT-PxIxIT peptides are in panels A of Figures 1.16 and 1.20, respectively; the dimeric complexes are in panels B; and the trimeric complexes are at the left hand side of panels C with either $^{15}$N-labeled LxVP or PxIxIT peptides. In the trimeric Cn-PxIxIT-LxVP complexes, unlike the bound conformation with excess of each peptide, only parts of the peptides are tightly bound to Cn surface, which is clearly seen from the uneven recovery of peptide signal intensities from the dimeric to the trimeric complex transition. It should also be noted that the trimetric condition resembles the binding event of full-length NFAT, where both PxIxIT and LxVP motifs are on the same polypeptide chain, and thus, concentrations of the both motifs are presented as equally (IV).

It’s unlikely, yet possible, that the isolated NFAT-LxVP motif we used in this study may have different biophysical characteristics and hence binding behavior, as compared to the LxVP motif in the intact NFAT NHR. The PxIxIT and the LxVP segments may bind sequentially, so steric constrains could direct the LxVP to another site once the PxIxIT segment is already in bound state. To resolve this, co-complex structures of Cn with the full-length NHR or even entire NFAT would be needed. As the NHR of NFAT is unstructured in both bound and unbound states with Cn, this remains a great challenge.
The NMR-derived location of the NFAT-LxVP binding site is depicted in a surface representation of the CnAB heterodimer in Figure 1.21B. Surprisingly, a previous report indicated that an LxVP-derived peptide inhibited dephosphorylation of the 19-residue phosphopeptide substrate for Cn (the phosphopeptide RII), while the NFAT-PxIxIT peptide did not show such an inhibitory effect on Cn dephosphorylation activity towards phosphorylated RII\textsuperscript{66}. This observation was used to support the notion that the LxVP and PxIxIT motifs bind at different sites, and based on \textit{in silico} docking studies a putative NFAT-LxVP binding epitope was predicted to reside at the interface of CnA and CnB, overlapping with the binding site of the two well-established immunosuppressive drugs, CsA and FK506\textsuperscript{45} (Figure 1.9B and 1.21B). However, the binding mode presented in this dissertation would also explain clearly how dephosphorylation of the RII peptide by Cn is inhibited by the LxVP peptide and not by the PxIxIT peptide. The former, LxVP peptide, directly competes with the RII peptide for binding to Cn, while the PxIxIT peptide, which binds to an overlapping but not identical site, allows for simultaneous binding. It was also shown that mutation of CnA residues 330-332 from NIR to AAA, which are in the core of the PxIxIT binding site, and therefore also in our NMR-derived LxVP binding site, and distant to the CnA/CnB interface and the active site, did alter calcineurin phosphatase activity towards the RII peptide as well\textsuperscript{68}. The fact that the RII peptide has an LxVP sequence suggests that the triple mutation of Cn disrupts the LxVP interaction at the overlapping epitope. In addition, we cannot rule out the possibility that the LxVP motif within the RII peptide, which lacks the neighboring WxK motif, would have a different binding mode as compared to the NFAT-LxVP peptide.

Our experiments reported here do not support the previous results that the LxVP peptide binds to a site between CnA and CnB subunits\textsuperscript{45}. The identification of the partially overlapping
but non-identical binding sites for the two NFAT segments is compatible with the different
effects of LxVP and PxIxIT on the dephosphorylation activities of Cn towards its substrates. It
should also be noted that inhibition of Cn’s activity by the LxVP sequence does not diminish the
importance of this site as a potential target for inhibiting Cn-dependent NFAT dephosphorylation.
Indeed, it was shown more than a decade ago that both binding motifs, PxIxIT and LxVP, need to
be deleted in order to completely block NFAT dephosphorylation by Cn, supporting the essential
role each of the sites plays in this interaction\textsuperscript{44}. We should also point out that our experimental
data place the LxVP binding site at a distance of \( \sim 20\text{Å} \) from calcineurin’s active center, which is
closer than the \( \sim 30\text{Å} \) distance for the binding site predicted by recent \textit{in silico} docking studies\textsuperscript{45}.
Considering the additional eight residues at the C-terminus of the LxVP peptide used in that
recent docking study, as well as the physical proximity of the LxVP binding site to the catalytic
center of Cn we found experimentally, the results presented here may explain how the LxVP
peptide might be able to reach a site close enough to Cn’s active site and prevent access of other
substrate peptides. Indeed, in the representation of Figure 1.22B, a groove can be seen
connecting the LxVP site to Cn’s catalytic center capable of accommodating the WxK segment
C-terminal to the LxVP motif used in our peptide.

The observation that the two NFAT segments bind Cn simultaneously and do not displace
each other at equimolar concentrations can readily explain earlier observations that addition of a
PxIxIT containing peptide cannot fully prevent the nuclear translocation process of NFAT\textsuperscript{39,44}. This
could be due to the observation that NFAT’s regulatory domain (NHR) remains bound to the
phosphatase by its LxVP motif. Our findings show that the LxVP-derived peptide binds to a site
overlapping with the PxIxIT binding epitope, which resolves previous observations and
establishes a distinct single Cn epitope as the key target for inhibiting the Cn-NFAT interaction.
Figure 1.23 Graphic summary of Chapter 1: Structural Delineation of the Calcineurin-NFAT Interaction by NMR. The same denotations and colors are used as in Figure 1.22. Adapted from Gal et al (2014)\textsuperscript{26}.

However, the observation that the NFAT peptides containing the LxVP and PxlxIT motifs do not fully displace each other reveals that the binding epitopes are not entirely identical. In particular, spectra of Cn-bound $^{15}$N-labeled NFAT-LxVP with and without unlabeled PxlxIT are
not identical (Figure 1.16). The same observation was made for Cn-bound $^{15}$N-labeled PRIEIT with and without unlabeled LxVP (Figure 1.20). This provides further supporting evidence that both NFAT regions containing the conserved sequence, LxVP and PxIxIT, respectively, bind to the overlapping epitope on CnA simultaneously, and form a single loop that contains all phosphorylation sites (Figure 1.22C). This loop formation can constrain the degrees of freedom of the bound NFAT regulatory domain and could induce formation of a localized structural architecture around the nuclear localization sequence (NLS) and relate to the mechanism controlling NFAT translocation. In addition, loop formation of NFAT's regulatory domain has the advantage of shortening the distance of the phosphorylated residues to the active site of Cn and is expected to enhance the dephosphorylation reaction on multiple sites (Figure 1.6 and 1.22B). Although the loop formation would also be expected if the LxVP and PxIxIT sites bind to distinct regions on Cn, presence of the groove connecting the NMR-derived PxIxIT/LxVP binding site to Cn’s catalytic center reveals an advantage of loop formation by the partially overlapping epitopes (Figure 1.21B). Further investigations are needed to gain structural understanding of this loop formation.

Translocation of NFAT to the nucleus is a decisive event for T-cell activation. It is triggered by calcium-induced activation of Cn and subsequent dephosphorylation of NFAT's regulatory domain by Cn. It is important to identify the key residues and/or binding region of the Cn-NFAT interface with atomic resolution. Elucidating the dynamic interplay of the two conserved NFAT segments provides crucial biophysical insight regarding the dephosphorylation process mediated by Cn. This in turn paves the way for understanding the nuclear translocation mechanism resulting in T-cell activation. The first Chapter of this dissertation is focused mainly on the structural delineation of the calcineurin-NFAT interaction using NMR spectroscopy and
biochemical assays. As shown in the graphic summary of Figure 1.23, we have identified the LxVP binding site on Cn, which overlaps with that of PxIxIT. In addition, our study opens a new avenue for finding selective immunosuppressive drugs by relying on the structural aspects of Cn-NFAT interactions. Indeed, we are actively pursuing these questions via biophysical approaches in studies described in Chapter 2.
CHAPTER 2: NMR-based Fragment Screening for Inhibitors of Cn-NFAT Interaction

2.1 Introduction

2.1.1 Inhibition of calcineurin by small molecules

As discussed in the previous chapter, the NFAT family of transcription factors plays pivotal roles in the development and function of the immune system, whose activation process is tightly regulated by the Ca\(^{2+}\)/CaM-dependent serine/threonine protein phosphatase calcineurin. Under normal circumstances, activation of the immune response is required to protect an organism from the attack of potential pathogens. However, on the other hand, it is also necessary to suppress the NFAT activation and subsequent immune response for therapeutic purposes in diseases such as organ and tissue transplantation, allergy, and the autoimmune disease\(^6\). Given the importance of the Cn-NFAT signaling pathway in the immune system, it has been the molecular target for a number of immunosuppressive drug development efforts. Indeed, targeting the Cn-NFAT signaling pathway, in particular, inhibiting the phosphatase activity of calcineurin has revolutionized clinical transplantation.

A summary of several types of inhibitors of the calcineurin-NFAT signaling pathway, which were discovered and at least partially characterized in the last few decades, has been presented in Figure 2.1\(^7\). As discusses in Chapter 1 and shown in this summary, the Cn-NFAT signaling pathway involves multiple sequential biological events, and therefore, can be inhibited at different levels by inhibitors employing different mode of actions (shown as red arrows in Figure 2.1). For example, the Cn-NFAT signaling pathway can be interrupted by selectively inhibiting (i) the receptor induced Ca\(^{2+}\) entry into cells, (ii) the NFAT-PxIxIT binding to CnA,
(iii) the enzymatic phosphatase activity of calcineurin (iv) the NFAT nuclear translocation, and (v) the NFAT binding to its transcriptional partners and/or DNA in the nucleus.

In this dissertation, we focus mainly on inhibition activities at the level of calcineurin, especially at the level of the Cn-NFAT interaction, which is necessary and specific to the immune response. Here, we review first several well-known small molecule inhibitors of calcineurin, including CsA and FK506, which inhibit Cn’s phosphatase activity, and INCAs, which blocks the Cn-NFAT interaction.

![Figure 2.1](image-url)

**Figure 2.1 Summary of inhibition of the calcineurin-NFAT pathway at multiple levels.** A selection of the most important and best characterized inhibitors of calcineurin-NFAT signaling is shown (in red) at their point of interference. Adapted from Sieber et al (2009).

The most well-known small molecule inhibitors of Cn are cyclosporin A (CsA), a fungal-derived cyclic lipophilic undecapeptide, and FK506, a bacterial tacrolimus (Figure 2.2). Both
CsA and FK506 are widely used immunosuppressive drugs in the clinic. The immunosuppressive activities of CsA were first discovered 1976 in animal models\textsuperscript{72}. CsA was first used as a post-transplant immunosuppressive therapy in 34 recipients of cadaveric organs in 1979\textsuperscript{73}. FK506 was first described as an alternative to CsA in 1987\textsuperscript{74}, followed by its first clinical application for liver, kidney, and pancreas transplantation in 1989\textsuperscript{75}. FK506 has also recently been approved for use in the treatment of atopic dermatitis\textsuperscript{80}.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{immunophilin_ligands.png}
\caption{The structure of immunophilin ligands. FK506 and rapamycin share some common structural features, while CsA has an entirely different chemical structure. Adapted from Schreiber et al (1992)\textsuperscript{71}.}
\end{figure}

Although CsA and FK506 differ considerably in their chemical structures (Figure 2.2), they share a quite similar mechanism of action and biological effects \textit{in vivo}. It has been shown that CsA and FK506 are not active calcineurin inhibitors by themselves and cannot bind directly to
Cn, but instead, they need to first bind to their endogenous matchmaker proteins, immunophilins, in order to be activated and gain inhibitory functions\textsuperscript{71,81}. Immunophilins are endogenous cytosolic peptidyl-prolyl isomerases (PPIases, also known as a rotamase) that interconvert between the \textit{cis} and \textit{trans} positions, and are involved in \textit{de novo} protein foldings, as well as many other cellular functions\textsuperscript{82}. The two particular immunophilins involved in calcineurin inhibition are cyclophilin A (CypA) and FK506 binding protein 12 (FKBP12), whose isomerase activities are blocked by the binding of CsA and FK506, respectively (Figure 2.3)\textsuperscript{71,83}. It has been proposed that the loss of isomerase activity is due to the fact that the two drugs contain a 'twisted amide surrogate' structure, which is a transition state mimic of a peptidyl-prolyl bond undergoing isomerization\textsuperscript{71}.

Structural investigations of the two immunophilin-drug complexes, CypA-CsA and FKBP12-FK506, by X-ray crystallography and nuclear magnetic resonance (NMR), have provided support for their interactions with Cn, and have greatly furthered our understanding of the action modes of these two drugs. As shown in Figure 2.3, the drug-immunophilin complexes bind to Cn at the interface of the two Cn subunits, the catalytic A (CnA) and regulatory B (CnB) subunits, and severely limit the access of protein substrates to the active site of Cn, thereby inhibiting the phosphatase activity of Cn\textsuperscript{71}. The CsA-immunophilin and FK506-immunophilin complexes are noncompetitive inhibitors of Cn, as they do not compete directly with Cn substrates for binding to the active center, but instead, bind at the CnA/CnB interface, which is over 30 Å away from the Cn active site. It has been shown that these drug complexes block NFAT dephosphorylation by Cn and its subsequent nuclear translocation, which appears to be the major mechanism of immunosuppression mediated by CsA and FK506 (Figure 2.4)\textsuperscript{71,84}. However, small molecular substrates of Cn, such as p-nitrophenyl phosphate (pNPP), can still be
dephosphorylated at the presence of the drug-immunophilin complexes\textsuperscript{85,86}. This suggests that the binding of the drug-immunophilin complexes to calcineurin, although impedes Cn-substrate interactions, does not alter the active site and thus, the enzymatic activity of Cn.

Figure 2.3 Binding of CypA-CsA and FKBP12-FK506 complexes to calcineurin. (A) The ribbon presentation of the Cn-FKBP12-FK506 trimeric complex structure (PDB entry: 1TCO). (B) Detailed interactions between the FKBP12-FK506 complex and Cn. The blue sticks-balls are the Cn residues interacting with FKBP12, while the pink sticks-balls are the residues for FK506 binding. (C) Ribbon presentation of the Cn-CypA-CsA trimeric complex structure. (D) Detailed binding of the CypA-CsA complex to Cn. The color codes for the ribbons are: golden for CnA, cyan for CnB, green for FKBP12, and red for CypA. FK506 and CsA are presented as blue and purple sticks-balls, respectively. Adapted from Ke et al (2003)\textsuperscript{83}. 

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The mechanism by which the CsA- and FK506-immunophilin complexes block substrates access to the active site of Cn is still unclear. It has been suggested that substrate access is simply blocked by the large immunophilin proteins, which prevent access by steric inhibition. A second possibility is that the drug-immunophilin complexes bind to the CnA/CnB interface and compete with the LxVP motif of substrates for binding to Cn. However, as we have shown in Chapter 1 that the NFAT-LxVP motif binds to CnA at an overlapping epitope with the PxlxIT docking site, which is away from the CnA/CnB interface. Therefore, this second hypothesis is inconsistent with our experimental results and has not been experimentally confirmed. It is also possible that the immunophilins simply serve to improve the binding properties of CsA and FK506 to Cn by providing additional protein-protein interactions, but may play little or no role in Cn inhibition.

Figure 2.4 The drug-immunophilin complex blocks nuclear translocation of NFAT. The drug-isomerase complex inhibits the dephosphorylation and nuclear translocation of NFAT. Adapted from Schreiber et al (1992)71.
More recently, it has been discovered that the CsA-CypA and FK506-FKBP complexes exert their immunosuppressive effects through targeting two distinct signaling pathways: besides the calcineurin-NFAT pathway, they also suppress the activation of JNK and p38 pathways at a level upstream of mitogen-activated protein kinase (MAPK) kinase kinase (MAP3K) (\textbf{Figure 2.5})\textsuperscript{87,88}. This finding may explain the experimental results that cells expressing a constitutively active NFAT are still sensitive to CsA or FK506.

\textbf{Figure 2.5} Schematic diagram showing the effects of drug-immunophilin complexes on the calcineurin-NFAT pathway and the JNK and p38 activation pathways. These two drug-immunophilin complexes exert immunosuppressive effects via targeting two distinct pathways. Adapted from Matsuda \textit{et al} (2000)\textsuperscript{88}.

Currently, CsA and FK506 are still widely used as effective immunosuppressive drugs in the clinic, especially in organ transplantation to prevent rejection, as well as in dermatology\textsuperscript{76-78}. Moreover, application of these two compounds as chemical probes in basic research has substantially advanced our understanding of the Cn-NFAT signaling pathway, and also contributed to the elucidation other Cn-dependent signaling processes\textsuperscript{4,79}. Despite their
effectiveness and wide application in the clinic, the CsA-CypA and FK506-FKBP12 complexes cannot distinguish between NFAT and other substrate proteins of Cn, and therefore, have dramatic side effects as immunosuppressive drugs. Moreover, the drug complexes also modulate other pathways than the calcineurin-NFAT signaling pathway, further compounding their undesired side effects and toxicity. One way to circumvent these side effects is to directly target and disrupt the protein-protein interaction between Cn and NFAT, while leaving the catalytic functionality of the enzyme and the access of other substrates intact. The discovery of INCAs, small molecule inhibitors of the NFAT-Cn association, followed this strategy, and has demonstrated that selective inhibition of the Cn-NFAT interaction is sufficient in preventing NFAT dephosphorylation and transcriptional activities in pre-clinical setting\textsuperscript{48,61-63}.

\textbf{Figure 2.6 Chemical structures of selected INCA compounds.} Note the chemical structure diversity of this class of Cn-NFAT inhibitors. INCAs are active compounds, while N-1 and N-2 are inactive compounds. Adapted from Roehrl et al (2004)\textsuperscript{48}. 
In an effort to develop more selective inhibitors for the calcineurin-NFAT interaction, our group had previously identified the INCA compounds, which are a group of chemically unrelated small molecules (Figure 2.6), selected in a high-throughput screening (HTS)\textsuperscript{48,61-63}. Briefly, Roehrl et al., had employed a HTS fluorescence polarization (FP) assay to screen a collection of \textasciitilde{}16,320 small molecules for their ability to disrupt the interaction between Cn and the affinity-enhanced PVIVIT peptide \textsuperscript{48,61-63}. Figure 2.6 lists a few examples of the most active (INCAs) and inactive compounds (N-1 and N-2), identified in that FP assay.

Although quite different in chemical structures, the INCA compounds fell into two functional classes: (i) compounds that could dissociate the VIVIT-Cn complex completely, such as INCA-1, INCA-2, and INCA-6, and (ii) compounds that have led to significant but partial dissociation of the Cn-VIVIT complex, such as INCA-3, INCA-5 and all other INCAs\textsuperscript{48}. The inhibitory effects of these INCA compounds \textit{in vivo} have been demonstrated using INCA-6 as an example. Specifically, INCA-6 was shown to inhibit the dephosphorylation of NFAT, and block its nuclear translocation in cells when administered at concentrations in the tens of \textmu{}M. INCA-6 also inhibited the induction of mRNAs for a set of cytokines known to be at the downstream of NFAT signaling pathway. Moreover, the selectivity and specificity of INCA-6 had also been confirmed as it had no effect on the catalytic activity of Cn toward a known phosphopeptide substrate, the RII peptide. However, INCA-2 and 7 do inhibit the enzymatic activity of Cn\textsuperscript{48}. Therefore, these INCA compounds, although identified in the same assay, may employ different mode of actions.

The most active compounds identified in this FP assay, INCA-1, 2 and 6, are all quinones or quinone derivatives, and this functionality was shown to be necessary for their inhibitory activity, suggesting the possibility that these quinones might act by covalently modifying Cn. Further, using MALDI-TOF mass spectrometry (MS), Kang et al., have revealed that the binding
site on calcineurin for INCA-1, INCA-2, and INCA-6 is centered on the Cys266 of calcineurin A subunit (Figure 2.7), and does not coincide with the core PxIxIT-binding site (β14 strand of CnA)\(^62\). In particular, INCA-1, 2 and 6 bind covalently but reversibly to Cn at the residue Cys266. It has also been suggested that the inhibition activity of INCAs arises through an allosteric change masking the PxIxIT docking site, which may be assisted by the covalent binding and modification of the Cys266 on CnA. As shown in Figure 2.7, the spatial arrangement of the binding sites for PVIVIT and INCA makes it possible that the change in conformation caused by INCA bindings involves the β11-β12 loop of CnA, and thus, the PVIVIT docking site.

**Figure 2.7 Structural context of Cys266 in calcineurin A.** (A) Structural location of Cys266 relative to the PxIxIT-binding site and the catalytic site of Cn (PDB entry: 1AUI). (B) Stereo view of the Cn-PVIVIT complex highlighting the distance of 15 Å from Cys266 to PVIVIT. (C) Stereo view of CnA backbone and sidechain packing around Cys266. Residues in the β11-β12 loop are in lavender, Phe299 and Pro300 are in red. Adapted from Kang *et al* (2005)\(^62\).
These INCA inhibitors may be effective in blocking the Cn-NFAT interaction. However, general cytotoxicity has been reported for all INCA compounds, ruling out their use in primary cells\textsuperscript{61-63,89}, and therefore, promoting the discovery of novel Cn-NFAT inhibitors, that are safer for in vivo applications.

**Table 2.1 Peptides and proteins inhibiting calcineurin-NFAT signaling\textsuperscript{70}**

<table>
<thead>
<tr>
<th>Inhibitor peptide</th>
<th>Mode of action</th>
<th>IC\textsubscript{50}/K\textsubscript{d}/K\textsubscript{i}</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaN\textsubscript{457-482-AID}</td>
<td>mask the active centre of Cn</td>
<td>10 (\mu\text{M})\textsuperscript{a}</td>
</tr>
<tr>
<td>CaN\textsubscript{424-521-AID}</td>
<td></td>
<td>2.5 (\mu\text{M})\textsuperscript{b}</td>
</tr>
<tr>
<td>mNFAT\textsubscript{c}2106-121 - SPRIEIT</td>
<td>block CaN-NFATc interaction</td>
<td>12 (\mu\text{M})\textsuperscript{1}</td>
</tr>
</tbody>
</table>
| VIVIT peptide                           |                                                        | 0.5 \(\mu\text{M}\)\textsuperscript{1}  \\
|                                          |                                                        | \(< 1 \mu\text{M}\)\textsuperscript{1}                  |
| AKAP\textsubscript{79330-357}            | blocks CaN-NFATc interaction                           | 1.5 \(\mu\text{M}\)\textsuperscript{d}                 |
| RCAN1                                    |                                                        | 60 \(\mu\text{M}\)\textsuperscript{k}                  |
| RCAN1-4141-197 - exon7                   | block CaN-NFATc interaction                           | 70 \(\mu\text{M}\)\textsuperscript{k}                  |
| RCAN1-4143-163 - CIC peptide            |                                                        | 1.25 \(\mu\text{M}\)\textsuperscript{k}                |
| LxVPc1 peptide                          | blocks CaN-NFATc interaction and modulates enzymatic activity of Cn | \(< 3 \mu\text{M}\)\textsuperscript{a} |
| RCAN1-495-118 - SP repeat peptide       | masks the active centre of Cn                         | 91.5 \(\mu\text{M}\)\textsuperscript{a}                |
| VacA                                     | inhibits translocation of NFATc                       | N.D.                                                    |
| A238L                                    | block CaN-NFATc interaction                           | N.D.                                                    |
| A238L-200-213                            |                                                        | 0.6 \(\mu\text{M}\)\textsuperscript{k}                 |

* IC\textsubscript{50}/K\textsubscript{d}/K\textsubscript{i}: ND - not determined; a - phosphatase assay on RII phosphopeptide; i - interference with Cn-NFAT binding assay; k - binding assay to Cn; l - phosphatase assay on NFAT.
2.1.2 Inhibition of calcineurin by peptides

Other than small molecule inhibitors, there are a growing number of inhibitory peptides and proteins from pathogen being discovered that can also inhibit the phosphatase activity of Cn, and thus Cn-NFAT-dependent pathways. The discovery process for these inhibitory peptides and pathogen proteins often starts with the identification of a specific binding motif from interaction partners of Cn. For example, the PxIxIT motif from NFAT, which interacts with CnA, has been the point of origin for several inhibitory peptides (Table 2.1).

In addition, modifications and affinity-driven optimization of these peptides, such as fragment shortening and point mutations, will further support these drug discovery efforts. However, one specific problem often associated with peptide inhibitors is that they usually have very low cell membrane permeability. Several strategies have been developed to overcome this hurdle. For example, a cell-permeable inhibitory peptide was synthesized with a C-terminal leading peptide sequence containing poly-Arginines.11

Let’s take VIVIT for example: the VIVIT peptide containing an amino acid sequence of MAGPHPVIVITGPHEE was designed based on the PxIxIT motif located near the N-terminus of NHR. This synthetic 16-mer oligopeptide, optimized by selective amino acid exchange, possesses ~25 times higher efficiency in inhibiting NFAT dephosphorylation by Cn and NFAT-dependent expression of endogenous cytokine genes in T cells compared to the original 16-mer NFAT1-SPRIEIT peptide.41,70,89 As shown in Figure 2.1, both VIVIT and CsA/FK506 inhibit the Cn-NFAT signaling pathway through inhibiting the dephosphorylation of NFAT by Cn. However, on a molecular basis, VIVIT employs a quite different mode of action compared to CsA and FK506 (Figure 2.8),89 and is thought to be more selective than the CsA- and FK506-
immunophilin complexes, as evidenced by the fact that overexpression of GFP-VIVIT fusion protein in Jurkat T cells inhibits NFAT- but not NFκB-dependent reporter gene expression, while the drug-immunophilin complexes inhibit the activation of both transcription factors. Moreover, many efforts have been put into improving the cell permeability of VIVIT. In particular, 11R-VIVIT peptide is shown to be cell-permeable in selected cell types and mice.

Figure 2.8 Action mode of VIVIT in comparison with CsA/FK506 in the regulation of calcineurin-NFAT signaling. CsA and FK506 disrupt Cn catalytic activity and thus affect all downstream signal transduction pathways of Cn, including NFAT and NFκB. VIVIT selectively inhibits the Cn-NFAT interaction, but does not impair Cn catalytic activity. Further, apart from interfering with the Cn-NFAT signaling, CsA also acts on other targets, such as MAPK, to promote NFAT nuclear export. Adapted from Sieber et al (2009).
Other than the PxIxIT peptide and its affinity-enhanced synthetic derivative, VIVIT, a growing number of endogenous or pathogen protein inhibitors of calcineurin have also been identified in recent years, such as Cabin1/cain, FKBP38, CHP, A238L, AKAP79, RCAN1, RCAN3, and DSCR1. These endogenous or pathogen Cn inhibitors are throwing new light on the function and regulation of Cn in a wide variety of cellular processes and cell types, and have as well provided us with new starting points for immunosuppressive drug discovery.

Figure 2.9 Different classes of calcineurin inhibitory proteins. Inhibitors of Cn, dual regulators of Cn, and anchoring proteins of Cn all serve as endogenous protein inhibitors of Cn. Adapted from Liu (2003).

To give a few examples: one of the first endogenous inhibitors of Cn to be identified was the 79 kDa A kinase associated protein (AKAP79), which contains a PxIxIT motifs for binding to Cn and targets Cn to Ca\(^{2+}\) channels in the cell membrane. The second endogenous Cn inhibitors
to be identified was calcineurin homologous protein (CHP), which shares 65% sequence similarity with CnB and 59% with CaM, and is shown to inhibit Cn catalytic activity through preventing the assembly of the CnA/CnB/CaM complex in an active conformation. A more recently identified endogenous Cn inhibitor is known as Cabin1 (mouse) or cain (rat), which is a 240 kDa phosphoprotein with no significant homology to any known protein that binds to and inhibits Cn in a noncompetitive manner. Lastly, A238L, which belongs to a different class of endogenous Cn inhibitor, is found in the genome of the African swine fever virus. Given that the C-terminal of A238L is a Cn-binding domain containing both the PxIxIT and LxVK sequences, this viral protein could probably inhibit Cn either by blocking its active site, or by competing directly with its substrates for Cn binding. These peptide inhibitors of Cn, along with some novel small molecule inhibitors, provide useful research probes for studying the Cn-dependent pathways, but are not good enough for clinical use, promoting the discovery of better, more selective Cn inhibitors.

2.1.3 Fragment-based drug design

In modern drug discovery, fragment-based drug design (FBDD) has emerged as an alternative to high-throughput screening (HTS). In fact, FBDD is gaining momentum in both large pharmaceutical companies and biotechnology laboratories. The is because HTS requires searching huge collections of drug-sized molecules, while FBDD only requires searching smaller collections of fragments, and are showing a high success rate in generating chemical series with lead-like properties. Compared with hits generated from traditional HTS screening, the starting fragments identified in FBDD have considerably lower molecular weight but high ligand efficiency. The detailed comparison of FBDD and HTS is summarized in Table 2.2.
Table 2.2 Comparison of fragment-based approaches and high-throughput screening

<table>
<thead>
<tr>
<th>Fragment-based approaches</th>
<th>High-throughput screening (HTS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Emphasis on efficiency</td>
<td>Emphasis on potency</td>
</tr>
<tr>
<td>Typically screen a few hundred–few thousand compounds</td>
<td>Typically screen hundreds of thousands of compounds</td>
</tr>
<tr>
<td>M&lt;sub&gt;i&lt;/sub&gt; range ~150–300</td>
<td>M&lt;sub&gt;i&lt;/sub&gt; range ~250–600</td>
</tr>
<tr>
<td>Hit activity in the range mM–30 μM</td>
<td>Hit activity in the range ~30 μM–nM</td>
</tr>
<tr>
<td>Hits have clearly defined binding interactions; high proportion of atoms directly involved in protein binding</td>
<td>Hits can contain functional groups that contribute poorly to protein binding or act primarily as scaffolding</td>
</tr>
<tr>
<td>Biophysical screening techniques (NMR, X-ray) are direct measurements of binding interaction. Can screen against ‘inactive’ forms of the target protein (for example, kinases)</td>
<td>In vitro bioassay-based screening. Can generate false positives and high attrition in hit-validation stage</td>
</tr>
<tr>
<td>Protein-structure-based information key in validating and prioritizing chemistry hits</td>
<td>Chemistry (re)synthesis resource usually required to validate and prioritize screening hits</td>
</tr>
<tr>
<td>Hit-to-lead chemistry usually requires synthesis of only a few compounds designed to add additional, specific binding interactions</td>
<td>High attrition of chemical series in hit-to-lead stage. Usually requires several iterations of high-throughput chemistry. Attrition rates can be improved with knowledge of protein structure</td>
</tr>
<tr>
<td>Design-intensive</td>
<td>Resource-intensive</td>
</tr>
<tr>
<td>Requires expertise and knowledge in protein structure, protein–ligand-binding interactions and fragment design</td>
<td>HTS requires extensive infrastructure for storing and handling compound collections, screening, automation, data processing and chemistry follow-up</td>
</tr>
</tbody>
</table>

As shown in Table 2.2-left column, fragment hits identified in FBDD typically have low binding affinities in the range of mM to 30 μM. Conventional assays’ usefulness is limited in detecting such low-affinity binding events. Instead, biophysical screening methods, such as NMR, X-ray crystallography, and surface plasmon resonance (SPR), are particularly suitable for FBDD, given their sensitivity and the range of binding affinities that they can detect. Moreover, these biophysical methods can provide structural insights into the ligand-protein binding event, which is crucial in prioritizing fragment hits and rapidly developing them into leads, as well as in structure-activity relationships (SAR) studies.

One particular example of these biophysical screening methods that has been used in FBDD, is the saturation-transfer difference (STD) NMR, which has emerged as one of the most popular ligand-based NMR techniques for fragment screening as well as for the characterization of
protein-ligand interactions (Figure 2.10). The underlying principle of this STD NMR experiment is that for a weak binding ligand with $K_d$ ranging from mM to 10 nM, there is an exchange between the bound and the free ligand state, which is suitable for NMR detection. Briefly, an STD experiment involves subtracting a ligand spectrum in which the protein was selectively saturated (on-resonance) from one recorded without protein saturation (off-resonance). In this regard, only the signals of the ligand hydrogens that are in close proximity to the protein ($\leq 5 \text{ Å}$) and receive magnetization transfer will appear in the difference spectrum and within those, the ones that are closer to the protein will have more intense difference signals, due to a more efficient magnetization transfer. Therefore, STD NMR is an efficient and attractive method for FBDD.

**Figure 2.10 Scheme of the STD-NMR experiment.** The exchange between free and bound ligand allows intermolecular transfer of magnetization from the receptor protein to the bound small molecule (red) for NMR detection. No signals from the unbound small molecules (blue) can be detected. Adapted from Viegas et al (2011).
Following initial FBDD screening and fragment hits identification, fragment linking and the hit to lead generation is carried out (Figure 2.11B)\(^98\), which relies on the identification of pairs of fragments that are close enough for chemical linking. A recently developed NMR-based technique that allows the identification of pairs of fragment hits, which sit in adjacent sites on the surface of a given receptor protein, is called structure-activity relationships (SAR) by interligand nuclear Overhauser effects (ILOEs) (Figure 2.11A)\(^100,101\).

**Figure 2.11 Structure-activity relationships by interligand NOE and fragment linking.** (A) Schematic representation of the SAR by ILOE approach. Adapted from Becattini et al (2006)\(^100\). (B) Fragment 1 and 2 bind to the receptor at adjacent sites and are joined together by a linking group that allows the lead molecule to span both sites. Adapted from Rees et al (2004)\(^98\).

FBDD’s contribution to drug discovery is increasingly recognized by the medicinal chemistry community, and in particular, it is increasingly being applied to challenging therapeutic targets for which conventional drug discovery methods have failed. One such class of
challenging targets in drug discovery is protein-protein interaction (PPI). In this chapter, we present an example of using the fragment-based approach to target PPI.

Protein-protein interactions (PPI) are integral to almost all cellular functions and aberrant PPI often leads to human diseases. Although the inhibition of protein-protein interaction by small molecules has been seen as challenging due to the difficulty in occluding large, often featureless protein-protein interfaces by relatively small organic molecules, the targeted modulation of PPI by small molecules is rapidly gaining pharmacological interest in the development of therapeutics and chemical probes, as these small molecule probes would offer previously unavailable opportunities to explore the relevance of PPI for cellular signaling as well as for providing clinical benefits to patients.\(^65,102,103\)

A particular example of PPI, which is gaining significant therapeutic importance in the treatment of graft transplant rejection, autoimmune diseases and cardiovascular disorders, is the interaction between Cn and NFAT. Here, we will discuss the development and validation of a direct (orthosteric) small molecule inhibitor targeting the Cn-NFAT interaction, which is discovered through fragment-based drug design using NMR techniques. Briefly, an initial NMR-based screen of the Maybridge Ro3 library of \(~1,000\) fragments against Cn using NMR saturation-transfer difference and interligand NOE led to the identification of two low affinity binders, 7F8 and 7B9, which sit in adjacent sites on the surface of Cn. Covalent linkage and iterative evolution of the two fragment hits produced a novel Cn-NFAT inhibitor (SL-3) with an IC50 value of \(~10\ \mu \text{M}\), as measured by fluorescence polarization assay. In vitro and in-cell assays demonstrate that this small molecule selectively inhibits NFAT dephosphorylation and NFAT-mediated gene expression without affecting Cn phosphatase activity in general. By measuring the paramagnetic relaxation enhancement (PRE) of Cn bound to a spin-labeled
derivative of SL-3 (SL-R1), we further demonstrate that the binding site on Cn for these inhibitors coincides with the core PxIxIT-binding site. These compounds are promising probes for the study of Cn-NFAT interaction, NFAT activation by Cn, and NFAT-mediated cell signaling and can also serve as lead scaffolds for the development of alternative immunosuppressive drugs.

Details of the experiment design and results are presented as follows.

**Figure 2.12 The results of the STD NMR screening.** (A, red) Initial STD NMR screening of ~1,000 fragments from the Maybridge Ro3 Library was carried out using 100 µM of each compound in the presence of 5 µM CnA alone. (B, blue) The same STD screening was performed with 5 µM CnA-DM complex. Compounds were ranked according to their signal intensities in the saturation transfer difference spectra. As we can see, results from the two STD screenings differ from each other.
2.2 Identification and development of compounds inhibiting calcineurin-NFAT interaction

2.2.1 Fragment hit identification by STD NMR screening

To identify the initial fragment hits for inhibiting the Cn-NFAT interaction, we started first by screening for fragments that bind to calcineurin in the absence of NFAT. Briefly, we screened ~1,000 fragments purchased from the Maybridge Ro3 Fragment Library at high concentrations (100 µM each) in cocktails of 10 with 5 µM non-labeled CnA by ligand-based saturation-transfer difference (STD) NMR. In such STD experiments, only the signals for compounds that are in close contact to the CnA protein (≤ 5Å), and experience magnetization transfer from selectively saturated CnA are observed in the STD spectrum, while no signals from nonbinding small molecules will be present.

This initial STD NMR screening allowed the identification of CnA binders, but did not differentiate competitive binders from noncompetitive binders. In an effort to distinguish fragment hits that bind Cn competitively and prevent NFAT binding from those that bind Cn independently, we repeated the STD screen at the presence of 5µM CnA-DM complex, where DM is a drug-sized compound we have identified internally to bind CnA covalently at residue Lys293 and inhibit VIVIT binding. The CnA-DM complex served as a mimic of the CnA-VIVIT complex. Only those that show a less STD effects at the presence of DM were selected for follow-up experiments. The STD NMR screening results are summarized in Figure 2.12, where the top 70 fragments from both screenings are ranked according to their signal intensities in the difference spectra, and thus, their closeness to the Cn protein. Cocktails containing the top 20 fragments that had the strongest signals in the CnA-alone STD screening, but showed less STD effects in the CnA-DM STD screening, were selected as initial hits for the next step.
Figure 2.13 Identification of fragment hits 7B9 and 7F8 in the initial STD NMR screening. (A) Identification of 7B9 as a Cn binder in the absence of NFAT. The difference spectrum overlaps (red) very well with the $^1$H-NMR spectrum of 7B9, suggesting 7B9 as a hit. (B) The difference spectrum (red) contains peaks from both 7F5 and 7F8 spectra, with the latter showing significantly more STD effects, indicating better binding with Cn.
As an example, the identification of fragments 7B9 and 7F8 as hits for Cn binding in the Cn-alone STD screening is illustrated in Figure 2.13A and B, respectively. As we can see from Figure 2.13A, the 1D $^1$H-NMR spectrum of 7B9 overlaps with the STD spectrum (red). Therefore, 7B9 binds to free Cn and was identified from the 7B cocktail as an initial hit. Similarly, fragments 7F5 and 7F8 were also identified from the 7F cocktail as initial hits for Cn binding. Moreover, as shown in Figure 2.13B, 7F8 clearly show more STD effects than 7F5, and thus, is a tighter binder for Cn. Based on this analysis, 7F8 was ranked higher in the STD screening results than 7F5 (Figure 2.12-top, red). One drawback associated with the STD NMR experiment is that STD relies on the fact that for a weak binding ligand, there is exchange between its bound and free state, and therefore, a ligand that binds too tightly may be missed. But in our screening, the fragments used here are expected to have weak affinities and can thus be detected in an STD-based screen.

2.2.2 Structure-activity relationships study by interligand NOE

Following initial hit identification, the top 20 fragment hits from the two STD NMR screens were then subject to structure-activity relationships (SAR) study by interligand NOE (ILOEs), which allows the identification of pairs of fragment hits that bind simultaneously to Cn and sit in adjacent sites on the CnA surface. Briefly, each pair of fragment hits from the STD screens was mixed at 100 µM concentrations in 0.5 × PBS D$_2$O buffer and their interligand NOEs were recorded on a Bruker Avance 500 MHz spectrometer in the presence of 5 µM CnA. Results of the SAR by INOEs are summarized in Table 2.3, where pairs that give ILOEs signals at both long and short mixing times are highlighted. In NOESY experiment, the larger the mixing time the more NOEs will be observed, but the less difference in intensity by distance. In addition, the mixing time is correlated to the size of the molecule, small molecule, long mixing time. In our
experiment, the mixing time was reduced from 600 ms to 200 ms, and then to 100 ms in order to differentiate distance based on signal intensity, as well as to eliminate artifacts caused by spin diffusion. Accordingly, we were able to identify a pair of low-affinity fragments, 7F8 and 7B9, which showed strong ILOEs signals, suggesting their simultaneous binding to neighboring sites on the surface of Cn. 7F8 and 7B9 were selected for the next step and served as building blocks for producing linked compounds.

Table 2.3 Results of structure-activity relationships study by interligand NOE

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* Y: interligand NOE signals detected; N: no ILOEs detected.
Figure 2.14 Interligand NOE between 7F8 and 7B9. (A) Interligand NOE signals are observed between (i) the protons of the –OCH$_3$ of 7F8 and the protons of the –SCH$_3$ and the aromatic ring of 7B9, (ii) the protons of the –C(CH$_3$)$_3$ of 7F8 and the protons of the aromatic rings of 7B9. Color code: intramolecular NOEs of 7B9 are shown in yellow; intramolecular NOEs of 7F8 are shown in red; interligand NOE signals between 7B9 and 7F8 are shown in cyan. (B) The relative position of 7F8 and 7B9 in bound states with Cn is proposed based on ILOEs (red arrows) in A.
The interligand NOE signals detected between fragments 7B9 and 7F8 also provide structural insights. Especially, they can be used to determine the relative orientation of the two fragments in their Cn-bound states and subsequently guide the synthesis of linked ligands from the individual fragments. As shown in Figure 2.14, strong interligand NOE signals were detected between (i) the protons of the –OCH$_3$ of 7F8 and the protons of –SCH$_3$ and the aromatic ring of 7B9, (ii) protons of the –C(CH$_3$)$_3$ of 7F8 and protons of the aromatic rings of 7B9, suggesting that these pairs of functional groups are in close proximity when docked on the surface of Cn. Based on these IδOE signals, our fragment linking strategy was designed and carried out.

Following SAR by ILOEs, 2D $^{15}$N, $^1$H TROSY-HSQC titration and virtual docking of the two fragments, 7F8 and 7B9, onto CnA were performed in order to gain structural insights on the Cn-7F8-7B9 complex and prioritize chemistry. However, because of the lack of significant NMR chemical shift perturbations (Figure 2.15, and Figure 2.16), we were unable to identify and map the exact binding sites of 7B9 and 7F8 on CnA. We then opted for the systematic synthesis of linked compounds with linkers of various lengths (one- to five-carbon chains) attached at different sites on the two fragment hits.

2.2.3 Hit-to-lead progression

Guided by the interligand NOEs illustrated in Figure 2.14, we have then designed and synthesized ~50 bidentate derivatives of fragment hits 7F8 and 7B9. These bidentate compounds share similar structure features as linked compounds and as derivatives of the initial hits, but their linker lengths, linking positions, and functional groups vary systemically. Among these linked compounds, SL-111 is the direct bidentate derivative of 7F8 and 7B9, SL-3 is the most active compound we have identified so far, and SL-R1 is a spin-labeled derivative of SL-3.
Figure 2.15 Titration of fragment 7B9 into $^2\text{H},^{15}\text{N}$-CnA protein. (A) 2D TROSY-HSQC NMR spectrum of free $^2\text{H},^{15}\text{N}$-CnA protein. (B) 2D TROSY-HSQC NMR spectrum of $^2\text{H},^{15}\text{N}$-CnA protein bound to fragment 7B9 in a 1:5 molar ratio. (C) Overlay of the HSQC spectrum in B onto spectrum in A. This shows that no chemical shift perturbation of the assigned $^2\text{H},^{15}\text{N}$-CnA resonances occurs upon 7B9 addition.
Figure 2.16 Titration of fragment 7F8 into $^2\text{H},^{15}\text{N}$-CnA protein. (A) 2D $^1\text{H}^{15}\text{N}$-TROSY-HSQC spectrum of free [U-$^2\text{H}^{15}\text{N}$] CnA recorded on Bruker Avance 600 MHz spectrometer at 298K. (B) 2D $^1\text{H}^{15}\text{N}$-TROSY-HSQC spectrum of [U-$^2\text{H}^{15}\text{N}$] CnA protein bound to fragment 7F8 in a 1:5 molar ratio. (C) Overlay of the $^1\text{H}^{15}\text{N}$-TROSY-HSQC spectrum in B onto the spectrum in A. The two spectra overlap very well with each other, indicating that no chemical shift perturbations of the assigned [U-$^2\text{H}^{15}\text{N}$] CnA resonances occurred upon 7F8 addition, and thus, no significant conformational change of CnA was caused by 7F8.
Synthesis, purification and analysis of linked compounds followed standard procedures. Briefly, commercially available reagents and solvents were used without further purification. All reactions were monitored by thin layer chromatography (TLC) with 0.25 mm E. Merck pre-coated silica gel plates (60 F254) and/or Waters LCMS system (Waters 2489 UV/Visible Detector, Waters 3100 Mass, Waters 515 HPLC pump, Waters 2545 Binary Gradient Module, Waters Reagent Manager, Waters 2767 Sample Manager) using SunFire C18 column (4.6 × 50 mm, 5 µm particle size): solvent A = 0.035% TFA in Water; solvent B = 0.035% TFA in MeOH; flow rate: 2.5 ml min⁻¹.

Purification of reaction products was carried out by flash chromatography using CombiFlash Rf with Teledyne Isco RediSep Rf High Performance Gold or Silicycle SiliaSep High Performance columns (4 g, 12 g or 24 g) and Waters LCMS system using SunFire Prep C18 column (19 × 50 mm, 5 µm particle size): solvent A = 0.035% TFA in Water; solvent B = 0.035% TFA in MeOH; flow rate: 25 ml min⁻¹. The purity of all compounds was over 95% and was analyzed with Waters LCMS system.

1D ¹H NMR and ¹³C NMR spectra of synthesized compounds were obtained using a Varian Inova 400 (400 MHz for 1H NMR and 100 MHz for 13C NMR) and/or Inova 600 (600 MHz for 1H NMR and 150 MHz for 13C) spectrometer at room temperature. Purified compounds were first dissolved in deuterated chloroform (CDCl₃) or deuterated dimethyl sulfoxide (DMSO). Chemical shifts are measured and reported relative to chloroform (δ = 7.26) for 1H NMR and chloroform (δ = 77.23) for 13C NMR, or dimethyl sulfoxide (δ = 2.50) for 1H NMR and dimethyl sulfoxide (δ = 39.51) for 13C NMR. Data are reported as: br = broad, s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet.
2.3 SL-3 inhibits calcineurin-NFAT interaction in FP assay

2.3.1 Fluorescence polarization assay development

To assess the efficacy of our linked compounds, the fluorescence polarization (FP) assay was used to measure their IC$_{50}$ values, taking advantage of the high affinity of the PVIVIT peptide for Cn binding. The FP assay used here is similar to the one described previously$^{48}$. The inhibitory activities of the fragment hits and the linked compounds were measured in FP competitive binding assays to gain insight into structure-activity relationships (SAR) as well as to facilitate hit-to-lead progression. Design and optimizations of the FP assay are described as follows:

![Figure 2.17 Z-factor of the FP assay.](image)

The C-terminal carboxytetramethylrhodamine (TAMRA)- or FITC-labeled PVIVIT peptide with amino acid sequence GPHPVIVITGPHEEK was synthesized by Peptide 2.0 Inc. The application of two different fluorophores allows eliminations of false positives caused by compounds auto-florescence. The FP assay set up, including buffer condition, sample volume, as well as peptide and protein concentrations, is optimized based on Z factor (Z’, Figure 2.17),

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which serves as an indication of high signal to background ratio\textsuperscript{104}. The optimized FP assay condition contains 1.2 μM GST-CnA, 30 nM fluorescently labeled VIVIT peptide, and 0.5 mM TCEP dissolved in 1/16 × PBS buffer pH 7.6 and has a Z-factor of as high as 0.85. FP measurements were made on 30 μl samples arrayed in black 384-well plates and the FP signals were recorded on an EnVision\textsuperscript{®} Multilabel Reader.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure2_18.png}
\caption{IC\textsubscript{50} measurement of SL-3 using FP assay. SL-3 inhibits the calcineurin-VIVIT interaction in FP assay with an inhibitory constant of ~10 μM, while the original fragments, 7F8 and 7B9, show no significant inhibition at the concentrations tested.}
\end{figure}

2.3.2 SL-3 inhibits calcineurin-VIVIT interaction in FP assay

SL-111, derived from the original fragment hits, 7B9 and 7F8, with a three-carbon linker connecting the -OCH\textsubscript{3} of 7F8 and the aromatic ring of 7B9, inhibited Cn-VIVIT interaction moderately in FP assay (IC\textsubscript{50} ~ 100μM). Analogues with different linker lengths (one, two, four, and five carbons) proved less effective in displacing the PVIVIT peptide. Further optimization of SL-111 yielded SL-3, which can displace PVIVIT from CnA at low micromolar concentrations.
and has an IC$_{50}$ of ~10µM (Figure 2.18). As shown in the FP curves above, 7F8 (red) and 7B9 (blue) showed no significant inhibitory activity toward the Cn-VIVIT interaction at concentrations of up to 1 mM. By linking the two fragments together, we have identified SL-3, which showed significant inhibition effects for the Cn-VIVIT interaction (black), and improved the efficacy of the original fragments by least ~100 times.

2.4 SL-3 inhibits calcineurin-NFAT interaction in vivo

2.4.1 SL-3 inhibits calcineurin dephosphorylation of NFAT in vivo

Dephosphorylation of NFAT in Jurkat T cells was assessed as described previously$^{40,48}$. Briefly, Jurkat T cells were treated with compounds at indicated concentrations for 12 hrs and then stimulated with 3 µM ionomycin for 15 min. Cells were washed 3 times with PBS, collected by centrifugation, and lysed in cell lysis buffer containing 25 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 5% glycerol, Roche PhosSTOP phosphatase inhibitor cocktail tablets and Roche Complete Protease inhibitor cocktail tablets. Samples were then boiled in Laemmli sample buffer, separated by SDS-PAGE using a 4-12% (wt/vol) separating gel, transferred to nitrocellulose membranes, and probed with anti-p-NFATc2 (Ser 326) antibody (Santa Cruz biotechnology).

As shown in Figure 2.19, stimulation of Jurkat T cells with the Ca$^{2+}$ ionophore agent, ionomycin, caused Cn-dependent dephosphorylation of NFAT and reduction in phosphorylated NFAT (p-NFAT) (lane 2 and lane 4). As discussed above, CsA is known to inhibit calcineurin’s phosphatase activity, and thus, it inhibits dephosphorylation of NFAT by Cn in vivo (lane 3), which serves as a positive control in our experiment settings. Pretreatment of the Jurkat T cells with linked compound, SL-3, resulted in a concentration-dependent blockade of NFAT
dephosphorylation that was partial with 20 μM SL-3 (lane 6), nearly complete (lane 5) and comparable to CsA (lane 3) with 50 μM SL-3.

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<td></td>
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**Figure 2.19 Western blot of phosphorylated NFAT1 (p-NFATc2).** SL-3 inhibits calcineurin dephosphorylation of NFAT in Jurkat T cells in a dose dependent manner.

2.4.2 SL-3 does not affect calcineurin phosphatase activity

To assess whether treatment of intact cells with SL-3 resulted in the inactivation of calcineurin towards substrates other than NFAT, a Cn phosphatase activity assay was performed. The Cn phosphatase activity assay was performed in Jurkat T cell lysates using the calcineurin Cellular Activity Assay Kit (Enzo Life Sciences) according to the manufacturer’s instructions. Briefly, we incubated Jurkat T cells with DMSO (control), and 20 μM and 50 μM SL-3, lysed the cells, and assayed Cn phosphatase activity in the cell lysates by measuring the free phosphate release from RII phosphopeptide. The experiments were performed in triplicate.

As shown in **Figure 2.20**, the phosphatase activity of Cn in Jurkat T cell lysates was not diminished in the presence of high concentrations of SL-3 as compared to that of DMSO-treated
cells. We then conclude that the block of NFAT dephosphorylation by SL-3 is not due to nonspecific inactivation of Cn or nonspecific blockade of substrate access, but rather targeted inhibition of the Cn-NFAT interaction.

Figure 2.20 Release of phosphate from the RII phosphopeptide. SL-3 does not affect calcineurin phosphatase activity at concentrations of 20 µM and 50 µM. The experiment was performed in triplicate. Phosphate release is shown normalized to the 5% (v/v) DMSO control.

2.5 SL-3 inhibits NFAT transcriptional activity in vivo

Finally, to assess whether the linked compound, SL-3, would also prevent the transcriptional activity of NFAT in vivo, an NFAT-driven luciferase reporter assay was performed in HEK293T human embryonic kidney cells. Briefly, HEK293T cells were transiently transfected with pGL3-NFAT luciferase plasmid (Addgene, Plasmid ID 17870) and treated with DMSO or the SL-3 compound at indicated concentrations for 12 hrs. Cells were then stimulated with 20 ng·ml⁻¹
phorbol 12-myristate 13-acetate (PMA) and harvested with either CellTiter Glo or Firefly luciferase reagent according to manufacturer’s instructions (Promega). The luciferase activity in cell extracts was measured. In order to measure the cytotoxicity of SL-3, HEK293T cell viability was also monitored.

**Figure 2.21 NFAT-luciferase assay.** SL-3 inhibits NFAT-driven transcriptional activity in a dose-dependent manner (black). SL-3 does not show significant toxicity to the HEK293T cells at concentrations ranging from 1 µM to 50 µM (red). Cell viability data is shown normalized to the 5% (v/v) DMSO control.

As shown in **Figure 2.21**, PMA-stimulated NFAT transcriptional activity was significantly inhibited by 50 µM SL-3 (the last data point on the black curve). Furthermore, SL-3 caused a remarkable dose-dependent reduction in NFAT-driven reporter activity (black) and showed minimum toxicity to cells (red) even at high concentrations. The nearly complete inhibition of NFAT transcriptional activity by SL-3, ~80% in the experiment shown, shows a good parallel with the immunoblotting result illustrated in **Figure 2.19**.
Figure 2.22 $^{15}$N, $^1$H-HSQC titration of SL-3 into $^{15}$N-labeled calcineurin A. (A) 2D $^{15}$N, $^1$H TROSY-HSQC NMR spectrum of free $^2$H,$^{15}$N-CnA protein (red). (B) 2D $^{15}$N, $^1$H TROSY-HSQC NMR spectrum of $^2$H,$^{15}$N-CnA protein bound to linked compound SL-3 in a 1:5 molar ratio (cyan). (C) Overlay of the TROSY-HSQC spectrum in B onto the spectrum in A. The two spectra overlap very well with each other. This shows that no chemical shift perturbation of the assigned $^2$H,$^{15}$N-CnA resonances occurs upon SL-3 addition, and thus, no significant conformational change of CnA was caused by SL-3.
2.6 Structural characterization of Calcineutin-SL-3 interaction

2.6.1 $^{15}$N, $^{1}$H-HSQC titration of SL-3 into calcineurin solution

To better characterize the interaction between linked compounds and Cn we first did NMR titration experiment by adding increasing amounts of SL-3 into $^{15}$N-labeled CnA (Figure 2.22). However, as we can see from the overlapping spectra (Figure 2.22C), no significant chemical shift perturbations (CSPs) occurred to the assigned CnA amide resonances upon SL-3 addition, suggesting, probably no significant conformational or environmental changes have happened to CnA upon SL-3 binding. Another possible explanation is that, since we only have partial assignments for CnA and are missing signals and assignments for residues near the active center due to severe line broadening caused by the Fe$^{3+}$, residues that are shifted by SL-3 may be completely missing from the HSQC spectrum. In the former case, SL-3 could bind to any of the blue region (assigned) shown in Figure 1.11A, while in the latter case SL-3 may bind to any of the grey region (unassigned). To distinguish between the two, other types of NMR experiments were performed, including 3D $^{1}$H$^{1}$H-NOESY-$^{15}$N-TROSY experiment and the cross-saturation experiment. However, neither of them gave significant cross peaks. As discussed in Chapter 1, another important type of NMR experiment for studying protein-protein or protein-small molecule interactions is paramagnetic relaxation enhancement (PRE) NMR, which reports distance-dependent direct effects and not allosteric effects. We then performed PRE NMR.

2.6.2 Paramagnetic relaxation enhancement NMR reveals direct inhibition

We then employed an alternative strategy to characterize the Cn-SL-3 interaction using paramagnetic relaxation enhancement (PRE) NMR. In order to perform PRE NMR experiment, we need to first spin label the compound. Subsequent derivatization of these linked Cn ligands
with 4-carboxy-TEMPO yielded SL-R1, which was confirmed to inhibit the Cn-VIVIT interaction in FP assay, and was used as a paramagnetic probe for PRE NMR measurements. We acquired 2D $^{15}\text{N}, \, ^1\text{H}$ TROSY HSQC spectra of $^{15}\text{N}$-labeled CnA in the presence of SL-R1 before (paramagnetic, $I_{\text{para}}$) and after (diamagnetic, $I_{\text{dia}}$) reduction of the nitroxide spin label with excess amount of ascorbic acid. As shown in **Figure 2.23A**, the two CnA residues that are affected most by compound SL-R1 are Met329 and Ile331 ($I_{\text{para}}/I_{\text{dia}} < 0.20$), coincident with the core PxIxIT-binding residues and are known to form intermolecular hydrogen bonds with PVIVIT\(^{43}\). Residue Phe299 was also affected by SL-R1 with $I_{\text{para}}/I_{\text{dia}}$ of ~0.60. The PRE result indicates that SL-R1 binds to CnA at a region centered around Met329 and Ile 331, and inhibits the Cn-NFAT interaction by competing directly with PxIxIT for its core binding residues on CnA surface.

As shown in **Figure 2.23B**, the two CnA residues that showed the strongest spin label effects in the PRE experiment are mapped onto the Cn-VIVIT complex structure as blue spheres. They are at the center of the VIVIT binding site. Residue Phe299, which showed moderate spin label effects, is shown as cyan sphere and is also part of the Cn-VIVIT interface. As we can see more clearly from **Figure 2.23C**, PVIVI interacts with three hydrophobic pockets of CnA. Residues Met329 and Ile331 form the hydrophobic floor of the second and third pockets and interact directly with residues Ile6 and Ile8 of the VIVIT peptide, respectively. Residue Phe299 is also part of the hydrophobic pockets. It separates the first and second pockets and sits between residues Pro4 and Ile6 of the PVIVIT sequence\(^{43}\). Therefore, residues Met329, Ile331 and Phe299 of CnA are the core residues that form the Cn-VIVIT interface and are necessary for the Cn-NFAT interaction. By interacting with these residues, our linked compound, SL-R1, competes directly with the VIVIT peptide for CnA binding, which is likely the major mechanism of action for our linked compounds to inhibit the Cn-NFAT interaction and subsequent NFAT activation.
Figure 2.23 PRE NMR of calcineurin in complex with SL-R1. (A) Intensity reduction ratio for each resonance in the PRE NMR experiment. The two residues with strongest spin label effects are Met329 and Ile331. Residue Phe299 also showed moderate spin label effects. (B) Mapping of the three most affected residues onto CnA-VIVIT complex structure. Met329 and Ile331 are shown as blue spheres and Phe299 in cyan (PDB entry: 2JOG). (C) Schematic representation of the interaction between VIVIT and hydrophobic pockets of CnA. Adapted from Takeuchi et al (2007)\textsuperscript{43}. The PRE reveals SL-3 binds to the same hydrophobic pockets as VIVIT and competes directly for CnA binding, which is probably the main action of this inhibitor.
As we have discussed earlier in this chapter, several allosteric inhibitors of the Cn-NFAT association have previously been reported, such as INCAs. INCAs binds to Cys266 of CnA, which is more than 15 Å away from the VIVIT binding site (Figure 2.7B)\(^6^2\), and inhibits Cn-NFAT interaction by allosteric effects. As far as we know, there have been no synthetic small molecules reported to bind directly at the VIVIT docking site of CnA. SL-3 and SL-R1 are the first examples of direct (orthosteric) small molecule inhibitor for the Cn-NFAT interaction. These linked compounds compete directly with the PxIxIT motif for CnA binding, and inhibits the Cn-NFAT interaction, subsequent NFAT dephosphorylation and transcriptional activity through masking the PxIxIT binding motif on CnA.

2.6.3 NMR-guided docking of SL-R1 onto calcineurin

Now we have shown that the linked compound, SL-R1, binds to the same site on CnA as VIVIT. We want to characterize further the structure of the CnA/SL-R1 or CnA/SL-3 complex. However, due to the low solubility of SL-R1 and SL-3 and their relatively low binding affinity to Cn, we were not able to solve the CnA/SL-R1 or CnA/SL-3 complex structure by solution NMR spectroscopy, which requires high concentrations of SL-R1 or SL-3 in the bound state. Further, as we recall from Figure 1.12, in the X-ray crystal structure of CnA, the VIVIT binding site, which is also the SL-R1 binding site, is no longer accessible due to crystal packing. Therefore, it may not be possible to solve the co-crystal structure of CnA in complex with SL-R1 or SL-3 using this protein construct. In light of these difficulties, we then turned to structural modeling and docking studies. In this docking experiment, structural modeling was based on the coordinates of calcineurin from PDB entries 1TCO and 1AUI. The coordinates of docked PVIVIT peptide were from PDB entries 2P6B and 2JOG. The docking studies were conducted on a Linus workstation using Glide (Schrödinger) configured by SBGrid\(^1^0^5\). Since we have
obtained structural insights of the CnA/SL-R1 complex from the PRE NMR experiments, the docking studies were performed with SL-R1 and guided by the PRE NMR experiment results described earlier.

As shown in **Figure 2.24**, PRE NMR-guided molecular docking of SL-R1 onto Cn gives light to the binding mode of these bidentate compounds, and how they fit into the core PxIxIT-binding site. As also can be seen in **Figure 2.24**, there is still room to ‘grow’ the compounds. Further optimization of these linked compounds based on the docking results may provide the first direct means of antagonizing Cn-NFAT interaction in preclinical and clinical models.
2.7 Conclusion

As we have discussed in Chapter 1, transcriptional signaling from the Ca\(^{2+}\)-calmodulin-activated serine/threonine phosphatase calcineurin (Cn) to the nuclear factor of activated T cells (NFAT), which has been well credentialed as a potential target in the treatment of graft transplant rejection, autoimmune diseases and cardiovascular disorders, is critically dependent on the protein-protein interaction between CnA and the PxIxIT motif in NFAT. Although effective in the disruption of Cn phosphatase activity, immunosuppressive drugs CsA and FK506 also result in undesired side effects and toxicity, promoting the discovery of alternative inhibitors which selectively inhibit the Cn-NFAT interaction without altering the enzymatic functioning of Cn towards other substrates. In light of this finding, several peptides directly inhibiting NFAT binding to Cn, such as VIVIT, as well as small molecule inhibitors of NFAT-Cn association, including INCAs, which target an allosteric site centered around Cys266 of CnA, have been developed. However, these peptide and small molecule inhibitors are not good enough for therapeutic use and to-date there have been no reported small molecule inhibitors directed against the PxIxIT binding site of Cn.

In this chapter, we have reported on the fragment-based design, synthesis and validation of the first direct-acting small molecule inhibitors that specifically target the core PxIxIT-binding site on CnA and inhibit Cn-NFAT interaction at low micromolar concentrations. We have demonstrated that these inhibitors selectively inhibit Cn-dependent NFAT dephosphorylation and subsequent NFAT-mediated gene expression without affecting Cn phosphatase activity in general. Our studies not only resulted in chemical tools that can be used to further elucidate the role of Cn-NFAT signaling in autoimmune and cardiovascular disorders, but they could also translate into potential lead compounds for further immunosuppressive drug development.
BIBLIOGRAPHY


