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Supplemental Data

A Conserved Docking Site Modulates Substrate Affinity for Calcineurin, Signaling Output, and In Vivo Function

Jagoree Roy, Huiming Li, Patrick G. Hogan, and Martha S. Cyert

Supplemental Experimental Procedures

Yeast Strains, Media, and General Methods

Yeast strains used in this study are listed in Table S1. Strain JRY3 was constructed by replacement of the *CRZ1* ORF in BY1001 with a *CRZ1::NAT* allele PCR-amplified from pJR27. Yeast media was prepared as described in (Sherman, 1991) except that twice the amount of amino acids were added to synthetic media, and 3.5% NH_4Cl was used instead of NH_4SO_4. Yeast were transformed by the lithium acetate method (Ausubel et al., 1987). FK506 (LC Laboratories, Woburn, MA) was dissolved in 90% ethanol, 10% Tween 20 and added to culture media at 0.01%. $CaCl_2$ was added to liquid culture to the desired concentration for 1-3 hours, as noted. Where relevant, FK506 was added to the culture at least 1 hour prior to the addition of $CaCl_2$.
Growth of yeast strains on various plates was scored by dilution plating: 0.5 to 1.0 OD_{600} units of cells (approximately 10^7 cells), from each culture, were serially diluted five to six-fold in distilled water and transferred to plates. Plates were incubated at 30^0C for 2-3 days unless otherwise noted.

Plasmids

Plasmids used in this study are described in Table S2. All plasmids constructed via PCR-based methods were sequenced to confirm the desired changes. pJR4 was created by PCR amplifying the *RCN1* open reading frame, from yeast genomic DNA and cloning it into the 2-hybrid vector pACT2 such that the GAL4 activation domain is in frame with the *RCN1* start codon. pJR9 was constructed by modifying *CNA1* nucleotides in BJP2001, such that amino acids 366-368 (NIR) were all changed to

alanine, using an overlapping PCR strategy. This allele is referred to as *cna1*$^{NIR\text{-}AAA}$. pJR16 was constructed by replacing a *SalI* fragment from BJP2014 with a *XhoI* – *SalI* fragment from pJR9. pJR15 was constructed by PCR amplification of *cna1*$^{NIR\text{-}AAA}$ from pJR9 and sub-cloning into pGEX5X-1 such that the *CNA1* start codon was in frame with GST. pJR21 and pJR22 were constructed by modifying relevant nucleotides in pAMS451 (containing HA-tagged *CRZ1*) using a ligase-free sub-cloning method (L. Liu, Y. Deng and M. Rexach, unpublished results) such that *CRZ1* codons 331-336 (PIISIQ) are changed to PVIAVN and PVIVIT, respectively. pJR23 and pJR24 were constructed by PCR-amplifying the inserts from pJR21 and pJR22 respectively, and sub-cloning them into pGEX5X-1 such that the *CRZ1* start codons are in frame with GST and the HA-tag is removed. pJR26 was constructed by modifying relevant nucleotides in pJR22, using overlapping PCR, such that CRZ1 codons C571, C574, H587 and H591 are all changed to alanine. These mutations affect the putative 1st zinc finger domain in *CRZ1* and are referred to as *-znfd*. pJR28 was constructed by replacing a *XhoI* - *HindIII* fragment containing the 3' end of *CRZ1* from pAMS451 with a similar fragment from pJR26. pJR31 and pJR32 were constructed by sub-cloning *PvuI* fragments from pJR26 and pJR28 respectively, into pRS425. pJR29 and pJR30 were constructed by replacing a *BseRI* - *EcoRI* fragment from pKK249 with similar fragments from pJR23 and pJR24, respectively. pJR27 was constructed by replacing the *SpeI* - *HindII* fragment in pAMS435 (Stathopoulos and Cyert, 1997) which contains the entire *CRZ1* ORF, with a PCR amplified *NAT*^{r} fragment from p4339 ((Tong et al., 2001)).

Purification of Yeast Calcineurin

Bacterially expressed truncated calcineurin A (residues 1-417) - calcineurin B hetero-dimer was purified as follows: BL21 *E.coli* cells transformed with pLys (Stratagene, La Jolla, CA), BJP3003 and *CNB1*-pET9a were grown to mid-log phase in LB medium with 100 μg/ml carbenicillin, 30 μg/ml kanamycin and 25 μg/ml chloramphenicol at 30^{o}C and then induced with 0.3 mM isopropyl β-d-thiogalactoside for 16-18 hours at 18^{o}C. Cells were pelleted and frozen at -80^{0}C for at least one hour. Thawed cell pellets were re-suspended gently in lysis buffer (50 mM Tris-HCl pH 8.0, 100 mM NaCl, 2 mM EDTA, 2 mM EGTA) supplemented with 5 mM DTT and protease

inhibitors (5 μg/ml pepstatin, 5 μg/ml leupeptin, 5 μg/ml aprotinin and 0.1 mg/ml PMSF) and lysed by sonication (four-30 second pulses at 40% output with 1 min. rests in between). NaCl was immediately added to 1.5 M. Unlysed cells and cell debris was removed by centrifugation (30,000g, 20 min.). 0.1% Tween-20 was added to the resulting extract, which was then incubated with equilibrated glutathione-sepharose beads (Amersham Biosciences) at 4°C for 4-6 hours, in batch method. The beads were washed twice in wash buffer (50 Mm Tris-HCl pH 8.0, 110 mM KOAc, 2 mM MgOAc, 0.1% Tween-20, 2 mM DTT), twice in wash buffer supplemented with 1 mM ATP and twice with Factor Xa buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM $CaCl_2$). Calcineurin was then removed from the beads by digestion with Factor Xa (New England Biolabs) in Factor Xa buffer supplemented with 10% glycerol. Protein concentration was determined using a Bio-Rad protein assay (Bio-Rad, Richmond, CA). Proteins were also analyzed by SDS-PAGE and visualized with Gel Code Blue Reagent (Pierce, Rockford. IL). Calcineurin activity was determined using the Biomol Quanitizyme assay kit (Biomol International, Plymouth Meeting, PA) with RII phospho-peptide as substrate and following the manufacturer's directions. Calcineurin containing the mutation *cna1*$^{NIR\text{-}AAA}$ was purified and assayed as above except that BL21 cells transformed with pLys, *CNB1*-pET9a and pJR15 were used for the purification.

Peptide and Protein Binding to Yeast Calcineurin

To verify that VIVIT binds saturably to yeast calcineurin, 100 nM fluorescent PVIVIT in PBS, 0.1% bovine IgG, was titrated with increasing concentrations of truncated calcineurin A-calcineurin B hetero-dimer. Polarization of Oregon Green fluorescence was monitored in 12-μl samples in a black 384-well HE micro-plate (Molecular Devices, Sunnyvale, CA) using an Analyst plate reader (Molecular Devices, Sunnyvale, CA). The K_d obtained for PVIVIT binding to yeast calcineurin, 2 μM, was consistently higher by ~4 fold than the value measured for human calcineurin catalytic domain or human truncated calcineurin A-calcineurin B hetero-dimer. In order to study the interaction of yeast calcineurin with synthetic peptides or recombinant GST-Crz1 protein, fluorescent PVIVIT probe was used in a competitive binding assay (Li et al., 2004). Here the incubations contained 100 nM fluorescent PVIVIT, a fixed concentration

(typically 5 μM) of calcineurin, and increasing amounts of competing peptide or protein. Polarization of fluorescence was monitored as above, and the K_i for binding of unlabelled peptide to calcineurin was estimated by fitting the equilibrium binding data to a three-state model ((Li et al., 2004), Roehrl et al). For these experiments, GST-Crz1, GST-Crz1PVIAVN, and GST-Crz1PVIVIT were expressed in *E. coli* strain BL21(DE3) and purified as follows: An overnight culture was diluted 1:500 into 1 liter of LB and grown to OD_{600} = 0.5 at 37°C, and induced for 5 hours with 0.1mM IPTG at 25°C. The bacterial pellet was lysed in Buffer A (50 mM Tris HCl, pH7.5, 50mM NaCl, 1mM EDTA and 2mM DTT) containing protease inhibitors (1mM PMSF, 10μg/ml leupeptin and 25μg/ml Aprotinin). The clarified lysate was loaded onto a SP column (SP sepharose high performance, GE Healthcare, Piscataway, NJ) that was pre-equilibrated with buffer A. GST-Crz1 was eluted with a gradient of NaCl from 50mM to 1M in buffer A. Fractions containing GST-Crz (eluting at approximately 200 mM NaCl) were pooled and concentrated using Amicon Ultra-15 with 10kD MWCO (Millipore Corporation, Billerica, MA) to about 5mg/mL and then further purified with a home-packed Superdex 200 size-exclusion column (GE Healthcare, Piscataway, NJ).

Table S1. Yeast Strains Used in This Study

Strain	Genotype	Source
PJ69-4A	*Mat a, ura3-52, leu2-3, 112, his3-Δ 200, gal8OΔ. GAL2-ADE2, LYS::GAL1-HIS3, met2::GAL7-Lac*	(James et al., 1996)
BY1001	*Mat a, ura3-52, leu2-Δ1, lys2-801amber, ade2-101ochre, trp1-Δ63, his3–Δ200, cna1Δ1::hisG, cna2Δ1::HIS3, 4X-CDRE-LacZ::TRP1*	(Jiang and Cyert, 1999)
JRY3	*Mat a, ura3-52, leu2-Δ 1, lys2-801amber, ade2-101ochre, trp1-Δ 63, his3-Δ200, cna1Δ1::hisG, cna2Δ1::HIS3, crz1::NAT^R, 4X-CDRE-LacZ::TRP1*	This study
KWY251	*Mat a, ura3-52, leu2-Δ1, lys2-801amber, ade2-101ochre, trp1- Δ63, his3-Δ200, crz1::lox, 4X-CDRE-LacZ::TRP1*	K. Williams, unpublished
KWY252	*Mat a, ura3-52, leu2-Δ1, lys2-801amber, ade2-101ochre, trp1-Δ 63, his3-Δ200, crz1::lox, cnb1Δ1::hisG, 4X-CDRE-LacZ::TRP1*	K. Williams, unpublished
LBY66	*Mat a, ura3-52, leu2-Δ1, lys2-801amber, ade2-101ochre, trp1-Δ63, his3-Δ200, 4X-CDRE-LacZ::TRP1*	L. Boustany, unpublished

Table S2. Plasmids Used in This Study

Plasmid	Description	Source/Reference
pACT2	Two- hybrid vector (GAL4 activation domain fusion)	(James, 2001)
pGBT9	Two-hybrid vector (GAL4 DNA binding domain fusion)	(James, 2001)
BJP2014	*CNA1*-pGBT9	(Jiang and Cyert, 1999)
pJR16	*cna1*$^{NIR\text{-}AAA}$-pGBT9	This study
pGB004	*SLM1*-pACT2	(Bultynck et al., 2006)
pGB003	*SLM2*-pACT2	(Bultynck et al., 2006)
	HPH1-pACT2	(Heath et al., 2004)
	CNB1-pACT2	(Jiang and Cyert, 1999)
pJR4	*RCN1*-pACT2	This study
BJP2001	*CNA1*-pRS314	(Jiang and Cyert, 1999)
pJR9	*cna1*$^{NIR\text{-}AAA}$-pRS314	This study
pJR15	*cna1*$^{NIR\text{-}AAA}$ trunc-pGEX5x-1	This study
BJP3003	*CNA1*trunc-pGEX5x-1	B. Jiang, unpublished
CNB1-pET9a	*CNB1* ORF in pET9a	V. Heath, unpublished
pAMS451	*HA-CRZ1*-pRS315	(Stathopoulos and Cyert, 1997)
pJR21	*HA--CRZ1*^{PVIAVN}-pRS315	This study
pJR22	*HA-CRZ1*^{PVIVIT}-pRS315	This study

pJR23	*CRZ1* ^{PVIAVN}-pGex5x-1	This study
pJR24	*CRZ1* ^{PVIVIT} pGex5x-1	This study
pJR26	*HA-crz1*^{PVIVIT} *znfd*-pRS315	This study
pJR27	*crz1::NAT*-YEP351	This study
pJR28	*HA-crz1-znfd*-pRS315	This study
pKK249	*CRZ1*-pUG36 (N-terminal 3X-GFP fusion)	K. Kafadar, unpublished
pJR29	*CRZ1*^{PVIAVN} pUG36 (N-terminal 3X-GFP fusion)	This study
pJR30	*CRZ1*^{PVIVIT} pUG36 (N-terminal 3X-GFP fusion)	This study
pKC60	*PMC1::HA*, *URA3*, 2μ	Kyle Cunningham

Supplemental References

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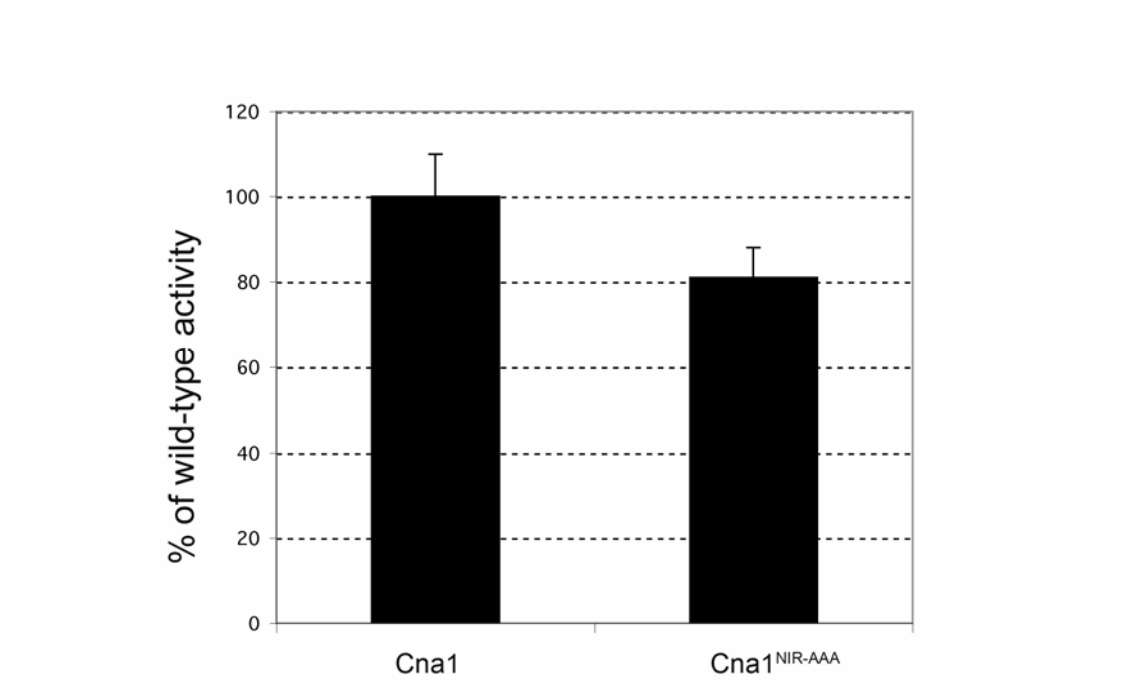

Figure S1. Cna1$^{NIR-AAA}$ Is Catalytically Active In Vitro

Phosphatase activity of bacterially purified calcineurin complex consisting of Cna1 or Cna1$^{NIR-AAA}$ and Cnb1, was measured using a RII-phospho-peptide substrate. Average activity, of three experiments, is shown relative to wild-type.