Identification of a Novel Region Critical for Calcineurin Function in Vivo and in Vitro*

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Calcineurin is a Ca$^{2+}$/calmodulin-regulated protein phosphatase that plays critical functional roles in T-cell activation and other Ca$^{2+}$-mediated signal transduction pathways in mammalian cells. In Saccharomyces cerevisiae, calcineurin regulates the transcription of several genes involved in maintaining ion homeostasis (PMC1, PMR1, and PMR2) and cell wall synthesis (FKS2). In this paper, we report the identification and characterization of 11 single amino acid substitutions in the yeast calcineurin catalytic subunit Cna1p. We show that six substitutions (R177G, F211S, S232F, D258V, L259P, and A262P) affect the stability of calcineurin and that two substitutions (V385D and M400R) disrupt the interaction between Cna1p and the calcineurin regulatory subunit Cnb1p. We also identify three mutations (S373P, H375L, and L379S) that are clustered between the catalytic and the calcineurin B subunit-binding domains. These mutations do not significantly affect the ability of Cna1p to interact with Cnb1p, calmodulin, or Fkb1p (FK506-binding protein). However, these residue substitutions dramatically affect calcineurin activity both in vitro and in vivo. Thus, by using a random mutagenesis approach, we have shown for the first time that the linker region of the calcineurin catalytic subunit, as defined by the Ser$^{373}$, His$^{375}$, and Leu$^{379}$ residues, is crucial for its function as a phosphatase.

Calcineurin, also known as PP2B, is a Ser/Thr-specific protein phosphatase (1). Calcineurin is tightly regulated by Ca$^{2+}$/calmodulin, and it plays critical functional roles in many calcium-mediated signal transduction pathways. For example, calcineurin is required during T-cell activation for the transcriptional regulation of interleukin 2 (IL2) and other cytokine genes (2, 3). In human T-cells, calcineurin directly binds to and dephosphorylates the transcription factor NFAT (4, 5). The immunosuppressive drugs FK506 and cyclosporine A, in association with their respective cellular receptor proteins FKBP12 and cyclophilin A, bind to calcineurin and inhibit its phosphatase activity (6), thus preventing the calcineurin-dependent transcriptional activation of cytokine genes and T-cell activation (7).

In the budding yeast Saccharomyces cerevisiae, calcineurin-deficient strains exhibit normal growth under standard conditions (8–10). However, calcineurin function is required for cell viability under some specific growth conditions. For example, mutations that disrupt calcineurin function are lethal in combination with several mutations that impair cell wall synthesis, including fks1Δ (11–13). FKS1 and FKS2 are a pair of homologous genes responsible for the synthesis of β1,3-glucan (11, 12, 14), which is a glucose homopolymer. Since β1,3-glucan is an essential cell wall component, yeast cells require both the FKS1 and FKS2 genes for viability. In a fks1Δ mutant, the transcription of FKS2 gene is up-regulated to compensate for the loss of FKS1 function (14). Because calcineurin is required for the activation of FKS2 transcript, strains lacking both FKS1 and calcineurin functions are inviable due to the lack of FKS2 expression (11–13). Recently, it has been shown that the calcineurin-dependent FKS2 transcriptional regulation is mediated by a zinc finger containing transcription factor CRZ1/TCN1, which associates with a DNA element (calcineurin-dependent responsive element (CDRE)) located in the promoter of the FKS2 gene (15).

Calcineurin is also required for the proper regulation of ion homeostasis in yeast; calcineurin-deficient cells fail to grow in the presence of high concentrations of Mn$^{2+}$, Na$^+$, Li$^+$ ions but are more tolerant to Ca$^{2+}$ than the wild type cells (16–19). Some of these ion sensitivities are due to a defect in the calcineurin-dependent transcription of several ion transporter genes, including PMR1, PMR2, and PMC1 (17, 20), whose expression are also regulated through the CRZ1/TCN1 transcription factor (15, 21).

The calcineurin holoenzyme consists of the following two subunits: a catalytic A subunit (CnA) and a regulatory B subunit (CnB) (1). The two subunits are tightly bound through hydrophobic interactions (22), and both CnA and CnB are essential for calcineurin function. The crystal structure of the full-length recombinant human calcineurin and that of a proteolytic fragment of bovine calcineurin (complexed with FKBP12-FK506) have been solved (23, 24). The CnA subunit contains four functional domains as follows: a catalytic domain at the N terminus of the protein, a CnB-binding domain, a CaM-binding domain, and an auto-inhibitory domain at the C terminus (25). The catalytic domain shares significant sequence homology with other phosphatases such as PP1, PP2A, and APP (1, 26, 27). Comparisons of the x-ray crystallographic data of calcineurin and PP1 have shown that the active site of calcineurin is extremely well conserved in both its amino acid composition and the three-dimensional structure (23, 24). The

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1 The abbreviations used are: NFAT, nuclear factor of activated T-cells; CnA, calcineurin A subunit; CnB, calcineurin B subunit; CaM, calmodulin; FKBP, FK506-binding protein; CDRE, calcineurin-dependent responsive element; RII, type II regulatory subunit of cAMP-dependent protein kinase; PCR, polymerase chain reaction; WT, wild type.
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active site of calcineurin also contains a binuclear (Fe$^{3+}$-Zn$^{2+}$) metal center, and site-specific mutagenesis studies have identified several active site amino acid residues that are critical for in vitro catalysis and/or metal ion binding (28, 29). The CnB-binding domain is composed of an amphipathic α-helix and is located downstream of the catalytic domain (25, 30–32). The amino acid residues located at the upper part of the α-helix are exclusively non-polar residues. These non-polar residues form a continuous hydrophobic surface to which the CnB subunit binds through hydrophobic interactions (23, 24). Four hydrophobic residues (Val$^{349}$, Phe$^{350}$, Phe$^{356}$, and Val$^{357}$) located within the CnB-binding domain of human CnA subunit have been shown to be essential for CnA and CnB interaction in vitro (31). The Cm-binding domain is located downstream of the CnB-binding domain (25, 33). This part of the protein is highly sensitive to proteolytic digestion. In addition, the Cm-binding domain is invisible in the electron density map of the full-length recombinant human calcineurin (23), suggesting that this region is quite flexible. The auto-inhibitory domain is located at the C terminus of calcineurin, downstream of the Cm-binding domain (25, 34). In the absence of calmodulin, the auto-inhibitory domain is situated in close proximity to the active site and inhibits the enzymatic activity (23). Ca$^{2+}$/calmodulin binding to the Cm-binding domain causes conformational changes to displace the auto-inhibitory domain from the active site, thus activating the phosphatase activity (1, 23, 24). Proteolytic removal of the autoinhibitory domain also activates the enzymatic activity and transforms the truncated calcineurin into a constitutively active, Ca$^{2+}$/calmodulin-independent protein phosphatase (1, 25).

Calcineurin is a very well conserved protein. The catalytic subunit of yeast calcineurin, which is encoded by two homologous and redundant genes CNA1 and CNA2, share 55% sequence identity with the human CnA subunit (8, 9). The yeast regulatory subunit, which is encoded by a single gene CNB1, is 56% identical to the human CnB subunit (10, 35). To understand better the structure and function of the CnA subunit, especially those concerning the catalytic domain, we conducted an extensive mutational analysis of the yeast CNA1 gene, using a PCR-based random mutagenesis approach (36). In this paper, we report the identification of 11 single mutations within the CNA1 gene that cause in vivo functional defects. We show that six of the mutations (R177G, F211S, S232F, D256V, L259P, and A262P) affect the steady-state levels of the mutant proteins. Two other mutations (V385D and M400R) specifically disrupt the interaction between the CnA and CnB subunits. Finally, we also identify three mutations (S373P, H375L, and L379S) that are clustered within the linker region between the catalytic domain and the CnB-binding domain. These three mutated CnA subunits were capable of binding to CnB and CaM as efficiently as the wild type protein, yet they displayed dramatic functional defects in vivo and biochemical defects in vitro. Thus, our random mutational analysis has defined this (Ser$^{373}$, His$^{375}$, Leu$^{379}$)-containing region as a novel region of the calcineurin catalytic subunit that is critical for its activity.

**EXPERIMENTAL PROCEDURES**

**Yeast Strains and Culture Conditions**—Yeast strain BJY301 (MATa, ura3-52, lys2-801, ade2-101, trp1-D85, his3-D200, leu2-3,1, can1Δ, can2Δ, ura3-52:4XCDRE-lacZ), a derivative of YPH499 (37), was used to generate and study CNA1 mutants. In addition to chromosomal disruptions for both CNA1 and CNA2 genes, BJY301 contains a lacZ reporter under the control of four tandem copies of CDRE (calcineurin-dependent responsive element) (15). Y190 (MATa, ura3-52, his3-D200, ade2-101, lys2-801, trp1-901, leu2-3, 112, gal4Δ, gal80Δ, URA3::GAL4-yGALAL-GAL1-TRP1-lacZ, cyh2, LYS2::GAL1-LAC::HIS3::GAL1-LAC::HIS3) was used for all two-hybrid analyses (38, 39). It contains a HIS3 reporter and a lacZ reporter, both of which are under control of a GAL4-responsive element. Yeast cells were grown on standard YPD or SCD media according to Sherman et al. (40). except that amino acid supplements were added in SCD media at twice of the recommended level. Standard procedures were used for genetic manipulation of yeast strains (40). Phenotypic characterization of the calcineurin mutant strains was performed as described by Pozos et al. (119).

**Plasmids**—The plasmids used in this study are shown in Table I. In some cases, multiple steps were employed for plasmid construction, and detailed procedures for plasmid construction are available upon request. BJP2001 is a pRS314-based plasmid containing the wild type (WT) CNA1 gene (37). Plasmids BJP2002–2012 are the same as BJP2001, except that each plasmid contains a single mutation, which leads to an amino acid substitution as indicated in parentheses. Plasmids BJP2013–2029 were employed for two-hybrid experiments. Plasmid BJP2013 contains the full-length CNB1 gene fused in-frame to the GAL4 activation domain. Plasmids BJP2014–2019 contain the full-length CNA1 (WT or mutant) gene fused in-frame to the GAL4 DNA-binding domain (41). Plasmids BJP2020–2029 contain the full-length CNA1 (WT or mutant) gene under the control of a GAL1-GAL10-inducible promoter.

**Random Mutagenesis and Isolation of CNA1 Mutations**—Random mutagenesis was performed by PCR essentially as described previously (36), except that we used equal amounts of dATP, dTTP, dGTP, and dCTP in our PCR reactions. In addition, a lower concentration of MnCl$_2$ (40 μM) was utilized to reduce the mutation rate to 1–2 changes per kilobase. The mutagenic PCR products from several independent reaction mixtures were pooled and used directly to transform, together with the Sure-NeI gapped pRS14-CNA1 plasmid (TRP1 marked), into the yeast strain BJY301 using the lithium acetate method (36, 43). Yeast transformants grown on SCD-Trp plates were replica-plated and incubated at 25 and 37 °C. The β-galactosidase activity of the transformants was determined by colony lift assays using the chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (15). We screened for transformants that were white (deficient in CDRE-lacZ activation) at 25 °C but blue (capable of CDRE-lacZ activation) at 25 °C. Plasmids from these colonies were then extracted, amplified in Escherichia coli,

| Plasmid | Description | Ref. |
|---------|-------------|-----|
| pRS314  | YCp vector with the TRP1 marker | 37  |
| BJP2001 | CNA1(WT) in pRS314 | This study |
| BJP2002 | CNA1(R177G) in pRS314 | This study |
| BJP2003 | CNA1(F211S) in pRS314 | This study |
| BJP2004 | CNA1(S232F) in pRS314 | This study |
| BJP2005 | CNA1(D256V) in pRS314 | This study |
| BJP2006 | CNA1(L259P) in pRS314 | This study |
| BJP2007 | CNA1(A262P) in pRS314 | This study |
| BJP2008 | CNA1(S373P) in pRS314 | This study |
| BJP2009 | CNA1(H375L) in pRS314 | This study |
| BJP2010 | CNA1(L379S) in pRS314 | This study |
| BJP2011 | CNA1(V385D) in pRS314 | This study |
| BJP2012 | CNA1(M400R) in pRS314 | This study |
| GTB9   | TRP1 marked two-hybrid vector expressing the GAL4 DNA-binding domain | 38, 39 |
| GAD2f  | LEU2 marked two-hybrid vector expressing the GAL4 activation domain | 38, 39 |
| BJP2013 | GAD2f-CNB1 | This study |
| BJP2014 | GBT9-CNA1(WT) | This study |
| BJP2015 | GBT9-CNA1(S373P) | This study |
| BJP2016 | GBT9-CNA1(H375L) | This study |
| BJP2017 | GBT9-CNA1(L379S) | This study |
| BJP2018 | GBT9-CNA1(V385D) | This study |
| BJP2019 | GBT9-CNA1(M400R) | This study |
| BJP2020 | GAD2f-CNA1(WT) | This study |
| BJP2021 | GAD2f-CNA1(L379S) | This study |
| BJP2022 | GAD2f-CNA1(H375L) | This study |
| BJP2023 | GAD2f-CNA1(L379S) | This study |
| pHs14  | GBT9-CMD1 | 41  |
| BJP2025 | YEp551-CNB1 | This study |
| BJP2026 | YEp552(gal::CNA1(WT)) | This study |
| BJP2027 | YEp552(gal::CNA1(S373P)) | This study |
| BJP2028 | YEp552(gal::CNA1(H375L)) | This study |
| BJP2029 | YEp552(gal::CNA1(L379S)) | This study |
and re-transformed into BJY301 for second round colony lift assays. Transformants that passed the second round test were collected, and the plasmids recovered from these colonies were subjected to DNA sequence analysis to identify mutations in the CNA1 gene. When multiple mutations were found within the CNA1 gene, further subcloning procedures were performed to obtain single mutations.

**Computer Modeling**—We generated the three-dimensional Cna1p structure using the COMPOSER module of SYBYL (Tripos, Inc.). COMPOSER is a knowledge-based homology modeling program. It compares the protein of interest with proteins whose three-dimensional structures are known and uses the x-ray crystallographic coordinates from homologous proteins (with at least 30% sequence identity) to build a three-dimensional model for your protein. In the case of Cna1p modeling, we have used the truncated bovine Cna subunit (which displays 59% identity) as the structural template (24). The images shown in Fig. 2 are generated by the computer graphic program InsightII (Molecular Simulations Inc.).

**Western Blot Analyses**—Procedures for preparation of yeast whole cell lysates, SDS-polyacrylamide gel electrophoresis, and immunodetection of yeast calcineurin catalytic subunits were performed as described previously (44). The signals from the Western blot were developed by enhanced chemiluminescence (ECL), scanned, and quantified using the Image/Histogram function of the Adobe Photoshop program (Adobe Systems). Protein concentrations were determined by the BCA method (Pierce), using bovine serum albumin as protein standards.

**Calcineurin Purification and Phosphatase Assay**—Yeast cells containing both YEp351-CNB1 and YEp352(gal)-CNA1(WT or mutant) plasmids were grown in 500 ml of SCD-Leu-Ura media at 25 °C. After galactose induction, cells were harvested, resuspended in lysis buffer, and quickly frozen by dropping the cell slurry directly into liquid nitrogen (10). Calcineurin (wild type or mutant) was partially purified by DE52 ion-exchange chromatography and CaM affinity column purification as described previously (45). Phosphatase activity from these affinity purified calcineurin preparations was measured using the BIOMOL GREEN Calcineurin Assay Kit (BIOMOL Research Laboratories, Inc.). Each reaction (50 μl total volume) contained 50 μM Tris, pH 8.0, 100 mM NaCl, 6 mM MgCl2, 0.5 mM dithiothreitol, 0.1 mg/ml bovine serum albumin, 0.1 mM CaCl2, 1 μM bovine calmodulin, and 0.3 mM RII phosphopeptide substrate. In the case of calcineurin inhibition, 2 mM EGTA or 1 μg/ml FK520 (Merck) plus 2 μg/ml human FKBP12 (Sigma) was included in the reaction mixture to determine the Km and Vmax for the wild type and selected mutant Cna1p proteins. The phosphatase activities were measured under the same reaction conditions described above but with variable concentration of the RII phosphopeptide substrate, ranging from 0.015 to 0.6 mM. Kinetic parameters Km and Vmax were determined from the Lineweaver-Burk double-reciprocal plot, using the data analysis/graphics software KaleidaGraph (Synergy Software).

**RESULTS**

**Isolation of CNA1 Point Mutants Defective for Calcineurin Activity**—The aim of this study was to identify functionally important amino acid residues within the calcineurin catalytic domain. We used error-prone PCR to generate random mutations within the CNA1 gene, and then we introduced these mutated genes into a cna1Δ (ΔURA3) strain (see "Experimental Procedures") (36). To screen for mutations that disrupt calcineurin function in vivo, we integrated a CDRE-lacZ reporter gene, which contains four copies of the 24-base pair calcineurin-dependent responsive element from the promoter of FKS2 gene (15), into the genome of the starting strain. Expression of this CDRE-lacZ reporter is completely dependent on calcineurin function; only those transformants harboring functional CNA1
plasmids express β-galactosidase and exhibit a dark blue color in the presence of the chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (15). The transformants containing mutations that disrupted CNA1 function were easily identified as white colonies. However, we expected that the majority of these white colonies would contain stop-codon or frameshift mutations. To eliminate these null mutations from our screen, we characterized mutations that caused temperature-sensitive calcineurin defects in vivo. Specifically, we screened for transformants that, in the presence of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, were blue at 25 °C but white at 37 °C.

After screening approximately 40,000 yeast transformants, we identified 19 colonies that contained point mutations within the CNA1 gene. Approximately equal numbers of transition mutations (11 of 19) and transversion mutations (8 of 19) were recovered from our screen, indicating that the PCR mutagenesis condition we employed did not introduce significant bias between transition and transversion. Since six mutations were recovered multiple times, these 19 point mutations resulted in single amino acid substitutions at 11 residues of the yeast Cna1p protein. As shown in Table II, these residue substitutions disrupted the ability of calcineurin to activate the CDRE-lacZ reporter in a temperature-dependent manner. At 37 °C, strains containing these mutant cna1 genes displayed very low CDRE-lacZ reporter activities, ranging from 0.6 to 4.7% of the wild type level. At 25 °C, however, significant higher CDRE-lacZ activities, ranging from 7 to 109% of the wild type level, were observed for each of the mutant strains.

Next, we conducted phenotypic analysis to determine if each of the cna1 mutants we identified also displayed calcineurin-specific phenotypes. The calcineurin null mutant (cna1Δ cna2Δ) strain is sensitive to MnCl₂ (18, 19). As shown in Fig. 1,
none of the *cna1* mutant strains identified in our screen displayed Mn\textsuperscript{2+} sensitivity at 25 °C. At 37 °C, all the mutant strains were more sensitive to MnCl\textsubscript{2} than the wild type control; however, strains containing the S232P, D258V, S373P, H375L, and L379S substitutions were slightly more Mn\textsuperscript{2+} tolerant than the other mutant strains. This collection of *cna1* mutants also displayed other calcineurin-related phenotypes at 37 °C (13, 16–19); they were sensitive to Li\textsuperscript{+}, tolerant to Ca\textsuperscript{2+}, and lethal in combination with the fksΔ mutation (data not shown). These results, taken together with the CDRE-lacZ data (Table II), demonstrated that the 11 single residue substitutions we identified indeed caused temperature-sensitive defects in Cna1p function.

The Mutations Are Localized in Three Distinct Clusters—In order to visualize where these point mutations were located within the protein structure, we constructed a three-dimensional model of the yeast Cna1p using the crystal structure of the truncated bovine CnA (containing the catalytic domain and the CnB-binding domain) as a template (see "Experimental Procedures" for details) (24). Since the yeast and bovine proteins are highly homologous, the computer-generated Cna1p three-dimensional structure is very similar to the bovine structure (Fig. 2). After mapping all the mutated residues on to the computer model, it was clear that these mutations were clustered in three distinct regions of the protein. Cluster I residues (including Arg177, Phe211, Ser232, Asp258, and D258V, and pGAD2 vectors were used to express the GALA DNA-binding domain and the GALA activation domain fusion proteins, respectively.

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in our screen using Western immunoblot analysis (Fig. 3). At 37 °C, very low levels of Cna1p were observed in lysates prepared from strains expressing *cna1*<sup>R177G</sup>, *cna1*<sup>F211S</sup>, *cna1*<sup>S232P</sup>, and *cna1*<sup>D258V</sup>, *cna1*<sup>L259F</sup>, and *cna1*<sup>A262P</sup>. In contrast, a significant amount of Cna1p was observed in strains harboring *cna1*<sup>S373P</sup>, *cna1*<sup>H375L</sup>, *cna1*<sup>L379S</sup>, *cna1*<sup>Y377S</sup>, and *cna1*<sup>M400R</sup> (approximately 25–40% as much as the wild type). At 25 °C, most of the mutant strains we characterized contained close to wild type levels of Cna1p. The exceptions were strains expressing *cna1*<sup>R177G</sup>, *cna1*<sup>F211S</sup>, and *cna1*<sup>L259F</sup>, which contained significantly reduced levels of Cna1p. These results indicated that substitutions R177G, F211S, S232P, D258V, L259P, and A262P, i.e. those predicted to be in the interior of the Cna1p protein, dramatically affected protein stability at 37 °C.

### Mutations V385D and M400R Disrupt Cna1p-Cnb1p Interactions—We further characterized Cna1p<sup>S373P</sup>, Cna1p<sup>H375L</sup>, Cna1p<sup>L379S</sup>, Cna1p<sup>V385D</sup>, and Cna1p<sup>M400R</sup> because these mutant proteins were more stable and also because they all contained substitutions in residues exposed on the protein surface that had the potential to take part in protein-protein interactions. Two of these substitutions (V385D and M400R) were located within the CnB-binding domain (residues 384–405) of Cna1p. Studies on mammalian calcineurin indicated that CnA binds to CnB through hydrophobic interactions (22–24). Since both V385D and M400R substitute charged residues for nonpolar residues, we investigated the interactions of Cna1p<sup>V385D</sup> and Cna1p<sup>M400R</sup> with Cnb1p <i>in vivo</i>, using the yeast two-hybrid analysis (38). Bait plasmids expressing GAL4BD-Cna1p fusion proteins, along with the prey plasmid expressing GALA4DB-Cnb1p, were co-transformed into the Y190 strain, which harbors both GALA-HIS3 and GALA-lacZ reporters. As expected, interaction between the GAL4BD-Cna1p<sup>WT</sup> fusion and the GALA4DB-Cnb1p fusion led to effective activation of the GALA-HIS3 reporter and strong cell growth on SCD-His plates at both 25 and 37 °C (Fig. 4A). However, cells expressing GALA4DB-Cnb1p along with GAL4BD-Cna1p<sup>V385D</sup> or GAL4BD-Cna1p<sup>M400R</sup> completely failed to grow at 37 °C on SCD-His plate, although the mutant fusion proteins were both expressed at the same level as that of the wild type fusion (Fig. 4B). At 25 °C, cells containing the GAL4BD-Cna1p<sup>V385D</sup> fusion displayed wild type growth on SCD-His plates, whereas GALA4DB-Cna1p<sup>V385D</sup> containing cells grew only slightly better than the vector alone negative control strain. These results demonstrated that the V385D substitution dramatically affected the interaction between CnA and CnB subunits at both temperatures and that the M400R mutation disrupted the CnA-CnB interaction at 37 °C only. Similar results were also observed by assaying β-galactosidase production from a GALA-lacZ reporter gene (data not shown).

### Mutations Buried within the Catalytic Subunit Core Affect Protein Stability—Since mutations, especially those located within the hydrophobic core of a protein, might significantly alter the protein conformation and destabilize the structure, we examined the stability of the mutant Cna1p proteins identified...
three surface residue substitutions (S373P, H375L, and L379S) did not appear to affect significantly the interactions between CnA and CnB; cells expressing GAL4AD-Cnb1p together with GAL4BD-Cna1pS373P, GAL4BD-Cna1pH375L, or GAL4BD-Cna1pL379S showed wild type growth on SC-D-His plates at both 25 and 37 °C (Fig. 4, panel A). Since CaM binding is also required for calcineurin function (1), we then tested if the S373P, H375L, and L379S substitutions affected the interaction between CnA and CaM, again using the two-hybrid system. To this end, we constructed plasmids expressing GAL4AD-Cna1p fusions, and we tested them in the two-hybrid assay with GAL4BD-Cmd1p. Fig. 5 showed clearly that the GAL4AD-Cna1pS373P, GAL4AD-Cna1pH375L, and GAL4AD-Cna1pL379S fusion proteins all activated the GALA-HIS3 reporter to levels comparable to that of the wild type fusion protein, indicating that these three mutant proteins were able to bind Cmd1p efficiently in vivo. Because the catalytic subunit of calcineurin also interacts with the immunosuppressive drug-immunophilin FK506-FKBP complex (1, 6), we examined whether the S373P, H375L, and L379S substitutions affected the interaction between Cna1p and the immunophilin protein Fkh1p, in the presence of FK520 (an analog of FK506), using the same GAL4AD-Cna1p fusion proteins. No significant defects were observed for the FK520-dependent interactions between GAL4BD-Fkh1p and the GAL4AD-Cna1pS373P, GAL4AD-Cna1pH375L, or GAL4AD-Cna1pL379S fusion proteins (data not shown).

In summary, these two-hybrid analyses indicated that the S373P, H375L, and L379S substitutions did not significantly affect the Cna1p-Cnb1p, Cna1p-Cmd1p, or Cna1p-Fkh1p interactions in vivo.

Substitutions S373P, H375L, L379S Affected Enzymatic Activity in Vitro—To understand better the defects caused by the S373P, H375L, and L379S substitutions, we directly examined their effects on phosphatase activity in vitro. DE52 ion-exchange and CaM affinity columns were employed to purify wild type Cna1p, Cna1pS373P, Cna1pH375L, and Cna1pL379S from yeast strains overexpressing each protein (see “Experimental Procedures”) (45). These affinity purified calcineurin preparations did not contain significant levels of contaminating enzymes and were subjected to protein phosphatase assays in vitro (Fig. 6, panel A, the phosphatase activity observed from the wild type Cna1p preparation was completely abolished by the addition of EGTA. More importantly, the phosphatase activity was efficiently inhibited by the calcineurin-specific inhibitor FK520-FKBP complex. These results demonstrated that the affinity purified calcineurin preparations did not contain significant levels of contaminating phosphatase activity, which is consistent with previously published reports. The residual phosphatase activity observed in the presence of FK520-FKBP complex is very likely to be caused by the use of human FKBP, which may lead to incomplete inhibition of the yeast calcineurin activity. The Cna1pS373P, Cna1pH375L, and Cna1pL379S proteins all showed dramatic defects in their phosphatase activity in vitro (Fig. 6, panel B). Cna1pS373P exhibited low levels of activity at both 37 and 25 °C (5 and 4% of the wild type level, respectively). Cna1pH375L exhibited 10% of wild type activity at 37 °C and 8% at 25 °C. Cna1pL379S showed 8 and 18% wild type activity at 37 and 25 °C, respectively. These results strongly indicated that the Ser373, His375, and Leu379 residues were critical for calcineurin phosphatase activity. To study further the effect of these residue substitutions on the enzyme, we conducted kinetic analyses at 37 °C, and we determined the $K_m$ and $V_{max}$ for the wild type Cna1p and Cna1pS373P, Cna1pH375L, and Cna1pL379S mutant proteins (Table III). Compared with the wild type Cna1p, all three mutant proteins displayed marked reductions in their $V_{max}$. The $V_{max}$ of Cna1pS373P was 40-fold lower than that of wild type, Cna1pH375L and Cna1pL379S showed 13- and 8-fold reductions in their $V_{max}$, respectively. These mutations also affected the $K_m$, although the changes were less severe. Cna1pS373P displayed an approximately 5-fold reduction in $K_m$, whereas both Cna1pH375L and Cna1pL379S showed slight increases (1.5- and 1.2-fold, respectively). These results indicated that the S373P, H375L, and L379S substitutions affected the turnover rate more severely than their effects on the enzyme-substrate interaction.

DISCUSSION

In this study, we conducted an extensive structure-function analysis of the yeast calcineurin catalytic subunit, and we identified 11 single amino acid substitutions that compromise calcineurin function. Cells harboring the mutant Cna1p proteins we identified all displayed similar, temperature-sensitive defects as follows: at 37 °C they fail to activate the CDRE-lacZ reporter, become more sensitive than wild type cells to Mn$^{2+}$ and Li$^+$ but more tolerant to Ca$^{2+}$, and display synthetic lethality with the fks1Δ mutation.

To understand the effects of these mutations on the structure of the calcineurin protein, we generated a three-dimensional computer model of Cna1p using the crystal structure of trun-
Table III: Kinetic parameters of the mutant Cna1p proteins

| Cna1p        | $K_m$ | $V_{max}$
|--------------|-------|--------|
|              | $\mu M$ | %     |
| Wild type    | 90 ± 8 | 100    |
| S373P        | 16 ± 2 | 2.5 ± 0.5 |
| H375L        | 134 ± 17 | 7.7 ± 1.8 |
| L379S        | 108 ± 10 | 13 ± 3.4 |

* $V_{max}$ is expressed as the percentage of the maximal activity observed for the wild type calcineurin, which is 14.2 ± 0.9 nmol of P/min/mg total protein of the CaM affinity purified preparation.

The CnA subunit present in the crystal complex is a proteolytic fragment that contains the catalytic and the CnB-binding domains but lacks the CaM-binding and autoinhibitory domains (24). Since the corresponding region of yeast Cna1p (from residue 49 to 408) is highly homologous to the bovine fragment (59 identity and 77% similarity), the computer-generated Cna1p model should closely resemble its genuine three-dimensional structure. This is true especially for the C-terminal part of the model (residues 360–408, containing the substitutions S373P, H375L, L379S, V385D, and M400R), where the yeast and bovine calcineurin share 86% identity and 94% similarity in amino acid sequences.

According to the computer-generated structure of Cna1p, 6 of the 11 substitutions we identified (R777G, F211S, S232F, D258V, L259P, and A262P) are located within the hydrophobic interior of the protein. Generally, the tightly packed hydrophobic core of a protein is very sensitive to mutational alterations. Since these six mutations are all structurally severe substitutions, they are very likely to cause significant conformational changes and destabilize the protein structure, especially at increased temperatures. Consistent with this prediction, our results clearly showed that proteins containing these mutations were present at extremely low levels at 37 °C. Alternatively, it is also possible that these mutations affected protein synthesis. Although strains containing any of these six mutations were quite sensitive to Mn$^{2+}$ at 37 °C, those containing the S232F and D258V substitutions were somewhat more Mn$^{2+}$-tolerant than the others. These results suggested that the Cna1pS232F and Cna1pD258V mutant proteins were less severely compromised in their enzymatic function, so that the residual proteins still present at 37 °C could function to support partially cell growth in the presence of MnCl$_2$. Consistent with this interpretation, we observed that at 25 °C, when the Cna1pS232F and Cna1pD258V mutant proteins were present at normal levels, strains containing the S232F and D258V substitutions displayed high levels of the CDRE-lacZ activity (69.6 and 70.8% of the wild type level, respectively). In contrast, strains containing the other four mutant proteins (Cna1pA177G, Cna1pF211S, Cna1pL259P, and Cna1pA262P) showed significantly lower levels of the CDRE-lacZ activity even at 25 °C. In any case, since these mutations dramatically reduced the steady-state protein level, we did not investigate them further. Rather, we concentrated on the five mutant proteins we identified that are more stable.

The V385D and M400R substitutions are both located within the CnB-binding domain. Based on the crystal structures of the bovine CnA-CnB-FKBP complex (24), both residues are predicted to be on the surface and to form close contacts with the CnB subunit (see Fig. 2, panels E and F). Our two-hybrid analyses clearly demonstrated that these substitutions affect the ability of Cna1p to bind Cnb1p. Of the two mutations, the V385D substitution is a structurally more severe alteration, as it substitutes a negatively charged residue (aspartate) for a highly hydrophobic residue (valine). Indeed, we observed more severe defects caused by the V385D mutation. The Cna1pV385D mutant protein shows a marked defect for Cnb1p binding and a significant reduction in CDRE-lacZ activation even at 25 °C. In comparison, the M400R mutation, which substitutes a positively charged residue (arginine) for a non-polar residue (methionine), is a structurally less severe alteration. As a consequence, the Cna1pM400R mutant protein displays defects for Cnb1p binding and CDRE-lacZ activation only at 37 °C, but remains largely functional at 25 °C. Previous site-specific mutagenesis studies on mammalian calcineurin have indicated that several hydrophobic residues within the CnB-binding domain are important for CnA-CnB interaction. Soderling and colleagues (22) simultaneously mutated four hydrophobic residues Val$^{385}$, Phe$^{390}$, Phe$^{396}$, and Val$^{395}$ (corresponding to Val$^{385}$, Phe$^{390}$, Phe$^{394}$, and Val$^{395}$ of yeast Cna1p) to glutamate residues or glutamine residues and showed that mutant Cna1p subunits containing these multiple substitutions fail to interact with the CnB subunit in vitro. Our observations that V385D and M400R single substitutions in yeast Cna1p affect the CnA-CnB interaction are consistent with, and provide in vivo experimental support for, the conclusion reached from the in vitro studies of mammalian calcineurin regarding the importance of hydrophobic interactions between the CnA and CnB subunits.

The S373P, H375L, and L379S mutations are clustered within the linker region that connects the catalytic domain and the CnB-binding domain, and they all cause similar and severe functional defects in vivo: at 37 °C, cells containing either Cna1pS373P, Cna1pH375L, or Cna1pL379S display more than 20-fold reduction in $\beta$-galactosidase production from the CDRE-lacZ reporter and exhibit multiple calcineurin-specific phenotypes. By using two-hybrid analyses, we have shown that these amino acid substitutions do not significantly affect the interactions of Cna1p with either Cnb1p, Cmd1p, or Fkb1p. Although we observe modest decreases in Cna1pS373P, Cna1pH375L, and Cna1pL379S protein levels at 37 °C, we do not think these reductions in protein levels account for the dramatic functional defects caused by these residue substitutions for the following reasons: at 37 °C, the mutant proteins Cna1pS373P, Cna1pH375L, and Cna1pL379S are present at approximately 25–40% of the wild type level, yet cells expressing these mutant proteins only produce 1.4–4.7% as much $\beta$-galactosidase from the CDRE-lacZ reporter as cells containing wild type Cna1p. These observations strongly suggest that Cna1pS373P, Cna1pH375L, and Cna1pL379S mutant proteins are present, but non-functional, at 37 °C. Consistent with this interpretation, these mutant proteins all exhibit strong enzymatic defects in vitro: the S373P substitution results in a 20-fold reduction in phosphatase activity, the H375L mutation causes a 10-fold reduction, and the L379S mutation leads to a 10-fold reduction at 37 °C, and a 5-fold decrease at 25 °C. Our kinetic analyses also showed that the S373P, H375L, and L379S substitutions affected both the $K_m$ and the $V_{max}$ of the mutant proteins, indicating that these mutations altered both the enzyme turnover rate and the enzyme-substrate interaction.

In the in vitro phosphatase assays, we do not detect any temperature dependence for Cna1pS373P and Cna1pH375L phosphatase activity and only a very slight degree of temperature dependence for Cna1pL379S activity. This is surprising because cells containing each of these mutant proteins exhibited clear temperature-sensitive calcineurin functional defects in vivo. These differences between the in vivo and in vitro behaviors could be substrate-dependent. The RII peptide, which is derived from the mammalian cAMP-dependent protein kinase, is used in our phosphatase assay in vitro (1). This 19-residue...
peptide is a relatively small substrate, and it only binds to a region that overlaps the active site of calcineurin. Recent studies on the interactions between mammalian calcineurin and its substrate NFAT have indicated that at least two separate regions of the CnA catalytic domain are involved in NFAT binding: a region that contains the active site (where the RII peptide binds), and a region that is distinct from the active site (46, 47). By analogy, the interactions between yeast Cna1p and its endogenous protein substrate(s) may also involve region(s) outside of the active site. Thus at 25°C, the mutant Cna1p proteins we studied might be able to dephosphorylate a protein substrate better than the RII peptide, due to the additional interaction(s) outside of the active site that might compensate for the structural effects of the S373P, H375L, or L379S substitutions.

In addition, the RII peptide may not be an optimal substrate for calcineurin, although it is an excellent substrate for mammalian calcineurin. This may also contribute to the different behaviors observed between the in vitro and in vivo analyses.

Taken together, our in vitro biochemical examinations and in vitro phenotypic analyses and in vivo studies indicate that the Ser373, His375, and Leu379-containing region plays a crucial role in calcineurin function. This (Ser373, His375, Leu379)-containing region is crucial for its function as a phosphatase. Since this (Ser373, His375, Leu379)-containing region is highly conserved (Fig. 7), we believe that the corresponding region is very likely to play an important functional role in human calcineurin as well.

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