Competitive inhibition of calcineurin phosphatase activity by its autoinhibitory domain

Jasbir K. SAGOO*, David A. FRUMAN*, Sebastian WESSELBORG*, Christopher T. WALSH† and Barbara E. BIERER*†‡§

*Division of Pediatric Oncology, Dana-Farber Cancer Institute, 44 Binney Street, Boston, MA 02115, †Departments of Biological Chemistry and Molecular Pharmacology and Medicine, Harvard Medical School, Boston, MA 02115, and ‡Hematology–Oncology Division, Department of Medicine, Brigham and Women's Hospital, Boston, MA 02115, U.S.A.

Calcineurin (protein phosphatase 2B), a calmodulin- and calcium-dependent serine/threonine phosphatase, appears to be regulated by a C-terminal autoinhibitory domain. A 25 amino acid peptide derived from this domain inhibits calcineurin phosphatase activity *in vitro*. Here we show that a 97 amino acid fragment of the calcineurin α C-terminus is approx. 8-fold more potent than the shorter peptide in calcineurin inhibition experiments. Mutation of an evolutionarily conserved Asp to Asn, previously shown to disrupt calcium-dependent signalling and calcineurin regulation in T-lymphocytes, greatly reduced inhibition by the autoinhibitory domain *in vitro*. Kinetic analysis

of wild-type and mutated autoinhibitory domains show that both are competitive inhibitors of calcineurin phosphatase activity with K_i values of $5.0 \pm 0.2 \mu\text{M}$ and $36.0 \pm 3.7 \mu\text{M}$ respectively. This suggests intrasteric regulation of calcineurin, with the autoinhibitory domains interacting at the active site of the enzyme. The competitive behaviour of the autoinhibitory domains contrasts with the mechanism of calcineurin inhibition by immunosuppressant–immunophilin complexes, which have been shown to bind to calcineurin at a region removed from the active site.

INTRODUCTION

Calcineurin (Cn; protein phosphatase 2B), a highly conserved Ca^{2+} /calmodulin (CaM)-dependent serine/threonine phosphatase, plays a critical role in the signalling pathways necessary for T-cell activation [1–5]. This heterodimeric phosphatase is composed of a 60 kDa catalytic subunit (CnA) and a 19 kDa calcium-binding regulatory subunit (CnB). The CnA subunit, depicted in Figure 1, consists of a catalytic core, a CnB-binding domain, a CaM-binding domain, and a C-terminal portion. The C-terminus appears to possess autoinhibitory activity, in that limited proteolysis of the enzyme, removing 17 kDa of the C-terminal region, renders the enzyme constitutively active [6]. The observation that synthetic peptides corresponding to regions of the C-terminus inhibit calcineurin phosphatase activity *in vitro* further supports the function of this domain in autoinhibition [7–9].

Calcineurin has been identified as the molecular target of the

immunosuppressive agents FK506 and cyclosporin A (CsA) [1,3–5]. Both immunosuppressive compounds associate with intracellular binding proteins, termed immunophilins; the immunosuppressant–immunophilin complexes [FK506–FKBP12 and CsA–cyclophilin (CyP)] bind to calcineurin and inhibit its phosphatase activity [10]. Previous studies have shown that the binding sites on calcineurin for these immunosuppressant–immunophilin complexes are not identical, but are overlapping [11], and that the immunosuppressant–immunophilin complexes compete in *in vitro* assays of activity [1]. Biochemical [12], molecular biological [11,13] and structural studies [14,15] show that FK506–FKBP and CsA–CyP complexes inhibit calcineurin activity in a non-competitive manner. Furthermore, mutational analysis had localized the binding site for FK506–FKBP12 to the latch region, a structure that forms when CnB binds to CnA [13]. Recently, the X-ray crystallographic structure of proteolysed CnA (lacking the C-terminal CaM binding and autoinhibitory domain), CnB, FK506 and FKBP12 confirmed that the FK506–

Figure 1 Structure of CnA and the sequence of the 97 amino acid WT and D477N autoinhibitory domains

The putative 25 amino acid autoinhibitory peptide is underlined and the point mutation from aspartic acid (D) to asparagine (N) is indicated by an arrow.

Abbreviations used: CnA, calcineurin catalytic subunit; CnB, calcineurin regulatory subunit; CaM, calmodulin; CsA, cyclosporin A; CyP, cyclophilin; FKBP12, FK506-binding protein; pNPP, *p*-nitrophenyl phosphate; PNP, *p*-nitrophenol; WT, wild type; PKA, cAMP-dependent protein kinase; TFA, trifluoroacetic acid; DTT, dithiothreitol; PP1A, protein phosphatase 1A; NFAT, nuclear factor of activated T-cells.

§ To whom correspondence should be addressed at, Division of Pediatric Oncology, Dana-Farber Cancer Institute.

FKBP12 complex binds 10 Å away from the phosphatase active site [14]. The FK506–FKBP12 complex inhibits calcineurin activity by sterically preventing calcineurin substrates from binding to the active site [14]. That immunosuppressant–immunophilin complexes did not bind to the active site had been suggested earlier by the observation that the calcineurin-dependent hydrolysis of *p*-nitrophenyl phosphate (pNPP) was enhanced rather than inhibited by the immunosuppressant–immunophilin complexes [1]. These studies are consistent with allosteric inhibition of calcineurin phosphatase activity by the immunosuppressant–immunophilin complexes.

There has been considerable debate concerning the mechanism by which the calcineurin autoinhibitory domain modulates phosphatase activity. Peptide inhibition experiments have produced conflicting results regarding competitive [16] versus non-competitive inhibition [9,17], depending perhaps upon the specific peptide involved, the substrate used for analysis or the specific experimental conditions used. We have previously characterized a mutation in murine calcineurin α gene expressed in the EL4 T-lymphoma cell line. This mutation changes an evolutionarily conserved Asp (D) to Asn (N) at position 477 [8]. The Asp-477 residue lies near the centre of the defined 25 amino acid autoinhibitory peptide [9] and correlates with increased responsiveness to calcium ionophore in T-cells. Inhibition studies using synthetic wild-type (WT) and D477N peptides showed that this specific mutation resulted in a decrease in autoinhibitory activity and suggested a pseudosubstrate model for autoinhibition. In this model, we proposed that the conserved Asp would serve as a phosphoserine or phosphothreonine mimic similar to the substrate consensus sequence, but lacking the phosphorylated residue.

We reasoned that the physiological behaviour of the autoinhibitory domain would be best represented not by a short peptide but rather by the complete autoinhibitory domain, particularly as other potential autoinhibitory regions within the C-terminus may exist [7]. We have generated and purified recombinant fragments of the WT and D477N mutant autoinhibitory domains and have determined that they act as competitive inhibitors of calcineurin phosphatase activity with respect to pNPP substrate, demonstrating that the active site is engaged by the autoinhibitory domain. In addition, we have shown that even in the context of the complete autoinhibitory domain, the single Asp to Asn mutation renders the D477N mutant autoinhibitory domain much weaker than the WT autoinhibitory domain. Characterization of the mechanism of calcineurin autoinhibition may provide insights into the design of novel immunosuppressant agents targeted to inhibit calcineurin activity by binding at an alternative site to immunosuppressant–immunophilin complexes.

MATERIALS AND METHODS

Construction and expression of recombinant autoinhibitory domains

The autoinhibitory domains were designed based on the sequence of calcineurin α previously described [18]. The CaM-binding site is reported to terminate at residue 414 and is followed by a region of conserved amino acids. The recombinant autoinhibitory domain was designed to begin within this conserved region at amino acid K424 and to end at the C-terminal Q521 (Figure 1). The pMH-Neo expression vector containing the WT Cn α cDNA (pMH-Neo-Cn α) and the cDNA containing the D to N mutation [pMH-Neo-Cn α (D477N)] [8] were used as templates to generate, by PCR, the WT and D477N mutant autoinhibitory domains respectively. The forward primer (5'-GACGCCATA-

TGAAGGGCCTGACCCCAACT-3') and the reverse primer (5'-GACGGGATGCTCACTGGATATTGCTGCT-3') were used to amplify a 291 nucleotide sequence corresponding to the 97 amino acid autoinhibitory domain. The PCR parameters used were 30 cycles of 94 °C for 1 min, 60 °C for 1 min and 72 °C for 2 min, with a 10 min extension at 72 °C after completion of the cycles. The amplified cDNA fragments were sequenced and directionally cloned into the *Bam*H1 and *Nde*I sites of the PET15b vector containing a hexa-histidine epitope tag (Novagen, Madison, WI, U.S.A.). The DE3 host strain, BL21, was then transformed with the resulting plasmids and incubated at 37 °C in Luria broth supplemented with 100 µg/ml ampicillin. The cultures were grown to $A_{600} = 0.8$ at which time 0.4 mM isopropyl β -D-thiogalactopyranoside (Sigma Chemical Co., St. Louis, MO, U.S.A.) was added and the cultures further incubated for 4 h at 37 °C. Bacterial cells were harvested by centrifugation for 20 min at 5800 g. Pellets were resuspended in binding buffer containing protease inhibitors [5 mM imidazole/0.5 M NaCl/20 mM Tris, pH 7.9/0.1 % Nonidet P40/200 µl (0.2 M) of PMSF/20 µg/ml pepstatin A/20 µg/ml leupeptin/1 % (v/v) aprotinin]. Resuspended cells were lysed by sonication and centrifuged at 39000 g for 20 min to remove cell debris. The supernatant was filtered through a 0.45 µm membrane and a 10 ml volume was loaded onto His-binding resin previously activated with 50 mM NiSO₄ (Novagen, Madison, WI, U.S.A.). The column (bed volume 2.5 ml) was washed with 10 column vol. of binding buffer, followed by 6 column vol. of wash buffer (60 mM imidazole/0.5 M NaCl/20 mM Tris, pH 7.9) to remove non-specifically bound proteins. Bound protein was eluted with 6 column vol. of elution buffer (1 M imidazole/0.5 M NaCl/20 mM Tris, pH 7.9) and collected in 1 ml fractions. Fractions containing the protein were pooled and buffer exchanged on Sephadex G25 resin into storage buffer (100 mM Tris, pH 7.5/100 mM NaCl). The protein was dialysed overnight at 4 °C against 5 litres of storage buffer to ensure that all traces of imidazole were removed. Collodion membranes (10 ml), with a molecular-mass cut-off of 12 kDa (Sartorius, Gottingen, Germany), were used to concentrate the protein samples to approx. 1.5 ml. The concentrations of the autoinhibitory domains were determined by amino acid analysis (Molecular Biology Core Facility, Dana-Farber Cancer Institute, Boston, MA, U.S.A.) and found to be 1.63 mM and 1.62 mM for WT and D477N respectively.

Peptide synthesis

Peptides were synthesized and HPLC-purified at the Molecular Biology Core facility of the Dana-Farber Cancer Institute. The 19 amino acid peptide DLDVPIGRFDRRVSVAEE (termed RII), corresponding to a portion of the RII subunit of cAMP-dependent protein kinase (PKA) [19], was phosphorylated and used as a substrate in calcineurin phosphatase assays (see below). Lyophilized RII was dissolved in water to a concentration of 2.0 mM, as determined by amino acid analysis. The calcineurin autoinhibitory peptide ITSFEAKGLDRINERMPPRRDAMP (WT autoinhibitory peptide), and the mutated peptide ITSFEAKGLNRINERMPPRRDAMP (D477N autoinhibitory peptide) were dissolved in water to a concentration of 1.12 mM and 1.56 mM respectively, as determined by amino acid analysis.

Preparation of [γ -³²P]RII peptide

Phosphorylation of the Ser residue of RII with [γ -³²P]ATP (3000 Ci/mmol; DuPont New England Nuclear, Boston, MA, U.S.A.) was performed with the catalytic subunit of PKA as described elsewhere [20]. The kinase reaction contained 400 µl of

reaction buffer (40 mM Mes buffer, pH 6.5/4 mM MgCl₂/0.1 mM CaCl₂/0.4 mM EGTA/0.8 mM EDTA/0.1 mg/ml BSA), 6 µl of 50 mM ATP, 30–60 µCi of [γ -³²P]ATP and 45 µl of 2.0 mM RII peptide, in a total volume of 900 µl. Labelling was initiated by the addition of 100 µl of reconstituted PKA catalytic subunit [250 units dissolved in 100 µl of water containing 6 mg/ml dithiothreitol (DTT)]. After incubation for 1 h at 30 °C, the phosphorylated peptide was purified by Sep-Pak C₁₈ chromatography. Columns (Waters/Millipore, Milford, MA, U.S.A.) were activated with 3 ml of 30 % acetonitrile in 0.1 % trifluoroacetic acid (TFA) followed by 5 ml of 0.1 % TFA. The contents of the kinase reaction were applied to the column and the column was washed with 75 ml of 0.1 % TFA to remove unincorporated ATP. The labelled [γ -³²P]RII peptide was eluted with 30 % acetonitrile in 0.1 % TFA in 6 × 0.5 ml fractions. Radioactive fractions were lyophilized and resuspended in 1 ml of resuspension buffer (50 mM Tris, pH 7.5/100 mM NaCl/0.5 mM DTT/100 µg/ml BSA). The concentration of the [γ -³²P]RII peptide solution was 150 µM. For enzyme kinetics experiments, higher concentrations of RII peptide were required for radiolabelling, providing a final concentration of 150–400 µM.

Calcineurin phosphatase assay using [γ -³²P]RII peptide substrate

Inhibition study of WT and D477N autoinhibitory domains

Calcineurin assays were performed in a 60 µl reaction volume using 50 nM purified bovine brain calcineurin (Upstate Biotechnology Inc., Lake Placid, NY, U.S.A.) and 200 nM calmodulin (Sigma). The 150 µM [γ -³²P]RII substrate was diluted to 15 µM in assay buffer (50 mM Tris, pH 7.5/100 mM NaCl/0.5 mM DTT/100 µg/ml BSA/0.4 mM CaCl₂). The WT and D477N autoinhibitory domains were prepared at concentrations ranging from 0.9 µM to 90 µM. A 20 µl volume of each concentration of autoinhibitory domain was added to 20 µl of calcineurin/CaM in duplicate tubes, and the tubes were equilibrated for 1 min at 30 °C. In each assay, blank tubes were prepared containing 40 µl of assay buffer to monitor background activity. Control tubes contained 20 µl of assay buffer and 20 µl of calcineurin/CaM. The reaction was initiated by the addition of 20 µl of [γ -³²P]RII substrate at 20 s intervals and incubated at 30 °C for 15 min. Stop solution [100 mM potassium phosphate buffer, pH 7.0, containing 5 % (v/v) trichloroacetic acid] was used to terminate the reactions at 20 s intervals. Released inorganic phosphate was isolated by cation-exchange chromatography using Dowex AG-50W-X8 cation-exchange resin (Bio-Rad) as described [20]. The phosphatase reactions were applied to the columns and the effluent collected in scintillation vials. The scintillation vials were then filled with 4 ml of ReadySafe scintillation fluid (Beckman, Fullerton, CA, U.S.A.) and radioactivity was measured in a Beckman scintillation counter. Data calculation is described elsewhere [20] and represented as a percentage of total calcineurin activity.

Kinetic analysis of WT and D477N autoinhibitory domains

For kinetic analysis, the concentration of [γ -³²P]RII peptide was varied from 8 µM to 200 µM for each concentration of autoinhibitory domain. The WT and D477N autoinhibitory domains were fixed at four different concentrations: 0 µM, 5 µM, 10 µM and 20 µM. The data were fitted to non-linear regression eqns. (1) and (2) for competitive and non-competitive inhibition respectively, using the program KINETASYST (Intellikinetics, State College, PA, U.S.A.).

$$v = V_{\max}[S]/\{K_m + [S](1 + [I]/K_i)\} \quad (1)$$

$$v = V_{\max}[S]/\{K_m(1 + [I]/K_{ii}) + [S](1 + [I]/K_{ii})\} \quad (2)$$

where v is the velocity, $V_{\max}$ is the maximum velocity, $[S]$ is substrate concentration, K_m is the Michaelis constant, $[I]$ is inhibitor concentration, and K_i and K_{ii} are the dissociation constants for the enzyme–inhibitor complex for competitive and non-competitive inhibitors respectively.

Calcineurin phosphatase assay using pNPP substrate

Inhibition study of WT and D477N autoinhibitory domains

Phosphatase assays using 60 mM pNPP (Sigma) were performed in a 50 µl reaction volume using 50 nM purified bovine brain calcineurin (Sigma) and 200 nM CaM (Sigma). The WT and D477N autoinhibitory domains were prepared at a concentration range of 0.9 µM–90 µM in assay buffer (100 mM Tris, pH 7.5/100 mM NaCl/0.5 mM DTT/100 µg/ml BSA/1 mM MnCl₂/0.4 mM CaCl₂). Each concentration of autoinhibitory domain was added to calcineurin/CaM in duplicate tubes. In each assay, blank tubes containing buffer only were prepared to determine background levels of hydrolysed pNPP. Control tubes contained buffer and calcineurin/CaM. After a 5 min incubation at 30 °C, the reactions were initiated by the addition of 60 mM pNPP at 20 s intervals. The tubes were incubated for a further 30 min at 30 °C, allowing for a 1–10 % product formation. This range of product formation is sufficient to determine a change in absorbance, but low enough not to cause end-product inhibition. Reactions were terminated by the addition of 950 µl of 1 M NaOH at 20 s intervals. The liberation of *p*-nitrophenol (PNP) was measured at 405 nm using a Beckman Du 64 spectrophotometer (Beckman). For data calculation, the average absorbance value for the duplicate samples was plotted against a standard curve of varying known concentrations of PNP versus absorbance at 405 nm. The average absorbance value for the duplicate samples was converted into the amount of PNP released, and the background PNP value was subtracted from the result. The final value was divided by the reaction time to give activity/min, which is represented as a percentage of the total calcineurin activity.

Kinetic analysis of WT and D477N autoinhibitory domains

For kinetic analysis, the concentration of pNPP was varied from 10 mM to 320 mM for each concentration of the WT and D477N autoinhibitory domain. The WT and D477N autoinhibitory domains were fixed at four different concentrations: 0 µM, 5 µM, 10 µM and 20 µM. The data were fitted to non-linear regression eqns. (1) and (2) for competitive and non-competitive inhibition respectively, using the program KINETASYST.

RESULTS AND DISCUSSION

Characterization of WT and D477N mutant autoinhibitory domains

The WT and D477N autoinhibitory domains (Figure 1) have a predicted molecular mass of 14 kDa and were expressed in BL21 *Escherichia coli* bacterial cells. These fusion proteins contained a hexa-histidine epitope tag to facilitate purification by nickel affinity chromatography. High-level protein expression of the autoinhibitory domains occurred 4 h after isopropyl β -D-thiogalactopyranoside induction; the majority of the protein was expressed in the soluble fraction. Bacterial lysates were loaded onto a nickel affinity column and the bound proteins were eluted from the column using different concentrations of imidazole. Each fraction for the purification of the WT (Figure 2) and D477N (results not shown) autoinhibitory domains was resolved

Figure 2 SDS/PAGE analysis of the WT autoinhibitory domain purified from BL21 *E. coli* bacterial cells

The proteins were separated by 16% SDS/PAGE and stained with Coomassie Brilliant Blue: *E. coli* lysate (lane 1); flowthrough (lane 2); 60 mM imidazole wash (lane 3); bound protein eluted with 1 M imidazole in 1 ml fractions (lanes 4–8). Positions of molecular mass markers (kDa) are indicated on the left.

by SDS/PAGE. A 14 kDa protein band did not appear in the flowthrough (Figure 2, lane 2) and non-specifically bound proteins were eluted with 60 mM imidazole (Figure 2, lane 3). The 14 kDa WT autoinhibitory domain was eluted with 1 M imidazole in 1 ml fractions, with the majority of the protein eluting as a single band in fractions 2 and 3 (Figure 2, lanes 5 and 6).

The biological activities of the purified WT and D477N autoinhibitory domains were assessed by their ability to inhibit calcineurin phosphatase activity *in vitro*. Inhibition studies were performed using two different calcineurin substrates, the 19 amino acid $[\gamma\text{-}^{32}\text{P}]\text{RII}$ phosphopeptide and the small pNPP molecule. The $[\gamma\text{-}^{32}\text{P}]\text{RII}$ phosphopeptide has been reported to be a reliable substrate for calcineurin [7,16,19–21]. Calcineurin is also able to hydrolyse pNPP, a low-molecular-mass compound which has been extensively used in calcineurin assays [22,23]. The small pNPP molecule binds directly at the active site, and the hydrolysis of pNPP by calcineurin generates PNP, which can be readily monitored spectrophotometrically. A distinct advantage of using the pNPP substrate was that, unlike the $[\gamma\text{-}^{32}\text{P}]\text{RII}$ phosphopeptide, high concentrations of the pNPP substrate could be obtained. These features proved to be important for the kinetic studies described later.

Using the $[\gamma\text{-}^{32}\text{P}]\text{RII}$ phosphopeptide as a substrate, the WT autoinhibitory domain inhibited calcineurin activity in a concentration-dependent manner with an IC_{50} of approx. $2.5\ \mu\text{M}$ (Figure 3A). The D477N autoinhibitory domain has an IC_{50} of $40\ \mu\text{M}$ and was approx. 20-fold less potent than the WT autoinhibitory domain in inhibiting calcineurin activity. At high concentrations ($> 90\ \mu\text{M}$) of D477N autoinhibitory domain, calcineurin activity was inhibited by approx. 70%. The 97 amino acid WT autoinhibitory domain ($\text{IC}_{50} \approx 2.5\ \mu\text{M}$) was approx. 8-fold more potent than the 25 amino acid WT autoinhibitory peptide ($\text{IC}_{50} \approx 20\ \mu\text{M}$) in inhibiting calcineurin phosphatase activity (Table 1). Our previous studies [8] demonstrated that the IC_{50} could not be approached using the D477N autoinhibitory peptide; at a D477N peptide concentration of $100\ \mu\text{M}$, only 30% of calcineurin activity was inhibited. Thus it was not possible to compare the potency of the D477N autoinhibitory peptide with the D477N autoinhibitory domain. The greater inhibition by the larger WT and D477N domains compared with

Figure 3 Inhibition of calcineurin phosphatase activity by the WT and D477N autoinhibitory domains

(A) Inhibition mediated by the autoinhibitory domains using $[\gamma\text{-}^{32}\text{P}]\text{RII}$ substrate in the presence of CaM (see the Materials and methods section). The determinations in each experiment were performed in duplicate and the data shown are means $\pm$ S.D. from three separate experiments. Phosphatase activity is expressed as a percentage of total calcineurin activity. (B) A similar inhibition study in which pNPP (60 mM) was used as a substrate, with the data presented as the means $\pm$ S.D. from three separate experiments.

Table 1 Comparison of IC_{50} (μM) values of the WT and D477N autoinhibitory domains versus the WT and D477N autoinhibitory peptides using $5\ \mu\text{M}$ $[\gamma\text{-}^{32}\text{P}]\text{RII}$ as substrate

ND = Not determined. At a D477N peptide concentration $> 100\ \mu\text{M}$ there was 30% inhibition of calcineurin phosphatase activity compared with the WT peptide [8].

	IC_{50} (μM)	
	WT	D477N
Peptide	20	ND
Domain	2.5	40

their respective peptides suggested that residues outside the 25 amino acid autoinhibitory peptide contribute to inhibition by interacting with the catalytic domain. Alternatively, the additional 72 amino acids may stabilize a high-affinity conformation

Figure 4 Kinetic analysis of the inhibitory mechanism of the WT and D477N autoinhibitory domains

Purified calcineurin was incubated with WT (top) and D477N (bottom) autoinhibitory domains at different concentrations [0 μM ($\bullet$), 5 μM ($\blacksquare$), 10 μM ($\blacklozenge$) and 20 μM ($\triangle$)] with the pNPP concentration increasing from 10 mM to 320 mM. Bottom: D477N autoinhibitory domain at higher concentrations, as indicated on the graph. Each graph represents the average of duplicate determinants from a single assay, with similar results being observed in two additional experiments. The K_i values for the WT and the D477N autoinhibitory domains are $5.0 \pm 0.2 \mu\text{M}$ ($n = 3$) and $36.0 \pm 3.7 \mu\text{M}$ ($n = 3$) respectively.

of the autoinhibitory peptide to increase interaction with the catalytic domain. It is worth noting that with 60 mM pNPP as a substrate, the WT ($\text{IC}_{50} \approx 2.5 \mu\text{M}$) and D477N ($\text{IC}_{50} \approx 60 \mu\text{M}$) autoinhibitory domains both inhibited the hydrolysis of pNPP (Figure 3B), with IC_{50} values comparable to the IC_{50} values observed using the $[\gamma\text{-}^{32}\text{P}]\text{RII}$ substrate (Figure 3A). The behaviour of the WT and D477N autoinhibitory domains differs from that of the FK506–FKBP12 complex in that calcineurin-dependent hydrolysis of pNPP is enhanced rather than inhibited by the FK506–FKBP complex ([1] and results not shown).

We have previously documented that the D477N mutation

alters the biological characteristics of calcineurin expressed in the EL4 T-lymphoma cell line, allowing activation at suboptimal concentrations of Ca^{2+} and CaM [8]. The results presented herein confirm that the Asp-477 of the autoinhibitory domain is involved in a critical interaction with the catalytic domain, since mutation to Asn significantly reduces the inhibitory potency of the domain. In both calcineurin and protein phosphatase 1A (PP1A), the catalytic domain consists of two sites: the substrate-binding groove and the phosphatase active site [14,15,24]. The crystallographic studies demonstrate that the active-site residues of calcineurin involved in catalysis are conserved within PP1A, and substrate dephosphorylation by both phosphatases appears to proceed via nucleophilic attack [14,15,24]. A characteristic Asp-His catalytic pair of amino acids has been implicated in the mechanism of nucleophilic attack, with particular emphasis on His-151 located in the phosphatase active site of calcineurin. His-151 is hydrogen-bonded to Asp-121 and a metal-bound water molecule. In this configuration, His-151 acts as a proton acceptor from the water molecule, generating a potential attacking nucleophile [15]. The negatively charged Asp group in the WT autoinhibitory domain may serve as a molecular mimic of a phosphorylated Ser or Thr and may interact electrostatically with the basic His-151 at the active site. Mutation of the Asp in the autoinhibitory domain to Asn results in the loss of charge which would reduce such an electrostatic interaction, thus contributing to the weakened action of the mutant autoinhibitory domain in the inhibition of calcineurin activity.

Kinetic mechanism of calcineurin inhibition by the WT and D477N autoinhibitory domains

We postulated that the autoinhibitory domain behaved as a pseudosubstrate and should inhibit calcineurin phosphatase activity competitively, thus implying intrasteric regulation. Enzyme kinetics experiments were used to investigate the mechanism of inhibition. We constantly observed a zero intercept in Lineweaver–Burk plots using the $[\gamma\text{-}^{32}\text{P}]\text{RII}$ substrate, even with concentrations of $[\gamma\text{-}^{32}\text{P}]\text{RII}$ as high as 400 μM (results not shown). Since we failed to achieve saturation of the enzyme with the $[\gamma\text{-}^{32}\text{P}]\text{RII}$ substrate, we were unable to use this substrate in kinetic experiments. In contrast we were able to achieve saturation of calcineurin activity using pNPP as a substrate. Furthermore, since pNPP binds at the active site, any displacement of this substrate by the autoinhibitory domain would imply direct interaction of the domain with the active site.

To determine the mechanism of inhibition by the autoinhibitory domains, calcineurin activity was assayed using increasing concentrations of the WT and D477N autoinhibitory domains and hydrolysis of pNPP was measured spectrophotometrically. For each concentration of purified autoinhibitory domain, the concentration of pNPP was varied from 10 mM to 320 mM and three independent experiments were performed for each domain. The kinetic data from one representative experiment were plotted as a double-reciprocal plot for both the WT (Figure 4, top) and D477N autoinhibitory domains (Figure 4, middle and bottom). Data points were analysed by non-linear regression using eqn. (2) for non-competitive inhibition, but provided neither a value for K_{ii} nor a comprehensive convergence of lines. The same data points were analysed using eqn. (1) for competitive inhibition; the lines converged on the ordinate, providing a $K_i = 5 \pm 0.2 \mu\text{M}$ ($n = 3$) for the WT autoinhibitory domain and a $K_i = 36 \pm 3.7 \mu\text{M}$ ($n = 3$) for the D477N autoinhibitory domain. Thus, the WT and D477N autoinhibitory domains inhibit calcineurin activity competitively with respect to pNPP, and the K_i values demonstrate

that the WT autoinhibitory domain is a more potent inhibitor of calcineurin phosphatase activity than the D477N autoinhibitory domain. These data are consistent with the IC_{50} values obtained for inhibition of calcineurin phosphatase activity using [γ - ^{32}P]RII and pNPP as substrates. Characteristic of competitive inhibition, the V_{max} values remained constant for the WT and D477N autoinhibitory domains, while the K_m values increased with increasing inhibitor concentration. At equivalent concentrations of the WT autoinhibitory domain (Figure 4, top), the inhibition mediated by the D477N autoinhibitory domain (Figure 4, middle) gave poor resolution of competitive inhibition; however, at higher D477N autoinhibitory domain concentrations competitive inhibition was readily observed (Figure 4, bottom). The IC_{50} value of the WT autoinhibitory peptide was 8-fold higher than that of the D477N autoinhibitory peptide and the difference between the WT autoinhibitory peptide and domain remained undetermined. It was therefore not possible to test the ability of the autoinhibitory peptides to inhibit pNPP in kinetic studies due to the high concentration of peptides required for such experiments.

Previous studies have reported conflicting data regarding the mechanism of inhibition with the WT autoinhibitory peptide. Non-competitive inhibition has been reported by Hashimoto et al. [9] using ^{32}P -labelled myosin light chain as a substrate, suggesting allosteric inhibition similar to inhibition by the FK506-FKBP12 complex. It has been suggested that since myosin light chain does not contain the preferred R-R-X-S/T sequence present within many substrates of calcineurin [9,21], it may behave differently than substrates containing this sequence. Differences in experimental conditions, such as the use of specific cations (Mg^{2+} , Ca^{2+} , Mn^{2+}), may produce altered kinetic behaviour of the calcineurin phosphatase enzyme [7,9,16,25]. Data analysis may also play a role in the differences observed. For instance, the non-linear regression equations used herein subject each data point to competitive and non-competitive models, which may prove to be more sensitive than simple first-order regression [9,17]. The X-ray crystallographic analysis of human calcineurin by Kissinger et al. [15], at 2.1 Å resolution, in the absence of the immunosuppressant-immunophilin complex demonstrates that Asp-477, Lys-474 and amino acids 470–485 of the autoinhibitory region form a binding interface with the catalytic domain. However, one caveat with this X-ray crystallographic analysis is its failure to show the amino acids between the CnB binding domain and the autoinhibitory domain, which remain disordered at 2.1 Å resolution. Our data confirm that the autoinhibitory domain of calcineurin acts as a competitive inhibitor, and extend the predictions made from the crystallographic structure by demonstrating the critical involvement of Asp-477 with the catalytic domain.

In conclusion, the kinetic data reported herein demonstrate that the autoinhibitory domain is an intrasteric inhibitor of calcineurin phosphatase activity and that Asp-477 plays a critical role in the interaction. The decreased IC_{50} of the autoinhibitory domains compared with the autoinhibitory peptides suggests that other elements of the larger domains contribute to the enhanced inhibition of phosphatase activity. Further mutational experiments will clarify which amino acids, outside of the 25 amino acid peptide, contribute to stabilizing the interaction between the autoinhibitory domain and calcineurin. In addition, mutational analysis will also identify specific amino acids involved in interactions, not only at the phosphatase active site, but also within the substrate-binding groove. Given the sequence similarity between the phosphatase active site of PP1A and calcineurin, the unique substrate-binding grooves appear to be

essential for defining substrate specificity. A direct substrate for calcineurin has recently been identified, nuclear factor of activated T-cells (NFAT), which, it is speculated, binds within the substrate-binding groove of calcineurin [26]. Identification of the binding domain of NFAT may provide a prototype for understanding substrate specificity; this molecular definition may enable the development of novel immunosuppressive agents that target specific signalling pathways rather than calcineurin itself. With this approach one would not only avoid unwanted toxicities of the drug, which are intrinsic to inhibition of calcineurin (nephrotoxicity, neurotoxicity), but also target specific pathways for inhibition involving calcineurin (for example, T-cell signal transduction via NFAT) as they are determined.

We thank Drs. Marty Bollinger, Philip Cole, Dewai Pai and Neeta Mukerjee for valuable discussion. This work was supported by grant AI 32514 from the National Institute of Health (B.E.B.). B.E.B. is the recipient of an Established Investigator Award from the American Heart Association.

REFERENCES

- Liu, J., Farmer, J. D. J., Lane, W. S., Friedman, J., Weissman, I. and Schreiber, S. L. (1991) *Cell* **66**, 807–815.
- Liu, J., Albers, M. W., Wandless, T. J., Luan, S., Alber, D. G., Belshaw, P. J., Cohen, P., MacKintosh, C., Klee, C. B. and Schreiber, S. L. (1992) *Biochemistry* **31**, 3896–3901.
- Fruman, D. A., Klee, C. B., Bierer, B. E. and Burakoff, S. J. (1992) *Proc. Natl. Acad. Sci. U.S.A.* **89**, 3686–3690.
- O'Keefe, S. J., Tamura, J., Kincaid, R. L., Tocci, M. J. and O'Neill, E. A. (1992) *Nature (London)* **357**, 692–694.
- Clipstone, N. A. and Crabtree, G. R. (1992) *Nature (London)* **357**, 695–697.
- Hubbard, M. J. and Klee, C. B. (1989) *Biochemistry* **28**, 1868–1874.
- Perrino, B. A., Ng, L. Y. and Soderling, T. R. (1995) *J. Biol. Chem.* **270**, 340–346.
- Fruman, D. A., Pai, S., Burakoff, S. J. and Bierer, B. E. (1995) *Mol. Cell. Biol.* **15**, 3857–3863.
- Hashimoto, Y., Perrino, B. A. and Soderling, T. R. (1990) *J. Biol. Chem.* **265**, 1924–1927.
- Bierer, B. E., Hollander, G., Fruman, D. A. and Burakoff, S. J. (1993) *Curr. Opin. Immunol.* **5**, 763–773.
- Cardenas, M. E., Muir, R. S., Breuder, T. and Heitman, J. (1995) *EMBO J.* **14**, 2772–2783.
- Etzkorn, F. A., Chang, Z., Stolz, L. A. and Walsh, C. T. (1994) *Biochemistry* **33**, 2380–2388.
- Milan, D., Griffith, J., Su, M., Price, E. R. and McKeon, F. (1994) *Cell* **79**, 437–447.
- Griffith, J. P., Kim, J. L., Kim, E. E., Sintchak, M. D., Thomson, J. A., Fitzgibbon, M. J., Fleming, M. A., Caron, P. R., Hsiao, K. and Navia, M. A. (1995) *Cell* **82**, 507–522.
- Kissinger, C. T., Parge, H. E., Knighton, D. R., Lewis, C. T., Pelletier, L. A., Tempczyk, A., Kalish, V. J., Tucker, K. D., Showalter, R. E., Moomaw, E. W. et al. (1995) *Nature (London)* **378**, 641–644.
- Parsons, J. N., Wiederrecht, G. J., Salowe, S., Burbaum, J. J., Rokosz, L. L., Kincaid, R. L. and O'Keefe, S. J. (1994) *J. Biol. Chem.* **269**, 19610–19616.
- Perrino, B. A., Hashimoto, Y. and Soderling, T. R. (1990) *FASEB J.* **4**, A2237.
- Kincaid, R. L., Giri, P. R., Higuchi, S., Tamura, J., Dixon, S. C., Marietta, C. A., Amorese, D. A. and Martin, B. M. (1990) *J. Biol. Chem.* **265**, 11312–11319.
- Blumenthal, D. R., Takio, K., Hanson, R. S. and Krebs, E. G. (1986) *J. Biol. Chem.* **261**, 8140–8145.
- Fruman, D. A., Pai, S.-Y., Klee, C. B., Burakoff, S. J. and Bierer, B. E. (1996) *Methods (Companion to Methods Enzymol.)* **9**, 146–154.
- Donella-Dean, A., Krinks, M. H., Ruzzene, M., Klee, C. B. and Pinna, L. A. (1994) *Eur. J. Biochem.* **219**, 109–117.
- Haddy, A., Swanson, S. K.-H., Born, T. L. and Rusnak, F. (1992) *FEBS Lett.* **314**, 37–40.
- Takai, A. and Mieskes, G. (1992) *Biochem. J.* **275**, 233–239.
- Goldberg, J., Huang, H., Kwon, Y., Greengard, P., Nairn, A. and Kuriyan, J. (1995) *Nature (London)* **376**, 745–753.
- Gupta, R. C., Khandelwal, R. L. and Sulakhe, P. V. (1990) *Mol. Cell. Biochem.* **97**, 53–66.
- Wesselborg, S., Fruman, D. A., Sagoo, J. K., Bierer, B. E. and Burakoff, S. (1996) *J. Biol. Chem.* **271**, 1274–1277.