Activation of Calcineurin A Subunit Phosphatase Activity by Its Calcium-Binding B Subunit†

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Received July 24, 1995; Revised Manuscript Received November 6, 1995⊗

ABSTRACT: The protein phosphatase activity of calcineurin (CaN) is activated through calcium binding to both calmodulin and the B subunit of CaN. The purpose of this study was to determine which domain(s) in the CaN B subnit is required for either binding to the CaN A subunit or for transducing the effects of B subunit Ca^{2+} binding to the stimulation of the CaN A subunit phosphatase activity. We have previously demonstrated that interaction of CaN B regulatory subunit with the CaN A catalytic subunit requires hydrophobic residues within the CaN A sequence 328−390 [Watanabe Y., Perrino, B. A., Chang, B. H., & Soderling, T. R. (1995) *J. Biol. Chem. 270*, 456−460]. In the present study, selected hydrophobic residues within the B subunit were mutated to Glu to Gln. CaN B subunit mutants BE-1 (Val^{115}/Leu^{116} to Glu), BE-2 ($Val^{156/157/168/169}$ to Glu), and BQ-2 ($Val^{156/157/168/169}$ to Gln) were expressed and purified. The three mutant B subunits bound $^{45}Ca^{2+}$ normally. Mutants BE-2 and BQ-2 interacted with a GST fusion protein containing the B subunit binding domain of the CaN A subunit (residues 328−390), and they stimulated the phosphatase activity of the CaN A subunit in an *in vitro* reconstitution assay. Mutant BE-1 had a 3-fold reduced affinity for binding CaN A, and this mutant, even at saturating concentrations, gave very little stimulation of CaN A phosphatase activity. We conclude that residues Val^{115}/Leu^{116} in the B subunit participate in high-affinity binding to the A subunit and are required for transducing the effects [i.e., decrease K_m and increase V_{max}; Perrino, B. A., Ng, L. Y., & Soderling, T. R. (1995) *J. Biol. Chem. 270*, 340−346] of B subunit Ca^{2+} binding to stimulation of CaN A phosphatase activity.

Calcineurin (CaN)[1] is an isozyme family of widely distributed Ca^{2+}/calmodulin-dependent Ser/Thr protein phosphatases (protein phosphatase 2B) [reviewed in Guerini and Klee (1991)] that modulate the functions of proteins such as the L-type Ca^{2+} channel (Hosey et al., 1986), the Na^+ channel (Murphy et al., 1993), the NMDA receptor channel (Lieberman & Mody, 1994), and the heat-stable inhibitors of protein phosphatase 1 (inhibitor 1 and DARPP-32) (Cohen, 1989). More recently it was determined that calcineurin is the major target of the immunosuppressants cyclosporin A and FK506 (O'Keefe et al., 1992). These drugs have been used as cell-permeable inhibitors to identify roles for calcineurin in the transcriptional regulation of interleukin 2 (Baldari et al., 1991), several immediate−early genes (Enslen & Soderling, 1994) and in the induction of synaptic long-term depression in hippocampus (Mulkey et al., 1994).

CaN is a heterodimer composed of A and B subunits (Guerini & Klee, 1991). The 57−61 kDa A subunit contains the catalytic elements (Winkler et al., 1984), a calmodulin (CaM)-binding domain (Kincaid et al., 1988), and autoinhibitory elements (Hashimoto et al., 1990). The 19 kDa CaN B subunit is an "EF-hand" Ca^{2+}-binding protein which remains tightly associated with the A subunit in the presence or absence of Ca^{2+} (Stewart et al., 1982). Expressed CaN A has very low phosphatase activity by itself, but addition of Mn^{2+}/CaM or Mn^{2+}/B subunit gives 5−10-fold activations (Perrino et al., 1992). However, addition of both B subunit and CaM to the A subunit in the presence of Mn^{2+} results in a synergistic 600-fold activation. Kinetica analysis of A subunit phosphatase activity indicates that Ca^{2+}/CaM increases the V_{max} 5−10-fold whereas Ca^{2+}/B subunit activates by decreasing the K_m 10-fold plus a 10-fold increase in V_{max} (Perrino et al., 1992, 1995; Stemmer & Klee, 1995).

We have previously shown that mutagenesis to Glu or Gln of several residues (Val^{349}, Phe^{350} and Phe^{356}, Val^{357}) in a strongly hydrophobic region of the A subunit blocks binding of and activation by wild-type B subunit (Watanabe et al., 1995). That study identified those hydrophobic residues between the catalytic domain and the CaM-binding motif in the A subunit as important for binding to the B subunit. In the present study we applied the same approach by mutating several hydrophobic regions in the B subunit and analyzing the ability of the mutants to bind to wild-type A subunit and to activate its protein phosphatase activity.

MATERIALS AND METHODS

cDNA Construction and Mutagenesis. The cDNAs encoding rat brain CaN A (α_δ isoform) and CaN B (Perrino et al., 1995) were introduced into *Sma*I/*Eco*RI-digested PVL1393 and *Bss*HII/*Bam*HI-digested modified pBlueBac fusion transfer vector, respectively. The latter vector expresses a fusion protein with Factor Xa cleavable NH_2-terminal six histidine residues. For mutagenesis (version 2.1 Amersham mutagenesis kit), CaN B was cloned into the *Bss*HII and *Bam*HI sites

† Supported in part by NIH Grants GM 41292 and DK 44239.

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⊗ Abstract published in *Advance ACS Abstracts*, December 15, 1995.

[1] Abbreviations: BSA, bovine serum albumin; CaM, calmodulin; CaN, calcineurin; DTT, dithiothreitol; EGTA [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; GST, glutathione-*S*-transferase; PCR, polymerase chain reaction; PVDF, polyvinylidene; PMSF, phenylmethylsulfonyl fluoride; ^{32}P-RII pep, ^{32}P-labeled peptide derived from the RII subunit of cAMP-kinase; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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of M13mp18. The CaN B mutants were sequenced using the Sequenase Version 2.0 sequencing kit. The mutant cDNAs were ligated into modified pBlueBac and transferred into Sf9 cells.

Cell Culture and Protein Purification. Expression in Sf9 cells and purification of expressed CaN A on CaM-Sepharose were performed as described previously (Perrino et al., 1992, 1995; Watanabe et al., 1994). For purification of recombinant wild-type and mutant CaN B, cells were harvested 45 h postinfection by centrifugation at 800*g* for 10 min. Cell pellets were suspended in extraction buffer (10 mM Tris-HCl, pH 7.5, 1 mM PMSF) and sonicated with 3 × 10 s bursts at 15 s intervals using a Branson cell disruptor. After centrifugation (15 000*g* for 60 min), the supernatant was mixed for 3 h at 4 °C with a Ni^{2+} chelator gel (Probond Resin, Invitrogen) that was equilibrated in buffer A (50 mM Tris-HCl, pH 7.5, 300 mM NaCl). The gel was washed with 10 bed volumes of buffer A, and the CaN B fusion protein was then eluted with buffer C (250 nM imidazole, pH 7.0). The purified proteins were digested by Factor Xa to produce a nonfusion CaN B. Protein concentration was determined by the method of Bradford (1976) using BSA as the standard.

Binding of CaN B to CaN A328−390. The fusion protein between glutathione-*S*-transferase and the B subunit binding of CaN A (residues 328−390) was constructed, expressed in *Escherichia coli*, and purified on glutathione-Sepharose as previously described (Watanabe et al., 1995). The GST/CaN A328−390 fusion protein (4 μg) was incubated (total volume, 500 μL) for 1 h at 4 °C with 1 μg of CaN B (wild-type or mutant), glutathione-Sepharose 4B [40 μL of 50% (v/v) slurry, Pharmacia Biotech Inc.], 40 mM Tris-HCl, pH 7.5, 6 mM $MgCl_2$, 0.5 mM $CaCl_2$, and 0.1 mg of BSA/mL. The Sepharose beads were collected by centrifugation, washed three times with 100 mM Tris-HCl, pH 7.5, containing 200 mM NaCl, resuspended in 40 μL of 2× SDS−PAGE sample buffer, and boiled for 3 min. Eluted proteins were analyzed by SDS−PAGE using 15% polyacrylamide.

Other Methods. Reconstitution of CaN A and CaN B was carried out as described previously (Perrino et al., 1992, 1995; Watanabe et al., 1995). RII peptide (DLDVPIPGRFDRRVSVAAE), a synthetic peptide substrate for CaN, was synthesized, and its purity, amino acid composition, and concentration were determined as described (Hashimoto & Soderling, 1987). It was phosphorylated by cAMP-kinase (Perrino et al., 1992) and used as substrate for CaN in 40 mM Tris-HCl, pH 7.5, 0.1 M KCl, 0.5 mM DTT, 0.5 mM $CaCl_2$, 6 mM magnesium acetate, 0.5 μM CaM, and 2 mg of BSA/mL. Calcium binding to CaN B on membranes was determined by the $^{45}Ca^{2+}$ overlay technique (Maruyama et al., 1984). One-dimensional SDS−PAGE was carried out according to the method of Laemmli (1970). The electrophoretic transfer of proteins from the SDS−PAGE to the PVDF membrane was performed as described by Towbin et al. (1979). For immunodetection of the transferred proteins, the procedure of Burnette (1981) was used except that the second antibody was linked to horseradish peroxidase. Antigen−antibody complexes were visualized by reacting the bound peroxidase with the chemiluminescence reagent (Du Pont).

Materials. The modified pBlueBac fusion transfer vector was kindly provided by Dr. R. Maurer (Department of Cell Biology and Anatomy, Oregon Health Sciences University).

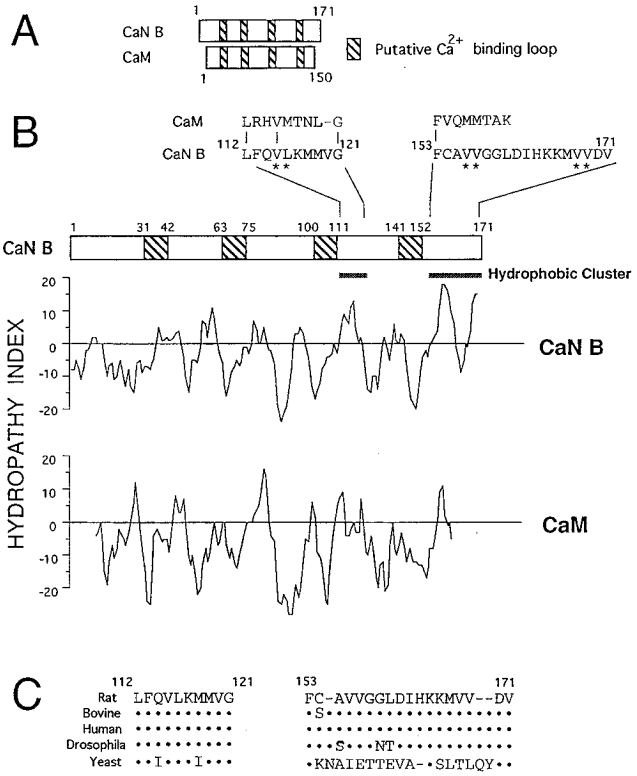

FIGURE 1: Comparisons of CaN B and CaM. Panel A: Schematic of rat brain CaN B subunit and CaM with Ca^{2+}-binding EF hands indicated by cross-hatching. Panel B: Hydropathy plots (window average of six residues) for CaN B and CaM. The hydrophobic domains selected for mutation are indicated by the shading, and the corresponding sequences for CaN B and CaM are shown above the schematic of CaN B. The asterisks indicate the residues mutated in this study. Panel C: Comparison of CaN B sequences 112−121 and 153−171 for the indicated species. Solid dots denote identical residues.

[γ-^{32}P]ATP (6000 Ci/mmol) was purchased from Dupont-New England Nuclear. Restriction enzymes and DNA-modifying enzymes were from Promega or Bethesda Research Laboratories. Grace's insect medium, lactalbumin hydrolysate, yeastolate, Pluronic F-68, and antibiotics were from Gibco/BRL. Fetal bovine serum was purchased from Hyclone. Dowex resins were from Bio-Rad. CaM-Sepharose and GSH-Sepharose were obtained from Pharmacia. Anti-CaN B monoclonal antibody was from Upstate Biotechnologies, Inc. All other materials and reagents were of the highest quality available from commercial suppliers.

RESULTS AND DISCUSSION

Engineering, Expression, and Purification of B Subunit Mutants. Although CaN B subunit has about 30%−35% sequence homology with CaM (Klee et al., 1988), these two Ca^{2+}-binding proteins do not substitute for or compete against each other in the stimulation of CaN A subunit phosphatase activity (Perrino et al., 1992, 1995; Stemmer & Klee, 1994; Watanabe et al., 1995). Figure 1A is a schematic of the CaN B subunit and CaM with the EF-hand motifs highlighted. Figure 1B shows a hydropathy index plot for CaN B subunit with two major hydrophobic regions indicated by shading. Because the region in the A subunit which binds B subunit is highly hydrophobic, we initially chose to make mutations in hydrophobic domains of the B subunit. The residues chosen for mutation, Val^{115}/Leu^{116} to Glu (mutant BE-1) and $Val^{156/157/168/169}$ to Glu (mutant BE-2) or Gln

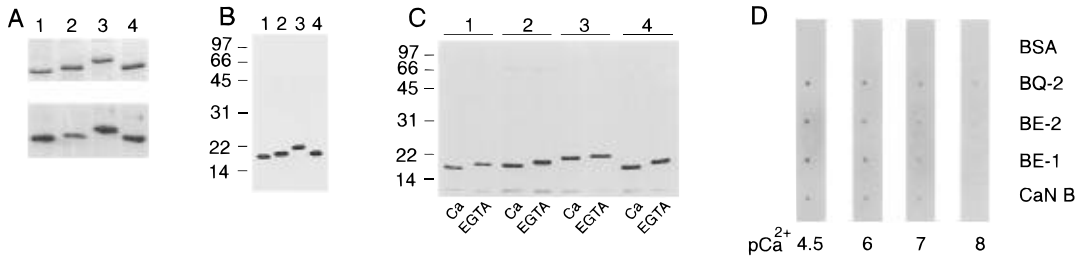

FIGURE 2: Characterization of mutant CaN B subunits. Proteins (2 μg) were separated by SDS−PAGE (15% acrylamide) and stained with Coomassie Blue (panel A, upper lanes, and panel C) or transferred to PVDF membrane and immunoblotted with anti-CaN B antibody (panel A, lower lanes) or $^{45}Ca^{2+}$ overlay (panel B). Lane 1, wild-type CaN B; lane 2, mutant BE-1; lane 3, mutant BE-2; lane 4, mutant BQ-2. In panel C the sample buffer contained either 2 mM Ca^{2+} or 2 mM EGTA as indicated. Panel D: $^{45}Ca^{2+}$ binding to proteins (0.5 μg) spotted on nitrocellulose membrane was determined at the indicated free Ca^{2+} concentration. Ca^{2+}/EGTA buffers were used to control free Ca^{2+} concentration (Stewart et al., 1982).

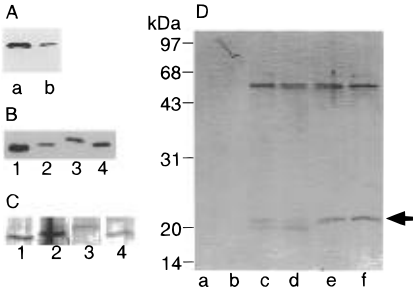

FIGURE 3: Binding of CaN B to CaN A. Panels A−C: CaN B (1 μg) was incubated for 1 h at 4 °C (Materials and Methods) in the presence of 8 μg of GST alone (panel A, lane b) or 4 μg of fusion protein GST/CaN A328−390 plus 4 μg of GST (all other lanes). Eluted proteins from glutathione-Sepharose were analyzed by SDS−15% PAGE, transferred to PVDF membrane, and immunoblotted with anti-CaN B antibody (panels A and B) or analyzed by silver stain (panel C). Panel A: Wild-type CaN B. Panels B and C: Wild-type CaN B (lane 1), BE-1 (lane 2), BE-2 (lane 3), and BQ-2 (lane 4). Panel D: Wild-type CaN B (lanes a, c, d) and mutant BE-1 (lanes b, e, f) were incubated without (lanes a, b) or with CaN A (5.5 μg) in 1:1 (lanes c, e) or 3:1 (lanes d, f) molar ratios (CaN B:CaN A) for 2 h at 30 °C under reconstitution conditions (Watanabe et al., 1995). The mixtures were adsorbed to CaM-Sepharose in the presence of 1 mM Ca^{2+}, washed in buffer containing 1 mM $CaCl_2$ and 1 M NaCl, and eluted with buffer containing 1 mM EGTA. The eluted proteins were analyzed by SDS−PAGE (15%) and Coomassie Blue, and the B subunit is indicated by the arrow.

(mutant BQ-2), are indicated by the asterisk, and the analogous sequences in CaM are given. The region containing Val^{115}/Leu^{116} is highly conserved in CaN B from human to *Drosophila* and yeast, whereas the region containing $Val^{156/157/168/169}$ is highly conserved except in yeast (Figure 1C). It has been demonstrated that mammalian B subunit can activate the catalytic A subunit from *Neurospora crassa* (Ueki & Kincaid, 1993). In these mutated regions CaN B is significantly more hydrophobic than CaM (Figure 1B). Hydropathy plots predict that the Glu/Gln mutations convert these hydrophobic motifs to hydrophilic ones (not shown).

The cDNA sequences for the wild-type and mutant CaN B subunits were inserted into an expression vector which contains a poly(His) sequence, and they were expressed in Sf9 cells (see Materials and Methods). The poly(His)/CaN B fusion proteins were purified on Ni^{2+} chelator gel and cleaved with Factor Xa. The purified wild-type B subunit migrated on SDS−PAGE with a mobility similar to non-myristoylated B subunit (not shown). We have previously demonstrated that when the B subunit (i.e., nonfusion protein) is expressed using the baculovirus/Sf9 cell system, both myristoylated and non-myristoylated B subunits are present (Perrino et al., 19920. The BE-1 and BQ-2 mutants migrated similarly to wild-type B subunit whereas the BE-2 mutant had a slightly reduced mobility (Figure 2A). Note that the immunoreactivity of the BE-1 mutant is reduced (Figure 2A, bottom of lane 2).

Binding of Ca^{2+} and CaN A Subunit by the Mutant B Subunits. The wild-type and mutant B subunits bound $^{45}Ca^{2+}$ as determined in a gel overlay experiment (Figure 2B). It is known that binding of Ca^{2+} to B subunit causes a conformational change that gives a small increase in mobility on SDS−PAGE (Klee et al., 1988), and wild-type and mutant B subunits all demonstrated a similar Ca^{2+}-induced mobility shift (Figure 2C). This suggests that the mutants not only bound Ca^{2+}, but they also underwent a conformation change. Using a dot−blot type assay, the apparent affinities of the mutants for $^{45}Ca^{2+}$ were similar to wild-type B subunit (Figure 2D). Thus, the mutants appear to have normal Ca^{2+}-binding capabilities.

We have shown that wild-type B subunit binds to a GST fusion protein containing residues 328−390 of the CaN A subunit (Watanabe et al., 1995), and the specificity of this binding for the CaN A residues 328−390 is demonstrated in Figure 3A. The GST/CaNA328−390 fusion protein was incubated with wild-type and mutant B subunits, applied to glutathione-Sepharose to remove unbound B subunit, eluted with SDS sample buffer, and analyzed by SDS−PAGE and Western blot. The mutant and wild-type B subunits all bound to the previously identified B subunit domain in the A subunit (Figure 3B). It appears that the binding of the BE-1 mutant (lane b) to the CaN A subunit domain is reduced relative to wild-type and the other mutants. This may be partially an artifact due to the lower immunoreactivity of the BE-1 mutant (see lane 2 of Figure 2A), and when an analogous experiment was analyzed by silver stain, equivalent amounts of wild-type and mutant B subunits were detected (Figure 3C). To further examine binding of the BE-1 mutant to A subunit, wild-type B subunit and BE-1 mutant were incubated *in vitro* at 1:1 and 3:1 molar ratios with full-length A subunit under reconstitution conditions (Watanabe et al., 1995). This mixture was then subjected to affinity chromatography on CaM-Sepharose which binds the A subunit in the presence of calcium. After elution with EGTA the proteins were analyzed by SDS−PAGE and Coomassie Blue staining (Figure 3D). In the absence of A subunit, there was no binding of wild-type or mutant BE-1 B subunits (lanes a and b). In lanes c−f there are equivalent amounts of 60 kDa A subunit, and at both molar ratios approximately equal amounts of wild-type B subunit and

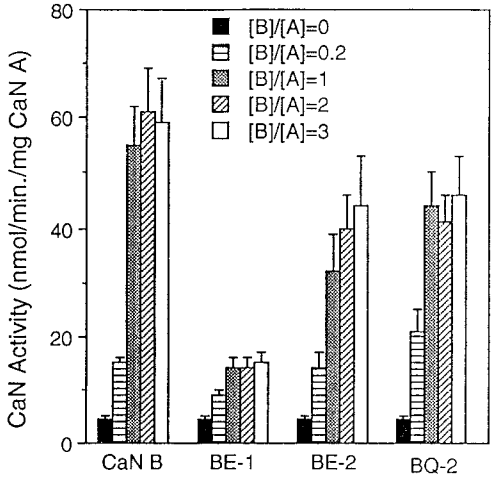

FIGURE 4: Effects of CaN B subunit mutagenesis on reconstitution of CaN A subunit phosphatase activity. Panel A: CaN A (100 nM) was incubated with purified wild-type or mutant CaN B at 0, 20, 100, 200, or 300 nM for 2 h at 30 °C and then assayed at 30 °C for 15 min with 500 nM CaM, 0.5 mM Ca^{2+}, 6 mM Mg^{2+}, and 60 μM ^{32}P-RII peptide as indicated under Materials and Methods.

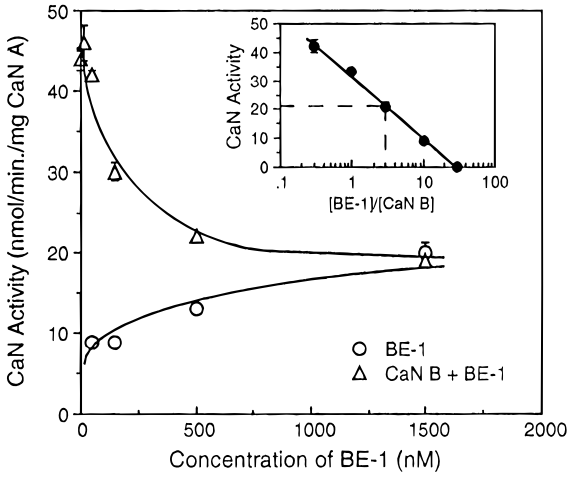

FIGURE 5: Competition of BE-1 mutant for reconstitution by wild-type B subunit of CaN A phosphatase activity. CaN A (50 nM) was incubated as in Figure 5A without (○) or with 50 nM (△) wild-type B subunit and 0−1500 nM BE-1 mutant for 2 h prior to determination of phosphatase activity. The inset shows the data for the competition between BE-1 and B subunit with the data for BE-1 alone subtracted. The dotted line indicates 50% inhibition at a ratio of BE-1/B of about 3.

BE-1 are bound to the A subunit. The lower M_r wild-type B subunit band is the result of limited proteolysis during storage of the B subunit. These results and those from the GST fusion construct clearly show that BE-1 can bind the A subunit, but neither of these methods is sufficiently quantitative to detect small differences in the affinity of the interactions. However, subsequent experiments (see Figure 5) indicate that binding of BE-1 to CaN A subunit may be somewhat impaired.

Activation of the CaN A Subunit Phosphatase Activity by Mutant B Subunits. Since the mutant B subunits were able to bind Ca^{2+} and bind the A subunit, we next determined their abilities to stimulate the phosphatase activity of the CaN A subunit. CaN A subunit by itself has very low phosphatase activity, but the phosphatase activity can be reconstituted by *in vitro* incubation for 2 h with wild-type B subunit (Perrino et al., 1992, 1995; Watanabe et al., 1995). We used this *in vitro* reconstitution assay to assess the ability of the mutant B subunits to activate the CaN A subunit. Figure 4 shows that wild-type B subunit at a 1:1 molar ratio to A subunit maximally stimulated the phosphatase activity assayed in the presence of Ca^{2+}/Mg^{2+}/CaM. The BE-1 mutant gave very poor reconstitution (25% compared to wild-type) of phosphatase activity, even at 3-fold molar excess over A subunit, whereas the BE-2 and BQ-2 mutants stimulated the phosphatase activity to 70%−80% that of wild-type. This reconstitution experiment was incubated for 2 h, which is near-optimal for wild-type B subunit. When the *in vitro* incubation was extended for up to 7 h, the BE-1 mutant still gave only 25% reconstitution of phosphatase activity (not shown).

The data of Figure 3 indicate that the mutant B subunits can interact with the fusion protein containing residues 328−390 of the CaN A subunit. In order to determine relative affinities of the BE-1 mutant and wild-type B subunit for CaN A, their abilities to compete during *in vitro* reconstitution of CaN A phosphatase activity were determined. CaN A was incubated with equimolar wild-type B subunit and increasing concentrations of BE-1. Since interaction of BE-1 with CaN A results in weak phosphatase activation (Figure 4), the increasing BE-1 should inhibit the phosphatase activation by whild-type CaN B. As shown in Figure 5, increasing concentrations of BE-1 did inhibit phosphatase reconstitution, and a 3-fold molar excess of BE-1 over wild-type B subunit was required for half-maximal inhibition (see Figure 5, inset). This result indicates that binding of BE-1 mutant to CaN A was of lower affinity compared to wild-type B subunit. Note that, although a 3-fold molar excess of BE-1 was able to compete with wild-type subunit for activation of CaN A (Figure 5), a 3-fold molar excess of BE-1 gave only 20−25% activation (Figures 4 and 5). This indicates that, although excess BE-1 subunit can bind to the CaN A subunit, it poorly transduces the effect of B subunit Ca^{2+} binding to stimulate the A subunit phosphatase activity.

We also tested wheterh interaction of BE-1 would stimulate the phosphatase activity of CaN A truncated at residue 420 (A420). We have previously shown that the heteromeric CaN containing A420 and wild-type B is fully active in the absence of Ca^{2+}/CaM (Perrino et al., 1995). Although CaN A420 does not require calcium for activity, it does still require B subunit binding (Perrino et al., 1995). Therefore, it was of interest to determine whether the phosphatase activity of A420, which does not require Ca^{2+} transduction through the B subunit, could be activated by BE-1. However, BE-1 was not able to activate A420 even though the wild-type B subunit did (not shown).

Conclusions. This study identifies a hydrophobic motif around residues Val^{115}/Leu^{116} (BE-1 mutant) that is involved in interaction with the A subunit and in transducing the effects of B subunit Ca^{2+} binding to the activation of the A subunit phosphatase activity. We and others have previously demonstrated that binding of Ca^{2+} to the B subunit results in a 10-fold decrease in K_m for substrate and a 5−10-fold increase in V_{max} (Perrino et al., 1992, 1995; Stemmer & Klee, 1994). This uncoupling of Ca^{2+}-dependent subunit transduction in the BE-1 mutant was not due to lack of Ca^{2+} binding to BE-1 (Figure 2). The BE-1 mutant can bind to CaN A (Figure 3), but this binding was of lower affinity than for wild-type B subunit to the A subunit (Figure 5). Binding of BE-1 to CaN A also was abnormal in that it did

not reconstitute phosphatase activity in an A420 truncation mutant that does not require Ca^{2+} binding for full phosphatase activity. While this paper was under review, the crystal structure of proteolyzed CaN complexed with FK506/FKBP12 was published (Griffith et al., 1995). This structure shows that the primary binding of the A and B subunits requires hydrophobic interactions of A subunit residues 350−370, confirming our previous mutagenesis study (Watanabe et al., 1995) that hydrophobic residues Val^{349}/Phe^{350} and Phe^{356}/Val^{357} are critical for interaction of the A and B subunits. From the published crystal structure it is concluded that one of the residues mutated in the present study, Leu^{116}, is involved in interaction of the B subunit with the FK506/FKBP12 complex. Since the crystal structure of CaN with bound Ca^{2+}/CaM in the absence of the inhibitory FK506/FKBP12 complex has not been published, the role of Val^{115}/Leu^{116} in the active structure of CaN is not known. A speculative hypothesis, which would be consistent with the conclusion of the present paper, is that interaction of Leu^{116} with the FK506/FKBP12 complex prevents interaction of Leu^{116} with the A subunit that is required for transducing the effect of B subunit Ca^{2+} binding to activation of the A subunit phosphatase activity. Further studies will be required to test this hypothesis.

Some of our results are similar to those described in a paper that appeared while this manuscript was in preparation. Milan et al. (1994) showed that mutation of B subunit residue Val^{120} (just four residues COOH-terminal from our BE-1 mutation) did not prevent binding of the B subunit to the A subunit, but this mutant B subunit poorly activated the A subunit. Furthermore, this same mutant did not bind the cyclosporin/cyclophilin complex. We determined that cyclosporin A/cyclophilin gave poor inhibition of our reconstituted CaN A/BE-1 phosphatase activity (not shown). However, our results are difficult to interpret since the CaN A/BE-1 complex had very low phosphatase activity even in the absence of immunosuppressants. Current evidence indicates that this immunosuppressant complex binds to both the B subunit and the A subunit (Husi et al., 1994; Clipstone et al., 1994).

Milan et al. (1994) also reported that the B subunit mutants F82K, F153A, and M166D failed to associate with the CaN A subunit. Their result for the latter two mutants is surprising since our mutants BE-2 and BQ-2, which are in the same region, bound the GST/CaN A328−390 and also gave 70%−80% activation of the phosphatase activity of CaN A. It is possible that F153 and M166 (their mutations) may play unique roles in binding to CaN A whereas valines at positions 155, 156, 168, and 169 (our mutations) are not involved.

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BI951703+