Protein Science (1996), 5:1917–1921. Cambridge University Press. Printed in the USA.

FOR THE RECORD

Dynamic NMR studies of ligand–receptor interactions: Design and analysis of a rapidly exchanging complex of FKBP-12/FK506 with a 24 kDa calcineurin fragment

JASNA FEJZO, CHRISTOPHER A. LEPRE, JEFFREY W. PENG, MICHAEL S.-S. SU, JOHN A. THOMSON, AND JONATHAN M. MOORE

Vertex Pharmaceuticals Incorporated, 130 Waverly Street, Cambridge, Massachusetts 02139-4242

(RECEIVED February 28, 1996; ACCEPTED June 26, 1996)

Abstract: Dynamic NMR methods, such as differential line broadening and transferred NOE spectroscopy, are normally reserved for the study of small molecule ligand interactions with large protein receptors. Using a combination of isotope labeling and isotope edited NMR, we have extended these techniques to characterize interactions of a much larger protein/drug complex, FKBP-12/FK506 with its receptor protein, calcineurin. In order to examine this multicomponent system by dynamic NMR methods, the 93 kDa, tightly bound FKBP-12/FK506/Cn complex was replaced with a lower affinity, rapidly exchanging system consisting of FKBP-12/FK506 (13 kDa), recombinant calcineurin subunit B (CnB) (20 kDa), and a synthetic peptide (4 kDa) corresponding to the B binding domain (BBD) of calcineurin catalytic subunit A (CnA).

Analysis of ^{1}H-^{13}C HSQC data acquired for the FKBP-12/^{13}C-FK506 and FKBP-12/^{13}C-FK506/CnB/BBD complexes indicates that FKBP-12/FK506 and CnB/BBD are in fast exchange in the quaternary complex. Comparison of proton line widths shows significant broadening of resonances along the macrocycle backbone at 13-CH, 13-OMe, 15-OMe, 18-CH_2, 20-CH, 21-CH, and 25-Me, as well as moderate broadening on the macrocycle backbone at 17-Me, 24-CH, and the pyranose 12-CH_2 protons. The tri-substituted olefin and cyclohexyl groups also show moderate broadening at the 27-Me, 28-CH, and 30-CH_2 positions, respectively. Unexpectedly, little line broadening was observed for the allyl resonances of FK506 in the quaternary complex, although ^{13}C longitudinal relaxation measurements suggest this group also makes contacts with calcineurin. In addition, intermolecular transfer NOE peaks were observed for the allyl 37-CH_2, 21-CH, 30-CH_2, 13-OMe, 15-OMe, 17-Me, 25-Me, and 27-Me groups, indicating that these are potential sites on the FK506 molecule that interact with calcineurin.

Keywords: calcineurin; FKBP-12; FK506; immunophilins; structure-based drug design; transferred NOE

Reprint requests to: Jonathan M. Moore, Vertex Pharmaceuticals Incorporated, 130 Waverly Street, Cambridge, Massachusetts 02139-4242.

Complexes of the potent immunosuppressants FK506 (Fig. 1) and cyclosporin A with the immunophilins FKBP-12 and cyclophilin, respectively, have been demonstrated to inhibit signal transduction pathways leading to T-cell activation by binding to and inhibiting the protein phosphatase, calcineurin (Cn) (Liu et al., 1991). Since the immunophilin/drug complex and not the drug or immunophilin protein alone is the inhibitory species, information regarding the interaction of this complex with its downstream target, calcineurin, is considered critical for the structure-based design of improved immunosuppressive agents. Unfortunately, the ternary complexes of both FKBP-12/FK506 and Cyp/CSA with calcineurin are both large (>93 kDa) and preclude the use of NMR techniques for a direct structure determination. Therefore, we have focused upon alternative methods to probe the interaction of FKBP-12/FK506 with calcineurin. The 93 kDa, tightly bound FKBP-12/FK506/Cn complex was replaced with a lower affinity, rapidly exchanging system consisting of FKBP-12/FK506 (13 kDa), recombinant calcineurin subunit B (CnB) (20 kDa), and a synthetic peptide (4 kDa) corresponding to the B binding domain (BBD) of calcineurin catalytic subunit A (CnA) (Fig. 2) Dynamic NMR methods, such as differential line broadening and transferred NOE spectroscopy, normally reserved for the study of small molecule interactions with large protein receptors (reviewed in Ni, 1994), have been extended to characterize interactions of a much larger protein/drug complex, FKBP-12/FK506 with a 24 kDa fragment of its receptor protein, calcineurin. Differential line broadening effects and additional NOE contacts for FK506 in the FKBP-12/FK506/CnB/BBD quaternary complex versus the binary FKBP-12/FK506 complex have been observed, and indicate which regions of the FK506 molecule are most influenced by interaction with calcineurin.

Several recent mutational analyses (Clipstone et al., 1994; Kawamura & Su, 1995; Watanabe et al., 1995) indicate that residues within the primary sequence 328–390 of CnA define the B-subunit binding domain (BBD), and that residues in the BBD sequence are critical as well for immunophilin/drug recognition. Based on recent studies (Kawamura & Su, 1995), a 34-residue sequence (S337-N370: SPHPYWLPNFMDVFTWSLPFVGE KVTEMLVNVLN) within the B binding domain region of CnA (Fig. 2) was chosen for synthesis. The binding affinity of the BBD peptide for CnB was

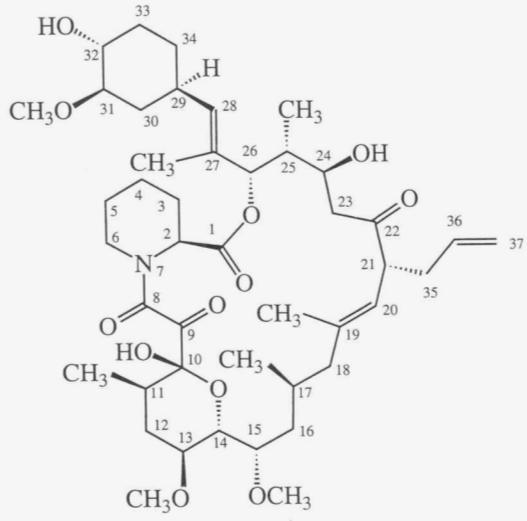

Fig. 1. Chemical structure and numbering of FK506.

measured by fluorescence titration, monitoring BBD fluorescence at 350 nm, and the K_d determined to be 400 ± 300 nM. The affinity of FK506 for FKBP-12 has been determined previously to be subnanomolar (Harding et al., 1989) and thus the binary system experiences extremely slow exchange. Accordingly, only one set of resonances corresponding to FKBP-12/FK506 complex is observed in the NMR spectrum. The affinity of FKBP-12/FK506 binary complex for the BBD/CnB complex was measured in a calcineurin competition assay (Aldape et al., 1992) and the K_i was determined to be approximately 25 μM. Although the competition assay does not directly measure BBD/CnB binding to FKBP-12/FK506, the K_i value indicates weak binding when compared with the nanomolar K_i measured for full length calcineurin (Aldape et al., 1992). Comparison of heteronuclear correlation spectra recorded for the FKBP-12/^{13}C-FK506 binary and FKBP-12/^{13}C-FK506/BBD/CnB quaternary complexes reveals that ^{1}H and ^{13}C resonances for FK506 appear at almost the same chemical shifts in both spectra (data not shown), but are slightly broadened in the spectrum for the quaternary complex. These results indicate that the two components are in fast exchange:

$$\text{FKBP-12/FK506} + \text{BBD/CnB} \underset{k_{off}}{\overset{k_{on}}{\rightleftarrows}} \text{FKBP-12/FK506/BBD/CnB}.$$

To characterize the exchange kinetics in more detail, simulations of exchange lineshapes were carried out,[1] assuming a diffusion limited on rate, $k_{on} = 10^8\ s^{-1}$, and $K_d = k_{off}/k_{on}$. In order to obtain the most conservative value of the K_d for all ^{13}C sites of FK506 examined, simulations were carried out for lines for which the minimum broadening was observed (i.e., $\Delta\Delta\nu_{1/2} = \Delta\nu_{1/2}^{bound} - \Delta\nu_{1/2}^{free} \cong 0$ Hz), and for which minimal differences in resonance maxima occurred (London, 1993). Using this approach, values of $k_{off} > 10^4\ s^{-1}$ and $K_d > 100\ \mu$M were necessary at the equimolar concentrations used. Proton line widths were measured for all resolved resonances in the HSQC (Bodenhausen & Ruben, 1980) spectra of the FKBP-12/^{13}C-FK506 binary and FKBP-12/^{13}C-FK506/BBD/CnB quaternary complexes. All signals for which the differential line broadening exceeds 5 Hz are listed in Table 1. In rapidly exchanging protein-ligand and protein-protein systems, line width differences will arise due to both correlation time effects (faster relaxation in the bound state) as well as from differences in chemical shift between the free and bound state (the $\Delta\omega$ effect). Unless there exist significant differences in intramolecular dynamics in the free state, these correlation time effects will be close to uniform for all sites in the ligand, and the observed differential broadening will be due primarily to differences in chemical shift between the free and bound state. Differences in chemical shift may arise from differences in chemical environment brought about by intramolecular local conformational changes in the bound state, or due to close packing of ligand atoms with receptor side chains. In practice, it is difficult or impossible to distinguish which of the two phenomena are responsible for the observed broadenings. Lineshape analysis of the HSQC data indicates significant broadening of resonances along the macrocycle backbone and cyclohexyl groups, as well as moderate broadening of protons from the pyranose, tri-substituted olefin, and additional cyclohexyl sites. All broadenings greater than 5 Hz are indicated schematically in Figure 3.

^{13}C-^{1}H sites that undergo exchange broadening in the present study correlate well with sites that have been determined in previous studies to possess moderate to high solvent exposure in the binary FKBP-12/^{13}C-FK506 complex (Lepre et al., 1993) and are

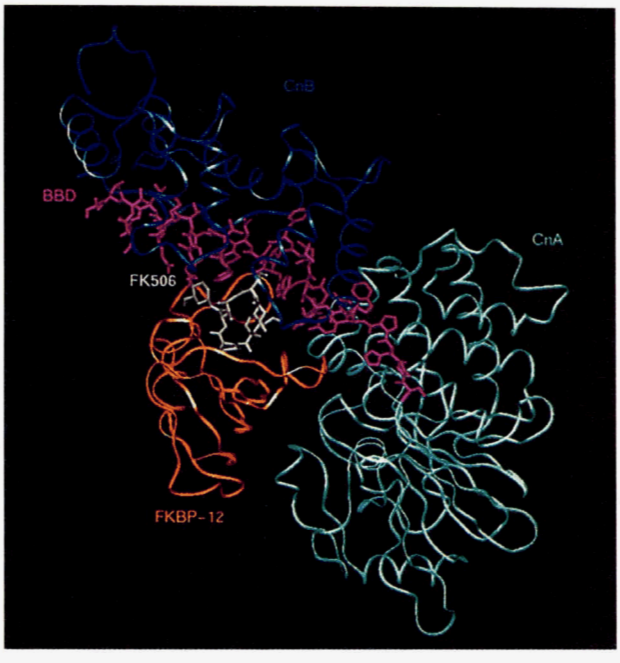

Fig. 2. The overall architecture of the calcineurin ternary complex (Griffith et al., 1995) showing individual components: CnA in cyan, CnB in blue, FKBP-12 in orange, FK506 in white. The B-subunit binding domain of CnA (BBD), located between the NH_2-terminal catalytic domain and the calmodulin binding domain, is shown in magenta. The BBD peptide was synthesized by the solid phase method (Advance ChemTech 396 MPS) using Fmoc-amino acid derivatives and HBtU/HOBt activation with Fmoc-Asn(Trityl)-Wang resin (0.49 mmol/g). The desired peptide (R_t = 38 min) was isolated as the TFA salt after lyophilization (24.2 mg; 3%). The sequence was confirmed by N-terminal sequencing and FABMS (glycerol/thioglycerol), *m/e* 4009 $(M+H)^+$.

[1] Software for simulating exchange lineshapes was kindly provided by Dr. Feng Ni, Biotechnology Research Institute, National Research Council Canada.

Table 1. *Line broadening of ^1H FK506 resonances measured in ^1H-^{13}C HSQC spectra of the FKBP-12/^{13}C-FK506 complex and FKBP-12/^{13}C-FK506/BBD/CnB exchange complexes*[a]

C-atom	$(FKBP/FK506)_f$ $\Delta\nu_{1/2}$[b]	FKBP/FK506/ CnB/BBD $\Delta\nu_{1/2}$	$\Delta\Delta\nu_{1/2}$	NOEs[c]
C12′[d]	45	51	6	–
C12″	53	61	8	–
C13	37	49	12	–
C18′[d]	43	54	11	–
C18″	41	55	14	+
C20	57	75	18	–
C21	35	45	10	+
C24	32	41	9	–
C28	37	43	6	–
C29	45	50	5	+
C30	41	51	10	+
13-OCH_3	24	35	11	+
15-OCH_3	26	39	13	+
17-CH_3	28	33	5	+
25-CH_3	37	47	10	+
27-CH_3	26	32	6	+

[a] All data were collected on a Bruker DMX-500 spectrometer at 303 K using 0.3 mM 1:1 binary complex and 0.3 mM 1:1:1:1 quaternary complex in 50 mM Tris/D_2O, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM β-mercaptoethanol at pD = 8.2. ^{13}C-labeled FK506 was prepared as described previously (Lepre et al., 1992).

[b] Linewidths are presented in Hz and determined to ±0.7 Hz uncertainty.

[c] Observation of transfer NOE cross peaks in the 2D (^{13}C, ^1H) HSQC-NOESY spectrum (τ_m = 60 ms) of the quaternary complex is indicated in the last column (+ indicates peaks observed in the ^1H dimension (ω_2) at the ^{13}C shift of the listed carbon).

[d] Prime and double prime indicate non-degenerate protons attached to the same carbon.

thus likely to interact with calcineurin. The present results can also be rationalized by comparison with the recently determined X-ray structure of the quaternary complex of FKBP-12/FK506 with active, truncated bovine calcineurin (Griffith et al., 1995). This structure reveals that the sites in the C13–C21 region that undergo significant broadening are in close proximity to the aromatic groups of B binding helix residues Trp 352 and Phe 356. Since previous ^{13}C relaxation studies (Lepre et al., 1993) indicate all macrocycle backbone CH sites are rigidly fixed in the binary FKBP-12/FK506 complex, and the FKBP-12/FK506 binary "ligand" structure retains its conformation in the calcineurin bound X-ray structure, it appears the differential line-broadening effects observed for the macrocycle backbone are more likely due to close packing interactions with the receptor, in agreement with our previously proposed model (Cheng et al., 1994), rather than conformational changes induced in the bound state. On the other hand, this distinction cannot be made for the "northwest corner" of FK506 (cyclohexyl group and linking olefin), since our previous work (Lepre et al., 1993) indicates the cyclohexyl group is flexible in the binary complex. Examination of the X-ray structure (Griffith et al., 1995) suggests a possible explanation. In this structure, the cyclohexyl lacks close contacts with Cn, but the side chain of His 87 of FKBP-12 is shifted ~2.8 Å closer to the cyclohexyl, and the centroid of the FK506 cyclohexyl shifts approximately 1.7 Å from its position in the binary complex. These observations suggest that line-broadening changes at 30-CH_2 and in the C27–C29 region are due more to conformational changes in FKBP-12 and in the "northwest corner" of FK506, than to direct interactions with, or ligand conformational changes directly induced by, calcineurin binding. Line broadening at 25-CH_3 may also be due to conformational changes in FKBP-12, since in the quaternary complex the side chain of Phe 46 of FKBP-12 is closer to this group than in the binary complex. Regardless of whether calcineurin interacts with the cyclohexyl directly, or indirectly through FKBP-12, such interactions are unlikely to be important since a wide variety of substitutions can be made in this region without significantly reducing immunosuppressive activity (Goulet et al., 1994).

Unexpectedly, no line broadening was observed for the allyl resonances of FK506 in the quaternary complex, although initial NMR titrations of unlabeled FKBP-12/FK506 with the BBD/CnB complex exhibited a diminishing of fine structure due to broadening of the ^1H allyl resonances at C36 and C37 (5.80 and 5.02 ppm, respectively (Fig. 4). This fine structure is not detectable in the HSQC spectrum of even the binary FKBP-12/^{13}C-FK506 complex due to ^{13}C relaxation effects on the attached proton. The small magnitude of the observed differential line broadening is surprising, since previous chemical modification studies indicated that truncations of the allyl group in the FK506 analogs FK520 and FK523 correlate with decreased calcineurin inhibition (Liu et al., 1992), implying this region of the drug is important for calcineurin recognition. To examine the allyl group behavior in more detail, ^{13}C longitudinal relaxation rates (R_1) were measured for resonances C26 and C36 in the binary and quaternary complexes.[2] For C26, relaxation rates were the same within error limits (2.4 ± 0.2 s^{-1}) in both the binary and quaternary complexes. C26, which is involved in binding to FKBP-12, was found to be very rigid in the binary complex (Lepre et al., 1993), and was expected to remain rigid in the quaternary complex. However, for the allyl group C36 site, a significantly slower relaxation rate was observed for the binary complex (0.74 ± 0.04 s^{-1}) than for the quaternary complex (2.16 ± 0.01 s^{-1}), suggesting that mobility at the C36 site, which is highly disordered and solvent-exposed in the binary complex (Lepre et al., 1993), is attenuated in the quaternary complex and interacts with calcineurin. The lack of significant line broadening for the allyl resonances must arise due to a near degeneracy of the bound and free chemical shifts, since ^{13}C relaxation experiments clearly indicate interaction with calcineurin.

Transfer NOE peaks between FK506 and non-FK506 resonances, which appear in the [^1H, ^{13}C] HSQC-NOESY spectrum of the quaternary complex but not in the spectrum of the binary complex, provide additional evidence of transient contacts between FK506 and calcineurin. Ligand protons involved in the contacts are indicated in Table 1. In comparison with the X-ray structure of the quaternary complex (Griffith et al., 1995), the contacts observed in the C27–C30 region most likely arise due to contacts with the side chains of His 87 and Tyr 82 of FKBP-12. Most of the resonances in C13–C21 region and the 37-CH_2 allyl resonance show NOE connectivities with aromatic side chains, and probably

[2] A 1D version of the proton detected heteronuclear inversion-recovery experiment (Nirmala & Wagner, 1988) was used to collect data for 13 delays ranging from 10 ms to 10 s. Relaxation rates were determined by non-linear least-squares fitting of the experimental peak intensities to a single three-term exponential. Only protons attached to the C36 and C26 carbons were examined, since these were the only isolated CH resonances in the ^{13}C edited 1D spectrum of FKBP-12/^{13}C-FK506.

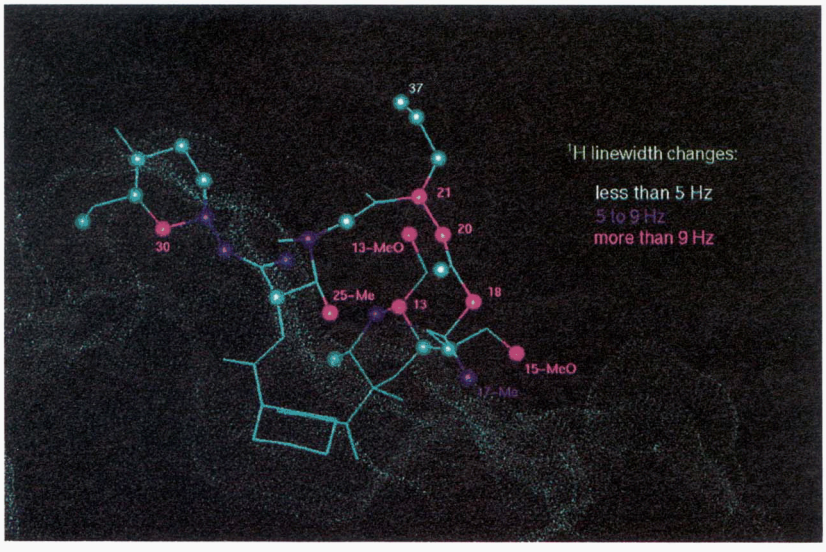

Fig. 3. Structure of FK506 in the FKBP-12 binding pocket. ^{13}C-^{1}H sites where observed line broadening is less than 5 Hz (cyan), 5 Hz to 9 Hz (purple), or greater than 9 Hz (magenta) are shown as colored spheres.

result from contacts with the aromatic groups of B binding helix residues Trp 352 and Phe 356. Unlike the line-broadening data, the complete set of transfer NOEs observed cannot be rationalized with the current crystallographic model, suggesting that the mode of interaction of FKBP-12/FK506 is different in the weaker affinity NMR complex than in the nanomolar crystallographic complex. These differences in binding mode are not surprising, given the severely truncated nature of the NMR complex (Fig. 2). For example, the conformation of the N-terminal residues of the BBD, which in the nanomolar crystallographic complex form a contiguous region that interacts with the catalytic domain of CnA, may be slightly altered in the NMR complex due to loss of these inter domain interactions (Fig. 2). Conformational differences in the BBD in the weakly interacting truncated complex, which result in a shift of sidechain positions relative to FKBP-12/FK506, could explain why contacts predicted for the C13–C21 region of FK506 from the X-ray structure are not observed in the truncated complex by NMR.

In conclusion, replacing the catalytic A subunit of calcineurin with the BBD peptide successfully converts the 93 kDa, tightly bound quaternary complex into a lower affinity, rapidly exchanging system suitable for NMR study. NMR differential line-broadening results are consistent with the X-ray structure of the quaternary complex, but transferred NOE data appear ambiguous, and suggest an alternative binding conformation in the rapidly exchanging NMR complex. The present work illustrates the utility

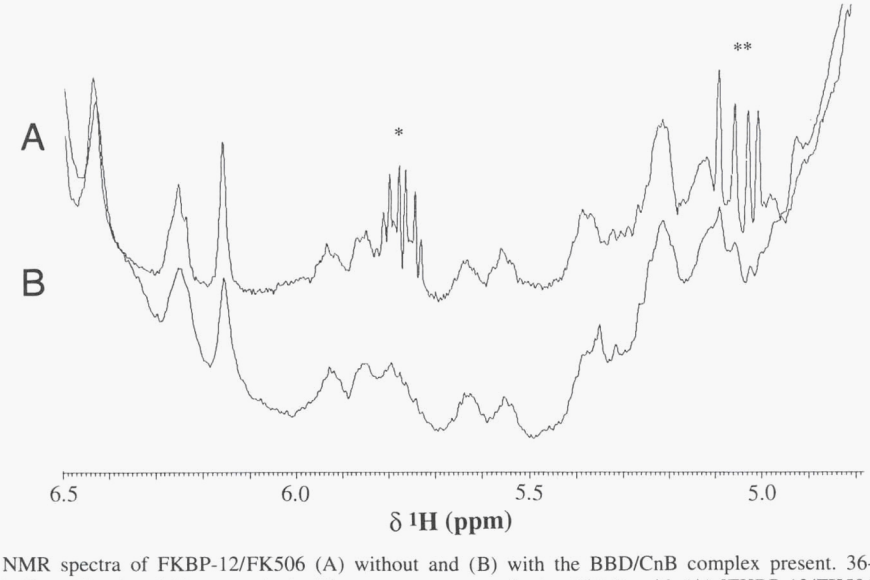

Fig. 4. Proton NMR spectra of FKBP-12/FK506 (A) without and (B) with the BBD/CnB complex present. 36-CH and 37-CH_2 resonances are indicated by * and **, respectively. The spectra were acquired at 303 K, with (**A**) [FKBP-12/FK506] = 0.8 mM and (**B**) [FKBP-12/FK506] = 0.3 mM, [BBD/CnB] = 0.3 mM.

of isotope edited transferred NOE and differential line-broadening measurements as an attractive and general NMR method for identifying and characterizing protein–protein or protein–ligand interactions in larger molecular weight systems. Although in the present study we are able to validate our results by comparison with an X-ray crystal structure, the methods will be most useful for obtaining rapid qualitative information regarding systems for which high resolution structural information is not available.

Acknowledgments: ^{13}C-FK506 was produced by H. Kuboniwa and T. Matsura of Chugai Pharmaceuticals, Inc. We are indebted to S. Harbeson for synthesis of the BBD peptide, J. Black and M. Fleming for assistance in preparing the quaternary complex, S. Chambers for protein expression, P. Connelly for help with fluorescence titrations, and M. DeCenzo for performing the calcineurin competition assay. We also thank Feng Ni of the Biotechnology Research Institute, National Research Council Canada, for software used in simulation of exchange lineshapes, and the Vertex crystallography group, particularly J. Griffith and M. Navia for helpful discussions.

References

Aldape RA, Futer O, DeCenzo MT, Jarrett BP, Murcko MA, Livingston DJ. 1992. Charged surface residues of FKBP12 participate in formation of the FKBP12-FK506-calcineurin complex. *J Biol Chem 267*:16029–16032.

Bodenhausen G, Ruben D. 1980. Natural abundance nitrogen-15 NMR by enhanced heteronuclear spectroscopy. *Chem Phys Lett 69*:185–189.

Cheng, JW, Lepre CA, Moore JM. 1994. ^{15}N NMR relaxation studies of the FK506 binding protein: Dynamic effects of ligand binding and implications for calcineurin recognition. *Biochemistry 33*:4093–4100.

Clipstone NA, Fiorentino DF, Crabtree GR. 1994. Molecular analysis of the interaction of calcineurin with drug-immunophilin complexes. *J Biol Chem 269*:26431–26437.

Goulet MT, Rupprecht KM, Sinclair PJ, Wyvratt MJ, Parsons, WH. 1994. The medicinal chemistry of FK-506. *Perspect Drug Discovery Design 2*:145–162.

Griffith JP, Kim JL, Kim EE, Sintchak MD, Thomson JA, Fitzgibbon MJ, Fleming MA, Caron PR, Hsiao K, Navia MA. 1995. X-ray structure of calcineurin inhibited by the immunophilin-immunosuppressant FKBP12-FK506 complex. *Cell 82*:507–522.

Harding MW, Galat A, Uehling DE, Schreiber SL. 1989. A receptor for the immunosuppressant FK506 is a cis-trans peptidyl-prolyl isomerase. *Nature 341*:758–760.

Kawamura A, Su MS-S. 1995. Interaction of FKBP12-FK506 with calcineurin A at the B subunit-binding domain. *J Biol Chem 270*:15463–15466.

Lepre CA, Cheng J-W, Moore, JM. 1993. Dynamics of a receptor-bound ligand by heteronuclear NMR: FK506 bound to FKBP-12. *J Am Chem Soc 115*:4929–4930.

Lepre CA, Thomson JA, Moore JM. 1992. Solution structure of FK506 bound to FKBP-12. *FEBS Lett 302*:89–96.

Liu J, Albers MW, Wandless TJ, Luan S, Alberg DG, Belshaw PJ, Cohen P, MacKintosh C, Klee CB, Schreiber SL. 1992. Inhibition of T cell signaling by immunophilin-ligand complexes correlates with loss of calcineurin phosphatase activity. *Biochemistry 31*:3896–3901.

Liu J, Farmer JD Jr, Lane WS, Friedman J, Weissman I, Schreiber SL. 1991. Calcineurin is a common target of cyclophilin-cyclosporin A and FKBP-FK506 complexes. *Cell 66*:807–815.

London RE 1993. Chemical-shift and linewidth characteristics of reversibly bound ligands. *J Magn Reson A 104*:190–196.

Ni F. 1994. Recent developments in transferred NOE methods. *Prog NMR Spectrosc 26*:517–606.

Nirmala NR, Wagner G. 1988. Measurement of ^{13}C relaxation times in proteins by two-dimensional heteronuclear ^{1}H-^{13}C correlation spectroscopy. *J Am Chem Soc 110*:7557–7558.

Watanabe V, Perrino BA, Chang BH, Soderling TR. 1995. Identification in the calcineurin A subunit of the domain that binds the regulatory B subunit. *J Biol Chem 270*:456–460.