A Composite FKBP12-FK506 Surface That Contacts Calcineurin

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Complexes of the immunosuppressant FK506 with its immunophilin protein receptor, FKBP12, block immune receptor-mediated signal transduction pathways by binding to and inhibiting the protein phosphatase calcineurin (CN).[1,2] Although structures of the ligated and unligated forms of FKBP12 have been determined,[3-5] the structural mechanism by which the natural product mediates formation of a receptor-ligand-receptor complex remains unclear. We have previously identified regions of FK506 that are important in contacting CN in the ternary complex.[6] We now report the identification by site-directed mutagenesis of residues on FKBP12 that are important for binding to CN. The results suggest that elements of both FK506 and FKBP12 are in contact with CN and identify the CN binding groove created by the composite FKBP12-FK506 surface.

The mutagenesis experiments were guided by comparisons between high-resolution structures of free[3] and FK506-bound[4] FKBP12 and by comparisons between the amino acid sequences of FKBP12[7] and the homologous, yet functionally distinct immunophilin, FKBP13.[8] The unligated solution structure of FKBP12 contains two loops, comprising residues 40-44 and 84-91 (termed the 40s loop and the 80s loop, respectively), that surround the ligand binding site and are poorly defined by the NMR data. In the crystal structure of the FKBP12-FK506 complex, however, both loops are well defined by the electron density.[9] Although the amino acid sequences of FKBP12 and FKBP13 are highly similar, the sequences are significantly different in both the 40s and 80s loops. The observation that the FKBP13-FK506 complex is an extremely weak inhibitor of CN[10] further suggests that these regions might be important in contacting CN.

These comparisons of structure and amino acid sequence led us to a mutagenesis strategy based on the construction of FKBP12-FKBP13 chimeras. These proteins consist of small segments of sequence from FKBP13 grafted into the analogous positions of FKBP12. Chimera **1** contains FKBP13 residues LPQNQ in place of RDRNK, and chimera **2** contains residues ERGAPPK in place of ATGHPGI (Figure 1). While both chimeric proteins are active rotamases and bind FK506 with high affinity, only chimera **1** is a potent inhibitor of CN (Table I). Surprisingly, however, the R42Q and R42A single-site mutants also show diminished CN binding (note that an R42Q mutation exists in chimera **1**).[11,12] These results indicate that the role of the amino acid at position 42 depends strongly on the residues surrounding it. In the context of the FKBP12 40s loop, residue 42 must be arginine; in the context of the FKBP13 40s loop, it can be glutamine. The fact that two very different sequences in the 40s loop are both able to support CN-inhibitory activity indicates that the exact positioning and nature of the amino acids in this region are not critical for interaction with CN. Rather, we propose that R42 may play an indirect role in orienting nearby regions of the FKBP12-FK506 complex and that these regions make direct contacts to CN.

In order to identify the individual residues in the 80s loop that are important for CN binding, several single and double FKBP12 mutants were prepared. Of the three mutants A84E/T85R, H87A, and G89P/I90K, only the third showed a significant decrease in CN-inhibitory activity. The single-point mutants G89P and I90K were also both found to be poor inhibitors of CN when complexed to FK506. The reduction in activity of the first of these may be due to the different conformational properties of glycine and proline.[14] However, the substitution of lysine for isoleucine

	40s Loop					80s Loop							
	40	41	42	43	44	84	85	86	87	88	89	90	91
FKBP12	R	D	R	N	K	A	T	G	H	P	G	I	I
FKBP13	L	P	Q	N	Q	E	R	G	A	P	P	K	I

Figure 1. Amino acid sequences of FKBP12 and FKBP13 in the 40s and 80s loops.

Table I. Biochemical Properties of FKBP Mutants[a]

protein	rotamase activity[b] (10 °C) k_{cat} K_M^{-1} ($\times 10^6$ M^{-1} s^{-1})	K_i (nM) FK506[b]	K_i (nM) calcineurin[c]
FKBP12 (wt)	2.2 ± 0.2	0.4 ± 0.2	7.9 ± 3.0
FKBP13 (wt)	1.5 ± 0.3	55 ± 5	1500 ± 400
chimera **1** (40s loop exchange)	0.57 ± 0.05	0.4 ± 0.2	19 ± 2
chimera **2** (80s loop exchange)	4.2 ± 0.4	2.1 ± 0.3	580 ± 120
R40A	1.2 ± 0.4	0.1 ± 0.1	8.1 ± 2.8
R42A	1.1 ± 0.2	0.2 ± 0.1	280 ± 80
R42Q	1.3 ± 0.3	1.7 ± 0.6	850 ± 250
K44A	1.4 ± 0.2	0.1 ± 0.1	1.0 ± 0.2
K35I	1.6 ± 0.2	0.6 ± 0.2	7.8 ± 2.2
Q53A	1.8 ± 0.3	0.2 ± 0.1	5.2 ± 0.5
A84E/T85R	2.2 ± 0.2	0.6 ± 0.2	8.7 ± 1.1
G89P/I90K	1.8 ± 0.2	0.6 ± 0.2	>5000
P88V	1.5 ± 0.2	1.1 ± 0.3	16 ± 6
G89P	1.5 ± 0.3	2.7 ± 0.8	87 ± 29
I90K	3.2 ± 0.3	0.1 ± 0.1	660 ± 60
H87A	1.9 ± 0.2	1.5 ± 0.2	3.1 ± 1.8

[a] Mutagenesis was performed using the megaprimer method.[15] Nucleotide sequencing of all constructs was performed by the dideoxynucleotide method.[16] Proteins were expressed in *Escherichia coli* strain XA-90 as glutathione S-transferase fusion proteins in vector pGEX-3X (Pharmacia). The fusion proteins were purified using a glutathione affinity matrix (Pharmacia) following the manufacturer's protocol and cleaved with factor Xa protease (New England Biolabs). The cleaved FKBPs were purified to homogeneity using anion exchange chromatography. [b] See ref 17. K_i for FK506 refers to inhibition of FKBP12 (mutant) rotamase activity by FK506. [c] See refs 1 and 6. K_i for calcineurin refers to inhibition of calcineurin phosphatase activity by FKBP12(mutant)-FK506 complexes. Calcineurin and calmodulin were purchased from Sigma. Reported K_i values are the average of at least three assays.

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(11) Further evidence that the LPQNQ 40s loop (that of FKBP13) can support CN inhibition comes from studies of reverse chimeras, where residues of FKBP12 are grafted into FKBP13 in an attempt to induce inhibitory activity. See: Rosen, M. K.; et al. *J. Am. Chem. Soc.*, following communication in this issue.
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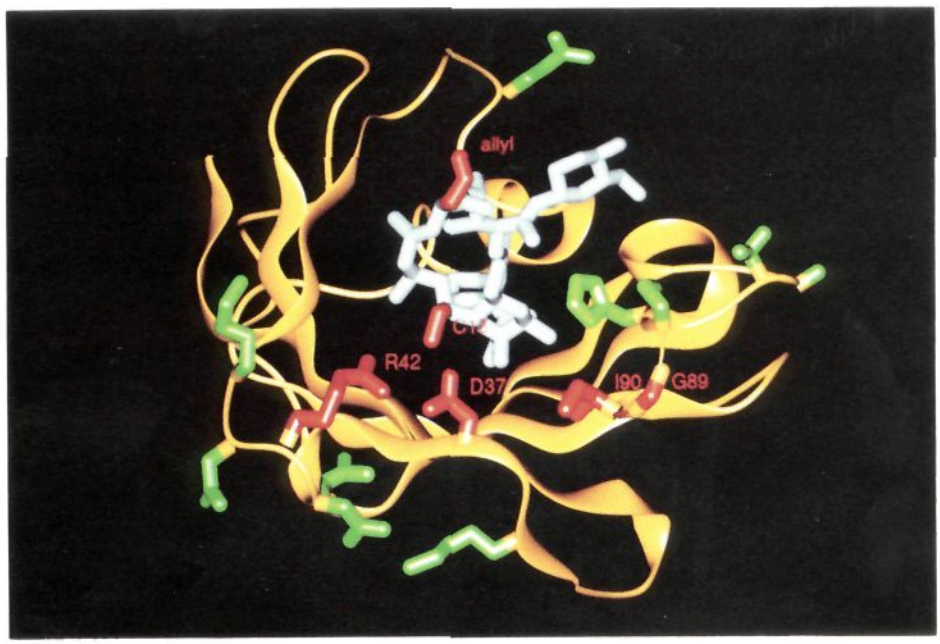

Figure 2. Structure of the FKBP12–FK506 complex showing side chains of residues mutated in this study. The side chain of aspartic acid 37 has also been included.[13] Side chains whose mutation does not significantly alter the calcineurin inhibitory activity of the immunophilin–ligand complex are green. Residues whose mutation results in significantly reduced calcineurin inhibition are red. Bound FK506 is light blue, with substituents that contact CN[6] in red.

in the second mutant should not have any obvious effect on the backbone conformation of the protein. The poor inhibition of this mutant and the location of the side chain of I90 in a hydrophobic depression near the C15 methoxyl group of bound FK506 (a major determinant of CN inhibition[6]) then suggest that I90 may contact CN in the ternary complex.

The mutagenesis experiments are summarized graphically in Figure 2. It is clear that the regions of the protein whose mutation most strongly affects CN inhibition, G89, I90, and R42, are proximal to two regions of FK506 (the C15 methoxyl and the C21 allyl groups) previously shown to be critical for CN binding.[6] Taken together, these moieties define a contiguous, largely hydrophobic region on the surface of the structure. We therefore surmise that this composite surface constitutes the CN binding site on the FKBP12–FK506 complex.

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