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Activation of an Inactive Immunophilin by Mutagenesis

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Table I. Biochemical Properties of FKBP13 Mutants[a]

protein	rotamase activity (10 °C) $k_{cat}\ K_M^{-1}$ ($\times 10^6$ M^{-1} s^{-1})	K_i (nM) FK506	K_i (nM) calcineurin
FKBP12 (wt)	2.2 ± 0.2	0.4 ± 0.2	7.9 ± 3.0
FKBP13 (wt)	1.5 ± 0.3	55 ± 5	1500 ± 400
reverse chimera **1** (80s loop)	0.63 ± 0.06	35 ± 5	27 ± 5
P89G/K90I	1.2 ± 0.2	44 ± 8	13 ± 1

[a] Construction and purification of proteins, as well as the performance of all assays, were as described in ref 9, except that FKBP13 mutants were expressed in the vector pGEX-1N.[8]

Signal transduction in cells is regulated by specific interactions between proteins. We have recently demonstrated that the complex of the immunophilin FKBP12 (12 kDa FK506 binding protein) with the natural product FK506 binds to and inhibits the protein phosphatase calcineurin (CN).[1] In T cells, this inhibition prevents the translocation of the cytoplasmic subunit of the transcription factor NF-AT into the nucleus, blocking the signal transduction pathway emanating from the T-cell receptor.[2] Although a number of FKBPs have now been identified,[3] including 13,[4] 25,[5] and 59[6] kDa family members, a variety of data suggest that the effects of FK506 in several cell lines are mediated by FKBP12.[7] Further, biochemical evidence indicates that complexes of the different FKBPs with FK506 have differing abilities to inhibit CN.[8] We now report insights into the structural basis for the specificity of interaction between the FKBP12–FK506 complex and CN gained through the construction of FKBP12–FKBP13 chimeric proteins. These studies demonstrate the use of site-directed mutagenesis not only to abrogate protein function but also to add new, potent activity to inactive proteins as well.

In the preceding communication,[9] we presented data indicating that two loops in FKBP12, composed of residues 40–44 and 84–91, are important in mediating interactions between the FKBP12–FK506 complex and CN. In addition to the construction of inactive FKBP12 mutants, it was also of interest to construct active FKBP13 mutants. In particular, we hoped to find the minimal number of amino acid substitutions necessary to impart CN-inhibitory activity to the FKBP13–FK506 complex. Following a strategy similar to that reported previously,[9] we began by constructing mutant reverse chimera **1**, which contains the FKBP12 sequence from residues 83–90 grafted into the FKBP13 framework (all residues are numbered according to their positions in the FKBP12 sequence). This molecule is approximately a 55-fold better inhibitor than FKBP13, with activity only 3-fold poorer than native FKBP12 (Table I). Data on FKBP12 mutants[9] modified in this region led us to believe that the amino acids at positions 89 and 90 might be the most critical determinants for CN binding. Accordingly, we constructed the FKBP13 double mutant, P89G/K90I. This molecule has inhibitory activity nearly identical to that of FKBP12. These data further support our earlier conclusion that the different FKBP12 and FKBP13 sequences in the region 40–44 are both fully capable of mediating CN inhibition. Thus, it appears that a difference of only two residues is responsible for the large difference in activity between these two immunophilins.

A possible molecular basis for some of these effects can be inferred from the structure of the FKBP12–FK506 complex.[10] As suggested in the preceding communication,[9] the side chain of I90 may contact CN directly in the ternary complex; its substitution by lysine in FKBP13 could disrupt hydrophobic immunophilin–phosphatase contacts. Mutagenesis of residues 87 and 89 suggests that the backbone conformation of the loop from H87 to I91 is also important for proper CN recognition. A mutation that introduces β-branching at residue 87 (H87V) causes a 4-fold decrease in CN-inhibitory activity,[11] while mutations that do not introduce β-branching (H87F, H87L,[11] and H87A[9]) have wild type activity. The energetically most favorable conformation of valine ($\chi_1 = 180°$, which minimizes gauche interactions between the methyl groups and the peptide backbone and is highly favored in statistical analyses of high-resolution protein crystal structures[12]) would place the V87 pro-S methyl group in close contact (~2.5 Å) with the aromatic ring of Y82. As the phenolic hydroxyl group of Y82 makes a hydrogen bond to FK506, it is likely that strain would be relieved by distortion of the 80s loop. The loop would also be distorted by mutation of G89, since this residue has a positive ϕ angle in the FKBP12–FK506 structure. Positive ϕ angles are rarely observed in high-resolution protein structures for amino acids other than glycine,[13] presumably due to steric interactions between the side chain of the residue in question and the carbonyl oxygen of the preceding residue in the polypeptide chain (see Figure 1). Thus, amino acids other than glycine at position 89 would most likely assume a different conformation. As both the H87V and G89P mutants show decreased CN inhibition, we conclude that the conformation of this loop is important in formation of the ternary complex.

Among the known FKBPs, only FKBP12 can bind with high affinity to CN and mediate the actions of FK506 in T cells.[8,14] These unique properties correlate with the important role played by G89 and I90 in FKBP12, which are replaced by different residues in all other mammalian FKBPs characterized to date. The current studies suggest that specific mutations can alter the potential biological actions of these immunophilins.

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(14) Reference 8 describes investigations of FKBP12, FKBP13, and FKBP25. Although FKBP59 has not yet been examined, we predict that its FK506 complex should not bind to CN since, like FKBP13, it has proline and lysine at positions 89 and 90.

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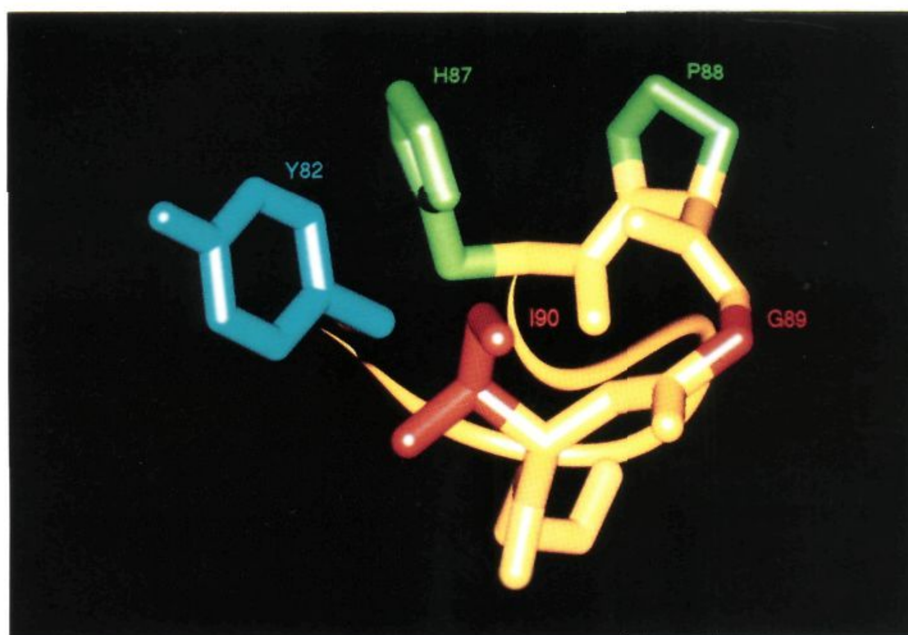

Figure 1. 80s loop of the FKBP12–FK506 complex. The side chains of residues 82 and 87–90 are shown. Residue coloring is as follows: H87, P88 green; G89, I90 red; Y82 blue.

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