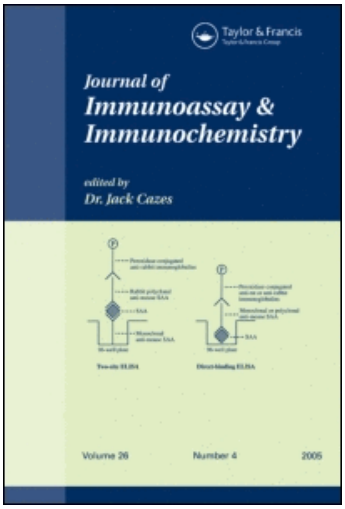


Journal of Immunoassay and Immunochemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597271

A Soluble Binding Assay for Measuring ^{3}H-FK506 Binding to the HSP56 Immunophilin

K. L. Leach[ab]; V. A. Ruff[a]; A. W. Yem[c]; M. R. Deibel Jr.[c]
[a] Department of Cell Biology, The Upjohn Company, Kalamazoo, Mi [b] Karen Leach Department of Cell Biology, The Upjohn Company, St. Kalamazoo, MI 49007 [c] Department of Biochemistry, The Upjohn Company, Kalamazoo, Mi

To cite this Article Leach, K. L. , Ruff, V. A. , Yem, A. W. and Deibel Jr., M. R.(1994) 'A Soluble Binding Assay for Measuring ^{3}H-FK506 Binding to the HSP56 Immunophilin', Journal of Immunoassay and Immunochemistry, 15: 4, 339 — 355
To link to this Article: DOI: 10.1080/15321819408009582
URL: http://dx.doi.org/10.1080/15321819408009582

JOURNAL OF IMMUNOASSAY, 15(4), 339-355 (1994)

A SOLUBLE BINDING ASSAY FOR MEASURING ^{3}H-FK506 BINDING TO THE HSP56 IMMUNOPHILIN.

Karen L. Leach*‡, Valerie A. Ruff‡,
Anthony W. Yem§, and Martin R. Deibel, Jr.§

‡Department of Cell Biology and §Department of Biochemistry
The Upjohn Company, Kalamazoo, Mi 49007

ABSTRACT

Heat shock protein 56 (hsp56) was previously identified as an immunophilin based on its ability to specifically bind to FK506-Affi-Gel 10. In this report, we have quantitated human Jurkat T cell hsp56 binding to ^{3}H-FK506, as well as to the immunosuppressant rapamycin. Binding was measured utilizing immunoadsorbed hsp56, and, in addition, we demonstrate that ^{3}H-FK506 binds to hsp56 in solution. Hsp56 bound to an antibody-Sepharose column binds ^{3}H-FK506 with an affinity of 19.4 ± 4.6 nM, as compared to 23.2 ± 6.8 nM for soluble hsp56. In competition experiments, the apparent affinity constant for rapamycin was 11.6 ± 2.8 nM, using immobilized hsp56, and 17.3 ± 7.7 nM, using the soluble hsp56 preparation. These results demonstrate that hsp56 binds FK506 and rapamycin with similar affinities, and suggest that hsp56 may play a role in mediating the cellular function of both of these drugs.

* To whom correspondence should be addressed:
Karen Leach
Department of Cell Biology
The Upjohn Company
301 Henrietta St.
Kalamazoo, MI 49007
(616) 385-5390
(616) 384-9308 (FAX)

(KEYWORDS: Immunosuppression, Rapamycin, FK506-binding protein, Heat shock)

INTRODUCTION

FK506 is a potent immunosuppressant drug that inhibits early steps in T cell activation in a calcium-dependent manner (reviewed in Reference 1). In T cells, FK506 binds to an immunophilin, called FKBP-12. Using FK506 affinity chromatography, additional FK506-binding proteins have been isolated, including FKBP-13 and FKBP-25 (2,3). The FK506-FKBP-12 complex binds to and inhibits the activity of calcineurin, a calcium and calmodulin-dependent serine/threonine phosphatase (4). Overexpression of calcineurin in human Jurkat T cells makes the cells more resistant to the effects of FK506, and the immunosuppressive activity of a number of FK506 analogs correlates with their ability to inhibit calcineurin activity (5,6,7). These results have led to the hypothesis that calcineurin is the primary cellular target for the FK506-FKBP-12 complex.

The most recent FK506 and rapamycin binding protein to be identified is a 60 kDa protein (8,9). Purification and N-terminal sequence analysis showed identity with a known 56 kDa heat shock protein, hsp56 (8). This protein was originally shown to interact with the 90 kDa heat shock protein in progesterone, estrogen, and glucocorticoid receptor complexes (10). In the case of the glucocorticoid receptor, hsp56 has been shown to be part of the heterocomplex

composed of glucocorticoid receptor, hsp90 and hsp70 (11,12). Following the demonstration that this protein is also an immunophilin, it has been referred to as FKBP59 (13), HBI (14), FKBP51 (15) and FKBP52 (16), as well as p56-59 (17,18). As in our previous work (8,19), we will refer to it as hsp56.

The genes for the human and rabbit hsp56 have been cloned, and they encode proteins of approximately 52 kDa that, like FKBP-12, have peptidyl-prolyl isomerase activity, which is inhibited by FK506 (16,20). The protein also contains two domains in its N-terminus (Domain I: residues 31-135; Domain II: residues 145-253) that share sequence homology to the FK506 binding domain of FKBP-12 (13,21). The homology of Domain I (54%) to the FKBP-12 sequence is higher than that of Domain II (26%). Several putative phosphorylation sites have also been identified by sequence analysis, suggesting that hsp56 function may be modulated by post-translational modification (16).

Although hsp56 binds FK506, its exact role, if any, in mediating the immunosuppressive effects of FK506 is not presently known. It does not bind to, or inhibit, calcineurin *in vitro* (15). Rapamycin can displace hsp56 binding to an FK506 affinity matrix (13), although the relative affinity of rapamycin for hsp56 has not been measured directly in binding assays.

A separate, but indirect method for determining the binding constant for FK506 and rapamycin has been applied based on the

knowledge that hsp56 is a rotamase, an enzyme which catalyzes the isomerization of cis and trans amide-bond rotamers of peptide and protein substrates. FK506 and rapamycin inhibit the rotamase activity of hsp56, and using this method, two groups have reported affinities of FK506 for hsp56. Wiederrecht et al. (15) estimated a K_D of 116 nM, while Peattie et al. (16) reported a K_D of 10 nM. These values are quite disparate, and may reflect differential inactivation of the enzymatic activity of hsp56 during purification. In addition, the complexity of the rotamase assay may be a contributing factor. Thus far, a binding constant of drug for hsp56, measured directly in a soluble binding assay, has not been reported.

Understanding the role of hsp56 as an immunophilin and as part of steroid receptor complexes is an important biological question to address. Previously, we generated a polyclonal antibody specific for hsp56, and used it to investigate the expression of hsp56 in tissues, and its localization within cells (19). In this paper, we have measured binding using an immobilized antibody method. Further we have developed a method for measuring binding of hsp56 directly to ^{3}H-FK506 in solution. Using these assays we demonstrate that hsp56 binds FK506 and rapamycin with similar affinities.

MATERIALS AND METHODS

Materials - FK506 and rapamycin were prepared at The Upjohn Company from fermentation broths of **Streptomyces tsukubaenis**

(Upjohn Culture Collection 11062) and **Streptomyces hygroscopicus** (Upjohn Collection 5931), respectively. ^{3}H-dihydroFK506 (^{3}H-FK506) (49.1 Ci/mmol) was custom synthesized at Amersham. Polyethylene glycol 8000 and bovine gamma globulin (fraction II) were purchased from Sigma. The antibody to FKBP-12 was as described in Ruff et al., (22).

Cell Culture - Human Jurkat T cells were grown as described (8) in RPMI 1640 medium containing 10% fetal calf serum.

Binding Assays - *Antibody-Sepharose Method*: The IgG fraction was purified from UPJ56 serum (19), using Protein A Sepharose, and coupled to Sepharose CL via CNBr activation (Sigma Chemical Co.). The final preparation of immobilized IgG was at a density of 3.5 mg per ml of Sepharose CL. Jurkat cells were washed twice in PBS, resuspended in 10 mM 4-(2-hydroxyethyl)-1-piperzineethanesulfonic acid (HEPES), pH 7.5, containing 10% glycerol (1×10^{8} cells/ml) and lysed by sonication, followed by centrifugation at 100,000 x g for 60 min. Aliquots (25 µl) of the supernatant were incubated with 100 µl of packed UPJ56-Sepharose for 2-3 hr in silanized microfuge tubes (Integrated Separation Systems, Siliconized SepraTubes #SA104011), followed by three washes with 10 mM HEPES, containing 0.5 M KCl, and two washes with 10 mM HEPES, pH 7.5. The washed Sepharose was

incubated with various concentrations of ^{3}H-FK506, in the presence or absence of 200-fold molar excess cold FK506 in a total volume of 200 µl. ^{3}H-FK506 stock solution was dried down in a silanized tube, and dissolved in 10 mM HEPES, pH 7.5, containing 0.02% Triton X-100. Various concentrations of ^{3}H-FK506 were obtained by further dilutions of the stock solution in 10 mM HEPES/0.02% Triton X-100. The use of the silanized tubes in the binding assay and the Triton-containing buffer were essential for maintaining FK506 in solution. Following incubation with ^{3}H-FK506 for 12-16 hr at 4 °C, the Sepharose was spun at 12,000 x g for 3 min, an aliquot of the supernatant was removed to quantitate free ^{3}H-FK506, and the pellet was washed three times with 10 mM HEPES, pH 7.5, containing 0.1 M KCl. The tip of the tube containing the pellet was cut off and counted to quantitate the bound ^{3}H-FK506. Results were analyzed using a nonlinear least squares fit program (23), provided by F. Kezdy (The Upjohn Co.).

Soluble binding assay Hsp56 was partially purified from Jurkat cytosol using Superose-12 chromatography. The column was eluted with 50 mM Tris-Cl, pH 7.5 containing 150 mM NaCl, 5 mM $CaCl_2$, and 5 mM $MgCl_2$, at a flow rate of 1 ml/min. Fractions containing hsp56 (assayed by Western blot using the UPJ56 antibody) were combined and aliquots were incubated with various concentrations of ^{3}H-FK506 in 10 mM HEPES, pH 7.5, containing 0.02% Triton X-100, in a final

concentration of 200 µl. After incubation for 12-16 hr at 4 °C, gamma globulin (400 µg) was added, followed by polyethylene glycol (PEG) (16%$_f$, w/v) to precipitate the protein-FK506 complex. The precipitates were collected by centrifugation for 15 min at 12,000 x g, and an aliquot of the supernatant was removed to quantitate the free ^{3}H-FK506. The remaining supernatant was removed, and the pellet was washed once with PBS. The tip of the tube was cut off and counted to quantitate the bound ^{3}H-FK506.

For the rapamycin competition experiments, binding assays were carried out using 25 nM ^{3}H-FK506 in the presence of various concentrations of rapamycin for 12-16 hr at 4 °C. The remainder of the binding assay was carried out as described above. The IC_{50} values for rapamycin were corrected to the K_i values by the method of Cheng and Prusoff (24).

Western Blots - Western blots were carried out as previously described (19) using the UPJ56 and FKBP-12 antibodies. Briefly, samples were electrophoresed on 10% (hsp56) or 18% (FKBP-12) polyacrylamide gels and transferred to Nitropure (Integrated Separation Systems). Blots were blocked with 5% nonfat dry milk (10 mM Tris, pH 7.5, containing 500 mM NaCl) and then probed with hsp56 or FKBP-12 antibodies diluted in TBS containing 3% (w/v) bovine serum albumin. Immunoreactivity was detected using alkaline phosphatase-conjugated second antibody.

RESULTS AND DISCUSSION

The exact biological role of hsp56 is not known. However, as demonstrated by the purification procedure of hsp56, it specifically binds to the immunosuppressant drug FK506 (8). Binding to FK506, therefore, can be used as a functional assay of hsp56 activity, allowing investigation of potential biological modulators. Towards this end, FK506 and rapamycin binding to hsp56 was characterized using two different methods. First, hsp56 was immunoadsorbed from human T cell (Jurkat) cytosol using UPJ56, an hsp56 antibody (19), immobilized to Sepharose. After extensive washing in buffer containing 0.5 M KCl, the Sepharose was incubated with ^{3}H-FK506, and after additional washing, the bound ^{3}H-FK506 was quantitated. Scatchard analysis of the binding data demonstrated one binding site for FK506, with an affinity of 19.4 ± 4.6 nM (mean ± SEM, n=3) (Figure 1). Using a similar method for measuring binding, Tai *et al.* reported a K_D of 66 nM for ^{3}H-FK506 binding to hsp56 from human IM-9 B cells (13). These differences in affinities may be the result of the different hsp56 antibodies used in the two studies, or they may reflect intrinsic differences in T cell versus B cell hsp56.

Rapamycin binding experiments were performed using the immobilized hsp56 method (Figure 2). Although it has been reported that rapamycin can effectively elute hsp56 from an FK506 affinity matrix (13), the affinity of rapamycin for hsp56, to our knowledge, has

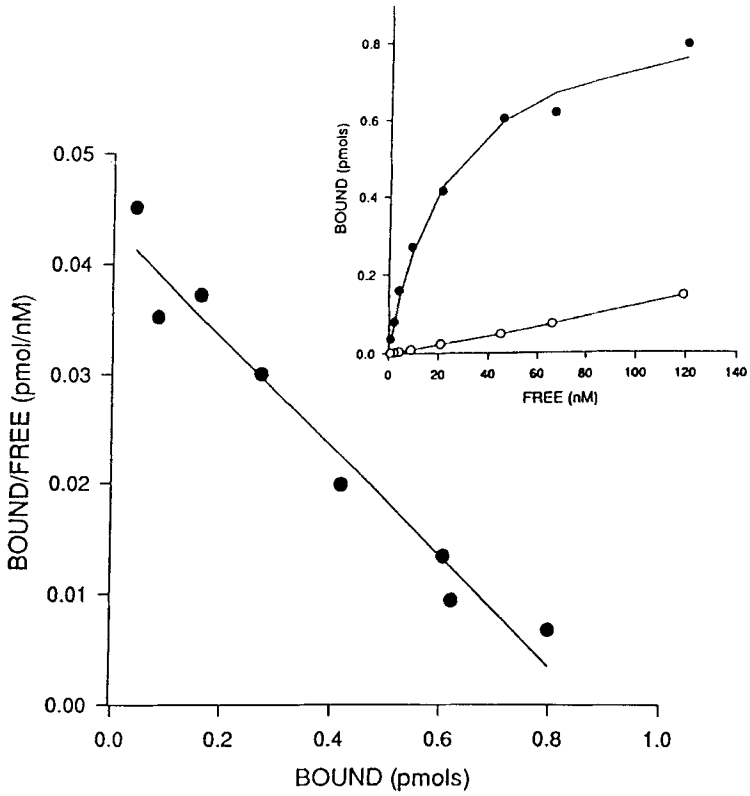


Figure 1. **Scatchard analysis of ^{3}H-FK506 binding to immobilized hsp56.** Human Jurkat lysate was immunoadsorbed to UPJ56-Sepharose, washed in HEPES buffer containing 0.5 M KCl, followed by incubation with various concentrations of ^{3}H-FK506, as described in "Materials and Methods." After additional washes, the bound ^{3}H-FK506 was quantitated. The results shown are from one of three representative experiments. The inset shows the saturation binding curve; specific (•) and non-specific (o) binding.

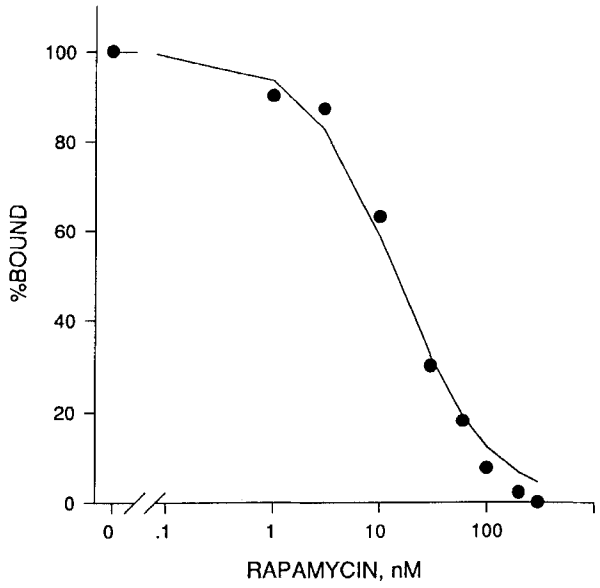


Figure 2. **Rapamycin competes with ^{3}H-FK506 for binding to immobilized hsp56.** Human Jurkat lysate was immunoadsorbed to UPJ56-Sepharose, washed in HEPES buffer containing 0.5 M KCl, followed by incubation with various concentrations of rapamycin plus 25 nM ^{3}H-FK506, as described in "Materials and Methods." After additional washes, the bound ^{3}H-FK506 was quantitated. The results shown are from one of three representative experiments.

not been directly measured in a binding assay. ^{3}H-FK506 binding to hsp56 was carried out in the presence of 0.01 - 200 nM rapamycin. The competition curve fit the theoretical curve for a competitive inhibitor, with an apparent affinity of 11.6 ± 2.8 nM (mean ± SEM). These results indicate that hsp56 binds FK506 and rapamycin with similar affinities, and may be involved in mediating the biological effects of both of these drugs. FKBP-12 also has similar affinities for FK506 and rapamycin,

while FKBP-13 and FKBP-25 bind FK506 with 10 - 150-fold lower affinity than rapamycin (25,26).

One concern about the Sepharose binding method is that immunoadsorption of hsp56 and/or interaction with the antibody may affect its ability to bind FK506. To test this directly we carried out experiments using a soluble binding assay, in which both hsp56 and ^{3}H-FK506 were in solution. Hsp56 was partially purified from Jurkat cell cytosol using gel filtration chromatography. As shown by Western blots using FKBP-12 and hsp56 antibodies, this step effectively separated hsp56 from FKBP-12, the other major FK506 binding protein in Jurkat cells (data not shown). The fractions containing hsp56 were pooled and incubated with ^{3}H-FK506. Scatchard analysis of binding performed by this method resulted in a K_D of 23.1 ± 6.8 nM (mean ± SEM) for ^{3}H-FK506 (Figure 3). A single class of binding sites was detected, similar to the immobilized hsp56 binding assay. The total amount of ^{3}H-FK506 bound was higher in the PEG assay than with the immunoabsorbed hsp56 method. The total pmol bound using the UPJ56-Sepharose method was somewhat variable, and the exact amount of hsp56 bound to the UPJ56-Sepharose that is available to bind ^{3}H-FK506 is difficult to estimate. However, quantitative ELISA assays were carried out on the hsp56 preparation used in the PEG assays, using the UPJ56 antibody. Results from these experiments indicated that the stoichiometry of ^{3}H-FK506 binding to hsp56 was 1:1 (data not shown).

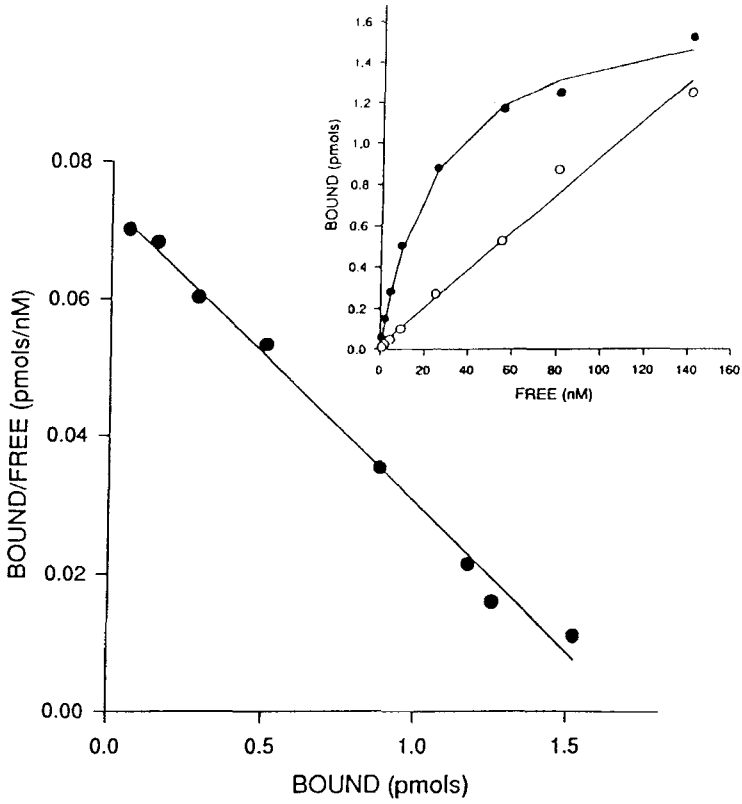
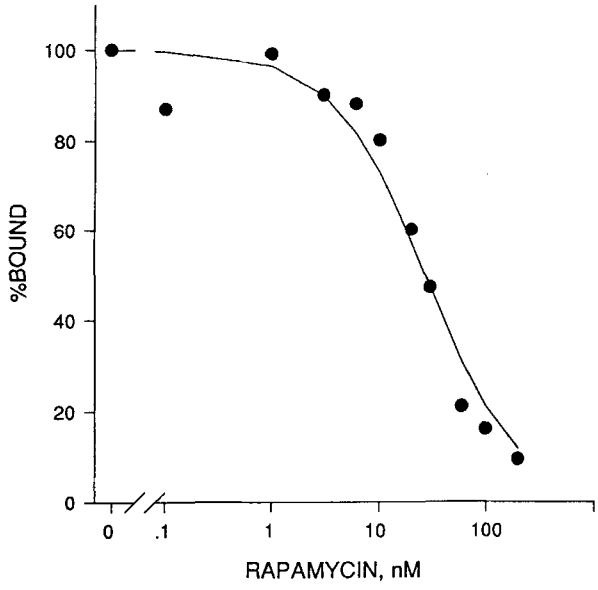


Figure 3. **Scatchard analysis of ^{3}H-FK506 binding to soluble hsp56.** Partially purified from Jurkat cytosol, was incubated with various concentrations of ^{3}H-FK506, as described in "Materials and Methods." After addition of gama globulin and precipitation by PEG, the bound ^{3}H-FK506 was quantified. The results shown are from one of three representative experiments. The inset shows the saturation binding curve; specific (•) and non-specific (o) binding.



4. Rapamycin competes with ^{3}H-FK506 for binding to hsp56 in solution. Hsp56, partially purified from Jurkat cytosol, was incubated with various concentrations of rapamycin in the presence of 25 nM ^{3}H-FK506, as described in "Materials and Methods." After addition of gamma globulin and precipitation by PEG, the bound ^{3}H-FK506 was quantitated. The results shown are from one of three representative experiments.

These results are consistent with the hypothesis that only Domain I mediates FK506 binding to hsp56. Furthermore, Domain I purified from a controlled Endo Lys C digestion of chicken thymus hsp56, has been shown to bind ^{3}H-FK506 with an affinity comparable to that of human hsp56 shown here (27).

The PEG precipitation binding assay was also used in rapamycin competition experiments (Figure 4). The competition curve was similar

to that obtained with the immobilized hsp56 binding assay, yielding an apparent affinity of 17.3 ± 7.7 nM (mean ± SEM).

The level of non-specific binding varied somewhat between the two assays. In the UPJ56-Sepharose method, non-specific binding was low, with a maximum of approximately 20%. In contrast, in the PEG assay, non-specific binding increased with increasing concentrations of ligand, and at 60 nM ^{3}H-FK506, non-specific binding was approximately 40%. This value increased even more at higher concentrations of ^{3}H-FK506, and thus the reliability of this assay is highest at concentrations of ^{3}H-FK506 below 100 nM.

There are advantages to each of these binding assays. The immunoadsorbed hsp56 method requires more manipulation, due to the extensive washing steps. However, since the assay is based on specific immunoadsorption of hsp56, crude cellular fractions can be used as the starting material. In contrast, the PEG precipitation method is well-suited to assays involving large numbers of samples, although prior partial purification is necessary to isolate hsp56.

Taken together, these results demonstrate that the two methods of measuring binding yielded comparable results. Importantly, these assays demonstrated that hsp56 binds ^{3}H-FK506 with an affinity in the nM range. Furthermore, hsp56 has a similar affinity for rapamycin, as indicated by both binding methods. This is the first report of direct measurement of ^{3}H-FK506 to hsp56 in a "solution phase" assay.

ACKNOWLEDGEMENTS

We wish to thank Dr. Rita Huff for help with analysis of the binding data, Dr. Binks Wattenberg for critical reading of the manuscript, and Jacque Hercik for her help in manuscript preparation.

REFERENCES

1. Schreiber, S.L., and Crabtree, G.R. The mechanism of action of cyclosporin A and FK506. Immunol. Today 1992; 13: 136-42.

2. Jin, Y-J., Albers, M.W., Lane, W.S., Bierer, B.E., Schreiber, S.L., and Burakoff, S.J. Molecular cloning of a membrane-associated human FK506- and rapamycin-binding protein, FKBP-13. Proc. Natl. Acad. Sci. USA, 1991; 88: 6677-81.

3. Jin, Y-J., Burakoff, S.J., and Bierer, B.E. Molecular cloning of a 25-kDa high affinity rapamycin binding protein, FKBP25. J. Biol. Chem. 1992; 16: 10942-5.

4. Liu, J., Farmer, J.D., Jr., Lane, W.S., et al. Calcineurin is a common target of cyclophilin-cyclosporin A and FKBP-FK506 complexes. Cell 1991; 66: 807-15.

5. O'Keefe, S.J., Tamura, J., Kincaid, R.L., Tocci, M.J., and O'Neil, E.A. FK506 and CSA-sensitive activation of the interleukin-2 promoter by calcineurin. Nature 1992; 357: 692-4.

6. Clipstone, N.A., and Crabtree, G.R. Identification of calcineurin as a key signalling enzyme in T-lymphocyte activation. Nature 1992; 357: 695-7.

7. Liu, J., Albers, M.W., Wandless, T.J., et al. Inhibition of T cell signalling by immunophilin-ligand complexes correlates with loss of calcineurin phosphatase activity. Biochemistry 1992; 31: 3896-901.

8. Yem, A.W., Tomasselli, A.G., Heinrikson, R.L., et al. The Hsp56 component of steroid receptor complexes binds to immobilized FK506 and shows homology to FKBP-12 and FKBP-13. J. Biol. Chem. 1992; 267: 2868-71.

9. Fretz, H., Albers, M.W., Galat, A., et al. Rapamycin and FK506 binding proteins (Imunophilins). J. Am. Chem. Soc. 1991; 113: 1409-11.

10. Smith, D.F., and Toft, D.O. Steroid receptors and their associated proteins. Mol. Endocrinol. 1993; 7: 4-12.

11. Pratt, W.B. Control of steroid receptor function and cytoplasmic-nuclear transport by heat shock proteins. Bioessays 1992; 14: 841-7.

12. Hutchinson, K.A., Scherrer, L.C., Czar, M.J., et al. FK506 binding to the 56-kilodalton immunophilin (Hsp56) in the glucocorticoid receptor heterocomplex has no effect on receptor folding or function. Biochemistry 1993; 32: 3953-7.

13. Tai, P.-K.K., Albers, M.W., Chang, H., Faber, L.E., and Schreiber, S.L. Association of a 59-kilodalton immunophilin with the glucocorticoid receptor complex. Science 1992; 256: 1313-18.

14. Massol, N., Lebeau, M-C, Renoir, J-M., Faber, L.E., and Baulieu, E.E. Rabbit FKBP59-heat shock protein binding immunophillin (HBI) is a calmodulin binding protein. Biochem. Biophys. Res. Commun. 1992; 187: 1330-5.

15. Wiederrecht, G., Hung, S., Chan, H.K., et al. Characterization of high molecular weight FK-506 binding activities reveals a novel FK-506-binding protein as well as a protein complex. J. Biol. Chem. 1992; 267: 21753-60.

16. Peattie, D.A., Harding, M.W., Fleming, M.A., et al. Expression and characterization of human FKBP52, an immunophilin that associates with the 90-kDa heat shock protein and is a component of steroid receptor complexes. Proc. Natl. Acad. Sci. USA, 1992; 89: 10974-8.

17. Tai, P-K.K., Maeda, Y., Nakao, K., Wakim, N.G., Duhring, J.L., and Faber, L.E. A 59-kilodalton protein associated with progestin, estrogen, androgen, and glucocorticoid receptors. Biochemistry 1986; 25: 5269-75.

18. Sanchez, E.R., Faber, L.E., Herzel, W.J., and Pratt, W.B. The 56-59-kilodalton protein identified in untransformed steroid receptor complexes is a unique protein that exists in cytosol in a complex with both the 70- and 90-kilodalton heat shock proteins. Biochemistry 1990; 29: 5145-52.

19. Ruff, V.A., Yem, A.W., Munns, P.L., et al. Tissue distribution and cellular localization of hsp56, an FK506-binding protein. J. Biol. Chem. 1992a; 267: 21285-8.

20. Lebeau, M-C., Massot, N., Herrick, J., et al. P59, an hsp 90-binding protein. J. Biol. Chem. 1992; 267: 4281-4.

21. Callebaut, I., Renoir, J-M., Lebeau, M-C., et al. An immunophilin that binds M_r 90,000 heat shock protein: Main structural features of a mammalian p59 protein. Proc. Natl. Acad. Sci. USA 1992; 89: 6270-4.

22. Ruff, V.A., McGee, J.E., Yem, A.W., Deibel, M.R., and Leach, K.L. FKBP-12 is not an inhibitor of protein kinase C. Immunol Invest. 1992b; 21: 259-73.

23. Chio, C.L., Lajiness, M.E., and Huff, R.M. Activation of heterologously expressed D3 dopamine receptors: Comparison with D2 dopamine receptors. Mol. Pharmacol., 1993; 45: 51-60.

24. Cheng, Y.C., and Prusoff, W.H. Relationship between the inhibition constant and the concentration of inhibitor which causes 50 percent inhibition of an enzymatic reaction. Biochem. Pharmacol. 1973; 22: 3099-108.

25. Rosborough, S.L., Fleming, M., Nelson, P.A., Boger, J. and Harding, M.W. Identification of FKBP-related proteins with antibodies of predetermined specificity and isolation by FK506 affinity chromatography. Transplant. Proc. 1991; 23: 2890-3.

26. Galat, A., Lane, W.S., Standaert, R.F., and Schreiber, S.L. A rapamycin-selective 25-kDa immunophilin. Biochemistry 1992; 31: 2427-34.

27. Yem, A.W., Reardon, I.M., Leone, J.W., Heinrikson, R.L., and Deibel, Jr. An active FK506-binding domain of 17000 daltons is isolated following limited proteolysis of chicken thymus HSP56. Biochemistry 1993; 32: 12571-6.