Correlation between Ligand−Receptor Affinity and the Transcription Readout in a Yeast Three-Hybrid System†

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ABSTRACT: The yeast two-hybrid assay has proven to be a powerful method to detect protein−protein interactions as well as to derive genome-wide protein interaction maps. More recently, three-hybrid assays have emerged as a means to detect both protein−RNA and protein−small molecule interactions. Despite the routine use of the two-hybrid assay and the potential of three-hybrid systems, there has been little quantitative characterization to understand how the strength of the protein interaction correlates with transcription activation. It is not known if the additional interaction in three-hybrid systems compromises the sensitivity of the system. Thus, here, we set out to determine the K_D cutoff of a small molecule three-hybrid system and to determine if there is a correlation between the K_D and the levels of transcription activation. A series of mutations to FK506-binding protein 12 (FKBP12) were designed to vary the affinity of this protein for the small molecule synthetic ligand for FK506-binding protein 12 (SLF). These FKBP12 variants were overexpressed and purified, and their K_D's for SLF were measured using a fluorescence polarization assay. Then the levels of transcription activation in a Mtx−DHFR yeast three-hybrid system were determined for these variants using a *lacZ* reporter gene. The K_D cutoff of the Mtx yeast three-hybrid system is found to be ca. 50 nM. Further, the levels of transcription activation correlate with the strength of the binding interaction, though the dynamic range is only 1 order of magnitude. These results establish that the three-hybrid assay has the requisite sensitivity for drug discovery. However, the small dynamic range highlights a limitation to equilibrium-based assays for discriminating interactions based on affinity.

The yeast two-hybrid assay provides an in vivo alternative to traditional biochemical techniques for detecting protein−protein interactions (*1*). In this assay, the two proteins of interest are fused to a DNA-binding domain (DBD)[1] and an activation domain (AD), respectively, such that if the two proteins interact they reconstitute a transcriptional activator (DBD-AD) and activate the transcription of a downstream reporter gene. Because the assay is carried out in vivo, it can be used not just to detect the interaction of two proteins but to map out entire protein−protein interaction networks in a high-throughput format (*2*, *3*). More recently, this approach was extended to the detection of small molecule−protein and RNA−protein interactions as a three-hybrid assay (*4*−*6*). Here, for example, a dimeric ligand ["chemical inducer of dimerization" (CID) (*7*, *8*)] bridges a DBD−receptor protein chimera and an AD−receptor chimera to reconstitute a transcriptional activator (Figure 1). One of the ligand−receptor pairs serves as an anchor, while the other is the small molecule protein interaction of interest. Several ligand−receptor pairs suitable for use as anchors have been developed (*6*, *9*−*11*). It has been suggested that three-hybrid assays could be used to discover the protein targets of drugs, to engineer ligand−receptor interactions, and to screen libraries of small molecules for drug discovery (*4*, *8*, *9*, *12*−*14*).

Despite the widespread use of two- and three-hybrid assays, very little is known about how the levels of transcription activation correlate with the strength of the binding interaction. Golemis and co-workers undertook a systematic study of the relationship between the affinity of the protein−protein interaction and the yeast two-hybrid readout using the eukaryotic proteins Myc, Max, and Mxi1 and the prokaryotic protein λ repressor cI (*15*). Myc, Max, and Mxi1 are known to form homo- and heterodimers with one another, and their K_D's have been determined in vitro by cosedimentation studies. The affinities for Myc-Max and Max-Mxi1 heterodimerization are both <5 nM, while for the formation of homodimers both K_D's are above 1 μM. The λ repressor cI is a homodimer, and for these studies the wild-type protein, $K_D = 20$ nM, and three mutants, one with an estimated K_D of 50−100 nM and the two others with a K_D of over 1 μM, were used. Using these well-studied homo- and heterodimers, they characterized their levels of transcrip-

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[1] Abbreviations: AD, activation domain; DBD, DNA-binding domain; Mtx, methotrexate; FKBP12, FK506-binding protein; DHFR, dihydrofolate reductase; ONPG, *o*-nitrophenyl β-D-galactopyranoside; IPTG, isopropyl β-D-thiogalactopyranoside.

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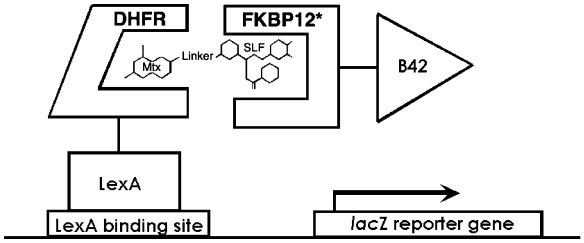

FIGURE 1: Yeast three-hybrid assay. A heterodimeric ligand (Mtx−SLF) bridges a DNA-binding protein−receptor protein chimera (LexA−DHFR) and a transcription activation protein−receptor protein chimera (B42−FKBP12), effectively reconstituting a transcriptional activator and stimulating transcription of a *lacZ* reporter gene. The levels of *lacZ* transcription serve as an indicator of the efficiency of FKBP12−Mtx-induced protein dimerization.

tion activation in the yeast two-hybrid assay using a series of *lacZ* and *LEU2* reporter genes with different numbers of DBD binding sites. From their studies they concluded that in the yeast two-hybrid assay it is not possible to detect interactions whose K_D's are over 1 μM. These studies provide important quantitative characterization of the yeast two-hybrid system. However, as the authors point out, because three different proteins, Myc, Max, and Mxi1, were used for these studies, differences in transcription activation may reflect differences, for example, in protein expression levels rather than affinity. The cI protein is not an ideal model because it is a homodimeric interaction in a heterodimeric system. For both systems, only a limited set of K_D's were available. To extend this work to a three-hybrid system and to provide a single model system with a broad range of K_D's, we set out to examine the correlation between the strength of the binding interaction and the *n*-hybrid readout using a series of mutants of a well-studied ligand−receptor pair.

We chose to carry out these studies using the methotrexate (Mtx)−dihydrofolate reductase (DHFR) yeast three-hybrid system previously reported by our laboratory (*10*, *12*). The sensitivity of a three-hybrid, as opposed to a two-hybrid, assay should be a function of the ligand−receptor pair being used as an anchor. Because Mtx binds DHFR with low picomolar affinity, the Mtx anchor should facilitate the detection of relatively weak interactions at the other end (*10*, *16*, *17*). To characterize the sensitivity of the Mtx three-hybrid system, we chose the well-studied interaction between the FK506-*b*inding *p*rotein (FKBP12) and the *s*ynthetic *l*igand for *F*KBP12 (SLF) (see Scheme 1). SLF is a synthetic analogue of the well-characterized natural product FK506 that binds FKBP12 with low nanomolar affinity (*18*, *19*). SLF has been used extensively as a CID (*9*, *18*). In vitro studies have shown that SLF binds FKBP12 with an IC_{50} of 86 nM, and the high-resolution structure of the SLF−FKBP12 complex has been determined at a resolution of 2 Å by X-ray crystallography (*18*, *20*). This nanomolar dissociation constant leaves ample room for the design of FKBP12 mutants with reduced but measurable affinity. FKBP12 is a well-behaved, monomeric protein, which should facilitate the characterization of the FKBP12 mutants. Finally, recently we reported the synthesis of the Mtx−SLF CID for use in a bacterial three-hybrid system (*9*).

Here we examine the relationship between the binding affinity of the SLF−FKBP12 ligand−receptor interaction and the transcription readout by determining the K_D of several FKBP12 mutants for SLF in vitro and then comparing them to the levels of transcription activation in the yeast three-hybrid assay in vivo. First, we showed that Mtx−SLF is an efficient CID in the yeast three-hybrid assay. Then, on the basis of both biochemical and high-resolution structural data, we designed mutants of FKBP12 intended to span several orders of magnitude in their K_D for SLF (*18*−*20*). The dissociation constants of the FKBP12 mutants for SLF were determined in vitro by using a fluorescence polarization assay (*21*). Then these same FKBP12 variants were put back in the yeast three-hybrid system, and their levels of transcription activation were detected using a *lacZ*, *LEU2*, and *URA3* reporter gene. Here we present and discuss the correlation between the K_D of the ligand−receptor interaction and the transcription readout.

EXPERIMENTAL PROCEDURES

Materials. Standard protocols for molecular biology and yeast genetics were used. Restrictions enzymes and T4 DNA ligase were purchased from New England Biolabs (Beverly, MA). PFU Turbo polymerase and TG1 cells were purchased from Stratagene (La Jolla, CA). The dNTPs used in site-

Scheme 1

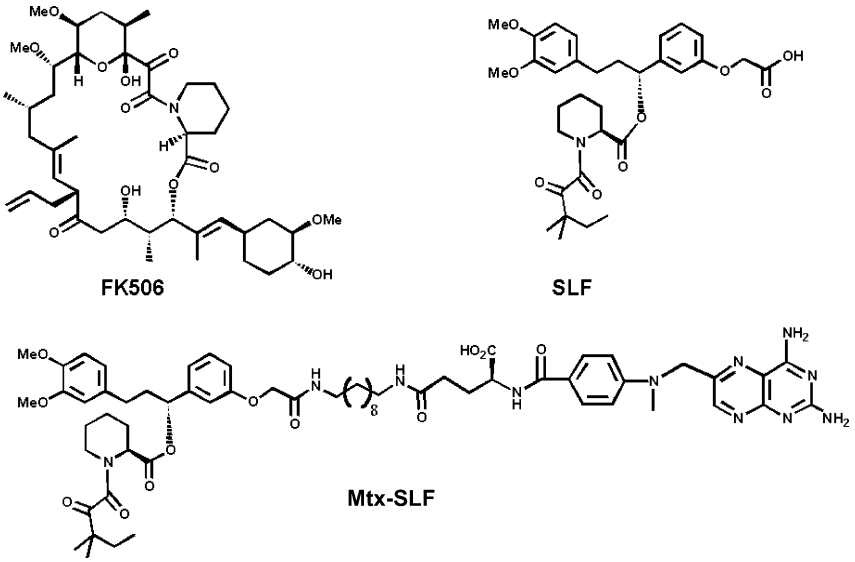

Table 1: Strains and Plasmids Used in This Study

name	description	source/ref
strain	genotype	
BL21(DE3)		Novagen
FY251	*MATa trp1Δ63 his3Δ200 ura3-52 leu2Δ1 GAL*$^+$	M. Carlson
V704Y	*MATa ade4::P$_{gal1}$-lexA-eDHFR(HIS3) ade2::ura3-52 GAL*$^+$	this study
plasmid	details	
PMW112	*8lexAop-lacZ* 2μ kan^R	*26*
PMW102-FKBP12	P_{GAL}/B42-HA-FKBP12 kan^R	this study
pBC1052	pMW102-FKBP12 with D37V mutation	this study
pKS1269	pMW102-FKBP12 with W59L mutation	this study
pKS1270	pMW102-FKBP12 with F36Y mutation	this study
pBC1053	pMW102-FKBP12 with R42Q mutation	this study
pBC1054	pMW102-FKBP12 with H87L mutation	this study
pKS1273	pMW102−FKBP12 with Y26F mutation	this study
pSG1205	P_{T7}/FKBP12-his$_6$ kan^R	this study
pKS1274	pSG1205 with D37V mutation	this study
pKS1275	pSG1205 with W59L mutation	this study
pKS1276	pSG1205 with F36Y mutation	this study
pKS1277	pSG1205 with R42Q mutation	this study
pKS1278	pSG1205 with H87L mutation	this study
pKS1279	pSG1205 with Y26F mutation	this study
PKS1293	P_{T7}/LexA-his$_6$ kan^R	this study

directed mutagenesis and PCR reactions were purchased from Amersham Pharmacia Biotech. The Tuner BL21(DE3) cells, pET26b plasmid, and the BugBuster protein extraction reagent used in protein purification were purchased from Novagen (Madison, WI). The yeast protein extraction reagent, Y-Per, was purchased from Pierce (Rockford, IL). The 5-bromo-4-chloro-3-indoyl β-D-galactopyranoside (X-gal) used in the X-gal indicator plates was purchased from Diagnostic Chemicals (Oxford, CT). The phrog used to transfer 48 droplets of yeast media onto X-gal plates was purchased from Dan-Kar Corp. (Wilmington, MA). The *o*-nitrophenyl β-D-galactopyranoside used in the liquid *lacZ* assays was purchased from Sigma. The transformations of *Escherichia coli* were carried out by electroporation using a Bio-Rad *E. coli* pulser. The Ni-NTA spin columns used to purify proteins as well as the miniprep spin kit and the gel extraction spin kit used to purify DNA were purchased from Qiagen (Valencia, CA). Centricon centrifugal filter units YM-3 for protein concentration and buffer change were purchased from Millipore (Billerica, MA). Anti-HA IgG antibodies for protein detection were purchased from Roche (Indianapolis, IN). Anti-FKBP12 IgG antibodies for protein detection was purchased from ABR Affinity Bio Reagents (Golden, CO). Anti-LexA IgG for protein detection was purchased from Invitrogen Corp. (Carlsbad, CA). The ECF Western blotting reagent pack, which included the anti-mouse and anti-rabbit IgG-linked alkaline phosphatase, was purchased from Amersham Pharmacia Biotech. Oligonucleotides were purchased from the Great American Gene Co. (Ramona, CA). Isopropyl β-D-thiogalactoside (IPTG) was purchased from American Bio-Organics. *N*,*N*,*N*′,*N*′-Tetramethylethylenediamine (TEMED) and acrylamide:bisacrylamide (37:5:1) for making 15% acrylamide/bisacrylamide gels were purchased from Fisher (Pittsburgh, PA). Methotrexate was a gift from the National Cancer Institute (NCI). FK506−fluorescein was a gift from Ariad (Cambridge, MA). Mtx−fluorescein was purchased from Molecular Probes (Eugene, OR). All other chemicals were purchased from Aldrich or Molecular Probes. All solutions were made from distilled water prepared from a Milli Q water purification system. For PCR, a MJ Research PTC-200 Pellier thermal cycler was used. For the fluorescence polarization assay a Wallac 1420 VICTOR^2 plate reader from Perkin-Elmer was used (Boston, MA). Ultraviolet−visible measurements were taken using a Molecular Devices Pectramax 384. Circular dichroism measurements were done on a Jasco J810 spectropolarimeter. Sequencing was performed by GeneWiz Inc. (North Brunswick, NJ). The *Saccharomyces cerevisiae* strain FY251 was provided by Marian Carlson.

Synthesis of SLF−Fluorescein (9, 22). The *tert*-butyl ester of SLF (50 mg, 78 μmol) was dissolved in dichloromethane (2 mL) and trifluoroacetic acid (2 mL) and stirred at room temperature for 1 h. Toluene (5 mL) was added, and the solvent was removed in vacuo. Toluene (5 mL) was added and removed in vacuo four additional times. The flask was placed under high vacuum for 3 h before back-filling with nitrogen and adding *N*,*N*′-disuccinimidyl carbonate (20 mg, 78 μmol), acetonitrile (2 mL), and pyridine (10 μL, ∼100 μmol). The reaction was allowed to stir for 16 h under nitrogen before water (50 mL) was added. The mixture was extracted with EtOAc (twice, 50 mL). The extracts were collected, washed with brine (10 mL), and dried over anhydrous sodium sulfate. The product was concentrated in vacuo and purified by silica gel column chromatography (100% EtOAc) to give the activated ester (28 mg, 41 μmol). 5-(Aminoacetamido)fluorescein (Molecular Probes, Eugene, OR) (5 mg, 12 μmol) in *N*,*N*-dimethylformamide (1 mL) was added to the activated ester followed by pyridine (10 μL, ∼100 μmol). The reaction mixture was stirred for 12 h under nitrogen before the solvent was removed in vacuo. The product was purified by preparative thin-layer chromatography (once with 1:1 EtOAc:hexanes and then four times with 100% EtOAc). The silica was excised and filtered with EtOAc to collect the product SLF−fluorescein (6 mg, 6.2 μmol).

Plasmid Construction. Plasmids used in this study are listed in Table 1. For expression and purification of FKPB12 in *E. coli* we created plasmid pSG1205. A 345 bp *Nde*1 to *Xho*1 fragment encoding the FKBP12 gene was amplified using the primers VWC1309, 5′-GCA TAC GTC CAT ATG GGA GTG CAG GTG GAA AC (*Nde*I), and VWC1310, 5′-CAG CTA GCC TCG AGT TTT AGA AGC TCC ACA

T (*Xho*I). This fragment was inserted between the *Nde*1 and *Xho*1 sites in the pET26b vector to generate the plasmid pSG1205, encoding a C-terminal fusion of the FKBP12 gene to a His_6 tag. For expression of the B42−FKBP12 fusion in the yeast three-hybrid assay, we created plasmid pMW102-FKBP12. A 345 bp *Eco*RI to *Xho*I fragment encoding the FKBP12 gene was created by using the primers VWC22, 5′-GCA TAC GTC GAA TTC ATG GGA GTG CAG GTG G (*Eco*RI), and VWC23, 5′-GCA TTG CTG CTC GAG TCA TTC CAG TTT TAG AAG C (*Xho*I). This fragment was inserted between the *Eco*RI and *Xho*I sites in the pMW102 vector to generate the pMW102-FKBP12 plasmid, encoding FKBP12 fused to the C-terminus of the HA epitope and B42. The FKBP12 mutants used in both the over-expression vector and the yeast three-hybrid vector were made using Stratagene's QuikChange site-directed mutagenesis kit. The following primers were used to make the FKBP12 variants. For D37V the primers VWC1022, 5′-GAA GAT GGA AAG AAA TTT GTT TCC TCC CGG GAC AGA AAC, and VWC1023, 5′-GTT TCT GTC CCG GGA GGA AAC AAA TTT CTT TCC ATC TTC, generated plasmids pBC1052 from pMW102-FKBP12 and pKS1274 from pSG1205. For W59L the primers VWC1364, 5′-GTG ATC CGA GGC TTG GAA GAA GGG GTT GCC, and VWC1365, 5′-GGC AAC CCC TTC TTC CAA GCC TCG GAT CAC, generated plasmids pKS1269 from pMW102-FKBP12 and pKS1275 from pSG1205. For F36Y the primers VWC1366, 5′-GAA GAT GGA AAG AAA TAT GAT TCC TCC CGG GAC AG, and VWC1367, 5′-CT GTC CCG GGA GGA ATC ATA TTT CTT TCC ATC TTC, generated plasmids pKS1270 from pMW102-FKBP12 and pKS1276 from pSG1205. For R42Q the primers VWC1015, 5′-AAA TTT GAT TCC TCC CGG GAC CAA AAC AAG CCC TTT AAG TTT ATG, and VWC1016, 5′-CAT AAA CTT AAA GGG CTT GTT TTG GTC CCG GGA GGA ATC AAA TTT, generated plasmids pBC1053 from pMW102-FKBP12 and pKS1277 from pSG1205. For H87L the primers VWC1017, 5′-GCC TAT GGT GCC ACT GGG TTG CCA GGC ATC ATC CCA CCA, and VWC1018, 5′-TGG TGG GAT GAT GCC TGG CAA CCC AGT GGC ACC ATA GGC, generated the plasmids pBC1054 from pMW102-FKBP12 and pKS1278 from pSG1205. And for Y26F the primers VWC1372, 5′-ACC TGC GTG GTG CAC TTC ACC GGG ATG CTT GAA, and VWC1373, 5′-TTC AAG CAT CCC GGT GAA GTG CAC CAC GCA GGT, generated plasmids pKS1273 from pMW102-FKBP12 and pKS1279 from pSG1205. The full coding region was sequenced by using the primers VWC422, 5′-CAA GAC CCG TTT AGA GGC, for the plasmids derived from pSG1205 and VWC1053, 5′-CAG CCT CTT GCT GAG TGG, for the plasmids derived from pMW102-FKBP12.

Strain Construction. The yeast strains used in this paper are listed in Table 1. The strain V704Y was prepared by integrating the gene encoding LexA−DHFR under the control of the *GAL1* promoter at the chromosomal loci *ade4* in the *S. cerevisiae* strain FY251 (MAT**a**). This strain was transformed using lithium acetate with the plasmid pMW112, which encodes the *lacZ* gene under the control of eight tandem LexA operators, and either the plasmid pMW102-FKBP12, pBC1052, pKS1269, pKS1270, pBC1053, pBC1054, or pKS1273 followed by selection on synthetic complete (SC) media containing 2% glucose and lacking the appropriate selective nutrients as described (*23*). Six transformants for each mutant were picked for use in the yeast three-hybrid assay.

Protein Purification. The wild-type and mutant FKBP12 variants were purified from the *E. coli* strain Tuner BL21-(DE3) carrying the FKBP12 expression vector pSG1205 and its derivatives (Table 1). Briefly, 1 mL of an LB overnight culture was used to inoculate 500 mL of LB. Both cultures contained 30 μg/mL kanamycin to select for the expression vector. The 500 mL culture was grown at 37 °C, shaking at 300 rpm, to an OD_{600} of 0.6, at which time expression of the protein was induced by the addition of IPTG to a final concentration of 0.5 mM. After growth for an additional 3 h, the cells were harvested by centrifugation. The cell pellet underwent one freeze−thaw cycle (from −80 °C to room temperature) before being lysed by 6 mL of Bug Buster protein extraction reagent on ice for 30 min. The protein was then purified under standard nondenaturing conditions using the Ni-NTA spin kit according to the manufacturer's protocol (Qiagen). The protein was judged to be >95% pure on the basis of Coomassie staining of a sodium dodecyl sulfate (SDS)−polyacrylamide gel electrophoresis (PAGE). The protein fraction was subsequently dialyzed against a buffer containing 450 mM Tris-HCl, pH 7.5, and 10 mM DTT and concentrated to approximately one-fourth of the original volume by using a Centricon filter. The enzyme concentration was determined on the basis of the A_{280} assuming $\epsilon = 9927\ cm^{-1}\ M^{-1}$ as reported for the wild-type protein (*24*). For the mutants D37V, R42Q, and H87L the extinction coefficient used was the same as for the wild type since there were no changes in the number of tryptophan, tyrosine, or cysteine residues. For the other mutants the assumption was made that a tryptophan residue contributes $5690\ cm^{-1}\ M^{-1}$ to the extinction coefficient, while a tyrosine residue contributes $1280\ cm^{-1}\ M^{-1}$ and a cysteine residue contributes $120\ cm^{-1}\ M^{-1}$ (*25*). Thus, the following values were used: for W59L, $\epsilon = 4237\ cm^{-1}\ M^{-1}$; for F36Y, $\epsilon = 11207\ cm^{-1}\ M^{-1}$; for Y26F, $\epsilon = 8647\ cm^{-1}\ M^{-1}$. The purified protein was stored at 4 °C.

His_6-tagged LexA was expressed from pKS1293 and purified essentially as described above, except using denaturing conditions of 8 M urea. The protein concentration was determined using a calculated extinction coefficient for the linearized protein sequence of $\epsilon = 17\,780\ cm^{-1}\ M^{-1}$.

Circular Dichroism. Wavelength scans were carried out for the purified proteins to ensure that each mutant's overall structure is similar to that of the wild-type enzyme. Proteins were diluted to 10 μM in a buffer containing 450 mM Tris-HCl, pH 7.5. CD wavelength scans were measured on a Jasco J-810 spectropolarimeter. Spectra were recorded from 190 to 300 nm, every 1 nm, with eight accumulations for each sample.

Binding Affinity Assay. The affinity of FK506−fluorescein and SLF−fluorescein for FKBP12 was determined by fluorescence polarization. Either FK506−fluorescein or SLF−fluorescein at a final concentration of 2.5 or 10 nM, respectively, was incubated with purified FKBP12 or the appropriate mutant at varying concentrations ranging from 0.5 nM to 5 μM in a buffer (50 mM KH_2PO_4, 150 mM NaCl, 0.1 mg/mL BSA, 0.002% Tween, pH 7.5, for FK506−fluorescien and 50 mM KH_2PO_4, 150 mM NaCl, 0.002% Tween, pH 7.5, for SLF−fluorescein) that minimized

nonspecific interactions and resulted in a strong fluorescent signal (*21*). By comparing the intensity of emitted fluorescence both parallel and perpendicular to incident light, it is possible to compute the amount of bound tracer. Using Kaleidagraph (Synergy Software, PA), the data were fit to the following equation in order to obtain the dissociation constant:

$$F = F_0 - (F_0 - F_{100})[(K_D + [F]_T + [P]_T) - (K_D + [F]_T + [P]_T)^2 - (4[F]_T[P]_T)]^{0.5}/(2[F]_T)$$

where F_0 is the polarization of the tracer with no receptor, F_{100} is the maximum polarization with an infinite amount of receptor, $[F]_T$ is the total amount of tracer used, and $[P]_T$ is the total amount of receptor used.

Protein Expression Levels. Western blots were carried out to assess the protein expression levels of FKBP12 and its mutant variants. The yeast strains were grown to saturation in the appropriate SC media containing 0.5% glucose, 1.5% galactose, and 2% raffinose and then lysed using acid-washed glass beads. Relative protein expression levels were determined by loading 25 μL of each crude lysate onto a SDS−PAGE followed by transfer to a PVDF membrane. The membrane was blocked with 5% nonfat milk, followed by incubation with primary antibody (anti-HA IgG) at a 1:1000 dilution followed by incubation with the secondary antibody (anti-mouse IgG linked to alkaline phosphatase) at a 1:2500 dilution. The secondary antibody was detected by incubation of the membrane with 1 mL of the fluorescent substrate ECF in solution (prepared according to Amersham Pharmacia Biotech's protocol). The membrane was visualized by fluorescence scanning using the Storm phosphorimager (Amersham).

To obtain an estimate on the concentration of both LexA−DHFR and B42−FKBP12 being expressed from the *GAL1* promoter, Western blots were carried out essentially as described above using varying amounts of crude cell lysate (0.015−15 μg of total cellular protein) along with varying concentrations of known protein standard. The LexA−DHFR concentration was assessed using varying concentrations of purified LexA (7 nM−7 μM) and detecting with a primary antibody (anit-LexA IgG) at a 1:5000 dilution, while the B42−FKBP12 concentration was determined using varying concentrations of purified FKBP12 (10 nM−10 μM) and detecting with a primary antibody (anti-FKBP12 IgG) at a 1:1000 dilution. Secondary antibody (anti-rabbit IgG linked to alkaline phosphate) was then added at a 1:5000 dilution and visualized using a solution of ECF substrate described above. Standard curves were determined by assessing the pixel density of each band using ImageQuant software version 5.2 (Amersham Biosciences) for both the purified LexA and FKBP12 standards, and protein concentrations for LexA−DHFR and B42−FKBP12 were determined on the basis of these standard curves.

Cellular Small Molecule Concentration. See Supporting Information.

Liquid lacZ Transcription Assays. The yeast strains expressing the three-hybrid constructs with wild-type and mutant versions of FKBP12 were assayed for β-galactosidase activity both on plates and on liquid cultures. Each of the yeast strains was inoculated from frozen glycerol stocks onto SC media containing 2% glucose and lacking the appropriate nutrients. Incubation at 30 °C, shaking at 250 rpm, was allowed to proceed until saturation (approximately 3 days). Plate assays were performed by transferring a droplet of the saturated yeast culture onto X-gal indicator plates, using a 48 prong phrogger. The X-gal indicator plates contained either 0, 1, or 10 μM Mtx−SLF along with 0.5% glucose, 1.5% galactose, and 2% raffinose and lacked the appropriate selective nutrients in order to induce expression of the LexA−DHFR and B42−FKBP12 chimeras, which were under the control of the *GAL1* promoter. Competition experiments were also performed using X-gal indicator plates containing 1 μM Mtx−SLF in the presence of 0, 10, or 100 μM SLF. The plates were allowed to grow at 30 °C for 3−5 days. For the liquid assays, 5 μL of the saturated yeast cultures was used to inoculate 95 μL of SC media containing 0.5% glucose, 1.5% galactose, and 2% raffinose and lacking the appropriate. Identical inoculations were done except that the media contained varying concentrations (0.01−30 μM) of the dimeric ligand Mtx−SLF. The cultures were allowed to grow for 3 days at 30 °C, shaking at 250 rpm. The cells were harvested by centrifugation, and the pellets were subsequently resuspended in 100 μL of distilled water. Next, the cultures were transferred to a flat-bottomed 96-well plate (Fisher) for reading at A_{600}. The cultures were centrifuged, and the pellets were resuspended in 100 μL of the Y-Per protein extraction reagent. Lysis was allowed to proceed for 30 min. Then, 8.5 μL of a 10 mg/mL ONPG solution was added to the extracts and allowed to incubate for 30 min at 37 °C, shaking at 300 rpm. The β-galactosidase activity reaction was stopped with 130 μL of 1 M sodium carbonate. The extracts were centrifuged, and the supernatant was transferred to a flat-bottomed 96-well plate, where the A_{420} was measured. The following equation was used to calculate β-galactosidase units: β-galactosidase $= 1000[A_{420}/(A_{600} \times$ time (in min) $\times$ volume assayed (in mL))].

RESULTS

To investigate the relationship between the binding affinity of a ligand−receptor pair and the transcription readout in a yeast three-hybrid assay, we created a system that took advantage of a tight anchor (Mtx−DHFR) previously developed by our laboratory and a well-characterized interaction in the mid-nanomolar range (SLF−FKBP12). We first confirmed that the yeast three-hybrid assay works with these chimeras, since this pair had only been used before in a bacterial three-hybrid system (*9*). Then, to obtain FKBP12 variants with a wide range of binding affinities for SLF, we designed point mutants of FKBP12 on the basis of previous biochemical studies and analysis of the high-resolution crystal structure of FKBP12 with SLF and FK506 (*19*, *20*). We then determined the dissociation constants for SLF and the FKBP12 mutant variants by using a fluorescence polarization assay (*21*). Finally, we inserted these FKBP12 point mutants into the yeast three-hybrid assay and measured the levels of transcription activation using a *lacZ* reporter gene. These data allowed us to delineate what type of correlation exists between the in vitro binding affinities of the ligand−receptor pair being studied and the transcription readout in a three-hybrid assay.

Since the ligand−receptor pair FKBP12−SLF had only been used in a bacterial three-hybrid system before, we first

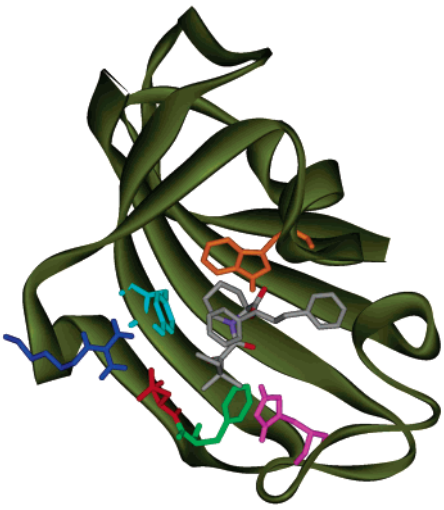

FIGURE 2: Ribbon diagram of FKBP12 bound to SLF highlighting the residues chosen for mutation in this study. SLF is shown as stick representation with coloring based on elements. The residues chosen for analysis are highlighted in different colors: R42, dark blue; D37, red; F36, green; H87 purple; Y26, light blue; W59, orange. The diagram is taken from the high-resolution FKBP12−SLF structure by Holt et al. (*20*). The graphic was prepared using WebLab Viewer Lite (Accelrys Inc.).

had to determine if it was compatible with the yeast three-hybrid system. After testing several conditions, we determined that robust activation of the *lacZ* reporter gene occurs when the media contains 0.5% glucose, 1.5% galactose, and 2.0% raffinose (data not shown). Presumably these conditions yield an optimum stoichiometric ratio of the protein chimeras LexA−DHFR and B42−FKBP12. Further, *lacZ* activation is maximized when the concentration of the DBD fusion protein is decreased by integrating the LexA−DHFR construct into the chromosome. We carried out standard controls to show that transcription activation is CID dependent. The original Mtx−Dex yeast three-hybrid system has been thoroughly characterized (*12*). However, the yeast three-hybrid system used in this study failed to significantly activate either a *LEU2* or *URA3* reporter gene under numerous different conditions tested (data not shown). Therefore, we used a *lacZ* reporter gene under the optimal concentration of sugars described above throughout this study.

Design of FKBP12 Mutants. Since we needed a series of FKBP12 variants with different K_D's for SLF in order to study the relationship between the transcription readout and ligand−receptor binding, we decided to rationally design point mutations in FKBP12 that were expected to result in a wide range of affinities for SLF. Despite the fact that there are few studies characterizing the effect of point mutations on the binding affinity of FKBP12 for SLF, there is a high-resolution crystal structure of SLF bound to FKBP12 (*18*, *20*, *27*, *28*). Furthermore, there are extensive reports of how active site mutations influence the interaction between FK506 and FKBP12 (*19*, *24*, *29*). FK506 and SLF bind a shallow, hyrdrophobic pocket between the α-helix and five-stranded antiparallel *β*-sheet of the protein (Figure 2) (*24*). One of the residues in the hydrophobic cleft, Trp59, has interesting properties: it is important for the binding of rapamycin but has a negative effect on protein stability. Therefore, we rationalized that the mutation of Trp59 to Leu, a much smaller, aliphatic residue, should severely disrupt binding to SLF. A second residue of interest was Phe36, the position exploited in previous work to engineer the so-called bump hole SLF/FKBP12 variants (*18*). We rationalized that placing a residue only slightly larger and more polar in this region should not significantly impair binding, effectively giving us a medium-strength binder; thus, we chose to mutate Phe36 to Tyr. Two hydrophilic residues, Arg42 and Asp37, are in the solvent-exposed interface of the binding pocket and in close enough proximity to form a salt bridge (*19*). We decided to make a mutation that would abolish the salt bridge and consequently modify the architecture of the binding pocket and one that could retain the electrostatic interaction. Thus, we changed Asp37 to Val, a drastic, nonconservative mutation, and Arg42 to Gln, a less drastic one. Another interesting residue for analysis was His87 since it is also on the solvent-exposed region of the binding pocket and in close proximity to the aliphatic side chain of the dicarbonyl moiety in SLF (Figure 2). We changed His87 to Leu, a small, hydrophobic residue, in the hope that it would cause very little disruption in binding or potentially make FKBP12 a better binder by increasing hydrophobic interactions with SLF.

Lastly, we were interested in Tyr26 since it appears to make a hydrogen bond to one of the dicarbonyls of SLF (Figure 2). To abolish this hydrogen bond, yet maintain an aromatic residue in this position, we chose to mutate Tyr26 to Phe in the hope of obtaining a mild defect in binding.

In Vitro Binding Affinities. After deciding what point mutations to use in this study, we needed to purify FKBP12 and its variants and determine their affinity for SLF in vitro. FKBP12 and its derivatives were overexpressed in *E. coli* from a T7 promoter and purified by a Ni affinity column using a His_6 tag. Each protein was judged to be >95% pure on the basis of SDS−PAGE and staining with Coomassie. Also, each protein was judged to be intact structurally, based on the comparison of the wavelength CD scan to that of the wild-type protein. Three different in vitro assays have been used to determine the affinity of small molecule ligands for FKBP12. The relative affinities (IC_{50}) of SLF for FKBP12 have been determined by competition of FK506−fluorescein from the FKBP12 binding pocket using fluorescence polarization measurements (*18*). The inhibition constants (K_I's) for inhibition of the prolyl isomerase activity of FKBP12 have also been found (*29*). Finally, the K_D has been determined directly on the basis of the change in fluorescence polarization of SLF−fluorescein (*21*). We chose to use the latter assay because it is a direct measure of K_D. Both the IC_{50} and K_I measurements reflect not only the effect of the mutant on SLF binding but also the effect on FK506 binding and substrate binding, respectively. We also determined the K_D's of the FKBP12 mutant variants for FK506 on the basis of the change in FP with FK506−fluorescein.

Reflecting our design, the FKBP12 mutant variants do in fact span several orders of magnitude in their K_D for SLF. The H87L, F36Y, and R42Q variants all have K_D's within an order of magnitude from that of the wild-type protein. H87L is the only variant studied here with higher affinity than the wild-type protein, with a 7.3 nM ± 0.5 K_D. The wild-type protein has a 15 nM ± 1 K_D. F36Y and R42Q have a 48 ± 5 nM and a 62 ± 5 nM K_D, respectively. The W59L, D37V, and Y26F variants all showed significant

defects in binding, with K_D's of 140 ± 9, 140 ± 9, and 170 ± 16 nM, respectively.

The effect of the mutants on FK506 binding did not necessarily correlate with that for SLF. We determined the wild-type protein to have a 1.3 ± 0.1 nM K_D. Similarly to SLF, the H87L and R42Q mutant variants have K_D's for FK506 similar to that of the wild-type protein, 1.1 ± 0.1 and 2.1 ± 0.2 nM, respectively. The Y26F variant also shows little defect in binding, with a 4.9 ± 0.5 nM K_D. The F36Y and W59L variants show medium defects in binding with K_D's of 14 ± 2 and 19 ± 1 nM, respectively. The mutant D37V showed a very strong defect in binding, about 100-fold lower than wild type with a K_D of 126 ± 9 nM. In summary, the K_D's for SLF−fluorescein had the following order from strongest to weakest binder: H87L > WT > F36Y ≈ R42Q > W59L = D37V > Y26F. For FK506−fluorescein the order was the following: H87L ≈ WT > R42Q > Y26F > F36Y > W59L > D37V.

To ensure that the altered binding affinities were a direct consequence of molecular interactions and not of a folding defect, we used circular dichroism (CD) to check if the wild-type and mutant proteins adopted a similar secondary structure. The CD wavelength spectra for the mutants W59L, H87L, R42Q, and Y26F were similar to that of the wild-type protein (data not shown). Meanwhile, the α-helicity peak for the mutant D37V was shifted, indicating some difference in structure. This observation was expected for this nonconservative mutation since it severely disrupts electrostatic interactions with R42 and consequently could modify the architecture of the surface of the binding pocket. Moreover, the point mutant F36Y also had a peak slightly shifted. Overall, however, the CD wavelength spectra for the mutant variants and wild-type FKBP12 were conserved, confirming that no point mutation globally disrupted the fold of FKBP12.

Liquid lacZ Assays. Next, we wanted to compare the levels of transcription activation of the FKBP12 variants in our Mtx−DHFR yeast three-hybrid system. Since the LexA−DHFR protein chimera was being expressed ectopically from a *GAL1* promoter, we had to optimize the combination and concentration of sugars in the media in order to obtain robust *lacZ* expression (data not shown). Then we determined the dependence of transcription activation on the concentration of the ligand Mtx−SLF. Also, it was important to know if wild-type and mutant proteins showed maximum transcription readout at different or equal concentrations of Mtx−SLF. Thus, we performed a liquid *lacZ* assay for wild-type and mutant proteins using varying concentrations of Mtx−SLF (0.01−30 μM) (Figure 3). We observed that the wild-type and mutant proteins showed maximum activation, as measured by the use of ONPG as a substrate for β-galactosidase, at a concentration of 10 μM Mtx−SLF. Values beyond this concentration decreased transcription readout. Interestingly, this conclusion was valid for all mutants. Also, we observed that transcription activation started when the concentration of Mtx−SLF was 100 nM; lower concentrations resulted in activity indistinguishable from background (when no Mtx−SLF is present). Again, this was valid for the wild-type and mutant proteins.

After determining that a concentration of 10 μM Mtx−SLF resulted in maximal transcription activation, we decided to compare the β-galactosidase readout of yeast containing wild-type and mutant FKBP12 side by side. Therefore, we

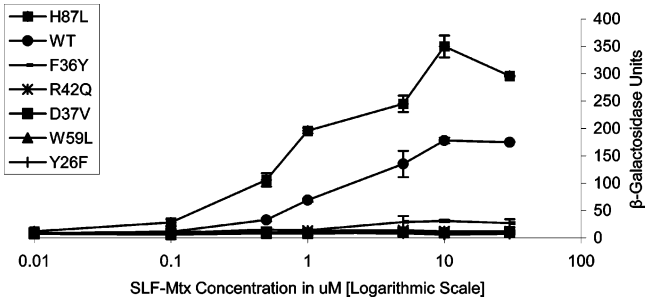

FIGURE 3: Determination of the dependence of the dimeric ligand Mtx−SLF on transcription activation in a yeast three-hybrid assay. Yeast cultures expressing the components of the yeast three-hybrid system using either wild-type FKBP12 or one of its variants were grown in the presence of different amounts of the dimeric ligand Mtx−SLF. The activity of the *lacZ* reporter gene was measured by using a standard liquid assay. Transcription activation for the wild-type and mutant proteins was detectable around 0.1 μM dimerizer and peaked at 10 μM. At higher concentrations of Mtx−SLF, transcription activation of the reporter gene decreased but in different relative amounts for each FKBP12 variant.

Table 2: Binding Affinity of FKBP12 and Its Variants for both SLF−Fluorescein and FK506−Fluorescein As Determined by a Fluorescence Polarization Assay

	binding affinity to FK506−fluorescein (nM)	binding affinity to SLF−fluorescein (nM)	transcription fold activation in yeast three-hybrid assay
H87L	1.1 ± 0.1	7.3 ± 0.5	44 ± 2.0
WT	1.3 ± 0.1	15 ± 1	17 ± 1.7
F36Y	14 ± 2	48 ± 5	2.9 ± 0.1
R42Q	2.1 ± 0.2	62 ± 5	2 ± 0.1
W59L	19 ± 1	140 ± 9	1.6 ± 0.1
D37V	126 ± 9	140 ± 9	1.2 ± 0.2
Y26F	4.9 ± 0.5	170 ± 20	1 ± 0.1

calculated the fold activation of wild-type and mutant proteins by dividing the peak value of transcription activation, in β-galactosidase units (obtained at 10 μM Mtx−SLF), by the background activity (the value at 0 μM Mtx−SLF) (Table 2). We observed that yeast expressing the FKBP12 mutant H87L had the highest value of fold activation (about 44-fold over background). Wild-type FKBP12 resulted in the second highest fold activation (about 17) followed by F36Y (2.9), R42Q (2.0), W59L (1.6), D37V (1.2), and Y26F (1), which showed no measurable activation over background.

As shown in Figure 4, a similar phenomenon was observed using the X-gal indicator plates to assess the level of β-galactosidase produced from strains possessing the different FKBP12 mutants. After 5 days at 30 °C, wild-type FKBP12-containing strains (column 2) all showed a clear blue coloration in the presence of both 1 and 10 μM Mtx−SLF but clearly could be distinguished from strains possessing the H87L FKBP12 mutant (column 7), which showed more intense blue coloration, and strains possessing the F36Y mutant (column 5), which showed a lighter blue coloration. Strains possessing R42Q (column 6), W59L (column 4), D37V (column 3), and Y26F (column 8) all failed to show any blue coloration in the plate assays. Competition experiments were also performed in the presence of 1 μM Mtx−SLF with a 10-fold and 100-fold excess of SLF, but at best a minor decrease in the β-galactosidase signal was observed for the wild-type FKBP12-containing strain or any of the mutant strains at the higher concentration (Supporting Information).

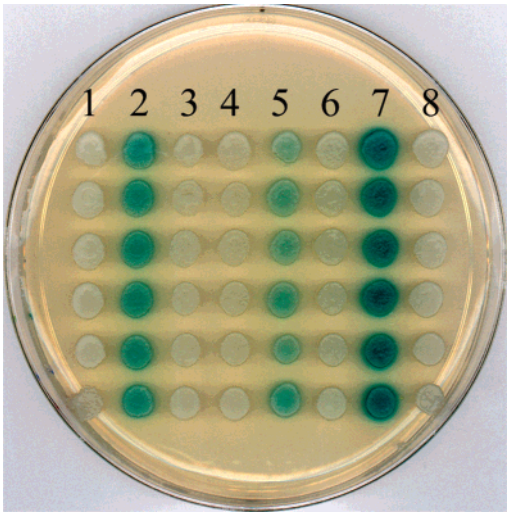

FIGURE 4: X-gal plate assay of Mtx−SLF-induced *lacZ* transcription from strains expressing either wild-type FKBP12 or a mutant of FKBP12. Six separate transformants of each yeast strain were grown in the presence of 10 μM Mtx−SLF for 3 days at 30 °C. All strains are derived from the parent strain V704Y possessing pMW112, but columns 1−8 differ in the protein that is fused to B42: 1, B42; 2, B42−FKBP12; 3, B42−FKBP12 D37V; 4, B42−FKBP12 W59L; 5, B42−FKBP12 F36Y; 6, B42−FKBP12 R42Q; 7, B42−FKBP12 H97L; 8, B42−FKBP12 Y26F.

Protein Expression Levels. To verify that the observed differences in transcription readout were a result of difference binding affinities and not shifted in vivo protein expression level, we prepared a Western blot using an anti-HA mouse antibody, since cloning FKBP12 into the plasmid pMW102 created a translational fusion to the HA epitope. By loading equal amounts of crude cell lysate onto each well, we determined that all proteins were being expressed at similar levels, ruling out the possibility of transcriptional readout varying due to different stability and/or expression levels of FKBP12 and its mutants in vivo (Supporting Information). Furthermore, using purified standards of either LexA or FKBP12, we were able to determine that both the LexA−DHFR and B42−FKBP12 proteins represented about 1% of the total cellular protein being expressed in the yeast cells under galactose induction. This corresponds to protein concentrations of ca. 10 μM for LexA−DHFR and ca. 30 μM for B42−FKBP12 and its mutants (Supporting Information).

Cellular Small Molecule Concentration. To obtain an estimate on the concentration of small molecules that entered yeast cells, we carried out a fluorescence-based approach using Mtx and SLF tagged with the same fluorescein amine probe. By growing the yeast in the presence of either fluorescein methotrexate or SLF−Fl and washing the cells repeatedly to remove any residual media, we were able to isolate the fluorescent small molecules which were contained within the yeast cell. We measured whole cell fluorescence as well as lysing the cells before taking fluorescence measurements by exciting at 496 nm and observing emission at 516 nm. By comparing the fluorescence observed to the fluorescence of known concentrations of SLF−Fl and determining the volume of cells used in the assay, we quantified the concentration of small molecules within the cell and determined that both fluorescein methotrexate and SLF−Fl were present at quite significant concentrations within the cell. At 1 μM small molecule, fluorescein methotrexate was shown to be present within the cells at nearly the same concentration as in the growth media, 1.8 μM, whereas SLF−Fl was shown to have a significantly higher intracellular concentration by almost an order of magnitude, 9 μM. These molecules are not identical to Mtx−SLF; however, we can thus safely assume that Mtx−SLF gets into the cells at about the same concentration as the concentration in the media based on the resemblance of the fluorescent small molecules to Mtx−SLF. This experiment does not indicate the concentration of the molecules within the nucleus of the cell where it is most important to our three-hybrid system and cannot rule out high concentrations with the cell membranes; however, our cellular small molecule concentration experiments do show that Mtx and SLF are quite successful at diffusing into the yeast cells.

DISCUSSION

First reported only in 1989, the yeast two-hybrid assay has rapidly become a commonplace technique for biochemistry and cell biology research (*1*). Focused two-hybrid assays are used both to discover and to define proteins that interact with a target protein of interest. High-throughput approaches have even allowed genome-wide analysis of protein−protein interactions. More recently, so-called three-hybrid assays have sought to extend this genetic assay to RNA− and small molecule−protein interactions. Small molecule three-hybrid systems should allow protein target identification by screening the small molecule drug against a cDNA library. Such an approach could be used to find out not only the target responsible for drug efficacy but also other targets responsible for drug toxicity. Similarly, the three-hybrid assay should provide a high-throughput assay for the directed evolution of proteins with new binding properties by screening a small molecule against a library of protein variants. Structure−activity relationship data for a known ligand−receptor interaction could be obtained by screening the ligand against defined receptor point mutations. Finally, small molecule libraries could be screened against a known protein target. For any of these approaches to succeed, the yeast three-hybrid assay must have a reasonable K_D cutoff and be able to detect a wide range of affinity interactions. For example, ideally lead compounds with micromolar K_D's could be used to identify their protein targets. For screening small molecule libraries, the K_D of the small molecule for the protein target needs to correlate with the levels of transcription activation. Little is known, however, about either criterion. Yeast three-hybrid systems reported to date have relied on very high-affinity ligand−receptor interactions. To our knowledge, there are no systematic studies of the relationship between the ligand−receptor K_D and the transcription readout in yeast three-hybrid systems. Moreover, these studies have bearing on the use of the yeast two-hybrid assay and may shed light on notorious false positives and false negatives.

To analyze the effects of the interaction between FKBP12 and its variants with SLF in vivo, we first had to fully characterize the interactions in vitro. In the course of designing the point mutants of FKBP12, we decided to determine its affinity for the two ligands, the natural product FK506 and the artificially synthesized molecule SLF. This comparison helped us to identify what sort of binding defect

was present as well as if it was specific for SLF or not. Contrary to a previous study, we found that the mutation H87L shows no binding defect in regard to FK506 and actually increases (by 2-fold) the affinity of the protein for SLF (*19*). We suspect that this discrepancy derives from the inhibition of the prolyl-isomerase activity of FKBP12 as a means to measure K_I as opposed to a direct in vitro binding method (fluorescence polarization). The point mutations F36Y and R42Q resulted in intermediate binders for SLF, while F36Y had a more severe binding defect when we used FK506. Y26F showed a moderate binding defect with FK506 (about 4-fold worse than wild type) and a more severe one with SLF (about 11-fold worse than wild type), possibly because this residue is more significant to the binding of SLF than FK506 (indeed, there is evidence for Y26 making a hydrogen bond to the C9 carbonyl of SLF). Moreover, D37V showed an extreme binding defect for FK506 (almost 100-fold worse than wild type), while the defect in binding SLF was not that pronounced (about 9-fold worse than wild type). Conversely, this residue may be more important for the binding of FK506 than SLF. Finally, W59L showed relatively similar binding defects for both ligands (about 9-fold worse than wild type for SLF and 14-fold worse for FK506). With these in vitro data it was then possible to delineate a relationship between binding affinity and transcription activation and to determine cutoff limits for the system.

Of particular interest was the K_D cutoff of the yeast three-hybrid assay. Little is known about the K_D cutoff from existing yeast three-hybrid studies. To date, only model systems with very high affinity interactions have been employed: Mtx−DHFR (K_D = 10 pM); FK506−FKBP12 (K_D = 0.4 nM); SLF−FKBP12 (K_D = 15 nM; IC_{50} = 80 nM); Dex−GR (K_D = 0.5 nM); biotin−streptavidin (K_D = 100 fM); estradiol−ER (K_D = 1.0 nM) (*4*, *10*, *18*, *30*, *31*). All of these pairs have K_D's <1 nM, with the exception of the SLF−FKBP12 pair. However, it should be noted that the Dex−GR K_D in yeast is uncertain since this interaction is dependent on associated heat shock factors. The studies here with SLF and FKBP12 variants suggest that the K_D cutoff is about 50 nM. Both wild-type FKBP12 (15 nM) and the FKBP12 H87L variant (7 nM) show robust transcription activation. The FKBP12 F36Y and R42Q variants (48 and 62 nM, respectively) show low-level activation, and all of the other FKBP12 variants, W59L (140 nM), D37V (140 nM), and Y26F (170 nM), show no detectable transcription activation over background. The K_D cutoff, of course, is dependent on the use of the Mtx−DHFR anchor, where at the concentrations of protein and small molecule assumed to be present in vivo, ca. 10 μM at high CID concentrations, the 10 pM K_D ensures the Mtx end of the CID is completely bound. Consistent with this explanation, the K_D cutoff determined here for the yeast three-hybrid assay agrees with that determined by Golemis and co-workers in their study of the yeast two-hybrid assay. There, the weakest affinity interaction that could be detected was the λ repressor cI variant with a K_D of 50−100 nM. While presumably the K_D cutoff could be lowered by using less sensitive reporter genes as was done for the yeast two-hybrid assay, it is not clear how that the sensitivity could be increased significantly. The most sensitive *lacZ* reporter gene available with eight tandem lexA operators was used here. Both the expression levels of the transcriptional activator fusion protein and the concentration of the small molecule CID were optimized for maximal transcription activation, presumably through optimization of the CID ternary complex concentration bound to DNA. It was ruled out that either a *LEU2* or *URA3* reporter gene could further increase the sensitivity. Given the high concentrations (ca. 10 μM) of both the LexA−DHFR and B42−FKBP12 fusion proteins and the Mtx−SLF CID in vivo, it seems unlikely that endogenous yDHFR (10 nM) or yFKBPs (700 nM for yFKBP12) (*32*) contribute to the K_D cutoff. These endogenous proteins could at most bind to a small percentage of total CID, no matter what their affinity.

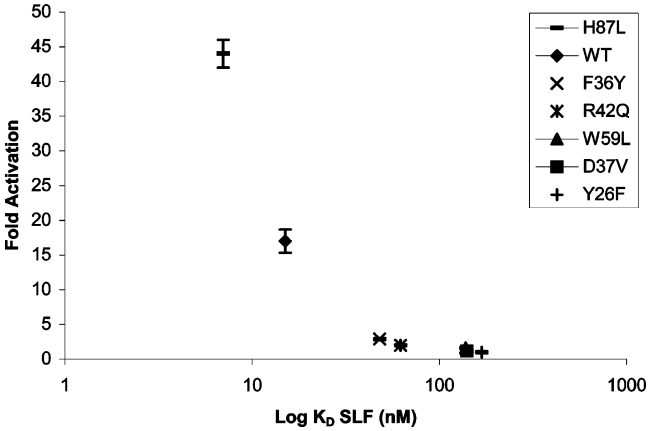

FIGURE 5: Binding affinity versus transcription activation. The dissociation constants obtained in the fluorescence polarization assay are plotted in logarithmic scale on the *X*-axis. Fold activation obtained from assaying β-galactosidase activity in the yeast three-hybrid system was plotted on the *Y*-axis. There is a correlation between transcription activation and binding affinity over a small range of K_D's.

Despite the complexity of transcription activation, in *S. cerevisiae*, the K_D of the SLF−FKBP12 interaction does correlate with the levels of transcription activation in the yeast three-hybrid assay (Figure 5). While the FKBP12 variants Y26F, D37V, W59L, and R42Q all cannot be detected by this assay, the levels of transcription activation increase going from the F36Y (48 nM) to the wild type (15 nM) to the H87L variant (7 nM) on a logarithmic scale. The dynamic range under the set of conditions employed here spans only 1 order of magnitude. As discussed above, the dynamic range presumably could be expanded as for the yeast two-hybrid assay by using reporter genes with different sensitivities. The levels of transcription activation, however, for the F36Y, wild type, and H87L variants are all well outside experimental error for one another. Interestingly, when the levels of transcription activation are plotted as a function of CID concentration for these three variants, it is not that the higher affinity interaction activates at lower concentrations but rather that all three variants show the same CID concentration profile, only the absolute levels of transcription activation are always greater the higher the affinity of the variant.

The mechanism of transcription activation in *S. cerevisiae* is complex and not completely understood (*33*), making it difficult to model the relationship between ligand−receptor K_D and transcription activation in the yeast three-hybrid assay. However, it is interesting to consider the relationship between the ligand−receptor K_D and transcription activation

by simply thinking about two-component binding. Transcription activation presumably occurs when a reporter gene−DBD−CID complex binds to the AD. Given the very high affinity of the Mtx−DHFR anchor pair, the high affinity of the LexA dimer for its operator, and the high concentrations of the three-hybrid components in vivo, it is reasonable to assume the system lies far toward reporter gene−DBD−CID complex formation. Thus, transcription activation can be described in terms of binding of the reporter gene−DBD−CID complex to the AD (with excess DBD and CID in essence as competitor). When the concentration of the AD is less than or equal to$\leq$ the K_D, then the amount of complex should reflect the K_D of the CID−AD interaction, here SLF−FKBP12. But when the concentration of the AD is greater than or equal to the K_D, the amount of complex simply reflects the concentration of the AD. The results obtained here are consistent with this latter scenario. The DBD and AD are being expressed at high levels in the cell, ≥ 10 μM. It is this concentration that is effectively limiting the K_D cutoff of both the two- and three-hybrid systems. This explanation accounts for the observation that ligand−receptor pairs with different K_D's all show the same optimal CID concentrations for transcription activation. For an equilibrium phenomenon, the K_D cutoff is bounded by the effective concentrations that can be achieved. This reasoning also explains why the full dynamic range of the *lacZ* transcription assay is spanned by FKBP12 variants that vary only 1 order of magnitude in their K_D for SLF. For an equilibrium phenomenon, small differences in free energy lead to large population differences. In its present form, the *n*-hybrid assay is not ideally suited for discriminating binding interactions on the basis of their affinity. To further increase the sensitivity and dynamic range of this assay, it will be important to think about the readout as an equilibrium phenomenon. It may be possible, for example, to exploit cooperative interactions to tune the K_D range. This simple two-component binding analysis, however, cannot explain our other observation of the transcription activation data, namely, that the magnitude of transcription activation varies as a function of the SLF−FKBP12 K_D.

CONCLUSION

In summary, by using a series of FKBP12 variants designed to span several orders of magnitude in their K_D for SLF, we have determined the sensitivity of the Mtx−DHFR yeast three-hybrid system. The upper limit of K_D's that can be detected by this assay is ca. 50 nM. This K_D cutoff is high enough to realistically use the three-hybrid assay for drug discovery. The levels of transcription activation correlate with the K_D of the ligand−receptor interaction as detected in vitro. The dynamic range, however, is limited to only an order of magnitude. For *n*-hybrid assays to be useful for discriminating interactions based on their affinities, likely several distinct systems will be needed to tune through different strength interactions. These results can be readily extrapolated to RNA yeast three-hybrid and yeast two-hybrid assays. Yeast *n*-hybrid assays have emerged as integral tools for the discovery and study of protein interactions. These results provide a quantitative framework for interpreting both directed and high-throughput *n*-hybrid experiments.

SUPPORTING INFORMATION AVAILABLE

Proton NMR spectrum of SLF−fluorescein, binding curves for FKBP12 and SLF−fluorescein and for FKBP12 and FK506−fluorescein, Western blot showing the protein expression level of the B42−FKBP12 variants, competition experiments, Western blots to quantify LexA−DHFR and B42−FKBP12, and an estimate of cellular small molecule concentration. This material is available free of charge via the Internet at http://pubs.acs.org.

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