Identification of an 11-kDa FKBP12–rapamycin-binding domain within the 289-kDa FKBP12–rapamycin-associated protein and characterization of a critical serine residue

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ABSTRACT   Complexed with its intracellular receptor, FKBP12, the natural product rapamycin inhibits G_1 progression of the cell cycle in a variety of mammalian cell lines and in the yeast *Saccharomyces cerevisae*. Previously, a mammalian protein that directly associates with FKBP12–rapamycin has been identified and its encoding gene has been cloned from both human (designated FRAP) [Brown, E. J., Albers, M. W., Shin, T. B., Ichikawa, K., Keith, C. T., Lane, W. S. & Schreiber, S. L. (1994) *Nature (London)* **369,** 756–758] and rat (designated RAFT) [Sabatini, D. M., Erdjument-Bromage, H., Lui, M., Tempst, P. & Snyder, S. H. (1994) *Cell* **78,** 35–43]. The full-length FRAP is a 289-kDa protein containing a putative phosphatidylinositol kinase domain. Using an *in vitro* transcription/translation assay method coupled with proteolysis studies, we have identified an 11-kDa FKBP12–rapamycin-binding domain within FRAP. This minimal binding domain lies N-terminal to the kinase domain and spans residues 2025–2114. In addition, we have carried out mutagenesis studies to investigate the role of Ser[2035], a potential phosphorylation site for protein kinase C within this domain. We now show that the FRAP Ser[2035] → Ala mutant displays similar binding affinity when compared with the wild-type protein, whereas all other mutations at this site, including mimics of phosphoserine, abolish binding, presumably due to either unfavorable steric interactions or induced conformational changes.

By inhibiting intermediate steps in signal transduction leading to G_1 progression of the cell cycle in a variety of mammalian cell lines (1–4) and in the yeast *Saccharomyces cerevisiae* (5–7), rapamycin may illuminate the events that link mitogenic signaling and the cell cycle machinery. Association with an intracellular FK506- and rapamycin-binding protein (FKBP12) is necessary for the actions of rapamycin (1, 2, 5–7), which has been shown to block the activation of p70 S6 kinase (p70[S6K]) (4, 8–10) and cyclin-dependent kinases (11–14). These downstream proteins, however, have been shown not to be the direct target of the FKBP12–rapamycin complex. Recently, a mammalian protein that directly associates with FKBP12–rapamycin was isolated and its encoding gene was cloned independently from human and rat by two research groups (15, 16). The human protein is designated FRAP (FKBP12–rapamycin-associated protein) (15), and the rat protein, RAFT1 (rapamycin and FKBP12 target 1) (16), has >98% sequence identity to FRAP. Human FRAP is a 289-kDa protein composed of 2549 aa. It is highly related to two gene products from *S. cerevisiae*, TOR1/DRR1 and TOR2/DRR2 (7, 17–19), identified through rapamycin-resistant mutations. FRAP is 43% identical to TOR1/DRR1 and 45% identical to TOR2/DRR2. The C-terminal 660 aa of FRAP display a

greater homology to TOR1/DRR1 (57% identity) and TOR2/DRR2 (59% identity), which also have homology to several known phosphatidylinositol (PI) kinases, including mammalian PI 3-kinase (20) and a yeast PI 3-kinase, VPS34 (21). These similarities imply that this C-terminal portion of FRAP may contain a lipid kinase (LK) domain. A potential site for phosphorylation by protein kinase C (PKC) is present in TOR1 (Ser[1972]-Arg) and TOR2 (Ser[1975]-Arg), and mutations at Ser[1972] [Ser[1972] → Arg (S1972R) and S1972N] in TOR1 or at Ser[1975] (S1975I) in TOR2 render yeast cells resistant to rapamycin (17, 19). This Ser-Arg site is conserved in FRAP, which gives rise to the possibility of its involvement in the interaction between FRAP and FKBP12–rapamycin.

Here we report mapping of the minimal human FKBP12–rapamycin-binding domain (FRB domain) in FRAP through a proteolysis approach and utilizing an *in vitro* transcription/translation assay system. A small, 90-aa fragment of FRAP is shown to be sufficient to confer specific FKBP12 binding in a rapamycin-dependent fashion, with undiminished binding affinity compared with larger fragments. Further, we have probed the role of Ser[2035] in the FKBP12–rapamycin–FRAP binding interactions; this residue is most probably located in the interface between FRAP and FKBP12–rapamycin, and replacement with any residue that contains a larger side chain, including mimics of phosphoserine, abolishes binding activity, presumably due to steric hindrance or an induced conformational change.

MATERIALS AND METHODS

Plasmids, Bacteria, and DNA Manipulations. The vector pGEX-2T (Pharmacia) was used for expression of glutathione *S*-transferase (GST) fusion proteins in *Escherichia coli* strain BL21. *In vitro* transcription/translation was carried out on the vector pET-5a, which contains a phage T7 promoter (22). Site-specific mutagenesis was performed according to Kunkel (23) with uracil-containing pBS/SK-derived DNA template isolated from the *E. coli* strain CJ236. Restriction enzymes were purchased from New England Biolabs.

Purification of GST Fusion Proteins. cDNAs encoding human FKBP12 (24) and the 12-kDa FRAP fragment (FRAP12) were subcloned into pGEX-2T for the expression and purification of GST-FKBP12 (GFK) and GST-FRAP12, as well as FRAP12, according to standard procedures (manual from Pharmacia).

Partial Purification of Proteolytic Bovine FRAP Fragments. FRAP was isolated from fresh bovine brain (15). Brain homogenate was loaded onto an S-Sepharose (Pharmacia) column equilibrated with PIP buffer (50 mM sodium phos-

phate/2 mM EDTA/2 mM EGTA/25 mM NaF/100 μM Na$_3$VO$_4$/25 mM β-glycerophosphate/2 mM dithiothreitol/ 0.3% Triton X-100) at pH 7.2 and eluted with PIP containing 1 M NaCl at pH 8.0. The eluate was treated with trypsin ($\approx$0.001%, wt/vol) at 4°C for 40 min, and GFK–rapamycin or GFK–FK506 was added at 100 nM. The mixture was incubated at 4°C for 15 min and then loaded onto a glutathione-Sepharose column. The column was subsequently washed and the Sepharose beads were suspended in SDS sample buffer and boiled for 5 min. After removal of the beads, the eluted proteins were analyzed by SDS/PAGE.

In Vitro **Transcription and Translation.** Various segments of the human FRAP cDNA were amplified by PCR and subcloned into the vector pET-5a between the *Bam*HI and *Eco*RI restriction sites. The resultant plasmids were linearized at the *Eco*RI site and used as templates for *in vitro* transcription (RiboMax T7 system; Promega). The mRNA thus obtained was purified by phenol/chloroform extraction and ethanol precipitation and then added to rabbit reticulocyte lysates containing [^{35}S]methionine for *in vitro* translation by standard procedures (Promega). The translated lysates were used directly in binding assays.

FKBP12–Rapamycin-Binding Assay. To 1 ml of TN buffer (50 mM Tris·HCl, pH 8.0/1 M NaCl) containing 1 μM GFK–rapamycin (1:1), 10 μl of translated lysate or protein purified from *E. coli* was added and incubated on ice for 5–15 min. As controls, GFK–FK506 (1:1) or GFK alone was used instead of GFK–rapamycin. The incubated binding mixtures were passed through 50 μl of glutathione-Sepharose in minicolumns. The column matrices were then washed with 1 ml of TN buffer three times, suspended in SDS sample buffer, and boiled for 5 min. The samples were subsequently analyzed by SDS/PAGE followed by autoradiography, phosphor imaging, or silver staining. To determine equilibrium binding constants, similar assays were carried out with various concentrations of GFK–rapamycin (1:1) and the same amount of translated lysates, which gave rise to a final concentration of FRAP fragments of <0.5 nM. The results were quantitated by phosphor imaging.

CD Spectroscopy. Spectroscopy was performed on a JASCO J-700 CD spectrometer with a 0.5-mm optical cell over the range 190–260 nm. The protein concentration was 1.2 mg/ml in 25 mM Tris·HCl, pH 7.5/150 mM NaCl/1 mM dithiothreitol.

RESULTS

C Terminus of FRAP Contains the FRB Domain. Partial purification of bovine brain FRAP by use of GFK rapamycin and glutathione affinity chromatography has been reported (15). Using the same strategy combined with tryptic digestion of crude proteins before addition of GFK–rapamycin, we were able to identify a spectrum of proteolytic fragments of FRAP with FKBP12–rapamycin binding affinity. In addition to full-length FRAP, which ran as a 220-kDa band in an SDS/7.5% polyacrylamide gel, protein bands of approximately 200, 140, 120, 75, and 40 kDa appeared after tryptic digestion (Fig. 1*A*). These proteins were retained on glutathione-Sepharose after incubation with GFK–rapamycin, but not with GFK–FK506. After electroblotting of the proteins to a poly(vinylidene difluoride) membrane, the 75-kDa fragment was isolated and subjected to N-terminal microsequencing, the result of which aligned the N terminus of this fragment with aa 1819 of human FRAP (Fig. 1*B*). The high degree of sequence identity between bovine and human FRAP has been shown previously (15). Therefore, the 75-kDa fragment that contains the FRB domain is composed of the last 730 aa of FRAP. Sequence alignment of FRAP and its yeast homolog TOR1 (19) in this region reveals a highly hydrophilic sequence, presumed to be a loop on FRAP, followed by sequences of strong homology
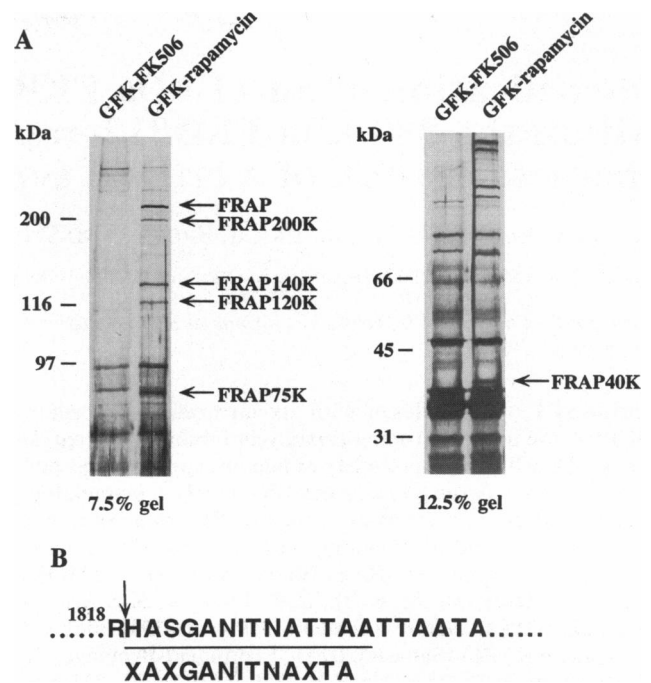

FIG. 1. Proteolytic analysis of FRAP. (*A*) FKBP12–rapamycin-binding FRAP fragments generated by digestion with trypsin are retained on glutathione-Sepharose in the presence of GFK–rapamycin (lane 2), but not in the presence of GFK–FK506 (lane 1). Bands corresponding to full-length FRAP and 200-kDa, 140-kDa, 120-kDa, and 75-kDa FRAP fragments (FRAP200K, etc.) are indicated (arrows) on the SDS/7.5% polyacrylamide gel (*Left*); the 40-kDa fragment is shown on the 12.5% gel (*Right*). (*B*) N terminus of the 75-kDa fragment aligns with residue 1819 of FRAP. The upper sequence was deduced from human FRAP cDNA; the lower sequence resulted from N-terminal microsequencing of the 75-kDa fragment. The original data obtained from microsequencing indicated contamination of two GST proteins (with and without the initial methionine) in the protein preparation. The sequence shown here was deduced from the original sequence by subtracting contaminating GST sequences. Arrow indicates the trypsin cleavage site.

between these two proteins, which is consistent with the susceptibility of this region to proteolysis and the existence of a conserved functional domain. We considered that the minimal FRB domain may be smaller than 75 kDa on the basis of the FKBP12–rapamycin-binding capacity of a 40-kDa fragment (Fig. 1*A*). However, analysis of this smaller proteolytic fragment was precluded by the fact that it migrated very closely with GFK in the SDS/polyacrylamide gel.

Characterization of the Minimal FRB Domain. To further map the FRB domain in FRAP, we have developed a rapid and convenient assay method. Various regions of the cDNA were amplified by PCR and subcloned into an *in vitro* transcription/ translation vector driven by the T7 promoter. Radiolabeled FRAP fragments were produced in reticulocyte lysates in the presence of [^{35}S]methionine and directly subjected to binding assays using GFK. Since the last 300 or so residues at the C terminus are predicted by sequence homology (17, 18) to form a LK domain, we dissected the 75-kDa fragment into an N-terminal 46-kDa and a C-terminal 29-kDa fragment and tested their FKBP12–rapamycin-binding affinity. The N-terminal 46-kDa fragment bound FKBP12 in a rapamycin-dependent manner; moreover, it did not bind FKBP12 alone or the FKBP12–FK506 complex (Fig. 2). The C-terminal fragment, the presumed LK domain, did not bind FKBP12 or either of the FKBP12–drug complexes. It has been reported that a point mutation converting Ser[1972] to arginine in TOR1 (or Ser[1975] in TOR2) leads to rapamycin resistance in yeast, and recent experiments have shown that the S1972R mutant
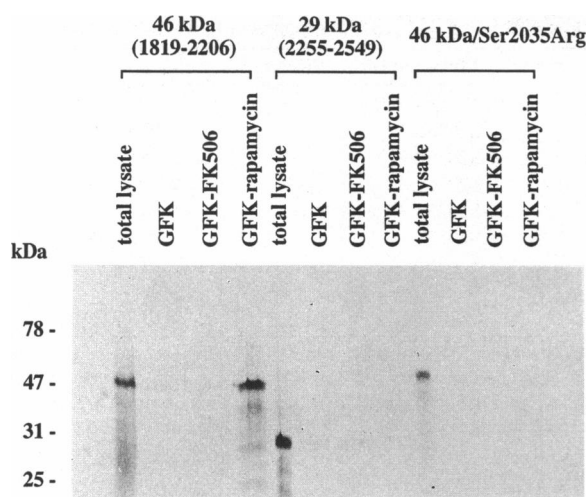
FIG. 2.   Assay of FKBP12–rapamycin binding by a 46-kDa FRAP fragment (aa 1819–2206), a 29-kDa fragment (aa 2255–2549, LK domain), and the 46-kDa fragment with a S2035R mutation. Assays were performed with 1 μM GFK or GFK–drug (1:1) and analyzed by SDS/12.5% PAGE. For each fragment, the translated lysate was loaded on the first lane, followed by samples treated with GFK alone, GFK–FK506, and GFK–rapamycin.

abrogates binding of the TOR1 protein to FKBP12–rapamycin (X.-F.Z., unpublished work). This serine is conserved in FRAP (residue 2035), suggesting its possible involvement in the interaction with FKBP12–rapamycin. Indeed, S2035R mutation in the 46-kDa fragment abolished its affinity for FKBP12–rapamycin (Fig. 2). Having identified this crucial residue for binding, we systematically truncated the 46-kDa fragment and carried out binding assays using fragments encompassing this serine (Fig. 3). The smallest fragment that binds FKBP12–rapamycin spanned aa 2025–2114, with a calculated molecular mass of 11 kDa. Further deletion of this fragment either from the N terminus (10 aa) or the C terminus (25 aa) abrogated binding activity completely. To determine whether or not this minimal FRB domain has similar affinity for its target when compared with larger fragments, we estimated the dissociation constants of the 46-kDa (aa 1819–2206), 20-kDa (aa 1995–2174), and 11-kDa fragments for binding to FKBP12–rapamycin. The binding curves were generated from binding assays as described above with subnanomolar concentrations of labeled FRAP fragments and various concentrations of GFK–rapamycin (1:1) (Fig. 4; autoradiographs of SDS/polyacrylamide gels are shown as insets). The three fragments displayed very similar FKBP12–rapamycin binding affinity, with equilibrium dissociation constants

around 5 nM (Fig. 3). Therefore, the minimal FRB domain has undiminished affinity for its target compared with larger fragments. Although it is possible that these affinities are overestimated due to the nature of our assay method, they should at least be useful in a comparative context.

Role of Ser[2035] in FKBP12–rapamycin–FRAP Binding Interactions. It has been postulated that the conserved serine residue at 2035 in FRAP (or RAFT) or 1972 in TOR1 (or 1975 in TOR2) may be a potential PKC phosphorylation substrate and thus a crucial site for regulation of FRAP and TOR biological activity (19). To probe the possible involvement of a phosphoserine at this site in the interaction between FRAP and FKBP12–rapamycin, we introduced several mutations and examined the mutants' ability to bind FKBP12–rapamycin. A 20-kDa fragment (aa 1995–2174) was used for the mutation studies (Table 1). The only mutation at Ser[2035] that still retained FKBP12–rapamycin-binding activity is alanine. The aspartate and glutamate mutants, intended mimics of phosphoserine, showed no binding affinity. Thus, all residues larger than serine at this site abolished binding activity, apparently due to a steric effect or an induced conformational change. When the binding affinity of the S2035A mutant was compared with that of the wild-type protein (Fig. 5), the mutant displayed an almost identical concentration dependence for FKBP12–rapamycin binding and had a comparable equilibrium dissociation constant (Table 1). From these results, although we cannot demonstrate whether Ser[2035] is a regulatory phosphorylation site, we can conclude that FKBP12–rapamycin binds FRAP with nonphosphorylated Ser[2035]. The W2027R mutation was also found to abrogate the binding of FRAP to FKBP12–rapamycin. This site resides in the minimal FRB domain, consistent with an involvement in FKBP12–rapamycin binding. In addition, conversion of Arg[2036], which is part of the potential PKC site, to leucine did not alter the binding affinity.

Secondary Structure of the Minimal Binding Domain. For the purpose of structural studies, a 12-kDa FRB domain (aa 2015–2114) of FRAP was expressed and purified from *E. coli* cells by standard GST-fusion-protein isolation methods. The fusion protein was highly soluble when expressed at room temperature, indicating its proper folding in *E. coli* cells. The purified 12-kDa protein was indeed active; it displayed specific FKBP12–rapamycin binding affinity (Fig. 6B). Since *E. coli* cells lack the eukaryotic machinery required for posttranslational modifications of mammalian proteins, the activity of this protein is again in concord with the model that phosphorylation of Ser[2035] of FRAP is not required for binding to FKBP12–rapamycin. The CD spectrum of this FRB domain between 200 and 230 nm is characteristic of a polypeptide composed mainly of α-helix (25, 26), which is consistent with a Chou–Fasman (software by Genetics Computer Group)

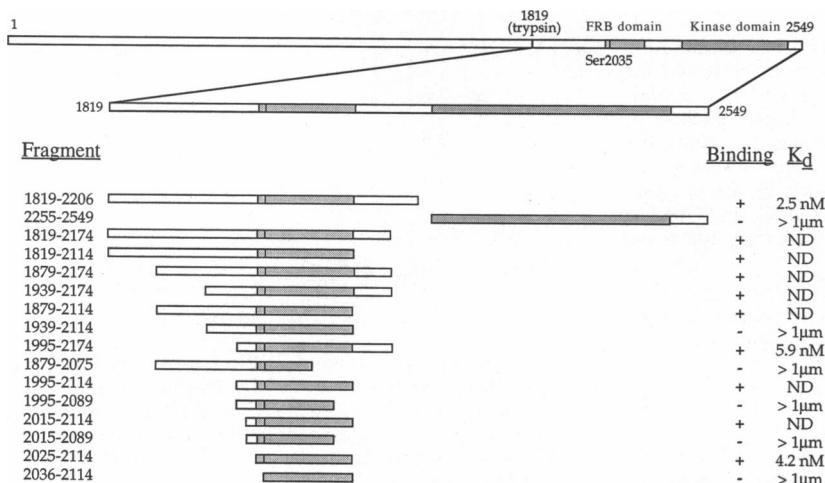| Fragment	Binding	K_d
1819–2206	+	2.5 nM
2255–2549	–	>1μM
1819–2174	+	ND
1819–2114	+	ND
1879–2174	+	ND
1939–2174	+	ND
1879–2114	+	ND
1939–2114	–	>1μM
1995–2174	+	5.9 nM
1879–2075	–	>1μM
1995–2114	+	ND
1995–2089	–	>1μM
2015–2114	+	ND
2015–2089	–	>1μM
2025–2114	+	4.2 nM
2036–2114	–	>1um

FIG. 3.   Mapping of the minimal FRB domain of FRAP by binding assays. Fragments are identified by their starting and ending residue numbers. Dissociation constants (K_d) for the fragments 1819–2206, 1995–2174, and 2025–2114 were derived from the data in Fig. 4. ND, not determined. The minimal binding domain spans residues 2025–2114, N-terminal of the putative kinase domain.
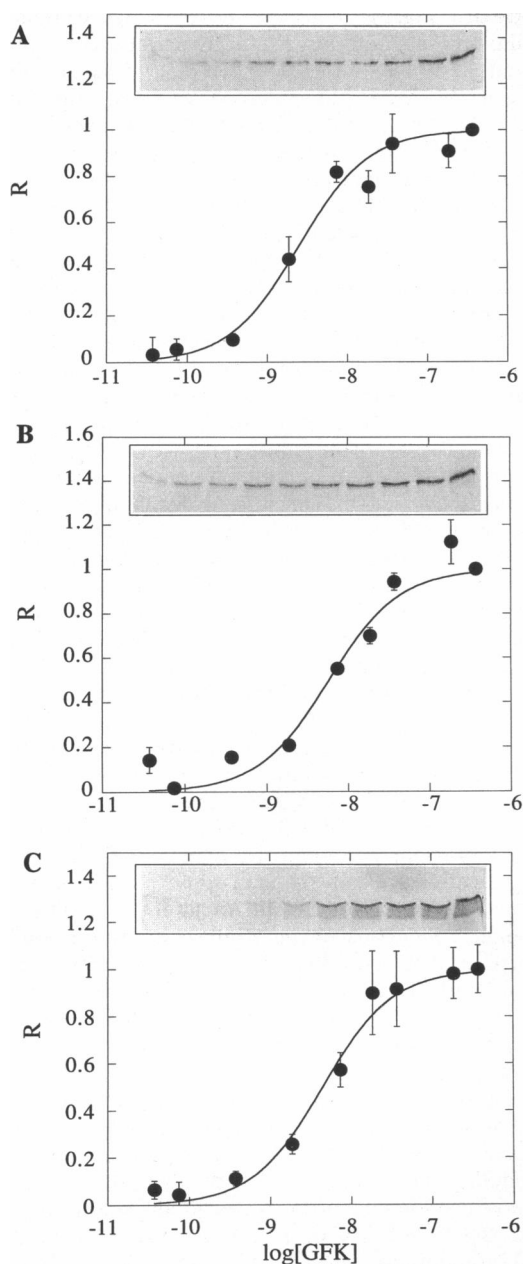
FIG. 4. Determination of FKBP12–rapamycin binding affinity for three FRAP fragments. Binding curves for the 46-kDa (aa 1819–2206) (*A*), 20-kDa (aa 1995–2174) (*B*), and 11-kDa (aa 2025–2114) (*C*) fragments are shown. The fractional saturation of binding, R, was determined as D/D_{max}, where D and D_{max} were the band densities of a bound fragment at the indicated concentration and a saturating concentration of GFK–rapamycin, respectively, both corrected for background in the absence of GFK–rapamycin. *Insets* show autoradiographs of the corresponding SDS/polyacrylamide gels. Each set of data was the average of three independent experimental results; the standard deviations are shown as error bars. The curves were generated by fitting the data to the following equation by the program KALEIDAGRAPH 3.0 (Synergy Software): $R = \log[\text{GFK}]/(\log[\text{GFK}] + \log K_d)$. The dissociation constants (K_d) obtained from the curve fittings are summarized in Table 1.

secondary-structure prediction based on the primary sequence indicating that 80% of the sequence forms α-helical structures.

DISCUSSION

Human FRAP (or rat RAFT) (15, 16) is a large single polypeptide of 2549 aa. Using a proteolysis approach, we have

Table 1. Binding of FKBP12–rapamycin by FRAP mutants

Mutation	Binding	K_d, nM
None	+	2.5*
S2035R	−	>1000
S2035D	−	>1000
S2035E	−	>1000
S2035T	−	>1000
S2035I	−	>1000
S2035A	+	2.9†
R2036L	+	ND
W2027R	−	>1000

Mutations were introduced into a 20-kDa fragment (aa 1995–2174) which was then subcloned into pET-5a for subsequent *in vitro* transcription and translation. The binding assays were carried out as described in *Materials and Methods*. ND, not determined.
*Derived from the data in Fig. 3.
†Derived from the data in Fig. 4.

dissected this protein and identified the FRB domain within the C-terminal 730 aa of the protein. Further analysis utilizing an *in vitro* transcription/translation system has allowed us to map the minimal binding domain to a fragment composed of only 90 aa, residues 2025–2114, with a calculated molecular mass of 11 kDa. This small fragment binds specifically to the FKBP12–rapamycin complex, with an affinity [equilibrium dissociation constant (K_d) of 3 nM] comparable to that of larger FRAP fragments. Interestingly, a longer fragment that contained this minimal binding domain did not show any observable binding (aa 1939–2114; Table 1), possibly due to improper folding caused by the extra N-terminal sequence. Proper folding of a 12-kDa FRB domain expressed in *E. coli* has been demonstrated by its ability to bind FKBP12–rapamycin and by its CD spectrum. The ability to produce large quantities of pure active protein from *E. coli* should greatly facilitate structural investigations of the FRB domain and the FKBP12–rapamycin–FRB domain complex.

On the basis of the rapamycin-resistant phenotype of yeast cells encoding an S1972R or S1972N mutation on their *TOR1* gene (or S1975I on *TOR2*) (17, 19), this serine residue has been speculated to play an important role in functional regulation. One possible mechanism is through serine phosphorylation, as it has been suggested to be a potential PKC site (19). *In vitro* experiments have demonstrated the binding of TOR1 to FKBP12–rapamycin and the inability of S1972R mutant to bind FKBP12–rapamycin (X.-F.Z., unpublished work). Since this residue is also conserved in FRAP, we have used muta-
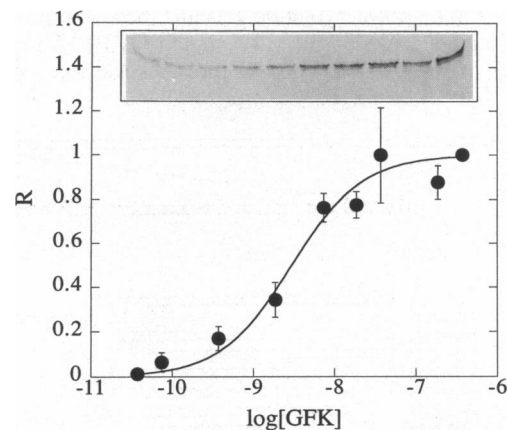

FIG. 5. Determination of FKBP12–rapamycin binding affinity for the S2035A mutant of the 20-kDa fragment (aa 1995–2174). Data processing was as described in Fig. 3 legend. Data were the average of three independent experimental results; the standard deviations are shown as error bars. *Inset* shows an autoradiograph of the corresponding SDS/polyacrylamide gel.
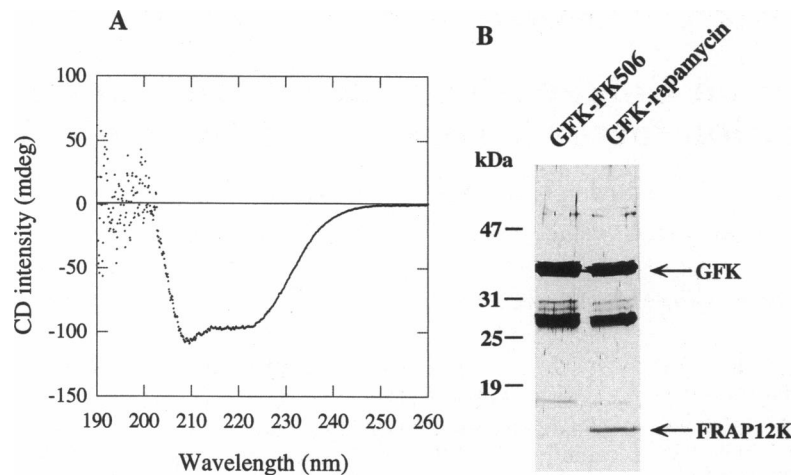
FIG. 6. Characterization of a 12-kDa FKBP12–rapamycin-binding domain (aa 2015–2114) expressed in *E. coli*. (*A*) CD spectrum measured between 190 and 260 nm. The protein concentration was 1.2 mg/ml in 25 mM Tris·HCl, pH 7.5/150 mM NaCl/1 mM dithiothreitol. mdeg, Millidegrees. (*B*) Binding to FKBP12–rapamycin was analyzed by SDS/PAGE followed by silver staining. Lane 1, GFK–FK506; lane 2, GFK–rapamycin. Multiple protein bands that are not identified here are contaminants from the GFK preparation, intensified due to the large amount being used.

tional studies to investigate its role in the binding of FRAP to FKBP12–rapamycin. As expected, the S2035R mutation abolished the FKBP12–rapamycin-binding activity of a 20-kDa fragment of FRAP. Furthermore, conversion of this serine to aspartate, glutamate, threonine, or isoleucine abrogated the binding activity, whereas an alanine mutant displayed the same FKBP12–rapamycin-binding activity as in the wild-type protein. Thus, Ser[2035] in FRAP appears to be at the interface of FRAP and the FKBP12–rapamycin complex, and residues with a larger side chain may generate steric hindrance or induce a conformational change that abolishes the binding completely. The possibility that Ser[2035] phosphorylation is required for FRAP to bind FKBP12–rapamycin (19) is eliminated. However, it is still possible that phosphorylation and dephosphorylation of this serine regulate FRAP biological function and that FKBP12–rapamycin binding of the dephosphorylated state prevents its phosphorylation and therefore blocks its signal-transducing function. It is noteworthy that the FRB domain and its critical serine residue lie outside, and adjacent to, FRAP's kinase domain. This situation is reminiscent of the regulatory subunit of the cAMP-dependent kinase, which has a serine residue that when altered to residues other than alanine prevents its inhibitory binding to the catalytic subunit (27). Whether or not the FRB domain has a similar regulatory function is an intriguing question.

Note Added in Proof. Two reports have been published describing the FKBP12–rapamycin-binding properties of a 133-aa fragment of mouse FRAP (28) and a 196-aa fragment of TOR2 (29), both using the yeast two-hybrid system. Our direct binding data are consistent with their observations.

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