The FKB12-Rapamycin-binding Domain Is Required for FKB12-Rapamycin-associated Protein Kinase Activity and G\textsubscript{1} Progression\textsuperscript{*}

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The immunosuppressant rapamycin, in complex with its cellular receptor FKB12, targets the cellular protein FKB12-rapamycin-associated protein/mammalian target of rapamycin/rapamycin and FKB12 target 1 (FRAP/mTOR/RAFT1) and inhibits/delays G\textsubscript{1} cell cycle progression in mammalian cells. As a member of the novel phosphatidylinositol kinase-related kinase family, FRAP's kinase activity is essential for its signaling function. The FKB12-rapamycin binding (FRB) domain in FRAP is also speculated to play an important role in FRAP function and signaling. However, the biochemical and physiological functions of FRB, as well as the mechanism for rapamycin inhibition, have been unclear. The present study focuses on investigation of FRB's role and the functional relationship between FRB domain and kinase domain in FRAP. Microinjection of purified FRB protein into human osteosarcoma MG63 cells results in a drastic blockage of the G\textsubscript{1} to S cell cycle progression; such a dominant negative effect is reversed by a point mutation (Trp\textsuperscript{2027} → Phe). The same mutation also abolishes kinase activity of FRAP without affecting ATP binding, and truncation studies suggest that upstream sequences including FRB are required for kinase activity in vitro. Given these data, we propose a model for FRAP function, in which the FRB domain is required for activation of the kinase domain, possibly through the interaction with an upstream activator. In addition, our observations provide direct evidence linking FRAP function to G\textsubscript{1} cell cycle progression.

Mammalian cell proliferation is regulated by extracellular mitogens via multiple signal transduction pathways. One such pathway leads to the up-regulation of protein synthesis, which is essential for G\textsubscript{1} progression of the cell cycle (1–3). At least two proteins involved in regulating the translational machinery have been found to lie downstream of this pathway: the p70 S6 kinase (p70\textsuperscript{euk})\textsuperscript{1} (4, 5) and an eIF4E binding protein (4E-BP1) (3, 6–8). The immunosuppressant rapamycin inhibits this pathway at a point upstream of p70\textsuperscript{euk} (9, 10) and 4E-BP1 (11–14); this inhibition requires the presence of the cellular protein FKB12 (15) and results in selective reduction of protein synthesis (16–19) and G\textsubscript{1} arrest in a variety of mammalian cells (15), as well as in the yeast Saccharomyces cerevisiae (20, 21).

A major player in the rapamycin-sensitive pathway has been identified as the cellular target of rapamycin-FKB12 complex, designated FRAP (22), RAFT1 (23), or mTOR (24). FRAP belongs to the novel family of phosphatidylinositol kinase (PIK)-related kinases which include Ataxia telangiectasia mutated. Members of this family are involved in a range of essential cellular functions, including cell cycle progression, cell cycle checkpoints, DNA repair, and DNA recombination (25–28). A kinase domain with sequence homology to lipid and protein kinases has been found at the C termini of all members in this family, and the kinase activity is crucial for the functions of these proteins. The FRAP protein is a 289-kDa single polypeptide containing the characteristic C-terminal kinase domain, the activity of which has been shown to be toward serine/threonine residues (10, 14, 29, 30) and it is required for signaling to p70\textsuperscript{euk} and 4E-BP1 (10, 14, 31). However, it is currently unclear how FRAP receives upstream signals and transduces them to the downstream components. Phosphatidylinositol 3-kinase and protein kinase B/Akt have been implicated to relay extracellular mitogenic stimuli to FRAP (32, 33), whereas evidence has also been presented to suggest that FRAP may regulate a nutrient (amino acid)-responsive pathway that is a prerequisite, but not an overlap, of the mitogenic pathway (34).

Further investigation is required to reconcile these observations. In vitro phosphorylations of both p70\textsuperscript{euk} and 4E-BP1 by FRAP have been reported (14, 30); however, definitive evidence is still needed to demonstrate which protein (if either) is the physiological substrate for FRAP.

The FKB12-rapamycin binding (FRB) domain in FRAP has been identified as an 11-kDa segment located N-terminal to the kinase domain (35, 36) (Fig. 1). A serine residue, Ser\textsuperscript{3085}, within this domain is crucial for the interaction between FRAP and FKB12-rapamycin; substitutions at this site with any residues that have larger side chains than serine abolish formation of the ternary complex (35). Consistently, full-length FRAP bearing these mutations displays rapamycin resistance in Jurkat cells (10). Similarly, in the yeast homologues of FRAP, TOR1 and TOR2, mutations at an equivalent serine residue were found by genetic analysis to confer rapamycin resistance (37–39). Crystal structure of the FRB domain indicates a well defined four-helical bundle of FRB is still needed to demonstrate which protein (if either) is the physiological substrate for FRAP.

The biochemical function of FRB and the mechanism for rapamycin-inhibition of FRAP are currently unclear. Although it has been a common speculation that rapamycin may inhibit...
the kinase activity of FRAP, micromolar concentrations of rapamycin required to inhibit FRAP activity in vitro (10, 14, 33) still need to be reconciled with the low IC50 observed in vivo (subnanomolar) and the high affinity of FKBP12-rapamycin-FRAP binding (35). Only 15–50% activity of FRAP toward pT706k and 4E-BP1 in vitro was inhibited by nanomolar concentrations of rapamycin (30). In addition, FRAP bound to rapamycin-FKBP12 is found to retain a robust autokinase activity (41). The absence of FRB domain in other PIK-related kinases also seems to imply that this domain is probably not required for the intrinsic catalytic activity of the kinase.

To understand FRAP function and the mechanism of rapamycin inhibition of its pathway, we have probed the physiological function of FRB in vivo and examined the functional relationship between FRB and the kinase domain in FRAP. Here we report experimental evidence suggesting that the kinase activity of FRAP requires a functional FRB, which may provide a binding site for an upstream activator; this putative function of FRB is necessary for G1 progression in mammalian cells.

EXPERIMENTAL PROCEDURES

Tissue Culture and Bacterial Strain—Both human osteosarcoma MG63 cells and human embryonic kidney 293 cells were maintained in DMEM containing 10% fetal bovine serum (FBS) and penicillin/streptomycin at 37 °C with 5% CO2. MG63 cells were plated onto coverslips in 12–16 h prior to transfection. Six microliters of SuperFect reagent (Qiagen) was used to transfect 2 μg of DNA for each plate. At 24 h after transfection, the cells were lysed in lysis buffer (20 mM sodium phosphate, pH 7.2, 1 mM NaVO3, 25 mM NaF, 25 mM β-glycerophosphate, 2 mM EDTA, 2 mM EGTA, 0.2% Triton X-100, 1 mM DTT, 1 mM p-nitrophenyl-methylsulfonyl fluoride), immunoprecipitated using M2 FLAG affinity gel (Sigma). The immune complexes were washed three times with lysis buffer, two times with lysis buffer containing 0.5 M NaCl, and once with kinase buffer (40 mM Tris-HCl, pH 7.5, 50 mM NaCl, 20% glycerol, 0.1% Triton X-100, 10 mM MnSO4, 1 mM DTT), followed by addition of 20 μl of kinase reaction mixture containing 100 μM ATP, 10 μCi of [γ-32P]ATP, and 2 μg of GST-4E-BP1 in the kinase buffer. The reaction was carried out at 30 °C with rocking for 15 min and stopped by addition of 20 μl of 2× SDS sample buffer. Samples were heated briefly before loaded on SDS-PAGE for autoradiography and Western analysis. To assess ATP binding activity of FRAP, transfection and cell lysis were carried out as above, followed by addition of ATP-agarose (C8-linked; Promega) at 4 °C for 2 h. The matrix was then washed with lysis buffer, eluted with SDS sample buffer, and analyzed by Western blotting.

RESULTS

Kinase Domain Defined by Homology May Not Be Sufficient for Activity of FRAP—To examine the primary structure requirement for FRAP kinase activity, we have generated a series of N-terminal deletion mutants of FRAP (Fig. 1A). These truncated mutant proteins were transiently expressed in HEK293 cells, immunoprecipitated, and assayed for their autophosphorylation activities. Surprisingly, a fragment (1362C) much larger than the predicted kinase domain was required for such activity (Fig. 1B). This fragment includes the FRB domain and a stretch of upstream sequences in addition to the C-terminal kinase domain. The kinase domain alone (2061C) was inactive, and so were the fragments containing both the kinase and FRB domains without additional upstream sequences (1819C and 1967C) (Fig. 1B). It was conceivable that the loss of autophosphorylation might be due to deletion of the autophosphorylation site(s). The autophosphorylation site is currently unknown. However, a loss of 4E-BP1 phosphorylation (data not shown) was observed simultaneously with the loss of autophosphorylation, suggesting that the catalytic activity was abolished in these deletion mutants. Although we cannot rule out the possibility of protein misfolding in these mutants, at least 1819C was still able to bind FKBP12-rapamycin (data not shown). These observations implicate potential involvement of FRB and/or upstream sequences in regulation of the FRAP catalytic activity.

Microinjection of FRB Protein Prevents Cell Cycle Progression into S Phase—Given the obvious significance of FRB domain in FRAP's signaling function as indicated by rapamycin effect, and considering the implications of the kinase activity data, we decided to investigate the function of FRB with a more direct approach. Taking advantage of the large quantities of soluble FRB protein purified from E. coli (35), we examined the effect of introducing excess exogenous FRB into cultured cells by microinjection. Human osteosarcoma MG63 cell line has been chosen for these experiments because of the previously demonstrated anti-cancer effect of rapamycin at 4E-BP1, a key phosphorylation substrate of FRAP, and the central role of the cell cycle (42). Cells synchronized at G0 by serum starvation re-entered the cell cycle upon sucrose stimulation and completed S phase (DNA synthesis) in 25 h (data not shown). As shown in Fig. 2, cells microinjected with 4.5 mg/ml FRB protein no longer entered S phase, as indicated by the lack of DNA synthesis. This effect was apparently not an artifact of
high protein concentrations injected and was specific to the FRB protein, as injection of a nonspecific protein (rabbit IgG) at the same concentration or the indicator fluorescein-dextran alone had no effect on the cell cycle (Fig. 2). The statistical significance of the FRB effect is indicated by data summarized in Table I; more than 80% of FRB injected cells were arrested at G1. The effect of FRB was concentration-dependent; injection of the protein at less than 4 mg/ml concentrations arrested a lower percentage of cells (data not shown). Due to the variation in injection flow rate, injected cells received various amounts of protein even when a constant concentration (4.5 mg/ml) of FRB was used. Normal cell cycle progression was almost always observed in cells with lower amounts of injected protein (as indicated by the fluorescence intensity), again suggesting a concentration dependence.

These observations imply that the free FRB protein acts as a dominant negative factor that inhibits FRAP's normal function, which is presumably essential for G1 progression. Assuming the observed FRB effect is specific, one simple explanation is that this domain in FRAP functions as a binding site for either an upstream activator or a downstream effector; an excess of free FRB titrates off this factor and therefore inhibits FRAP signaling. To confirm the specificity of the FRB effect as well as to characterize the structure-function relationship in FRB, we set out to construct and examine the following FRB mutants.

FKBP12-Rapamycin Binding of Mutant FRB Proteins—Serine 2035 in FRB has been shown to be crucial for rapamycin binding; all mutations at this site containing larger side chains abolish the formation of FKBP12-rapamycin-FRB complex (35). It is obviously of great interest to study the cell cycle effect of FRB mutated at Ser2035. However, given that full-length FRAP carrying Ser2035 mutations can signal to p70S6k and 4E-BP1 normally (10, 14, 31), this mutant is expected to retain wild-type functions other than rapamycin binding. Using the crystal structure of FKBP12-rapamycin-FRB ternary complex (40) as a guide, we designed two other mutants of FRB (Fig. 3A) that could potentially interfere with the presumed effector/activator binding. Tyrosine 2105 is located at the interface between FRB and FKBP12 in the complex and forms interactions with both rapamycin and FKBP12 (40); mutation at this residue may disrupt FRB's normal function if the putative
effect binding is molecularly similar to FKBP12-rapamycin binding. Another site under consideration was tryptophan 2027; a Trp2027 → Arg mutation FRAP was found unable to bind rapamycin-FKBP12. Since this residue is not situated in the vicinity of the rapamycin binding pocket or FRB-FKBP12 interface (35), it is unlikely to be directly involved in rapamycin binding. It is therefore possible that mutations at Trp2027 may perturb the tertiary structure of FRB; such mutants would be expected to abrogate the cellular function of FRB.

Ser2035 → Ile, Tyr2105 → Ala, and Trp2027 → Phe were separately introduced into the FRB cDNA by site-directed mutagenesis using PCR. The mutant FRB proteins were expressed and purified from E. coli and tested for rapamycin binding in a pull-down assay using GST-FKBP12 (35). As shown in Fig. 3B, Ser2035 → Ile FRB did not bind to rapamycin-GST-FKBP12, consistent with earlier observations (36, 43). The loss of binding activity in the Trp2027 → Phe mutant suggests a crucial role of this tryptophan residue in maintaining the tertiary structure of FRB, since this residue is not directly involved in rapamycin binding (40). The Tyr2105 → Ala mutant FRB bound rapamycin-FKBP12 equally as well as did the wild-type protein, which is somehow unexpected, as the crystal structure predicted a critical role for this residue (40). On the other hand, there might be certain degree of affinity decrease resulted from this mutation that was not detected by our qualitative binding assays, which were carried out with concentrations much higher than the wild-type dissociation constant (~5 nM; Ref. 35).

**Trp2027, but Not Ser2035, Is Crucial for FRB’s Cellular Function**—The various FRB mutant proteins analyzed above were microinjected into MG63 cells under the same conditions as described earlier, and the cell cycle progression into S phase was examined in the injected cells. Similar to the wild-type protein, both Ser2035 → Ile and Tyr2105 → Ala mutants caused G1 arrest in cells upon injection (Fig. 4; Table I). The conservation of residues subjected to mutational studies are indicated, and the residues subjected to mutational studies are indicated. B, the binding assays were carried out as described under “Experimental Procedures,” with purified wild-type and mutant FRB proteins. In addition to binding assays in the absence or presence of rapamycin, the pure protein for each FRB was loaded as a control (no rapamycin, no GST-FKBP).

![Figure 3](http://www.jbc.org/)

**Statistical data of cell cycle progression upon microinjection of wild-type and mutant FRB proteins**

| Protein injected | Percentage of cells in S phase |
|------------------|--------------------------------|
| No injection     | 97                             |
| Dextran          | 91.0 ± 3.6                     |
| IgG              | 88.7 ± 6.1                     |
| Wild-type FRB    | 16.2 ± 7.2                     |
| Ser2035 → Ile FRB| 22.4 ± 5.0                     |
| Tyr2105 → Ala FRB| 18.8 ± 6.0                     |
| Trp2027 → Phe FRB| 86.1 ± 9.7                     |

Microinjections of synchronized MG63 cells were carried out as described under “Experimental Procedures” and in legends to Figs. 2 and 4. Three to eight independent experiments were carried out for each injection condition, and 50–200 cells were successfully injected in each experiment. All injected cells were scored for S phase entry by BrdUrd incorporation, and data are presented as the percentage of injected cells in S phase. On average, about 97% cells without injection are in S phase under the same growth conditions.

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2 J. Chen, unpublished observation.
proteins tagged with a FLAG epitope were transiently expressed in HEK293 cells, immunoprecipitated, and subjected to kinase assays. As shown in Fig. 5, the Trp2027→Phe mutation abolished FRAP’s activity in both autophosphorylation and phosphorylation of purified 4E-BP1, whereas the Ser2035→Thr mutation did not affect the kinase activity. A point mutation (Asp2357→Glu) at a conserved catalytic site also inactivated FRAP kinase activity, consistent with previous reports (10, 30).

We further examined the ATP binding capacity of these mutant FRAP proteins using ATP-agarose. As shown in Fig. 6, wild-type and all mutant proteins bound the ATP matrix equally well; addition of free ATP abolished the matrix binding of both the wild-type and Trp2027→Phe mutant proteins, confirming the specificity of the binding. In comparison to protein kinase A, the Asp2357 site in the kinase domain is thought to be crucial for catalysis but not to be involved in ATP binding (44). As expected, Asp2357→Glu mutant FRAP retains wild-type affinity for ATP (Fig. 6). The ATP binding of the Trp2027→Phe mutant FRAP indicates that inactivation of kinase activity by this mutation is not due to a direct perturbation on the structure of the kinase domain. Taken together, an attractive model for FRAP function emerges, where the FRB domain may serve as a binding site for a positive regulator of FRAP kinase activity, although it cannot be excluded that FRB may be an integral part of the kinase domain. Since the kinase activity of FRAP has been shown to be essential for FRAP signaling (10, 14, 31), the behavior of Trp2027→Phe mutation provides a direct link between the observed cell cycle arrest effect upon FRB microinjection and the function of FRAP.

**DISCUSSION**

Rapamycin blocks/delays G1 cell cycle progression in various mammalian cells and S. cerevisiae, presumably through inhibition of a pathway leading to translational regulation. In yeast, rapamycin depletes the pool of polysomes (45) and abolishes protein translation via inhibition of the targets of rapamycin-FKBP12, TOR1 and TOR2. These two proteins have been shown to be absolutely required for G1 progression (20, 21, 39, 45). The mammalian homolog FRAP is believed to function similarly, as deduced from rapamycin inhibition of G1 progression. However, the rapamycin effect in mammalian cells is less straightforward. Different cell lines display varied sensitivity to rapamycin in their growths; only translation of a small subset of mammalian mRNAs is affected by rapamycin (16–19), and to a lesser extent; rapamycin inhibition of p70S6K does not always correlate with cell cycle blockage (46). Our observations of a dominant negative effect on the G1 cell cycle elicited by microinjection of the FRB domain in FRAP provide direct evidence linking FRAP function to G1 progression.

As for all members of the PIK-related kinase family, the kinase activity of FRAP is essential for its function (10, 14, 31). The FRB domain located N-terminal to the kinase domain has been speculated to play an important role in FRAP signaling since it is the target site for rapamycin. Although rapamycin...
inhibition of FRAP kinase activity in vitro has been reported (10, 14, 33), more definitive evidence was needed to link FRB directly to the kinase in FRAP, given that high concentrations of rapamycin were required for such inhibition, and that FRAP bound to FKBP12-rapamycin matrix was still active (41). Our observation that a point mutation (Trp2027 → Phe) in the FRB domain abolishes FRAP kinase activity, both in autophosphorylation and in phosphorylation of 4E-BP1, provides compelling evidence for the involvement of FRB in the function of the kinase domain. The dominant negative effect on the G1 cell cycle elicited by microinjection of the wild-type (but not Trp2027 → Phe) FRB suggests a plausible mechanism for FRB function, in which the FRB domain interacts with an activator of FRAP kinase in vivo and an excess of free FRB titrates off this activator. The observed concentration dependence of FRB effect upon microinjection is consistent with such a model; a threshold concentration would be determined by both the cellular abundance of the putative activator and the affinity of FRAP/activator interaction. The different roles of the two critical residues, Trp2027 and Ser2035, in the FRB domain have provided insight into the interaction between FRB and the putative activator. Although serine 2035 has been shown to locate in the rapamycin binding cleft (35), mutation at this site (Ser2035 → Ile) does not seem to change the physiological function of FRB, whereas Trp2027, with no direct contact with rapamycin or FKBP12, is crucial for FRB function. Thus, the presumed interaction with an activator and rapamycin binding of FRB may not share the same molecular mechanisms. Nonetheless, one can envision that the functional interaction between FRB and the putative activator is abolished by the binding of rapamycin-FKBP12 complex, due to either steric hindrance or conformational change. The lack of complete rapamycin inhibition of FRAP kinase activity in vitro may be due to artifacts of the in vitro systems. Alternatively, partial inhibition may be sufficient for bringing FRAP activity below a threshold level critical for signaling. Finally, we cannot exclude the possibility that FRB has yet another function, which is targeted by rapamycin.

It should be noted that the FRB domain and the kinase domain are highly conserved in the yeast homologs TOR1 and TOR2, where TOR2 has two separate functions: one shared with TOR1 (G1 function) (38, 47) and one unique (actin cytoskeleton function) (48, 49); both functions require the kinase activity, but only the G1 function is sensitive to rapamycin (39). Our model of FRB’s involvement in kinase activity does not seem to apply to TOR2’s function on actin cytoskeleton. It is conceivable that the yeast proteins may differ from the mammalian homologue in their functions despite high sequence homology. However, it is also possible that the regulation of TORs’ G1 function mirrors that of FRAP, whereas TOR2’s actin cytoskeleton function is regulated differently. For instance, the latter function may involve a lipid substrate rather than a protein substrate, thus differentiating two modes of regulation.

An alternative mode of regulation that would explain our current observations equally well is an intramolecular interaction involving FRB and another domain of FRAP (e.g. the kinase domain), rather than another factor. However, we have extensively examined potential interactions between FRB and FRAP or various domains/segments of FRAP (including FRB self-association) using co-immunoprecipitation and yeast two-hybrid methods, and no interaction has been detected. Therefore, although we cannot completely exclude the possibility of intramolecular interactions, it is likely that the FRB domain in FRAP interacts with another factor in vivo. In view of the recent report that insulin stimulates mTOR (FRAP) kinase activity via the protein kinase B pathway (33), the activator-binding model for FRB function is particularly attractive as it may provide a molecular basis for such stimulations. Several scenarios can be envisioned for the mechanism of kinase regulation via FRB. For instance, activator binding at FRB may elicit conformational changes of the kinase domain favorable for catalysis or binding of a substrate; chemical modification (e.g. phosphorylation) of FRB, rather than physical interaction with the activator, may have an impact on the kinase domain; the activator may also be some intracellular component instead of a soluble factor, and localization of FRAP through FRB may be required for kinase activity. Further investigations are under way to examine these possible mechanisms of FRAP regulation.

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