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FKBP12-Rapamycin-associated Protein (FRAP) Autophosphorylates at Serine 2481 under Translationally Repressive Conditions*

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The FKBP12-rapamycin associated protein (FRAP, also RAFT, mTOR) belongs to a family of phosphatidylinositol kinase-related kinases. These kinases mediate cellular responses to stresses such as DNA damage and nutrient deprivation in a variety of eukaryotes from yeast to humans. FRAP regulates G_1 cell cycle progression and translation initiation in part by controlling the phosphorylation states of a number of translational and cell cycle regulators. Although FRAP is known to be phosphorylated *in vivo* and to phosphorylate several proteins (including itself) *in vitro*, FRAP's phosphorylation sites and substrate specificity are unknown. We report here the identification of a FRAP autophosphorylation site. This site, Ser-2481, is located in a hydrophobic region near the conserved carboxyl-terminal FRAP tail. We demonstrate that the COOH-terminal tail is required for FRAP kinase activity and for signaling to the translational regulator $p70^{s6k}$ (ribosomal subunit S6 kinase). Phosphorylation of wild-type but not kinase-inactive FRAP occurs at Ser-2481 *in vivo*, suggesting that Ser-2481 phosphorylation is a marker of FRAP autokinase activity in cells. FRAP autophosphorylation is blocked completely by wortmannin treatment but not by rapamycin treatment, amino acid deprivation, or serum withdrawal, treatments that lead to acute dephosphorylation of eIF4E-binding protein (4E-BP1) and $p70^{s6k}$. Ser-2481 phosphorylation increases slightly upon c-Akt/PKB activation and dramatically upon calyculin A treatment of T-cells. These results suggest that FRAP-responsive dephosphorylation of 4E-BP1 and $p70^{s6k}$ occurs through a mechanism other than inhibition of intrinsic FRAP kinase activity.

In many mammalian cell types, progression through the G_1 stage of the cell cycle is dependent upon the FKBP12-rapamycin associated protein (FRAP,[1] also RAFT, mTOR); inhibition of FRAP by the small molecule rapamycin following its intracellular binding to the immunophilin FKBP12 causes G_1 arrest in these cells (1). The importance of FRAP in cell cycle progression appears to be conserved in most eukaryotic cells including yeast, where inhibition of FRAP homologs Tor1p and Tor2p by rapamycin treatment also results in G_1 cell cycle arrest (1). FRAP may exert its influence over cell cycle progression by indirect regulation of the phosphorylation states of a number of proteins including translational regulators such as the ribosomal subunit S6 kinase ($p70^{s6k}$) (2) and the eIF4E-binding protein (4E-BP1) (3). The binding of FKBP-rapamycin to FRAP causes rapid dephosphorylation of $p70^{s6k}$ and 4E-BP1, resulting in decreased translation of mRNA transcripts possessing polypyrimidine tracts or extensive secondary structure in their 5′- untranslated regions (4). Rapamycin treatment also affects translation in yeast, decreasing protein synthesis to about 10% of untreated levels (5).

FRAP possesses a kinase domain with significant sequence similarity to the lipid kinases, and the known functions of FRAP depend upon FRAP kinase activity. Rapamycin-resistant FRAP point mutants are able to protect $p70^{s6k}$ and 4E-BP1 from rapamycin-induced, FRAP-responsive dephosphorylation, but rapamycin-resistant mutants that are kinase-inactive do not offer this protection (2, 6). In yeast, the known rapamycin-inhibitable functions of Tor1p and Tor2p all depend upon an intact kinase domain, as does the rapamycin-resistant, cell cycle-independent essential function of Tor2p (7).

Although the FRAP kinase domain is closely related to the phosphatidylinositol kinases (PIKs), no lipid kinase activity has been detected, and it appears that FRAP possesses serine/threonine protein kinase activity. Direct phosphorylation of 4E-BP1 by FRAP or an associated kinase has been demonstrated *in vitro* (6, 8). The phosphorylation occurs at Thr-37 and Thr-46, two residues followed by prolines that are known to be phosphorylated *in vivo* (9). FRAP has also been reported to phosphorylate a bacterially expressed fragment of $p70^{s6k}$ at Thr-389, a site known to be dephosphorylated *in vivo* upon rapamycin treatment (8). Thr-389 is surrounded by bulky, hydrophobic residues, and phosphorylation of Thr-389 is reported to be more efficient than the somewhat weak phosphorylation of the 4E-BP1 proline-directed sites (8). Because FRAP can phosphorylate these translational regulators *in vitro*, it has been proposed that FRAP is the mitogen-stimulated kinase responsible for the mitogen-stimulated phosphorylation of 4E-BP1 and $p70^{s6k}$. However, serum stimulation causes a greater than 20-fold activation of $p70^{s6k}$, but the *in vitro* kinase activity of FRAP toward 4E-BP1 is increased only 2–4-fold under similar conditions (8, 10).

It is also possible that FRAP regulates 4E-BP1 and $p70^{s6k}$ phosphorylation indirectly through a serine/threonine protein

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[1] The abbreviations used are: FRAP, FKBP12-rapamycin associated protein; s6k, S6 kinase; 4E-BP1, eIF4E- binding protein; PIK, phosphatidylinositol kinase; PI3K, phosphatidylinositol-3-kinase; PP, protein phosphatase; TAg Jurkat cells, Jurkat T-cells overexpressing the SV40 large T antigen; FBS, fetal bovine serum; HEK, human embryonic kidney; PAGE, polyacrylamide gel electrophoresis; MES, 4-morpholineethanesulfonic acid; HPLC, high performance liquid chromatography.

phosphatase as suggested by several lines of evidence. The kinases that phosphorylate rapamycin-sensitive sites on $p70^{s6k}$ remain active under conditions of FRAP inhibition (11–13). FRAP inhibition results in activation of the phosphatase PP2A (14), and PP2A associates with $p70^{s6k}$ but not with a rapamycin-resistant $p70^{s6k}$ mutant (14, 15). FRAP can also phosphorylate PP2A *in vitro* (14), and Tor2p has been shown to phosphorylate the PP2A regulator Tap42p *in vitro* (16). Therefore, it seems likely that regulation of PP2A is a significant part of FRAP's function.

Although FRAP kinase activity is critical for FRAP function, little is known about how FRAP activity is regulated. Because mitogenic stimuli, especially those mediated by phosphatidylinositol-3-kinase (PI3K), affect $p70^{s6k}$ and 4E-BP1 activity, it has been proposed that mitogens regulate FRAP activity *in vivo* (6, 8, 10). FRAP is also posited to be a sensor of cellular amino acid levels, permitting translation when amino acids are abundant and preventing translation of specific mRNA transcripts when amino acids are scarce (17). Like rapamycin treatment, amino acid deprivation causes rapid dephosphorylation of $p70^{s6k}$ and 4E-BP1, and a rapamycin-resistant truncation of $p70^{s6k}$ is also resistant to amino acid deprivation-induced dephosphorylation (17). Therefore, like other PIK-related kinases including ATM, ATR, and DNA-PK, FRAP may serve as a checkpoint protein by sensing intracellular abnormalities and transmitting signals to halt cell cycle progression (18). However, identification of the factors that regulate FRAP activity has been complicated by the lack of a robust assay of FRAP activity *in vivo*. Purified 4E-BP1 has been used as a substrate for assaying FRAP kinase activity *in vitro*, but cell treatments that cause dramatic changes in $p70^{s6k}$ activity have only minor or negligible effects on the *in vitro* activity of FRAP (8, 10), possibly because immunoprecipitation and washing of FRAP remove factors that regulate FRAP activity *in vivo*. A more direct assay of FRAP *in vivo* activity may prove useful in determining the mechanism of FRAP regulation.

FRAP autophosphorylates *in vitro* and is phosphorylated *in vivo* (2). Autophosphorylation is a common feature of protein kinases, often serving as a marker for kinase activity. Autophosphorylation can also play an important role in kinase regulation, altering not only kinase catalytic activity but also specificity for allosteric effectors, proteolysis, membrane association, and binding to other proteins (19). Here we report the identification of a FRAP autophosphorylation site and generation of phosphospecific antibodies that allow direct detection of FRAP phosphorylation *in vivo*. We also report initial efforts to assess the phosphorylation of FRAP under various cellular conditions and demonstrate that the Ser-2481 autokinase activity of FRAP is not inhibited under translationally repressive conditions.

EXPERIMENTAL PROCEDURES

Cell Culture—Jurkat T-cells overexpressing the SV40 large T antigen (TAg Jurkat cells) were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and 2 mM L-glutamine. Human embryonic kidney 293 (HEK293) cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% FBS and 2 mM L-glutamine. Sf9 insect cells were maintained in TNM-FH supplemented with 10% FBS.

Expression of Recombinant Proteins—TAg Jurkat cells were transiently transfected by electroporation. 10^7 cells were washed with RPMI without phenol red and resuspended in 0.25 ml of the same in a 4-mm electroporation cuvette. 5 μg of the plasmid pBJ5 containing NH_2-terminally FLAG epitope-tagged FRAP (pBF-FRAP) was added, and cells were electroporated at 250 V, 129 ohms with a BTX electroporation system. Cells were then resuspended in 10 ml of RPMI supplemented with 10% FBS and 2 mM L-glutamine, and protein was expressed for 24 h. HEK293 cells were transiently transfected with 10 μg of pBF-FRAP in 10-cm dishes using LipofectAMINE PLUS reagent (Life Technologies, Inc.) following the manufacturer's instructions. Recombinant FRAP was expressed in Sf9 insect cells using a baculovirus generated by the Baculogold system (Pharmingen) for expression of the FLAG epitope tag fused to amino acids 1362–2549 of FRAP (FRAP1362C). 10^9 Sf9 cells were infected with 57 ml of concentrated virus in 0.5 liter and incubated at 27 °C for 54 h before harvesting recombinant FLAG-FRAP.

Phosphoamino Acid Analysis—TAg Jurkat cells (4×10^7) were transfected with wild-type, full-length FRAP as described above and allowed to express FRAP for 24 h. Cells were then washed twice with phosphate-free RPMI 1640 and resuspended in 4 ml of phosphate-free RPMI supplemented with 10% dialyzed FBS. After addition of 5 mCi of $^{32}P_i$, cells were incubated for a further 5 h to allow for phosphate incorporation. Cells were then washed twice in ice-cold phosphate-buffered saline and lysed in 0.5 ml of MIPP lysis buffer (20 mM NaH_2PO_4, pH 7.2, 1 mM Na_3VO_4, 5 mM NaF, 25 mM β-glycerophosphate, 2 mM EGTA, 2 mM EDTA, 0.5% Triton X-100, 1 mM dithiothreitol, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 0.2 mM phenylmethylsulfonyl fluoride) for 20 min. The cell lysate was clarified by centrifugation at $18,000 \times g$ for 15 min, and FLAG-FRAP was immunoprecipitated with 15 μl of M2-agarose beads (Eastman Kodak). Immune complexes were washed three times with 1 ml of MIPP lysis buffer and subjected to 5% SDS-PAGE. Proteins were transferred to Immobilon P (Millipore), and a band corresponding to ^{32}P-labeled FRAP was excised. Phosphoamino acid analysis was performed as described previously (20).

In Vitro Autokinase Reactions—TAg Jurkat cells were transfected, grown, and lysed, and recombinant FRAP was immunoprecipitated and washed as described for phosphoamino acid analysis. FRAP immune complexes were then washed with 1 ml of 0.5 M LiCl, 50 mM Tris, pH 7.5, and washed again with 1 ml of kinase reaction buffer (25 mM Hepes, pH 7.7, 50 mM KCl, 10 mM $MgCl_2$, 20% glycerol, 0.1% Nonidet P-40, 1 mM dithiothreitol). FRAP immune complexes were then incubated with 30 μl of kinase reaction buffer containing 100 μM ATP with 10 μCi of [γ-^{32}P]ATP for 5 min at 30 °C. Immune complexes were then subjected to SDS-PAGE and autoradiography.

Purification and Sequencing of a FRAP Phosphopeptide—FRAP1362C was expressed in Sf9 cells, immunoprecipitated with 0.7 ml of M2-agarose, and washed as described above. *In vitro* kinase reactions were performed in 0.5 ml of kinase reaction buffer containing 100 μM ATP with 125 μCi of [γ-^{32}P]ATP for 5 min at 37 °C. FRAP1362C was purified by preparative SDS-PAGE, transferred to Immobilon P, and stained with Ponceau S. The band corresponding to FRAP1362C was excised, washed with water, and blocked with 100 mM acetic acid, 0.5% polyvinylpyrrolidone 360,000 for 30 min at 37 °C. The membrane was then washed with water and resuspended in 200 μl of 50 mM ammonium bicarbonate. FRAP1362C was digested with 5 μg of modified sequencing grade porcine trypsin (Promega) for 12 h at 37 °C. Tryptic peptides were then dried with a SpeedVac and resuspended in 200 μl of 50 mM MES, 1 M NaCl, pH 5.5. Phosphopeptides were separated from the bulk of unphosphorylated peptides with a ferric chelating column as described previously (21). Radiolabeled fractions eluted from the ferric chelating column were separated further by reversed phase HPLC using a 1×150-mm Zorbax C18 column. The radiolabeled peaks eluting from this column were then collected and subjected to liquid chromatography-tandem mass spectrometry.

Mutagenesis of FRAP—FRAP cDNA encoding amino acids 1967–2549 containing the S2035T mutation was cloned into the mutagenesis vector pALTER-1 (Promega) using *Kpn*I and *Eco*RI restriction sites. Mutants were then generated following the manufacturer's instructions. *Kpn*I and *Bss*HII restriction sites were then used to move the mutated cDNA fragment into pBF-FRAP for full-length mutant FRAP expression. Mutation was confirmed by vector sequencing.

$p70^{s6k}$ Assay—Assays of $p70^{s6k}$ activity were performed as described previously (2).

Antibody Production—Phosphorylation state-specific (phosphospecific) antibodies directed against FRAP were produced by immunizing New Zealand White rabbits with the synthetic phosphopeptide (TVPESIHS*FIGDGLVC where S* represents the site of phosphorylation) coupled to keyhole limpet hemocyanin. IgG was purified using protein A from the best responding rabbits as determined by enzyme-linked immunosorbent assay against the phosphopeptide and corresponding non-phosphopeptide. Antibodies reactive with the non-phosphopeptide were removed by adsorption using a non-phosphopeptide Ser-2481 (TVPESIHSFIGDGLVC) affinity column. Antibodies that flowed through this column were next passed over a column of immobilized phosphopeptide and eluted at low pH and dialyzed. The eluted antibodies were characterized by enzyme-linked immunosorbent assay against phospho- and non-phosphopeptides to determine the extent of phosphospecificity and subsequently by Western blotting to determine specificity against FRAP in whole cell extracts.

ATP Binding—FLAG epitope-tagged wild-type and mutant FRAP were expressed in TAg Jurkat cells and immunoprecipitated as de-

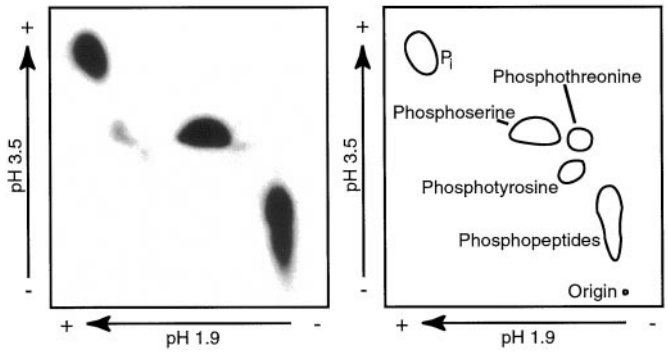

FIG. 1. **FRAP is phosphorylated on serine *in vivo*.** FLAG epitope-tagged FRAP was expressed in proliferating TAg Jurkat cells in the presence of ^{32}P. The ^{32}P-labeled FRAP was purified by immunoprecipitation and SDS-PAGE and hydrolyzed to amino acids with HCl. Phosphoamino acids were separated electrophoretically and identified by autoradiography. The positions of phosphoamino acid standards, as determined by ninhydrin staining, are indicated on the *right*.

scribed above. FLAG-FRAP was then eluted with 160 μl of 200 μM FLAG peptide, 0.1 M NaCl, 50 mM Hepes, pH 7.4, 0.1% Triton X-100, 1 mM dithiothreitol for 1.5 h at 4 °C. The supernatant was then added to 20 μl of C-8-linked ATP-agarose (Sigma), and $MgCl_2$ was added to a concentration of 25 mM. ATP binding was allowed to occur in the presence or absence of 5 mM free ATP for 1.5 h at 4 °C after which the ATP-agarose was washed twice in 0.1 M NaCl, 50 mM Hepes, pH 7.4, 0.1% Triton X-100, 20 mM $MgCl_2$, 1 mM dithiothreitol. After washing the ATP-agarose resin, FRAP binding was determined by SDS-PAGE and immunoblotting with an anti-FLAG antibody.

RESULTS

Identification of a FRAP Autophosphorylation Site—FRAP has been shown to autophosphorylate on a serine residue(s) *in vitro* (2). Using phosphoamino acid analysis, we determined that FRAP is phosphorylated on serine *in vivo* as well (Fig. 1), consistent with the possibility that FRAP autophosphorylation may occur *in vivo*. No phosphothreonine or phosphotyrosine was detected in ^{32}P-labeled FRAP from TAg Jurkat cells under normal growth conditions.

To identify the serine(s) that is(are) phosphorylated on FRAP *in vitro*, a FLAG epitope-tagged, 136-kDa fragment of FRAP comprised of residues 1362–2549 was expressed using the baculovirus system in Sf9 insect cells. This fragment of FRAP (FRAP1362C) includes the FRAP kinase domain and, like full-length FRAP, autophosphorylates *in vitro* but is expressed at much higher levels than full-length FRAP. FRAP1362C was immunoprecipitated with anti-FLAG antibodies, washed, and incubated with [γ-^{32}P]ATP. Autophosphorylated FRAP1362C was resolved by SDS-PAGE and transferred to polyvinylidene difluoride membrane, from which the band containing ^{32}P-labeled FRAP1362C was excised and digested with trypsin. The phosphopeptides produced were separated from the bulk of unphosphorylated peptides with an immobilized iminodiacetic acid column charged with Fe^{3+}. ^{32}P-Labeled phosphopeptides eluted in two peaks (Fig. 2*A*) that were collected and individually purified further by reversed phase HPLC (Fig. 2*B*). Each iron column peak contained only one phosphopeptide as determined by HPLC, and both phosphopeptides exhibited the same retention by HPLC. The HPLC-purified peptides were sequenced by liquid chromatography-tandem mass spectrometry. No sequence was obtained for the phosphopeptide from the later eluting iron column peak, but the spectrum obtained from the earlier eluting peak corresponds to the phosphorylated FRAP sequence ^{2471}TGTTVPESIHSFIGDGLVKPEALNK^{2495}. This phosphopeptide is derived from a region near the COOH terminus of FRAP between the kinase catalytic domain and the highly conserved COOH-terminal tail that is a defining characteristic of PIK-related kinases (22) (Fig. 2*C*). The phosphopeptide possesses two serine residues (Ser-2478 and Ser-2481), and because of their close proximity and the weakness of the ion series, it was not possible to determine unambiguously which of the two serines was phosphorylated.

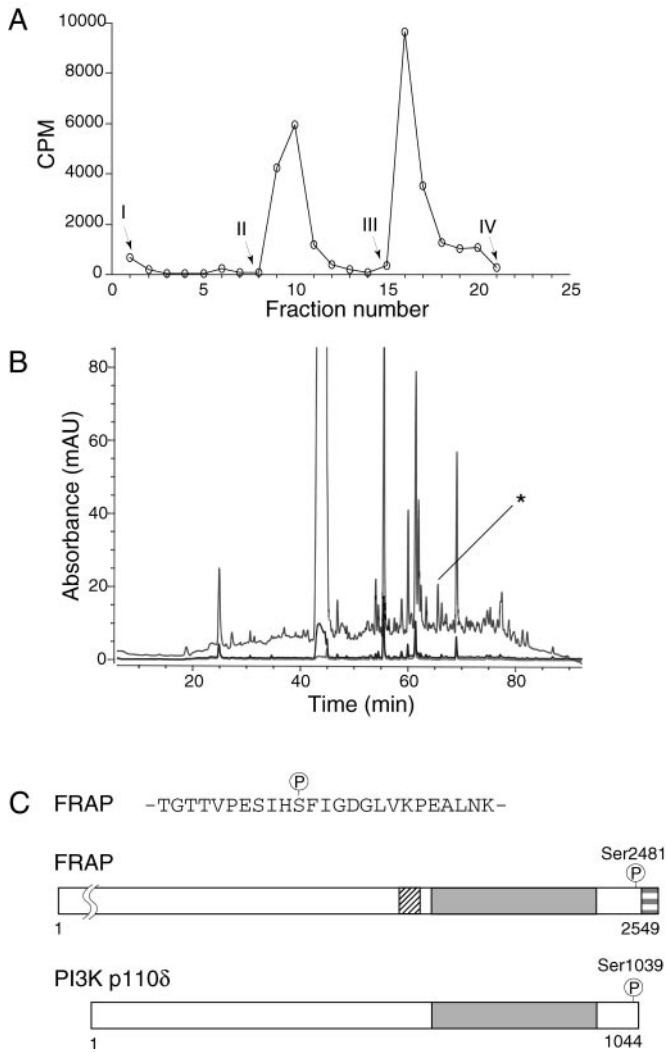

FIG. 2. **FRAP autophosphorylates Ser-2481.** Baculovirus-expressed FRAP1362C was purified, autophosphorylated, and digested with trypsin. *Panel A*, phosphopeptides generated by digestion were separated from the bulk of unphosphorylated peptides using a ferric chelation column. Peptides were eluted with buffer I (50 mM MES, 1 M NaCl, pH 5.5) followed by buffer II (2 mM MES, pH 6.0), buffer III (0.5 M NH_4HCO_3), and finally buffer IV (0.1 M EDTA, pH 8.0), added at the points indicated. Fractions of 0.75 ml were collected and Cerenkov counted. *Panel B*, fractions 9 and 10 from the ferric chelation column were combined and separated further by RP-HPLC. A peptide eluting at 67 min (indicated by *) was found to be radiolabeled by Cerenkov counting. The *top*, *middle*, and *bottom* HPLC traces indicate absorbance at 205, 277, and 292 nm, respectively. *Panel C*, the radiolabeled HPLC fraction was subjected to liquid chromatography-tandem mass spectrometry sequencing and yielded a spectrum consistent with the phosphorylated peptide comprised of FRAP residues 2471–2495. Autophosphorylation sites Ser-2481 of FRAP and Ser-1039 of PI3K p110δ (indicated by *P*) are located at comparable positions relative to the related FRAP and PI3K kinase domains (*solid gray bar*). *Diagonal lines* indicate the FKBP12-rapamycin binding domain, and *horizontal lines* indicate the COOH-terminal tail characteristic of PIK-related kinases.

Effect of Autophosphorylation Site Mutation—In an effort to determine whether Ser-2478 or Ser-2481 is phosphorylated *in vitro* and to assess the role of autophosphorylation in FRAP signaling, Ser-2478 and Ser-2481 were replaced individually with alanine or acidic amino acids by site-directed mutagenesis. A mutant was also generated in which Ser-2478 and Ser-2481 were both replaced with alanine. Full-length FRAP clones

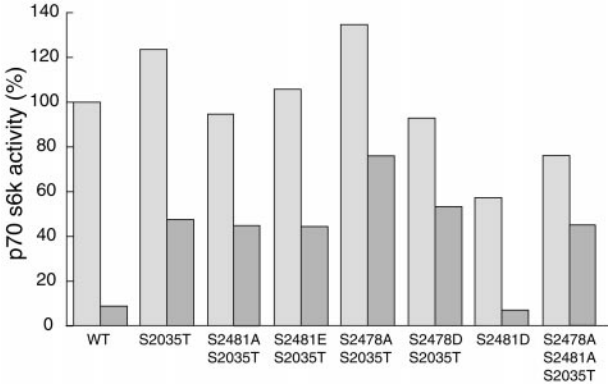

FIG. 3. **Autophosphorylation site mutation does not affect the ability of FRAP S2035T to protect p70^{s6k} from rapamycin-induced dephosphorylation.** FRAP or the single or double FRAP mutants indicated were cotransfected with hemagglutinin epitope-tagged p70^{s6k} into TAg Jurkat cells. Cells were untreated (*light gray bars*) or treated with 25 nM rapamycin for 25 min (*dark gray bars*) before the hemagglutinin-tagged p70^{s6k} was immunoprecipitated and assayed for activity against a synthetic peptide. Activity was normalized to the activity of p70^{s6k} in the presence of wild-type (*WT*) recombinant FRAP from untreated cells.

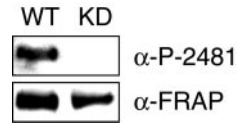

FIG. 4. **Phosphospecific antibodies raised against phosphoserine 2481 recognize wild-type but not kinase-inactive FRAP.** Wild-type (*WT*) and kinase-dead (*KD*) FRAP expressed in TAg Jurkat cells were immunoprecipitated and incubated with Mg^{2+}/ATP *in vitro*. Samples were subjected to SDS-PAGE and immunoblotting with phosphospecific (α-P-2481) antiserum. Antibodies recognized wild-type but not kinase-inactive FRAP. Expression of kinase-inactive FRAP was confirmed by stripping the polyvinylidene difluoride membrane and reprobing with a non-phosphospecific FRAP antibody (α-FRAP).

were prepared possessing these mutations as well as the mutation of Ser-2035 to threonine (S2035T), which confers rapamycin resistance to the encoded protein (2). These mutants were then tested for their ability to autophosphorylate *in vitro* and to protect p70^{s6k} from rapamycin-induced dephosphorylation. All of the mutants remained capable of autophosphorylation (data not shown), suggesting the existence of an additional autophosphorylation site not identified by the process described above. Such an unidentified autophosphorylation site may have been responsible for the later eluting iron column peak or may reside in the NH_2-terminal 1361 amino acids of FRAP which were not present in the baculovirus-expressed FRAP. Mutants at Ser-2478 and Ser-2481 also retained the ability to protect p70^{s6k} from rapamycin-induced dephosphorylation when they also possessed the S2035T mutation (Fig. 3). These results suggest that autophosphorylation at Ser-2478 or Ser-2481 is not required for FRAP signaling to p70^{s6k}. FRAP autophosphorylation may be involved in an uncharacterized FRAP function distinct from signaling to p70^{s6k}, although it is also possible that autophosphorylation has no functional effect on FRAP. Whether autophosphorylation plays a significant but uncharacterized role in FRAP function or has no functional effect on FRAP, we reasoned that it may serve as a useful marker of FRAP kinase activity *in vivo* and prove useful in determining the mechanism of FRAP regulation. We therefore attempted to generate phosphospecific FRAP antibodies.

Generation and Testing of Phosphospecific FRAP Antibodies—Phosphospecific antibodies directed against FRAP were produced by immunizing New Zealand White rabbits with the synthetic phosphopeptide (TVPESIHS*FIGDGLVC where S* represents the site of phosphorylation) coupled to keyhole limpet hemocyanin. IgG was purified, and phosphospecificity was confirmed by enzyme-linked immunosorbent assay and Western blotting. To investigate whether Ser-2481 is in fact a site of autophosphorylation, TAg Jurkat cells were transfected with epitope-tagged wild-type and kinase-inactive FRAP. The recombinant FRAP was immunoprecipitated, washed, and incubated with Mg^{2+}/ATP. The protein samples were then subjected to SDS-PAGE and transferred to polyvinylidene difluoride membrane. Western blotting with a non-phosphospecific antibody (anti-FRB) revealed comparable levels of wild-type and kinase-inactive FRAP, yet the phosphospecific FRAP antibody (P-2481) recognized only wild-type FRAP (Fig. 4). Therefore, it appears that Ser-2481 is autophosphorylated *in vitro* and that phosphorylation is dependent upon intact FRAP kinase activity. The ability of the P-2481 antiserum to distinguish between wild-type and kinase-inactive FRAP also suggests that the antiserum is specific for FRAP phosphorylated at Ser-2481.

The Carboxyl Terminus of FRAP Is Required for Kinase Activity and Signaling to p70^{s6k}—Autophosphorylation of PI3K p110δ occurs on serine 1039, located 128 amino acids COOH-terminal to the catalytic DFG aspartate of its kinase domain (23). Similarly, the FRAP autophosphorylation site Ser-2481 is located 124 amino acids COOH-terminal to its homologous catalytic aspartate (Fig. 2*C*). However, unlike PI3K, which terminates 5 residues after its autophosphorylation site, FRAP extends for an additional 63 amino acids in a sequence that is highly conserved among the PIK-related kinases. To explore the role of this conserved COOH-terminal tail in FRAP function, mutations of FRAP were made that cause truncation of 30, 20, 10, or 1 amino acid from the COOH terminus (Fig. 5A). Truncation of 10 or 20 amino acids from the COOH terminus reduces FRAP autokinase activity to the level of kinase-inactive FRAP (Fig. 5*B*). When mutations were made in rapamycin-resistant FRAP S2035T resulting in truncation of as few as one amino acid from the COOH terminus, the mutants were unable to protect p70^{s6k} from rapamycin-induced dephosphorylation (Fig. 5*C*). These results suggest that FRAP autokinase activity and signaling to p70^{s6k} are very sensitive to truncation of the conserved COOH-terminal tail.

It is possible that COOH-terminal truncation disrupts FRAP function by preventing proper folding of FRAP. We tested the ability of the FRAP mutants to bind ATP using ATP coupled to agarose via the C-8 position of adenosine. The mutants retained the ability to bind ATP-agarose, binding as well as wild-type FRAP, and were competed off the ATP-agarose by free ATP (Fig. 5*D*). Therefore, it appears that COOH-terminal truncation blocks FRAP activity by some mechanism other than disruption of ATP binding.

Serine 2481 Autophosphorylation Occurs in Vivo—To test if FRAP Ser-2481 is phosphorylated *in vivo*, cell lysates were prepared from TAg Jurkat, CTLL-2, and HEK293 cells and separated by SDS-PAGE prior to Western blotting with the phosphospecific P-2481 antiserum. In all cell types tested, phosphoserine 2481 was detected, indicating that Ser-2481 is phosphorylated *in vivo*. Phosphorylation of serine 2481 detected in cells could be a result of FRAP-catalyzed phosphorylation, as occurs *in vitro* (Fig. 4), or Ser-2481 could be phosphorylated by a separate kinase *in vivo*. The relative contribution of FRAP kinase activity to Ser-2481 phosphorylation was measured by transfecting TAg Jurkat and HEK293 cells with wild-type and kinase-inactive epitope-tagged (FLAG) FRAP. The recombinant FRAP was immunoprecipitated, and phosphorylation of Ser-2481 was measured by Western blotting with the phosphospecific P-2481 antiserum. In both cell lines, phosphorylation of Ser-2481 was detected readily in wild-type FRAP but not detected in kinase-inactive FRAP (Fig. 6). Therefore,

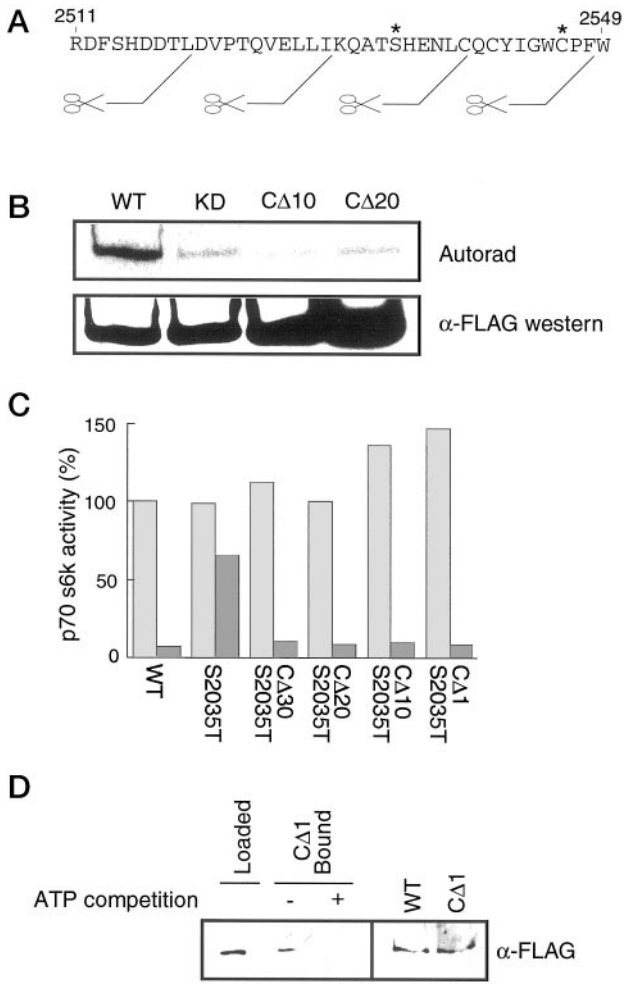

FIG. 5. **The COOH terminus of FRAP is required for autokinase activity and signaling to p70^{s6k}.** The PIK-related kinases are characterized by a highly conserved, 30-amino acid carboxyl-terminal domain of unknown function. *Panel A*, site-directed mutations resulting in truncations of varying lengths (indicated with *scissors*) or point mutations (indicated by *asterisks*) were generated. *Panel B*, truncation of 10 or 20 amino acids from the COOH terminus (*CΔ10*, *CΔ20*) reduced FRAP autokinase activity to the level of a kinase-dead (*KD*, D2357E) mutant as measured in an *in vitro* autokinase reaction. *WT*, wild-type. *Panel C*, carboxyl-terminal truncation blocks the ability of FRAP S2035T to protect p70^{s6k} from rapamycin-induced dephosphorylation. FRAP or the single or double FRAP mutants indicated were cotransfected with hemagglutinin epitope-tagged p70^{s6k} into TAg Jurkat cells. Cells were untreated (*light gray bars*) or treated with 25 nM rapamycin for 25 min (*dark gray bars*) before the hemagglutinin-tagged p70^{s6k} was immunoprecipitated and assayed for activity against a synthetic peptide. Activity was normalized to the activity of p70^{s6k} in the presence of wild-type recombinant FRAP from untreated cells. *Panel D*, truncation of one amino acid from the COOH terminus of FRAP (*CΔ1*) does not prevent FRAP from binding ATP. FLAG epitope-tagged wild-type or CΔ1 FRAP was expressed in TAg Jurkat cells, immunoprecipitated, and eluted with FLAG peptide before binding to C-8-linked ATP-agarose in the presence or absence of competition with 5 mM free ATP. After washing the ATP-agarose resin, FRAP binding was determined by SDS-PAGE and immunoblotting with an anti-FLAG antibody.

the majority of Ser-2481 phosphorylation that occurs *in vivo* is autocatalyzed and phosphorylation at Ser-2481 serves as a reporter of *in vivo* FRAP kinase activity.

Serine 2481 Phosphorylation Is Inhibited by Wortmannin but Not by Rapamycin or Amino Acid Deprivation—Wortmannin is a small molecule that inhibits PI3K with an IC_{50} of 2–5 nM. It has also been shown to inhibit FRAP autokinase activity at higher concentrations (~200 nM IC_{50}) (24). HEK293 cells were treated with wortmannin, and cell lysates from these cells, were probed with P-2481 antiserum. Wortmannin blocks Ser-2481

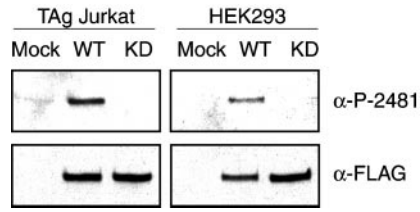

FIG. 6. **Autophosphorylation of FRAP Ser-2481 occurs *in vivo*.** FLAG epitope-tagged wild-type (*WT*) and kinase-dead (*KD*) FRAP were expressed in TAg Jurkat and HEK293 cells. Recombinant FRAP was then immunoprecipitated with anti-FLAG (M2) antibody and probed for Ser-2481 phosphorylation and total FLAG-FRAP expression by immunoblotting with antibodies against phosphoserine 2481 (α-P-2481) and the FLAG epitope (α-FLAG).

phosphorylation almost completely in HEK293 cells at concentrations above 100 nM, and concentrations as low as 10 nM cause a noticeable dephosphorylation of FRAP Ser-2481 (Fig. 7*A*). Similar results were obtained with TAg Jurkat cells, although slightly higher concentrations of wortmannin were required to obtain a similar effect (Fig. 7*B*). These results indicate that wortmannin does inhibit FRAP kinase activity *in vivo*, most likely by direct binding to the kinase active site of FRAP.

The FKBP12-rapamycin complex has been shown to inhibit FRAP autokinase activity *in vitro* at high concentration (500 nM) (2). FRAP immune complex-mediated phosphorylation of 4E-BP1 and fragments of p70^{s6k} are also inhibited by FKBP12-rapamycin *in vitro*, although in many cases only a fraction of the kinase activity is inhibited by FKBP12-rapamycin (8). Amino acid deprivation has also been posited to inhibit FRAP (17, 25). To determine if rapamycin treatment or amino acid deprivation inhibits FRAP autophosphorylation of Ser-2481 *in vivo*, HEK293 and TAg Jurkat cells were treated with rapamycin for 30 min or deprived of amino acid for 60 min. Cell lysates from treated and untreated cells were then probed for phosphoserine 2481. No significant change in the relative amount of phosphoserine 2481 was detected after rapamycin treatment or amino acid deprivation (Fig. 7, *A* and *B*). Histidinol, an amino alcohol known to cause accumulation of non-aminoacylated tRNA molecules and to cause some cellular responses characteristic of amino acid deprivation, had only a minor effect on Ser-2481 phosphorylation at high concentration (Fig. 7*A*) (26). Therefore, it seems likely that *in vivo*, rapamycin, and possibly amino acid deprivation, inhibit FRAP by some means other than direct kinase inhibition. This is consistent with the observation that autophosphorylation is equally robust for FRAP immunoprecipitated from proliferating or amino acid-deprived cells (Fig. 7*C*). One possibility is that the above treatments result in the inability of a subset of FRAP substrates to associate, and therefore be phosphorylated, by the kinase. This model is analogous to that proposed by the Tor proteins based on a completely different experimental approach (7).

To eliminate the possibility that some kinase other than FRAP phosphorylates Ser-2481 under the translationally repressive conditions of rapamycin treatment and amino acid deprivation, TAg Jurkat cells were transfected with wild-type or kinase-inactive FLAG-FRAP. After 24 h of expression, transfected cells were treated with rapamycin for 30 min or deprived of amino acids for 60 min before lysis and immunoprecipitation of recombinant FRAP. No phosphorylation of Ser-2481 was detected in kinase-inactive FRAP under any of these conditions (Fig. 7*D*), suggesting that autophosphorylation is the only significant source of Ser-2481 phosphorylation in TAg Jurkat cells under these conditions. As observed with endogenous FRAP, rapamycin treatment and amino acid deprivation resulted in little, if any, decrease in *in vivo* autophosphorylation of recombinant FRAP (Fig. 7*D*).

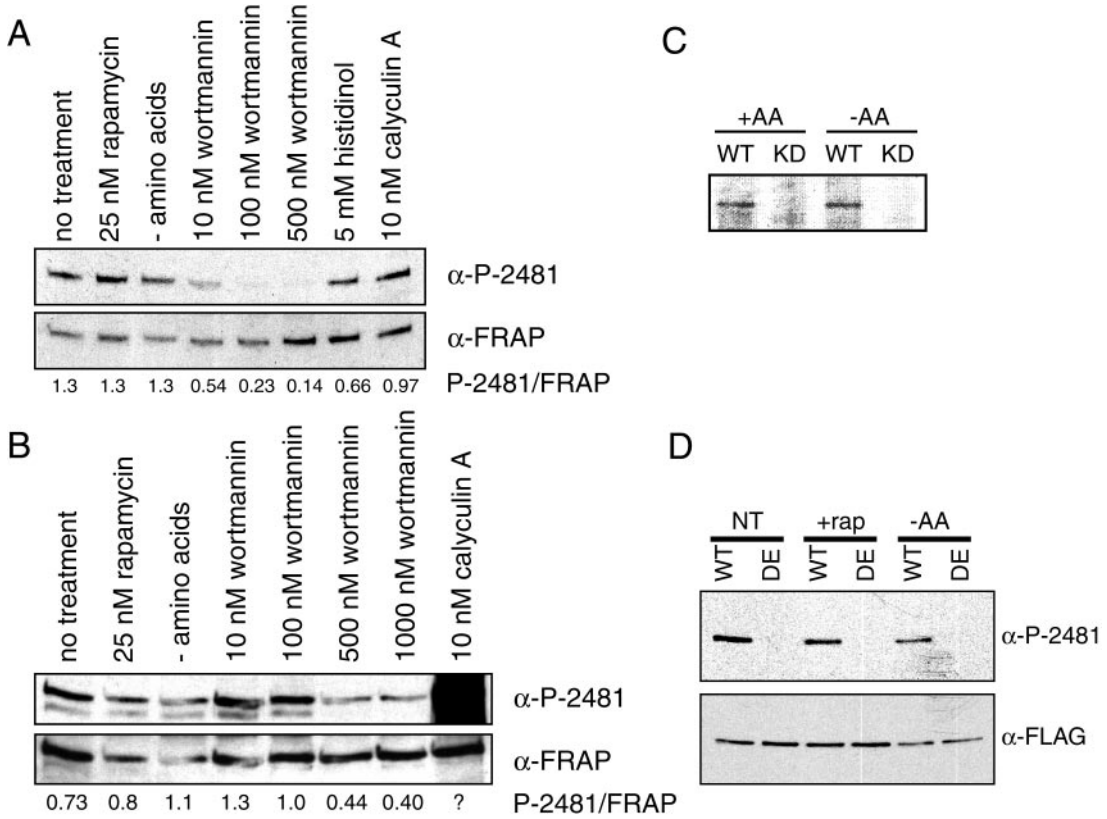

FIG. 7. **FRAP autophosphorylation of Ser-2481 is inhibited by wortmannin but not by rapamycin treatment or amino acid deprivation.** HEK293 cells (*panel A*) or TAg Jurkat cells (*panel B*) were either untreated, treated with 25 nM rapamycin for 30 min, grown in Dulbecco's phosphate-buffered saline supplemented with 10% dialyzed FBS for 1 h (−amino acids), treated with wortmannin for 45 min, treated with 5 mM histidinol for 1 h, or treated with 10 nM calyculin A for 15 min. After treatment, cells were lysed, and cell lysates were subjected to 5% SDS-PAGE, transferred to Immobilon P, and the extent of autophosphorylation was determined by immunoblotting with antibodies against phosphoserine 2481 (α-P-2481). The membrane was then stripped and reprobed for total FRAP using a non-phosphospecific FRAP antibody (α-FRAP). Approximate immunoreactivity ratios for phosphospecific *versus* non-phosphospecific FRAP antibodies are shown (P-2481/FRAP) and were obtained with the program NIH Image 1.62. *Panel C*, wild-type (*WT*) and kinase-inactive (*KD*) FRAP were expressed in TAg Jurkat cells for 24 h. Cells were then maintained in amino acid-containing media or deprived of amino acids for 2 h prior to lysis. FRAP was then immunoprecipitated and incubated with Mg^{2+}/[γ-^{32}P]ATP *in vitro*. After SDS-PAGE, autophosphorylation was detected by autoradiography. *Panel D*, wild-type and kinase-inactive FRAP were expressed in TAg Jurkat cells for 24 h. Cells were treated as indicated before immunoprecipitation of recombinant FRAP and immunoblotting as in *panel A*.

The effect of calyculin A treatment on Ser-2481 phosphorylation was also tested. Calyculin A is a potent inhibitor of the serine/threonine phosphatases PP1 and PP2A. Treatment of HEK293 cells with 10 nM calyculin A for 15 min had no discernible effect on Ser-2481 phosphorylation (Fig. 7*A*). However, similar calyculin A treatment of TAg Jurkat cells caused a dramatic increase in Ser-2481 phosphorylation (Fig. 7*B*). This robust increase in Ser-2481 phosphorylation was also observed in another T-cell line, CTLL-2 cells (data not shown). The reason for the difference in calyculin A responsiveness between HEK293 and TAg Jurkat cells is not known but may result from reduced protein phosphatase activity in HEK293 cells. To address the possibility that rapamycin does indeed inhibit FRAP autokinase activity but is not detectable because of a compensatory decrease in FRAP phosphatase activity, we treated TAg Jurkat cells with rapamycin after a brief (15 min) pretreatment with calyculin A. As observed in the absence of calyculin A, rapamycin had no detectable effect on the level of FRAP Ser-2481 phosphorylation (data not shown).

Mitogenic Stimuli Have Minor Effects on Ser-2481 Phosphorylation—It has been proposed that FRAP kinase activity is serum-stimulated and that this increase in kinase activity is responsible for the hyperphosphorylation of $p70^{s6k}$ and 4E-BP1 which occurs after serum stimulation (8, 10). To examine the effect of serum stimulation on Ser-2481 phosphorylation, HEK293 cells were serum deprived for 36 h prior to stimulation with 10% serum for 0, 10, 20, or 30 min. After stimulation for the times indicated, cell lysates were prepared and subjected to SDS-PAGE. Western blotting with the phosphospecific P-2481 antiserum revealed that FRAP is phosphorylated at Ser-2481 in serum-deprived cells, and serum stimulation increases phosphorylation less than 2-fold after 30 min (Fig. 8). Serum deprivation blocks 4E-BP1 and $p70^{s6k}$ phosphorylation at rapamycin-sensitive sites almost completely, and serum stimulation causes rapid and pronounced phosphorylation of these sites (27). Because FRAP kinase activity appears to be largely intact after serum deprivation, it seems unlikely that the pronounced effects of serum on 4E-BP1 and $p70^{s6k}$ phosphorylation result solely from changes in FRAP kinase activity.

Because activated mutants of the protooncogene c-Akt/PKB have been shown to promote hyperphosphorylation of 4E-BP1(28) and $p70^{s6k}$ (29), we also examined the effect of c-Akt on FRAP autophosphorylation. We probed for phosphoserine 2481 in cell lysates from HEK293 cells and HEK293 cells stably expressing myristoylated Akt. FRAP Ser-2481 from cells expressing myristoylated Akt are somewhat hyperphosphorylated relative to FRAP Ser-2481 from cells not expressing myristoylated Akt (Fig. 8).

DISCUSSION

The PIK-related kinases have emerged as key regulators of cellular responses to DNA damage and nutrient deprivation.

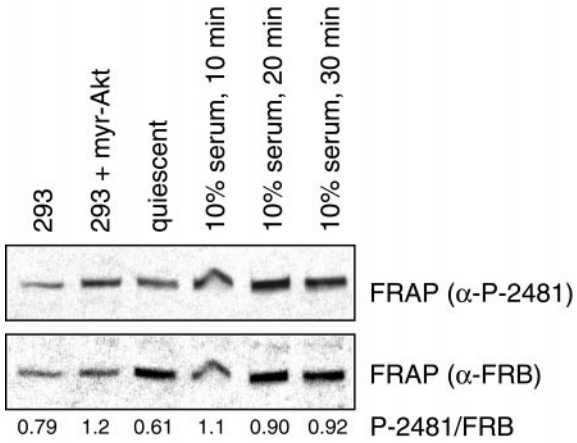

FIG. 8. **Serum stimulates FRAP autophosphorylation slightly.** HEK293 cells were grown to confluence in the presence of 10% FBS before the growth medium was replaced with medium containing 0.5% FBS. Cells were incubated for a further 36 h and then stimulated with 10% FBS for the times indicated after which cells were lysed. Proliferating HEK293 cells and HEK293 cells stably overexpressing myristoylated Akt were also lysed. Cell lysates were subjected to 5% SDS-PAGE, transferred to Immobilon P, and the extent of autophosphorylation was determined by immunoblotting with antibodies against phosphoserine 2481 (α-P-2481). The membrane was then stripped and reprobed for total FRAP using a non-phosphospecific FRAP antibody (α-FRAP). Approximate immunoreactivity ratios for phosphospecific *versus* non-phosphospecific FRAP antibodies are shown (P-2481/FRB) and were obtained with the program NIH Image 1.62.

Despite indications that the kinase activity of these proteins is required for their cellular functions and that, at least in the case of DNA-PK, phosphorylation regulates function (30), no phosphorylation sites have been identified for any PIK-related kinase, possibly because of technical difficulties caused by their large size. The identification of Ser-2481 as a FRAP autophosphorylation site *in vitro* and *in vivo* provides new insight about FRAP kinase activity under growth conditions and under conditions inhibitory to growth. Autophosphorylation has been observed in numerous protein kinases. In some cases, the significance of autophosphorylation remains unknown, whereas in others, autophosphorylation regulates kinase proteolysis, membrane localization, binding to other proteins, substrate specificity, and catalytic activity (19). In the case of FRAP, the consequences of autophosphorylation are not yet known. Mutation of Ser-2481 to alanine or glutamate does not appear to have any effect on $p70^{s6k}$ activity nor to abolish completely FRAP autokinase activity. However, it is possible that autophosphorylation at this site influences a separate, untested FRAP function. Whether autophosphorylation plays a significant role in FRAP function or simply serves as a marker for FRAP kinase activity in cells (see below), identification of an autophosphorylation site has allowed increased understanding of FRAP's *in vivo* kinase activity and the regulation of FRAP's function.

Ser-2481 is surrounded by bulky, hydrophobic residues, possibly revealing a preference for phosphorylation of such sites by FRAP. The five 4E-BP1 sites reported to be phosphorylated by FRAP *in vitro* are not bulky, hydrophobic sites but are all Ser-Pro or Thr-Pro sites (8, 9, 31). However, a comparison between FRAP phosphorylation of these sites and a bulky, hydrophobic site derived from $p70^{s6k}$ suggests a strong preference for phosphorylation of bulky, hydrophobic sites *in vitro* (8). The nature of the FRAP Ser-2481 autophosphorylation site is consistent with this preference. It has been noted that the sequence surrounding Ser-2481 is similar to the sequences surrounding the activation loop phosphorylation sites in the catalytic domains of the AGC kinases (32). Several of these kinases, including $p70^{s6k}$, c-Akt/PKB, and protein kinase C are believed to be involved in the FRAP signaling pathway. The significance, if any, of the sequence similarity between these activation loops and the FRAP autophosphorylation site is unknown. Perhaps these phosphorylated sequences are recognized by a common phosphatase or binding protein, but it appears unlikely that the AGC kinase activation loops are FRAP substrates because another kinase, PDK1, is responsible for AGC kinase activation loop phosphorylation (33).

FRAP autophosphorylation may share some features with the autophosphorylation of PI3K p110δ reported recently (23). Both FRAP and PI3K p110δ autophosphorylate on serines located comparable distances COOH-terminal to the catalytic domains; Ser-1039 of PI3K is located 128 amino acids COOH-terminal to the catalytic DFG aspartate of its kinase domain, and Ser-2481 of FRAP is located 124 amino acids COOH-terminal to its homologous catalytic aspartate (Fig. 2*C*). Autophosphorylation of PI3K has been proposed to inhibit its lipid kinase activity while leaving the protein kinase activity of PI3K p110δ intact (23). Thus autophosphorylation may convert PI3K p110δ from a kinase with lipid and protein specificity to a kinase with specificity for protein substrates only. No lipid kinase activity has yet been observed for any of the PIK-related kinases, despite their homology to the lipid kinases. As more is learned about autophosphorylation of PI3K and FRAP, it will be interesting to discover if the structural similarity of their autophosphorylation sites reflects any functional similarity between the two kinases.

PI3K terminates just a few amino acids COOH-terminal to its autophosphorylation site, whereas FRAP extends beyond its autophosphorylation site in a COOH-terminal tail that we demonstrate to be absolutely critical for FRAP kinase activity and function. Other PIK-related kinases appear to be similarly intolerant of COOH-terminal truncations or additions. Naturally occurring mutations resulting in COOH-terminal truncations or additions to the mammalian PIK-related kinases DNA-PK and ATM result in severe combined immune deficiency (34) and ataxia telangiectasia disease phenotypes (35), respectively.

FRAP is known to mediate rather pronounced changes in the phosphorylation and activity of 4E-BP1 and $p70^{s6k}$ upon rapamycin treatment (4). It has been widely accepted that rapamycin inactivates FRAP by inhibiting its kinase activity. FRAP autokinase activity and intermolecular kinase activity have been shown to be inhibited by the FKBP12-rapamycin complex *in vitro*, although the extent of inhibition has been disputed (2, 6, 8). We now show that *in vivo*, FRAP autophosphorylation of Ser-2481 is not inhibited detectably by rapamycin treatment. Ser-2481 phosphorylation appears to be completely dependent upon FRAP kinase activity; kinase-inactive FRAP is not phosphorylated at Ser-2481 *in vitro* or *in vivo*. Therefore, the absence of change in Ser-2481 phosphorylation after rapamycin treatment suggests that FRAP autokinase activity is intact. (It should be noted that phosphoserine 2481 is a substrate for cellular phosphatases *in vivo* as judged by our experiments with calyculin A. Therefore, if FKBP12-rapamycin inhibits the kinase activity of FRAP, previously phosphorylated Ser-2481 would be expected to be dephosphorylated.) In contrast, wortmannin treatment causes the complete dephosphorylation of Ser-2481, suggesting that wortmannin disrupts FRAP autokinase activity. Wortmannin-induced FRAP inactivation may be an indirect consequence of PI3K inhibition, but this seems unlikely because wortmannin concentrations in the range of the PI3K IC_{50} (2–5 nM) do not inhibit FRAP Ser-2481 phosphorylation (data not shown). A more likely possibility is that *in vivo*, wortmannin inhibits FRAP kinase activity directly as has been shown *in vitro* (24).

It is not altogether surprising that rapamycin does not significantly inhibit FRAP kinase activity *in vivo* given the non-equivalence of rapamycin-treated and kinase-inactive Tor2p in

yeast. Kinase-inactive Tor2p is unable to carry out its cell cycle-independent essential function, but this function remains intact upon rapamycin treatment (7). Just as rapamycin treatment is not equivalent to kinase inactivation of Tor2p in yeast, rapamycin treatment and amino acid deprivation have effects on Ser-2481 autophosphorylation which are not equivalent to FRAP kinase inactivation. This does not mean, however, that rapamycin does not inhibit FRAP function. Rather, it suggests that rapamycin influences FRAP function through some mechanism other than direct kinase inhibition. Given that rapamycin does not disrupt FRAP's intrinsic kinase activity, how does rapamycin inhibit FRAP function? A number of possibilities exist, including the possibility that the FKBP12-rapamycin complex alters FRAP localization or disrupts association between FRAP and a subset of its regulatory proteins or kinase substrates. If amino acid deprivation inhibits FRAP function as has been proposed (17), it is likely to do so by a mechanism similar to that of FKBP12-rapamycin inhibition, likely involving the FRB domain of FRAP, because amino acid deprivation also has little effect on FRAP autophosphorylation.

FRAP also retains autokinase activity upon serum deprivation. FRAP autophosphorylation in quiescent HEK293 cells is much greater than that observed from kinase-inactive or wortmannin-treated FRAP and is only increased slightly upon serum stimulation. It is possible that the actual serum-induced increase in FRAP kinase activity is quite pronounced *in vivo* but is not observed because of saturation of the intramolecular substrate. However, this seems unlikely because this *in vivo* result is consistent with earlier *in vitro* results showing that FRAP immunoprecipitated from serum-stimulated cells is only about 2- or 3-fold more active toward 4E-BP1 than FRAP immunoprecipitated from serum-deprived cells (8, 10).

Serum deprivation inactivates $p70^{s6k}$ almost completely, and serum stimulation activates $p70^{s6k}$ by greater than 20-fold, whereas serum effects on FRAP activity are much less pronounced and seem to occur more slowly than effects on $p70^{s6k}$ (8). Therefore, it seems likely that some factor other than FRAP is primarily responsible for direct serum-induced phosphorylation of 4E-BP1 and $p70^{s6k}$. This idea is supported by the observation that a $p70^{s6k}$ mutant that is unresponsive to FRAP regulation is still sensitive to the effects of serum deprivation and stimulation (12, 13). Furthermore, PDK1, a rapamycin-resistant, serum-responsive, PIP_3-dependent protein kinase, has been shown to phosphorylate one of the rapamycin-sensitive sites on $p70^{s6k}$ (36, 37) and may also be responsible for phosphorylation of other rapamycin-sensitive sites (38).

If changes in FRAP kinase activity do not correlate directly with 4E-BP1 and $p70^{s6k}$ phosphorylation, how does rapamycin treatment cause such pronounced dephosphorylation of 4E-BP1 and $p70^{s6k}$? It seems that this is achieved to a significant degree through FRAP regulation of a serine/threonine phosphatase. FRAP inhibition increases the activity of the phosphatase PP2A by an unknown mechanism, although FRAP may phosphorylate PP2A directly (14). In yeast, Tor2p can phosphorylate the phosphatase regulator Tap42p (16), and rapamycin treatment causes dissociation of Tap42p and the phosphatase Sit4p (39). Association between the mammalian Tap42p homolog (α4) and several phosphatases has been demonstrated and may be rapamycin-sensitive, although this point remains controversial (40, 41). Whether FRAP regulates phosphatase activity through phosphorylation or by some other means is unknown, but the involvement of a phosphatase in FRAP signaling may explain how FRAP-mediated dephosphorylation of 4E-BP1 and $p70^{s6k}$ can occur without inhibition of intrinsic FRAP kinase activity.

This study underscores the complexity of the FRAP signaling pathway and the inadequacy of linear kinase cascades in describing FRAP signaling. Although FRAP kinase activity is affected somewhat by mitogenic stimulation, FRAP is not simply a serum-induced kinase whose activity reflects the presence or absence of a given mitogen. Rather, FRAP appears able to mediate protein dephosphorylation while maintaining kinase activity, likely through regulation of PP2A. Discovering how FRAP achieves this may be a critical step toward understanding the checkpoint functions of the PIK-related kinases.

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FKBP12-Rapamycin-associated Protein (FRAP) Autophosphorylates at Serine 2481 under Translationally Repressive Conditions

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