FKBP12-rapamycin-associated protein (FRAP) or mammalian target of rapamycin (mTOR) and its effector proteins form a critical signaling pathway that regulates eukaryotic cell growth and proliferation. Although the protein components in this pathway have begun to be identified, little is known about their subcellular localization or the physiological significance of their localization. By immunofluorescence, we find that both endogenous and recombinant FRAP/mTOR proteins show localization predominantly in the endoplasmic reticulum (ER) and the Golgi apparatus. Consistent with this finding, FRAP/mTOR is cofractionated with calnexin, an ER marker protein. Biochemical characterization suggests that FRAP/mTOR is a peripheral ER/Golgi protein with tight membrane association. Finally, we have identified domains of FRAP/mTOR which may mediate its association with the ER and the Golgi apparatus.

Rapamycin is a macrolide isolated from the Easter Island soil bacterium Streptomyces hygroscopicus and is currently used as an immunosuppressant in kidney transplantation (1) and in drug-eluting stents to prevent restenosis after angioplasty (2). In addition, rapamycin ester derivatives are in clinical trials for cancer treatment (3). The FKB12-rapamycin-associated protein (FRAP), also known as mammalian target of rapamycin (mTOR), is a 289-kDa phosphatidylinositol 3-kinase-related kinase family, FAT and FATC, flank the kinase domain to the N and C terminus, respectively (16).

FRAP is linked to translational control through its two main effector proteins, the ribosomal S6 kinases (S6Ks) and the translation regulator eIF4E-binding protein 1 (17–21). S6Ks promote protein synthesis by phosphorylation of ribosomal protein S6, allowing the translation of 5′-oligopyrimidine tract-containing mRNAs that encode for ribosomal proteins and translational regulators (17–21). eIF4E-binding protein 1 binds to eIF4E and inhibits CAP-dependent translation under unfavorable growth conditions. eIF4E-binding protein 1 becomes phosphorylated by FRAP and is released from eIF4E when growth conditions become favorable (17–21). FRAP also controls protein synthesis by regulating ribosome biogenesis (22–24). FRAP signaling is regulated both by nutrients, including amino acids and glucose, and by mitogens (17–21).

Both the endoplasmic reticulum (ER) and the Golgi apparatus are internal membrane structures constituting more than one-half of the total membranes in a cell. The ER is known to have two essential functions. First, proteins destined for transport to other organelles and the cell surface or for secretion are synthesized in the ER. During translation, they are translocated into the ER lumen through a pore in the ER membrane and are folded and become glycosylated inside the ER lumen. Second, synthesis of lipids and cholesterol takes place on the cytoplasmic side of the ER membrane. The ER is also an important participant in cell signaling, including the unfolded protein response, the ER overload response, and the ER-to-nuclear sterol signaling by SREBP (25). Several recent studies suggest that the ER is also involved in signal transduction pathways traditionally thought to occur solely through the plasma membrane. For example, the small GTPase Ras that is restricted to the ER and the Golgi apparatus can actively engage in signal transduction to mitogen-activated protein kinases (26). These studies indicate that the ER and the Golgi apparatus can serve as anchors for diverse cell signaling events.

A large body of evidence shows that proper localization plays a crucial role in the specificity and/or regulation of cell signaling events. Despite the importance of FRAP in cell growth and therapeutic intervention, little is known about its precise subcellular localization. We have examined common mammalian cell lines for FRAP localization. We find that FRAP is localized predominantly in the ER and the Golgi apparatus. Biochemical analyses suggest that FRAP is a peripheral ER membrane protein but tightly anchored to the ER/Golgi membranes. Fi-
nally, we found two discrete domains in the N terminus which are crucial for targeting to the ER and the Golgi apparatus, respectively. Like the sterol-SREBP pathway, FRAP may use the ER/Golgi as anchors for nutrient sensing and signaling.

### EXPERIMENTAL PROCEDURES

**Plasmids, Cell Lines, Antibodies, and Chemicals—FLAG-FRAP plasmids have been described previously (12). Mammalian cell lines were obtained from American Type Culture Center (ATCC) and were maintained as recommended by ATCC. FRAP antibodies were produced (Harlan) by immunizing rabbits with polypeptides corresponding to the amino acids 1–120 of human FRAP. The rabbit FLAG polyclonal antibody was purchased from Sigma, calnexin antibodies were purchased from BD Transduction Laboratories, Golgin-97 antibodies and Mitotracker were purchased from Molecular Probes, phospho-Thr389 antibody was purchased from Cell Signaling, FRAP-H9262-KBP12-rapamycin binding-specific antibody was purchased from Santa Cruz Biotechnology, and horseradish peroxidase-conjugated secondary antibodies were purchased from Pierce. Leptomycin B, sodium azide, and protease inhibitor mixture (Roche Diagnostics, GmbH) were added to protein extract at 4 °C, which were washed three times with wash buffer containing 0.5 M HEPES-KOH, pH 7.4, 1 M LiCl, 0.1% Triton X-100, 5% glycerol, 10 mM sodium pyrophosphate, 10 mM β-glycerophosphate, 1.5 mM Na3VO4, 50 mM NaF, and 1 x protease inhibitor mixture. Protein A-bound materials were eluted by boiling in SDS protein sample buffer. For Western blot, protein samples were separated on SDS-polyacrylamide gels and transferred onto an Immobilon-P membrane. After blocking with 5% dry milk in TBST, the membrane was incubated with primary antibodies for 1 h to overnight, followed by incubation with horseradish peroxidase-conjugated secondary antibodies (1:10,000 for 30 min and with ECL (Amersham Biosciences).

**FRAP Kinase Assay—**After immunoprecipitation, FRAP or control immunocomplexes were incubated at 30 °C with the FRAP kinase reaction mixture (50 mM HEPES-KOH, pH 7.4, 10 mM MgCl2, 1 mM dithiothreitol, 2 mM ATP, 0.5 μg of GST-p70S6K1 μl of kinase buffer). Aliquots of samples were withdrawn at different times, and the kinase reaction was stopped by boiling in SDS sample buffer. Phosphorylation of GST-p70S6K1 at Thr389 was analyzed by Western blot with a phospho-Thr389-specific antibody. A preimmune serum immunocomplex was used as a negative control.

**Transfection and Immunofluorescence (IF) Microscopy—**HELa cells were transfected with plasmids using the calcium phosphate method. For indirect IF, cells were fixed in 3% paraformaldehyde, 2% sucrose in H2O for 10 min at 37 °C, permeabilized with ice-cold Triton buffer (0.5% Triton X-100 in 20 mM HEPES, pH 7.4, 50 mM NaCl, 3 mM MgCl2, 300 mM sucrose) for 5 min on ice, blocked with 0.1% bovine serum albumin and 1% trypsin and chymotrypsin were purchased from Sigma.

**Subcellular Localization of FRAP**—The N-terminal FRAP antigenic polypeptide was conjugated to CNBr-activated Sepharose beads (Amersham Biosciences) according to manufacturer’s instruction. The N-terminal FRAP antigen (150 mg of IgG) was equilibrated in binding buffer (Bio-Rad) and was loaded onto the N-terminal FRAP-Sepharose affinity column. After washing with binding buffer, FRAP-specific IgG was eluted with elution buffer (Bio-Rad). Fractions were collected, analyzed, and subsequently dialyzed against 10 mM HEPES, pH 7.5, 150 mM NaCl, 50% glycerol until further use. Purification and dialysis were performed at 4 °C.

**Immunoprecipitation and Western Blot—**Cell lysis for immunoprecipitation and Western blot were prepared using ice-cold lysis buffer containing 50 mM HEPES-KOH, pH 7.4, 40 mM NaCl, 1 mM EDTA, 0.1% Triton X-100, 5% glycerol, 10 mM sodium pyrophosphate, 10 mM β-glycerophosphate, 1.5 mM Na2VO4, 50 mM NaF, and 1 x protease inhibitor mixture (Roche Diagnostics, GmbH). Immunoprecipitation was performed by incubating the cell lysates with 10 μl of FRAP antibody/mg of protein extract at 4 °C for 90 min. The immune complexes were bound to protein A-agarose beads (Amersham Biosciences) for 1 h at 4 °C, which were washed three times with wash buffer containing 50 mM HEPES-KOH, pH 7.4, 1 M LiCl, 0.1% Triton X-100, 5% glycerol, 10 mM sodium pyrophosphate, 10 mM β-glycerophosphate, 1.5 mM Na2VO4, 50 mM NaF, and 1 x protease inhibitor mixture. Protein A-bound materials were eluted by boiling in SDS protein sample buffer. For Western blot, protein samples were separated on SDS-polyacrylamide gels and transferred onto an Immobilon-P membrane. After blocking with 5% dry milk in TBST, the membrane was incubated with primary antibodies for 1 h to overnight, followed by incubation with horseradish peroxidase-conjugated secondary antibodies (1:10,000 for 30 min and with ECL (Amersham Biosciences).

**Western Blot—**Lysates were collected, analyzed, and subsequently dialyzed against 10 mM HEPES-KOH, pH 7.4, 40 mM NaCl, 1 mM EDTA, 0.1% Triton X-100, 5% glycerol, 10 mM sodium pyrophosphate, 10 mM β-glycerophosphate, 1.5 mM Na2VO4, 50 mM NaF, and 1 x protease inhibitor mixture (Roche Diagnostics, GmbH). Immunoprecipitation was performed by incubating the cell lysates with 10 μl of FRAP antibody/mg of protein extract at 4 °C for 90 min. The immune complexes were bound to protein A-agarose beads (Amersham Biosciences) for 1 h at 4 °C, which were washed three times with wash buffer containing 50 mM HEPES-KOH, pH 7.4, 1 M LiCl, 0.1% Triton X-100, 5% glycerol, 10 mM sodium pyrophosphate, 10 mM β-glycerophosphate, 1.5 mM Na2VO4, 50 mM NaF, and 1 x protease inhibitor mixture. Protein A-bound materials were eluted by boiling in SDS protein sample buffer. For Western blot, protein samples were separated on SDS-polyacrylamide gels and transferred onto an Immobilon-P membrane. After blocking with 5% dry milk in TBST, the membrane was incubated with primary antibodies for 1 h to overnight, followed by incubation with horseradish peroxidase-conjugated secondary antibodies (1:10,000 for 30 min and with ECL (Amersham Biosciences).

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Room temperature and washed as with the primary antibodies. Glass cover slips carrying treated cells were mounted with Cytoseal mounting medium onto glass slides and analyzed using a Zeiss laser scanning confocal microscope (Zeiss Axiosvert 200 m microscope and LSM 5 Pascal laser scanning confocal) or an Olympus BX51 fluorescence microscope equipped with a Qimaging Retiga EXi digital camera. No significant difference in IF staining was observed with the N-terminal FRAP antibody or affinity-purified N-terminal FRAP antibody. Mitotracker Red was used to stain HeLa cells according to manufacturer’s instructions.

Subcellular Fractionation and Related Biochemistry—HeLa cells were washed with ice-cold HME buffer (10 mM HEPES, 250 mM mannitol, 0.5 mM EDTA, pH 7.4), resuspended in 5 volumes of ice-cold HME buffer containing 0.1 mM phenylmethylsulfonyl fluoride, and Dounce homogenized with 10 gentle strokes. Nuclei and unbroken cells were pelleted at 1,500 \( \times g \), followed by a spin at 10,000 \( \times g \) (10 min, 4 °C). The pellets (P10) were resuspended in HME buffer. The S10 supernatant (after saving an aliquot) was overlaid on a 20% sucrose cushion and further centrifuged at 100,000 \( g \) (60 min, 4 °C). The pellets were washed with ice-cold HME buffer (10 mM HEPES, 250 mM mannitol, 0.5 mM EDTA, pH 7.4), resuspended in 5 volumes of ice-cold HME buffer, and centrifuged at 10,000 \( g \) (10 min, 4 °C). The pellets (P100) were resuspended in HME, 2 M KCl, 4 M urea in 10 mM MOPS, or 0.1 M sodium carbonate, pH 11.5, and incubated for 40 min at 4 °C prior to centrifugation. Two consecutive washes (S1, S2) were performed for each condition. For protease protection assays, the P100 pellets were resuspended in HME plus 10 mM CaCl2 with or without 1% Triton X-100, and then incubated with different amounts of trypsin/chymotrypsin (0, 10, 20 \( \mu g \)) for 40 min at 4 °C. Reactions were stopped by the addition of aprotonin and boiling in SDS protein sample buffer.

RESULTS

To characterize FRAP subcellular localization, we have generated a polyclonal antibody against the N-terminal 120 amino acids of FRAP. This antibody specifically recognized a protein of ~250 kDa in all cell types examined, including HeLa, HEK293 (C2C12, Rh30, and MRC5) (normal human lung fibroblasts) (Fig. 1A). A minor species of 140 kDa was occasionally seen, which appears to be a FRAP proteolytic product during cell lysis. This Western blot signal was completely blocked by the FRAP antigenic polypeptide (Fig. 1A), and this Western blot signal was completely absent when a preserum was used (Fig. 1B). In addition, the 250-kDa polypeptide was immunoprecipitated by the FRAP antibody, but not a preimmune serum (Fig. 1C). To establish further the specificity of the FRAP antibody, we assayed the ability of the anti-FRAP immunocomplex to phosphorylate p70S6K1 using a system recently developed by Thomas and co-workers (27). In this assay, the anti-FRAP immunocomplex was incubated with recombinant GST-p70S6K1 (amino acids 333–412) and a high ATP concentration (2 mM). Phosphorylation of p70S6K1 at Thr389, a FRAP-dependent phosphorylation site, was assayed by Western blot with a phospho-Thr389-specific antibody. We found that Thr389 was rapidly phosphorylated by the FRAP immunocomplex, but not by an immunoprecipitate with the preimmune serum (Fig. 1D). These results demonstrate the high specificity of the anti-FRAP antibody.
We next used the FRAP antibody to study FRAP subcellular localization using indirect IF microscopy. We analyzed the staining pattern of HeLa cells with this antibody in the absence or presence of FRAP antigenic polypeptide. A strong and highly reproducible perinuclear staining pattern was observed with the FRAP antibody (Fig. 1E). A minor nuclear staining signal was also noticeable (Fig. 1E). This IF signal was completely removed by incubation with the FRAP antigenic polypeptide (Fig. 1E). In addition, no significant signal was observed with the preserum (Fig. 1E). Therefore, FRAP is distributed predominantly in the perinuclear region. In contrast, no FRAP staining was detected on the periphery of the cells, indicating that FRAP is not localized to the plasma membrane. In addition, only a small fraction of FRAP was found in the nucleus (Fig. 1E). We have compared the FRAP antiserum with an affinity-purified FRAP antibody but observed no difference in FRAP IF staining pattern, immunoprecipitation, or Western blot.

The perinuclear staining pattern of FRAP shows strong similarity to that of the ER and the Golgi apparatus. To verify this, we performed double IF staining with the FRAP antibody and an antibody specific for the ER (calnexin) or the Golgi apparatus (Golgin-97) in HeLa cells. By laser scanning confocal microscopy, we found that the FRAP staining strongly overlapped with that of calnexin, a common ER marker protein and Golgin-97, a common Golgi marker protein (Fig. 2A). A recent report suggests that FRAP is localized to mitochondria (28). We therefore examined the relationship between FRAP and mitochondria in HeLa cells by costaining the cells with the FRAP antibody and Mitotracker Red, a mitochondrial membrane-specific dye. Although there appeared to be some colocalization between FRAP and mitochondria, their distribution patterns were largely distinctive, suggesting that only a small fraction of FRAP is localized in mitochondria in these cells (Fig. 2B). To corroborate this in situ staining pattern for endogenous FRAP, we transfected HeLa cells with a FLAG-tagged FRAP. IF staining with anti-FLAG antibodies showed the same perinuclear pattern that was observed with calnexin (Fig. 2C). Additionally, an anti-FRAP C-terminal antibody showed similar codistribution of FRAP with calnexin (Fig. 2D). These results suggest that FRAP is primarily localized to the ER and Golgi apparatus in HeLa cells. Next, we investigated FRAP distribution in other common mammalian cell lines with both N- and C-terminal FRAP antibodies. We found essentially identical FRAP distribution pattern in C2C12 murine myoblasts, MRC5 human diploid fibroblasts, rat embryonic fibroblasts, and Rh30 rhabdomyosarcoma cancer cells (Fig. 3A), suggesting that the ER/Golgi localization is not cell type-specific.

Modulation of cell signaling can occur by changing subcellular localization of key components of the corresponding pathways. We investigated whether FRAP distribution changed in response to cellular conditions that modulate FRAP signaling. We found that FRAP remained in the perinuclear pattern during serum starvation or rapamycin treatment, conditions that inhibit FRAP signal transduction (Fig. 3B). A similar pattern was observed in serum-stimulated cells that had been deprived from serum for an extended period (Fig. 3B). Mitochondria have recently been linked to FRAP functions because sodium azide, an inhibitor of the mitochondrial respiratory function, has been shown to block p70S6K1 phosphorylation (28, 29). However, treatment of HeLa cells with sodium azide did not change the FRAP perinuclear staining pattern (Fig.
A recent report indicates that FRAP shuttles between the cytoplasm and nucleus (30). However, short term treatment (2.5 h) with leptomycin B, an inhibitor of Crm1-dependent nuclear export, failed to elicit substantial FRAP accumulation in the nucleus in HeLa cells (Fig. 3B). This observation suggests that the proposed nucleocytoplasmic shuttling mechanism is unlikely a fast one.

To characterize FRAP localization further, we performed biochemical fractionation of HeLa cell lysates. After mild homogenization and pelleting of nuclei and unbroken cells, heavy membranes including mitochondria, lysosomes, peroxisomes, and some ER membranes were pelleted by low speed centrifugation (10,000 × g). FRAP was found in both the supernatant (S10) and pellet (P10) (Fig. 4A). The S10 supernatant was further fractionated by high speed centrifugation (100,000 × g) to pellet light membranes, including the ER and Golgi apparatus membranes. As expected, the ER marker protein calnexin was found in the pellet (P100) but not in the supernatant (S100) (Fig. 4A). We also found that FRAP was in the P100 but not the S100 fraction (Fig. 4A). These results are in agreement with our IF data and suggest that FRAP is associated with the ER and the Golgi apparatus.

FRAP may be associated with the outer or inner surface of the ER as a peripheral or integral membrane protein. Alterna-

Fig. 5. The N-terminal regions are important for FRAP targeting to the ER and the Golgi apparatus. A, schematic representation of FLAG-FRAP deletion mutants used in this study. B, the full-length and truncated FLAG-FRAP proteins were transiently expressed in HeLa cells. Their localization was determined by IF using a FLAG-specific antibody. DAPI was used to stain the nuclei. C, exclusive localization of the P6 FLAG-FRAP mutant in the Golgi apparatus. The P6-expressing HeLa cells as in B were analyzed for P6 FLAG-FRAP colocalization with the Golgi apparatus by IF using FLAG-specific and Golgin-97 antibodies. DAPI was used to stain the nuclei.
Regulate translation and ribosome biogenesis. These results suggest that the ER and the Golgi are common sites for nutrient sensing and signaling.

So it becomes detached from the ER and the Golgi apparatus, leading to nuclear relocation of SREBP and activation of SREBP-dependent genes. Because FRAP is also anchored onto ER membranes, the ER and the Golgi apparatus may be used for amino acid sensing/signal by FRAP to regulate translation and ribosome biogenesis. These results suggest that the ER and the Golgi are common sites for nutrient sensing and signaling.

The nature of FRAP association with the ER, we treated the P100 fraction with conditions known to disrupt selectively the membrane association of different types of membrane-bound proteins. A high salt condition is commonly used to dissociate peripheral membrane proteins whose membrane interaction is largely electrostatic in nature. As expected, washing with 2 M KCl significantly disrupted the binding of the peripheral Golgi membrane protein Golgin-97 to membranes but had no effect on association of the integral membrane protein calnexin with ER/Golgi membranes (Fig. 4B). This condition only removed a minor fraction of FRAP from the P100 membranes (Fig. 4B). Washing with 4 M urea completely removed Golgin-97, but not calnexin, from the P100 membranes (Fig. 4B). This washing condition significantly, but not completely, removed FRAP from the P100 membranes (Fig. 4B). Finally, a high pH (sodium carbonate, pH 11) condition completely dissociated Golgin-97 and FRAP from the P100 membranes, although only partially removing calnexin (Fig. 4B). These results suggest that FRAP is an outer ER membrane protein that is tightly associated with the membrane.

To determine further the topology of FRAP ER localization, we performed the protease protection assay. We reasoned that if FRAP were an outer membrane protein exposed to the cytoplasm, it should be sensitive to protease treatment. On the other hand, an inner ER protein should be protected from proteolysis unless the ER is first dissolved with detergent. We incubated the P100 fraction with a combination of trypsin and chymotrypsin in the absence or presence of Triton X-100. Calnexin is an integral membrane protein with the bulk of its sequence in the ER lumen. However, it has ~100 amino acids exposed to the cytoplasm. When treated with protease in the absence of detergent, calnexin was processed to a smaller polypeptide with molecular weight corresponding to its inner ER fragment (Fig. 4C). In the presence of detergent, calnexin was further degraded (Fig. 4C). In contrast, we found that FRAP was completely digested regardless of whether Triton X-100 was present or not (Fig. 4C). Therefore, FRAP appears to be an outer ER protein.

To determine the sequence(s) that specify FRAP localization, we analyzed the subcellular distribution of systematically generated FLAG-FRAP deletion mutants by IF (Fig. 5A). After transient transfection of the wild type and mutant FLAG-FRAP into HeLa cells, IF staining was performed using FLAG-specific antibodies. As shown earlier, the full-length FLAG-FRAP (P1) showed the typical ER/Golgi localization pattern (Fig. 1C). Deletion of the N terminus up to amino acid 1361 (P2, P3, and P4) did not affect the normal FRAP distribution pattern (Fig. 5B). Similarly, removing the C terminus starting from amino acid 1444 (P5) did not change FRAP localization (Fig. 5B). Strikingly, further deletion of 404 amino acids (from amino acid 1040) from the C terminus (P6), however, led to apparently exclusive Golgi localization of FRAP (Fig. 5, B and C). Also interestingly, FRAP lost either ER or Golgi localization with a further deletion of 109 amino acids from the C terminus (P7 and P8) (Fig. 5B). The two C-terminal deletion mutants (P7 and P8) became distributed throughout the cytoplasm and the nucleus (Fig. 5B). These results indicate that amino acids 1362–1443 and amino acids 931–1039 are important for ER and Golgi localization, respectively.

**DISCUSSION**

In this study, we show that both endogenous and recombinant FRAP proteins are predominantly localized to the ER and Golgi apparatus in HeLa cells, as judged by IP and cell fractionation experiments. A similar FRAP distribution pattern was observed with several other common mammalian cell lines, including C2C12 murine myoblasts, MRC5 human diploid fibroblasts, rat embryonic fibroblasts, and Rh30 rhabdomyosarcoma cells, suggesting that ER/Golgi localization is a common theme of FRAP distribution. Huang and Houghton (3) recently reported predominant nuclear localization of FRAP/mTOR in certain mammalian cells. Interestingly, in the same cell lines (C2C12 murine myoblasts and Rh30 rhabdomyosarcoma cells), we still observed that the majority of FRAP showed ER/Golgi localization (Fig. 3A). The apparent discrepancy between the two studies could reflect the specificity of different antibodies used. For example, one antibody may only recognize a subpopulation of FRAP proteins because of the availability of the corresponding epitope. This latter hypothesis is supported by the observation that the C-terminal FRAP antibody does show slightly more nuclear staining in C2C12 murine myoblasts and Rh30 rhabdomyosarcoma cells (Fig. 3A).

Our results are also in agreement with the biochemical properties of FRAP/TOR signaling complexes reported recently. It has been found that FRAP and the yeast TOR proteins, and their associated cofactors such as Raptor/Kog1 and GβL/Lst8, are bound to light intracellular membranes (13, 14, 31, 32). Electron microscopy study indicates that the yeast Raptor/Kog1 protein is colocalized with TOR at an intracellular location that appears to be the ER in budding yeast (32). The interaction between FRAP/TOR and Raptor/Kog1 is highly sensitive to detergent (13, 14, 31, 32), suggesting that the ER membrane is important in facilitating the assembly of the...
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FRAP/TOR signaling complex. Taken together, these observations indicate that the ER localization of FRAP/TOR is an evolutionarily conserved phenomenon and is likely to play an important role in FRAP/TOR signaling/functioning.

Our results further indicate that FRAP is associated with the outer surface of the ER because treatment of the P100 fraction leads to complete proteolysis of FRAP by trypsin/chymotrypsin. Unlike the peripheral Golgi protein Golgin-97, FRAP is highly resistant to high salt washes and moderately resistant to 4 M urea or high pH, suggesting that FRAP is much more tightly associated with the ER membrane than a typical peripheral membrane protein. FRAP may be lipid-modified or an integral membrane protein with the bulk of its sequence exposed to the cytoplasm. We have taken a deletional approach to map the regions important for FRAP ER/Golgi targeting. Our results indicate that amino acids 1362–1443 are critical for ER targeting because FRAP mutants missing this region were unable to localize to the ER (Fig. 5B). On the other hand, amino acids 931–1039 are essential for localization to the Golgi apparatus (Fig. 5B). Interestingly, these two regions appear to be competing with each other: the P6 mutant missing the region necessary for ER targeting becomes exclusively localized to the Golgi apparatus (Fig. 5B).

The fact that FRAP is predominantly localized at the ER or Golgi is intriguing in light of the fact that the sterol-SREBP signaling pathway is anchored at the ER and utilizes the Golgi apparatus as well (25, 33). Both the FRAP and SREBP pathways sense the level of nutrients, amino acids and sterol, respectively (for a model see Fig. 6). Analogous to the sterol sensing mechanism, FRAP may base on the ER/Golgi to sense amino acid levels and transduce amino acid signals. FRAP signaling is likely to be mediated by interaction with downstream effectors and could also relocate to other subcellular compartments such as the nucleus in response to signaling input. Like the SCAP/SREBP system that utilizes both the ER and the Golgi apparatus for regulation of sterol levels, FRAP may also exhibit a dynamic localization pattern between these organelles which is important for its signaling functions. Deletion of the sequences important for ER/Golgi localization results in cytoplasmic as well as nuclear localization (Fig. 5B). Therefore, similar to the SCAP/SREBP system, the ER and the Golgi apparatus may be used to regulate the availability of FRAP proteins to the cytoplasm and nucleus, which may be important for diverse FRAP/TOR-dependent events such as translation and ribosome biogenesis.

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