Cytoplasmic–nuclear shuttling of FKBP12-rapamycin-associated protein is involved in rapamycin-sensitive signaling and translation initiation

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Translation initiation is one of the key events regulated in response to mitogenic stimulation and nutrient availability, tightly coupled to mammalian cell cycle progression and growth. FKBP12-rapamycin-associated protein (FRAP; also named mTOR or RAFT1), a member of the *ataxia telangiectasia* mutated (ATM)-related kinase family, governs a rapamycin-sensitive membrane-to-cytoplasm signaling cascade that modulates translation initiation via p70 S6 kinase ($p70^{s6k}$) and eIF-4E binding protein 1 (4E-BP1). Our studies reported here reveal a surprising regulatory mechanism of this signaling, which involves cytoplasmic–nuclear shuttling of FRAP. By using leptomycin B (LMB), a specific inhibitor of nuclear export receptor Crm1, we show that FRAP is a cytoplasmic–nuclear shuttling protein. Inhibition of FRAP nuclear export by LMB coincides with diminished $p70^{s6k}$ activation and 4E-BP1 phosphorylation. Further investigation by altering FRAP's nuclear shuttling activity with exogenous nuclear import and export signals has yielded results that are consistent with a direct link between nuclear shuttling of FRAP and mitogenic stimulation of $p70^{s6k}$ activation and 4E-BP1 phosphorylation. Furthermore, by using a reporter system, we provide evidence suggesting that nuclear shuttling of FRAP regulates mitogen-stimulated rapamycin-sensitive translation initiation. These findings uncover a function for the nucleus in the direct regulation of the protein synthesis machinery via extracellular signals.

Regulation of translation initiation in mammalian cells is an essential response to mitogenic stimulation and nutrient availability. One of the signaling cascades emanating from growth factor receptors in the plasma membrane to the translational machinery in the cytoplasm is characterized by its sensitivity to the immunosuppressant rapamycin (1, 2). The direct mammalian target of rapamycin has been identified as FRAP (also named mTOR/RAFT1) (3–5), a member of the ATM-related kinase family with sequence homology to phosphatidylinositol kinases (6, 7). FRAP is required for mitogenic regulation of $p70^{s6k}$ (8) and 4E-BP1 (also known as PHAS-I) (9), both of which are involved in the regulation of translation initiation. Phosphorylation of the S6 subunit of 40S ribosome by $p70^{s6k}$ is correlated with mitogenic stimulation and increased translation initiation of mRNAs containing 5′-terminal oligopyrimidine tract (5′-TOP) (10–12). This subset of mRNAs codes for ribosomal proteins and translation elongation factors, suggesting that 5′-TOP-dependent translation is involved in the regulation of the translational machinery, which is an essential process for both cell growth and cell cycle progression. 4E-BP1 inhibits 5′-mRNA cap binding complex formation by binding to eIF4E in quiescent cells; on mitogenic stimulation, phosphorylation of 4E-BP1 dissociates it from eIF4E and thus allows translation initiation of a majority of mammalian mRNAs (13, 14). Both $p70^{s6k}$ activity and 4E-BP1 phosphorylation require kinase-active FRAP and are abolished by nanomolar concentrations of rapamycin (15–17). Consistently, both proteins have been shown to be phosphorylated by FRAP *in vitro* (16, 18, 19), although other components in this pathway have yet to be shown to account for the regulation of both downstream effectors by multiple phosphorylation.

An emerging concept based on recent evidence involves FRAP playing a permissive role on the mitogenic stimulation of $p70^{s6k}$ activation and 4E-BP1 phosphorylation by sensing amino acid sufficiency (20–22), although a direct link between mitogens and FRAP is also possible (19, 23). The regulatory mechanism of FRAP function, however, has been elusive. The essential kinase activity of FRAP is only marginally increased by mitogenic stimulation (19, 23) and unaffected by amino acid deprivation. As expected for its role in regulating the translational machinery, FRAP is thought to be a cytoplasmic protein localized to intracellular membranes (24, 25). But it is not known what role this subcellular localization plays in FRAP function. The studies reported here reveal a surprising mechanism of FRAP regulation, which involves cytoplasmic–nuclear shuttling of the FRAP protein. We present evidence suggesting that nuclear shuttling of FRAP regulates mitogenic stimulation of $p70^{s6k}$ activation and 4E-BP1 phosphorylation. Furthermore, we demonstrate that this nuclear shuttling is involved in mitogenic regulation of rapamycin-sensitive translation initiation.

Materials and Methods

Cell Culture and Transfection. Both human embryonic kidney (HEK) 293 cells and monkey kidney epithelial CV-1 cells were maintained in DMEM containing 10% FBS at 37°C with 5% CO_2. Transient transfection was performed by using SuperFect (Qiagen, Chatsworth, CA) according to the manufacturer's recommendations. HEK293 cells at ≈60% confluency were transfected in six-well plates; the amount of DNA per well was (whenever applicable) 1 μg FRAP, 1 μg $p70^{s6k}$, 0.4 μg 4E-BP1, and 10 ng luciferase. CV-1 cells were transfected in 12-well plates with 2 μg of FRAP cDNA per well.

Antibodies. The following antibodies were obtained from commercial sources: M2 anti-FLAG epitope from Sigma; 16B12 anti-HA from Berkeley Antibody Company (Richmond, CA); anti-4E-BP1 from Zymed; anti-$p70^{s6k}$ from Upstate Biotechnology (Lake Placid, NY); all secondary antibodies from Jackson ImmunoResearch. A polyclonal anti-FRAP antibody raised against the FKBP12-rapamycin-binding domain (26) and 9E10.2 anti-Myc ascites were generated by the Immunological Research Facilities at University of Illinois at Urbana–Champaign. Anti-

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Abbreviations: FRAP, FKBP12-rapamycin associated protein; $p70^{s6k}$, p70 S6 kinase; 4E-BP1, eIF4E binding protein 1; 5′-TOP, 5′-terminal oligopyrimidine tract; ATM, *ataxia telangiectasia* mutated; LMB, leptomycin B; NLS, nuclear localization signal; NES, nuclear export signal; HEK cells, human embryonic kidney cells.

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tubulin ascites was generously provided by Dr. Vladimir Gelfand (University of Illinois at Urbana–Champaign).

Plasmid Construction. cDNA plasmids expressing nuclear localization signal (NLS)-, NLS′-, nuclear export signal (NES)-, and NES′-FRAP were generated by inserting oligonucleotide linkers encoding the corresponding peptides at *Not*I site between a Myc epitope and the start codon of FRAP in pCDNA-Myc-FRAP(S2035T). The signal peptide sequences are as follows: NLS: GPKKKRKVESG; NLS′: GPK*T*KRKVESG; NES: LQLPPLERLTL; NES′: LQLPP*DL*RLTL. pCDNA3-FLAG-FRAP (27) and pBJ5-HA-p70^{s6k} (15) were described previously. FLAG-4E-BP1 cDNA was obtained by PCR and inserted into pCDNA3. Luciferase cDNA was inserted into pCDNA3 via *Bam*HI and *Xho*I sites to yield pCDNA3-luciferase. An 80-bp 5′ untranslated region sequence from hamster eEF2 gene (24) was inserted between the cytomegalovirus promoter and the 5′ end of luciferase cDNA via *Hin*dIII and *Bam*HI sites to yield pCDNA3-TOP-luciferase.

Cell Fractionation. Subconfluent cells grown on 10-cm plates, untreated or treated with 10 ng/ml leptomycin B (LMB) for 12 h, were dounced in hypotonic buffer (20 mM Tris·Cl, pH 7.5/10 mM KCl/1 mM DTT/0.5 mM PMSF) until at least 99% of the cells were disrupted. The cytoplasmic fraction was obtained as supernatant after centrifugation at 2,000 × *g* for 3 min. The pellet was washed in hypotonic buffer and extracted with hypertonic buffer (20 mM Tris·Cl, pH 7.5/500 mM KCl/1 mM DTT/0.5 mM PMSF), yielding the nuclear fraction. Both cytoplasmic and nuclear fractions were analyzed by Western blotting.

Indirect Immunofluorescent Staining. CV-1 cells cultured on glass coverslips were transfected with various FRAP cDNAs, by using SuperFect (Qiagen). After 24 h, cells were fixed in 3.7% formaldehyde (in PBS), followed by permeabilization in wash buffer (0.1% Triton X-100/1% BSA, in PBS). Incubation with M2 anti-FLAG or 9E10.2 anti-Myc antibodies was carried out in wash buffer at room temperature, followed by incubation with FITC–anti-mouse antibody in wash buffer. Confocal microscopy was performed with a Zeiss laser scanning confocal microscope (model LSM210).

Kinase Assays. For p70 S6 kinase assays, transfected or untransfected HEK293 cells were lysed in lysis buffer (20 mM Tris·Cl, pH 7.5/0.1 mM Na_3VO_4/25 mM NaF/25 mM β-glycerophosphate/2 mM EGTA/2 mM EDTA/1 mM DTT/0.5 mM PMSF/0.3% Triton X-100), followed by immunoprecipitation. The immune complexes were washed three times in lysis buffer and once in S6 kinase buffer (50 mM MOPS, pH 7.2/10 mM $MgCl_2$/10 mM paranitrophosphate/0.1% Triton X-100/1 mM DTT). The kinase reaction was carried out at 37°C for 15 min in kinase buffer containing 100 μM ATP, 1 μCi [γ-^{32}P]ATP, and 5 μM S6 kinase peptide substrate (sequence: RRRLSSLRA; Upstate Biotechnology). The reactions were stopped by adding EDTA to a final concentration of 0.2 M, which was blotted onto p81 papers and washed in 0.425% phosphoric acid, and radioactivity was measured by scintillation counting. For FRAP autokinase assays, cells were lysed in lysis buffer containing 0.5 M KCl, followed by immunoprecipitation. Kinase assays were performed in immune complexes as previously described (27) and analyzed by SDS/PAGE and phosphorimaging (Cyclone; Packard).

Results

FRAP Is a Cytoplasmic–Nuclear Shuttling Protein. FRAP has been reported to be a cytoplasmic protein localized to intracellular membranes (24, 25). However, we have consistently observed a faint but clear nuclear staining, exclusive of nucleolus, by confocal microscopy in CV-1 cells expressing FLAG-tagged FRAP (Fig. 1*A*). One of the possible explanations for a low amount of nuclear protein is a rapid nuclear export relative to nuclear import. To probe this possibility, we treated cells expressing FLAG-FRAP with leptomycin B (LMB), a specific inhibitor of nuclear export receptor Crm1 (28, 29). As shown in Fig. 1*A*, LMB treatment resulted in a significant increase in FRAP nuclear staining, suggesting that FRAP may indeed be undergoing shuttling between the cytoplasm and the nucleus. To eliminate potential artifacts that might result from the recombinant protein, we examined the endogenous FRAP protein. Immunostaining of the endogenous protein is not reliable because no FRAP-deficient cell line is available as a negative control. We therefore chose cell fractionation to assess the distribution of FRAP between the two compartments. It should be pointed out that throughout this study we used HEK293 cells for function and signaling analyses, but we relied on CV-1 cells for immunocytochemical experiments because of their more discernible morphology and better tolerance of growth conditions on glass coverslips. To confirm that FRAP behaved similarly in these two cell lines, we examined endogenous FRAP by fractionation in both CV-1 and HEK293 cells. As shown in Fig. 1*B*, the nuclear fraction contained a low level of FRAP, which was increased on treatment with LMB. The two cell lines yielded similar results. As a control, tubulin was not found in the nuclear fractions in the presence or absence of LMB. These data suggest that FRAP is a nuclear shuttling protein. No conventional nuclear import signal or nuclear export signal has been found in the FRAP sequence, but it is possible that FRAP

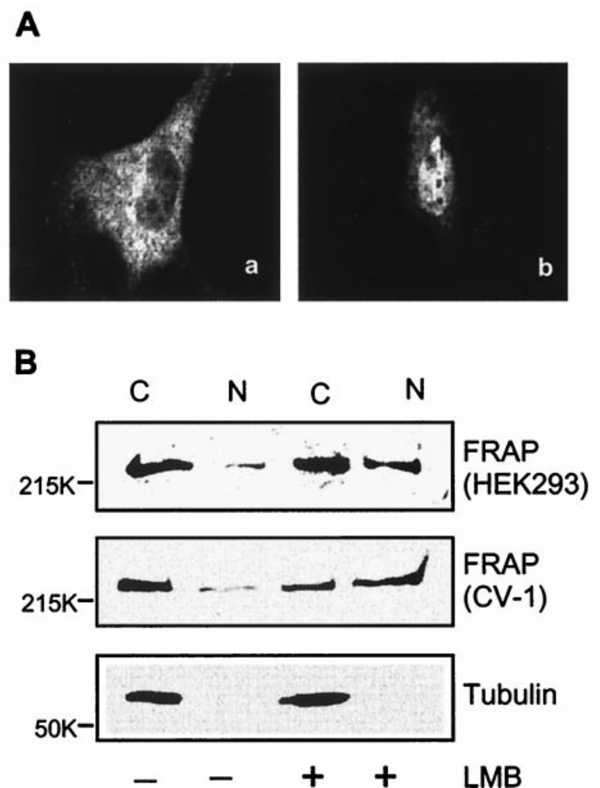

Fig. 1. FRAP is a cytoplasmic–nuclear shuttling protein. (*A*) CV-1 cells transiently transfected with FLAG-FRAP cDNA without (*a*) or with (*b*) treatment with 10 ng/ml LMB for 12 h were analyzed by immunocytochemistry and subsequent confocal microscopy. (*B*) Endogenous FRAP was analyzed on Western blotting on subcellular fractionation of both CV-1 and HEK293 cells. Anti-tubulin blot of the same fractions from CV-1 cells served as a control for the fractionation. C, cytoplasmic; N, nuclear.

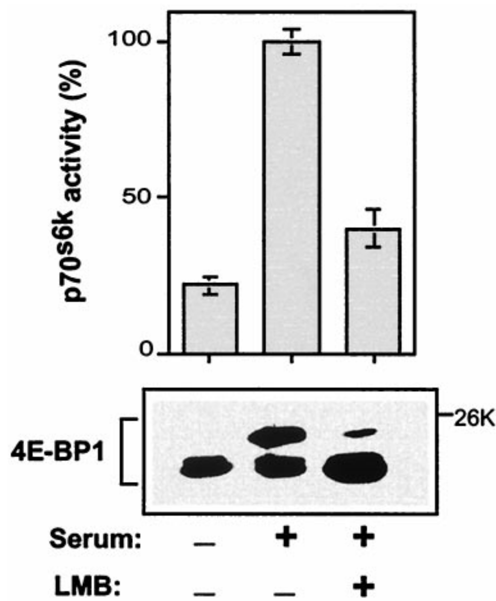

Fig. 2. LMB inhibits mitogenic stimulation of $p70^{s6k}$ activation and 4E-BP1 phosphorylation. Quiescent HEK293 cells were stimulated with 15% serum for 1 h, with or without prior treatment with 10 ng/ml LMB for 12 h. Endogenous $p70^{s6k}$ was immunoprecipitated and kinase assays were carried out in the immune complexes. 4E-BP1 phosphorylation was analyzed by Western blotting of whole cell lysates. Hyperphosphorylation of 4E-BP1 resulted in slower mobility on 13% SDS/PAGE.

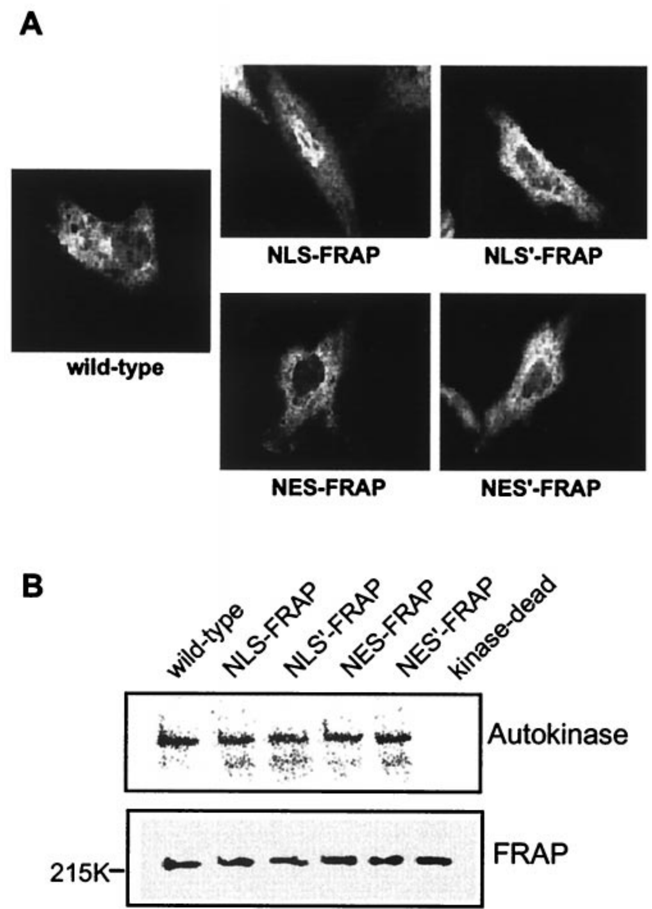

Fig. 3. Subcellular localization and kinase activity of FRAP proteins tagged with various translocation signals. (*A*) Myc-tagged NLS-, NLS′-, NES-, and NES′-FRAP proteins were transiently expressed in CV-1 cells, followed by immunostaining and confocal microscopic analysis. (*B*) The same recombinant FRAP proteins were transiently expressed in HEK293 cells and immunoprecipitated by an anti-Myc antibody, and *in vitro* autokinase assays were performed in the immune complexes. All FRAP constructs, including wild-type, contained the S2035T mutation.

carries unconventional translocation signals (30). This is not unusual for the ATM-related kinase family; ATM has been shown to be a *bona fide* nuclear protein without a clearly identifiable nuclear localization sequence. Alternatively, FRAP could translocate by means of associating with another nuclear shuttling protein.

LMB Inhibits $p70^{s6k}$ Activation and 4E-BP1 Phosphorylation. To assess the potential downstream effects of FRAP nuclear shuttling, we examined the effect of LMB on $p70^{s6k}$ and 4E-BP1. Interestingly, LMB treatment led to a significant inhibition of $p70^{s6k}$ activity and 4E-BP1 phosphorylation (Fig. 2). Several explanations were possible for these observations. A provocative notion is that nuclear shuttling of FRAP was required for the cytoplasmic pathway leading to activation of $p70^{s6k}$ and 4E-BP1, both of which are cytoplasmic proteins. Alternatively, nuclear FRAP could be inaccessible or inactive for cytoplasmic $p70^{s6k}$ and 4E-BP1; LMB may simply sequester active FRAP from the cytoplasm. Finally, it should be noted that LMB is a general inhibitor of nuclear export. All cellular proteins undergoing Crm1-dependent nuclear export would be affected by this drug. Thus the effect of LMB on $p70^{s6k}$ and 4E-BP1 might not be due to its effect on FRAP. To establish any direct connection between FRAP nuclear shuttling and downstream signaling, a more specific approach was required.

Various Translocation Signals Alter FRAP Nuclear Localization and Nuclear Shuttling Activity. To probe the function of nuclear FRAP and to test the possibility that nuclear shuttling of FRAP is required for its cytoplasmic signaling, we engineered recombinant proteins to alter the nuclear shuttling activity of FRAP. Two strong translocation signals, the NLS from simian virus 40 (31) and the NES from the HIV protein Rev (32), were individually tagged to the N terminus of FRAP containing a Myc epitope. FRAP proteins fused to mutated NLS and NES sequences were also generated (designated NLS′ and NES′). The effect of these signal peptides on the localization of recombinant FRAP was investigated in CV-1 cells on transient expression followed by immunostaining (Fig. 3*A*). NLS-FRAP displayed significantly increased nuclear staining compared with the wild-type protein. The intensity of nuclear staining of NLS′-FRAP was between those of wild-type and NLS-FRAP, consistent with the fact that the single mutation in NLS′ attenuates, but does not abolish, its nuclear import capacity (33). NES decreased FRAP nuclear staining, whereas NES′-FRAP behaved in a manner similar to that of the wild-type protein. Despite the tagging of exogenous signals, the intrinsic translocation signals (unidentified NLS and NES) on FRAP remained, and the engineered FRAP proteins were still expected to shuttle; it was the balance between import and export that was changed by such manipulation.

The kinase activity of these proteins was assayed by *in vitro* autophosphorylation; all of them displayed activity similar to that of wild-type (Fig. 3*B*). The D2357E mutant of FRAP (kinase-dead) (15) was used as a negative control. Therefore, the tagging of signal peptides at the N terminus did not affect the intrinsic activity of these proteins. In addition, these data suggest that the catalytic activity of FRAP was not affected by its nuclear translocation. This notion was further supported by the fact that LMB treatment did not change FRAP kinase activity (data not shown).

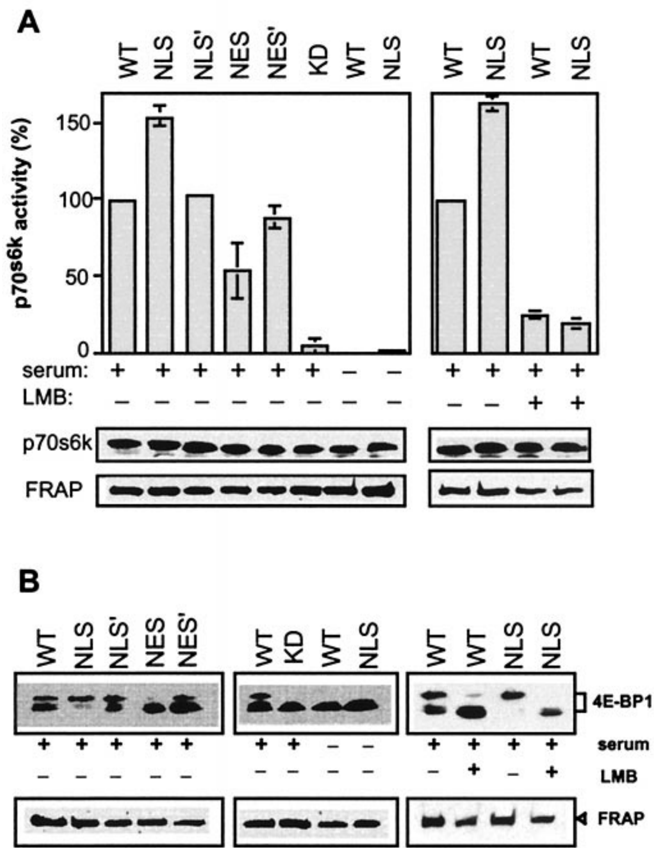

Fig. 4. Cytoplasmic–nuclear shuttling of FRAP regulates downstream signaling. Myc-tagged FRAP proteins with various translocation signals were coexpressed with HA-p70^{s6k} or FLAG-4E-BP1 in HEK293 cells. Various FRAP proteins are designated as follows: WT, wild-type; NLS, NLS-FRAP; NLS′, NLS′-FRAP; NES, NES-FRAP; NES′, NES′-FRAP; KD, kinase-dead. All FRAP constructs, including wild type, contained the S2035T mutation. All cells were pretreated with 100 nM rapamycin for 30 min before serum stimulation for 1 h. LMB treatment was carried out for 12 h before lysis. (*A*) *In vitro* kinase assays were performed with immunoprecipitated HA-p70^{s6k}. Results are shown as activities relative to that for wild type. Expressions of Myc-FRAP and HA-p70^{s6k} were monitored by Western blotting, by using epitope tag antibodies. (*B*) Phosphorylation of FLAG-4E-BP1 as reflected by mobility shift was examined by Western blotting, using M2 anti-FLAG antibody.

Nuclear Shuttling of FRAP Regulates Downstream Signaling. A mutation at Ser2035 in FRAP abolishes rapamycin-binding without interfering with FRAP's signaling activity and thus confers resistance to rapamycin (15, 26). This mutation (S2035T) was present in all of the recombinant FRAP proteins carrying various localization signals, allowing us to probe the signaling function of these proteins in the presence of rapamycin in cells containing endogenous FRAP. Mitogen-stimulated p70^{s6k} activation was examined by cotransfection of HA-p70^{s6k} with each of these engineered FRAPs, followed by immunoprecipitation and *in vitro* kinase assays of HA-p70^{s6k}. Compared with wild-type FRAP, NLS-FRAP displayed an enhanced ability to activate p70^{s6k}, whereas NES-FRAP was unable to activate p70^{s6k} to the same extent as wild type (Fig. 4*A*). NLS′-FRAP and NES′-FRAP behaved similarly to the wild-type protein, which is consistent with the nature of these mutated signals. 4E-BP1 phosphorylation responded to the various FRAP proteins in a fashion identical to that of p70^{s6k} activity, i.e., increased nuclear import of FRAP enhanced 4E-BP1 phosphorylation, whereas FRAP nuclear export attenuated the phosphorylation (Fig. 4*B*). These data strongly suggest that both activation of p70^{s6k} and phosphorylation of 4E-BP1 require nuclear FRAP. However, NLS-FRAP failed to activate p70^{s6k} and 4E-BP1 in the absence of serum stimulation (Fig. 4), indicating that nuclear localization of FRAP did not result in constitutive activation. Furthermore, nuclear import of FRAP was probably not sufficient for the downstream activation; nuclear export might also play a critical role, as inhibition of nuclear export by LMB abolished NLS-FRAP's ability to activate p70^{s6k} and 4E-BP1 (Fig. 4). Taking all of these observations into consideration, we propose that FRAP nuclear shuttling is required for p70^{s6k} activation and 4E-BP1 phosphorylation.

To gain further insight into the mechanisms of p70^{s6k} and 4E-BP1 regulation by nuclear shuttling of FRAP, we examined the effect of LMB on the subcellular distribution of all three proteins simultaneously. As assessed by cell fractionation (Fig. 5), p70^{s6k} was mostly in the cytoplasm but accumulated in the nucleus on LMB treatment, suggesting that it may also be a Crm1-dependent nuclear shuttling protein. 4E-BP1 was exclusively cytoplasmic and did not appear in the nuclear fraction, even in the presence of LMB. Thus the regulation of p70^{s6k} and that of 4E-BP1 may be different. Although it cannot be ruled out that the communication between p70^{s6k} and FRAP may occur in the nucleus, phosphorylation of 4E-BP1 by FRAP most likely occurs in the cytoplasm, but before that FRAP must traverse the nucleus.

Nuclear Shuttling of FRAP Is Involved in Rapamycin-Sensitive Translation Initiation. To further examine the biological outcome of FRAP nuclear translocation, we established a reporter assay in which the *in vivo* expression of luciferase was driven by a constitutive promoter (cytomegalovirus) and was under the control of eEF2's 5′ untranslated region (34), which contains a TOP sequence and directs rapamycin-sensitive translation initiation (10). As expected, 5′-TOP-dependent expression of luciferase was decreased ≈40% on rapamycin treatment (Fig. 6*A*). 5′-TOP-independent translation was slightly increased on treatment by rapamycin (Fig. 6*A*), which is consistent with observations reported in *Xenopus* oocytes (35) and might reflect a competition among different classes of mRNAs for the translational machinery (35, 36). By using this reporter system, we examined the effect of altering FRAP nuclear translocation on rapamycin-sensitive translation initiation. Significantly, we observed that 5′-TOP-dependent translation initiation in response to mitogenic stimulation was decreased when FRAP was exported from the nucleus (NES-FRAP) (Fig. 6*B*), suggesting a

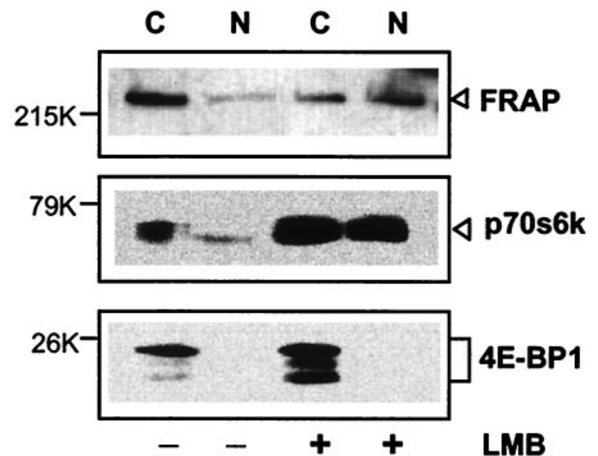

Fig. 5. LMB effect on subcellular localization of p70^{s6k} and 4E-BP1. Endogenous FRAP, p70^{s6k}, and 4E-BP1 proteins were analyzed by Western blotting on subcellular fractionation of CV-1 cells. C, cytoplasmic; N, nuclear.

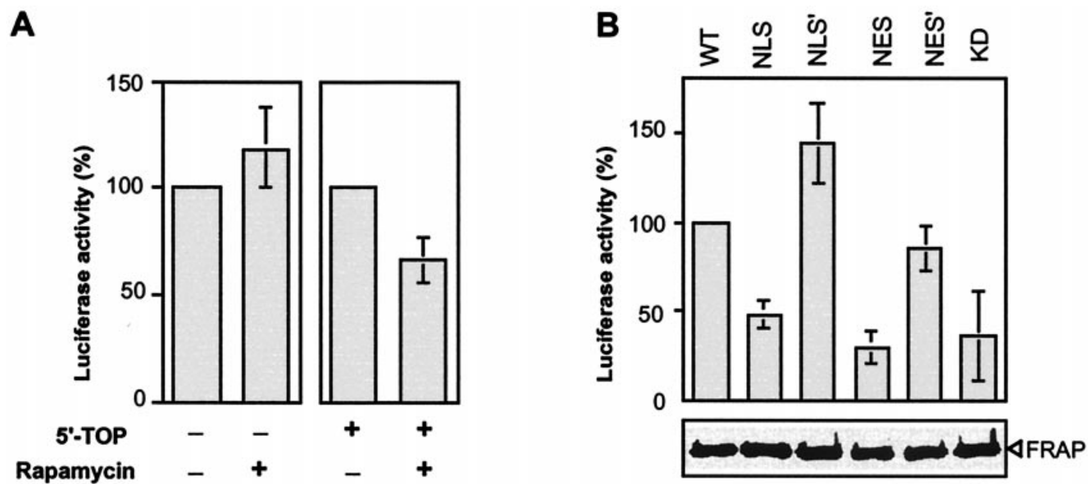

Fig. 6. Cytoplasmic–nuclear shuttling of FRAP regulates serum-stimulated rapamycin-sensitive translation. pCDNA3-luciferase cDNAs were transiently transfected into HEK293 cells. Transfected cells were serum-starved for 36 h, followed by serum stimulation for 3 h before cell lysis. Luciferase assays were carried out by using the luciferase assay system (Promega) according to the manufacturer's manual. Activities shown here correspond to luciferase expression during the 3-h serum stimulation. (*A*) Rapamycin (100 nM) was added during the 3-h serum stimulation of cells transfected with luciferase cDNA with or without eEF2 5′ untranslated region containing the 5′-TOP. The data shown are for activities relative to that in untreated cells. (*B*) Cotransfection of 5′-TOP-luciferase with variously tagged Myc-FRAPs was followed by luciferase assays performed after serum stimulation for 3 h in the presence of 100 nM rapamycin. Designation of various FRAP proteins is described in the legend to Fig. 4. All FRAP constructs, including wild type, contained the S2035T mutation. The data shown are for activities relative to that for wild type. FRAP protein expression was monitored by Western analysis.

link between nuclear FRAP and translational control. Interestingly, a moderate increase in FRAP nuclear import (NLS′) boosted 5′-TOP-dependent translation, whereas a strong nuclear import signal (NLS) led to a decrease in translation (Fig. 6*B*). Taken together, these data strongly suggest that nuclear shuttling of FRAP is required for the regulation of rapamycin-sensitive translation initiation. As both nuclear import and subsequent export would be required for FRAP function, maximal activation would be the result of an optimal balance between the two events. A moderate increase in nuclear import may result in the perfect balance, whereas a strong nuclear localization signal may disrupt that balance. It is noted that the effects of NLS and NLS′ on $p70^{s6k}$ activity and 4E-BP1 (Fig. 4) do not exactly match the outcome of translation, as one would expect, given that $p70^{s6k}$ and 4E-BP1 are considered mediators of rapamycin-sensitive translation initiation. However, it is possible that FRAP's role in translational regulation involves more than $p70^{s6k}$ activation and 4E-BP1 phosphorylation; the optimal biological outcome would be the result of a balance among several FRAP functions and may not necessarily coincide with maximal activation of a subset of effectors.

Discussion

Our findings reveal a functional nuclear residence for FRAP, the only member (and its yeast homologues) of the ATM family previously thought not to have any nuclear function. Inhibition of nuclear export by LMB has revealed a cytoplasmic–nuclear shuttling for FRAP (Fig. 1). Collective evidence from the manipulation of FRAP nuclear localization by various transport signals suggests that nuclear FRAP is required for signaling to $p70^{s6k}$ and 4E-BP1 (Figs. 3 and 4). LMB effects on $p70^{s6k}$ and 4E-BP1 (Figs. 2 and 4) further imply that nuclear shuttling of FRAP is the key to its downstream signaling. The involvement of nuclear shuttling in the activation of the FRAP-$p70^{s6k}$/4E-BP1 pathway is a surprising revelation, as this pathway regulates a cytoplasmic target, the protein synthesis machinery. Several possible mechanisms could explain the nuclear regulation of this pathway. One possibility is that FRAP, as the kinase for $p70^{s6k}$ and 4E-BP1, carries out its function in the nucleus. $p70^{s6k}$ and 4E-BP1 would shuttle between nucleus and cytoplasm to be fully phosphorylated: first phosphorylated by FRAP in the nucleus, then exported into the cytoplasm to be further phosphorylated by other kinases. This model may apply to the activation of $p70^{s6k}$, which appears to also shuttle through the nucleus (Fig. 5). However, the signal transduction between FRAP and 4E-BP1 most likely occurs in the cytoplasm because 4E-BP1 is exclusively cytoplasmic, even in the presence of LMB (Fig. 5). A compelling hypothesis is that FRAP must traverse the nucleus, where some type of modification occurs, to activate 4E-BP1 phosphorylation in the cytoplasm. The exact nature or consequence of this modification is unknown; intrinsic kinase activity of FRAP is apparently not affected by its localization (Fig. 3*B*). This is reminiscent of the recently proposed activation mechanism for Ste5, a scaffold protein in the mitogen-activated protein kinase pathway regulating yeast mating (33). Mahanty and colleagues have demonstrated that as a prerequisite for membrane localization and activation, Ste5 shuttles into and out of the nucleus, where a modification of unknown nature is speculated to occur (33). Regulation of FRAP by nuclear shuttling provides the second example of this regulatory mechanism, which may be a recurring theme in signal transduction.

We did not find any direct impact of mitogenic or nutrient signals on FRAP nuclear shuttling activity (data not shown). The finding that the enhanced signaling capacity of NLS-FRAP is dependent on serum stimulation (Fig. 4) implies that nuclear localization or shuttling may not be the consequence of mitogenic signaling. The relationship between FRAP's association with intracellular membranes (24, 25) and its nuclear shuttling is also unknown and presents an intriguing puzzle.

A positive connection between FRAP nuclear shuttling and rapamycin-sensitive translation initiation has been established by using a reporter system (Fig. 6). It is intriguing that translation initiation and $p70^{s6k}$ activation do not respond to FRAP subcellular distribution in an identical way, even though each behaves in agreement with the nuclear shuttling model. This apparent discrepancy may be attributed to FRAP's multifunctional role in translational regulation. There may be an analogy between FRAP and its family member ATM, which has been demonstrated to have

multiple downstream targets (37). The concept of FRAP participating in multiple pathways is further implicated by the recent discovery that its yeast homologues, TOR1 and TOR2, are directly involved in transcriptional regulation in addition to translational regulation (38–40). Two other S6 kinases, $p85^{s6k}$ (an isoform of $p70^{s6k}$) and the newly discovered S6K2 (41, 42), are both found in the nucleus (42, 43). It will be interesting to see whether nuclear FRAP also regulates these nuclear S6 kinases and, if so, how they contribute to the regulation of translation initiation.

The seemingly laborious nuclear shuttling mechanism may be devised by nature to best orchestrate the regulation of pleiotropic functions that FRAP may have and ensure specificity of signaling. The current studies extend our understanding of the nucleus's role in protein synthesis from mRNA production and ribosome biogenesis to a direct participation in the regulation of the translational machinery by extracellular signals.

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