Phosphorylation of the ribosomal S6 subunit is tightly correlated with enhanced translation initiation of a subset of mRNAs that encodes components of the protein synthesis machinery, which is an important early event that controls mammalian cell growth and proliferation. The recently identified S6 kinase 2 (S6K2), together with its homologue S6K1, is likely responsible for the mitogen-stimulated phosphorylation of S6. Like S6K1, the activation of S6K2 requires signaling from both the phosphatidylinositol 3-kinase and the mammalian target of rapamycin (mTOR). Here we report the investigation of the mechanisms of S6K2 regulation by mTOR. We demonstrate that similar to S6K1 the serum activation of S6K2 in cells is dependent on mTOR kinase activity, amino acid sufficiency, and phosphatidic acid. Previously we have shown that mTOR is a cytoplasmic-nuclear shuttling protein. As a predominantly nuclear protein, S6K2 activation was facilitated by enhanced mTOR nuclear import with the tagging of an exogenous nuclear localization signal and diminished by enhanced mTOR nuclear export with the tagging of a nuclear export sequence. However, further increase of mTOR nuclear import by the tagging of four copies of nuclear localization signal resulted in its decreased ability to activate S6K2, suggesting that mTOR nuclear export may also be an integral part of the activation process. Consistently, the nuclear export inhibitor leptomycin B inhibited S6K2 activation. Taken together, our observations suggest a novel regulatory mechanism in which an optimal cytoplasmic-nuclear distribution or shuttling rate for mTOR is required for maximal activation of the nuclear S6K2.

One of the critical events involved in mitogenic stimulation of mammalian cell growth and proliferation is the increased translation initiation of 5’-terminal oligopyrimidine tract-containing mRNAs, which encode components of the protein synthesis machinery (1). Phosphorylation of the ribosomal S6 subunit is correlated with 5’-terminal oligopyrimidine tract-dependent translation initiation, and the 70-kDa S6 kinase 1 (S6K1) is a serine/threonine protein kinase responsible for mitogen-stimulated S6 phosphorylation (2). In addition to playing an essential role in regulating cell growth, S6K1 appears to be a multifunctional protein involved in other cellular processes such as anti-apoptosis (3) and RNA processing (4). Two parallel pathways are both required for activation of S6K1, and they are mediated by the phosphatidylinositol 3-kinase (PI3K) and the mammalian target of rapamycin (mTOR), respectively (2, 5). While the PI3K pathway transduces mitogenic signals to S6K1, the mTOR pathway is believed to sense amino acid sufficiency and play a permissive role to govern S6K1 activation by PI3K signals (6–9).

mTOR is a serine/threonine protein kinase that belongs to the family of phosphatidylinositol kinase-related kinases (10). The kinase activity of mTOR is required, but not sufficient, for signaling to downstream effectors including S6K1 (11, 12). Most recently we have found that phosphatidic acid, likely produced by phospholipase D, directly mediates mitogenic stimulation of mTOR signaling to S6K1 (13). Thus, mTOR appears to regulate S6K1 by integrating nutrient and mitogen signals. We have also reported that mTOR is a cytoplasmic-nuclear shuttling protein (14), and this shuttling is involved in S6K1 regulation. Specifically, the constant nuclear entry and exit of mTOR is necessary for mitogenic activation of S6K1 (14), although the shuttling itself does not seem to be regulated by any known upstream signals.'

Targeted gene disruption of S6K1 in mice led to a reduced animal size, implicating S6K1 in cell growth and cell size regulation (15), but S6 phosphorylation and 5’-terminal oligopyrimidine tract-dependent translation were normal in the S6K1-deficient cells (15), suggesting the existence of a redundant kinase(s). Indeed, a homologue of S6K1 has been identified and named S6K2 (15–19). S6K1 and S6K2 are highly homologous in the kinase domain and adjacent regulatory region, and sequence diversity occurs mainly in the N and C termini. The most notable difference between these two proteins is their subcellular localization. Alternative splicing at the N terminus gives rise to two isoforms for both S6K1 (p70 S6K1a/I and p85 S6K1a/I) and S6K2 (p60 S6K2b/I and p54 S6K2b/I). p70 S6K1 is cytosolic, whereas p85 S6K1 is nuclear due to the unique N-terminal nuclear localization signal (NLS) (20). On the other hand, both S6K2 isoforms are predominantly nuclear due to an NLS in the C termini of the proteins (18). The differential subcellular localization of S6K1 (p70, p60, and p85) and S6K2 suggests potentially distinct regulatory mechanisms and/or diverse downstream effectors.

Like S6K1, the activation of S6K2 requires both the PI3K pathway and the mTOR pathway (17, 18, 21) and also involves the mitogen-activated protein kinase Erk (22, 23). While the PI3K and Erk pathways upstream of S6K2 have been characterized recently (21–23), the mTOR pathway has not been fully understood.
examined in relation to S6K2. Here we report the investigation of S6K2 regulation by mTOR. We show that S6K2 activation requires the kinase activity of mTOR, is dependent on amino acid sufficiency, and involves phosphatidic acid (PA). Furthermore, our data suggest that an optimal rate of mTOR cytoplasmic-nuclear shuttling gives rise to maximal activation of S6K2.

Experimental Procedures

Materials and Reagents—All cell culture media were from Invitrogen. Leptomycin B (LMB) was a generous gift from Dr. Minoru Yoshida at the University of Tokyo. Rapamycin and wortmannin were purchased from Calbiochem. Phosphatidic acid (1-palmitoyl 2-oleoyl) was from Avanti Polar Lipids. The following antibodies were obtained from commercial sources: M2 anti-FLAG (Sigma) and 16B12 anti-HA (Berkley Antibody Co., Richmond, CA). Anti-phospho-p44/42 (Thr202/Tyr204), anti-phospho-Akt (Ser473), anti-Erk, and anti-Akt antibodies were all obtained from Cell Signaling. All secondary antibodies were from Jackson ImmunoResearch Laboratories. 9E10.2 anti-Myc ascites were generated by the Immunological Research Facilities at the University of Illinois at Urbana-Champaign.

Plasmids—All the expression plasmids were constructed in pCDNA3 (Invitrogen). FLAG-mTOR-S203T, FLAG-mTOR-S203T/D2357E, Myc-mTOR-S203T, Myc-NLS-mTOR-S203T, and Myc-NES-mTOR-S203T were described previously (14, 24). Myc-mTOR cDNA with two and four copies of NLS were generated by sequentially inserting oligonucleotide linkers encoding NLS at a NotI site before the start codon of mTOR. FLAG-tagged NLS-mTOR, 2xNLS-mTOR, and 4xNLS-mTOR were constructed by inserting a linker encoding the FLAG epitope into the Myc-tagged mTOR constructs at a HindIII site at the 5’-end of mTOR cDNA. HA-S6K2 was kindly provided by Dr. John Blenis at Harvard Medical School (17).

Cell Culture and Transfection—Human embryonic kidney (HEK) 293 cells and monkey kidney epithelial CV-1 cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum (FBS) at 37 °C with 5% CO2. HEK293 cells or CV-1 cells were transfected in six-well plates at ~60% confluency with PolyFect (Qiagen). For co-transfection, 1.5 μg of mTOR DNA and 0.5 μg of S6K2 DNA were used. Whenever applicable, the cells were treated with 10 nM LMB for various times prior to fixation. The transfected cells were fixed in 3.7% formaldehyde (in phosphate-buffered saline), permeabilized in 0.1% Triton X-100 (in phosphate-buffered saline), and incubated with the primary antibody (2 μg/ml in 3% bovine serum albumin and phosphate-buffered saline) followed by incubation with FITC-anti-mouse IgG antibody (10 μg/ml in 3% bovine serum albumin and phosphate-buffered saline). The fluorescent images were obtained with a Leica fluorescence microscope or a Zeiss LSM510 confocal microscope.

Indirect Immunofluorescent Staining—CV-1 cells grown on glass cover slips were transfected for 24 h in 12-well plates with 1 μg of mTOR DNA or 0.5 μg of S6K2 DNA using PolyFect (Qiagen). Whenever applicable, the cells were treated with 10 nM LMB for various times prior to fixation. The transfected cells were fixed in 3.7% formaldehyde (in phosphate-buffered saline), permeabilized in 0.1% Triton X-100 (in phosphate-buffered saline), and incubated with the primary antibody (2 μg/ml in 3% bovine serum albumin and phosphate-buffered saline) followed by incubation with FITC-anti-mouse IgG antibody (10 μg/ml in 3% bovine serum albumin and phosphate-buffered saline). The fluorescent images were obtained with a Leica fluorescence microscope or a Zeiss LSM510 confocal microscope.

Kinase Assays—For S6 kinase assays, transfected or untransfected HEK293 or CV-1 cells were lysed in lysis buffer (20 mM Tris-Cl, pH 7.5, 0.1 mM Na3VO4, 25 mM NaF, 25 μM β-glycerophosphate, 2 mM EGTA, 2 mM EDTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, (pH 8.0). The freshly made PA solution was immediately added to cell culture for stimulation at a final concentration of 100 μM.

Fig. 1. mTOR kinase activity is required for S6K2 activation. Transfected HEK293 cells were serum-starved for 24 h and then stimulated with 20% FBS for 45 min with or without pretreatment by 100 nM rapamycin (Rap) followed by immunoprecipitation of recombinant S6K2 and S6 kinase assays. A, HA-S6K2 alone was transfected. B, HA-S6K2 was co-expressed with FLAG-mTOR constructs: RR, rapamycin-resistant (S2035T); KD, kinase-dead (D2357E). All cells were stimulated with FBS in the presence of rapamycin. Expressions of recombinant S6K2 and mTOR were examined by Western blotting using the anti-HA and anti-FLAG antibodies, respectively.

Fig. 2. Amino acid sufficiency is required for S6K2 activation. HEK293 cells were transfected with HA-S6K2, serum-starved (−S) for 24 h, deprived of amino acid (−AA) for 2 h, and then incubated with 20% dialyzed FBS in Dulbecco’s phosphate-buffered solution (+S+AA) or 20% FBS in Dulbecco’s modified Eagle’s medium (+S+AA) for 45 min. S6 kinase assays were performed with the immunoprecipitated recombinant S6K2. The expression of recombinant S6K2 was examined by Western blotting using the anti-HA antibody.
0.5 M KCl, and 0.3% Triton X-100) followed by immunoprecipitation using the 16B12 anti-HA antibody. The kinase assays were performed with the immune complexes as described previously for S6K1 (14).

For mTOR auto-kinase assays, transfected HEK293 cells were lysed in lysis buffer followed by immunoprecipitation using the M2 anti-FLAG antibody. Kinase assays were performed as described previously (24) and analyzed by SDS-PAGE and phosphorimaging.

**RESULTS**

**S6K2 Activation Requires mTOR and Its Kinase Activity**—In HEK293 cells, activation of a recombinant S6K2 by serum was abolished by rapamycin treatment (Fig. 1A), consistent with several previous reports (17–19). To confirm the critical role of mTOR in S6K2 activation, we introduced a rapamycin-resistant mTOR (S2035T) together with S6K2 by transient transfection. Indeed, S6K2 activation in the presence of rapamycin was significantly restored by the rapamycin-resistant mTOR (Fig. 1B), indicating that mTOR is the sole mediator of the rapamycin effect. A kinase-dead mTOR mutant (D2357E) failed to confer rapamycin resistance to S6K2 (Fig. 1B), suggesting that the kinase activity of mTOR is required for activation of S6K2.

**Amino Acid Sufficiency Is Required for Mitogenic Activation of S6K2**—Amino acid depletion leads to dephosphorylation and inactivation of S6K1 in a reversible manner via the mTOR pathway (6–8). To test whether amino acid sufficiency is also required for S6K2 activation, we examined the effect of amino acid deprivation on S6K2 activity. Amino acid withdrawal for 2 h in serum-starved HEK293 cells completely inhibited the ability of serum to stimulate S6K2 activation, and amino acid readdition restored the activation of S6K2 by serum (Fig. 2). These observations suggest that the response of S6K2 to mitogens is dependent on amino acid sufficiency.

**PA Is Involved in S6K2 Regulation**—Most recently we have shown that phosphatidic acid mediates mitogenic activation of mTOR signaling to S6K1 and 4E-BP1, likely by binding to the FKBP12-rapamycin binding domain of mTOR (13). To further investigate the mechanism of S6K2 regulation by mTOR, we examined the involvement of PA in S6K2 activation. Mitogenic stimulation may result in phospholipase D-mediated increase in PA production, which can be blocked by low concentrations of primary alcohols due to the production of phosphatidylalcohol.
at the expense of PA (25, 26). As shown in Fig. 3A, the serum-stimulated S6K2 activation was significantly blocked by 0.3% 1-butanol, which inhibited serum-induced PA production in these cells (Fig. 3B) (13). Under identical conditions, the activation of Erk and Akt was analyzed by Western blotting using phosphospecific antibodies. Neither Erk nor Akt was affected by butanol treatment (Fig. 3C). Hence, the butanol effect was highly specific for S6K2, and it suggested the role of PA in the regulation of S6K2. In addition, exogenous PA stimulated S6K2 activation in the absence of serum (Fig. 3D), further supporting the direct involvement of PA in S6K2 activation. The activation of S6K2 by PA was abolished by rapamycin, confirming the involvement of mTOR. Wortmannin, a specific inhibitor of PI3K, also completely inhibited S6K2 activation by PA (Fig. 3D), implying that a basal level of PI3K activity is required for the PA effect since under the same conditions PA does not stimulate PI3K (13). Furthermore, the ability of PA to stimulate S6K2 activity is fully dependent on amino acid sufficiency as PA had no effect in the absence of amino acids with or without serum (Fig. 3E). Therefore, the activation of S6K2 likely requires three parallel pathways: the amino acid-sensing mTOR pathway, the mitogen-activated PI3K pathway, and the mitogen-activated PA-mTOR pathway (presumably mediated by phospholipase D).

**LMB Inhibits S6K2 Activation**—Previously we have reported that mTOR is a cytoplasmic-nuclear shuttling protein and that this shuttling appears to be required for S6K1 activation (14). Given the distinct subcellular localization of S6K1 and S6K2, the effect of mTOR localization on S6K2 activity might be different from that on S6K1. Interestingly, LMB (27), a specific inhibitor of the nuclear export Crm1 (28), inhibited S6K2 activity by about 50% (Fig. 4A). The recombinant S6K2 was mostly localized in the nucleus, and the localization was not changed by LMB (Fig. 4B). It is thus possible that the nuclear export or shuttling of an upstream activator of S6K2 may be required for S6K2 activation. mTOR is a candidate for such
an activator since LMB sequestered mTOR in the nucleus (Fig. 4C).

Altered mTOR Nuclear Translocation Affects S6K2 Activation—To specifically examine the potential involvement of cytoplasmic-nuclear shuttling of mTOR in S6K2 activation, we made use of two previously engineered mTOR constructs (14) tagged with the SV40 NLS (29) and the human immunodeficiency virus Rev nuclear export sequence (NES) (30), respectively. The ability of these mTOR variants to activate S6K2 in vivo was examined by co-transfection with epitope-tagged S6K2 into CV-1 cells followed by immunoprecipitation of recombinant S6K2 and subsequent in vitro kinase assays. All the mTOR cDNA constructs contained the rapamycin resistance S2035T mutation, and all experiments were carried out in rapamycin-treated cells to eliminate endogenous mTOR signaling (11). Compared with the wild type, NLS-mTOR enhanced S6K2 kinase activation, whereas NES-mTOR led to a decreased S6K2 kinase activity (Fig. 5). These observations are similar to those made with S6K1, although S6K1 and S6K2 are differentially localized in the cell.

Cytoplasmic-Nuclear Shuttling of mTOR Is Required for S6K2 Activation—Since S6K2 is primarily a nuclear protein, its enhanced activation by NLS-mTOR and diminished activation by NES-mTOR would be simply due to mTOR activation of S6K2 in the nucleus. An alternative mechanism involves the cytoplasmic-nuclear shuttling of mTOR. To distinguish between these possibilities, we constructed mTOR in which two or four copies of NLS (2xNLS or 4xNLS, respectively) were tagged at the N terminus. If the nuclear entry of mTOR is sufficient to activate S6K2, increased S6K2 activation would be expected to correlate with enhanced mTOR nuclear import. On the other hand, if nuclear export of mTOR is also required, overenhanced mTOR nuclear import might have a negative impact on S6K2.

The subcellular localization of the recombinant mTOR engineered to alter its nuclear import activity was examined in CV-1 cells by indirect immunofluorescent staining (Fig. 6A). As reported previously (14) a small fraction of the wild-type mTOR protein was found in the nucleus. An even distribution between cytoplasm and nucleus was found for NLS-mTOR in 60% of the cells, whereas 60% of the 4xNLS-mTOR-expressing cells displayed mostly nuclear (30%) or almost exclusively nuclear (30%) staining. In both the NLS-mTOR- and 4xNLS-mTOR-expressing cells, about 40% of the population displayed mTOR localization similar to the wild type (data not shown). The extent of the nuclear localization of 2xNLS-mTOR was intermediate compared with those of NLS-mTOR and 4xNLS-mTOR (data not shown). Therefore, all the mTOR variants behaved as expected in their subcellular localization. The change in subcellular localization did not affect the catalytic activity of mTOR as all the mutants displayed in vitro autokinase activity comparable to the wild type when transiently expressed in HEK293 cells and immunoprecipitated (Fig. 6B).

The ability of the nuclear import-enhanced mTOR mutants to activate S6K2 was then examined by co-expressing mTOR with recombinant S6K2 in CV-1 cells that were treated with rapamycin, again taking advantage of the S2035T mutation in all the cDNA constructs to eliminate endogenous mTOR signaling. The tagging of one and two copies of NLS progressively increased the extent of S6K2 activation, but 4xNLS activated S6K2 to a lesser degree than NLS (Fig. 7). These observations suggest that the nuclear entry of mTOR alone is not sufficient to activate S6K2. Instead, an optimal rate of cytoplasmic-nuclear shuttling of mTOR may be required.

**DISCUSSION**

The rapamycin sensitivity of S6K2 has been a controversial issue. While several groups reported a complete blockage of S6K2 activity by low concentrations of rapamycin (17–19), one group observed a partial resistance of S6K2 activity to rapamycin at concentrations up to 200 nM (16, 31), and persistence of S6K2 activity upon amino acid withdrawal (31). We have found that in HEK293 and CV-1 cells S6K2 (βII isoform) activity was completely inhibited by 100 nM rapamycin (Fig. 1A and data not shown). In addition, amino acid withdrawal abolished S6K2 activation by serum, contrary to the report by Minami et al. (31), and readdition of amino acids restored serum stimulation of S6K2 (Fig. 2). Furthermore, we have demonstrated that the activation of S6K2 is dependent on mTOR kinase activity (Fig. 1B). The difference in amino acid requirement may be attributed to the two different isoforms of S6K2 that we (βII) and Minami et al. (βI, Ref. 31) studied. However, it is not clear what gave rise to the discrepancy in the effect of rapamycin on S6K2. The βII isoform with an optimal Kozak sequence surrounding the start codon (16) was examined by all groups (16–18). In addition, all reported assays (16–18, 31), as well as ours, were carried out with transiently expressed S6K2 in HEK293 cells. Thus, no obvious explanation can be found for the discrepancy in rapamycin sensitivity of S6K2; subtle differences in cell culture and/or assay conditions may be responsible but are not assessable from the published information.

The recent finding that PA mediates mitogenic activation of mTOR signaling to S6K1 and 4E-BP1 has uncovered a previously unexpected regulatory mode for mTOR (13). We now report that S6K2 is also regulated by PA as S6K2 was inhibited by a low concentration of butanol in serum-stimulated cells and activated by exogenous PA in serum-starved cells (Fig. 3). Although implicated by the effect of 1-butanol, the involvement of phospholipase D in S6K2 and S6K1 activation is yet to be definitively proven and is currently under investigation.

The cytoplasmic-nuclear shuttling of mTOR, both the nuclear entry and subsequent nuclear exit, appears to be required for the activation of S6K1 and 4E-BP1 (14). The predominantly nuclear localization of S6K2 (17, 18) (Fig. 4B), as opposed to the...
cytoplasmic localization of S6K1 and 4E-BP1, might suggest a distinct requirement for mTOR localization. Increased mTOR nuclear import (NLS-mTOR) led to enhanced S6K2 activation, whereas increased mTOR nuclear export (NES-mTOR) resulted in reduced S6K2 activity (Fig. 5), which may simply reflect a correlation between nuclear mTOR and the activation of nuclear S6K2. However, S6K2 activation was inhibited by LMB (Fig. 4A), suggesting that the nuclear export of an upstream component is required. Strong evidence for the critical role of mTOR shuttling came from the observations that while two copies of NLS tagged to mTOR further enhanced S6K2 activation in vivo, additional increase of nuclear entry by tagging four copies of NLS to mTOR reduced S6K2 activation (Fig. 7). Similar results were also obtained with S6K1 (data not shown). It is thus likely that a balanced distribution of mTOR between the cytoplasm and nucleus, or an optimal shuttling rate for mTOR, may be essential for maximal activation of downstream signaling. The fact that mTOR with two exogenous copies of NLS is most active does not necessarily suggest that nature has designed a suboptimal mTOR for downstream signaling. Since these experiments rely on overexpression of recombinant proteins, the stoichiometry of various components in the pathway may be different from that of the endogenous proteins. Nevertheless, the outcome of the multiple NLS tagging experiments has proven as a principle the importance of mTOR shuttling in activating downstream signaling.

Lee-Fruman et al. (17) reported that S6K2II was in a detergent-soluble fraction, whereas S6K2I stayed in the particulate fraction, suggesting that the two isoforms may be localized to different nuclear compartments. Interestingly, the localization of S6K2II appears identical to that of S6K1α/β (p85s6k) (17, 20), the activation of which is also dependent on mTOR shuttling (data not shown). It would be intriguing to examine the regulation of S6K2β in the context of mTOR localization. It remains a puzzle why activation of S6K2, a nuclear protein, requires the cytoplasmic-nuclear shuttling (and not just nuclear entry) of mTOR, a predominantly cytoplasmic protein. One simple possibility would be that upon activation of S6K2 in the nucleus mTOR is inactivated, and it is necessary for mTOR to be reactivated in the cytoplasm to allow maximal S6K2 activation. However, this hypothesis is not supported by the observation that nuclear entry of mTOR is
much slower than the rate of full S6K2 activation in the cell: while S6K2 is maximally activated at 30 min (17) (data not shown), mTOR nuclear entry, as indicated by sequestration by LMB, required more than 3 h to complete (Fig. 4C). It is not known whether activation of S6K2 occurs in the cytoplasm or nucleus or in both as a multistep process. Both PI3K and Akt, upstream regulators of S6K2 (17, 18, 21), have been found to translocate into the nucleus upon stimulation (e.g. see Refs. 32 and 33), and it cannot be ruled out that S6K2 itself may also shuttle between the two compartments. Therefore, many possibilities exist for the activation process of S6K2. The regulation of mTOR is also a complex process; the relationship between PA binding (presumably in the intracellular membranes) and nuclear translocation of mTOR is currently unclear and awaits future investigations.

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