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Glutamine is a key regulator for amino acid-controlled cell growth through the mTOR signaling pathway in rat intestinal epithelial cells

Tohru Nakajo[a], Tomoki Yamatsuji[a], Hidetoshi Ban[a], Kaori Shigemitsu[a], Minoru Haisa[a], Takayuki Motoki[a], Kazuhiro Noma[a], Tetsuji Nobuhisa[a], Junji Matsuoka[a], Mehmet Gunduz[b], Kazuyoshi Yonezawa[c], Noriaki Tanaka[a], Yoshio Naomoto[a,*]

[a] *Department of Gastroenterological Surgery, Transplant, and Surgical Oncology, Graduate School of Medicine and Dentistry, Okayama University, 2-5-1 Shikatacho, Okayama 700-8558, Japan*
[b] *Department of Oral Pathology and Medicine, Graduate School of Medicine and Dentistry, Okayama University, 2-5-1 Shikatacho, Okayama 700-8558, Japan*
[c] *Biosignal Research Center, Kobe University, Kobe 657-8501, Japan*

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Abstract

Amino acids, especially branched-chain amino acids such as L-leucine, have been shown to regulate activation of p70 S6 kinase and phosphorylation of 4E-BP1 through the mTOR signaling pathway. In our recent study, L-arginine was also shown to activate the mTOR signaling pathway in rat intestinal epithelial cells. L-Glutamine is an amino acid that is required for culturing of numerous cell types, including rat intestinal epithelial cells. In this study, we showed that L-glutamine inhibited the activation of p70 S6 kinase and phosphorylation of 4E-BP1 induced by arginine or leucine in rat intestinal epithelial cells. Although the molecular mechanism of L-glutamine-induced inhibition of the mTOR signaling pathway is still unknown, the presence of this novel signal pathway may indicate that individual amino acids play specific roles for cellular proliferation and growth.

Keywords: p70 S6 kinase; 4E-BP1; L-Glutamine; L-Arginine; L-Leucine; mTOR; IEC16; IEC18

Amino acids are essential nutrients for mammalian cells. Classically, they have been considered to constitute functional proteins that mediate various kinds of vital activities of cells, tissues, or organs, to be the building blocks of the structural proteins that make up cells or tissues, and to be metabolized as an energy source. Recent studies have shown that amino acids are also important signaling regulators, especially for p70 S6 kinase ($p70^{S6k}$) and eIF-4E binding protein 1 (4E-BP1), via mammalian target of rapamycin (mTOR) [1–7].

$p70^{S6k}$ is activated through phosphorylation of an array of Ser/Thr sites situated in the autoinhibitory domain in the carboxyl-terminal tail (Ser-434, Ser-441, Thr-444, Ser-447, and Ser-452), in the activation loop of the catalytic domain (Thr-252), and in the segment immediately carboxyl-terminal to the catalytic domain (Ser-392 and Thr-412), and phosphorylates S6 protein in the 40S ribosomal subunit in vivo, thereby driving the translation of mRNAs with a 5′ terminal oligopyrimidine tract (TOP) [8–10]. $p70^{S6k}$ is activated in response to diverse stimuli in vivo [11] and in vitro [12].

eIF-4E, a 7-methylguanosine cap-binding protein, binds to two other initiation factor proteins, eIF-4A

* Corresponding author. Fax: +81 86 221 8775.
E-mail address: ynaomoto@md.okayama-u.ac.jp (Y. Naomoto).

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and eIF-4G, to form the cap-dependent mRNA helicase complex known as eIF-4F. eIF-4F promotes the unwinding of the mRNA structure near the 5′ cap, which in turn facilitates binding by the 40S ribosomal subunit, thereby activating translational initiation. 4E-BP1 in nonphosphorylated form binds to eIF-4E and inhibits cap-dependent translation of mRNA in vitro and in vivo by preventing formation of the eIF-4F complex [10,13–16].

Recent studies have demonstrated that mTOR controls the translation machinery by regulating the phosphorylation of both 4E-BP1 and $p70^{S6k}$ [12,15–18]. Some branched chain amino acids, such as L-leucine, have been shown to be essential elements in the regulation of 4E-BP1 and $p70^{S6k}$ through the mTOR signaling pathway in hepatic cells, ovarian cells, muscular cells, and pancreatic β cells in a rapamycin-sensitive manner [6,19–21].

According to the traditional theory of nutrition reported in many gastroenterological researches, specific amino acids play important roles in proliferation or growth of intestinal cells. For example, glutamine has been reported to work as an important factor for metabolic function of intestinal mucosa [22], and for proliferation of colon cancer cells [23]. Another amino acid, arginine, improved the immunometabolic response and outcome of colorectal resection for cancer [24].

We tried to investigate the involvement of the mTOR signaling pathway in the intestinal cell lines, IEC6 and 18. In these cell lines, not only L-leucine but also L-arginine activates the mTOR signaling pathway [25]. In this study, we analyzed the biological signals induced by leucine and arginine, and discovered a regulatory effect induced by glutamine, which is well known as an essential amino acid for cell culture [26–30]. We must develop a new hypothesis to distinguish the signal for cellular proliferation from the signal for cell growth. Intestinal cells may utilize amino acids not only as nutrients, but also as specific and active biological molecules, for cellular proliferation or growth.

Materials and methods

Reagents and antibodies. Dulbecco's modified Eagle's medium (DMEM) and the MEM SELECT-AMINE Kit were purchased from Gibco-BRL (Grand Island, NY). Fetal bovine serum (FBS) was purchased from Sanko (Tokyo, Japan). Protein G–Sepharose 4 Fast Flow and [γ-^{32}P]ATP were from Amersham–Pharmacia Biotech. A rabbit polyclonal antibody raised against the C-terminal 18 amino acids of $p70^{S6k}$, sc-230, a polyclonal antibody against 4E-BP1, sc-6936, and an anti-rabbit IgG-HRP, sc-2004, were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). A rabbit polyclonal antibody against phospho-Ser2448 of mTOR, #2971, was obtained from Cell Signaling. Other ordinary reagents were available on the market.

Cell culture and treatments. Rat intestinal epithelial cells, IEC18, were obtained from Dainippon Pharmaceutical Co. Ltd. (Tokyo, Japan) and grown in DMEM with 10% FBS. Cells were incubated in DMEM without FBS for 24 h, rinsed twice with PBS, incubated in DMEM without either FBS or amino acids for 40 min, and then incubated in the indicated concentration of amino acids for 30 min. Then, cells were washed once with ice-cold PBS and frozen in liquid nitrogen. Frozen cells were lysed in ice-cold buffer A (50 mM Tris–HCl (pH 8.0), 1% Nonidet P-40, 120 mM NaCl, 20 mM NaF, 1 mM EDTA, 6 mM EGTA, 20 mM β-glycerophosphate, 0.5 mM dithiothreitol, 1 mM α-phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, and 1 μg/ml leupeptin). The cell extracts were centrifuged at 10,000*g* for 20 min at 4 °C, and the supernatants were used for the assays. The concentration of each amino acid in DMEM is designated as 1X [7,25], as follows (in mM): L-arginine, 0.398; L-cystine, 0.201; L-glutamine, 4.00; L-histidine, 0.200; L-isoleucine, 0.801; L-leucine, 0.801; L-lysine, 0.798; L-methionine, 0.201; L-phenylalanine, 0.400; L-serine, 0.400; L-threonine, 0.798; L-tryptophan, 0.0784; L-tyrosine, 0.399; and L-valine, 0.803.

Immunoprecipitation and p70 S6 kinase assay. The activity of $p70^{S6k}$ was determined by an immunocomplex assay using 40S ribosomal subunits as substrates, as described previously [31,32]. Briefly, supernatants were subjected to immunoprecipitation with anti-$p70^{S6k}$ antibody (sc-230) for 2 h at 4 °C, absorbed to protein G–Sepharose 4FF, and washed twice with buffer A containing 0.5 M NaCl and twice with buffer B (20 mM Mops (pH 7.2), 10 mM β-glycerophosphate). Thirty-five microliters of the reaction mixture (50 mM Mops (pH 7.2), 12 mM $MgCl_2$, 2 mM EGTA, 1 mM dithiothreitol, 60 μM ATP, 10 mM β-glycerophosphate, 0.5 μM protein kinase inhibitor, 0.5 A_{260} units of 40S ribosomal subunits, and 5 μCi [γ-^{32}P]ATP) was added to each sample, and the samples were incubated for 20 min at 30 °C. The reaction was terminated by adding 30 μl of the SDS buffer to each sample. The samples were separated by SDS–PAGE on 12% acrylamide gel, and the radioactivity incorporated into 40S S6 protein of each sample was quantified with a BAS-2000 Bioimaging analyzer (Fuji Photo Film, Tokyo, Japan).

Immunoblot analysis of 4E-BP1. Aliquots of the supernatants of the cell extracts were boiled for 7 min at 90 °C and cooled on ice, and then precipitated by centrifugation at 10,000*g* for 30 min at 4 °C. The heat-soluble proteins were separated by SDS–PAGE on 15% polyacrylamide gels, transferred onto a polyvinylidene difluoride membrane, immunoblotted with the polyclonal antibody against 4E-BP1 (sc-6936; 1:500 dilution) followed by anti-rabbit IgG-HRP (sc-2004; 1:2000 dilution), and then visualized using an ECL kit according to the manufacturer's protocol (Amersham–Pharmacia Biotech, Tokyo, Japan).

Immunoblot analysis of phospho-mTOR. Cells were lysed in 1× SDS sample buffer (62.5 mM Tris–HCl (pH 6.8 at 25 °C), 2% w/v SDS, 10% glycerol, 50 mM DTT, and 0.01% w/v bromophenol blue or phenol red) and centrifuged at 4 °C. The sample viscosity was reduced by pipetting. The samples were then boiled for 5 min and cooled on ice. The samples were separated by SDS–PAGE on 7.5% polyacrylamide gels, transferred onto a polyvinylidene difluoride membrane, immunoblotted with the polyclonal antibody against phospho-mTOR (#2971; 1:1000 dilution) followed by anti-rabbit IgG-HRP (sc-2004; 1:2000 dilution), and then visualized using an ECL kit according to the manufacturer's protocol (Amersham–Pharmacia Biotech).

Results

L-Arginine and L-leucine activated p70 S6 kinase

Withdrawal of amino acids from the medium results in a rapid but reversible inactivation of the $p70^{S6k}$ activity. We observed this response in both IEC6 and IEC18 cells. After 24 h of serum deprivation and 40 min of amino acid withdrawal, IEC6 and IEC18 cells were

incubated with medium containing each amino acid at 4× concentration for 30 min. L-leucine, which was previously reported to significantly activate $p70^{S6k}$ in other cells [6,19,20], and L-arginine also to activate $p70^{S6k}$ as compared with other amino acids (Fig. 1).

Effect of L-glutamine on L-arginine- and L-leucine-induced activation of p70 S6 kinase in IEC18 cells

After 24 h of serum deprivation, IEC18 cells were incubated in amino acid-depleted medium for 40 min and then incubated in a medium containing the indicated concentrations of L-arginine and L-leucine with or without L-glutamine at the indicated concentrations for 30 min. L-arginine and L-leucine significantly activated $p70^{S6k}$. In contrast, L-glutamine induced little or no activation of $p70^{S6k}$. When added to L-leucine and L-arginine, L-glutamine reduced L-arginine-induced $p70^{S6k}$ activity to approximately one-fortieth of the baseline value (Fig. 2), and reduced L-leucine-induced $p70^{S6k}$ activity to about one-fifth of the baseline value (Fig. 3).

Effect of L-glutamine on the ability of L-arginine and L-leucine to phosphorylate 4E-BP1

Phosphorylated 4E-BP1 displays slower electrophoretic migration when separated on SDS–PAGE. This multisite phosphorylated protein is divided into at least three electrophoretic forms according to its phosphorylation state: hyperphosphorylated γ, which migrates most slowly, intermediary β, and nonphosphorylated α, which migrates fastest [33].

We examined the ability of L-arginine and L-leucine to regulate 4E-BP1 phosphorylation and the additional effect of L-glutamine in IEC18 cells. After 24 h of serum deprivation, cells were incubated in amino acid-depleted medium for 40 min and incubated for 30 min with medium containing a 4× concentration of L-arginine and L-leucine with or without L-glutamine at the indicated concentrations. Addition of L-glutamine to L-arginine-containing medium reduced a hyperphosphorylated γ state in a dose-dependent manner. In contrast, addition of L-glutamine to L-leucine-containing medium had no effect on the phosphorylation state of 4E-BP1 (Fig. 4).

Effect of L-glutamine on the ability of L-arginine and L-leucine to phosphorylate mTOR

The mammalian target of rapamycin, mTOR, also known as FRAP or RAFT, is a serine/threonine protein kinase. mTOR acts as a sensor for amino acids, balancing the availability of nutrients and cell growth. When sufficient nutrients are available, mTOR transmits a positive signal to p70 S6 kinase and 4E-BP1 [17]. Until recently, phosphorylation of mTOR could not be assessed. Therefore, we examined p70 S6 kinase activity and 4E-BP1 phosphorylation as useful markers for the activation of the mTOR signaling pathway. Recently, an

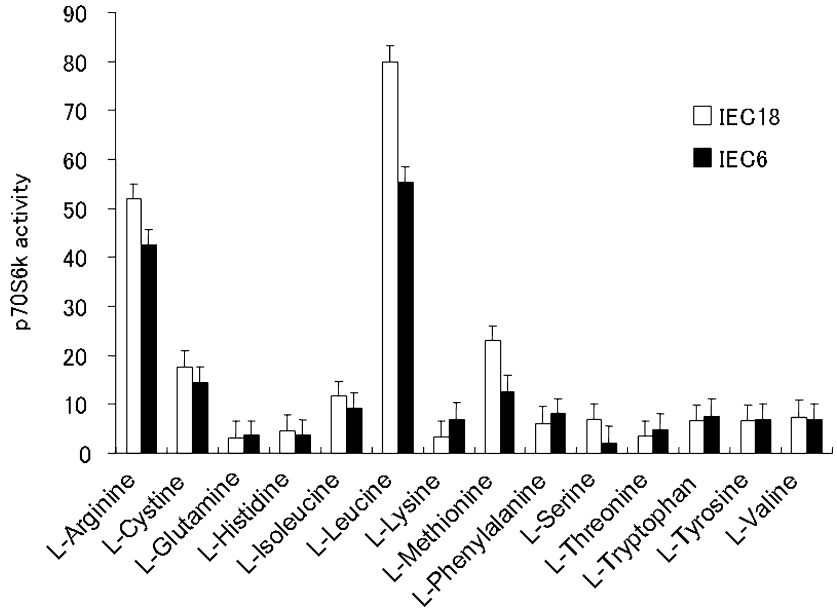

Fig. 1. Activity of p70 S6 kinase induced by addition of each amino acid. After 24 h of serum deprivation and 40 min of amino acid depletion, IEC18 cells and IEC6 cells were incubated with medium containing each amino acid at a 4× concentration for 30 min, and their endogenous p70 S6 kinase activities were measured using a p70 S6 kinase assay. Data are expressed as a percentage of the p70 S6 kinase activity when the cells were treated with complete amino acids at a 1× concentration for 30 min after 40 min of amino-acid deprivation. The p70 S6 kinase activity data are presented as means ± SD of three separate experiments. 1× concentration of each amino acid is equivalent to that in DMEM as described under Materials and methods.

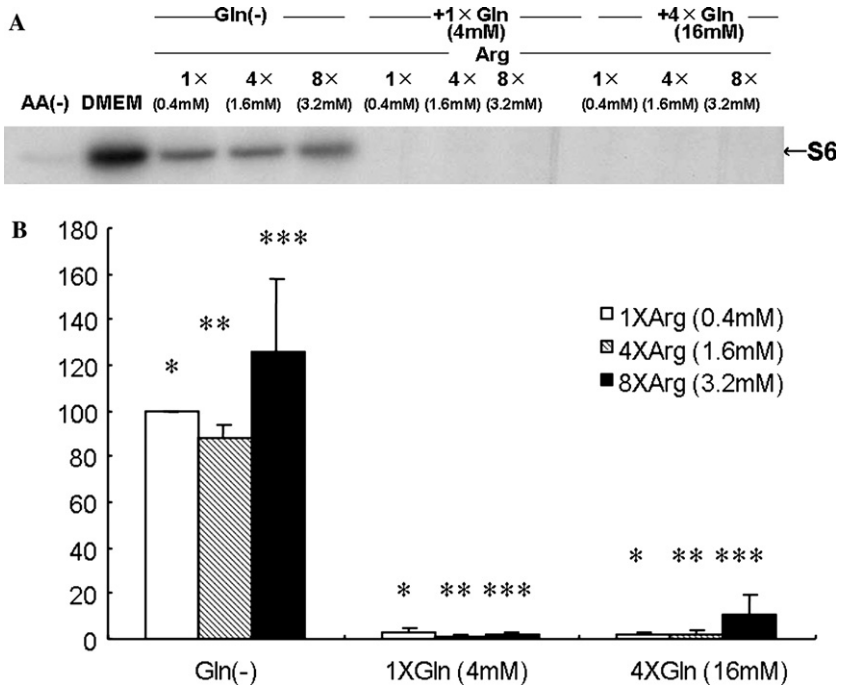

Fig. 2. (A) Inhibitory effect of L-glutamine on the ability of L-arginine to activate p70 S6 kinase in IEC18 cells. Twenty-four hours of serum deprivation and depletion of amino acids for 40 min were followed by incubation with medium containing the indicated concentration of L-arginine with or without L-glutamine for 30 min. And their endogenous p70 S6 kinase activities were measured using a p70 S6 kinase assay. The samples were separated by SDS–PAGE on 15% acrylamide gel, and the radioactivity incorporated into 40S S6 protein of each sample is shown. (B) The radioactivity incorporated into 40S S6 protein shown in (A) was quantified with a BAS-2000 Bioimaging analyzer. Data are expressed as a percentage of the p70 S6 kinase activity when the cells were treated with complete amino acids at a 1× concentration for 30 min after 40 min of amino-acid deprivation (*, **, ***$p < 0.01$).

antibody which could detect mTOR phosphorylation was developed. A rabbit polyclonal antibody against phospho-Ser2448 of mTOR was reported to detect the activation of mTOR signaling [34].

We examined the ability of L-arginine and L-leucine to phosphorylate mTOR using the above-described antibody, in addition to examining the effect of L-glutamine, in IEC18 cells. After 24 h of serum deprivation, cells were incubated in amino acid-depleted medium for 40 min and incubated for 30 min with medium containing a 4× concentration of L-arginine and L-leucine with or without a 4× concentration of L-glutamine. Addition of L-glutamine to L-arginine-containing medium reduced mTOR phosphorylation. On the other hand, addition of L-glutamine to L-leucine-containing medium had little effect on the phosphorylation of mTOR (Fig. 5).

Discussion

In this report we showed that glutamine had an inhibitory effect on leucine- or arginine-induced activation of the mTOR signaling pathway. Leucine is one of the so-called "essential" amino acids, a group of nine amino acids that cannot be synthesized by adult vertebrates. And glutamine has been found to be functionally essential for the survival and growth of most cultured cells [26,27]. In several animal species, glutamine was shown to be the major respiratory fuel for the intestinal tract [28–30]. For the rat intestinal epithelial cell line (IEC18) used here, glutamine was also essential for growth and protection against stresses [35]. Amino acids had been thought as just a nutrient for cell growth. Intracellular signaling through amino acids was not known.

Recent studies have demonstrated that mTOR controls the translational machinery, and its phosphorylation plays a very important role for cell proliferation and growth [17]. And we reported that mTOR is regulated by specific amino acids [7,19]. We examined the effect of each amino acids on mTOR in a rat intestinal epithelial cell line, IEC18. Of these, leucine and arginine have been shown to activate the mTOR signaling pathway, while glutamine does not [25].

Here we demonstrated the inhibitory effect of glutamine on leucine- or arginine-induced activation of the mTOR signaling pathway. Our recent study suggested that manner of mTOR activation induced by leucine is identical with arginine [25]. Signal transduction of arginine-induced stimulation is inhibited by the blockers of cationic amino acid transporters. In our current data, while glutamine dramatically suppressed arginine-induced signal transduction, its effect on leucine-induced

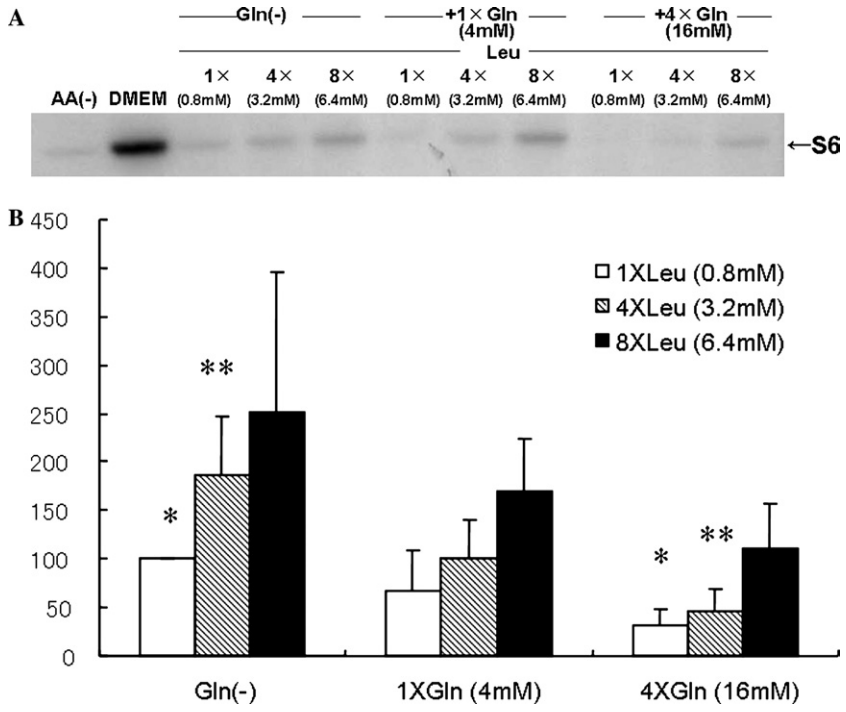

Fig. 3. (A) Inhibitory effect of L-glutamine on the ability of L-leucine to activate p70 S6 kinase in IEC18 cells. Twenty-four hours of serum deprivation and depletion of amino acids for 40 min were followed by incubation with medium containing the indicated concentrations of L-leucine with or without L-glutamine at the indicated concentration for 30 min. And their endogenous p70 S6 kinase activities were measured using a p70 S6 kinase assay. The samples were separated by SDS–PAGE on 15% acrylamide gel, and the radioactivity incorporated into 40S S6 protein of each sample is shown. (B) The radioactivity incorporated into 40S S6 protein shown in (A) was quantified with a Bioimaging analyzer as described in Fig. 2B (*, **$p < 0.01$).

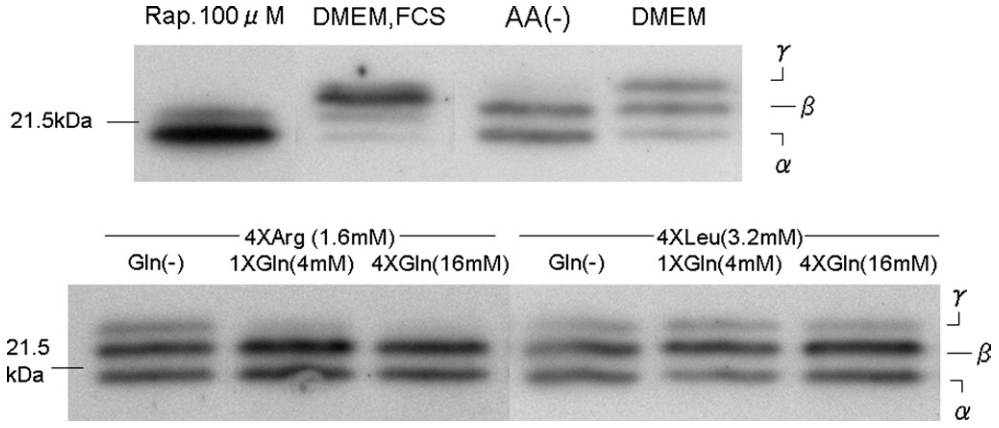

Fig. 4. Inhibitory effect of L-glutamine on the ability of L-arginine and L-leucine to phosphorylate 4E-BP1. After 24 h of serum deprivation and depletion of amino acids for 40 min, IEC18 cells were incubated with medium containing a 4× concentration of L-arginine or L-leucine with or without L-glutamine at the indicated concentration for 30 min. Immunoblot analysis of 4E-BP1 was performed as described under Materials and methods.

signaling was minimal. These data suggest that glutamine may function as a more specific inhibitor of mTOR signaling pathway induced by arginine, a cationic amino acid, possibly through blocking of its transporters, whereas its suppressive effect was not very high and specific on a neutral amino acid, leucine-induced signaling. This is the first report to argue that a specific amino acid, i.e., glutamine, can regulate a cellular signaling pathway induced by other amino acids. It is believed that glutamine is the most important amino acid for cellular proliferation and growth. For example, glutamine has been proven essential for the proliferation of intestinal cells [26–30] and cancer cells [23]. Our data may demonstrate a new concept for the biological role of phosphorylation of mTOR and intestinal cell growth. Although glutamine was expected to stimulate cellular

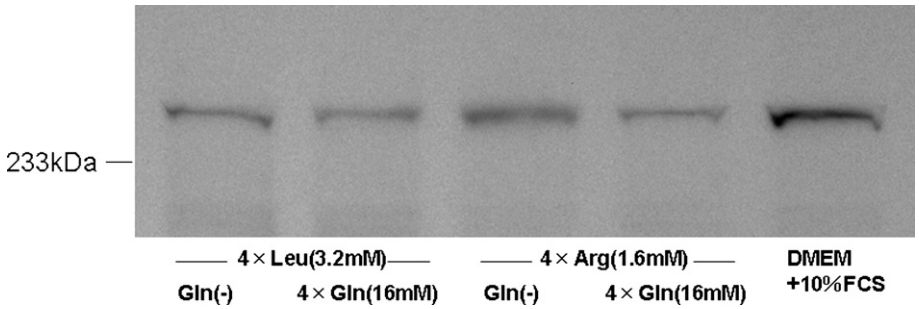

Fig. 5. Effect of L-glutamine on the ability of L-arginine and L-leucine to phosphorylate mTOR. After 24 h of serum deprivation and depletion of amino acids for 40 min, IEC18 cells were incubated with medium containing a 4× concentration of L-arginine or L-leucine with or without a 4× concentration of L-glutamine for 30 min. Immunoblot analysis of phospho-mTOR was performed as described under Materials and methods.

proliferation, our results showed that it suppressed mTOR signaling for cell growth. To explain such a discrepancy of signaling pathway, we should distinguish the signal for cellular proliferation from the signal for cell growth. Fingar et al. [36] demonstrated that mammalian cell size is controlled via mTOR and its downstream targets S6K1 and $p70^{S6k}$. And the outcome of mTOR signaling for cellular growth is not an increase in cell number, but rather an increase in cell size. This conclusion of the study is compatible with our data. This may suggest an answer to our question regarding the relation between amino-acid signaling and mTOR activation. In IEC18 intestinal cells, glutamine may function as an essential fuel, accelerating the cell cycle and increasing the cell number, while also inhibiting mTOR signals and suppressing the growth of cell size. Our new hypothesis is that the signal induced by leucine or arginine may stimulate the growth of cell size. On the other hand, the other signal induced by glutamine may stimulate cellular proliferation and increase cell number, but inhibit the growth of cell size. Based on this hypothesis, we can distinguish the signal for cellular proliferation from the signal for cell growth. Therefore, intestinal cells may utilize individual amino acids not only as nutrients but also as specific and active biological molecules for cellular proliferation or growth.

Another important point is that the key molecule of this specific signal is mTOR, which is a target of rapamycin. It means that this amino acid signal may play an important role for immunonutrition or intestinal immunity. These biological events investigated in our study might explain not only the mechanism of intestinal nutrition but also a universal system of signal transduction for cellular proliferation or growth.

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