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## **An in vivo and in vitro assessment of TOR signaling cascade in rainbow trout (*Oncorhynchus mykiss*)**

Iban Seiliez, Jean-Charles Gabillard, Sandrine Skiba-Cassy, Daniel Garcia-Serrana, Joaquim Gutiérrez, Sadasivam S. Kaushik, Stéphane Panserat, Sophie Tesseraud

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*Am J Physiol Regulatory Integrative Comp Physiol* 295:329-335, 2008. First published Apr 23, 2008; doi:10.1152/ajpregu.00146.2008

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S. Skiba-Cassy, M. Lansard, S. Panserat and F. Medale

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# An in vivo and in vitro assessment of TOR signaling cascade in rainbow trout (*Oncorhynchus mykiss*)

Iban Seiliez,<sup>1</sup> Jean-Charles Gabillard,<sup>2</sup> Sandrine Skiba-Cassy,<sup>1</sup> Daniel Garcia-Serrana,<sup>3</sup> Joaquim Gutiérrez,<sup>3</sup> Sadasivam Kaushik,<sup>1</sup> Stephane Panserat,<sup>1</sup> and Sophie Tesseraud<sup>4</sup>

<sup>1</sup>INRA, UMR1067 Nutrition Aquaculture et Génomique, F-64310 Saint-Pée-sur-Nivelle, France; <sup>2</sup>INRA, UR1037 Station Commune de Recherches en Ichtyophysiologie Biodiversité et Environnement, Campus de Beaulieu, Rennes, France;

<sup>3</sup>Departament de Fisiologia, Facultat de Biologia, Universitat de Barcelona, Barcelona, Spain; and <sup>4</sup>Institut National de la Recherche Agronomique, UR83 Recherches Avicoles, Nouzilly, France

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**Seiliez I, Gabillard J-C, Skiba-Cassy S, Garcia-Serrana D, Gutiérrez J, Kaushik S, Panserat S, Tesseraud S.** An in vivo and in vitro assessment of TOR signaling cascade in rainbow trout (*Oncorhynchus mykiss*). *Am J Physiol Regul Integr Comp Physiol* 295: R329–R335, 2008. First published April 23, 2008; doi:10.1152/ajpregu.00146.2008.—In mammals, feeding promotes protein accretion in skeletal muscle through a stimulation of the insulin- and amino acid-sensitive mammalian target of rapamycin (mTOR) signaling pathway, leading to the induction of mRNA translation. The purpose of the present study was to characterize both in vivo and in vitro the activation of several major kinases involved in the mTOR pathway in the muscle of the carnivorous rainbow trout. Our results showed that meal feeding enhanced the phosphorylation of the target of rapamycin (TOR), PKB, p70 S6 kinase, and eIF4E-binding protein-1, suggesting that the mechanisms involved in the regulation of mRNA translation are well conserved between lower and higher vertebrates. Our in vitro studies on primary culture of trout muscle cells indicate that insulin and amino acids regulate TOR signaling and thus may be involved in meal feeding effect in this species as in mammals. In conclusion, we report here for the first time in a fish species, the existence and the nutritional regulation of several major kinases involved in the TOR pathway, opening a new area of research on the molecular bases of amino acid utilization in teleosts.

feeding; signaling; protein synthesis; muscle; fish

TISSUE PROTEINS ARE CONSTANTLY synthesized and degraded, and the respective intensities of protein synthesis and proteolysis determine protein balance: there is protein accretion if protein synthesis is higher than proteolysis, loss if the opposite occurs, and maintenance if protein synthesis and proteolysis are equal. Among tissues, skeletal muscles are of main interest with regard to studies on protein metabolism to improve and control muscle growth and meat quality in animal production and to reduce muscle wasting under physiological and physiopathological situations such as aging and infection. In the white muscle of trout, despite the reportedly low-protein synthesis rates, almost all of the protein synthesized accumulates as accretion (13). The fact that rainbow trout requires high levels of dietary proteins (30–50% of the diet) (40) represents another interesting feature of protein metabolism compared with mammals. Therefore, a better understanding of the molecular bases involved in the nutritional regulation of muscle protein synthesis and growth is essential for optimizing dietary protein utilization in farmed fish.

In mammals, the synthesis of skeletal muscle proteins is rapidly stimulated after oral intake of nutrients through an acceleration of the initiation of mRNA translation (60). A principal site in the regulation of translation initiation involves the binding of mRNA to the 43S preinitiation complex, catalyzed by a multisubunit complex of eukaryotic factors referred to as eukaryotic initiation factor 4F (eIF4F) (49, 50, 52). The assembly of the eIF4F complex is dependent, in part, on the translation repressor protein eIF4E-binding protein-1 (4E-BP1). When hypophosphorylated, 4E-BP1 prevents the formation of the eIF4F complex by sequestering the mRNA cap-binding protein, eIF4E, into an inactive complex. The hyperphosphorylation of 4E-BP1 promotes the assembly of the eIF4F complex and thus increases the translation of capped mRNAs.

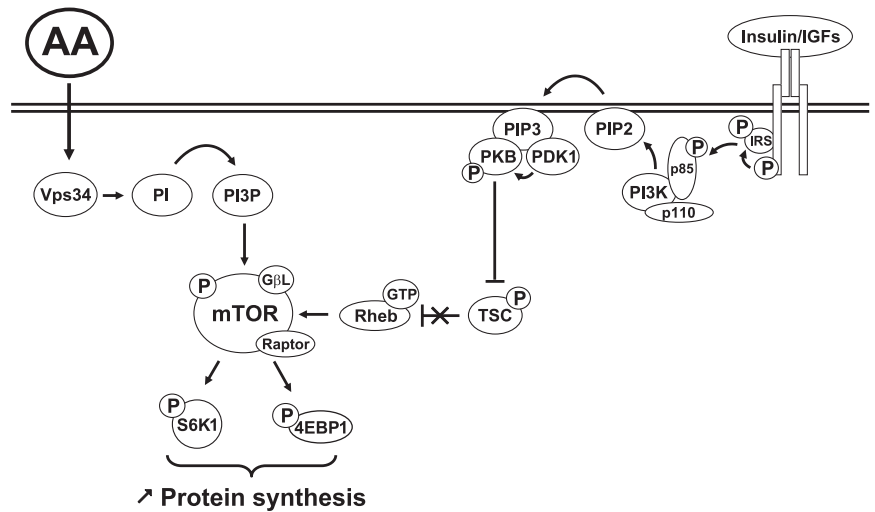
The increased activity of the 70-kDa ribosomal protein S6 kinase (S6K1) has also been implicated in stimulating protein synthesis under conditions that promote 4E-BP1 phosphorylation (51). Once S6K1 becomes phosphorylated on multiple serine and threonine residues, it becomes activated, and then phosphorylates several targets linked to translation, including the ribosomal protein S6, which is thought to increase the translation of mRNAs containing a terminal oligopyrimidine sequence adjacent to the m7GTP cap structure at the 5' end of the message (26), the eukaryotic initiation factor 4B (eIF4B) (18) and also the eukaryotic elongation factor 2 (eEF2) kinase (57). The consequence of eIF4B and eEF2 kinase phosphorylations on translation, however, remains unclear (18, 57).

The cellular pathways by which meal feeding modulates protein synthesis are beginning to be elucidated. The meal is composed of several nutrients, including carbohydrates and amino acids. Carbohydrates, besides providing a source of energy, lead to enhanced insulin secretion. Insulin, in turn, can stimulate the serine/threonine protein kinase Akt (also known as protein kinase B, PKB) through a phosphatidylinositol (PI) 3-kinase-dependent pathway (Fig. 1), followed by the phosphorylation of the protein termed tuberin (also called tuberous sclerosis complex 2, TSC2), which forms a tumor suppressor heterodimer with TSC 1 (24, 34). PKB-mediated phosphorylation inhibits the ability of TSC1/2 to promote hydrolysis of GTP bound to the G-protein Rheb, leading to its activation and then that of the mammalian target of rapamycin (mTOR), affecting the phosphorylation of some major effectors involved

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Fig. 1. Growth factor and nutrient-signaling pathway leading to the stimulation of protein synthesis. 4E-BP1, eIF4E-binding protein-1; AA, amino acids; GβL, G protein β subunit-like; GTP, guanosine-5'-triphosphate; IGFs, insulin-like growth factors; IRS, insulin receptor substrate; mTOR, mammalian target of rapamycin; P, phosphate; p85 and p110, regulatory subunits of PI3K; PI3K, phosphoinositide-3 kinase; PDK-1, phosphoinositide-dependent kinase-1; PI, phosphatidylinositol; PI3P, phosphatidylinositol-3'-phosphate; PIP2, phosphatidylinositol-3', 4-bisphosphate; PIP3, phosphatidylinositol-3', 4, 5-triphosphate; PKB, protein kinase B; Raptor, regulatory associated protein of mTOR; Rheb, Ras homolog-enriched in brain; S6K1, ribosomal protein S6 kinase-1; TSC, tuberous sclerosis complex; Vps34, vacuolar protein sorting 34.



in the regulation of translation initiation, e.g., S6K1 and 4E-BP1 (16, 54, 61). The amino acid-induced signaling cascade also originates from mTOR and promotes S6K1 and 4E-BP1 activation (32, 33). Recent studies in mammals point out that a major amino acid input to mTOR signaling is through a pathway parallel to that of the insulin-mediated TSC1/2-Rheb signaling axis mediated by class 3 PI3K and hVps34 (7, 42). However, the mechanisms of regulation are complex and little understood.

Recently, *in vitro* studies have shown the presence and the hormonal (insulin and/or IGFs) regulation of PKB in rainbow trout and zebrafish (8, 46), suggesting the existence of the above-mentioned mechanisms in teleosts. However, none of the downstream components have been studied in any fish species. The purpose of the present study was hence to characterize the effect of a single meal on the phosphorylation or activity of the main protein kinases involved in the TOR cellular pathway in the muscle of rainbow trout (*Oncorhynchus mykiss*). In addition, to analyze more specifically the effects of amino acids and insulin on the phosphorylation status of the main protein kinases involved in the TOR cellular pathway, *in vitro* studies were performed using primary cultures of trout muscle cells.

## MATERIALS AND METHODS

### Chemicals

BSA (fraction V, radioimmunoassay grade), leupeptin, aprotinin, and anti-carboxyl terminal mTOR antibody were purchased from Sigma Chemical (Saint-Quentin Fallavier, France). Nitrocellulose membrane, protein G-agarose, and S6K1 assay kit were purchased from Upstate Biotechnology (Euromedex, Mundolsheim, France) and 30% acrylamide/bis solution was from Bio-Rad (Marnes La Coquette, France). Anti-phospho PKB (Ser473), anticarboxyl terminal PKB, antiphospho-mTOR (Ser 2448), antiphospho-S6K1 (Thr 389), and antiphospho-4E-BP1 (Thr37/Thr46) were obtained from Cell Signaling (Ozyme, Saint Quentin Yvelines, France). Anticarboxyl terminal 4E-BP1 was obtained from Lab Vision (Interchim, Montluçon, France), and anticarboxyl terminal S6K1 was obtained from Santa Cruz (Tebu, Le Perray-en-Yvelines, France). [ $\gamma$ - $^{32}$ P]ATP was obtained from Amersham Health SA (Pantin, France).

### Animals and Experimental Procedures

Two tanks each containing 50 juvenile immature rainbow trout (weights ranging from 35 to 40 g) were maintained in our experimental farm (Donzacq, France) at 18°C under natural photoperiods and fed ad libitum with a commercial diet (Skretting, France; crude protein: 49.8% dry matter, crude fat: 13.8% dry matter; gross energy: 22 kJ/g dry matter) before the experiment. They were food deprived for 60 h (time necessary to entirely empty the digestive tract of fish of the size used at this rearing temperature), refed ad libitum, and sampled at 0.5 h, 1 h, 1.5 h, 2 h, 2.5 h, 3 h, 5 h, 8 h, 12 h, and 24 h after food administration. To limit handling stress in our successive samplings, the required number of fish ( $n = 6$  per sampling) was withdrawn from one of the two tanks at each sampling time. As a control, a group of fish ( $n = 6$ ) were sampled prior to refeeding. Blood was withdrawn using heparinized syringes from the caudal vein and centrifuged; the plasma was stored at  $-20^{\circ}\text{C}$  prior to the analysis of amino acids and insulin levels. After blood sampling, trout were killed, and laterodorsal white muscles were removed, quickly frozen in liquid nitrogen, and stored at  $-80^{\circ}\text{C}$ . Western blot analysis and activity assay were performed in muscle samples to monitor the meal feeding effect on the phosphorylation and/or activity of major kinases of the TOR pathway.

### Determination of Plasma Amino Acids and Insulin Levels

Total plasma free amino acid levels were determined by the ninhydrin reaction according to Moore (37) with glycine as a standard. Plasma insulin levels were measured by radioimmunoassay using bonito insulin as a standard and rabbit antiserum to bonito insulin as antiserum (21).

### Cell Cultures

For primary culture of muscle cells, we used rainbow trout maintained at the "Station Commune de Recherches en Ichtyo-physiologie, Biodiversité et Environnement" (SCRIBE, Rennes, France) in 0.6-m<sup>3</sup> tanks in a recirculated system. Primary cultures of skeletal muscle cells were carried out as follows: for each culture, 30 to 60 animals, each weighing ~5 g, were killed by a blow to the head and then immersed for 30 s in 70% ethanol to sterilize external surfaces. Laterodorsal muscle without skin was quickly removed. Cells were isolated, pooled, and cultured following previously described protocols (9, 48). All experiments were conducted with cells seeded at a density of 1.5 to  $2 \times 10^6$  per well, in six-well plastic plates (9.6 cm<sup>2</sup>/well, Nunc, Roskilde, Denmark).

Cells were incubated at 18°C, the optimal temperature for culture. After 7 days of culture, the cells (myotubes, as verified by visual microscopy) were kept overnight in serum-free medium, washed once with amino acid-deprived medium (Earle's balanced salt solution containing MEM vitamins and 2 g/l glucose), and incubated in the same medium for 2 h. Cells were then incubated for 30 min in fresh medium containing 1  $\mu$ M of bovine insulin with or without amino acids. We used a mixture of amino acids in a concentration (MEM as reference) defined as the following (in mM): 1.1 L-arginine HCl, 0.2 L-cysteine, 0.2 L-histidine HCl H<sub>2</sub>O, 0.4 L-isoleucine, 0.4 L-leucine, 0.4 L-lysine HCl, 0.1 L-methionine, 0.2 L-phenylalanine, 0.4 L-threonine, 0.05 L-tryptophan, 0.2 L-tyrosine, 0.4 L-valine, 0.2 L-alanine, 0.2 L-asparagine, 0.2 L-aspartic acid, 0.2 L-glutamic acid, 0.2 glycine, 0.2 L-proline, 0.2 L-serine, and 2.0 L-glutamine. Subsequently, the medium was aspirated, the wells were washed with ice-cold PBS, and the cells were lysed with lysis buffer (137 mM NaCl, 20 mM Tris·HCl, 1 mM MgCl<sub>2</sub>, 6H<sub>2</sub>O, 1 mM CaCl<sub>2</sub>, 2H<sub>2</sub>O, 0.15 mM sodium orthovanadate, 10  $\mu$ g/ml aprotinin, and 1% IGEPAL CA-630, 2 mM PMSF).

We also analyzed the effects of rapamycin (a specific inhibitor of the TOR protein) in the culture medium. Cells were incubated in serum-free medium overnight. Afterward, they were preincubated for 30 min with or without rapamycin (100 nM) and 1  $\mu$ M of insulin was added for 30 additional minutes. The medium was then aspirated, the wells were washed with ice-cold PBS, and the cells were lysed with lysis buffer.

#### Western Blot Analysis

Protein homogenates from muscles and cells were prepared as described by Dupont et al. (12) and Taouis et al. (53), respectively. Protein concentrations were determined with Bradford reagent method (5). Muscle or cell lysates (40  $\mu$ g and 10  $\mu$ g of protein, respectively) were subjected to SDS-PAGE gel electrophoresis and Western blot analysis using the appropriate antibody specific for the phosphorylated form of the protein of interest. Blots were then stripped and rehybridized with the respective antibody that recognizes both the phosphorylated and nonphosphorylated forms of the protein analyzed. Bands were revealed by enhanced chemiluminescence after the action of horseradish peroxidase-linked anti-rabbit  $\gamma$ -globulin. Nitrocellulose membranes were then scanned using the Epson Perfection 4990 scanner (Epson, Levallois-Perret, France), and the bands were quantified using ImagePro Plus software (Media Cybernetics, Imasys, Suresnes, France). The results were presented as means of individual densitometric analyses of several Western blots of the phosphorylated form corrected for the total content in samples.

Since mammalian antibodies were used, amino acid sequences of studied proteins from trout were monitored in the SIGENAE database (<http://www.sigena.org/>) to check the specificity of the antibodies in our samples (See supplemental Table 1 in the online version of this article.). Then, preliminary experiments were performed with murine fibroblast cell lysates as a control to identify the presence of the TOR signaling pathway components in rainbow trout muscle (see supplemental Table 1 online).

#### S6K1 Activity Assay

S6K1 activity was measured in muscle extracts by immune kinase assay according to the procedure in the S6 kinase assay kit (Euromedex, Mundolsheim, France). Briefly, muscle homogenates were immunoprecipitated with anti-S6K1 antibody, and the specific enzyme activity of the protein was measured by estimating the phosphorylation of an artificial substrate (AKRRRLSSLRA) corresponding to 11 amino acid sequence of the ribosomal protein S6 in the presence of labeled ATP. The radioactivity counts were measured by using a

Packard Tri-Carb 1900 TR liquid scintillation analyzer (Packard Bioscience, Rungis, France).

#### Statistical Analysis

Results are expressed as means  $\pm$  SE. Statistical analysis was performed by one-way ANOVA (Statview Software program, ver. 5; SAS Institute, Cary, NC) to detect significant intergroup differences. The Newman-Keuls multiple-range test was used to compare means in case of a significant ( $P < 0.05$ ) or a highly significant ( $P < 0.01$ ) effect.

## RESULTS

### Meal Feeding Induces an Increase of Plasma Insulin and Amino Acid Levels

Plasma insulin concentration reached its maximum level after 0.5 h of refeeding (Fig. 2A), with about a twofold increase compared with food-deprived trout ( $19.3 \pm 6.7$  ng/ml vs.  $8.5 \pm 0.7$  ng/ml,  $P < 0.01$ ), then declined after 24 h of refeeding to reach concentrations similar to those found in unfed trout ( $10.9 \pm 4.4$  ng/ml). Plasma total free amino acids reached significantly higher levels than those of unfed trout after 2.5 h of refeeding, remained higher until 12 h postfeeding and then reached levels of unfed trout by 24 h (Fig. 2B).

### Meal Feeding Induces the Phosphorylation and Activity of Several Major Kinases Involved in the TOR Pathway in Trout Muscle

**PKB phosphorylation.** Several reports suggest that PKB lies upstream of TOR signaling (41). PKB is a serine/threonine

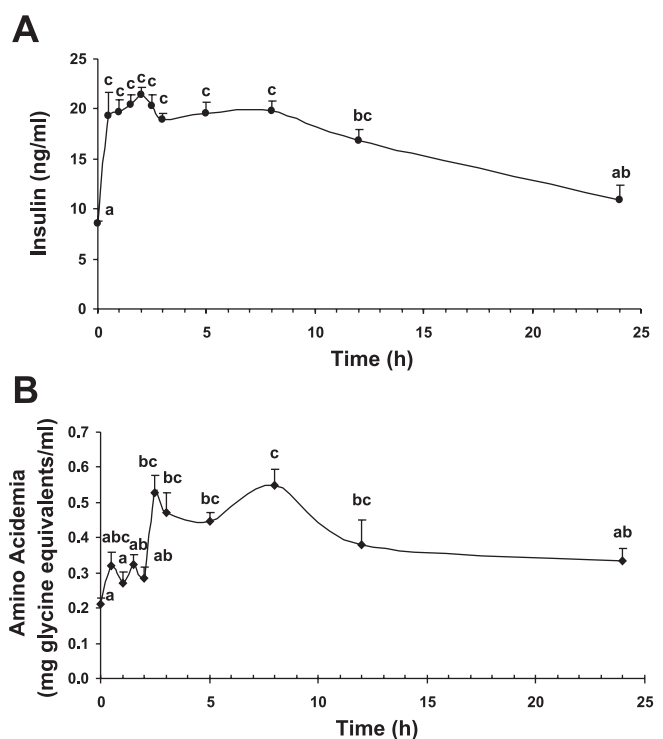


Fig. 2. Impact of refeeding on insulinemia (A) and amino acidemia (B) in rainbow trout deprived of food for 60 h, re-fed ad libitum and sampled at different times after food administration. Values are expressed as means  $\pm$  SE;  $n = 6$ . <sup>a,b,c</sup>Means not sharing a common superscript are significantly different ( $P < 0.05$ ) by the Student-Newman-Keuls test.

kinase that is activated by phosphorylation within the carboxy-terminus at serine<sup>473</sup> (1). Immediately after the initiation of the refeeding (0.5 h), the phosphorylation of PKB at serine<sup>473</sup> rose over twofold ( $P < 0.01$ ) and remained significantly higher than the phosphorylation level of unfed trout until 12 h after refeeding (Fig. 3A).

**TOR phosphorylation.** We next examined the potential role of TOR protein in mediating the effect of meal feeding downstream of PKB. In various species (*Drosophila* to mammals), TOR is believed to be an upstream kinase responsible for phosphorylating both S6K1 and 4E-BP1 (6, 47). Phosphorylation of TOR on residue Ser<sup>2448</sup> has been used to monitor the activation of TOR (3, 45). Therefore, we examined the phosphorylation state of Ser<sup>2448</sup> following refeeding and showed for the first time that TOR was expressed and phosphorylated in fish (Fig. 3B). The extent of phosphorylation of TOR was significantly increased 1 h following meal ( $P < 0.01$ ) and remained significantly elevated until 8 h after refeeding before declining.

**S6K1 phosphorylation and activity.** S6K1 is a threonine/serine kinase that phosphorylates several targets linked to translation, including the ribosomal protein S6, eIF4B, and eEF2 kinase. Analysis of the multisite phosphorylations of S6K1 indicates that its activity is dependent on phosphorylation of Thr<sup>389</sup> (58, 59). Therefore, phosphorylation of S6K1(Thr<sup>389</sup>) following meal feeding was examined in parallel to its activity (Fig. 3C and supplemental Figure 1 online). Prior to feeding, phosphorylation on S6K1 (Thr<sup>389</sup>) was below the level of detection. After refeeding, the phosphorylation of the S6K1 (Thr<sup>389</sup>) increased very slightly and showed a significant induction only at 5 h (values approximately twofold higher compared with food-deprived trout,  $P < 0.05$ ) before declining thereafter to reach a level not significantly different to that observed in

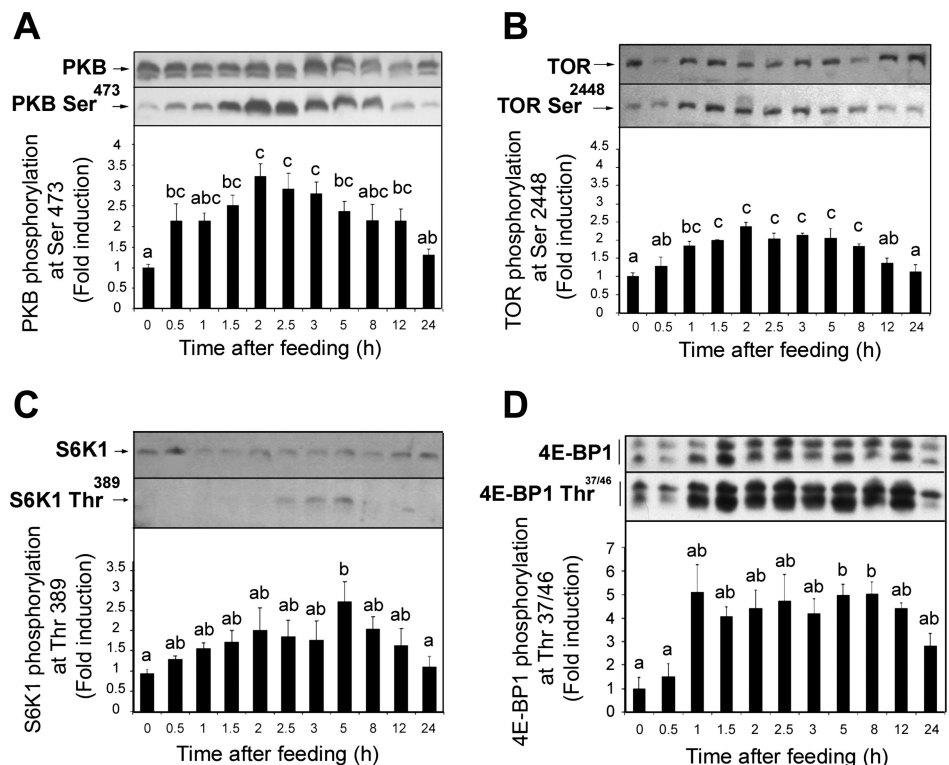
unfed trout. A different pattern was observed for S6K1 activity with a first increase at 2 h after refeeding and a second rise from 5 h to 12 h before declining.

**4E-BP1 phosphorylation.** Multiple 4E-BP1 residues are phosphorylated in vivo (38, 39). While phosphorylation by mTOR on Thr37 and Thr46 does not prevent the binding of 4E-BP1 to eIF4E, it is thought to prime 4E-BP1 for subsequent phosphorylation at Ser65 and Thr70 (17). Therefore, here, the phosphorylation of 4E-BP1 (Thr<sup>37/46</sup>) was examined following refeeding, and a significant induction was detected at 5 and 8 h (values ~5-fold higher compared with food-deprived trout,  $P < 0.01$ ) (Fig. 3D).

#### Specific and Combined Effects of Insulin and Amino Acids on TOR Pathway in Trout Myotubes

The mechanisms by which meal feeding modulates the phosphorylation and activity of the main kinases involved in the TOR pathway are beginning to be elucidated in mammals, and both insulin and amino acids have been shown to play key roles in this process. Therefore, to clarify the specific and combined effects of amino acids and insulin on the TOR signal transduction pathway, primary cultures of trout muscle cells were stimulated with insulin or insulin plus amino acids, and the phosphorylation status of PKB (Ser<sup>473</sup>), 4E-BP1(Thr<sup>37/46</sup>), and S6K1 (Thr<sup>389</sup>) were examined. Insulin improved the phosphorylation of all tested proteins (Fig. 4, A and B). The effect on S6K1 and 4E-BP1 was abolished by rapamycin treatment (Fig. 4B), suggesting the involvement of TOR in insulin action in fish as in other species. A combination of the two effectors (insulin and amino acids) caused an even greater stimulation than insulin

Fig. 3. Effect of meal feeding on phosphorylation of PKB at Ser<sup>473</sup> (A), TOR at Ser<sup>2448</sup> (B), S6K1 at Thr<sup>389</sup> (C), and 4E-BP1 at Thr<sup>37/46</sup> (D). Phosphorylation levels of PKB at Ser<sup>473</sup>, TOR at Ser<sup>2448</sup>, S6K1 at Thr<sup>389</sup>, and 4E-BP1 at Thr<sup>37/46</sup> were normalized for the content of the corresponding protein in samples. Values are expressed as means  $\pm$  SE ( $n = 6-8$  in each group) and are expressed as fold stimulation above basal level (unfed trout). Means not sharing a common superscript are significantly different ( $P < 0.05$ ) by the Student-Newman-Keuls test.



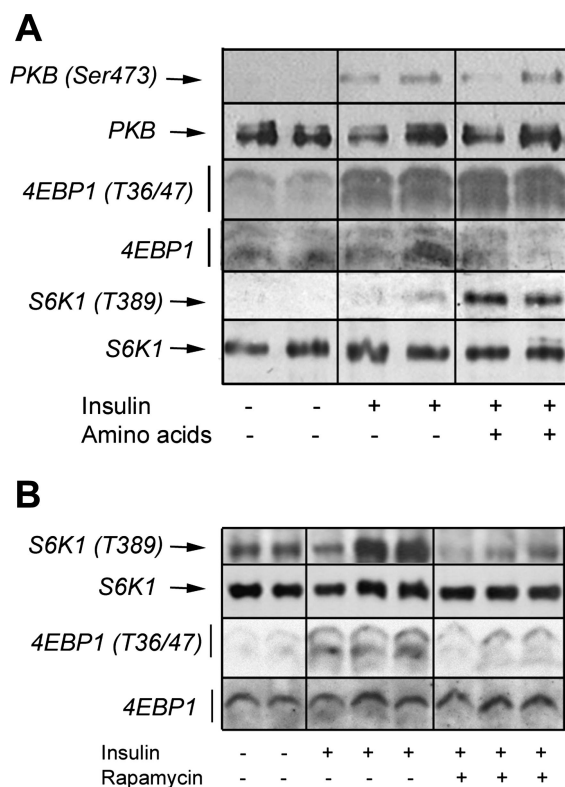


Fig. 4. Effects of insulin, insulin plus amino acids, or insulin plus rapamycin on the TOR pathway in trout myotubes. *A*: cells were incubated overnight in serum-free medium and subjected to amino acid deprivation for 2 h. The culture medium was then replaced for 30 min with an amino acid-free medium, an amino acid-free medium containing 1  $\mu$ M insulin or a mixture of amino acids containing 1  $\mu$ M insulin. *B*: cells were incubated overnight in serum-free medium. After a pretreatment with rapamycin (100 nM) or DMSO for 30 min, 1  $\mu$ M of insulin was added for 30 additional minutes. In *A* and *B*, cell lysates were analyzed by Western blot analysis with the indicated antibodies.

alone with regard to S6K1 phosphorylation (Fig. 4A), this effect not being observed for 4E-BP1 and PKB.

## DISCUSSION

Here, we report for the first time in a fish species the existence and nutritional regulation of the major kinases involved in the TOR pathway. The predicted amino acid sequences of rainbow trout PKB, TOR, S6K1, and 4EBP1 revealed that the peptides contain the specific phosphorylated sites known to regulate the activity of these kinases in response to feeding. In this regard, our results show that feed intake induces the activation of the TOR pathway in rainbow trout muscle by enhancing the phosphorylation and/or activity of PKB, TOR, S6K1, and 4EBP1. While the level of phosphorylation of PKB and TOR reached a significant induction 0.5–1 h after a meal, that of S6K1 and 4EBP1 showed a more delayed rise at 5–8 h. A different pattern was observed for S6K1 activity with a first increase at 2 h after refeeding and a second rise from 5 to 12 h. These results contrast with data in mammals showing that phosphorylation of S6K1 at Thr389 is critical and a rate-limiting step in the activation of the kinase (25, 43, 59). However, activation of S6K1 is controlled through a complex mechanism that involves the phosphorylation of at least eight Ser/Thr residues, such as Thr229, Ser371, Thr389, Ser404, Ser411, Ser418, Thr421, and Ser424 (23), with possi-

bly some interspecies differences. A possible explanation for the above observation could also be due to the well-established high variation of individual food intakes in rainbow trout (35), leading to high variability in responses between individuals and limiting the interpretation of the results when the effects are very subtle. However, and irrespective of the above considerations, our data suggest that the mechanisms involved in the regulation of the TOR pathway are well conserved between lower and higher vertebrates. Our data based on primary culture of trout muscle cells show that insulin and/or amino acids regulate TOR signaling at least *in vitro* and thus may play key roles in meal feeding effect in this species as in mammals.

Interestingly, the long-term stimulation of S6K1 and 4EBP1 following a meal (after 5–8 h) contrasts with observations in mammals (55). This is in good agreement with previous data showing that the refeeding of rainbow trout or Atlantic salmon after a fasting period stimulates protein synthesis in white muscle only 6–9 h after a meal (15, 36). These findings, therefore, suggest that in teleosts, as in mammals, S6K1 and 4EBP1 are important factors controlling protein synthesis. The reason for the difference between species is likely due to the delayed rise of plasma amino acid concentrations in trout that was significant only after 2.5 h of refeeding vs. 30 min normally encountered in rats (55). The late amino acid change is explained by the low rate of flow of foodstuffs through the digestive tract of juvenile trout as used here and reared at 18°C (transit rate in poikilotherms is affected by rearing temperature and body size) (20). According to numerous studies, the effect of feeding on protein synthesis is likely mediated through insulin- and nutrient-signaling parallel pathways both leading to mTOR activation (22, 29, 44, 56). Recent data also suggest that insulin and amino acids may act synergistically to enhance mRNA translation. For example, in human skeletal muscle, hyperphosphorylation of S6K1 is enhanced in response to increased plasma concentrations of either leucine or insulin, but a combination of the two effectors causes an even greater stimulation than either alone (19). These findings are in keeping with our *in vitro* results since the effect of insulin on myotube S6K1 phosphorylation is mild, but it is more prominent in the presence of amino acids. In rats, the increase in plasma insulin after refeeding an amino acid/protein-free meal has also been shown to be sufficient to stimulate PKB and mTOR through the PI3-kinase-dependent pathway but not to increase the phosphorylation state of S6K1 (4). However, feeding an amino acid/protein-rich meal had no effect on the phosphorylation state of both S6K1 and 4EBP1 when the insulin level was reduced by diazoxide injections (4), indicating that insulin is required to activate these proteins. Insulin may thus have a permissive effect for the amino acid-induced stimulation of protein synthesis via the phosphorylation of 4E-BP1 and S6K1, as reported by Kimball et al. (27). To our knowledge, the time course of changes of both postprandial plasma insulin and free amino acid levels have never been measured in the same animals in teleosts. In the present study, we observe a delay in the rise of plasma amino acid concentration (2.5 h) compared with that of insulin (0.5 h). The observed induction in phosphorylation of PKB and TOR early after refeeding correlate with the rise in circulating insulin concentrations, whereas the more delayed activation of S6K1 and 4EBP1 (5–8 h following meal feeding) is most likely attributed to higher concentrations of both insulin and amino acids.

In rainbow trout, the ability of amino acids to stimulate the TOR pathway can at first sight seem surprising. Indeed, the protein synthesis rates and more particularly the efficiency of translation are low in the muscle of trout compared with those in mammals (13, 14, 31), despite the high amino acid intake normally encountered (10, 40). We cannot exclude that major translation factors not yet characterized in fish (e.g., eIF2B) are rate-limiting for protein synthesis. However, some peculiarities of fish nutrient utilization may contribute to this species-specific regulation of protein metabolism. In fish, less than half of total amino acid pool available for protein synthesis is derived from intracellular protein degradation (11), whereas in mammals, this amount is almost 80% (free amino acid levels originate from both dietary protein intake and endogenous protein degradation). Because the contribution of ingested amino acids to the total intracellular pool is greater in fish than in mammals, differences in dietary protein content between species are bound to have an influence on protein metabolism. It is also worth stating that in teleosts, amino acids have a stronger insulinotropic action than carbohydrates (2) and thus may account for the likewise sustained postprandial plasma insulin levels as observed here, thereby achieving the permissive role of the hormone in stimulating protein synthesis. Taken together, these findings suggest that in trout, the protein synthesis response relies essentially on dietary protein supply and may explain the prolonged activation of the TOR signaling as reported here. Whether the TOR signaling is altered in fish fed decreasing levels of dietary protein is a fundamental issue that remains unexplored. Future research in these lines will provide insights on teleosts normally confronted with high protein levels and compare data from other animals generally having low protein intakes.

#### Perspectives and Significance

Amino acids that have long been considered simply precursors of protein synthesis are now recognized to exert other significant influences, that is, as precursors of essential molecules, acting as mediators or signal molecules affecting several other functions. There is an increasing body of evidence to support the key role of amino acids as signaling molecules in the regulation of protein synthesis and that dietary intake of proteins is essential for normal growth and development, as well as for effective therapeutic approaches to many pathophysiological circumstances that result in loss of skeletal muscle tissue (28). There is also increasing evidence that elevated dietary protein consumption contributes to obesity, insulin resistance, and type 2 diabetes (30). Therefore, the mechanisms by which excess amino acids lead to a high muscle protein accretion and/or the development of metabolic disorders have important implications for optimizing the growth and development of farmed animals, as well as for public health and clinical medicine. The present study provides the first ever data in a carnivorous species, known to require a high dietary amino acid supply, that feeding enhances the phosphorylation and/or activity of PKB, TOR, S6K1, and 4E-BP1 in muscle. These findings suggest that the mechanisms involved in the regulation of mRNA translation are well conserved between lower and higher vertebrates, even though other key initiation factors controlling protein synthesis are worth investigation. Further studies are warranted to follow this specific pathway as af-

ected by nutritional factors, especially dietary amino acid levels in a dose-dependent manner.

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