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Zargar S, Moreira TS, Samimi-Seisan H, Jeganathan S, Kakade D, Islam N, Campbell J, Adegoke OA. Skeletal muscle protein synthesis and the abundance of the mRNA translation initiation repressor PDCD4 are inversely regulated by fasting and refeeding in rats. *Am J Physiol Endocrinol Metab* 300: E986–E992, 2011. First published March 15, 2011; doi:10.1152/ajpendo.00642.2010.—Optimal skeletal muscle mass is vital to human health, because defects in muscle protein metabolism underlie or exacerbate human diseases. The mammalian target of rapamycin complex 1 is critical in the regulation of mRNA translation and protein synthesis. These functions are mediated in part by the ribosomal protein S6 kinase 1 (S6K1) through mechanisms that are poorly understood. The tumor suppressor programmed cell death 4 (PDCD4) has been identified as a novel substrate of S6K1. Here, we examined *1*) the expression of PDCD4 in skeletal muscle and *2*) its regulation by feed deprivation (FD) and refeeding. Male rats (~100 g; $n = 6$) were subjected to FD for 48 h; some rats were refed for 2 h. FD suppressed muscle fractional rates of protein synthesis and Ser^{67} phosphorylation of PDCD4 (−50%) but increased PDCD4 abundance ($P < 0.05$); refeeding reversed these changes ($P < 0.05$). Consistent with these effects being regulated by S6K1, activation of this kinase was suppressed by FD (−91%, $P < 0.05$) but was increased by refeeding. Gavaging rats subjected to FD with a mixture of amino acids partially restored muscle fractional rates of protein synthesis and reduced PDCD4 abundance relative to FD. Finally, when myoblasts were grown in amino acid- and serum-free medium, phenylalanine incorporation into proteins in cells depleted of PDCD4 more than doubled the values in cells with a normal level of PDCD4 ($P < 0.0001$). Thus feeding stimulates fractional protein synthesis in skeletal muscle in parallel with the reduction of the abundance of this mRNA translation inhibitor.

programmed cell death 4; protein metabolism; ribosomal protein S6 kinase; mRNA translation; mammalian target of rapamycin complex 1

OPTIMAL SKELETAL MUSCLE MASS AND METABOLISM are critical to the regulation of whole body substrate homeostasis and health. Indeed, in several diseases, including obesity, diabetes, and cancer, defects in muscle metabolism underlie or exacerbate the metabolic outcomes (21, 24, 41).

The growth factor- and nutrient-sensitive kinase complex mammalian target of rapamycin complex 1 (mTORC1) is now recognized as a master regulator of skeletal muscle mass (6, 7, 42, 43). Upon activation, this complex phosphorylates two principal substrates, the ribosomal S6 protein kinase 1 (S6K1) and the eukaryotic initiation factor (eIF)4E-binding protein 1 (4E-BP1) (20, 40). Phosphorylation of S6K1 leads to its activation, whereas 4E-BP1, an inhibitor of cap-dependent mRNA translation initiation, is inhibited by phosphorylation. S6K1 mediates the growth-promoting action of mTORC1, whereas inhibition of 4E-BP1 increases cell proliferation (9, 16).

S6K1 is critical to the regulation of skeletal muscle mass because mice lacking this enzyme have reduced muscle mass (3, 38). In several studies, stimulation of skeletal muscle protein synthesis by amino acids and insulin occurs in concert with increased Thr^{389} phosphorylation of this kinase, whereas the level of phosphorylated S6K1 is reduced by nutrient deprivation (3, 5, 8, 19, 22, 34, 37, 38).

The exact mechanisms by which activated S6K1 promotes skeletal muscle protein metabolism and mass are not clear but appear to include at least two pathways. First, S6K1 can phosphorylate the ribosomal protein S6 (33). This is thought to lead to the translation of a subset of ribosomal proteins critical for protein synthesis. This view has been challenged, because S6 phosphorylation is not reduced in the absence of S6K1, nor does restoration of phosphorylated S6 levels rescue growth defects seen in S6K1-null muscle cells (37, 44). Second, S6K1 may promote protein synthesis by phosphorylating eIF4B (28). This stimulates the association of the latter with the eukaryotic initiation factor 3 complex, leading to increased mRNA translation.

Recently, programmed cell death 4 (PDCD4) was described as a substrate of S6K1 (15). In its unphosphorylated state, PDCD4 inhibits mRNA translation by binding to and inhibiting eIF4A (25, 27) and -4G (30, 51). Upon growth factor stimulation, PDCD4 is phosphorylated by S6K1. This modification targets it for degradation by the ubiquitin protein ligase β-transducin repeat-containing protein (15). As a result its inhibition of mRNA translation is relieved, and protein synthesis can proceed (15). It is presently unknown whether PDCD4 protein is expressed in skeletal muscle, nor is it clear whether it is regulated by nutrients. These questions are critical because muscle is the single largest contributor to whole body protein metabolism, and the mTORC1/S6K1 pathway that regulates its growth is nutrient sensitive (31).

Overactivation of S6K1 is implicated in experimental and human insulin resistance, obesity, and cancer (9, 17, 48, 49). It is crucial to identify the mechanisms by which this kinase regulates protein metabolism in skeletal muscle. Such an understanding can lead to the dissection of the anabolic effects of this kinase from the undesirable consequences of its activities. Our objectives were to use the feed deprivation refeeding model in the rat as well as in vitro muscle cell culture to examine the regulation of PDCD4 in skeletal muscle and to relate changes in fractional rates of protein synthesis to the regulation of PDCD4. We also investigated whether depleting

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PDCD4 by RNA interference would modulate phenylalanine incorporation into proteins in myoblasts.

MATERIALS AND METHODS

Chemicals and Reagents

Horseradish peroxidase chemiluminescence substrate was obtained from Millipore (Billerica, MA), ECL Plus reagents from GE Healthcare (Piscataway, NJ), L-[2,3,4,5,6,-^{3}H]phenylalanine from GE Healthcare or American Radiolabeled Chemicals (St. Louis, MO), protease inhibitor cocktail, phosphatase inhibitor cocktail, L-tyrosine decarboxylase, pyridoxal phosphate, benzamidine, L-leucine, L-leucyl-L-alanine, sodium vanadate, ninhydrin, chloroform, and n-heptane from Sigma-Aldrich (St. Louis, MO), Pierce bicinchoninic acid protein assay kit from Thermo Fisher Scientific (Rockford, IL), L-phenylalanine from Calbiochem (San Diego, CA), and α-modification of Eagle's medium (AMEM), antibiotic and antimycotic preparation, and trypsin from Wisent (St. Bruno, QC, Canada). Other reagents were of analytical grade.

Antibodies

Antibodies to PDCD4, S6K1, Thr^{389} S6K1, ribosomal protein S6 (S6), Ser$^{235/236}$ S6, mTOR, Ser^{2448} mTOR, Ser^{473} Akt, horseradish peroxidise-conjugated anti-mouse, and anti-rabbit IgG were purchased from Cell Signaling Technology (Danvers, MA). Antibodies to Ser^{67} PDCD4 and γ-tubulin were obtained from Sigma-Aldrich.

Animals

Male Sprague-Dawley rats ranging from 50 to 70 g were purchased from Charles River Laboratories. We chose rats of this age because in them rates of muscle protein synthesis are high and sensitive to nutrient deprivation/provision (14). Rats were acclimatized in the animal facility at York University while being maintained at the standard 12:12-h light-dark cycle (lights on at 0700). They had free access to a nonpurified rat diet (50) and water. From 6 wk of age, rats were handled daily to minimize handling stress on the day of the experiment. All experiments were approved by the York University Institutional Animal Welfare Committee, and the guidelines of the Canadian Council on Animal Care were followed.

Study Design

Effects of feed deprsivation and refeeding on PDCD4 expression and regulation in skeletal muscle. Rats were assigned to one of two groups: *1*) fed (CTL) or *2*) 48-h feed deprivation (FD). Water was provided at all times. A subgroup of the FD group ($n = 6$) was studied at the end of deprivation. Other FD rats were refed for 2 h (2-h RFD; $n = 6$). To determine the fractional rates of protein synthesis, we used the flooding dose technique (23) as modified by Jepson et al. (29) for the intraperitoneal delivery of radioactive phenylalanine. Ten to fifteen minutes later they were euthanized by decapitation, and gastrocnemius muscles were dissected out and flash-frozen in liquid nitrogen.

Effects of amino acids on PDCD4 regulation in skeletal muscle. Rats were subjected to FD as described above. A subgroup of FD ($n = 7–8$/ group) was studied after deprivation or at different times (0.5–6 h) after being gavaged with a mixture of essential and nonessential L-amino acids (AA) (in g/l: isoleucine 11.7, leucine 20, lysine 11.7, methionine 4.86, phenylalanine 6.86, threonine 6, tryptophan 2.86, valine 11.18, cysteine 0.2, histidine 6.86, alanine 7.78, arginine 17.14, proline 9.72, serine 5.46, glycine 5.18, aspartic acid 4.54, glutamic acid 7.1). Rats were administered volumes of the solution that corresponded to 0.48 g L-leucine/kg [24 ml/kg of body wt (12)] or an equivalent volume of water. Ten to fifteen minutes before decapitation, fractional rates of protein synthesis were measured as described above.

Sample analysis. Gastrocnemius muscle samples were processed for the analyses of mRNA translation initiation factors, as described previously (1). Immunoblot membranes were probed for phosphorylated PDCD4 (Ser^{67}), S6K1 (Thr^{389}), S6 (Ser$^{235/236}$), mTOR (Ser^{2448}) or Akt (Ser^{473}). Immunoblot signals were quantified using the Carestream Molecular Imaging software (version 5.0.2.30; Carestream Health, Rochester, NY). Membranes were then stripped and reprobed with antibodies against total PDCD4, mTOR, S6K1, S6, or γ-tubulin (loading control).

Analysis of samples for the determination of fractional rates of protein synthesis. Samples were processed as described previously (2, 23). Briefly, muscle samples were homogenized on ice in ice-cold 2% $HClO_4$ (1 ml/100 mg) and then centrifuged (2,800 *g* for 15 min at 4°C). The pellet was washed four times with 4 ml of 2% $HClO_4$ and then hydrolyzed overnight in 6 M HCl. To determine the specific radioactivity of the tracer, phenylalanine in both the free (supernatant) and protein-bound (pellet) AA pools was enzymatically converted to β-phenethylamine (PEA). The resulting PEA was extracted from the other AAs, and its specific radioactivity was determined after its concentration (using a fluorimeter) and radioactivity were measured in a liquid scintillation counter. Fractional rates of protein synthesis (K_{syn}, %/day) were calculated according to Jepson et al. (29), $K_{syn} = 100x[S_b/0.9x(S_ixt)]$, where S_b represents the specific radioactivity of protein-bound phenylalanine, S_i is the specific radioactivity of tissue-free phenylalanine, and *t* denotes time (in days) of incorporation. In this context, this is the interval between the time the flooding dose was delivered and the time the dissected muscles were frozen. The factor 0.9 is used to correct for the delay in the attainment of plateau of tissue free phenylalanine-specific activity due to intraperitoneal delivery of the flooding dose (29).

Other assays. The insulin assay was performed using Ultra Sensitive Rat Insulin ELISA Kit (Crystal Chem, Downers Grove, IL). Total branched-chain AA (BCAA) were measured by rapid enzymatic fluorometric assay (11).

Cell culture. L6 myoblasts were grown in six-well plates in AMEM supplemented with FBS and antibiotic-antimycotic agents to final concentrations of 10 and 1%, respectively. The cells were starved for 24 h by incubation in serum-free AMEM. A group of starved cells was later incubated in a serum-replete medium for different lengths of time. Cells were then harvested in a lysis buffer (in mM: 25 Tris, pH 7.5, 1 EDTA, 1 DTT, 2.5% SDS), and lysates were probed for total and phosphorylated PDCD4, as described above. For RNA interference, myoblasts were transfected using Lipofectamine RNAi max (Invitrogen) with 30 nM siRNA oligonucleotides designed against PDCD4 (Sigma-Aldrich) or with a proprietary scrambled oligonucleotide (IDT, San Diego, CA). We used the following PDCD4 siRNA oligonucleotides: PDCD4 no. 1 sense [GUCUUCUACUAUUACCAUA (dT) (dT)], PDCD4 no. 1 antisense [5′-UAUGGUAAUAGUAGAAGAC (dT) (dT)], PDCD4 no. 2 sense [CUACUAUUACCAUAGACCA (dT) (dT)], and PDCD4 no. 2 antisense [UGGUCUAUGGUAAUAGUAG (dT) (dT)]. Seventy-two hours after transfection, cells were harvested and lysates probed for total PDCD4 and γ-tubulin. In other experiments, 60 h after transfection, cells were grown in an AA- and serum-free medium (RPMI 1640; US Biologicals, Swampscott, MA) for 12 h. Phenylalanine incorporation into proteins was then measured by assessing the incorporation of radioactive phenylalanine into TCA-precipitable proteins (26).

Statistical analysis. Values are presented as means ± SE. Treatment means were compared by one-way ANOVA and differences among means assessed using the Bonferroni multiple comparison test. When analyses revealed that variances were different (as was the case for Fig. 2, *C* and *D*), we performed nonparametric analyses (Kruskal-Wallis) and used Dunn's post hoc test to identify means that differed from one another. For comparison of the effect of PDCD4 depletion on phenylalanine incorporation into proteins, we used an unpaired *t*-test. Analyses were done using GraphPAD (version 3; GraphPad Software, La Jolla, CA). The level of significance was set at $P \leq 0.05$.

RESULTS

PDCD4 expression in skeletal muscle and its regulation during a feed deprivation and refeeding cycle. PDCD4 is a 464-AA residue protein (35) that migrates with the 55-kDa protein marker (Fig. 1*A*). Since its expression at the protein level has not been shown in muscle cells, and to confirm that

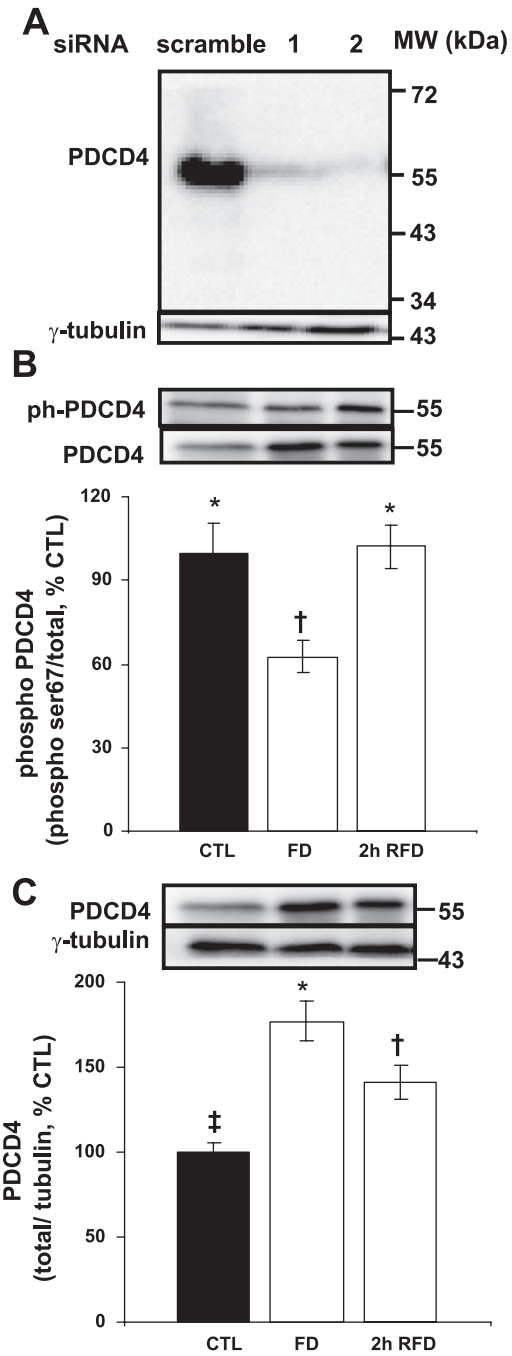

Fig. 1. Programmed cell death 4 (PDCD4) expression and regulation in skeletal muscle in response to feed deprivation and refeeding. *A*: PDCD4 protein level in L6 myoblasts treated with scramble siRNA or 1 of 2 siRNA oligonucleotides designed against PDCD4. *B* and *C*: PDCD4 phosphorylation (*B*) and abundance (*C*) in rat skeletal muscle in CTL (fed), in response to 48-h feed deprivation (FD), and FD followed by refeeding. Values are means ± SE; $n = 6$. Means without a common symbol differ; $P < 0.05$. RFD, refeeding; MW, molecular weight.

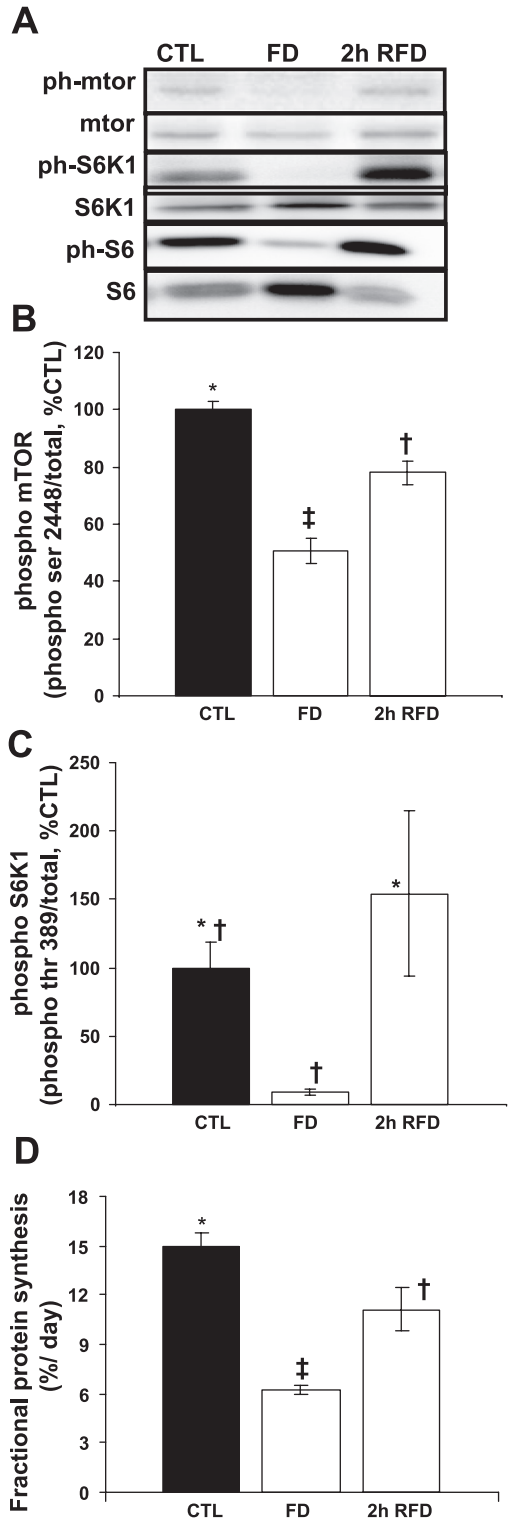

Fig. 2. Mammalian target of rapamycin (mTOR) signaling and fractional rates of protein synthesis in skeletal muscle in response to FD and RFD. Phosphorylation (ph) of mTOR (*A* and *B*) and ribosomal S6 protein kinase 1 (S6K1; *A* and *C*) and protein synthesis (*D*) in rat skeletal muscle in CTL, in response to FD, and FD followed by RFD. Values are means ± SE; $n = 6$. Means without a common symbol differ; $P < 0.05$.

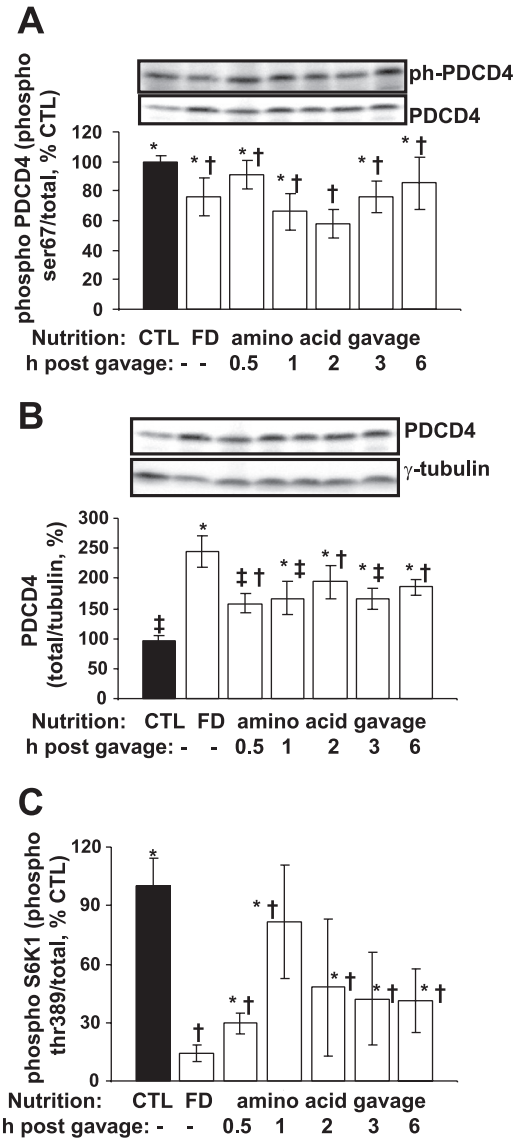

Fig. 3. PDCD4 phosphorylation and abundance in response to FD and amino acid gavage. PDCD4 phosphorylation (*A*) and abundance (*B*) and S6K1 phosphorylation (*C*) in rat skeletal muscle in CTL, in response to FD, and in FD followed by amino acid gavage. Values are means ± SE; $n = 7–8$ for *A* and *B*; $n = 5$ for *C*. Means without a common symbol differ; $P < 0.05$.

the detected signal is for the authentic protein, we knocked down PDCD4 using two different siRNA oligonucleotides. PDCD4 levels were lower only in L6 myoblasts treated with siRNA designed against this protein. We reproducibly detected PDCD4 in skeletal (mixed gastrocnemius) muscle (Fig. 1, *B* and *C*). FD significantly suppressed its Ser^{67} phosphorylation ($P < 0.05$). This was reversed during refeeding. When expressed relative to γ-tubulin, phosphorylation of PDCD4 tended to be lower in FD than in CTL ($P = 0.10$; data not shown). Upon phosphorylation, PDCD4 is degraded (15). Compared with CTL, FD increased the abundance of PDCD4 by ~60% ($P < 0.05$), but this was attenuated in 2-h RFD (Fig. 1*C*). In another experiment with rats that were subjected to an 18-h FD, leucine gavage tended to suppress soleus muscle PDCD4 abundance relative to FD ($P = 0.08$; Supplemental Fig. S1; Supplemental Material for this article can be found online at the *AJP-Endocrinology and Metabolism* website).

The observed changes in PDCD4 occurred concurrently with the activation of mTOR (as measured in Ser^{2448} phosphorylation) and in particular with the activation of S6K1 (Thr^{389} phosphorylation), the presumed kinase for PDCD4. Indeed, in RFD, increased muscle fractional rates of protein synthesis, as well as the phosphorylation of mTOR, S6K1, and S6, occurred in parallel to one another and to the phosphorylation of PDCD4 (Fig. 1*B* and 2, *A–D*, and Supplemental Fig. S2).

Oral amino acid administration can mediate the effect of feeding on PDCD4 abundance. Rather than refeeding feed-deprived rats, we gavaged them with a mixture of essential and nonessential AA. Relative to CTL, FD did not have a significant effect on PDCD4 phosphorylation but significantly increased its abundance (Fig. 3, *A* and *B*). Compared with CTL, PDCD4 phosphorylation was lower at 2 h after the AA gavage but was not different from CTL at other time points. Relative to FD, AA gavage, just like refeeding, significantly reduced PDCD4 abundance at 30 min after the gavage. At other time points after the gavage, except at 2 and 6 h, PDCD4 abundance was not different from control. In addition, AA gavage stimulated the phosphorylation of S6K1 and S6, although the response was variable (Fig. 3*C* and Supplemental Fig. S3). There was no effect of AA gavage on Akt Ser^{473} phosphorylation (Supplemental Fig. S4). Fractional rates of protein synthesis were suppressed by FD, but the rates at 1 and 2 h after AA gavage were not different from CTL; however, these values were also not different from FD (Table 1).

Plasma insulin and BCAA levels. The plasma insulin concentration (in pM) was suppressed by FD (36 ± 2) compared with the CTL (198 ± 1) and 2-h RFD (192 ± 31). In the AA gavage experiment, the plasma insulin level in FD was 62 ± 14 pM. This value was not significantly different from the concentration at 1 (50 ± 10) or 2 h (71 ± 10) after the AA gavage. The plasma BCAA concentrations did not differ among the CTL, FD, and 2-h RFD groups (data not shown). In the gavage

Table 1. *Skeletal muscle fractional rates of protein synthesis and plasma total BCAA in CTL and FD rats and in FD rats after administration of AA by gavage*

			AA				
	CTL	FD	30 min	1 h	2 h	3 h	6 h
Protein synthesis, %/day	13.9 ± 1.3*	7.3 ± 1.6†	6.6 ± 1.6†	9.8 ± 1.9*†	8.5 ± 1.6*†	8.0 ± 2.1†	7.8 ± 1.3†
BCAA, μmol/l	447 ± 75†	469 ± 80†	1,378 ± 140*	1,498 ± 204*	759 ± 59†	617 ± 40†	452 ± 109†

Values are means ± SE. BCAA, branched-chain amino acids; CTL, fed; FD, feed-deprived; AA, amino acids. Skeletal muscle (mixed gastrocnemius) fractional rates of protein synthesis and plasma levels of BCAA were determined as described in MATERIALS AND METHODS. For protein synthesis, $n = 7–8$, except for 30 min of AA, for which $n = 6$. For BCAA, $n = 5–6$, except for 6 h of AA, for which $n = 4$. Means within a row and without a common symbol differ ($P < 0.05$).

experiment, 30 min and 1 h after AA gavage, the total BCAA concentrations more than doubled the values in FD (Table 1).

In muscle cells, PDCD4 abundance is negatively regulated by serum. To show that our observations of the expression and regulation of this protein in skeletal muscle were not due to contamination with nonmuscle cells, we studied L6 myoblasts, a model that has been used by others to examine the regulation of S6K1 and mRNA translation by leucine (32). Serum deprivation for 24 h did not have any significant effect on PDCD4 phosphorylation or abundance (Fig. 4, *A–C*). Compared with serum deprivation, readdition of serum significantly increased PDCD4 phosphorylation and reduced its abundance in a time-dependent manner (Fig. 4, *A–C*).

Phenylalanine incorporation into proteins is higher in starved myoblasts depleted of PDCD4. When cultured in growth medium, phenylalanine incorporation into proteins tended to be higher in L6 myoblasts depleted of PDCD4 (by 13%, $P = 0.08$). However, when cells were grown in an AA- and serum-free medium, this measurement more than doubled in cells depleted of PDCD4 ($P < 0.0001$; Fig. 4*D*). Similar results were obtained with a second siRNA oligonucleotide designed against a different region of PDCD4 (Supplemental Fig. S5), indicating the specificity of the effect.

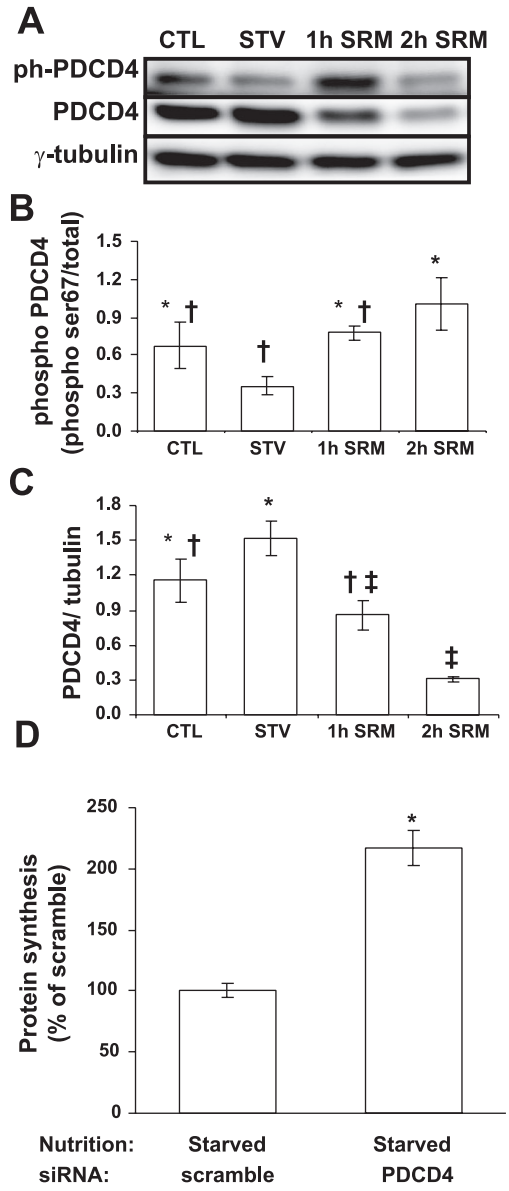

Fig. 4. Regulation of PDCD4 abundance and phenylalanine incorporation into proteins in L6 myoblasts in response to nutrition. *A–C*: PDCD4 phosphorylation (*A* and *B*) and abundance (*A* and *C*) in myoblasts in response to serum-free α-modification of Eagle's medium (STV) and serum-replete medium (SRM). *D*: phenylalanine incorporation into proteins in myoblasts depleted of PDCD4. Cells were treated with siRNA designed against PDCD4. They were then cultured in amino acid- and serum-deprived medium before phenylalanine incorporation into proteins was measured. Values are means ± SE. *B* and *C*: CTL, $n = 3$; for each of STV, 1-h SRM, and 2-h SRM, $n = 4$. *D*: $n = 6$. Means without a symbol letter differ; $P < 0.05$. *Different from scramble; $P < 0.0001$.

DISCUSSION

We have demonstrated that, in rat skeletal muscle, fractional rates of protein synthesis and the levels of PDCD4 were inversely regulated by feed deprivation/refeeding. In rats that were either fed or feed deprived and then refed, fractional rates of protein synthesis and the activation of S6K1 were higher, whereas the amount of PDCD4 was lower. The reverse was the case in feed-deprived rats. Moreover, when L6 cells were cultured in an AA-free medium, the rate of phenylalanine incorporation into proteins was significantly higher in cells depleted of PDCD4 compared with cells with normal levels of the protein. Thus, our data indicate that PDCD4 is a nutrient- and growth factor-sensitive negative regulator of protein synthesis in skeletal muscle and in myoblasts.

That S6K1 is important in regulating muscle mass is incontrovertible (3, 38). What has been unclear is the mechanism by which it acts. This is an important question because S6K1 activities are implicated in insulin resistance, obesity, and cancer (9, 17, 48, 49). Identifying the specific pathways by which S6K1 regulates protein synthesis can permit the isolation of the muscle growth-promoting functions of the kinase from its undesirable effects in insulin resistance. A previous study identified S6K1 as the kinase that, upon mitogen stimulation, phosphorylates PDCD4 and targets it for ubiquitination and degradation (15). Our data demonstrating the expression of PDCD4 in skeletal muscle and in muscle cells, and the fact that it is regulated by feed deprivation/refeeding, indicate that this protein likely plays an important role in regulating muscle protein status. We also showed that amino acids, in the absence of elevated systemic insulin, regulated PDCD4. This further highlights the significance of this protein as a possible nutrient-responsive regulator of protein synthesis. However, the overactivation of mTORC1 and S6K1, kinases that are critical in regulating muscle mass, is implicated in cancer (13), and PDCD4 is a tumor suppressor (47). A preferred strategy to increase muscle protein synthesis and mass could involve interventions that selectively promote PDCD4 degradation in skeletal muscle so that muscle protein anabolism is promoted without increased tumor risk in nonskeletal muscle tissues.

Our attempt to delineate the components of food that mediated the changes in PDCD4 and S6K1 regulation and protein synthesis revealed some differences between the effects of refeeding vs. AA gavage. Whereas increased phosphorylation of PDCD4 was seen at 2 h after the start of refeeding, the effect of AA gavage was less clear. However, in both studies, the

abundance of PDCD4 was suppressed by refeeding or AA gavage. In addition, fractional rates of protein synthesis increased from the feed-deprived state to 2 h postrefeeding. There was some interanimal variability in the amino acid gavage groups. This was not due to differential enrichments of phenylalanine in the tissue free pools in the amino acid gavage groups, because these measurements were not different from CTL (data not shown). The variability was likely due to differential responses of the rats to the amino acid gavage. Nevertheless, fractional rates of protein synthesis at 1 and 2 h after AA gavage were not different from CTL. Thus, refeeding and, to a lesser extent, amino acid gavage induced fractional rate of protein synthesis; both suppressed PDCD4 abundance. Notably, there was no insulin response to the AA gavage, although we did not measure the level of the hormone at 30 min postgavage. The changes in PDCD4 phosphorylation induced by refeeding vs. amino acid gavage suggest a role for Akt (45) and insulin (and perhaps components of the feed other than amino acids) (4) in mediating the effects of feeding on PDCD4 phosphorylation. Our finding that changes in PDCD4 abundance preceded changes in muscle fractional rates of protein synthesis (Fig. 3*B* and Table 1) suggests that, irrespective of what those other factors might be, they regulate protein synthesis and PDCD4 abundance in tandem.

Although both the phosphorylation status and the abundance of PDCD4 are regulated by mitogen-induced activation of S6K1 (15), it is not clear which is more important. In the case of 4E-BP1, which like PDCD4 inhibits eIF4F formation and cap-dependent mRNA translation, its phosphorylation is enough to relieve its inhibition of eIF4E (40). PDCD4 inhibits eIF4A in a dominant manner, since one molecule of this protein binds to and inactivates two molecules of eIF4A. It also prevents the binding of eIF4A to eIF4G (10, 35, 36). The site of S6K1 phosphorylation on PDCD4, Ser^{67}, is not involved in the interaction of the protein with eIF4A (10, 36). In fact, a truncated protein that lacks the NH_2-terminal region within which Ser^{67} is located efficiently binds to and inhibits eIF4A helicase activity (10). Our findings indicate that the abundance of PDCD4, rather than its phosphorylation per se, may be the critical factor in the regulation of mRNA translation. Indeed, in many of the conditions tested, changes in the abundance of PDCD4 mirrored the alterations in fractional rates of protein synthesis and phenylalanine incorporation into proteins more closely than changes in PDCD4 phosphorylation (compare Fig. 1, *B* and *C*, with Fig. 2*D* and Fig. 3*B* with Table 1; also see Fig. 4).

PDCD4 mRNA is abundant in several tissues, especially liver, lung, and kidney, but only very low levels are detected in skeletal and heart muscles (39, 46). To our knowledge, this is the first report of the expression of PDCD4 protein in skeletal muscle. Although we cannot completely rule out the expression of this protein in other cell types, our data from L6 myoblasts indicate that the expression of PDCD4 seen in rat skeletal muscle could not be ascribed solely to other cell types. We further demonstrated that, in myoblasts cultured in an amino acid- and serum-free medium, phenylalanine incorporation into proteins was significantly higher in cells depleted of PDCD4 compared with cells with normal levels of this protein. Thus, our data indicate that the abundance of PDCD4 is implicated, at least in part, in the suppression of muscle fractional rate of protein synthesis seen during feed deprivation. An extrapolation of our findings in myoblasts to whole muscle and animal protein metabolism is not possible. This is because cell incubation media do not accurately replicate the in vivo environment (18). As such, an assessment of the precise contribution of PDCD4 to skeletal and whole body protein status will require studying protein metabolism in PDCD4 knockout animals. However, our data provide a starting point on which such studies can be based.

In conclusion, our data indicate that the abundance of PDCD4 may be important in regulating skeletal muscle fractional rates of protein synthesis in response to feed deprivation and refeeding. When S6K1 levels are depleted, PDCD4 degradation is delayed, although at later times its levels decrease (15). If our findings in the cell culture experiments are confirmed in animal (or muscle specific) knockouts of PDCD4 or in studies where siRNA/shRNA that targets PDCD4 is transfected into a limb muscle, strategies that diminish the abundance of this protein without a requirement for S6K1 could constitute attractive interventions in enhancing muscle protein anabolism and mass.

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GRANTS

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DISCLOSURES

No conflicts of interest, financial, or otherwise, are reported by the authors.

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