Phosphorylation of p70\textsuperscript{S6k} correlates with increased skeletal muscle mass following resistance exercise

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Baar, Keith, and Karyn Esser. Phosphorylation of p70\textsuperscript{S6k} correlates with increased skeletal muscle mass following resistance exercise. Am. J. Physiol. 276 (Cell Physiol. 45): C120–C127, 1999.—High-resistance exercise training results in an increase in muscle wet mass and protein content. To begin to address the acute changes following a single bout of high-resistance exercise, a new model has been developed. Training rats twice a week for 6 wk resulted in 13.9 and 14.4% hypertrophy in the extensor digitorum longus (EDL) and tibialis anterior (TA) muscles, respectively. Polysome profiles after high-resistance lengthening contractions suggest that the rate of initiation is increased. The activity of the 70-kDa S6 protein kinase (p70\textsuperscript{S6k}), a regulator of translation initiation, is also increased following high-resistance lengthening contractions (TA, 363 ± 29%; EDL, 353 ± 39%). Furthermore, the increase in p70\textsuperscript{S6k} activity 6 h after exercise correlates with the percent change in muscle mass after 6 wk of training (r = 0.998). The tight correlation between the activation of p70\textsuperscript{S6k} and the long-term increase in muscle mass suggests that p70\textsuperscript{S6k} phosphorylation may be a good marker for the phenotypic changes that characterize muscle hypertrophy and may play a role in load-induced skeletal muscle growth.

THE EXTERNAL LOAD APPLIED to a muscle is a primary determinant of its size (5). This relationship has been clearly illustrated in a number of different species using models such as tenotomy, stretch, synergist ablation, and high-resistance exercise (36). In any of these models, a common response to the increased loading is an increase in muscle wet mass and protein content (36). Furthermore, the increase in skeletal muscle mass following increased loading correlates with increases in protein synthesis (14). This suggests that the control of protein synthesis may be important in regulating load-induced changes in muscle mass.

Laurent et al. (20) were the first to demonstrate that changes in the rate of protein synthesis following increased loading occur before changes in RNA accumulation. These authors concluded that, in chicken skeletal muscle, a hypertrophic stimulus results in increased RNA activity (the amount of protein synthesized per quantity of RNA). Smith et al. (31) extended this finding by showing that rabbit muscle stretched in vitro underwent a twofold increase in the rate of translation before changes in the rate of transcription. Wong and Booth (38, 39) demonstrated in rats that both shortening and lengthening contraction protocols increased protein synthesis rates 12–17 h after the exercise bout at a time when the level of RNA and DNA accumulation was unchanged. Finally, a similar rapid increase in the rate of protein synthesis is seen in humans (7, 23, 24), suggesting that one of the initial responses following an increased workload is the activation of muscle protein synthesis. Because the changes in protein synthesis appear to be independent of quantitative changes in RNA, the activation of synthesis must occur through a posttranscriptional mechanism.

To understand the regulation of protein synthesis, the contributions of initiation, elongation, and termination must be considered (3). Although all three steps are potential points of control, initiation has been shown to be rate limiting in the regulation of overall protein synthesis (25, 32). The rate of initiation of protein synthesis is regulated by a number of proteins that are in turn controlled by posttranslational modification (27). Three key proteins expressed in all cell types [eukaryotic initiation factor 2 (elF-2), 4E binding proteins (4E-BP), and 70-kDa S6 protein kinase (p70\textsuperscript{S6k})] have been identified, and their role in controlling translation is being elucidated (25). elF-2 is thought to regulate general protein synthesis, whereas 4E-BP and p70\textsuperscript{S6k} control growth-related protein synthesis (26). Furthermore, p70\textsuperscript{S6k} has been shown to be activated in cardiomyocytes and smooth muscle in response to a hypertrophic stimulus (4, 13, 30, 33), suggesting that the activation of p70\textsuperscript{S6k} may be important in regulating skeletal muscle growth.

The kinase activity of p70\textsuperscript{S6k} is controlled by a series of phosphorylation steps (28). Phosphorylation of p70\textsuperscript{S6k} in the carboxy terminal autoinhibitory domain and at Thr-229 results in increased protein synthesis (25, 28). Phosphorylation of Thr-229 appears to be independent of the percent change in muscle mass after 6 wk of training (r = 0.998). The tight correlation between the activation of p70\textsuperscript{S6k} and the long-term increase in muscle mass suggests that p70\textsuperscript{S6k} phosphorylation may be a good marker for the phenotypic changes that characterize muscle hypertrophy and may play a role in load-induced skeletal muscle growth.

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stimulated protein synthesis, p70S6k may be important in the regulation of protein synthesis following high-
resistance exercise.

Most studies of muscle growth have analyzed chronic changes in muscle phenotype, whereas only a few have addressed acute changes such as protein synthesis (5). A primary impediment has been the development of a model that provides control over muscle activation without introducing confounding variables. The most promising studies of the acute response to increased workload have used a field stimulation protocol (38, 39) to activate the muscles of the distal hindlimb. This model uses antagonist muscle action resulting in lengthening contractions in the dorsiflexor muscles and shortening contractions in the plantar flexor muscles. Due to the force-velocity relationship, it can be inferred that the dorsiflexors experience a higher force than the plantar flexors. An additional strength of the model is that the onset and termination of the exercise are controlled, thereby allowing the study of both acute and chronic responses to high-resistance contractions in vivo. This protocol was modified for this study such that muscle activation occurs through the innervating nerve. Such a modification ensures that all motor units of the distal hindlimb are recruited and assures that the muscles are untouched before collection.

The aims of this study were 1) to establish the modified model of skeletal muscle stimulation for studies of muscle growth, 2) to determine whether translation initiation is increased following a single bout of exercise, and 3) to determine whether a growth-related regulatory protein (i.e., p70S6k) is posttranslationally modified in response to exercise. Here, we report a model that uses high-resistance lengthening contractions to increase the wet mass of the rat dorsiflexor muscles. After an acute bout of these high-resistance lengthening contractions, polysome profiles indicate that translation initiation is elevated. Using Western blots, we demonstrate that p70S6k is phosphorylated following exercise and that the phosphorylation of p70S6k correlates with skeletal muscle growth. The strong correlation between the phosphorylation of p70S6k and the increase in muscle wet mass suggests that p70S6k phosphorylation may play a critical role in skeletal muscle growth and is a promising marker for the acute characterization of growth stimuli.

METHODS

Surgical implantation of electrodes and electrical stimulation. All experimental procedures were approved by the University of Illinois at Chicago Animal Care Committee. Animals were housed individually and allowed free access to food and water throughout the experimental period. For all surgical procedures, female Wistar rats (Charles River Laboratories, Wilmington, MA) were anesthetized with an intraperitoneal injection of pentobarbital sodium (50 mg/kg, supplemented as necessary). The implantation procedure involved surgically exposing the sciatic nerve before its point of trifurcation and suturing multistrand stainless steel electrode wires (Medwire, Mount Vernon, NY) on either side. The stimulating wires were then run subcutaneously to the base of the neck, where they were secured.

One week following the implantation of the electrodes, animals were chemically restrained using an intraperitoneal injection of a ketamine (75 mg/kg) and xylazine (25 mg/kg) cocktail and prepared for stimulation. The body of the rat was secured to the stimulation apparatus, and the right foot was allowed to move freely. External stimulating electrodes were attached to the neck cap, and the voltage applied (5–7 V) to the electrodes was adjusted to produce maximal palpable contractile force (Fig. 1). Muscle contractions were generated by stimulating the nerve at 100 Hz; 100 Hz was selected because at this frequency all motor units (both fast and slow) are recruited (6, 40). The contractions lasted 3 s and were followed by a 10-s rest (40), during which time the foot was returned to the neutral position. After the sixth repetition, there was an additional 50 s of rest. This pattern of stimulation was repeated for 10 sets of 6 repetitions, resulting in 60 contractions over a 22-min period. Due to the positioning of the electrode, above the point of sciatic nerve trifurcation, all of the muscles of the distal compartment contracted simultaneously. The result was a net plantar flexion of the ankle because the plantar flexor muscles (gastrocnemius, soleus, and plantaris) produce more force than the dorsiflexors (tibialis anterior (TA) and extensor digitorum longus (EDL)) (40). This ankle plantar flexion results in a shortening contraction of the plantar flexors and a lengthening contraction of the dorsiflexors.

At the time of death, experimental and contralateral control muscles were removed, trimmed of external lipid and connective tissue, blotted of excess moisture, weighed, and frozen in liquid nitrogen. Animals from the training study were killed 4 days after the last stimulation bout to eliminate possible acute effects of the stimulation protocol such as edema. Muscle growth was determined as the difference in wet mass between the experimental and contralateral control muscles relative to the body mass of the animal. The muscle protein concentration was determined using the DC protein assay (Bio-Rad, Hercules, CA). Animals for the acute studies were killed immediately after and at 3, 6, 12, 17, and 36 h after a single bout of resistance exercise.

Fig. 1. External stimulating electrodes were attached at neck, and voltage was applied to nerve to produce high-resistance contractions. Sciatic nerve was stimulated at 100 Hz in 3-s pulses with a 10-s rest period between repetitions. After 6th repetition, an additional 50-s rest was allowed. This cycle of stimulation was continued for 10 sets of 6 stimulations, resulting in 60 contractions over a 22-min period.
Sucrose gradient separation of polysomes. Polysome profiles were analyzed using a modification of the method of Agrawal and Bowman (1). Briefly, muscles were homogenized in a buffer (100 mg muscle mass/ml buffer) containing 50 mM Tris·HCl (pH 7.4), 250 mM KCl, and 25 mM MgCl₂. Before homogenization, 0.25 mM dithiothreitol, 1 mM cycloheximide, and 4 U/ml RNasin were added to the buffer. The homogenate was centrifuged for 5 min at 4°C and 5,000 g. The supernatant was removed and centrifuged for 15 min at 4°C and 15,000 g. Eight hundred microliters of the resulting supernatant were applied to a 0.4–1.2 M linear sucrose gradient (sucrose dissolved in homogenization buffer). The gradients underwent ultracentrifugation for 120 min at 40,000 rpm and 2°C in a Beckman swinging bucket rotor (SW41Ti). After ultracentrifugation, the bottom of the tube was punctured and an 80% sucrose solution was pumped in. The effluent from the top of the tube flowed through a SPECTRA/CHROM Flow Thru ultraviolet (UV) monitor measuring absorbance at 254 nm. The results of the UV quantification were displayed using a Perkin-Elmer 561 chart recorder. As described in Agrawal and Bowman (1), the tracing reflects the amount of RNA from the lightest (nontranslated mRNA in the ribonuclear pool) to the heaviest (mRNA with many ribosomes and their nascent polypeptide chains).

The supernatant (containing p70S6k) was removed (4), and its protein concentration was determined by the DC protein assay (Bio-Rad). Ten micrograms of the supernatant were separated on a 7.5% gel (18). After electrophoretic separation, the proteins were transferred to a poly(vinylidene difluoride) (Millipore, Bedford, MA) membrane for Western blot analysis (4, 37). The membrane was incubated in a 5% Blotto (5% powdered milk in 1× Tris-buffered saline) solution overnight at 4°C. The blocking solution was poured off, and the p70S6k antibody (Santa Cruz Biotechnologies, Santa Cruz, CA) was added and incubated for 45 min with gentle rocking. The blot was washed, and the peroxidase-conjugated horse anti-rat IgG secondary antibody (Vector Laboratories, Burlingame, CA) was added and incubated for 30 min. Antibody binding was detected using the Amersham Life Sciences enhanced chemiluminescence detection kit.

Quantification of p70S6k phosphorylation. The level of p70S6k phosphorylation was quantified using a Bio-Rad model GS-670 scanning densitometer and Molecular Analyst software (Bio-Rad). Each sample (insulin treated, exercised, and contralateral control) contained two to five bands that were individually quantified. The relative amount of phosphorylated p70S6k was calculated for each sample by summing the total amount of phosphorylated p70S6k (the one to four slower migrating bands) and dividing that by the total p70S6k (all bands). To determine the effect of insulin or exercise on p70S6k phosphorylation, we calculated the percent change in p70S6k phosphorylation between the experimental soleus, plantaris, EDL, and TA muscles and the respective untreated or contralateral control muscles.
size in a muscle that did not grow. At no time point studied were the polysome profiles of the stimulated soleus muscles different from those of the contralateral control muscles (Fig. 3A). Conversely, profiles from stimulated EDL muscles demonstrated a shift toward a heavier polysome pool, with the maximal effect of the stimulus on polysome size seen 6 h after the end of the bout (Fig. 3). Polysome profiles from the EDL at 17 and 36 h after high-resistance lengthening contractions were similar to those of the contralateral control muscles.

Fig. 3. Polysome profiles were generated following sucrose gradient ultracentrifugation of experimental and control muscle homogenates. Bottom of ultracentrifugation tube was punctured, and an 80% sucrose solution was used to float gradient through an ultraviolet flow cell. Right: absorbance at 254 nm (A254) from soleus 6 h following an acute bout of stimulation (A) and from EDL muscles 6 h (B), 17 h (C), and 36 h (D) following an acute bout of stimulation. Left: contralateral control muscles. Arrow indicates 80S peak, and therefore absorbance after this point is representative of polysomal RNA.
Because a change in polysome size was observed at 6 h specifically in the EDL muscle, this suggests that high-resistance lengthening contractions and not contractile activity alone results in a transient increase in the size of the polysome pool.

p70S6k phosphorylation. Increased p70S6k activity has been correlated with the rate of translation initiation (10). Because the kinase activity of p70S6k is correlated with its phosphorylation, the phosphorylation of p70S6k following acute exercise was investigated (8). As seen in Fig. 4 (A–D, lane 2), p70S6k is expressed in all of the muscles studied. As a positive control for p70S6k activation/phosphorylation, nonexercised animals were given an intraperitoneal injection of insulin (0.2 U/kg body wt) (15) to maximally phosphorylate/activate p70S6k (Fig. 4, A–D, compare lanes 1 and 2). As demonstrated previously (8), activation by insulin results in phosphorylation of p70S6k, causing a shift from the faster migrating form to the various slower migrating states.

Immediately after high-resistance lengthening contractions, p70S6k phosphorylation in EDL and TA muscle was not significantly different from that of the contralateral control muscle (Fig. 4, C and D, compare lanes 3 and 2). However, at 3 and 6 h (Fig. 4, C and D, compare lanes 4 and 5 to lane 2) the level of p70S6k phosphorylation was increased to its maximal levels (TA, 363.2 ± 29.4%; EDL, 353.4 ± 39.5%). This increase in p70S6k phosphorylation occurred independently of changes in the total level of p70S6k (TA, 93.8 ± 11.0% of control; EDL, 100.3 ± 31.0% of control). The phosphorylation of p70S6k in the EDL and TA following the exercise stimulus was comparable to that seen following insulin stimulation (TA and EDL, 85.4 and 93.2% of the insulin-stimulated value, respectively). In addition, elevated levels of p70S6k phosphorylation in the EDL and TA muscles are sustained through 36 h after stimulation (TA, 204.1 ± 28.3%; EDL, 135.7 ± 20.7%), with no change in total p70S6k (TA, 94.4 ± 23.3% of control; EDL, 98.1 ± 21.7% of control). These data suggest that high-resistance lengthening contractions result in maximal phosphorylation of p70S6k and that the increase in phosphorylation is maintained over a long period of time.

The phosphorylation of p70S6k following resistance exercise was also significantly increased over the contralateral control muscle in the plantaris following stimulation. In the plantaris, the phosphorylation of p70S6k was increased at all time points (Fig. 4B, compare lanes 3–7 to lane 2), with the greatest level of p70S6k phosphorylation in the plantaris occurring 3 h after stimulation (209.2 ± 34.5%). Interestingly, p70S6k phosphorylation in the plantaris only attained 56% of the insulin-stimulated value, suggesting that the signal for p70S6k phosphorylation in the plantaris is less potent than that in the EDL or TA muscles. In the soleus muscle, the basal level of p70S6k phosphorylation was greater than in the other muscles studied (Fig. 4, A–D, compare lane 2). However, stimulation of the soleus via the sciatic nerve had no effect on the relative phosphorylation state of p70S6k at any time point studied (Fig. 4, A and E).

**DISCUSSION**

Through modification of the protocol of Wong and Booth (40), an exercise model has been developed that produces significant growth in the dorsiflexor muscles of the rat hindlimb. This model of muscle growth is
particularly useful for the study of the early response to a single bout of resistance exercise for four reasons. First, by stimulation of the muscles through their innervating nerve, the possibility that the electrodes directly affect the study parameters has been eliminated. Second, because the muscles of the right distal hindlimb are unilaterally activated, the contralateral (left) leg provides unstimulated control muscles. Third, the lack of a surgical intervention in the distal hindlimb eliminates the possibility that the responses observed are the byproduct of postoperative stress. Finally, the ability to control the onset and termination of the stimulus ensures that all experimental animals receive the same exercise stimulus and allows the investigation of temporal events following a single bout of exercise.

The results of this study demonstrate that, after 6 wk of training, this model produces a significant increase in wet mass in the EDL (13.9%) and TA (14.4%) muscles, a slight increase in the plantaris muscle mass (6.9%; P = 0.3), and no change in soleus muscle mass (−2.3%). Although the increase in TA mass attained using this model (14.4%) was less than the 30% obtained by Wong and Booth (39), this is most likely due to the fact that the intensity of the stimulus differed between the two protocols. This model used 60 stimulations in each of 12 bouts, whereas the Wong and Booth (39) model used 192 stimulations in each of their 20 stimulation bouts. Although the intensity and duration of the stimulus used in this study was less than that of Wong and Booth (39), it was sufficient to produce a significant increase in wet mass and validate its use as a model of skeletal muscle growth.

It is important to note that the slow fiber-type soleus muscle, unlike the fast fiber-type plantaris muscle, did not exhibit any trend toward an increase in wet mass. Because many studies have demonstrated that the rat soleus muscle is capable of hypertrophy (5, 36), this finding suggests that the magnitude of the exercise stimulus was insufficient to induce growth. This observation is important because it suggests that the exercised soleus muscle can be used to assess the effect of neuromuscular activity independently of long-term changes in muscle size.

Acute increases in the rate of protein synthesis are common to all models of exercise-induced muscle growth. Thus the first goal of the study was to identify the stage at which protein synthesis is regulated following one bout of exercise. Only in the exercised EDL muscles is there a change in polysome distribution. At 6 h after high-resistance lengthening contractions, the EDL shows a shift toward heavy polysomes (Fig. 3). This finding suggests that more ribosomes were associated with mRNA in muscles following lengthening contractions. On the basis of the arguments of Bergmann and Lodish (3), the increased ribosome association seen in this study could be due to an increase in the rate at which new ribosomes are added to the mRNA (initiation), a decrease in the rate at which the ribosomes travel the length of the mRNA (elongation), and/or a decrease in the rate at which ribosomes were released from the mRNA (termination). Decreases in the rate of elongation and/or termination would result in an overall decrease in the rate of protein synthesis, whereas increasing the rate of initiation would increase the overall rate of protein synthesis. Because a hypertrophic stimulus has been shown to increase the overall rate of protein synthesis (7, 20, 23, 24, 31, 38, 39), we interpret the increase in polysomes to be the result of an increase in the rate of initiation of protein synthesis. This does not mean that initiation is the only site regulated by exercise; increases in the rate of elongation and/or termination are also possible in support of the increased rate of initiation.

Consistent with an increase in the rate of initiation, the phosphorylation of p70S6K in the TA and EDL was significantly increased by high-resistance lengthening contractions. This relationship between changes in polysome profiles and p70S6K phosphorylation was most evident 6 h after exercise. However, at 17 and 36 h, the increased phosphorylation of p70S6K was not paralleled by significantly heavier polysomes. The return of polysome size toward control levels at 17 and 36 h suggests that the rates of initiation, elongation, and termination in the experimental muscles are proportional to those in the contralateral control muscles. Because Wong and Booth (39) reported increased RNA activity at 17 and 41 h after acute exercise (34 and 30%, respectively), the normalization of the polysome profiles at 17 and 36 h suggests that the rates of elongation and termination are increased. Increased rates of elongation and termination may occur independently of p70S6K activity or may be a downstream effect of p70S6K activation. The

![Graph](http://apcell.physiology.org/)

**Fig. 5.** Correlation between change in phosphorylation of p70S6K at 6 h after stimulation and increased wet mass measured after muscles were stimulated 2 days/wk for 6 wk (means ± SE of change in p70S6K phosphorylation and percent increase in wet mass of muscles studied; n = 3). Correlation between change in phosphorylation of p70S6K at 6 h after stimulation and increased wet mass (r = 0.998) is significant (P < 0.05). Line illustrates a hypothesized relationship between acute change in p70S6K phosphorylation and increased muscle mass following training.
activation of p70\textsuperscript{56k} results in the selective translation of a set of growth-related mRNA containing a 5\textsuperscript{\prime} terminal polyuridine tract (5\textsuperscript{\prime} TOP) (16, 21, 34). This 5\textsuperscript{\prime} TOP motif is found in the 5\textsuperscript{\prime} untranslated region of elongation factors (eEF; e.g., eEF-1\alpha and eEF-2) and ribosomal proteins (e.g., S3, S14, and S24) (16, 17, 21, 34). Selective translation of these mRNAs would result in increased accumulation of elongation factors and ribosomal proteins and might subsequently play a role in increasing the overall rate of protein synthesis.

The activation/phosphorylation pattern of p70\textsuperscript{56k} across the different muscles was also evaluated 6 h after resistance exercise. These data indicate that high-resistance lengthening contractions and not exercise alone increased the phosphorylation of p70\textsuperscript{56k}. Maximal phosphorylation of p70\textsuperscript{56k} was observed in the EDL and TA, half-maximal phosphorylation was seen in the plantaris muscle, and no change in phosphorylation was found in the soleus muscle. Interestingly, this differential phosphorylation among the muscles mimicked the change in muscle wet mass after 6 wk of training. This raised the possibility that a correlation existed between the change in phosphorylation and the increase in muscle mass. Therefore, the mean percent increase in muscle wet mass after 6 wk of training was plotted against the mean percent increase in p70\textsuperscript{56k} phosphorylation measured at 6 h (Fig. 5). The results revealed a correlation coefficient between the two variables of 0.998 (P < 0.05), suggesting that a relationship between increased wet mass and p70\textsuperscript{56k} phosphorylation exists. If true, this implies that the acute increase in p70\textsuperscript{56k} phosphorylation may be important for the long-term increase of muscle mass. It is, however, important to note that, although the correlation coefficient is high, the increase in plantaris mass following training is not statistically significant, whereas the acute increase in p70\textsuperscript{56k} phosphorylation is. Further studies are required to determine whether the phosphorylation of p70\textsuperscript{56k} is required or causative for increased muscle mass following resistance exercise training.

It is important to note that the type of contractions produced by the EDL and TA (lengthening) are different from those produced by the plantaris and soleus muscles (shortening). Because the EDL and TA lengthen as they are activated, by definition the force per cross-sectional area across these muscles must be greater than that in the plantaris and soleus muscles. However, lengthening contractions also produce a higher incidence of fiber injury resulting from membrane damage, myofibrillar damage, and infiltration of the muscle by mononuclear cells (2, 22). It is possible that any or all of these factors are involved in the activation of p70\textsuperscript{56k}.

This is the first report of the phosphorylation of p70\textsuperscript{56k} following an exercise stimulus in skeletal muscle. This finding is consistent with observations in cardiac myocytes in which mechanical stretch results in hypertrophy (29). Stretch of cardiac myocytes also results in the activation (phosphorylation) of p70\textsuperscript{56k} in a time course similar to what we have observed in skeletal muscle (30, 33). Furthermore, cardiac myocytes also require the activation of p70\textsuperscript{56k} for increased protein synthesis following stretch (4, 30, 33). Because the activation of p70\textsuperscript{56k} is required for increased protein synthesis in response to a hypertrophic stimulus in cardiac myocytes and because p70\textsuperscript{56k} is phosphorylated following high-resistance lengthening contractions in skeletal muscle, the possibility exists that cardiac and skeletal muscles undergo hypertrophy via a conserved mechanism.

The results presented here provide a model for studying the acute effects of a growth stimulus on skeletal muscle. In this model, an acute bout of high-resistance lengthening contractions results in an increase in the rate of translation initiation. The high-resistance exercise bout also increases p70\textsuperscript{56k} phosphorylation. Furthermore, the correlation that exists between the phosphorylation of p70\textsuperscript{56k} and the long-term increase in muscle mass suggests that p70\textsuperscript{56k} phosphorylation may play a key role in skeletal muscle growth and also demonstrates promise as a marker for the phenotypic changes that characterize this growth.

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