Transient induction of cyclin A in loaded chicken skeletal muscle

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Flück, Martin, Magali Kitzmann, Christoph Däpp, Matthias Chiquet, Frank W. Booth, and Anne Fernandez. Transient induction of cyclin A in loaded chicken skeletal muscle. J Appl Physiol 95: 1664–1671, 2003. First published June 20, 2003; 10.1152/japplphysiol.00276.2003.—Cell proliferation is believed to contribute to the increased synthesis rate during load-induced growth of avian anterior latissimus dorsi (ALD) skeletal muscle, but the relative contribution of different cell types to this proliferative response and the time course of cell activation are not well documented. The present investigation measured the abundance and localization of cyclin A protein, which is uniquely present in proliferating cells and required for the entry of vertebrate cells into the DNA synthesis phase during the time course of chicken ALD loading. Total protein content in 1.5-, 7-, and 13-day loaded ALD increased by 60, 191, and 294%, respectively. Immunoblotting analysis identified that cyclin A protein per total protein was dramatically increased in ALD muscle after 1.5 days of loading but returned to control level at 7 days. In vitro kinase assays demonstrated a corresponding massive activation of the cyclin A-regulated, cyclin-dependent kinase 2 but not of cyclin-dependent kinase 2 protein level in muscle homogenates after 1.5 days of muscle loading. Immunofluorescence experiments demonstrated that the increase of cyclin A in 1.5 days of loaded ALD was primarily confined to nuclei of interstitial cells (92%) but was also found in fiber-associated cells (8%). In situ hybridization demonstrated an increased number of nuclei of interstitial cells expressing collagen I transcripts after 1.5 days of loading. These data show that the cell cycle protein cyclin A is induced in fiber-associated cells during the early growth response in loaded ALD and also implicate an activation of interstitial cells as playing an early role in this model for muscle growth.

Example, the content of DNA increases more rapidly than the protein content in growing avian anterior latissimus dorsi muscle (ALD) (24). Furthermore, introduction of DNA breaks by irradiation prevents hypertrophy of overloaded rat soleus muscle (38) and rat soleus muscle recovering from atrophy (31) and reduces the degree of muscle growth in loaded quail ALD muscle (27). Thus an increase in myonuclear number is needed to provide the synthesis rate necessary for the increase in protein in growing skeletal muscle (24, 31, 44).

Multiple cell types that compose skeletal muscle tissue are activated by loading and may contribute to increased muscle growth. Rapidly, after onset of loading of avian ALD, an increase in the number of proliferating connective tissue fibroblasts and satellite cells is observed (24, 43). In addition, loading of rat soleus has been shown to rapidly activate RNA synthesis and proliferating cells of the connective tissue. For example, fibroblast and capillaries have been demonstrated to be the major site of the increased new RNA in loaded rat and chicken skeletal muscle (8, 19, 39). To date, the respective contribution of an activation of myogenic vs. other (interstitial) cell types to total cell proliferation in load-induced muscle growth is still poorly documented, and the activating molecular mechanism is little defined.

In vertebrates, DNA is synthesized (replicated) only in proliferating cells during passage of the DNA synthesis phase of the cell cycle, before they divide during mitosis and give rise to two daughter cells. Decision of vertebrate cells to enter the cell cycle, synthesize DNA, and finally divide is controlled by a family of proteins, called cyclins (36). The concentration of cyclins rises and falls in a regular pattern during the cell cycle, enabling them to bind and activate at the appropriate moment to cyclin-dependent kinases (cdk), whose activity is needed to propel cells through the cell cycle (4). In particular, cyclin A is induced and required at S phase for DNA replication (13). It is at that point when

VARIOUS ANIMAL MODELS THAT augment loading of vertebrate skeletal muscle cause a rapid increase in muscle mass due to hypertrophy and/or hyperplasia of muscle fibers (5, 19, 40). Earlier work shows that an increase in nuclei, as indicated by an increase in DNA, is a prevalent feature of growing rat and avian muscle. For

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cycdin A forms complexes with cdk2, thereby inducing its serine-threonine kinase activity (26). Cyclin A-mediated phosphorylation of numerous nuclear targets, including the retinoblastoma protein pRb by catalytic active cdk2-cyclin A complexes, are believed to drive the onset of DNA replication (22).

The specific induction of cyclin A protein and cdk2 activity during the DNA synthesis phase of both proliferating myogenic cells (42) and fibroblasts (14) makes them suitable markers to assess entry of these cell types into the proliferative cell cycle in intact skeletal muscle. The purpose of the presented study was to examine the expression of kinetics and localization of cyclin A protein and the activity of its target cdk2 during loading of avian ALD muscle. Additionally, it was verified that the induction of interstitial fibroblast proliferation correlates with increased transcription in these cells of the major extracellular matrix protein, collagen I, whose expression is coupled to mitotic activity (17, 34).

MATERIALS AND METHODS

Overloading of skeletal muscle. Two-month-old white leghorn chickens (Texas A&M, College Station, TX, or Wüthrich Farm, Belp, Switzerland) were subjected to the loading protocol, as described previously (11). The left wing of the chicken was loaded with a weight corresponding to 10% of the animal’s body weight for either 1.5, 7, or 13 days. Control and loaded ALD muscles were harvested after anesthesia (subcutaneous injection of ketamine-HCl-xylazine-acepromazine (100:4:6 mg/kg body wt), snap-frozen in liquid nitrogen, and stored in sealed tubes at −80°C until use. After surgery, animals were killed by cervical dislocation. All procedures have been carried out according to the newest guidelines for animal research (3). The protocols were approved by the Institutional Animal Welfare Committee at the University of Texas–Health Science Center at Houston and the State Animal Protection Commission (Amt für Landwirtschaft des Kantons, Bern, Switzerland).

Isolation of total protein homogenate. Frozen ALD muscle was homogenized with a polytron homogenizer (Kinematica) on ice in Mueller buffer (50 mM HEPES, pH 7.4, 0.1% Triton X-100, 4 mM EGTA, 10 mM EDTA, 15 mM Na3P2O7, 100 mM β-glycerophosphate, 25 mM NaF, 1 mM Na3VO4, 0.5 μg/ml leupeptin, 0.5 μg/ml pepstatin, 0.3 μg/ml aprotinin, 5 ml buffer/700 mg tissue). Protein concentration was estimated by using a Lowry-based protein assay (DC protein assay, Bio-Rad), and aliquots corresponding to 50 μg of total protein each were run on a 8% SDS-PAGE followed by Coomassie blue staining of the gel to check estimated protein concentration and verify the integrity of extracted proteins. Total protein per control and loaded chicken ALD muscles was calculated from the muscle weight subjected to homogenization, the dilution factor (i.e., 5 ml buffer/700 mg tissue), was calculated from the muscle weight subjected to homogenization, the dilution factor (i.e., 5 ml buffer/700 mg tissue), and the protein concentration in muscle homogenate (9).

Immunoprecipitations and histone 1 kinase assay. Total protein homogenate was cleared by centrifugation at 13,000 rpm, and protein concentration was determined (DC protein assay, Bio-Rad). Protein (200 μg) was subjected to immunoprecipitation with polyclonal anti-cdk2 antibody (Santa Cruz Biotechnology) and protein A-Sepharose, as described previously (20). The selected antibody was chosen based on its capability to recognize both active and nonactive cdk2. Purified beads containing cdk2 were incubated in 20 μl of kinase buffer containing 50 μM ATP and 5 μCi of [γ-32P]ATP (3,000 Ci/mmol; Amersham) and 2 μg of histone H1 (Boehringer-Mannheim) for 40 min at room temperature, and the reaction was stopped by the addition of 5 μl of 5× Laemmli buffer (250 mM Tris-HCl, pH 6.8, 5% SDS, 35% glycerol, 40 mM DTT, and 1% bromophenol blue in ethanol) and boiling for 5 min. Terminated reactions were separated by 12% SDS-PAGE and Western blot on nitrocellulose membranes (Schleicher and Schuell), and the radiophosphate incorporation was visualized by audio-radiography by exposing the membrane to Kodak X-ray films (Kodak, Marne La Vallée, France). Thereafter, membranes were used for immunodetection analyses. Quantification of pixel signals (3 individual experiments) was carried out as described below for immunoblotting experiments.

Immunoblotting. Total protein homogenate was separated by SDS-PAGE, Western blotted on nitrocellulose membrane, Ponceau S stained to verify equal loading, and subjected to immunodetection analysis (see Ref. 11 for details). Membranes were blocked with PBS containing 10% dry milk and incubated either with cdk2 (Santa Cruz Biotechnology) or cyclin A antibody (13), respectively, in PBS containing 0.5% bovine serum albumin for 1 h at room temperature. After three washes in PBS, blots were incubated with secondary antibodies (horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse; Amersham, les Ulis, France). Alternatively, cyclin A immunoblotting was carried out by using antibody no. C24230 from Transduction Laboratories (Beckton Dickinson) and peroxidase-conjugated goat anti-rabbit IgG (ICN Biomedicals) as first and secondary immunoreagents. The blots were developed by using enhanced chemiluminescence reagents (Amersham), and the signal was recorded on KODAK XAR-5 film.

Quantification of protein abundance in immunoblots. Multiple loaded and contralateral control samples from the same loading treatment(s) were analyzed on the same immunoblot to test for the effect of loading on the abundance of the individual protein (cyclin A or cdk2) or cdk2 activity. The exposed films were scanned at a resolution of 300 × 300 dpi, imported into COREL-Photopaint 10, and saved under the tag image file format. Then the sum of pixels for each band of interest was estimated with AIDA Array Easy software (Raytest Schweiz, Urdorf, Switzerland). The pixel signals derived from films exposed for nonsaturating durations only were used for calculation of protein abundance. The pixel signals of the same band of interest from the scanned films were statistically analyzed for an effect of loading treatment, with a one-way ANOVA applying the Fisher least significance difference test as post hoc test (Statistica version 6.1 for Windows, StatSoft (Europe), Hamburg, Germany). The effect of duration of loading was analyzed by separating loaded and contralateral control samples from different loading treatments on the same immunoblot. Pixel signals from different Western blot experiments with comparable background were combined and analyzed by using a one-way ANOVA. Finally, the signals were expressed as the mean of pixel signals.

Immunofluorescence. Cryosections (12 μm thick) were prepared from the belly portion of the ALD, mounted on glass slides (SuperFrost Plus, Menzel-Glaser), air dried, and fixed in 4% paraformaldehyde. Cyclin A protein was detected essentially as described (13). Briefly, the slides were treated for 15 min in 0.1% Triton X-100 in PBS, rinsed in PBS, and fixed for 1 min in −20°C acetone (analysis grade), and the sections were rehydrated in PBS containing 0.5% BSA (BSA-PBS). Then the sections were incubated overnight at room temperature with anti-cyclin A antibody diluted 1:100 in PBS-BSA. After a brief wash in PBS, incubation with fluorescein-
conjugated anti-rabbit antibody for 2–3 h at 37°C was carried out, and nuclei were counterstained with Hoechst (0.1 μg/ml; Sigma). The signal was digitally recorded as described (25). Immunohistochemical analysis of laminin expression identified that the integrity of the basal lamina was not affected by sectioning of frozen ALD muscle.

**Semi-quantitative analysis of cyclin A positive cells.** For 1.5 and 7 days of loading treatment, one section from the belly portion of control and loaded ALD from two individual animals each was stained. Fluorescent photomicrographs were taken from three randomly chosen and nonoverlapping fields of the cyclin A- and Hoechst-stained sections. The fluorescent photomicrographs were overlaid with the phase-contrast image of immunofluorescent-stained sections. Then, the total number of nuclei, the number of cyclin A positive (and negative) nuclei in the interstitial tissue, and the number of cyclin A positive (and negative) nuclei associated to the fiber (i.e., <5 μm distance to fiber periphery) were counted. An average of 200 nuclei were counted per field. Significance of association of cyclin A positive nuclei to interstitial tissue or the muscle fibers for the 1.5 days of loading treatment was verified using a χ² test for statistical significance (Statistica version 6.1 for Windows).

**In situ hybridization.** Cryosections (12 μm thick) were prepared, stored over night at −70°C, and subjected to transcript detection essentially as described (11). To detect collagen I mRNA, a 186-bp cDNA fragment comprising the first exon (coding for 5′-untranslated region and signal peptide) of the chick collagen α1(I) gene was used (7). Digoxigenin (DIG)-labeled antisense and sense RNA probes were synthesized from linearized bluescript vector containing the corresponding cDNA with the DIG High Prime Labeling and Detection Starter Kit I (Roche Diagnostics, Rotkreuz, Switzerland).

After fixation (4% paraformaldehyde in PBS, 20 min), sections were washed in PBS, digested with 5 mg/ml proteinase K (Roche Diagnostics) in 40 mM EDTA and 0.1 M Tris·HCl, pH 8, for 20 min. Digestion was stopped with 2 mg/ml glycine in PBS for 1 min, and slides were fixed again with 4% paraformaldehyde in PBS for 20 min. Sections were acetylated for 5 min in 0.25% acetic anhydride and 0.1 M triethanolamine, pH 8. The slides were prehybridized with 350 μl hybridization buffer [50% formamide, 4× saline sodium citrate (SSC), 2× Denhardt’s, 2% dextran sulfate, and 1 mg/ml yeast tRNA] in a moist chamber for 1.5 h. Then 40-μl hybridization buffer containing heat-denatured (3 min at 80°C) DIG-labeled RNA probes (diluted 1:20) was applied per slide, covered with a coverslip, sealed, and incubated for 16 h at 60°C. Slides were washed in 0.2× SSC at 60°C for 30 min followed by digestion with RNase A (10 μg/ml) in 2× SSC for 5 min at 37°C. Slides were submerged in 2× SSC containing 50% formamide for 30 min and then equilibrated in 0.2× SSC at 20°C. For detection of DIG-labeled RNA, the DIG High Prime Labeling and Detection Starter Kit I (Roche Diagnostics) was used. After development, sections were washed in water and mounted in Kaiser’s glycerol gelatin (Merck). For publication purposes, fields were visualized on a slide film (Ektachrom 64T, Kodak) by using a microscope-photograph system (VANOX-S, Olympus). The slides were scanned by using a Nikon SF-200 slide-scanner operated by a Power Macintosh G3 using the Nikon Scan 2.0 interface and imported in Tiff format into COREL-Photopaint 10.

**Semi-quantitative analysis of collagen I-expressing nuclei.** The signals for collagen I mRNA were predominantly confined to nuclei of connective tissue cells (see Fig. 4). Transcript expression was, therefore, determined by counting the number and positive nuclei per fibers. Sections from 1.5-day loaded and corresponding contralateral control ALD muscle were analyzed on the same slide. Nonoverlapping, random fields from the sections were visualized with light microscopy at an objective magnification of ×40. Then the muscle fibers and the stained nuclei in the matching muscle fiber areas (<5-μm distance to the fiber periphery) were counted. Clearly distinguishable collagen I positive capillaries were excluded from the analysis. Typically, 30 fibers were counted per field until a total of 250 counted fibers was reached on each section. One section per 1.5-day loaded and contralateral control ALD muscle from three individual animals was analyzed. For each field, the ratio of collagen I positive nuclei per muscle fibers was calculated, and the mean for each loading period was determined. Significance of differences in number of positive nuclei between control and loaded ALD was verified by using a χ² test for statistical significance (Statistica version 6.1 for Windows).

**Results**

**Induction of muscle growth by loading.** After 1.5, 7, and 13 days of loading, the total protein in loaded relative to contralateral control ALD muscle was increased by 60, 191, and 294%, respectively, thus demonstrating effective induction of ALD growth (Table 1). Similarly, the wet weight of loaded ALD muscle relative to contralateral control muscle was increased after all duration of loading. This is in agreement with previous studies on the same system with young animals (9, 11, 24).

**Early and transient increase in cyclin A protein in loaded ALD.** Immunoblotting experiments of total homogenates revealed that cyclin A protein is low to absent in contralateral control ALD muscle, whereas its level is strongly induced in ALD muscle after 1.5 days of loading (Fig. 1A). Cyclin A protein stayed ele-

<table>
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<tr>
<th>Treatment</th>
<th>Muscle Weight, mg</th>
<th>Total Protein, mg</th>
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<tbody>
<tr>
<td>Control</td>
<td>296 ± 29</td>
<td>29.7 ± 6.9</td>
</tr>
<tr>
<td>Loaded</td>
<td>447 ± 45*</td>
<td>47.4 ± 8.5*</td>
</tr>
<tr>
<td>Difference, %</td>
<td>+51</td>
<td>+60</td>
</tr>
<tr>
<td>7 Days</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>275 ± 13</td>
<td>34.0 ± 4.2</td>
</tr>
<tr>
<td>Loaded</td>
<td>600 ± 45*</td>
<td>99.2 ± 11.1*</td>
</tr>
<tr>
<td>Difference, %</td>
<td>+106</td>
<td>+191</td>
</tr>
<tr>
<td>13 Days</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>286 ± 5</td>
<td>35.6 ± 6.3</td>
</tr>
<tr>
<td>Loaded</td>
<td>629 ± 12*</td>
<td>140.1 ± 25.1*</td>
</tr>
<tr>
<td>Difference, %</td>
<td>+120</td>
<td>+294</td>
</tr>
</tbody>
</table>

Values are means ± SE (n = 3–6) of muscle wet weight and total protein content in contralateral control and loaded anterior latissimus dorsi (ALD) muscle. * Significant difference between values of control and loaded ALD (P < 0.05).
vated after 7 days of loading, whereas it was reduced below detectable levels in 13-day loaded muscle (Fig. 1C). The level of cyclin A protein was significantly reduced in 7- vs. 1.5-day loaded ALD muscle. The level of cyclin A protein in contralateral control muscles was not different between loading treatments.

The cdk2 activity is induced in loaded ALD muscle. We analyzed whether the phosphotransfer activity of cdk2 correlates with the increase in its positive regulator, cyclin A, in loaded ALD muscle. Immunoprecipitation analysis identified that cdk2 activity is strongly (>40-fold, \( P < 0.001, n = 3 \)) induced after 1.5 and 7 days of loading (Fig. 2A). Thirteen days after loading, cdk2 activity in ALD homogenates appeared reduced (not shown). The cdk2 protein was not changed relative to contralateral controls with loading of ALD muscle (Fig. 2B).

Cyclin A increases in nuclei of the connective tissue. We carried out immunofluorescence experiments to identify the population of cells that express cyclin A protein on loading. Cyclin A positive cells were never observed in contralateral control ALD muscle, whereas 1.5 days after loading, cyclin A positive nuclei were detected principally outside the muscle fibers in the connective tissue compartment of ALD muscle (Fig. 3). Seven days after loading, no nuclei were found to stain cyclin A positive (not shown). Semiquantitative analysis identified that 10.8% of total nuclei in 1.5-day loaded ALD stained cyclin A positive. Cyclin A positive nuclei were primarily found in the interstitial tissue compartment, whereas <8% of cyclin A positive nuclei were associated with muscle fibers (Table 2). Relative to the number of nuclei per compartment, cyclin A positive nuclei were more abundant in the interstitial tissue than they were associated with muscle fibers.

Collagen I expression in the interstitium is increased. In situ hybridization experiments were carried out to test whether the activation of interstitial cell proliferation correlates with an increase in the transcript level for the extracellular matrix protein collagen I. These experiments demonstrated a significant increase in the number of interstitial fibroblasts expressing collagen \( \alpha_1(I) \) mRNA transcripts in ALD muscle loaded for 36 h (Fig. 4).

DISCUSSION

An increase in the number of nuclei contributes to rapid growth of avian slow-tonic skeletal muscle after
increased loading (24, 27). The intracellular events that trigger such nuclear proliferation in intact skeletal muscle tissue in response to external loading are largely undefined. Presented experiments demonstrate that a dramatic induction of the obligatory regulators of the proliferative cell cycle and DNA synthesis, cyclin A, and the phosphotransfer activity of cdk2 contributes to the molecular machinery that induces DNA synthesis and nuclear proliferation in loaded avian ALD muscle.

The time course of cyclin A induction precedes the reported increase in DNA content in loaded chicken ALD, which is maximal after 7 days of loading and drops thereafter (24). In vertebrates, cyclin A protein is only synthesized at the beginning of S phase, i.e., the DNA synthesis phase, and degraded at mitosis (30, 33). Consequently, cyclin A expression and cdk2 activity are specific markers of proliferation when detected in ALD muscle sections. The transient induction of cyclin A protein and cdk2 activity in 1.5-day loaded ALD muscle, therefore, implies that the cell proliferative response in loaded chicken ALD muscle occurs mainly in the rapid growth phase (1.5 days), whereas it is gone in the slow-growth phase (7 days, see Table 1) (10).

Immunofluorescence experiments demonstrate that the increase in cyclin A in 1.5-day loaded ALD is primarily confined to the connective tissue compartment (Fig. 3). Division of fibroblast and muscle precursor cells in culture depends on an increase in cyclin A (13, 42). Consistently, cyclin A positive interstitial cells showed occasional signs of cell division (not shown). Connective tissue fibroblasts have been reported to proliferate in loaded ALD (24), and interstitial cells that were not determined between fibroblasts and eventually invading infiltrating cells (11, 45) contribute a greater percentage to DNA synthesizing cells during the first 3 days of ALD loading than do fiber-associated cells (27). Moreover, the elevation of nonmuscle tissue accounts for the increase in quail ALD mass after 2 days of loading in quail (2). Our data suggest, in contrast to the suggestion of Laurent et al. (24), that proliferating nonmuscle cells significantly contribute to increased DNA content in loaded chicken

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Table 2. Distribution of cyclin A positive nuclei in 1.5-day loaded muscle

<table>
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<tr>
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<th>Cyclin A Positive Nuclei</th>
<th>Fiber Associated</th>
<th>Interstitial Tissue</th>
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<tbody>
<tr>
<td>Per cyclin A positive nuclei</td>
<td>7.6</td>
<td>92.4*</td>
<td></td>
</tr>
<tr>
<td>Per compartment</td>
<td>1.8</td>
<td>18.9*</td>
<td></td>
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Values are means ± SE in percentage of cyclin A positive nuclei in 1.5-day loaded ALD muscle. * Significant difference between the values in interstitial tissue and those associated with fibers ($\chi^2$ test, $P < 0.0001$).
ALD muscle and demonstrate that the proliferative response of interstitial cells is higher in the early phase (1.5 days) than after 7 days of loading. The strong reduction of cyclin A at 7 days of loading (Fig. 1), together with the similar percentage of proliferating interstitial nuclei between 3- and 7-day stretched ALD muscle of adult quails [16.71 vs. 18.72% (27)], indicates that proliferation of interstitial cells has likely ceased at 3 days of loading and does not play a major role in protein accretion and hypertrophy during chicken ALD muscle growth.

Proliferating fibroblasts secrete pivotal substrates and growth factors of skeletal muscle. In situ hybridization experiments demonstrated an increase in the number of interstitial nuclei expressing high mRNA levels for the matrix protein collagen I in 1.5-day loaded ALD (Fig. 4). This early increase of collagen I mRNA after 36 h of loading implies that transcriptional mechanisms contribute to the fivefold increase in the protein synthesis rate of this extracellular matrix protein in 2-day loaded chicken ALD (23). This observation is reminiscent of our laboratory’s recent observation that mRNA levels of tenascin C and collagen XII (and fibronectin) in endomysial cells are increased with 1.5 days of ALD loading in the same muscles (11). Notably, expression of both collagen I and tenasin C is regulated in parallel to mitotic activity, and fibronectin is expressed in proliferating fibroblasts (12, 17). Interestingly, collagen I and fibronectin control muscle differentiation (6, 16) and tenasin isoforms have been implicated to promote satellite cell proliferation (15). It has also been shown that loading of avian ALD for 2 days induces the level of transcripts coding for FGF-2 and -4 and causes accumulation of FGF-2 and -4 protein in the connective tissue (32). FGF-2 is expressed in fibroblasts, and FGFs stimulate proliferation of satellite cells (1, 18). These observations indicate that connective tissue fibroblasts, which are activated to enter the cell cycle early in loaded avian ALD muscle, may secrete factors that control proliferation of muscle stem cells.

In agreement with this hypothesis, we observed a certain percentage, i.e., 7.6%, of cyclin A positive cells in loaded ALD muscle at the periphery of muscle fibers. These cyclin A positive nuclei could represent proliferating satellite cells that are present under the basal lamina of muscle fibers but that, in loaded ALD, may also be present outside the basal lamina to form new myotubes (29, 45). This conclusion is supported by the observation that the percentage (1.8%) of cyclin A positive nuclei at the periphery of fibers of 1.5 day loaded ALD in this study (Fig. 3, Table 2) is similar to the reported percentage (0.3–1.7%) of satellite cells in quail ALD that enter the cell cycle and synthesize DNA in 1-day loaded ALD (43). In addition, activation of satellite cells in the interstitial space outside the basal lamina to form new myotubes in loaded avian slow-tonic ALD muscle is apparent, thereby leading to an increase in muscle fiber number after 5 days of loading, which plateaus after 2 wk of loading (2, 40, 45). The presence of proliferating muscle stem cells outside the basal lamina during fiber hyperplasia is further indicated by the activation of interstitial myogenic cells distinct from satellite cells during the rapid postnatal
growth of rat plantaris muscle when new fibers are formed in the interstitial space (41). Seven days after loading, cyclin A protein levels in loaded ALD, as determined by immunoblotting, were strongly reduced relative to 1.5 days of loading but remained higher than in contralateral control muscle (Fig. 1). Thirteen days after loading, the cyclin A and cdk2 activity levels were not different from contralateral controls. In this regard, our observation on cyclin A protein and cdk2 activity in ALD muscle is consistent with the report of Poolman and Brooks (37) in heart muscle cells, which shows a downregulation of cdk2 activity during the transition from hyperplasia to hypertrophy. This suggests that cellular activation of fiber-associated nuclei could reflect, to some extent, activation of muscle precursor cells 1) to form new fibers in this model or 2) to maintain the pool of residual satellite cells after their number is reduced because of increased fusion with muscle fibers.

The cellular adaptations in the chicken ALD overload are unique in the sense that satellite cell involvement (based on the irradiation approach) is not essential for compensatory muscle enlargement, as is the case in mammalian models (27), and that the degree of hyperplasia appears to be more prevalent in avian (2, 40) than rat models of compensatory muscle growth (35). The findings of early cyclin A protein increase and interstitial cell proliferation in loaded ALD muscle, therefore, may relate to the particularly high rate of new muscle fiber formation in the avian species with stretch-induced muscle growth.

The chickens used in this study were growing, and the muscles that reside in contralateral position to the ones being subjected to loading were shown to undergo a limited amount of muscular changes, including hyperplasia and hypertrophy in the rat (28). Accordingly, the rare detection of cyclin A in immunoblots of homogenates from 1.5- and 7-day contralateral control muscle, therefore, can be expected. The absence of cyclin A positive nuclei in immunofluorescence experiments of 7-day loaded ALD when proliferating satellite cells are detected in 7-day loaded quail ALD (43) can be explained by the absence of interstitial proliferation and a potentially lower sensitivity of the staining technique toward satellite cells that reside inside the basal lamina than those myogenic cells present outside muscle fibers.

Extracellular factors determine whether a cell will begin to enter the cell cycle, i.e., proliferate and synthesize DNA. Both growth factors and mechanical stimuli by a mechanism unrelated to autocrine growth factor action can induce DNA synthesis in fibroblasts (21). This implies that de novo induction of cyclin A protein in interstitial cells of loaded ALD muscle may involve the cooperative action of growth factors whose production and/or secretion is initiated soon after onset of loading, as well as a direct action of mechanical stress.

In summary, mechanical loading of chicken ALD muscle induces a transient increase in proliferation of interstitial and fiber-associated cells 1.5 days after load application through an increase in cyclin A and cdk2 activity. The activated cells may be cooperatively involved in cellular proliferation of interstitial cells and fiber hyperplasia occurring in this model of skeletal muscle growth.

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DISCLOSURES

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