Transient Induction of Cyclin A in Loaded Chicken Skeletal Muscle

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Running head: Cell cycle regulators in loaded muscle
ABSTRACT

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 is not well documented. The current 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 days 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 (cdk2) but not of cdk2 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 loaded ALD was primarily confined to nuclei of interstitial cells (92%) but 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 but also implicate an activation of interstitial cells as playing an early role in this model for muscle growth.

Keywords: nucleus, proliferation, fibroblast, hyperplasia, hypertrophy
INTRODUCTION

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 example the content of DNA increases more rapid than the protein content in loaded avian anterior latissimus dorsi muscle (ALD) (24). Furthermore introduction of DNA-breaks by irradiation prevents hypertrophy of overloaded rat soleus muscle (38), rat soleus muscle recovering from atrophy (32), 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;32;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 versus other (interstitial) cells 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 rise and fall in a regular pattern during the cell cycle enabling them to bind and activate at the
appropriate moment cyclin-dependent kinases (cdks) 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 then when cyclin A forms complexes with cyclin-dependent kinase 2 (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 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), make 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 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*—2-month-old white leghorn chicken (Texas A&M, College Station, U.S.A. or Wüthrich Farm, Belp, Switzerland) were subjected to the loading protocol as described previously (11). The left wing of chicken was loaded with a weight corresponding to 10% of 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 weight)], snap-frozen in liquid nitrogen, and stored in sealed tubes at -
80°C until use. After surgery, animals were sacrificed by cervical dislocation. All procedures have been carried out according to the newest guiding principles for animal research (1). The protocols were approved by the Institutional Animal Welfare Committee at the University of Texas-Health Science Center at Houston, USA 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, Switzerland) on ice in Mueller buffer (50 mM Hepes pH 7.4, 0.1% Triton X-100, 4 mM EGTA, 10 mM EDTA, 15 mM Na₄P₂O₇·10H₂O, 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 using a Lowry-based protein assay (DC protein assay, Bio-Rad), and aliquots corresponding to 50 μg 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), 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 determined (DC protein assay, Bio-Rad). 200 μg protein 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 upon its capability to recognize both active and non-active 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 stopped by addition of 5 μl of 5x Laemmli buffer (250 mM Tris-HCl pH 6.8, 5% SDS, 35% glycerol, 40 mM DTT, and 1% of bromophenol blue in ethanol) and boiling for 5 min. Terminated reactions were separated by 12% SDS-PAGE, western blotted on nitro-cellulose membranes (Schleicher and Schuell, Germany) and the radiophosphate incorporation was visualised by Audioradiography 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 nitro-cellulose membrane, Ponceau S stained to verify equal loading, and subjected to immunodetection analysis (see (11) for details). Membranes were blocked with phosphate-buffered saline (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 using antibody #C24230 from Transduction Laboratories (Beckton Dickinson GmbH, Germany) and peroxidase-conjugated goat anti-rabbit IgG (ICN Biomedicals GMBH, Germany) as first and secondary immunoreagents. The blots were developed 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 x 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 A. G., Urdorf, Switzerland). The pixel signals derived from films exposed for non-saturating 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) GmbH, 20253 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 analysed using a one-way ANOVA. Finally the signals were expressed as 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-Gläser, Germany), air dried and fixed in 4% paraformaldehyde. Cyclin A protein was detected essentially as described (13). Briefly, the slides were treated for 15 minutes in 0.1 % Triton X-100 in PBS, rinsed in PBS, fixed for 1 minute in -20°C acetone (analysis grade) and the sections rehydrated in PBS 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 hours at 37°C was carried out and nuclei
were counter stained 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.

Semiquantitative analysis of cyclin A positive cells—For 1.5 and 7 days loading treatment one section from the belly portion of control and loaded ALD from 2 individual animals each was stained. Fluorescent photomicrographs were taken from 3 randomly chosen and non-overlapping 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) was counted. An average of 200 nuclei was counted per field. Significance of association of cyclin A positive nuclei to interstitial tissue or the muscle fibers for the 1.5 days loading treatment, respectively, was verified in the following way. Data from 3 different fields each of the same muscles were pooled and mean percentages of 1) cyclin A positive nuclei derived from the 2 compartments (interstitial tissue or fiber-associated), and 2) cyclin A positive nuclei per compartment was calculated. Difference in the percentage of cyclin A positive nuclei between interstitial tissue and the fiber-associated compartment for the 1.5 day loading treatment was verified using a $\chi^2$-test for statistical significance (Statistica version 6.1 for Windows).

In situ hybridization—12 µm thick cryosections were prepared, stored over night at –70°C, and subjected to transcript detection essentially as described (11). To detect collagen type I mRNA, a 196 bp cDNA fragment comprising the first exon (coding for 5'-untranslated region and signal
peptide) of the chick collagen alpha 1(I) gene was used (7). Digoxygenin (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 Ltd., Rotkreuz, Switzerland).

After fixation (4% paraformaldehyde in PBS, 20 minutes), sections were washed in PBS, digested with 5 mg/ml proteinase K (Roche Diagnostics Ltd., Rotkreuz, Switzerland) in 40 mM EDTA, 0.1 M Tris-HCl, pH 8, for 20 minutes. Digestion was stopped with 2 mg/ml glycine in PBS for 1 minute, and slides were fixed again with 4% paraformaldehyde in PBS for 20 minutes. Sections were acetylated for 5 minutes in 0.25% acetic anhydride, 0.1 M triethanolamine, pH 8. The slides were prehybridized with 350 µl hybridization buffer (50% formamide, 4x SSC, 2x Denhardt’s, 2% dextran sulfate and 1 mg/ml yeast tRNA) in a moist chamber for 5 hours. Then 40 µl hybridization buffer containing heat-denatured (3 minutes at 80°C) DIG-labeled RNA probes (diluted 1:20) was applied per slide, covered with a coverslip, sealed and incubated for 16 hours at 60°C. Slides were washed in 0.2x SSC at 60°C for 30 minutes followed by digestion with RNase A (10 µg/ml) in 2x SSC for 5 minutes at 37°C. Slides were submerged in 2x SSC containing 50% formamide for 30 minutes and then equilibrated in 0.2x SSC at 20°C. For detection of DIG-labeled RNA, the DIG High prime labeling and detection Starter kit I (Roche Diagnostics Ltd., Rotkreuz, Switzerland) was used. After development, sections were washed in water and mounted in Kaiser’s glycerol gelatine (Merck AG, Switzerland). For publication purposes, fields were visualized on a slide film (Ektachrom 64T, Kodak) using a microscope-photograph system (VANOX-S, Olympus). The slides were scanned 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.
Semiquantitative analysis of collagen I expressing nuclei-The signals for collagen I mRNA were predominately confined to nuclei of connective tissue cells (see Figure 4). Transcript expression was therefore determined by counting the number of positive nuclei per fibers. Sections from 1.5 days loaded and corresponding contralateral control ALD muscle were analysed on the same slide. Non-overlapping, random fields from the sections were visualized with light microscopy at an objective magnification of x40. 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 days loaded and contralateral control ALD muscle from 3 individual animals was analyzed. For each field, the ratio of collagen I positive nuclei per muscle fibers was calculated and the mean for each muscle determined. Significance of differences in numbers of positive nuclei between control and loaded ALD was verified using a $\chi^2$-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 while it’s level is strongly induced in ALD muscle after 1.5 days of loading (Figure 1A). Cyclin A protein stayed elevated after 7 days of loading while it was reduced below detectable levels in 13 days loaded muscle (Figure 1C). The level of cyclin A protein was significantly reduced in 7 days versus 1.5 days loaded ALD muscle. The level of cyclin A protein in contralateral control muscles was not different between loading treatments.

*cdk2 activity is induced in loaded ALD muscle*—We analysed if 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 (Figure 2A). 13 days after loading, cdk2 activity in ALD homogenates appeared reduced (not shown). cdk2 protein was not changed relative to contralateral controls with loading of ALD muscle (Figure 2B).
Cyclin A increases in nuclei of the connective tissue—We carried out immunofluorescence experiments to identify the population of cells which express cyclin A protein upon 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 (Figure 3). 7 days after loading no nuclei were found to stain cyclin A positive (not shown). Semi-quantitative analysis identified that 10.8% of total nuclei in 1.5 days loaded ALD stained cyclin A positive. Cyclin A positive nuclei were primarily found in the interstitial tissue compartment while less than 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 hybridisation 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 hours (Figure 4).

DISCUSSION

An increase in the number of nuclei contributes to rapid growth of avian slow tonic skeletal muscle following 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 cyclin-dependent kinase 2, 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 seven 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 days 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) while 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 days loaded ALD is primarily confined to the connective tissue compartment (Figure 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 to a greater percentage to DNA synthesizing cells during the first 3 days of ALD loading than fiber-associated cells do (27). Moreover, the elevation of non-muscle tissue accounts for the increase in quail ALD mass after 2 days of loading in quail (3). Our data suggest, in contrast to the suggestion of Laurent et al. (24), that proliferating non-muscle cells
significantly contribute to increased DNA content in loaded chicken 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 (Figure
1) together with the similar percentage of proliferating interstitial nuclei between 3 and 7 days
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 days loaded ALD (Figure
4)(34). This early increase of collagen I mRNA after 36 hours of loading implies that
transcriptional mechanisms contribute to the 5-fold increase in the protein synthesis rate of this
extracellular matrix protein in 2 days loaded chicken ALD (23). This observation is reminiscent
to our recent observation that mRNA levels of tenascin-C, 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 tenascin-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 tenascin 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 fibroblast growth factor-2 (FGF-2) and –4 (FGF-
4), and causes accumulation of FGF-2 and –4 protein in the connective tissue (31). FGF-2 is
expressed in fibroblasts and FGFs stimulate proliferation of satellite cells (2;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 which 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 days loaded ALD in this study (Figure 3, Table 2) is similar to the reported percentage (0.3-1.7%) of satellite cells in quail ALD which enter the cell cycle and synthesize DNA in one 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 and which plateaus after 2 weeks of loading (3;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). 7 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 (Figure 1). 13 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 it is the case in mammalian models (27) and that the degree of hyperplasia appears to be more prevalent in avian (3;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 chicken used in this study were growing and muscles which 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 contralateral control muscle therefore can be expected. The absence of cyclin A-positive nuclei in immunofluorescence experiments of 7 days loaded ALD when proliferating satellite cells are detected in 7 days loaded quail ALD (43) can be explained by the absence of interstitial proliferation and a potentially lower sensitivity of the staining technique towards satellite cells which reside inside the basal lamina than those myogenic cells being present outside muscle fibers.

Extracellular factors determine whether a cell will begin to enter the cell cycle, i.e. proliferate and synthesise 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 which 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 anterior latissimus dorsi muscle induces a
 transient increase of proliferation of interstitial and fiber-associated cells between 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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References


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**FIGURE LEGENDS**

**Figure 1: Cyclin A protein in loaded muscle.** Equal amounts of protein in total homogenate were analyzed by immunoblotting for cyclin A protein as described in the methods. A) Representative result of immunodetected cyclin A protein (arrow) in homogenate from 1.5 days loaded and contralateral control ALD. B) Loading control showing Ponceau S stained membrane before probing with antibody. C) Mean and standard error of pixel signal of detected cyclin A protein (per total protein) in contralateral control (white bars) and loaded ALD (black bars). Asterisk indicates significant effect at p<0.05 (and 0.0001) of loading relative to the respective contralateral control ALD on cyclin A level. The cross indicates a significant difference between 1.5 and 7 days loaded ALD (P<0.001, n=4).

**Figure 2: cdk2 protein and activity in loaded muscle.** cdk2 was immunoprecipitated from equal amounts of protein in total homogenate and analysed for kinase activity against Histone 1 (designated as cdk2 activity) and amount of cdk2 by immunoblotting as described in the methods. A. Audioradiogram displaying a representative result from 3 individual experiments of cdk2 activity in homogenates of 1.5 and 7-days loaded and contralateral control ALD. B. Film displaying immunodetected cdk2 protein in cdk2 immunoprecipitates from homogenates of 1.5 and 7-days loaded and contralateral control ALD. Note the strong induction of cdk2 activity in 1.5 and 7 days loaded ALD.

**Figure 3: Cyclin A localization in loaded ALD muscle.** Cryosections from contralateral control and 1.5 days loaded ALD was analyzed for cyclin A protein as described in the methods and DNA stained with Hoechst. Top. Immunfluorescence stain for cyclin A protein (whitish green); Middle, Cyclin A staining and Hoechst-stain; Bottom, Hoechst-stained nuclei (blue) and phase-
contrast visualizing cellular structures. The presented images show representative results of cyclin A staining in different fields from the belly portion of the muscle. Bar indicates 50 µm. Note the absence of cyclin A positive nuclei in control ALD and the induction of cyclin A protein in nuclei of interstitial cells (arrowheads) and fiber-associated cells (white arrows).

**Figure 4: Collagen I transcripts in loaded ALD muscle.** A) Example of in situ hybridization experiments for collagen I transcript expression in 1.5 days loaded and contralateral control ALD. Arrows and arrowheads indicate collagen I mRNA positive interstitial nuclei and endoneurium of nerves, respectively. Bar, 100 µm. B) Mean and standard error of the number of collagen I expressing nuclei in the interstitium of contralateral control (white bar) and 1.5 days loaded ALD (black bar) as determined from in situ hybridization experiments on sections from 3 animals (for details see materials and methods). Asterisk indicates significant effect at p<0.005 (χ²-test).
**A**

1.5 days

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**B**

1.5 days

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**C**

![Graph showing Cyclin A pixel levels at 1.5 days, 7 days, and 13 days with asterisk and dagger symbols.](http://jap.physiology.org/)
A

1.5 days  
control  loaded  

7 days  
control  loaded  

Histone 1

B

1.5 days  
control  loaded  

7 days  
control  loaded  

CDK2
1.5 days control

Cyclin A

1.5 days loaded

Cyclin A & Hoechst

Phase-contrast & Hoechst
A

1.5 days control

1.5 days loaded

Collagen I mRNA

B

Endomysial collagen I mRNA

[particle/fiber]

control

1.5 days loaded

*
### Table 1: Protein content and wet weight of loaded and contralateral control ALD muscle

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</tbody>
</table>

| 7 days    | 275 ± 13           | 34.0 ± 4.2         | +106%      |          |         |        |            |
| loaded    | 600 ± 45 *         | 99.2 ± 11.1 *      | +191%      |          |         |        |            |

| 13 days   | 286 ± 5            | 35.6 ± 6.3         | +120%      |          |         |        |            |
| loaded    | 629 ± 12 *         | 140.1± 25.1 *      | +294%      |          |         |        |            |

Data are Means ± SE of muscle wet weight and total protein content in contralateral control and loaded ALD muscle. An asterisk indicates significant difference between values of control and loaded ALD (p < 0.05, n=3-6).
Table 2: Distribution of Cyclin A positive nuclei in 1.5 days loaded muscle

<table>
<thead>
<tr>
<th>Cyclin A positive nuclei</th>
<th>fiber-associated</th>
<th>interstitial tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>per Cyclin A positive nuclei</td>
<td>7.6%</td>
<td>92.4% *</td>
</tr>
<tr>
<td>per compartment</td>
<td>1.8%</td>
<td>18.9% *</td>
</tr>
</tbody>
</table>

Values are Means and SE of cyclin A positive nuclei in 1.5 days loaded ALD muscle. An asterisk indicates significant difference between the values in interstitial tissue and those associated with fibers ($\chi^2$-test, p < 0.0001).