Plasticity in Skeletal, Cardiac, and Smooth Muscle

Selected Contribution: Skeletal muscle focal adhesion kinase, paxillin, and serum response factor are loading dependent

SCOTT E. GORDON,1,2 MARTIN FLÜCK,1 AND FRANK W. BOOTH1,2
1Department of Integrative Biology and Pharmacology, University of Texas-Houston Health Science Center, Houston, Texas 77030; and 2Department of Veterinary Biomedical Sciences, University of Missouri, Columbia, Missouri 65211

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Gordon, Scott E., Martin Flück, and Frank W. Booth. Selected Contribution: Skeletal muscle focal adhesion kinase, paxillin, and serum response factor are loading dependent. J Appl Physiol 90: 1174–1183, 2001.—This investigation examined the effect of mechanical loading state on focal adhesion kinase (FAK), paxillin, and serum response factor (SRF) in rat skeletal muscle. We found that FAK concentration and tyrosine phosphorylation, paxillin concentration, and SRF concentration are all lower in the lesser load-bearing fast-twitch plantaris and gastrocnemius muscles compared with the greater load-bearing slow-twitch soleus muscle. Of these three muscles, 7 days of mechanical unloading via tail suspension elicited a decrease in FAK tyrosine phosphorylation only in the soleus muscle and decreases in FAK and paxillin concentrations only in the plantaris and gastrocnemius muscles. Unloading decreased SRF concentration in all three muscles. Mechanical overloading (via bilateral gastrocnemius ablation) for 1 or 8 days increased FAK and paxillin concentrations in the soleus and plantaris muscles. Additionally, whereas FAK tyrosine phosphorylation and SRF concentration were increased by ≤1 day of overloading in the soleus muscle, these increases did not occur until somewhere between 1 and 8 days of overloading in the plantaris muscle. These data indicate that, in the skeletal muscles of rats, the focal adhesion complex proteins FAK and paxillin and the transcription factor SRF are generally modulated in association with the mechanical loading state of the muscle. However, the somewhat different patterns of adaptation of these proteins to altered loading in slow- vs. fast-twitch skeletal muscles indicate that the mechanisms and time course of adaptation may partly depend on the prior loading state of the muscle.

compensatory hypertrophy; atrophy; mechanical stress; fiber type; muscle growth

THE MECHANISM(S) BY WHICH skeletal muscles sense mechanical loading of the extracellular matrix (ECM) and transduce this information to the intracellular compartment to signal adaptations in nuclear gene transcription are unclear. This phenomenon of “mechanotransduction” may be partly mediated via focal adhesion complexes (FACs), which are sites of force transmission across the cell membrane consisting of complicated assemblies of structural and signaling proteins related to the control of cell growth (9, 30, 31, 32). FACs are thought to be important for the maintenance of cell “tensegrity,” a model in which FACs serve as anchor points for cytoskeletal proteins to interconnect with the ECM (26, 27). Ingber (27) recently explained that “tensegrity structures maintain shape stability within a tensed network of structural members by incorporating other support elements that resist compression.” However, this model remains controversial for some (27).

We believe that if the tensegrity model is correct, then the density of support elements such as FACs would vary with differences in cell “compression” (i.e., loading state) in skeletal muscle. Therefore, we hypothesized that the skeletal muscle concentrations of two representative FAC proteins, focal adhesion kinase (FAK) and paxillin, would vary in association with the mechanical loading state of the muscle. Specifically, we hypothesized that FAK and paxillin concentrations would be lower in the plantaris or gastrocnemius muscles compared with the soleus muscle under conditions of normal muscle loading in rats. This hypothesis was based on the fact that the rat soleus muscle is primarily composed of slow-twitch fibers (5), which are typically much higher in their load bearing...
and/or recruitment levels than fast-twitch fibers during daily cage activities (22). We further hypothesized that soleus unloading would elicit a downregulation of FAK and paxillin concentrations to levels approaching those of the plantaris and gastrocnemius, whereas plantaris overloading (by gastrocnemius ablation) would elicit an upregulation of these proteins to concentrations approaching those of the soleus. Another reason for our choice of the fast-twitch rat plantaris muscle for overloading was to extend our laboratory’s previous observations in the rooster anterior latissimus dorsi (ALD) and rat soleus muscles, both slow-twitch (5, 28), that FAK and paxillin concentrations are increased during overload-induced hypertrophy (18). In that previous study, overloading also increased FAK autokinase activity (per unit of FAK mass) in the rooster ALD, whereas FAK autokinase activity was not reported for the rat soleus (18). Therefore, we chose to examine FAK tyrosine phosphorylation (as a measure of FAK activity) in the present investigation, hypothesizing that FAK tyrosine phosphorylation would be modulated in association with loading status in the soleus, plantaris, and gastrocnemius muscles. This hypothesis is supported by the fact that FAK tyrosine phosphorylation is induced by pressure overload in the heart (30) and mechanical strain in cultured smooth muscle cells and other cell types (9, 38, 39).

Because it is well established that unloading elicits skeletal muscle atrophy while overloading elicits skeletal muscle hypertrophy, we were curious as to whether FAK responses to altered muscle loading would also be associated with changes in the concentration of the transcription factor serum response factor (SRF). The binding of SRF to the serum response element 1 (SRE1) region of the skeletal α-actin promoter has been previously shown to be necessary and sufficient for skeletal α-actin gene expression (a marker of protein synthesis and cell hypertrophy) in skeletal muscle cells in culture as well as the whole animal (10, 13, 15, 41). The rationale for the potential relationship between FAK and SRF during altered muscle loading is that SRF exhibits increased mRNA abundance (13), increased protein concentration (19), and altered SRF-SRE1 binding on the skeletal α-actin promoter in the overloaded hypertrophying rooster ALD (11, 13). Moreover, according to a recent hypothesis, FAK signaling may lie upstream of SRF-mediated skeletal α-actin expression during overload-induced skeletal muscle hypertrophy (14). This hypothesis is based on findings that SRF-mediated skeletal α-actin promoter activity is dependent on the activation of RhoA and potentiated by the coactivation of β1D-integrin (both located in the FAC) in cultured C2C12 mouse myoblasts (41). Furthermore, this skeletal α-actin promoter activation by β1D-integrin-RhoA appears to be dependent on FAK, because cotransfection with FRNK (a dominant negative inactive FAK mutant) attenuated most of the promoter activity (42). Thus, as with FAK, we hypothesized that SRF concentration would be lower in the plantaris and gastrocnemius muscles compared with the soleus muscle and that unloading of the soleus, plantaris, and gastrocnemius muscles would elicit a downregulation of their SRF concentrations, whereas overloading of the soleus and plantaris muscles would elicit an upregulation of their SRF concentrations.

**METHODS**

**Animals.** All animals were individually housed, kept on a 12:12-h light-dark cycle, and given ad libitum access to water and rodent chow. Female (~180 g) and male (~290 g) Sprague-Dawley rats (Harlan) of similar ages were used for the skeletal muscle unloading and overloading experiments, respectively. Female rats were chosen for the muscle unloading experiment because tail suspension has been shown to reduce testosterone output in male rats due to the falling of the testicles into the abdominal cavity (16). Male rats were chosen for muscle overloading in an attempt to reproduce and extend our FAK, paxillin, and SRF findings in male chickens (18, 19). All procedures were approved by the University of Texas-Houston Health Science Center Animal Care and Use Committee.

**Skeletal muscle unloading.** Unloading of the soleus, plantaris, and gastrocnemius muscles was performed using a modification of the hindlimb-unloading protocol used by Babij and Booth (6). Eight hindlimb-unloaded animals and eight weight-bearing control animals were used for this experiment. Rats were lightly anesthetized with an intraperitoneal injection of ketamine (~50 mg/kg body wt) for tail wrap. After the rat recovered from the anesthetic, a swivel hook was placed through the bandage just distal to the tip of the tail. The swivel hook was then raised such that the hindlimbs were elevated just off the cage floor (this produces ~30° head-down tilt). Forelimbs remained in contact with the cage floor, allowing the rat to move through a 360° circle around the tail suspension apparatus. The unloading period lasted 7 days. Rats had ad libitum access to chow and water throughout the hindlimb-unloading protocol.

**Skeletal muscle overloading.** Functional overload of the plantaris and soleus muscles was induced via bilateral surgical ablation of the synergistic gastrocnemius muscle using the methods of Baldwin et al. (7) as previously modified by our laboratory (18). With the exception of gastrocnemius excision, sham surgeries consisted of the exact same procedure as the ablation surgeries. The overloading period lasted for either 1 or 8 days starting at the time when the rats were able to move independently about their cage after recovery from the anesthesia used for the ablation or sham surgery. There were seven animals per group at each time point.

**Muscle preservation and determination of muscle masses.** At the end of the unloading and overloading periods, rats were anesthetized with an intraperitoneal injection of a cocktail containing ketamine, xylazine, and acepromazine (75, 3, and 5 mg/kg body wt, respectively). The soleus, plantaris, and gastrocnemius muscles were then excised from all animals (gastrocnemius samples were obtained in the unloading experiment only). Hindlimb-unloaded animals were not allowed to bear weight on the unloaded muscles before excision. Muscle samples were immediately trimmed of excess fat and connective tissue, weighed on an analytic balance, and frozen in liquid nitrogen. All tissue was stored at −80°C until further analysis. Rats were killed by cervical dislocation while still anesthetized.

**Total protein isolation and determination.** Whole muscles were homogenized in a buffer containing 50 mM HEPES (pH 7.4), 0.1% Triton X-100, 10 mM EDTA, 4 mM EGTA, 15 mM \(\text{Na}_2\text{PO}_4\cdot10\text{H}_2\text{O}\), 100 mM β-glycerophosphate, 25 mM NaF,
50 µg/ml leupeptin, 50 µg/ml pepstatin, and 33 µg/ml apro tinin (700 mg tissue/5.0 ml buffer). Homogenization was accomplished on ice with a Polytron mixer (Kinematica, Littau/Luzern, Switzerland) using three 15-s pulses at a low setting, with aliquots subsequently being frozen in liquid nitrogen and stored at −80°C. Protein concentrations of the sample homogenates were determined in triplicate using BSA as a standard by a modified Lowry procedure (DC Protein Assay, Bio-Rad, Hercules, CA) and then used to calculate total protein per whole muscle.

**SDS-PAGE, Western blotting, and immunodetection.** Protein homogenate samples were solubilized in sample loading buffer (50 mM Tris-HCl, pH 6.8, 1.0% glycerol, 2% SDS, 2% β-mercaptoethanol, and 0.1% bromophenol blue) at a concentration of 1 mg/ml and boiled for 5 min. Proteins were then separated by 8% SDS-PAGE and Western blotted overnight at 4°C onto a nitrocellulose membrane at 33 V in transfer buffer (25 mM Tris-base, pH ~8.3, 192 mM glycine, and 20% methanol). Membranes were stained by Ponceau S to verify equal loading among lanes, after which immunodetection was accomplished at room temperature. For immunodetection, membranes were blocked for 1 h in blocking buffer (2.5% nonfat dry milk and 1% BSA in TBS-T (20 mM Tris-base, 150 mM NaCl, 0.05% Tween-20, pH 7.6), incubated with primary antibody in blocking buffer for 2 h, serially washed in TBS-T, incubated with horseradish peroxidase (HRP)-conjugated secondary antibody in blocking buffer for 1 h, and again serially washed in TBS-T. The HRP activity was detected using enhanced chemiluminescence reagent (ECL, NEN Life Science Products, Boston, MA) and exposure to Kodak-XAR5 autoradiographic film. Exposure time was adjusted to keep the integrated optical densities (IODs) within a linear and nonsaturated range for all bands of each gel. The IODs were then quantified using densitometry software (NIH Image), and the concentration of the antigen present in each muscle was calculated as the IOD normalized to units of total muscle protein initially loaded on the gel. All experimental groups were equally represented on every gel; however, a quality control sample homogenate sample was also run on each gel to correct sample IODs for exposure time differences between films. For samples requiring it, the total mass of each antigen per whole muscle was estimated by multiplying the normalized IOD values by the total protein content of the respective muscle. The rabbit anti-FAK antisera (used at a dilution of 1:2,000) was a generous gift of Dr. Andrew Ziemiecki (University of Bern, Bern, Switzerland) and has been previously characterized (18). The mouse anti-paxillin antibody was obtained from Transduction Laboratories (Lexington, KY) and used at a 1:10,000 dilution. The rabbit anti-SRF antibody (1:300 dilution) was generated and affinity purified as previously described (19). The HRP-conjugated anti-antibody and anti-mouse secondary antibodies (Amersham Pharmacia Biotech, Piscataway, NJ) were used at dilutions of 1:7,500 and 1:5,000, respectively.

**FAK immunoprecipitation and phosphotyrosine detection.** Muscle homogenate aliquots containing 1.5 mg of total protein were diluted to a volume of 750 µl in RIPA buffer [10 mM Tris-HCl (pH 7.4), 1.0% Triton X-100, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1.5 mM MgCl2, 2.5 µg/ml leupeptin, 2.5 µg/ml aprotinin, 0.2 mM phenylmethylsulfonyl fluoride, and 0.2 mM activated Na3VO4] and rocked for 45 min at 4°C. Tubes were then centrifuged at full speed in a microcentrifuge at 4°C for 20 min, after which the supernatant was removed, combined with 10 µl FAK antisera (described above), and rocked for 2.5 h at 4°C. Precipitation of antigen-antibody complexes was then accomplished at 4°C by adding 25 µl of protein G-Sepharose (Amersham Pharmacia Biotech; suspended in RIPA buffer), rocking for 1 h, and centrifuging at full speed in a microcentrifuge four times for 5 min each. The supernatant was removed, and the Sepharose pellet was resuspended in 1 ml RIPA buffer after each centrifugation except for the final one, after which the pellet was resuspended in 50 µl of 2X sample loading buffer (described above). Samples were then boiled for 5 min and centrifuged briefly, and the supernatant was loaded and run on a 6% SDS-PAGE gel for protein separation. Samples were then Western blotted at 45 V for 6 h onto a polyvinylidene fluoride membrane at 4°C in transfer buffer (5% BSA (Cohn Fraction V, Intergen, Purchase, NY) and 0.1 mM activated Na3VO4 in PBS-T (PBS with 0.05% Tween 20, pH 7.5), incubating for 1.5 h with mouse anti-phosphotyrosine antibody (clone 4G10 (Upstate Biotechnology, Lake Placid, NY), 1.0 µg/ml in PBS-T with 1% BSA and 0.1 mM activated Na3VO4, pH 7.5), serially washing in PBS-T with 0.1 mM activated Na3VO4 (pH 7.5), incubating for 1 h with HRP-conjugated anti-mouse antibody (Amersham; 1:10,000 in PBS-T with 1% BSA and 0.1 mM activated Na3VO4, pH 7.5), and then serially washing in PBS-T with 0.1 mM activated Na3VO4 (pH 7.5). The amount of phosphotyrosine was determined using ECL reagent, autoradiographic film, and the IOD readings as described above. Antibodies bound to the membrane were stripped at room temperature overnight by incubating in a solution containing 3 M NaSCN, 0.1 M boric acid, and 10 mM dithiothreitol (pH 8.0), after which the membranes were washed for several hours in distilled demineralized H2O. After the absence of HRP activity on the membranes was verified by ECL, the membranes were washed in TBS-T and subjected to FAK immunodetection as described above. The phosphotyrosine IOD was then normalized to FAK immunoprecipitate IOD to assess FAK tyrosine phosphorylation per unit FAK mass in the samples.

**Statistical analyses.** Multivariate analyses of variance with Fisher’s least significant difference post hoc tests were used in this investigation (Statistica, StatSoft, Tulsa, OK). Statistical significance was chosen as P ≤ 0.05.

**RESULTS**

**Skeletal muscle wet weights and protein contents per whole muscle.** As indicated both by wet weight and total protein content per whole muscle, significant atrophy was induced by 7 days of unloading in the soleus, plantar, and gastrocnemius muscles (Table 1). With 1 and 8 days of unloading, a significant increase in wet weight was observed in both the soleus and plantaris muscles (Table 2). However, this was reflected by a significant increase in total protein content only at 8 days of overloading in both muscles.

**Muscle-specific differences and unloading-induced changes in skeletal muscle FAK, paxillin, and SRF.** FAK concentration (Fig. 1), FAK tyrosine phosphorylation (Fig. 2), paxillin concentration (Fig. 3), and SRF concentration (Fig. 4) were all significantly lower in the control plantaris or gastrocnemius muscles than in the control soleus muscle (under normally loaded conditions). This confirmed our hypothesis that these proteins would be maintained at a lower level in fast-twitch vs. slow-twitch skeletal muscle because of the lesser loading of fast-twitch muscle fibers during daily...
cage activities (22). With 7 days of unloading, the FAK and paxillin concentrations and masses per whole muscle decreased in the plantaris and gastrocnemius muscles (Figs. 1 and 3). In contrast, unloading of the soleus resulted in increases in the FAK and paxillin concentrations and no change in their masses per whole soleus muscle. Whereas FAK tyrosine phosphorylation was dramatically decreased by unloading in the soleus, it was unaffected by unloading in the plantaris and gastrocnemius muscles (Fig. 2). Last, unloading resulted in a significant decrease in SRF concentration and mass per whole muscle in all three muscles (Fig. 4).

Overloading-induced changes in skeletal muscle FAK, paxillin, and SRF. FAK concentration (Fig. 5), FAK tyrosine phosphorylation (Fig. 6), paxillin concentration (Fig. 7), and SRF concentration (Fig. 8) were all significantly elevated by overloading in the soleus and plantaris muscles, but they differed in the time courses of their responses. Increases in FAK and paxillin concentrations were observed in the soleus and plantaris muscles after both 1 and 8 days of overloading, with FAK concentration in the soleus being greater at day 8 than at day 1. Furthermore, soleus paxillin concentration in sham-operated animals was lower at 8 days than at 1 day postsurgery. Note that the soleus FAK and paxillin concentration results presented here have been previously published (18), but they are included in the figures to enable comparison against the previously unpublished plantaris results as well as to complement the previously unpublished FAK tyrosine phosphorylation and SRF results for both muscles (all data are from the same groups of animals). One day of soleus overloading elicited an increase in FAK tyrosine phosphorylation, which was less elevated by day 8 of overloading. In the plantaris, FAK tyrosine phosphorylation was not increased by overloading until day 8.

Table 1. Muscle wet weights and protein contents per whole muscle after the 7-day unloading protocol

<table>
<thead>
<tr>
<th></th>
<th>Soleus</th>
<th>Plantaris</th>
<th>Gastrocnemius</th>
</tr>
</thead>
<tbody>
<tr>
<td>Muscle wet weight, mg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>78.7 ± 3.1</td>
<td>226.2 ± 5.7</td>
<td>1148.4 ± 18.1</td>
</tr>
<tr>
<td>Unloaded</td>
<td>48.7 ± 1.9</td>
<td>160.2 ± 3.9</td>
<td>810.1 ± 20.6</td>
</tr>
<tr>
<td>Percent difference</td>
<td>-38.1*</td>
<td>-29.2*</td>
<td>-29.5*</td>
</tr>
<tr>
<td>Muscle protein content, mg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>12.1 ± 1.1</td>
<td>41.2 ± 2.3</td>
<td>232.9 ± 10.6</td>
</tr>
<tr>
<td>Unloaded</td>
<td>6.8 ± 0.5</td>
<td>27.3 ± 1.1</td>
<td>158.6 ± 4.5</td>
</tr>
<tr>
<td>Percent difference</td>
<td>-43.8*</td>
<td>-33.7*</td>
<td>-31.9*</td>
</tr>
</tbody>
</table>

Values are means ± SE for 8 rats/group. *Significant difference between control and unloaded groups for specified muscle, P ≤ 0.05.

Table 2. Muscle wet weights and protein contents per whole muscle after the 1- and 8-day overloading protocols

<table>
<thead>
<tr>
<th></th>
<th>Soleus</th>
<th>Plantaris</th>
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<tbody>
<tr>
<td>Muscle wet weight, mg</td>
<td></td>
<td></td>
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<tr>
<td>Sham operated</td>
<td>126.3 ± 1.7</td>
<td>120.6 ± 4.8</td>
</tr>
<tr>
<td>Overloaded</td>
<td>157.0 ± 6.2</td>
<td>159.0 ± 5.4</td>
</tr>
<tr>
<td>Percent difference</td>
<td>24.3*</td>
<td>31.8*</td>
</tr>
<tr>
<td>Muscle protein content, mg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sham operated</td>
<td>17.3 ± 0.8</td>
<td>14.3 ± 1.2</td>
</tr>
<tr>
<td>Overloaded</td>
<td>19.8 ± 1.9</td>
<td>19.0 ± 1.6</td>
</tr>
<tr>
<td>Percent difference</td>
<td>14.5</td>
<td>32.9*</td>
</tr>
</tbody>
</table>

Values are means ± SE for 8 rats/group. *Significant difference between control and overloaded groups for specified muscle at specified time point, P ≤ 0.05.
Furthermore, FAK tyrosine phosphorylation in plan-
tar muscle of the sham-operated animals was higher
at 1 day than at 8 days postsurgery. Last, SRF concen-
tration was increased by overloading at both 1 and 8
days in the soleus, but only at 8 days in the plantaris.
In both muscles, SRF concentration was higher at
day 8 than at day 1 of overloading.

**DISCUSSION**

Because of the potential importance in FAC proteins
and SRF in mechanical signaling and maintenance of
skeletal muscle mass (9, 10, 13, 15, 41), we hypothe-
sized that these proteins would vary in skeletal muscle
in association with the mechanical loading state of the
muscle. In accordance with this hypothesis, we found
that FAK concentration and tyrosine phosphorylation,
paxillin concentration, and SRF concentration are all
lower in the lesser load-bearing plantaris and gastro-
cnemius muscles compared with the greater load-bear-
ing soleus muscles. Seven days of mechanical unloading
elicited the hypothesized decrease in SRF concentra-
tion in all three muscles, but FAK and paxillin concen-
trations were only decreased in the plantaris and gas-
tocnemius muscles. Remarkably, unloading decreased
FAK tyrosine phosphorylation only in the soleus muscle.
With mechanical overloading of the rat plantaris,
we were able to extend to fast-twitch muscle our labo-
rary’s previous findings in slow-twitch muscle of sim-
ilar rapid (≤1 day) increases in FAK and paxillin con-
centrations (18). Our laboratory’s previous over-
loading studies (18, 19) did not examine FAK tyrosine
phosphorylation or SRF concentration in the rat soleus
or plantaris muscles. In the present investigation, FAK
tyrosine phosphorylation and SRF concentration both
exhibited a rapid (≤1 day) increase with overloading in
the soleus muscle; however, these two responses were
delayed in the plantaris muscle, occurring somewhere
between 1 and 8 days of overloading. Thus the time
course and mechanisms by which FAC proteins and
SRF adapt to altered loading in skeletal muscle may
depend on the prior loading state of the muscle, as
observed with the differences between slow-twitch and
fast-twitch muscles.

This investigation lends more support to the hypoth-
esis that skeletal muscle FAK and paxillin concen-
trations are associated with, and modulated by, the load-
ing status of the muscle. The lower expression of these
two proteins in the lesser load-bearing plantaris and

![Figure 2](http://jap.physiology.org/)

**Fig. 2.** Top: muscle-specific differences and 7-day unloading-induced changes in skeletal muscle FAK tyrosine phosphorylation. Values are means ± SE for 8 rats/group. Individual values were calculated as the ratios of phosphotyrosine IOD values to FAK IOD values obtained after Western blot analysis of FAK immunoprecipitates (IPs). †Significantly different from the soleus muscle (differences reported only for concentrations in the control condition), $P ≤ 0.05$. *Significantly different from control condition for specified muscle, $P ≤ 0.05$. Representative phosphotyrosine and FAK immunoblots (middle and bottom, respectively) from C and UL muscles are also presented. FAK immunoreactivity was detected on the same mem-
brane as phosphotyrosine immunoreactivity after the membrane was stripped of anti-phosphotyrosine antibody complexes. Phospho-
tyrosine and FAK immunoreactivity bands both appeared at −120–
125 kDa.

![Figure 3](http://jap.physiology.org/)

**Fig. 3.** Top: muscle-specific differences and 7-day unloading-induced changes in skeletal muscle paxillin concentration and mass per
whole muscle (inset at top). Values are means ± SE of the IOD values obtained after Western blot analysis for 8 rats/group. Mass per whole
muscle was calculated by multiplying individual paxillin concentra-
tion IOD values by the total protein content of each respective
muscle. †Significantly different from the soleus muscle (differences reported only for concentrations in the control condition), $P ≤ 0.05$. *Significantly different from control condition for specified muscle, $P ≤ 0.05$. Bottom: representative paxillin immunoblot from C and UL
muscles. Paxillin appeared as diffuse doublet bands of immunoreac-
tivity in the range of 65–70 kDa.
gastrocnemius muscles vs. the greater load-bearing soleus muscle is similar in pattern to a vast number of other proteins that have been shown to be of lower abundance in fast-twitch than in slow-twitch skeletal muscle fiber types. This includes the myosin heavy chain (MHC) type I isoform (5), which is downregulated in slow-twitch muscle by decreased loading (40). Additionally, muscle LIM protein, a reported regulator of myogenic differentiation (3) possibly associated with FACs (8), has lower expression in fast-twitch than in slow-twitch skeletal muscles (37), as do FAK and paxillin. Not surprisingly, both MHC I (36, 40) and muscle LIM protein (37) are upregulated in fast-twitch muscle by increased loading and/or contractile activity, similar to the upregulation in FAK and paxillin concentrations induced by skeletal muscle overloading. Furthermore, we show in the present investigation that FAK and paxillin concentrations are elevated within 24 h of overload in the soleus and plantaris muscles, even earlier than any reported substantial change in MHC isoform phenotype with increased loading (40). Still, not all of the differences in FAK and paxillin concentrations can be explained purely by muscle loading state, because FAK and paxillin concentrations were inexplicably increased (and their masses per whole muscle unchanged) in the soleus with 7 days of unloading.

When compared with the unloading-induced decreases in FAK and paxillin concentrations in the plantaris and gastrocnemius muscles, the increased concentrations of these two proteins in the unloaded soleus muscle appear paradoxical. However, different rates of FAK and paxillin degradation may exist between muscles of varying fiber types and/or loading patterns (i.e., the soleus as opposed to the plantaris or gastrocnemius). Moreover, the FAK and paxillin masses per whole muscle were not affected by unloading in the soleus, indicating that the increases in their concentrations per unit total protein were a result of the total muscle protein loss and not a net synthesis of FAK or paxillin. It must be noted that the increased FAK concentration with soleus unloading is not necessarily indicative of an increased FAK activity, because overall FAK activity is also determined by FAK tyrosine phosphorylation per unit of FAK protein (9). In this respect, FAK tyrosine phosphorylation was much lower in the control plantaris and gastrocnemius muscles than in the control soleus muscle, and it fell dramatically in the unloaded soleus to a level more resembling those of control plantaris and gastrocnemius muscles. These findings confirmed our hypotheses,

![Fig. 4](image)

**Fig. 4.** Top: muscle-specific differences and 7-day unloading-induced changes in skeletal muscle serum response factor (SRF) concentration and mass per whole muscle (inset at top). Values are means ± SEs of the IOD values obtained after Western blot analysis for 8 rats/group. Mass per whole muscle was calculated by multiplying individual SRF concentration IOD values by the total protein content of each respective muscle. †Significantly different from the soleus muscle (differences reported only for concentrations in the control condition), P ≤ 0.05. ‡Significantly different from control condition for specified muscle, P ≤ 0.05. Bottom: representative SRF immunoblot from C and UL muscles. SRF appeared as doublet bands of immunoreactivity in the range of 55–58 kDa.

![Fig. 5](image)

**Fig. 5.** Top: increases in skeletal muscle FAK concentration with 1 and 8 days of overloading. Soleus and plantaris muscle overloading was induced via the bilateral surgical ablation of the gastrocnemius muscle. Values are means ± SE of the IOD values obtained after Western blot analysis for 7 rats/group. *Significantly different from sham-operated condition for specified muscle and time point, P ≤ 0.05. †Significantly different from 1-day time point for specified muscle and loading condition, P ≤ 0.05. Bottom: representative FAK immunoblot from sham-operated (S) and overloaded (OL) muscles. FAK appeared as doublet bands of immunoreactivity in the range of 120–125 kDa. The soleus data have previously appeared elsewhere (18) and are presented here for comparison.
which were based on the fact that phosphorylation of FAK on tyrosine residues is evoked by mechanical strain in the heart, cultured smooth muscle cells, and other cell types (9, 30, 38, 39). Interestingly, FAK tyrosine phosphorylation remained unchanged in the plantaris and gastrocnemius muscles with unloading. Although this observation contradicts our hypothesis, we speculate that the plantaris and gastrocnemius muscles are already at basal levels of FAK tyrosine phosphorylation because of the minimal loading of fast-twitch fibers in the rat (22). Thus it appears that FAK is downregulated by unloading in the slow-twitch soleus muscle via a reduction in tyrosine phosphorylation because of the minimal loading of fast-twitch fibers in the rat (22). It thus appears that FAK is downregulated by unloading in slow-twitch soleus muscle via a reduction in tyrosine phosphorylation, whereas it is downregulated by unloading in the fast-twitch plantaris and gastrocnemius muscles via a reduction in its protein concentration.

Although unloading decreased FAK tyrosine phosphorylation in only the slow-twitch soleus muscle, overloading increased FAK tyrosine phosphorylation in both the soleus and the fast-twitch plantaris muscle. Whereas our laboratory has previously shown an increase in FAK activity [reflective of tyrosine phosphorylation (9)] in the slow-twitch rooster ALD muscle with overloading (18), the effect of overloading on FAK tyrosine phosphorylation in fast-twitch skeletal muscle has not been previously explored. Interestingly, FAK tyrosine phosphorylation was not increased by overloading until day 8 in the plantaris; however, this may have been partly due to the fact that FAK tyrosine phosphorylation in plantaris muscles of the sham-operated animals was higher at 1 day than at 8 days postsurgery. Nevertheless, it is possible that fast-twitch skeletal muscle is simply slower to respond to altered loading because of less organized FAs (and therefore less FAK autophosphorylation) (9) or because of the higher fatigability of fast-twitch fibers (and therefore less initial ability to maintain constant recruitment and/or loading) (35). The variable levels of FAK tyrosine phosphorylation and paxillin concentration in the 1- vs. 8-day sham-operated animals lead us to believe that the surgical procedure itself may have affected these measures in these animals, possibly because of temporarily altered cage activity or muscle recruitment and/or loading patterns during the days postsurgery. These observations led us to choose the control animals in the unloading experiment as a more valid comparison between muscles of different fiber types.

**Fig. 6.** Top: increases in skeletal muscle FAK tyrosine phosphorylation with 1 and 8 days of overloading. Soleus and plantaris muscle overloading was induced via the bilateral surgical ablation of the gastrocnemius muscle. Values are means ± SE for 7 rats/group. Individual values were calculated as the ratios of phosphotyrosine IOD values to FAK IOD values obtained after Western blot analysis of FAK IPs. *Significantly different from sham-operated condition for specified muscle and time point, *P* ≤ 0.05. †Significantly different from 1-day time point for specified muscle and loading condition, †*P* ≤ 0.05. Representative phosphotyrosine and FAK immunoblots (middle and bottom, respectively) from S and OL muscles are also presented. FAK immunoreactivity was detected on the same membrane as phosphotyrosine immunoreactivity after the membrane was stripped of anti-phosphotyrosine antibody complexes. Phosphotyrosine and FAK immunoreactivity bands both appeared at ~120–125 kDa.

**Fig. 7.** Top: increases in skeletal muscle paxillin concentration with 1 and 8 days of overloading. Soleus and plantaris muscle overloading was induced via the bilateral surgical ablation of the gastrocnemius muscle. Values are means ± SE of the IOD values obtained after Western blot analysis for 7 rats/group. *Significantly different from sham-operated condition for specified muscle and time point, †*P* ≤ 0.05. †Significantly different from 1-day time point for specified muscle and loading condition, ‡*P* ≤ 0.05. Bottom: representative paxillin immunoblot from S and OL muscles. Paxillin appeared as diffuse doublet bands of immunoreactivity in the range of 65–70 kDa. The soleus data have previously appeared elsewhere (18) and are presented here for comparison.
types in normal animals. The fact that male and female rats were respectively used in the overloading and unloading experiments also cannot be ruled out as a potential cause of the different findings between experiments.

In the overloaded soleus and plantaris muscles, the striking increases in skeletal muscle FAK and paxillin concentrations before any increase in whole muscle protein content (≤1 day) may reflect rapid FAC assembly in response to increased cell mechanical loading. As integral components of the FAC that are recruited to the cell membrane during FAC assembly (9), FAK and paxillin have previously served as indexes of FAC density (18). Because FACs are one means by which connections can be drawn. Additionally, an increase in SRF concentration does not necessarily translate into

The fact that skeletal α-actin mRNA abundance decreases with unloading (6) and increases with overloading (13) in skeletal muscle may be related in part to such changes in SRF concentration under these conditions. To our knowledge, skeletal α-actin expression has not been directly compared between muscles of different fiber types. However, the higher SRF expression in the soleus may support a greater skeletal α-actin turnover, because myofibrillar turnover rate is higher in slow-twitch than in fast-twitch muscle (20). Last, whereas our finding of an overload-induced increase in SRF protein concentration in the fast-twitch plantaris muscle is novel, the increase in SRF protein concentration in the overloaded slow-twitch soleus muscle supports a similar observation in the overloaded slow-twitch ALD muscle of roosters (19). In that same study (19), skeletal muscle SRF was shown to be primarily localized to the nucleus. Therefore, the lower nuclear density (as measured by DNA concentration) in fast-twitch vs. slow-twitch muscle (6) and gain of nuclear density with fast-twitch muscle overloading (4) may be contributing factors underlying some of the observed SRF differences in this muscle type. On the other hand, nuclear density might not explain the decrease in SRF concentration with either slow- or fast-twitch muscle unloading, because DNA content increases per unit protein (and remains unchanged per whole muscle) in both fiber types in this model (6) despite observed increases in apoptotic nuclei (2, 24).

The fact that SRF concentration increased in the slow-twitch soleus muscle but not the fast-twitch plantaris muscle within 1 day of overload is interesting considering that SRF-SRE1 binding on the skeletal α-actin promoter is altered in 3- and 6-day overloaded slow-twitch ALD muscle, but not in the 6-day overloaded fast-twitch patagialis muscle, in roosters (12, 13). Yet, despite this time course similarity, differences between the rat synergist ablation and avian wing-stretch models must be taken into account before any connections can be drawn. Additionally, an increase in SRF concentration does not necessarily translate into

<table>
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<th>SRF Concentration (IOD per unit total protein)</th>
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<tr>
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<td>2</td>
<td>3</td>
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<td>8 Days</td>
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Fig. 8. Top: increases in skeletal muscle SRF concentration with 1 and 8 days of overloading. Soleus and plantaris muscle overloading was induced via the bilateral surgical ablation of the gastrocnemius muscle. Values are means ± SE of the IOD values obtained after Western blot analysis for 7 rats/group. *Significantly different from sham-operated condition for specified muscle and time point, P < 0.05. †Significantly different from 1-day time point for specified muscle and loading condition, P < 0.05. Bottom: representative SRF immunoblot from S and OL muscles. SRF appeared as doublet bands of immunoreactivity in the range of 55–58 kDa.
an increased binding to the skeletal α-actin promoter. The mechanism for enhanced SRF-SRE1 binding on the promoter regions of skeletal α-actin or other genes is unknown, although a posttranslational modification such as phosphorylation is one possibility (17). In the present investigation, the reason for the doublet bands (~2 kDa apart) in our SRF Western blots remains to be elucidated; however, the percentage of the IOD attributed to the upper band in these blots was increased by two- to threefold after 1 and 8 days of overloading in both the soleus and plantaris muscles (data not shown).

Because SRF-mediated skeletal α-actin expression is at least partly regulated by FAK signaling in cultured C2C12 myoblasts (41, 42), it is tempting to speculate about a potential association between changes in SRF concentration and changes in either FAK concentration or FAK tyrosine phosphorylation. However, it is important to note that neither past nor present data establish true causality regarding a potential FAK-SRF pathway in the skeletal muscles of whole animals, especially with respect to changes in loading status. Additionally, a multitude of other potential pathways are possible that may entail direct or indirect FAK or SRF involvement independent of one another. These include pathways involving intermediates such as phosphatidylinositol 3-kinase (23, 34, 42), mitogen-activated protein kinases (21, 30, 34), and Ca²⁺/calmodulin-dependent protein kinases (17) as well as other pathways stimulated by growth factors, mechanical loading, and/or interaction with the ECM (1, 10, 31, 33, 43). Future experiments must attempt to establish a causal link between changes in FAK concentration and/or tyrosine phosphorylation and changes in SRF concentration and/or SRF-mediated skeletal α-actin expression during states of altered loading in the skeletal muscles of whole animals.

It is possible that the FAK, paxillin, and SRF proteins detected by our immunoblots may have originated from other cell types found within whole skeletal muscles, such as fibroblasts, endothelial cells, or smooth muscle cells. Leukocyte invasion of hypertrophying skeletal muscle after synergist ablation has also been reported (4). However, our laboratory has previously demonstrated that most of the FAK content in skeletal muscle can be immunohistochemically localized to skeletal muscle cells themselves (18). Furthermore, SRF protein can be immunologically detected in fused skeletal myotubes after fibroblast depletion in culture (19). Because of the high percentage of total cell and nuclear volume attributable to skeletal muscle cells in whole skeletal muscle, we therefore propose that skeletal muscle cells are responsible for at least some, if not most, of the FAK and SRF responses observed in this investigation.

In summary, we found that the FAC proteins FAK and paxillin and the transcription factor SRF in rat skeletal muscle are all modulated in association with the mechanical loading state of the muscle. These proteins exhibited slightly different patterns of adaptation to altered loading in slow- vs. fast-twitch skeletal muscles, and it appears that FAK adaptations can occur in its concentration, tyrosine phosphorylation state, or both. We believe that the differences in FAK and paxillin concentrations with various loading states in skeletal muscle may reflect differences in myocyte FAC density. Last, it remains to be determined whether FAK adaptations to altered skeletal muscle loading either induce adaptations in SRF concentration (such as those observed in the present investigation) or alter SRF binding activity on target genes such as skeletal α-actin.

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REFERENCES

15. Croissant JD, Kim JH, Eichele G, Goering L, Lough J, Prywes R, and Schwartz RJ. Avian serum response factor expression restricted primarily to muscle cell lineages is re-
FAK, PAXillin, SRF, and Altered Skeletal Muscle Loading


