SHP-2 activates signaling of the nuclear factor of activated T cells to promote skeletal muscle growth

Mara Fornaro,1 Peter M. Burch,1 Wentian Yang,3 Lei Zhang,1 Claire E. Hamilton,1 Jung H. Kim,2 Benjamin G. Neel,3 and Anton M. Bennett1

1Department of Pharmacology and 2Department of Pathology, Yale University School of Medicine, New Haven, CT 06520
3Division of Hematology and Oncology, Department of Medicine, Cancer Biology Program, Beth Israel Deaconess Center, Boston, MA 02115

The formation of multinucleated myofibers is essential for the growth of skeletal muscle. The nuclear factor of activated T cells (NFAT) promotes skeletal muscle growth. How NFAT responds to changes in extracellular cues to regulate skeletal muscle growth remains to be fully defined. In this study, we demonstrate that mice containing a skeletal muscle–specific deletion of the tyrosine phosphatase SHP-2 (muscle creatine kinase [MCK]–SHP-2 null) exhibited a reduction in both myofiber size and type I slow myofiber number. We found that interleukin-4, an NFAT-regulated cytokine known to stimulate myofiber growth, was reduced in its expression in skeletal muscles of MCK–SHP-2–null mice. When SHP-2 was deleted during the differentiation of primary myoblasts, NFAT transcriptional activity and myotube multinucleation were impaired. Finally, SHP-2 coupled myotube multinucleation to an integrin-dependent pathway and activated NFAT by stimulating c-Src. Thus, SHP-2 transduces extracellular matrix stimuli to intracellular signaling pathways to promote skeletal muscle growth.

Introduction

Skeletal muscle mass is determined by both muscle fiber number and muscle fiber size. Skeletal muscle fibers are syncytial in nature, and an increase in myonuclear number is required for myofiber growth not only during muscle development (Horsley et al., 2001; Mitchell and Pavlath, 2001) but also during postnatal muscle growth (Darr and Schultz, 1989; Schultz and McCormick, 1994; Mozdziak et al., 1997). Postnatal muscle growth is dependent on myonuclei addition via myoblast proliferation and fusion to preexisting myofibers (Allen et al., 1999). However, the signaling cascades that regulate myofiber growth during development and postnatal myofiber growth remain to be fully defined.

Skeletal muscle mass is determined by both muscle fiber number and muscle fiber size. Skeletal muscle fibers are syncytial in nature, and an increase in myonuclear number is required for myofiber growth not only during muscle development (Horsley et al., 2001; Mitchell and Pavlath, 2001) but also during postnatal muscle growth (Darr and Schultz, 1989; Schultz and McCormick, 1994; Mozdziak et al., 1997). Postnatal muscle growth is dependent on myonuclei addition via myoblast proliferation and fusion to preexisting myofibers (Allen et al., 1999). However, the signaling cascades that regulate myofiber growth during development and postnatal myofiber growth remain to be fully defined.

Protein tyrosine phosphatases (PTPs) play an essential role in regulating the balance of cellular protein tyrosyl phosphorylation. PTPs have been implicated in processes such as cell proliferation, differentiation, and development (Alonso et al., 2004).

Although a complete understanding of how SHP-2 propagates intracellular signals remains to be determined, the basis for some of its positive signaling effects has been uncovered. SHP-2 controls the localization of C-terminal Src kinase (CSK), a negative regulator of c-Src, by dephosphorylating CSK-binding proteins such as Pag/CSK-binding protein and paxillin, thereby positively regulating c-Src activation (Ren et al., 2004; Zhang et al., 2004). SHP-2 can control the recruitment of p120 Ras–GTPase-activating protein to the plasma membrane in order to affect Ras activation (Klinghoffer and Kazlauskas, 1995; Agazie and Hayman, 2003; Montagner et al., 2005). In myoblasts, SHP-2 positively regulates RhoA signaling by dephosphorylating...
p190-B Rho–GTPase-activating protein, leading to the activation of RhoA-dependent muscle-specific gene expression (Kontaridis et al., 2004). These observations provide important mechanistic insights into the pleiotropic signaling effects of SHP-2.

The nuclear factor of activated T cells (NFAT) family is comprised of five members (NFAT1–5) that are implicated in a variety of developmental and disease processes (Crabtree and Olson, 2002; Hogan et al., 2003). NFAT1, 2, and 4 are highly expressed in skeletal muscle. The NFATs appear to play differential roles during skeletal muscle development, as indicated by the distinct skeletal muscle defects displayed by mice lacking individual NFAT family members. NFAT4(NFATc3)−/− mice have reduced muscle mass as a result of decreased fiber number (Kegley et al., 2001), whereas NFAT1(NFATc2)−/− mice exhibit reduced muscle mass because of smaller myofibers (Horsley et al., 2001). During myogenesis, the NFATs translocate into the nucleus by the calcium-activated serine/threonine phosphatase calcineurin. When activated, calcineurin dephosphorylates NFAT, causing it to translocate to the nucleus to stimulate gene expression.

NFAT has been proposed to mediate skeletal muscle growth by activating the transcription of interleukin-4 (IL-4), which acts as a myoblast recruitment factor to facilitate myotube multinucleation (Horsley et al., 2003; Schulze et al., 2005). Although these observations provide insight into the growth-promoting effects of NFAT on skeletal muscle, how NFAT is regulated in skeletal muscle remains to be fully defined.

Because of the early embryonic lethality observed in mice lacking SHP-2 (Saxton et al., 1997; Yang et al., 2006), we have used the Cre-loxP system to disrupt the Shp-2 gene in order to study its role in skeletal muscle function. We show that SHP-2 coordinates signals that stem from the extracellular matrix to target the NFAT signaling pathway, which promotes myofiber type formation and skeletal muscle growth.

Results

Generation of mice containing the skeletal muscle-specific deletion of SHP-2

To investigate the function of SHP-2 in skeletal muscle, we used the Cre-loxP system to conditionally inactivate Shp2 in the myogenic lineage. SHP-2 (lox/lox) mice were generated by engineering loxP sites flanking exon 11 of SHP-2 (Fig. 1 A). We bred SHP-2 (lox/lox) mice with transgenic mice expressing Cre recombinase from the muscle creatine kinase (MCK) promoter (Bruning et al., 1998) to generate mice with a skeletal muscle-specific disruption of Shp2, designated herein as MCK–SHP-2 null.

We found that MCK–SHP-2–null mice at 1 wk of age were phenotypically indistinguishable from their SHP-2 (lox/lox) littermates. Skeletal muscle extracts from the hind limb showed that SHP-2 was expressed to comparable levels in MCK–SHP-2–null and SHP-2 (lox/lox) control mice at 1 wk of age (Fig. 1 B). These results demonstrated that SHP-2 is not deleted embryonically. Consistent with this, MCK–SHP-2–null mice exhibited neither embryonic lethality nor developmental skeletal muscle defects. MCK–SHP-2–null mice also appeared to grow normally (Table I). As MCK–SHP-2–null mice aged, SHP-2 expression in skeletal muscles declined, whereas in SHP-2 (lox/lox) mice, SHP-2 expression remained constant (Fig. 1 B). By 11 wk of age, the expression of SHP-2 in the soleus, tibialis anterior (TA), and gastrocnemius (Gn) muscles was undetectable in MCK–SHP-2–null mice (Fig. 1 C). SHP-2 was expressed at equivalent levels in tissues other than striated muscles, such as the liver, as compared with SHP-2 (lox/lox) controls (Fig. 1 D). Thus, MCK–SHP-2–null mice exhibit the postnatal skeletal muscle–specific deletion of SHP-2.
Decreased myofiber size in MCK–SHP-2–null mice

At the histological level, skeletal muscle from MCK–SHP-2–null mice did not reveal any striking abnormalities (Fig. 2 A). Immunofluorescence staining of skeletal muscle sections also revealed no apparent differences in the expression of either β-dystroglycan or dystrophin between MCK–SHP-2–null and SHP-2 (lox/lox) mice (Fig. 2 A). However, in skeletal muscle sections of MCK–SHP-2–null mice, the integrity of laminin staining was slightly irregular (Fig. 2 A). The altered laminin staining did not appear to be indicative of muscle damage because there were no differences in the number of centrally located myonuclei between MCK–SHP-2–null and SHP-2 (lox/lox) mice (unpublished data).

We noted that the myofibers from MCK–SHP-2–null mice appeared smaller as compared with SHP-2 (lox/lox) controls (Fig. 2 A). Therefore, we measured the cross-sectional area (CSA) of myofibers in skeletal muscles from male MCK–SHP-2–null and SHP-2 (lox/lox) mice. MCK–SHP-2–null male mice had a significant (P < 0.01) reduction in CSA of the TA and diaphragm myofibers as compared with SHP-2 (lox/lox) mice (Fig. 2 B). Similar reductions in myofiber CSAs were also observed in the TA and diaphragm of MCK–SHP-2–null female mice (Fig. 2 C). The fiber number in the TA muscles of male MCK–SHP-2–null mice was also reduced (unpublished data). The size distribution of myofibers in the TA muscles of male MCK–SHP-2–null mice showed an increase in small myofibers (CSA < 1,200 μm²) and a reduction in large myofibers (CSA > 2,400 μm²; Fig. 2 D, left). An increase in small myofibers (CSA < 500 μm²) and a decrease in large myofibers (CSA > 1,000 μm²) in the MCK–SHP-2–null diaphragm was also observed (Fig. 2 D, right). The reduced myofiber CSA seen in MCK–SHP-2–null mice suggests that impaired myofiber growth occurs through a defect in myoblast fusion. To examine this in more detail, we quantitated myonuclear number in cross sections derived from the soleus muscle of MCK–SHP-2–null and SHP-2 (lox/lox) mice. Fig. 2 E shows that MCK–SHP-2–null mice contained ~25% less myonuclei than SHP-2 (lox/lox) controls. Collectively, these results suggest that SHP-2 is required for skeletal muscle growth by promoting myotube multinucleation.

Reduced type I slow muscle fibers in MCK–SHP-2–null mice

We next examined whether muscle fiber type composition was perturbed in MCK–SHP-2–null mice. Depending on the oxidative...
capacity, muscle fiber types are classified as either type I slow or type II fast fibers. We examined fiber type composition by performing immunostaining on skeletal muscle sections derived from SHP-2 (lox/lox) and MCK–SHP-2–null mice with antibodies directed to either type I slow or type II fast myosin heavy chain (MHC). MCK–SHP-2–null mice exhibited a marked reduction (\( \approx 25\% \)) in the number of type I slow fibers as compared with SHP-2 (lox/lox) mice (Fig. 3 A). Neither the regulatory nor catalytic subunits of calcineurin or NFAT1 were altered in their expression levels in MCK–SHP-2–null mice (unpublished data).

The observation that IL-4 levels were reduced in MCK–SHP-2–null mice suggests that the defect in myofiber size occurs because of a failure of myoblasts being recruited to the nascent myofiber to fuse. To test this, we compared the ability of myoblasts from MCK–SHP-2–null and SHP-2 (lox/lox) mice to form multinucleated myotubes. SHP-2 expression in proliferating myoblasts isolated from MCK–SHP-2–null mice was equivalent to that of myoblasts from SHP-2 (lox/lox) mice (Fig. 4 B). Proliferating myoblasts have low levels of MCK activity (Chamberlain et al., 1985; Jaynes et al., 1986), resulting in an insufficient level of Cre-mediated SHP-2 deletion. Similar results have been reported previously using this MCK-Cre transgene (Cohn et al., 2002). Even when myoblasts from MCK–SHP-2–null mice were induced to differentiate, the level of MCK-mediated Cre expression was still too low to result in the deletion of SHP-2 (unpublished data).

In vivo SHP-2 would be expected to be deleted from MCK–SHP-2–null myoblasts during myogenesis. To recapitulate the in vivo elimination of SHP-2 during differentiation, we infected myoblasts with an adenoviral vector expressing Cre recombinase concurrent with the initiation of myoblast differentiation. As shown in Fig. 4 C, Ad-Cre infection of myoblasts isolated from floxed SHP-2 mice concurrent with the initiation of myoblast differentiation resulted in the maximal expression of Cre recombinase within 48 h. Concomitant with the increased expression of Cre recombinase, SHP-2 was deleted during myogenesis, and, by 72 h, its expression was reduced by \( \approx 80\% \) as compared with Ad-GFP–infected myoblasts (Fig. 4 C). Using these conditions, SHP-2 floxed myoblasts were infected with Ad-GFP as a control or with Ad-Cre concurrent with the onset of myoblast differentiation. After 8 d of differentiation, control myoblasts formed prominent multinucleated myotubes (Fig. 4 D). However, SHP-2–deleted myoblasts were dramatically deficient in their ability to form myotubes (Fig. 4 D). Of the myotubes formed in the Ad-Cre–infected cultures, \( \approx 9\% \) contained more than five nuclei, whereas \( \approx 58\% \) of control myotubes contained more than five nuclei (Fig. 4 D). Concomitantly, differentiated SHP-2–deleted myoblasts accumulated myotubes with two to four nuclei to a significantly (\( P < 0.05 \)) greater extent than control myotubes (Fig. 4 D). These data demonstrate that SHP-2 is required to mediate myotube multinucleation.

Because the expression of IL-4 is diminished in MCK–SHP-2–null mice (Fig. 4 A), we tested whether NFAT signaling in skeletal muscle. Interestingly, the reduction in myofiber size in MCK–SHP-2–null mice was similar to that of skeletal muscle defects described in mice lacking the calcineurin substrate NFAT1 (Horsley et al., 2001). NFAT1 regulates IL-4 expression, which promotes muscle growth by stimulating fusion (Horsley et al., 2003; Schulze et al., 2005). We hypothesized that the smaller myofibers in MCK–SHP-2–null mice might be caused by the reduced NFAT-mediated transcriptional expression of IL-4. RT-PCR analysis showed that IL-4 mRNA expression was virtually undetectable in MCK–SHP-2 null as compared with SHP-2 (lox/lox) muscle (Fig. 4 A). Neither the regulatory nor catalytic subunits of calcineurin or NFAT1 were altered in their expression levels in MCK–SHP-2–null mice (unpublished data).

Figure 3. Reduced number of slow type I myofibers in MCK–SHP-2–null mice. Representative sections of soleus muscle immunostained with antibodies specific for either type I (A) or type II (B) MyHC. Data represent the mean percentage ± SEM (error bars) of the total number of fibers positively staining for each fiber type from four mice of each genotype (*, \( P < 0.01 \)). Bar, 50 \( \mu \text{m} \).
transcriptional activity is reduced in the absence of SHP-2. An adenoviral NFAT-luciferase reporter (Ad-NFAT-luc) that contained the NFAT-binding site from the IL-4 promoter (Wilkins et al., 2004) was used to determine NFAT activity in differentiating myoblasts. SHP-2 expression was eliminated during myoblast differentiation as described in Fig. 4 C. After 2 d in differentiation medium (DM), NFAT activity was significantly (P < 0.05) lower by up to 60% as compared with Ad-GFP–infected myoblasts (Fig. 4 E). These results indicate that SHP-2 stimulates NFAT activation during myoblast differentiation.

NFAT transcriptional activity in differentiating myoblasts requires SHP-2 catalysis

We examined whether SHP-2 promotes NFAT activity through its phosphatase domain by determining the effects on NFAT transcriptional activity in differentiating myoblasts upon the overexpression of a dominant-negative (DN) mutant of SHP-2 that lacks catalytic activity. For these experiments, we used C2C12 myoblasts because primary myoblasts were not conducive to transient transfection. C2C12 myoblasts were transiently transfected with vector control, wild-type (WT) SHP-2, or a catalytically inactive mutant of SHP-2 (SHP-2 cysteine to serine [CS]). NFAT transcriptional activity was assessed 48 h later during differentiation using the IL-2 minimal promoter containing NFAT-binding sites fused to the luciferase gene. The expression of SHP-2–CS in differentiating C2C12 myoblasts suppressed NFAT transcriptional activity significantly (P < 0.05) by ~50% as compared with SHP-2 WT (Fig. 5). Because SHP-2 only partially interfered with NFAT activity, it suggests that additional signaling components are required to provide the optimal activation of NFAT.

SHP-2 stimulates a c-Src-dependent pathway leading to NFAT activation

SHP-2 is required for integrin-induced activation of the Src family kinases (SFKs; Tsuda et al., 1998; Oh et al., 1999). In addition, we have shown that SHP-2 catalytic activity increases during C2C12 differentiation (Kontaridis et al., 2004). We hypothesized that SHP-2 positively regulates NFAT via c-Src to promote myotube multinucleation. First, we examined c-Src activity during C2C12 differentiation. Cell lysates obtained from C2C12 myoblasts cultured in growth medium (GM) for 24 h or in DM for 24, 48, and 72 h were subjected to immunoprecipitation with c-Src antibodies, and immune complexes were immunoblotted with antibodies against total c-Src and the activating tyrosine 416 phosphorylation site (c-Src–pY416). We found that c-Src expression was up-regulated, and, when normalized...
to total c-Src, c-Src–pY416 levels were induced by approximately sixfold during C2C12 myogenesis (Fig. 6 A). Thus, c-Src–specific activity and total expression levels increase during myogenesis.

To determine whether c-Src activates NFAT during myogenesis, we used a DN mutant of c-Src that renders it kinase inactive (Src-DN). C2C12 myoblasts were differentiated for 24 h and were then transfected with Src-WT and Src-DN. When overexpressed in differentiating C2C12 myoblasts, Src-WT stimulated NFAT transcriptional activity (Fig. 6 B). In contrast, the overexpression of Src-DN inhibited NFAT transcriptional activity by ~40% when transfected into differentiating C2C12 myoblasts as compared with vector control transfectants (Fig. 6 B). Moreover, c-Src–pY416 phosphorylation was markedly decreased in C2C12 myoblasts infected with a catalytically inactive/nonsubstrate-trapping mutant of SHP-2 (Ad–SHP-2–RM) but not with SHP-2–WT (Fig. 6 C).

Therefore, in myoblasts, SHP-2 acts upstream of c-Src. To establish the relationship between SHP-2 and c-Src for NFAT activation in differentiating myoblasts, we tested whether a gain of function mutant of c-Src could rescue the inhibitory actions of a catalytically inactive mutant of SHP-2 on NFAT activity. Again, differentiated C2C12 myoblasts were transfected, and an assessment of NFAT activity was performed 48 h later. Compared with vector control, constitutively active c-Src (c-Src–Y527F) stimulated NFAT activity approximately fourfold (Fig. 6 D). The inhibitory effect exerted by SHP-2–CS on NFAT was rescued upon coexpression with c-Src–Y527F but not to levels equivalent to that of c-Src–Y527F alone (Fig. 6 D). In light of the results shown in Fig. 6 C, this partial rescue of NFAT activity by c-Src–Y527F suggests that c-Src lies downstream of SHP-2 in the NFAT pathway. Failure to completely restore NFAT activity to levels obtained by c-Y527F alone again raises the possibility that multiple pathways cooperate with SHP-2 to promote NFAT activity in differentiating myoblasts.

Integrin-dependent myotube formation is facilitated by SHP-2 and c-Src

It has been suggested that the signal regulatory protein-1α (SIRP-1α; Fujitaka et al., 1996; Kharitonenkov et al., 1997; van Beek et al., 2005; van den Berg et al., 2005), a transmembrane glycoprotein, participates in a positive-feed forward pathway to promote SHP-2 signaling in response to integrins (Oh et al., 1999). Moreover, SIRP-1α becomes tyrosyl phosphorylated and forms a complex with SHP-2 during myogenesis (Kontaridis et al., 2004). We hypothesized that in differentiating myoblasts, SIRP-1α may serve to recruit SHP-2/c-Src signaling.

We first determined whether SIRP-1α tyrosyl phosphorylation and association with SHP-2 were dependent on SFK activity in differentiating myoblasts. C2C12 myoblasts induced to differentiate for 24 h were treated with the SFK inhibitor PP2.
for an additional 24 and 48 h (Fig. 7 A). We found that PP2 treatment dramatically decreased SIRP-1α tyrosyl phosphorylation and association with SHP-2, demonstrating that SHP-2 complexes with SIRP-1α in myoblasts in an SFK-dependent manner. If our hypothesis that c-Src–mediated SIRP-1α tyrosyl phosphorylation and subsequent recruitment of SHP-2 are critical for propagating SHP-2/c-Src signaling, multinucleated myotube formation should be reduced upon inhibition of the SFKs. To test this, C2C12 myoblasts were induced to differentiate for 24 h and were treated either with or without PP2 for an additional 48 h. PP2 inhibited multinucleated myotube formation both phenotypically (Fig. 7 B, top) and quantitatively (Fig. 7 B, bottom). These data imply that SFKs stimulate the recruitment of SHP-2 to tyrosyl-phosphorylated SIRP-1α, resulting in the initiation of SHP-2 signaling to promote myoblast fusion.

Next, we investigated whether integrin-dependent engagement couples SIRP-1α to SHP-2 to promote myotube formation. SIRP-1α tyrosyl phosphorylation in myoblasts was induced in response to integrin engagement (Fig. 7 C). We next determined whether the DN mutant of SHP-2 disrupts integrin-mediated multinucleated myotube formation. C2C12 myoblasts were infected with adenoviruses encoding for Ad-GFP, Ad–SHP-2–WT, or the catalytically inactive/nonsubstrate-trapping mutant of SHP-2, Ad–SHP-2–RM. Adhesion to fibronectin was essential for C2C12 myogenesis because C2C12 myoblasts were unable to form either multinucleated myotubes or express MHC when plated on poly-l-lysine (unpublished data). Although SHP-2–WT expression did not affect multinucleated myotube formation, the expression of SHP-2–RM prevented both MHC expression and multinucleated myotube formation on fibronectin (Fig. 7, D and E). Altogether, these data argue that in differentiating myoblasts, integrin engagement promotes SIRP-1α tyrosyl phosphorylation through the SFKs, resulting in the recruitment of SHP-2, which facilitates c-Src–mediated multinucleated myotube formation.

Discussion

Skeletal muscle growth occurs during embryonic development and through the early stages of life. In mammals, two waves of myogenesis termed primary and secondary myogenesis occur (Ontell et al., 1988; Buckingham et al., 2003). In mice, by day 11 of embryogenesis, primary myogenesis takes place. Muscle growth during embryogenesis after day 14 then proceeds through secondary myogenesis, in which secondary myoblasts form. Postnatally, myoblasts continue to fuse with the existing myofiber, thus contributing to muscle growth (Ontell et al., 1988; Buckingham et al., 2003). The signaling pathways that participate in controlling muscle growth postnatally remain poorly defined. In this study, we have examined the in vivo role of SHP-2 in skeletal muscle by generating a skeletal muscle–specific deletion of SHP-2. We found that skeletal muscle development in MCK–SHP-2–null mice was normal. The lack of a skeletal muscle developmental defect in MCK–SHP-2–null mice is attributable to the observation that MCK–SHP-2–null mice ablate SHP-2 expression postnatally.

In postnatal skeletal muscles of MCK–SHP-2–null mice, we observed that myofiber size was reduced, demonstrating that SHP-2 is required for the growth of skeletal muscle. Postnatal skeletal muscle growth is mediated by a muscle somatic stem cell called a satellite cell. Satellite cells remain quiescent, but, when activated, they proliferate, differentiate, and fuse with the existing myofiber. Presumably, satellite cells in MCK–SHP-2–null mice, when activated, first proliferate and then differentiate, where upon they express MCK (Chamberlain et al., 1985), resulting in the deletion of SHP-2. Therefore, it is reasonable to...
conclude that the skeletal muscle phenotype of MCK–SHP-2–null mice is reflective of SHP-2 deficiency during myogenesis.

Our results demonstrate that MCK–SHP-2–null mice accumulate smaller myofibers concomitant with a reduction in larger myofibers as well as a decrease in myonuclear number. These results are consistent with the interpretation that MCK–SHP-2–null mice have smaller myofibers as a consequence of a failure of nascent myofibers to grow into larger ones by recruiting myoblasts to fuse. This interpretation is supported by the result that cultured myoblasts in which SHP-2 was deleted during differentiation fail to undergo myotube multinucleation. There are other potential explanations for our results: a reduction in myoblast proliferation could result in a failure to fuse. However, we think this less likely because MCK is only expressed in differentiating myoblasts. Another possibility is that postnatal myofibers undergo apoptosis, resulting in a decrease in myofiber size in MCK–SHP-2–null mice. However, no observable differences in apoptosis were observed in skeletal muscles of MCK–SHP-2–null mice (unpublished data). Thus, the most plausible explanation for the reduced myofiber size in MCK–SHP-2–null mice is a failure of differentiating myoblasts to form multinucleated myotubes.

Interestingly, MCK–SHP-2–null mice and NFAT1-deficient mice bear some similarities. Like NFAT1-deficient mice, MCK–SHP-2–null mice were found to have smaller myofibers and reduced levels of IL-4 expression in skeletal muscle. These results support the idea that NFAT1 is a target for positive regulation by SHP-2 in skeletal muscle. However, it remains to be determined whether the phenotype of MCK–SHP-2–null mice is a consequence of disrupting NFAT1 solely as opposed to other (or additional) NFAT family members. PTPs have been reported previously to regulate NFAT activity (Fortin et al., 2001). However, these studies were performed in hematopoietic cells and demonstrated the negative regulation of NFAT activation by PTPs (Fortin et al., 2001). Our results provide evidence for a positive role of PTPs in NFAT signaling.

The precise mechanism through which SHP-2 regulates NFAT in skeletal muscle remains to be defined. SHP-2 plays an important role in the oscillatory control of calcium and NFAT in cardiomyocytes (Uhlen et al., 2006). It is conceivable that in skeletal muscle, SHP-2 also participates in the regulation of calcium signaling, raising the possibility that it controls NFAT via activation of the calcium-dependent serine/threonine phosphatase calcineurin. Both the magnitude and duration of calcium signals in skeletal muscle are thought to be important for the regulation of calcineurin activity, which is thought to control muscle fiber type composition. A combination of genetic and biochemical data support a role for calcineurin in slow muscle fiber type formation (Chin et al., 1998; Naya et al., 2000). Therefore, our results, which show a reduction in type I slow muscle fiber in MCK–SHP-2–null mice, suggest that SHP-2 may act upstream of calcineurin.

SHP-2 indirectly activates c-Src by controlling Pag/CSK-binding protein or paxillin tyrosyl phosphorylation and, thus, the recruitment of CSK (Ren et al., 2004; Zhang et al., 2004). Engagement of the SH2 domains of SHP-2 is essential for its activation (Neel et al., 2003). Previously, we demonstrated that SIRP-1α becomes tyrosyl phosphorylated and associates with SHP-2 during myogenesis (Kontaridis et al., 2001). Moreover, the kinetics of SIRP-1α–SHP-2 complex formation correlates with the induction of SHP-2 activation during C2C12 myogenesis (Kontaridis et al., 2004). In this study, we found that c-Src was active basally in proliferating myoblasts, and its activity increased during differentiation. Furthermore, c-Src activation was dependent on the catalytic activity of SHP-2. We propose that during myogenesis, SHP-2 is required for the activation of c-Src–dependent NFAT activation (Fig. 8). We have also shown that the c-Src–mediated activation of NFAT is reduced by the pharmacological inhibition of mitogen-activated extracellular-regulated kinase (Erk) kinase, suggesting that c-Src promotes NFAT transcriptional activity in myoblasts via Erk (unpublished data). Recent findings support both direct and indirect roles of Erk in the regulation of NFAT activity (Sanna et al., 2005).

We have also found that SHP-2 promotes NFAT activation by stimulating the RhoA pathway in myoblasts (unpublished data). Thus, calcineurin-independent pathways exist through which SHP-2 stimulates NFAT activity in myoblasts (Fig. 8). The importance of the SFKs in myogenesis has been suggested previously (Farrell and Ingram, 1983; Lu et al., 2002), and, here, we show that the inhibition of SFKs impairs myoblast fusion. We did observe that inhibition of the SFKs induced myoblast death, although the extent of myoblast death was not dramatic (unpublished data). Nevertheless, we cannot exclude the possibility that in addition to activating NFAT, c-Src promotes myotube formation by also maintaining myoblast survival. Although it is likely that NFAT may be only one of the downstream targets of c-Src involved in myogenesis, it is the first target of c-Src, which was defined here to be important for skeletal muscle growth.

A considerable amount of data supports a critical role for integrin-mediated signals in the regulation of muscle development and myofiber integrity (Danen and Sonnenberg, 2003). We have established a requirement for SHP-2 in integrin-mediated myogenesis. These results implicate SHP-2 as an early conduit linking extracellular matrix cues to downstream pathways.
involved in regulating muscle function (Fig. 8). When c-Src activity is abrogated in myoblasts, SIRP-1α tyrosyl phosphorylation and association with SHP-2 are inhibited, NFAT activation is diminished, and multinucleated myotube formation is blocked. These data predict that NFAT activation should be regulated in response to integrin engagement. In support of this, it has been shown that integrins can activate the NFATs (Jauliac et al., 2002). One pathway through which the integrin-dependent activation of c-Src/SHP-2 signalling and, subsequently, NFAT-mediated gene expression might occur is through the activation of Erk, but, additionally, calcium may also play a role.

In summary, we have identified SHP-2 as a novel regulator of skeletal muscle growth and slow type I skeletal muscle fiber formation. We have established a pathway through which SHP-2 regulates muscle growth by stimulating NFAT transcriptional activity. Furthermore, we show that SHP-2 stimulates c-Src in differentiating myoblasts to link extracellular matrix stimuli to intracellular signaling cascades that converge on NFAT. These results may provide the basis for further investigation into whether these signaling pathways are targets for dysregulation in skeletal muscle pathogenesis.

Materials and methods

Generation of floxed SHP-2 mice

The Shp2 floxed allele was generated by introducing two loxP sites that flanked exon 11 encoding 55 amino acids containing the PTP signature motif. This Shp2 floxed allele was generated in a 129Sv/B6 mixed background, crossed with MCK-Cre transgenic mice to obtain SHP-2 flox/flox; MCK-Cre mice, and maintained by intercrossing with SHP-2 (lox/lox) mice. SHP-2 (lox/lox) litters were used as controls in all experiments. All genotyping was performed by PCR using genomic DNA isolated from the mouse tail using primers for identifying mice carrying the Cre transgene (5′-AGTCCAAATTACTGACC-3′ and 5′-CCGCCTACAACCTGGAGAAG-3′) or primers for the SHP-2 floxed allele (5′-TAGCTGCTTAAACCCTCCTGTTG-3′ and 5′-CATCAGAGCAGCGCATATCAGG-3′). All histological and morphometric analyses were performed on male mice aged 11–13 wk unless otherwise indicated.

Antibodies, expression reagents, and cell lines

The following antibodies were used: mouse monoclonal antibodies to SHP-2 and caveolin-3 (BD Transduction Laboratories); rabbit polyclonal to laminin, c-Met, and Erk (Santa Cruz Biotechnology, Inc.); rabbit polyclonal and caveolin-3 (BD Transduction Laboratories) and to Src and phospho-

Histological and morphometric measurements

For histological analysis, muscles from MCK–SHP-2–null and SHP-2 (lox/lox) controls were dissected and fixed in formalin, embedded in paraffin, and stained with hematoxylin and eosin. Transverse muscle sections were visualized by using a microscope (Axiovert 25; Carl Zeiss Micro-Imaging, Inc.) and photographed by using a charge-coupled device camera (DAGE 330; Meyer Instruments). The CSA of myofibers was measured using ImageJ (version 1.36b; National Institutes of Health [NIH]) and anti–β-dystroglycan (1:25) or ant dystrophin (1:50) antibodies diluted in 10% NGS/PBS for 1 h. Sections were washed with PBS and incubated for 1 h with 1 μg/ml goat anti–rabbit antibody conjugated to AlexaFluor488 (Invitrogen) diluted in 10% NGS/PBS containing 2 μM TOTO-3 (Invitrogen). After washing, sections were mounted with Vectashield (Vector Laboratories) and visualized using a confocal microscope (510 META; Carl Zeiss Micromaging, Inc.). Myonuclear number was determined by counting the nuclei within the dystrophin-stained sarcolemmal of at least 100 myofibers per animal using Image software (version 1.36b) and is expressed as the number of myonuclei per myofiber.

Fiber type staining

For fiber type analysis, sections of frozen soleus muscle were prepared as described in the previous section. Sections were fixed with 4% PFA in PBS for 10 min at room temperature and immunostained with anti–type I and anti–type II MH antibodies for 1 h at room temperature. Primary antibodies were detected using the Vectastain Elite ABC reagent and 3,3-diaminobenzidine tetra chloride (Vector Laboratories) according to the manufacturer’s instructions. Sections were photographed using a microscope (Axiovert S100; Carl Zeiss Micromaging, Inc.) fitted with a digital camera (Spot; Diagnostic Instruments) and analyzed by counting the number of positively staining fibers in >300 fibers from each animal using Image software (version 1.36b).

Primary myoblast isolation and adenoviral infection

Primary myoblasts were prepared from the fore and hind limb skeletal muscle of 5–8-wk-old SHP-2 (lox/lox) mice. Muscle tissue was isolated, minced, and enzymatically digested in Ham’s F-10 medium containing 1.25 mg/ml protease type XIV and 2.5 mg/ml trypsin type Ix K for 2 h at 37°C. The digested tissue was strained through 40 μm of nylon mesh and centrifuged at 1,500 g for 3 min at 4°C. The resulting cell pellet was washed and re suspended in Ham’s F-10 medium, layered on a Percoll gradient consisting of 40 and 70% Percoll (GE Healthcare) in PBS, and centrifuged at 2,500 g for 20 min at 4°C. The purified cells were washed and resuspended in GM consisting of Ham’s F-10 with 20% PBS, 5 ng/ml FGF-2, 50 μU/ml penicillin, and 50 ng/ml streptomycin. Myoblasts were expanded and enriched as described previously (Rando and Blau, 1994), and the purity was assessed by MyoD and c-Met staining. To assess the effect of SHP-2 deletion on primary myoblast differentiation, the cells were plated at 10^5 cells per 35-mm dish and infected with either Ad-GFP or Ad-Cre at an MOI of 50 for 2 h at 37°C in Ham’s F-10 containing 2% PBS. Cultures were then switched to DM containing DME supplemented with 0.1% FBS, 1% sodium pyruvate, 5 μg/ml insulin, and 5 μg/ml transferrin. To determine the fusion index, cells were fixed with cold methanol for 5 min at −20°C, washed with PBS, and stained with modified Wright Giemsa (Sigma-Aldrich) stain. The number of nuclei in individual myotubes was counted for 40–100 myotubes. For the NFAT-Luciferase assays, primary myoblasts were infected sequentially with Ad-NFAT-Luc for 2 h followed by infection with either Ad-ΔC-er or Ad-Cre for an additional 2 h. Luciferase activity was determined using the Luciferase Assay System (Promega) and normalized to the total protein concentration for each sample as determined by Coomassie Protein Assay Reagent (Pierce Chemical Co.).

RT-PCR

RNA was isolated from hind limbs of 11-wk-old mice using Triazol reagent. IL4 amplification was performed by incubation at 94°C for 2 min followed
by 35 cycles at 94°C for 30 s, 72°C for 45 s, and 1 cycle at 72°C for 10 min using the following primers: sense, 5'-AACCCCGAGCTAGTGTGATC-3'; and antisense, 5'-CAGCGAAGGCGCCCGAAGTGC-3'. GAPDH was used as a control for each sample and was amplified at 60°C using the following primers: sense, 5'-GGTTGGAAGCACAAGGCTTC-3'; and antisense, 5'-GGAGTGGTCTGGAAGTC-3'.

Transient transfection and luciferase reporter assay

NFAT activity was measured using the NFATGL3 plasmid (Timmerman et al., 1994). C2C12 myoblasts were plated at a density of 4–8 × 10⁴ cells per well of a 12-well plate and were cotransfected with 0.1 μg NFAT-luc and 4 ng pRL-Renilla (Promega) along with 1–2 μg of the indicated expression plasmids using LipofectAMINE 2000 [Invitrogen]. Cells were initiated to undergo differentiation for 48 h. Cells were harvested, and luciferase activities were measured using the Dual Luciferase Assay System Kit (Promega).

Immunoprecipitation and immunoblotting

Tissues were homogenized in radioimmunoprecipitation assay buffer (1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate, 150 mM NaCl, 20 mM Hepes, pH 7.4, 10% glycerol, 10 mM NaF, 2 mM Na3VO4, 2 mM EDTA, 5 μg/ml leupeptin, 1 mM benzamidine, 5 μg/ml aprotinin, 1 μg/ml phosphatase A, 1 mM PMSF, and 1 mM DTT). Primary and C2C12 myoblasts were lysed in NP-40 lysis buffer (1% NP-40, 150 mM NaCl, 50 mM TrisHCl, pH 7.4, 10 mM NaF, 1 mM Na3VO4, 1 μg/ml leupeptin, 1 mM benzamidine, 1 μg/ml aprotinin, 1 μg/ml phosphatase A, and 1 mM PMSF). Lysates were subjected to centrifugation at 20,800 rpm for 30 min at 4°C. Protein concentrations were determined using the BCA Protein Assay Kit (Pierce Chemical Co.) before immunoblotting. For immunoprecipitations, supernatants were precleared for 15 min at 4°C with protein A– or protein G–Sepharose, washed five times with lysis buffer, and resuspended in sample buffer. Immunoblotting was performed as described previously (Kontaridis et al., 2004). Densitometric analysis on immunoblots was performed by using the LabWorks 4.0 Image Analysis software (UVP Bioimaging Systems).

Cell adhesion assay

C2C12 myoblasts were detached using trypsin/EDTA, washed three times in serum-free medium, resuspended in sample buffer. Immunoblotting was performed as described previously (Kontaridis et al., 2004). Densitometric analysis on immunoblots was performed by using the LabWorks 4.0 Image Analysis software (UVP Bioimaging Systems).

Fusion assays in C2C12 myoblasts

C2C12 myoblasts maintained in GM were plated at a density of 1.25 × 10⁵ cells per 35-mm plate. Cells were induced to differentiate by transfer to DM for 24 h and were treated with 2 μM P2 or DMSO in DM for an additional 48 h. Cells were fixed in 2.5% glutaraldehyde in PBS for 10 min at room temperature, mounted in Vectashield with DAPI, and visualized by glutation-dehyde-induced autofluorescence. Fusion index was calculated as the percentage of cells that had two or more than two myonuclei. The number of nuclei within individual myotubes was counted for 70–150 myotubes.

Statistical analyses

Data were assessed for statistical significance by either the application of a two-way t test assuming unequal variances or by analysis of variance.
