SHP-2 complex formation with the SHP-2 substrate-1 during C2C12 myogenesis

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SUMMARY

Myogenesis is a highly ordered process that involves the expression of muscle-specific genes, cell-cell recognition and multinucleated myotube formation. Although protein tyrosine kinases have figured prominently in myogenesis, the involvement of tyrosine phosphatases in this process is unknown. SHP-2 is an SH2 domain-containing tyrosine phosphatase, which positively regulates growth and differentiation. We show that in C2C12 myoblasts, SHP-2 becomes upregulated early on during myogenesis and associates with a 120 kDa tyrosyl-phosphorylated complex. We have identified that the 120 kDa complex consists of the SHP-2 substrate-1 (SHPS-1) and the Grb2-associated binder-1 (Gab-1). SHPS-1, but not Gab-1, undergoes tyrosyl phosphorylation and association with SHP-2 during myogenesis, the kinetics of which correlate with the expression of MyoD. Either constitutive expression or inducible activation of MyoD in 10T½ fibroblasts promotes SHPS-1 tyrosyl phosphorylation and its association with SHP-2. It has been shown that p38 mitogen-activated protein kinase (MAPK) activity is required for the expression/activation of MyoD and MyoD-responsive genes. Inhibition of p38 MAPK by SB203580 in differentiating C2C12 myoblasts blocks MyoD expression, SHPS-1 tyrosyl phosphorylation and the association of SHPS-1 with SHP-2. These data suggest that SHPS-1/SHP-2 complex formation is an integral signaling component of skeletal muscle differentiation.

Key words: SHP-2, Tyrosine phosphatase, Myogenesis, Signalling, Myoblast, SHPS-1

INTRODUCTION

Skeletal muscle differentiation is a multi-step process that involves the coordinated expression of muscle-specific genes, cell cycle withdrawal and fusion of myoblasts into multinucleated myotubes. The early stages of myogenesis involve the activation of muscle-specific transcription factors of the E-box family, such as MyoD, myogenin, Myf-5 and MRF4 (Dias et al., 1994; Lassar et al., 1994; Olson and Klein, 1994; Weintraub, 1993). Although previous studies have defined extensively the mechanisms of myogenesis at the transcriptional level, the intracellular signaling mechanisms involved are still poorly understood. Work from several laboratories has shown that intracellular signaling cascades regulated by tyrosyl phosphorylation are critical components of myogenic progression. For example, the mitogen-activated protein kinases (MAPKs), specifically the extracellular-regulated protein kinases (Erks 1 and 2), have been implicated as critical for early myogenic progression (Bennett and Tonks, 1997; Coolican et al., 1997; Gredinger et al., 1998; Weyman and Wolfman, 1998; Wu et al., 2000). The p38 MAPK and Erk5/BMK function to activate MEF2C, another muscle-specific gene (Kato et al., 1997; Zetser et al., 1999; Zhao et al., 1999b). In addition, SAPK3/Erk6/p38γ is induced during myogenesis and promotes myotube formation (Lechner et al., 1996). Other signaling molecules involved in myogenesis and regulated by protein tyrosine kinases (PTKs) include phosphatidylinositol 3′-kinase (PI-3K) (Coolican et al., 1997; Kaliman et al., 1996), its downstream serine/threonine kinase target PKB/Akt (Fujio et al., 1999; Jiang et al., 1999), and the integrins (Sastry et al., 1996). Given that tyrosyl phosphorylation is a balance between the opposing actions of PTKs and protein tyrosine phosphatases (PTPs), it is likely that PTPs will also play a critical role in myogenic progression.

We have focused on determining whether SHP-2, an Src homology 2 (SH2) domain-containing PTP, which is expressed ubiquitously and is highly abundant in skeletal muscle, is involved in myogenesis (Adachi et al., 1992; Ahmad et al., 1993; Feng et al., 1993; Freeman et al., 1992; Vogel et al., 1993). Studies in *Drosophila* (Perkins et al., 1992), *Xenopus* (Tang et al., 1995), *C. elegans* (Gutch et al., 1998) and mice (Saxton et al., 1997) demonstrate that SHP-2 is required for development. The PTP activity of SHP-2 mediates positive signaling (growth and differentiation promoting) and functions either upstream of, and/or parallel to, Ras in the Raf/MEK/Erk pathway (Neel, 1993; Neel and Tonks, 1997; Tonks and Neel, 1996). In addition to signaling to the Erks, SHP-2 plays an important role in cell migration/motility, morphogenesis and cell spreading via integrin-dependent mechanisms (O'Reilly et al., 2000; Oh et al., 1999; Tsuda et al., 1998; Yu et al., 1998).

In most cases, if not all, SHP-2 propagates its downstream signal(s) by first interacting via its SH2 domains with tyrosyl-phosphorylated proteins. This event serves both to localize and to activate SHP-2, and ensures that the target of SHP-2 is appropriately activated in a coordinated and specific manner. To begin defining the role of SHP-2 in skeletal muscle

differentiation, we have attempted to identify proteins with which it may interact during myogenesis. Such interacting proteins will likely dictate how and when SHP-2 is activated during myogenesis. The SH2 domains of SHP-2 mediate its direct interaction with the activated platelet-derived growth factor (PDGF) receptor β (Lechleider et al., 1993) as well as multi-substrate adapter proteins such as IRS-1 (Kuhné et al., 1993), Gab-1 (Holado-Madruga et al., 1997) Gab-2 (Gu et al., 1998; Zhao et al., 1999a), FRS-2/SNT-1, SNT-2 (Kouhara et al., 1997; Xu et al., 1998) and DOS (Herbst et al., 1996; Raabe et al., 1996). SHP-2 also interacts with transmembrane glycoproteins, such as the signal regulatory proteins (SIRPs) (Kharitonenkov et al., 1997) (also known as SHP-2 substrate-1 (SHPS-1) (Fujioka et al., 1996), brain-immunoglobulin like molecule (BIT) (Sano et al., 1997), p84 (Comu et al., 1997) and macrophage fusion receptor (MFR) (Saginario et al., 1998) and the platelet/endothelial cell adhesion molecule (PECAM) (Jackson et al., 1997; Sagawa et al., 1997). The basis for how and when SHP-2 participates in cell-type and growth factor-specific signaling is likely regulated by the mutually exclusive complement of protein-protein interactions that SHP-2 forms. We have identified that SHP-2 interacts with Grb-2-associated binder-1 (Gab-1) and SHPS-1 in the C2C12 myoblast cell line. We show that SHPS-1/SHP-2 complex formation is an integral signaling event during C2C12 myoblast differentiation, suggesting a functional link between SHP-2 and myogenic progression.

MATERIALS AND METHODS

Cell lines

C2C12 myoblasts were purchased from the American Tissue Culture Collection (ATCC, Rockville, MD, USA) and cultured at 37°C and 5% CO_2 in growth medium (GM) containing DMEM (Gibco, Grand Island, NY, USA) supplemented with 10% FBS (Sigma, St Louis, MO, USA), 1% sodium pyruvate and 1% penicillin/streptomycin. C3H10T½ (10T½) and C3H10T½-MyoD (10T½-MyoD) fibroblasts were provided by Dr Andrew Lassar (Harvard Medical School, Boston, MA, USA) and maintained in DMEM supplemented by 10% FBS, 1% sodium pyruvate and 1% penicillin/streptomycin. Cells were induced to differentiate by transfer to differentiation medium (DM) containing DMEM supplemented with 0.1% FBS, 1% sodium pyruvate, 1% penicillin/streptomycin and 5 μg/ml each of insulin (Sigma) and transferrin (Sigma). The L8 rat myogenic cell line was obtained from ATCC and cultured as described for C2C12 myoblasts. C2C12 myoblasts were seeded at 5×10^5 cells per 10 cm dish for 24 hours prior to the initiation of differentiation. C2C12 myoblasts at various stages during myogenesis were visualized with a Zeis Axiovert 100 inverted microscope and photographed using a SPOT CCD camera (Diagnostic Instruments, Sterling Heights, MI, USA). The MyoD-ER 10T½ (clone 9-5) inducible cell line was a gift from Stephen Tapscott (Fred Hutchinson Cancer Center, Seattle, WA, USA) and Stephen Konieczny (Purdue University, Purdue, IN, USA) (Hollenberg et al., 1993). MyoD-ER 10T½ cell lines were maintained in DMEM plus 10% FCS (Hyclone) on gelatin-coated plates. Experiments with the MyoD-ER 10T½ inducible cell lines were performed on non-coated plates and MyoD activation was induced by the addition of 0.1 μM 17β-estradiol for 24 hours.

Immunoprecipitation and immunoblotting

SHPS-1 and Gab-1 polyclonal antibodies were purchased from Upstate Biotechnology (Lake Placid, NY, USA). SHPS-1 polyclonal antibodies were also provided by Dr Benjamin Neel (Beth Israel Deaconess Hospital, Boston, MA, USA). Anti-SIRP-α1/MFR (10C4) monoclonal antibodies were provided by Dr Agnes Vignery (Yale University School of Medicine, New Haven, CT, USA) (Saginario et al., 1995). SHP-2 polyclonal antibodies used for immunoprecipitation experiments were obtained from Gen-Shen Feng (Burnham Institute, La Jolla, CA, USA) or were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). SHP-2 immunoblots were performed using a monoclonal antibody purchased from Transduction Laboratories (Lexington, KY, USA). Dr Peter Houghton (St Jude Children's Research Hospital, Memphis, TN, USA) provided antibodies to MyoD. Anti-phosphotyrosine immunoblots were carried out using the 4G10 antibody purchased from Upstate Biotechnology and myosin heavy chain antibodies (MF20) were purchased from the Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA, USA). Phospho-specific antibodies to Akt and MAPK were obtained from New England Biolabs (Beverley, MA, USA). Anti-Akt antibodies were purchased from Santa Cruz Biotechnology and anti-MAPK (C1) antibodies were a kind gift from John Blenis (Harvard Medical School, Boston, MA, USA). Insulin-like growth factor-I and II, and SB203580 were purchased from Calbiochem (La Jolla, CA, USA). All other chemicals were purchased from Sigma.

Immunoprecipitation and immunoblotting experiments were performed by lysing cells on ice in 1 ml of NP-40 lysis buffer containing 1.0% NP-40, 5 μg/ml leupeptin, 5 μg/ml aprotinin, 1 μg/ml pepstatin A, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine and 0.2 mM sodium orthovanadate. Lysates were centrifuged at 14,000 r.p.m. for 15 minutes at 4°C and supernatants were precleared for 15 minutes with 2 μl Pansorbin (Calbiochem-Novabiochem, La Jolla, CA, USA). Protein concentration was determined using the Bradford Assay (Pierce, Rockford, IL, USA). Approximately 0.5-1.0 mg of total cell lysates was used for each immunoprecipitation and approximately 50 μg of total cell lysates were used for immunoblotting. Immunoprecipitation experiments were carried out overnight at 4°C. For immunoprecipitation experiments, 6 μg of SHP-2 antibodies, 10 μg SHPS-1 antibodies, 15 μg of Gab-1 antibodies were used to immunoprecipitate the respective proteins. Immune complexes were collected on Protein A-sepharose beads, which were washed three times with lysis buffer containing 0.1 mM sodium orthovanadate, with a final wash in STE (150 mM NaCl, 20 mM Tris, pH 7.4, and 5 mM EDTA) plus 0.2 mM sodium orthovanadate. Immune complexes were resolved by SDS-PAGE and transferred onto Immobilon-P membranes (Millipore, Bedford, MA, USA). Immobilon-P membranes were washed three times for 10 minutes in TBST (20 mM Tris-HCl, pH 8.0, 150 mM NaCl and 0.05% Tween-20) and were blocked for at least 1 hour with 5% non-fat dry milk in TBST at 4°C. Immunoblots that were incubated with anti-phosphotyrosine 4G10 antibodies (pTyr) were blocked for at least 1 hour in 5% BSA (Boehringer-Mannheim, Indianapolis, IN, USA) in TBST. After blocking, membranes were washed three times for 10 minutes in TBST. Membranes were then incubated with primary antibodies (anti-SHP-2 at 1:1,000; anti-SHPS-1 at 1:5,000; anti-myosin heavy chain antibody at 1:12; anti-Gab-1 at 1:1,000; anti-MyoD at 1:1,000; anti-Akt at 1:1,000; and anti-myogenin at 1:12) diluted in 2.5% non-fat dry milk, for 2-4 hours at 4°C. Anti-phosphotyrosine 4G10 antibodies were diluted to 1:2,000 in 2.5% BSA/TBST and anti-phospho-MAPK and anti-phospho-Akt antibodies were diluted to 1:1,000 in 2.5% nonfat dry milk/TBST. Primary antibodies were incubated with membranes for either 3 hours at room temperature or overnight at 4°C. Following incubation with primary antibodies, membranes were washed three times for 10 minutes in TBST and primary antibodies were detected with either anti-rabbit or anti-mouse horseradish peroxidase-conjugated antibodies at a 1:5,000 dilution in TBST for 30 minutes. Membranes were washed and primary antibodies visualized using enhanced chemiluminescence detection (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

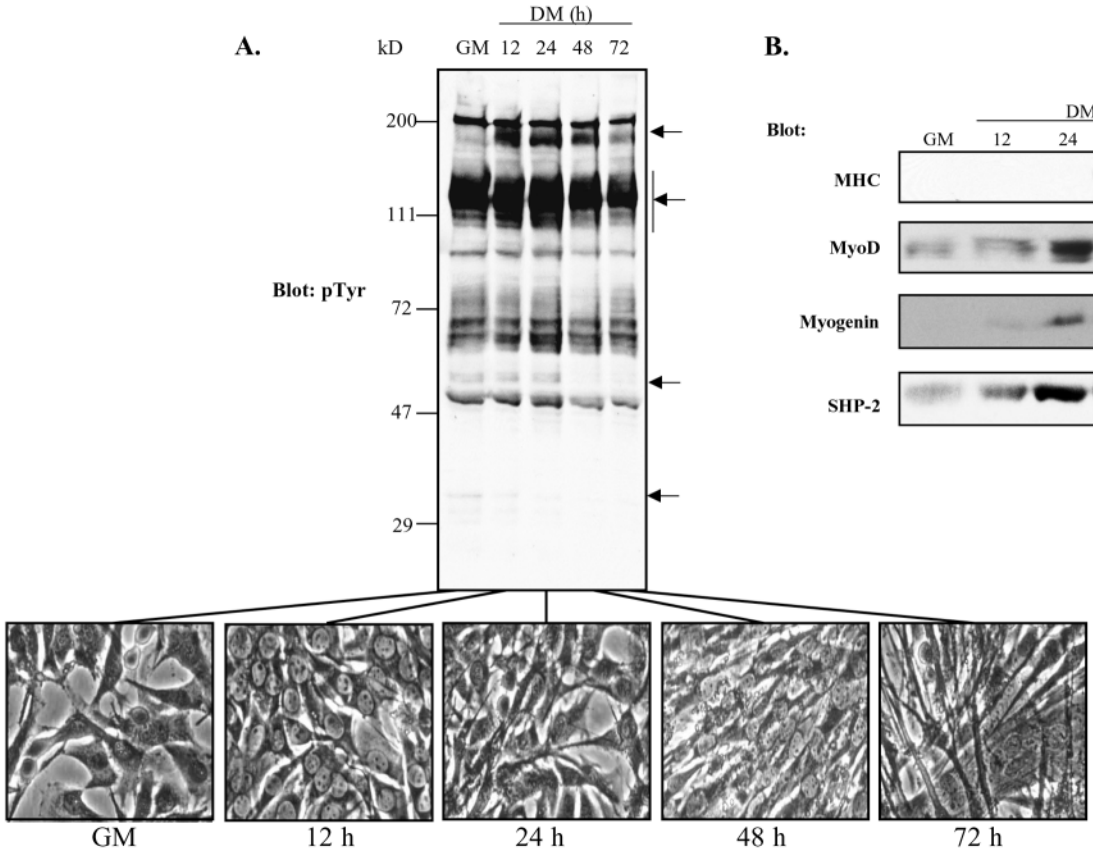


Fig. 1. Tyrosyl phosphorylation and SHP-2 protein expression during myogenic differentiation in the murine C2C12 cell line. C2C12 myoblasts were cultured either in the presence of growth medium (GM) or in differentiation medium (DM) for the indicated times as described in Materials and Methods. (A) (Top) Total cell lysates were prepared, separated by SDS-PAGE and immunoblotted with 4G10 anti-phosphotyrosine antibodies (pTyr). The arrows to the right indicate either specific proteins or a molecular mass range in which total tyrosyl-phosphorylation changes are observed during differentiation. The molecular mass markers (Gibco-BRL) are depicted on the left. (Bottom) Representative photomicrographs of C2C12 myoblasts, fixed in methanol and visualized with Wright Giemsa Stain, at each stage of differentiation corresponding to the total tyrosyl-phosphorylation analysis in top panel. (B) C2C12 cell lysates (50 μg) prepared from A were immunoblotted with the indicated antibodies. The positions of molecular mass markers are shown.

RESULTS

Changes in protein tyrosyl phosphorylation and induction of SHP-2 protein expression during C2C12 myogenesis

To characterize the involvement of SHP-2 in myogenesis, we analyzed the expression of SHP-2 protein and total protein tyrosyl phosphorylation during differentiation of the C2C12 myoblast cell line. Upon mitogen withdrawal, C2C12 myoblasts exit the cell cycle and express muscle-specific genes. At this stage of myogenesis (12-24 hours), we observe an increase in tyrosyl phosphorylation of several proteins, notably a 180 kDa protein and proteins within the 100-130 kDa range (Fig. 1A). In contrast, a 35 and a 50 kDa protein undergo tyrosyl dephosphorylation (Fig. 1A). During the early periods of myogenesis (12-24 hours), the levels of SHP-2 protein expression induced are concomitant with the induction of MyoD and myogenin (Fig. 1B). SHP-2 protein expression remains elevated throughout the later stages of myogenesis (48-72 hours), as C2C12 myoblasts undergo terminal differentiation, express myosin heavy chain (Fig. 1B) and fuse to form multinucleated myotubes (Fig. 1A). These data indicate that during C2C12 myogenesis, both tyrosyl phosphorylation and dephosphorylation events occur, and that the expression of SHP-2 is induced concomitant with that of early muscle-specific gene expression.

SHP-2 interacts with a 120 kDa tyrosyl-phosphorylated protein during C2C12 myogenesis

We hypothesized that if SHP-2 is involved in myogenic progression it would interact with tyrosyl-phosphorylated protein(s), via its SH2 domains, during differentiation. To test this possibility, SHP-2 was immunoprecipitated from undifferentiated and differentiating C2C12 myoblasts. The recovered SHP-2 immune complexes were resolved and immunoblotted with anti-phosphotyrosine antibodies. These experiments reveal that SHP-2 complexes with a 120 kDa (p120) tyrosyl-phosphorylated protein in undifferentiated myoblasts (Fig. 2A). In myoblasts undergoing differentiation, there is a transient increase in the mass of this p120 complex between 24 and 72 hours of differentiation (Fig. 2A). SHP-2 itself is tyrosyl-phosphorylated in undifferentiated and in differentiating myoblasts, but undergoes tyrosyl dephosphorylation as terminally differentiated multinucleated

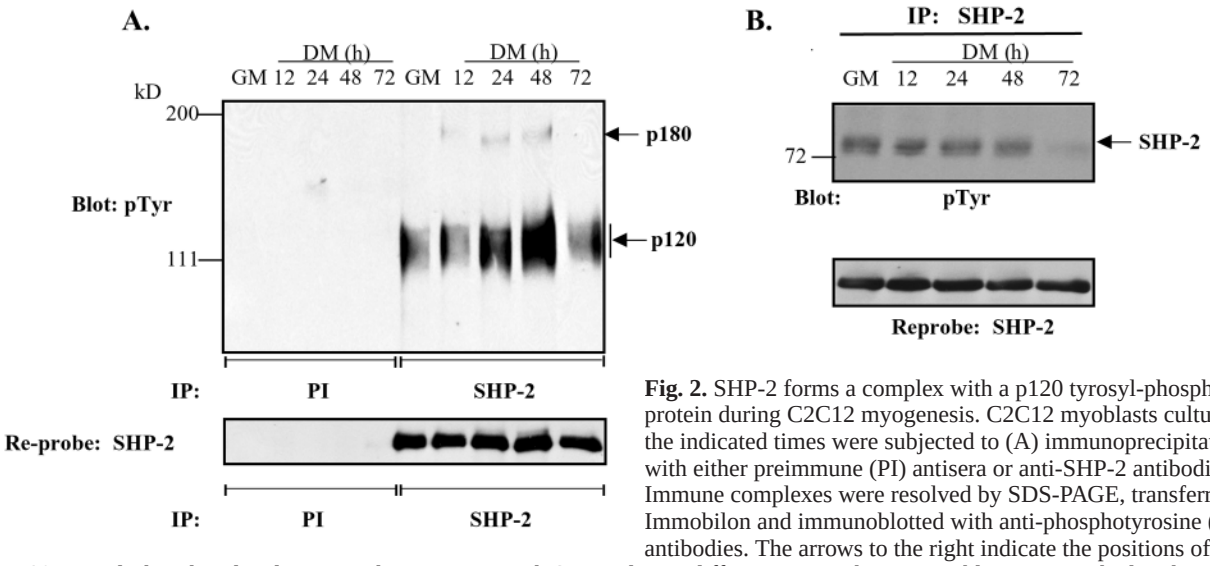


Fig. 2. SHP-2 forms a complex with a p120 tyrosyl-phosphorylated protein during C2C12 myogenesis. C2C12 myoblasts cultured at the indicated times were subjected to (A) immunoprecipitation (IP) with either preimmune (PI) antisera or anti-SHP-2 antibodies. Immune complexes were resolved by SDS-PAGE, transferred to Immobilon and immunoblotted with anti-phosphotyrosine (pTyr) antibodies. The arrows to the right indicate the positions of the p120 and p180 tyrosyl-phosphorylated proteins that associate with SHP-2 during differentiation. The immunoblot was reprobed with anti-SHP-2 antibodies (bottom). (B) Cell lysates from C2C12 myoblasts cultured as indicated were immunoprecipitated with anti-SHP-2 antibodies and immunoblotted with pTyr antibodies. As a control, this immunoblot was reprobed with anti-SHP-2 antibodies (bottom). The positions of molecular mass markers are shown.

myotubes form (Fig. 2B). In addition, we observe the presence of a 180 kDa (p180) tyrosyl-phosphorylated protein in these SHP-2 immune complexes (Fig. 2A). This p180 tyrosyl-phosphorylated protein associates with SHP-2 early on during myogenesis, and then undergoes either tyrosyl dephosphorylation and/or dissociation with SHP-2 between 48 to 72 hours as multinucleated myotubes form (Fig. 2A). These observations demonstrate that during C2C12 myogenic differentiation, SHP-2 forms a complex with at least two tyrosyl-phosphorylated proteins of 120 kDa and 180 kDa.

SHP-2-associated p120 tyrosyl-phosphorylated protein in C2C12 myoblasts is SHPS-1

SHP-2 interacts with a family of tyrosyl-phosphorylated transmembrane glycoproteins known as the SIRPs (Fujioka et al., 1996; Kharitonenkov et al., 1997; Saginario et al., 1998). Due to the glycosylated characteristics of the SIRPs, this family of proteins migrates with a molecular mass ranging from 100 to 150 kDa (Kharitonenkov et al., 1997). To test the possibility that the p120 was a SIRP family member, lysates were prepared from undifferentiated C2C12 myoblasts, and were subjected to immunoprecipitation with anti-SHP-2 antibodies. The immune complexes were immunoblotted using an antibody generated to the conserved cytoplasmic domain of SHPS-1/SIRP-α1, which detects all SIRP-related family members. This SHPS-1/SIRP-α1 antibody cross-reacts in an immune-specific manner with a protein of identical apparent molecular mass as the tyrosyl-phosphorylated p120 protein found in SHP-2 immune complexes (Fig. 3A, left). SHP-2 was recovered in an immune-specific manner, as confirmed by reprobing these immunoblots with anti-SHP-2 antibodies (Fig. 3A, bottom). These data suggest that the p120 SHP-2-interacting protein is a SIRP family member.

The antibodies to SHPS-1 used in the previous experiment are directed to the conserved cytoplasmic domain of the SIRPs, raising the possibility that SHPS-1 may not constitute the specific SIRP isoform bound by SHP-2 in myoblasts (Fig. 3A, left). To determine whether the SHPS-1 isoform binds to SHP-2 in myoblasts, we asked whether SHP-2 could be coimmunoprecipitated using a monoclonal antibody (10C4) that recognizes specifically the first hypervariable Ig (V1) domain of SHPS-1/MFR (Han et al., 2000). For these experiments, we utilized L8 rat myoblasts because this SHPS-1 monoclonal antibody (10C4) crossreacts only with rat SHPS-1 (data not shown). Lysates prepared from undifferentiated L8 myoblasts were subjected to immunoprecipitation with SHPS-1-specific antibodies and immune complexes were immunoblotted either with SHPS-1 antibodies that recognize all SIRP isoforms, or with anti-SHP-2 antibodies. Fig. 3A (right) demonstrates that SHP-2 is present in immune complexes derived from SHPS-1-specific antibodies. Moreover, these immune complexes were also recognized by antibodies directed to the conserved cytoplasmic domain of the SIRPs (Fig. 3A, right). These data indicate that SHP-2 associates specifically with SHPS-1 in C2C12 myoblasts. We also performed RT-PCR analysis for SIRP family members in C2C12 myoblasts, to determine which isoforms are expressed. A single PCR product was generated using degenerate V1 loop primers from cDNA prepared from terminally differentiated C2C12 cultures. Of the ten individual clones isolated, all contained the SHPS-1-specific V1 loop sequence (data not shown). These data, in conjunction with those presented in Fig. 3, indicate that SHPS-1 is the predominant, if not the sole, SIRP family member that forms a complex specifically with SHP-2 in C2C12 myoblasts.

Induction of SHPS-1 protein expression, tyrosyl phosphorylation and association with SHP-2 during C2C12 myogenesis

We next examined the protein expression and tyrosyl phosphorylation of SHPS-1 during myogenic differentiation. Early on during C2C12 myogenesis, the expression of SHPS-1 increases and plateaus by 48 hours into differentiation (Fig. 3B). In order to determine the kinetics of SHPS-1 tyrosyl phosphorylation, SHPS-1 was immunoprecipitated at various

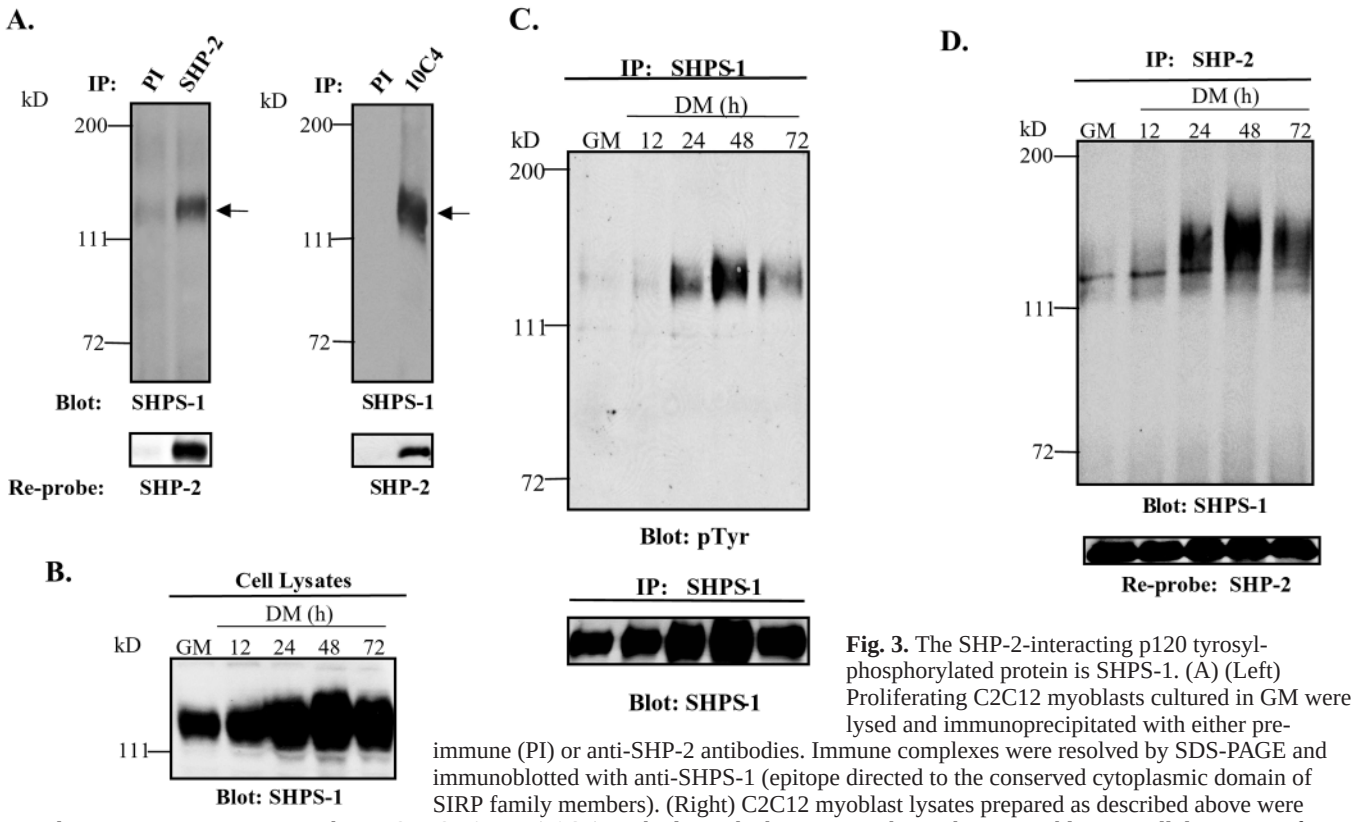


Fig. 3. The SHP-2-interacting p120 tyrosyl-phosphorylated protein is SHPS-1. (A) (Left) Proliferating C2C12 myoblasts cultured in GM were lysed and immunoprecipitated with either pre-immune (PI) or anti-SHP-2 antibodies. Immune complexes were resolved by SDS-PAGE and immunoblotted with anti-SHPS-1 (epitope directed to the conserved cytoplasmic domain of SIRP family members). (Right) C2C12 myoblast lysates prepared as described above were subjected to immunoprecipitation with anti-SHPS-1/MFR (10C4) antibodies, which recognize the V1 hypervariable extracellular region of SHPS-1. Immune complexes were immunoblotted with anti-SHPS-1 antibodies. (Bottom) The above immunoblots were reprobed with anti-SHP-2 antibodies. (B) Lysates were prepared from C2C12 myoblasts cultured in either GM or DM for the indicated times and were immunoblotted with anti-SHPS-1 antibodies. (C,D) Tyrosyl phosphorylation of SHPS-1 and association with SHP-2 during C2C12 myogenesis. Lysates prepared from C2C12 myoblasts cultured in either GM or DM for the indicated times were subjected to immunoprecipitation with either anti-SHPS-1 antibodies, followed by pTyr immunoblotting (C) or immunoprecipitation with anti-SHP-2 antibodies followed by anti-SHPS-1 immunoblotting (D). The lower panel in C represents an equal fraction of the lysates from the experiment above, which was immunoprecipitated for SHPS-1 and immunoblotted with anti-SHPS-1 antibodies as a control. The lower panel in D is a reprobe of the above experiment with anti-SHP-2 antibodies. The positions of molecular mass markers are shown.

times during myogenic differentiation. These immune complexes were immunoblotted with anti-phosphotyrosine antibodies. In addition, SHP-2 was immunoprecipitated from these lysates and immune complexes were immunoblotted with anti-SHPS-1 antibodies. We found that SHPS-1 is basally tyrosyl-phosphorylated, and forms a complex with SHP-2 in undifferentiated myoblasts (Fig. 3C,D, top). Upon initiation of myogenesis, SHPS-1 becomes tyrosyl-phosphorylated between 12 and 24 hours into differentiation (Fig. 3C, top), which correlates with an increase in the level of SHPS-1 in SHP-2 immune complexes (Fig. 3D, top). The level of tyrosyl phosphorylation on SHPS-1 peaks at 48 hours and declines slightly by 72 hours as myoblasts undergo fusion to form multinucleated myotubes (Fig. 3C, top). These data demonstrate that SHPS-1 undergoes an increase in protein expression, becomes tyrosyl-phosphorylated and associates with SHP-2 during C2C12 myogenesis.

SHP-2 interacting p120 tyrosyl-phosphorylated protein is a complex that also contains Gab-1

SHPS-1 is a glycoprotein containing 15 N-glycosylation sites. Upon treatment of SHP-2 immune complexes with endoglycosidase F, SHPS-1 can be deglycosylated, resulting in the generation of a core protein of an approximate molecular mass 60 kDa (Fujioka et al., 1996; Kharitonenkov et al., 1997; Saginario et al., 1995). Our experiments indicate that although SHPS-1 could be deglycoslyated to its 60 kDa core protein, we were unable to reduce completely the mass of the p120 complex to a lower core molecular mass (data not shown). These results suggest that other non-glycosylated tyrosyl-phosphorylated protein(s) might also constitute a component of the p120 complex that associates with SHP-2. Gab-1 is a tyrosyl-phosphorylated multi-substrate docking protein of 120 kDa that binds SH2 domain-containing proteins such as PI-3 kinase, Grb2 and SHP-2. Therefore, we tested whether the p120 complex also contains Gab-1. In Fig. 4A, we show that when lysates from undifferentiated C2C12 myoblasts are subjected to immunoprecipitation with anti-SHP-2 antibodies, the immune complexes contain a protein that is recognized in an immune-specific manner by antibodies to Gab-1. These data indicate that in undifferentiated C2C12 myoblasts, SHP-2 also forms a complex with Gab-1.

Tyrosyl dephosphorylation and constitutive association of Gab-1 with SHP-2 during C2C12 myogenesis

Analysis of Gab-1 during C2C12 differentiation reveals that Gab-1 protein expression remains constant throughout

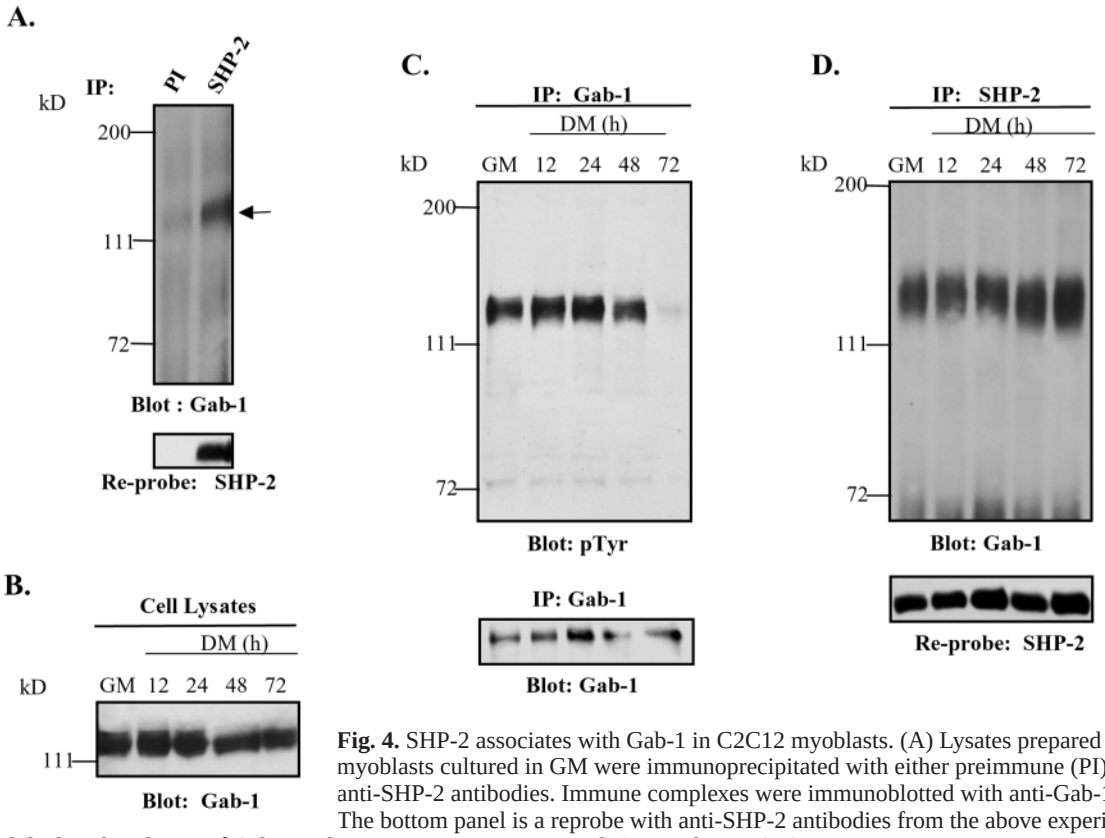


Fig. 4. SHP-2 associates with Gab-1 in C2C12 myoblasts. (A) Lysates prepared from C2C12 myoblasts cultured in GM were immunoprecipitated with either preimmune (PI) antisera or anti-SHP-2 antibodies. Immune complexes were immunoblotted with anti-Gab-1 antibodies. The bottom panel is a reprobe with anti-SHP-2 antibodies from the above experiment. (B,C,D) Tyrosyl dephosphorylation of Gab-1 and constitutive association with SHP-2 during C2C12 myogenesis. Lysates prepared from C2C12 myoblasts were cultured in either GM or DM for the indicated times. (B) Total cell lysates were immunoblotted with anti-Gab-1 antibodies. Lysates prepared from C2C12 myoblasts under the indicated conditions were also subjected to immunoprecipitation with either (C) anti-Gab-1 antibodies followed by pTyr immunoblotting or (D) immunoprecipitation with anti-SHP-2 antibodies followed by anti-Gab-1 immunoblotting. The bottom panel in C represents an equal fraction of the lysates from the experiment above that was immunoprecipitated for Gab-1 and immunoblotted with anti-Gab-1 antibodies as a control. Bottom panel in D is a reprobe with anti-SHP-2 antibodies of the above experiment. The positions of molecular mass markers are shown.

myogenic differentiation (Fig. 4B), in contrast to SHPS-1 (Fig. 3B). To determine the kinetics of Gab-1 tyrosyl phosphorylation during myogenesis, lysates prepared from C2C12 myoblasts at various stages during differentiation were subjected to immunoprecipitation with anti-Gab-1 antibodies. These immune complexes were immunoblotted with antibodies to detect for phosphotyrosine on Gab-1. In undifferentiated myoblasts, Gab-1 is heavily tyrosyl-phosphorylated (Fig. 4C, top). However, later on during myogenesis, Gab-1 undergoes a dramatic decrease in tyrosyl phosphorylation between 48 and 72 hours (Fig. 4C), when multinucleated myotubes form. Surprisingly, when SHP-2 was immunoprecipitated from these same cell lysates, and immune complexes were immunoblotted with antibodies to Gab-1, we found that Gab-1 associates with SHP-2 throughout the entire course of differentiation, even as Gab-1 undergoes tyrosyl dephosphorylation (Fig. 4D). Unlike SHPS-1 (Fig. 3C,D) complex formation between Gab-1 and SHP-2 does not correlate with myogenic progression. Nevertheless, these results show that Gab-1 is a component of the SHP-2-associated p120 complex in differentiating myoblasts.

MyoD induces the association of SHPS-1 and SHP-2 in 10T½ fibroblasts

The data presented in Fig. 3 indicate that the induction of SHPS-1 tyrosyl phosphorylation and association with SHP-2 correlates with the progression of myogenesis. In contrast, Gab-1 tyrosyl phosphorylation and association with SHP-2 does not (Fig. 4). We hypothesized that the initiation and subsequent progression of myogenesis in C2C12 myoblasts induces tyrosyl phosphorylation of SHPS-1 and subsequently its association with SHP-2. To test this, we compared SHP-2 complex formation with SHPS-1 in 10T½ fibroblasts and 10T½ fibroblasts that were converted to the myogenic lineage by stable expression of MyoD (10T½-MyoD). In Fig. 5A, SHP-2 was immunoprecipitated from 10T½ and 10T½-MyoD fibroblasts; the resultant immune complexes were immunoblotted with anti-phosphotyrosine antibodies. In 10T½-MyoD fibroblasts, we observed a dramatic increase in the amount of both p120 and p180 tyrosyl-phosphorylated proteins associated with SHP-2, as well as an increase in the levels of tyrosyl-phosphorylated SHP-2, as compared to 10T½ fibroblasts (Fig. 5A). Importantly, the levels of SHP-2 that were immunoprecipitated between 10T½ and 10T½-MyoD fibroblasts were equivalent (Fig. 5A, bottom panel), as was the total amount of SHPS-1 protein between these two cell lines (Fig. 5B). In addition, SHP-2 immune complexes also contained higher levels of SHPS-1 in 10T½-MyoD as compared to 10T½ fibroblasts (Fig. 5C). This was consistent with the observation that SHPS-1 itself was hypertyrosyl-

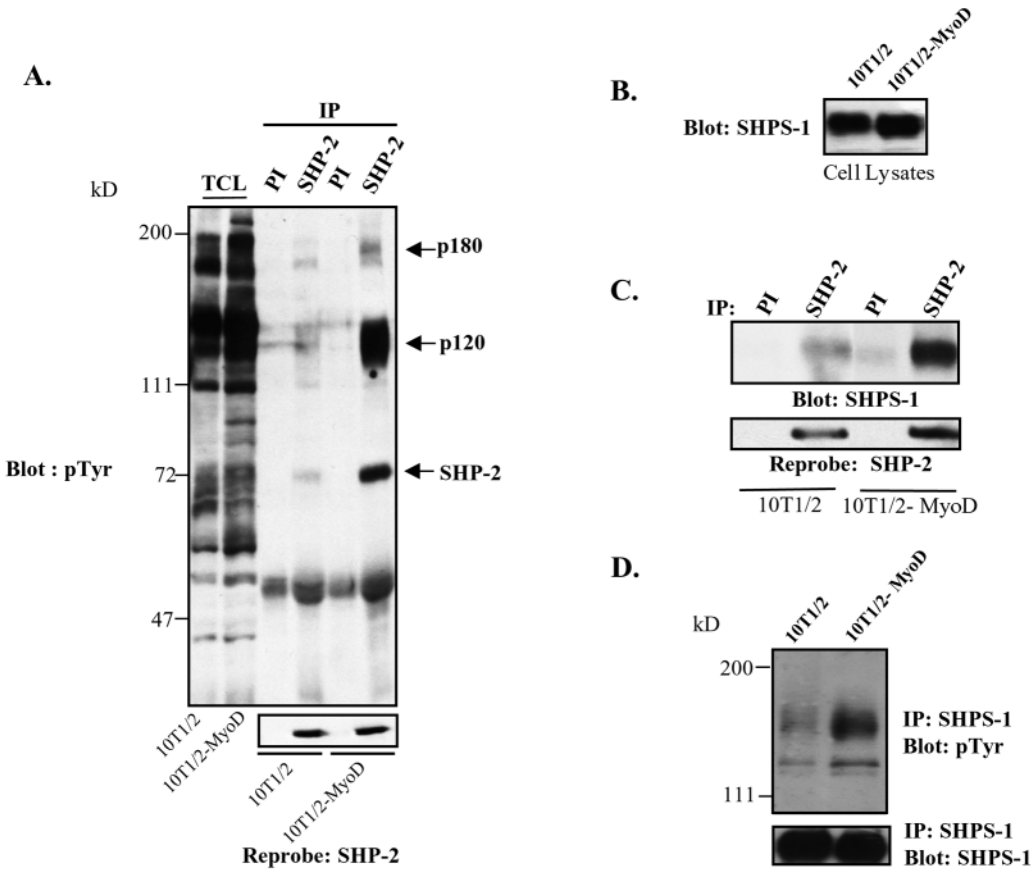


Fig. 5. Induction of SHPS-1 tyrosyl phosphorylation by constitutive expression of MyoD in 10T½ fibroblasts. (A) 10T½ and 10T½-MyoD fibroblasts cultured in GM were subjected to immunoprecipitation with either pre-immune (PI) antisera or anti-SHP-2 specific antibodies. Immune complexes and one-tenth of the total cell lysates (TCL) were resolved and immunoblotted with pTyr antibodies. The bottom panel represents a reprobe of the above immunoblot with anti-SHP-2 antibodies. (B) Equal amounts (50 μg) of total cell lysates from 10T½ and 10T½-MyoD cells were immunoblotted with anti-SHPS-1 antibodies. (C) 10T½ and 10T½-MyoD cells were immunoprecipitated with either pre-immune (PI) or anti-SHP-2 antibodies and immunoblotted for SHPS-1. The bottom panel shows the immunoblot reprobed for SHP-2. (D) Lysates prepared from 10T½ and 10T½-MyoD cells were immunoprecipitated with anti-SHPS-1 antibodies and immune complexes were immunoblotted with pTyr antibodies. (Bottom) SHPS-1 was also immunoprecipitated from a fraction of these same cell lysates and immune complexes were immunoblotted with anti-SHPS-1 antibodies as a control. The positions of molecular mass markers are shown.

phosphorylated in 10T½-MyoD fibroblasts as compared to 10T½ fibroblasts (Fig. 5D). These data demonstrate that the initiation of the muscle differentiation program in fibroblasts, by the expression of MyoD, is sufficient to induce SHPS-1/SHP-2 complex formation.

To substantiate further our observation that C2C12 myogenesis is sufficient to promote SHPS-1 tyrosyl phosphorylation and association with SHP-2, we utilized a 10T½ fibroblast cell line that stably expresses an estradiol-inducible MyoD chimera (MyoD-ER 10T½). These MyoD-ER 10T½ cells can be induced to activate MyoD upon treatment with estradiol. Fig. 6A shows that MyoD-ER 10T½ cells treated with estradiol activate MyoD, as reflected by the induction of MHC expression (Fig. 6A). Activation of MyoD in these MyoD-ER 10T½ cells results in an increase in SHPS-1, but not Gab-1, tyrosyl phosphorylation (Fig. 6B). In addition, an increase in the association of tyrosyl-phosphorylated SHPS-1 with SHP-2 was observed when MyoD-ER 10T½ cells were induced to activate MyoD (Fig. 6C). Collectively, the data presented in Figs 5 and 6 demonstrate that SHPS-1 tyrosyl phosphorylation and its association with SHP-2 occurs as a direct response to the initiation of myogenesis.

p38 MAPK is required for SHPS-1 tyrosyl phosphorylation and association with SHP-2 during myogenesis

In culture, the p38 MAPK inhibitor, SB203580, blocks muscle-specific gene expression and subsequently myogenesis (Kato et al., 1997; Zetser et al., 1999; Zhao et al., 1999b). If SHPS-1 tyrosyl phosphorylation and its association with SHP-2 occur as integral signaling events of myogenic progression, then inhibition of p38 MAPK should block SHPS-1/SHP-2 complex assembly in differentiating C2C12 myoblasts treated with the p38 MAPK inhibitor. Treatment of C2C12 myoblasts prior to the initiation of differentiation with SB203580 (30 μM) blocks myogenesis completely, as indicated by the absence of MyoD induction and multinucleated myotubes (Fig. 7A,B). Despite the fact that MyoD induction and myotube formation are prevented in differentiating C2C12 myoblasts treated with SB203580, the early induction of both SHP-2 and SHPS-1 protein expression is not. This observation suggests that both SHP-2 and SHPS-1 are induced early on during myogenesis independently of the p38 MAPK pathway. When SHPS-1 is immunoprecipitated from C2C12 myoblasts treated with SB203580 the levels of SHPS-1 tyrosyl phosphorylation are inhibited, as compared to control differentiating C2C12 myoblasts (Fig. 7C). Significantly, SHP-2 fails to associate with SHPS-1 in differentiating myoblasts treated with SB203580 (Fig. 7D). Immunoprecipitation of SHPS-1 (Fig. 7C, bottom panel) and immunoblotting with SHPS-1 antibodies or reprobing of the SHP-2 immunoprecipitates with SHP-2 antibodies (Fig. 7D, bottom panel) confirmed that comparable levels of these proteins between control and SB203580-treated cultures were analyzed. Although a

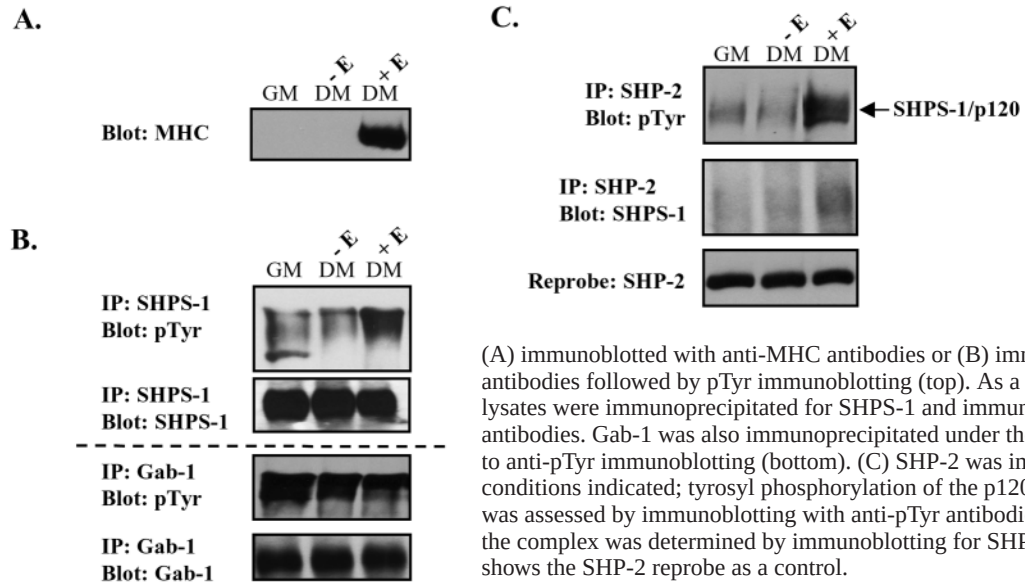
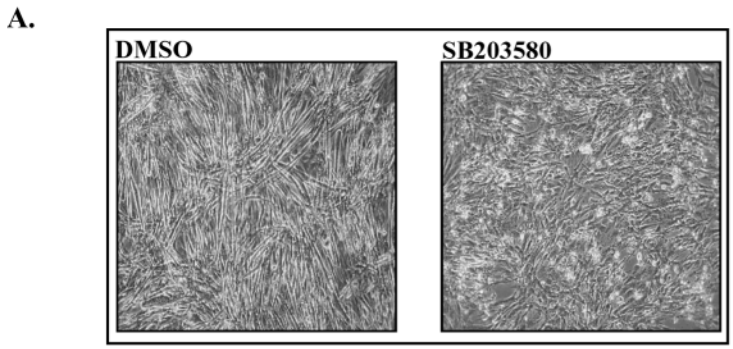
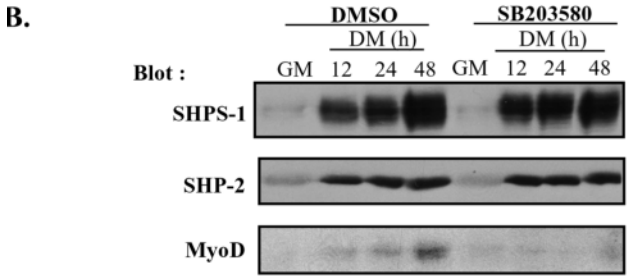


Fig. 6. Inducible activation of MyoD in 10T½ MyoD-ER fibroblasts promotes SHPS-1 tyrosyl phosphorylation and association with SHP-2. 10T½ MyoD-ER cells were cultured in GM or DM in the absence (–E) or presence (+E) of estradiol (0.1 μM) for 18 hours. Cell lysates prepared from these cultures were (A) immunoblotted with anti-MHC antibodies or (B) immunoprecipitated with anti-SHPS-1 antibodies followed by pTyr immunoblotting (top). As a control an equal fraction of these lysates were immunoprecipitated for SHPS-1 and immunoblotted with anti-SHPS-1 antibodies. Gab-1 was also immunoprecipitated under the indicated conditions and subjected to anti-pTyr immunoblotting (bottom). (C) SHP-2 was immunoprecipitated under the conditions indicated; tyrosyl phosphorylation of the p120/SHPS-1 complexed with SHP-2 was assessed by immunoblotting with anti-pTyr antibodies (top) and the amount of SHPS-1 in the complex was determined by immunoblotting for SHPS-1 (middle). The bottom panel shows the SHP-2 reprobe as a control.





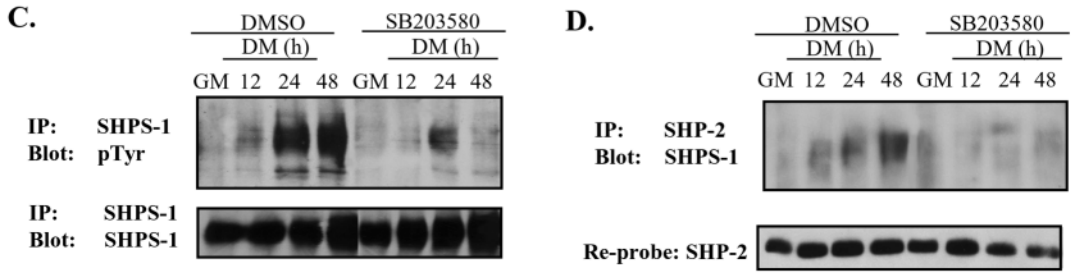


Fig. 7. p38 MAPK promotes MyoD induction, SHPS-1 tyrosyl phosphorylation and association with SHP-2 during myogenesis. (A) Photomicrographs of C2C12 myoblasts treated either with DMSO (0.2% (v/v)) or with 30 μM SB203580 for 72 hours in differentiation medium (DM). (B) C2C12 myoblasts were induced to differentiate in the presence of DMSO as control or SB203580 (30 μM). Cell lysates were prepared from these cultures at the indicated times. These lysates were subjected to immunoblotting with (B) SHPS-1, SHP-2 or MyoD antibodies, (C) SHPS-1 immunoprecipitation followed by pTyr immunoblotting or (D) SHP-2 immunoprecipitation followed by SHPS-1 immunoblotting. The bottom panel in C shows a fraction of the same cell lysates from the above experiments immunoprecipitated with anti-SHPS-1 antibodies and immunoblotted with SHPS-1 antibodies. The bottom panel in D shows the reprobe with anti-SHP-2 antibodies.

residual level of SHPS-1 tyrosyl phosphorylation is observed at 24 hours in SB203580 treated cultures (Fig. 7C), SHP-2 is not complexed with SHPS-1 at this time point. A likely explanation for these results is that SB203580 inhibits tyrosyl phosphorylation on SHPS-1 at a specific residue(s) that mediates complex formation with SHP-2. These experiments demonstrate that p38 MAPK promotes the association of SHPS-1 with SHP-2 in C2C12 myoblasts, and suggest

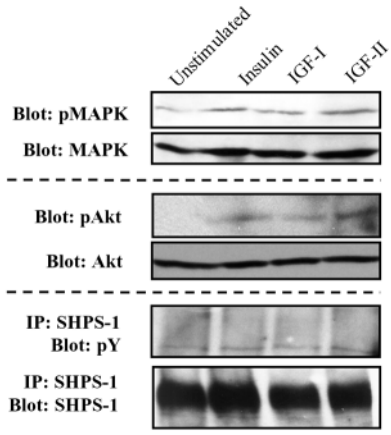


Fig. 8. Insulin, IGF-I and IGF-II fail to induce SHPS-1 tyrosyl phosphorylation. C2C12 myoblasts were either left unstimulated, or were stimulated with insulin, IGF-I or IGF-II following serum deprivation for 6 hours. Cell lysates prepared from C2C12 myoblasts following stimulation with the indicated growth factor were immunoblotted with anti-phospho MAPK and reprobed with anti-MAPK antibodies (top). These cell lysates were also immunoblotted with anti-phospho-Akt antibodies and reprobed with anti-Akt antibodies (middle). These immunoblots were also reprobed with anti-Akt antibodies. SHPS-1 immunoprecipitated from the cell lysates shown in the top panels were immunoblotted with either anti-pTyr or anti-SHPS-1 antibodies (bottom).

that SHPS-1/SHP-2 interactions are linked to the p38 MAPK/MyoD pathway in myoblasts.

Insulin and the insulin-like growth factors fail to induce SHPS-1 tyrosyl phosphorylation in C2C12 myoblasts

The insulin-like growth factors (IGFs) are potent mediators of muscle differentiation, and expression of the IGFs is regulated in an autocrine manner by muscle-specific genes (Florini et al., 1996; Florini et al., 1993; Kou and Rotwein, 1993; Rosen et al., 1993; Stewart and Rotwein, 1996). The IGFs are therefore attractive candidates for mediating SHPS-1 tyrosyl phosphorylation during C2C12 differentiation. To test this, serum-starved (6 hours) C2C12 myoblasts were stimulated with either insulin, IGF-I or IGF-II for 15 minutes. Insulin and the IGFs transduce their downstream signals via both the Ras/Raf/MAPK and the PI 3-K/Akt pathways (Coolican et al., 1997; Fujio et al., 1999; Kaliman et al., 1998; Pinset et al., 1997; Rommel et al., 1999). As expected, stimulation of C2C12 myoblasts with either insulin, IGF-I or IGF-II led to the activation of both MAPK and Akt, as indicated by the detection of Akt and MAPK phosphospecific immunoreactivity (Fig. 8, top and middle). Anti-phosphotyrosine immunoblotting of SHPS-1 immune complexes recovered from these same cells did not, however, result in any detectable SHPS-1 tyrosyl phosphorylation following insulin, IGF-I or IGF-II stimulation (Fig. 8, bottom). These data indicate that in myoblasts, SHPS-1 is not tyrosyl-phosphorylated in response to either insulin, IGF-I or IGF-II.

DISCUSSION

In this report, we present data that lays the foundation towards elucidating the role of SHP-2 in muscle function. We show that SHP-2, an SH2 domain-containing tyrosine phosphatase, interacts with SHPS-1, Gab-1 and a p180 protein in C2C12 myoblasts. Our results further demonstrate that the interaction of SHP-2 with SHPS-1 is a direct consequence of the myogenic process in C2C12 myoblasts. Together, these data suggest a role for SHP-2 and/or SHPS-1 in skeletal muscle differentiation.

SHP-2 is highly abundant in skeletal muscle and may play an important role in muscle development and/or post-developmental muscle function. Our observation that the expression of SHP-2 is induced during the initial periods of differentiation, concomitant with that of MyoD and myogenin induction (Fig. 1), is consistent with data from Mei et al. (1996). This group showed that SHP-2 is induced in differentiating myoblasts prepared from both embryonic mouse limbs and in developing rat muscle in vivo (Mei et al., 1996). In addition to SHP-2 becoming upregulated, the levels of SHPS-1 protein expression and tyrosyl phosphorylation increase during C2C12 differentiation (Figs 3 and 7). This correlates with an increase in the level of SHPS-1 that associates with SHP-2. Originally identified as a hypertyrosyl-phosphorylated protein in fibroblasts overexpressing a catalytically inactive mutant of SHP-2, SHPS-1 has been shown to bind and serve as a substrate for SHP-2 (Fujioka et al., 1996; Timms et al., 1998). The extracellular domain of SHPS-1 contains three Ig domains and a cytoplasmic domain comprising four tyrosyl-phosphorylation sites. Two of these tyrosyl residues are in the sequence context that describes an immunoreceptor tyrosine-based inhibitory motif (ITIM; I/V-X-pY-X-X-L/V/I). These ITIMs bind the SH2 domains of both SHP-2, and its related hematopoietic counterpart SHP-1 (Fujioka et al., 1996; Kharitonenkov et al., 1997; Saginario et al., 1998). SHPS-1 becomes tyrosyl-phosphorylated in response to a variety of growth factors and by integrin-mediated cell adhesion (Fujioka et al., 1996; Kharitonenkov et al., 1997; Ochi et al., 1997; Oh et al., 1999; Takeda et al., 1998; Tsuda et al., 1998). SHPS-1 is suggested to be involved in positive (Fujioka et al., 1996) and negative (Inagaki et al., 2000; Kharitonenkov et al., 1997) growth factor signaling, integrin-mediated signaling (Fujioka et al., 1996; Oh et al., 1999; Tsuda et al., 1998), neurite outgrowth (Abosch and Lagenaur, 1993), inhibition of IgE-induced mast cell activation (Lienard et al., 1999) and macrophage multinucleation (Saginario et al., 1995; Saginario et al., 1998). The functional consequences of SHPS-1 induction in expression, and tyrosyl phosphorylation during differentiation of C2C12 myoblasts, remain to be determined.

In addition to the p180 tyrosyl-phosphorylated protein (Fig. 2), which remains to be identified, we found that SHP-2 forms a complex with Gab-1 in C2C12 myoblasts (Fig. 4). Gab-1 tyrosyl-phosphorylation levels do not change when myoblasts are induced to differentiate (Fig. 3), or if MyoD is activated in the MyoD-ER 10T$\frac{1}{2}$ cells (Fig. 6). However, as myotubes form, Gab-1 undergoes tyrosyl dephosphorylation (Fig. 3), concomitant with that of tyrosyl dephosphorylation of SHP-2 (Fig. 2). Interestingly, we found that the amount of Gab-1 in SHP-2 immune complexes remains unchanged even when Gab-1 becomes dephosphorylated in terminally differentiated multinucleated myotubes (Fig. 4). Three possibilities can be proposed to explain this result. First, it is possible that SHP-2

interacts with Gab-1 in a non-phosphotyrosyl-dependent manner. Second, in myoblasts, SHP-2 could interact indirectly with Gab-1 via a third component. Finally, SHP-2 may interact directly with Gab-1 (Lehr et al., 1999), while SHP-2 and/or other PTPs dephosphorylate Gab-1 at sites other than those with which SHP-2 interacts. Since the level of Gab-1 protein during myogenesis remains constant (Fig. 4), the reduced levels of tyrosyl phosphorylation on Gab-1 during myogenesis are likely catalyzed through the actions of a PTP. There is both genetic and biochemical evidence implicating Gab-1 as an SHP-2 substrate (Herbst et al., 1996; Nishida et al., 1999; Raabe et al., 1996). Gab-1 mediates branching tubulogenesis in epithelial cells (Maroun et al., 1999; Weidner et al., 1996), and may also be involved in similar processes such as branching and/or multinucleation in myoblasts.

The most striking observation of this study is the result that tyrosyl phosphorylation of SHPS-1 and subsequently SHPS-1/SHP-2 complex formation are integral processes of the differentiation of C2C12 myoblasts (Figs 5-7). Three lines of evidence support this conclusion: (1) SHPS-1/SHP-2 association correlates with the induction of MyoD during differentiation (Figs 1 and 3); (2) constitutive expression and inducible activation of MyoD in fibroblasts result in SHPS-1 tyrosyl phosphorylation and association with SHP-2 (Figs 5 and 6) and (3) inhibition of p38 MAPK activity by SB203580 in C2C12 myoblasts blocks SHPS-1 tyrosyl phosphorylation and its association with SHP-2 (Fig. 7). Previous studies from several groups have shown that p38 MAPK is required for skeletal muscle differentiation. (Cuenda and Cohen, 1999; Han et al., 1997; Zetser et al., 1999; Zhao et al., 1999b). Our results support these observations, and further demonstrate that the interaction between SHPS-1 and SHP-2 is also dependent upon p38 MAPK activity in C2C12 myoblasts. As SHP-2 is recruited to SHPS-1 during differentiation, it may subsequently participate in multiple signaling pathways during myogenesis. For example, SHP-2 has been implicated in regulating cell adhesion and cell motility, which are important components of the myogenic process. Indeed, if SHPS-1/SHP-2 complex formation functions downstream of MyoD, then it is reasonable to propose that this complex initiates signaling events which occur in concert with muscle-specific gene expression during myogenesis. It is clear that a substantial amount of work is needed in order to uncover the precise role(s) that SHP-2 plays in skeletal muscle differentiation.

How does SHPS-1 become tyrosyl-phosphorylated during C2C12 differentiation? Several candidate autocrine growth factors that promote myogenesis have been identified. These include primarily the insulin-like growth factors (IGFs) (Florini et al., 1996; Florini et al., 1993). Moreover, a positive relationship between muscle-specific gene expression and IGF production during myogenesis has been reported (Florini et al., 1991; Kou and Rotwein, 1993; Rosen et al., 1993; Stewart and Rotwein, 1996). Interestingly, neither insulin, IGF-I nor IGF-II induce tyrosyl phosphorylation of SHPS-1 in C2C12 myoblasts, despite the activation of both the Erk and PI-3K/Akt pathways (Fig. 8). These observations suggest that growth factors other than insulin/IGFs, and/or other signaling mechanisms regulate SHPS-1 tyrosyl phosphorylation in myoblasts. Consistent with the inability of the IGFs to stimulate SHPS-1 tyrosyl phosphorylation, IGF-I also fails to induce p38 MAPK activation in C2C12 myoblasts (Wu et al., 2000). It will be important to establish how SHPS-1 becomes tyrosyl-phosphorylated during C2C12 myogenesis; this information may also yield insight into how the p38 MAPK pathway is activated.

In summary, we have identified that during C2C12 myogenesis, SHP-2 forms a complex with SHPS-1 and Gab-1. We have found that SHP-2/SHPS-1 interactions are induced as a specific consequence of myogenic progression in C2C12 myoblasts. Further investigation is now underway to determine the functional role of SHP-2, in the context of its interactions with both Gab-1 and SHPS-1, in myoblast growth and differentiation.

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