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Insulin Receptor Substrate protein 53 kDa (IRSp53) is a negative regulator of myogenic differentiation

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ABSTRACT

Fusion of mononucleated myoblasts to generate multinucleated myotubes is a critical step in skeletal muscle development. Filopodia, the actin cytoskeleton based membrane protrusions, have been observed early during myoblast fusion, indicating that they could play a direct role in myogenic differentiation. The control of filopodia formation in myoblasts remains poorly understood. Here we show that the expression of IRSp53 (Insulin Receptor Substrate protein 53 kDa), a known regulator of filopodia formation, is down-regulated during differentiation of both mouse primary myoblasts and a mouse myoblast cell line C2C12. Over-expression of IRSp53 in C2C12 cells led to induction of filopodia and decrease in cell adhesion, concomitantly with inhibition of myogenic differentiation. In contrast, knocking down the IRSp53 expression in C2C12 cells led to a small but significant increase in myotube development. The decreased cell adhesion of C2C12 cells over-expressing IRSp53 is correlated with a reduction in the number of vinculin patches in these cells. Mutations in the conserved IMD domain (*I*RSp53 and *M*IM (missing in metastasis) homology domain) or SH3 domain of IRSp53 abolished the ability of this protein to inhibit myogenic differentiation and reduce cell adhesion. Over-expression of the IMD domain alone was sufficient to decrease the cell–extracellular matrix adhesion and to inhibit myogenesis in a manner dependent on its function in membrane shaping. Based on our data, we propose that IRSp53 is a negative regulator of myogenic differentiation which correlates with the observed down regulation of IRSp53 expression during myoblast differentiation to myotubes.

1. Introduction

Skeletal muscle formation and regeneration depend on the fusion of mono-nucleated cells to generate multinucleated myotubes that further differentiate into myofibers. Skeletal muscle formation can be divided into a series of steps, namely, formation of muscle precursor cells, myoblast proliferation, cell cycle arrest, fusion of myoblast and the formation of multinucleated myotubes (Andres and Walsh, 1996; Charge and Rudnicki, 2004; Rochlin et al., 2010; Nowak et al., 2009). The fusion of myoblasts requires a series of cellular events, including cell–extracellular matrix (ECM) adhesion, cell migration, cell–cell adhesion and membrane fusion (Knudsen and Horwitz, 1977). The actin cytoskeleton plays an essential role in cell adhesion, cell migration and muscle formation (Nowak et al., 2009; Kim et al., 2007; Le Clainche and Carlier, 2008).

Myogenesis is regulated by a number of growth factors including insulin-like growth factors, IGF-I and IGF-II, which play a vital role in skeletal muscle differentiation and growth (Ren et al., 2008). IRSp53 (Insulin Receptor Substrate protein 53 kDa) was initially identified due to phosphorylation on its tyrosine residues upon stimulation by IGF-I (Yeh et al., 1996) and subsequently identified as a WAVE1 interacting protein in a yeast two-hybrid screen (Miki et al., 2000). The formation of myotubes from myoblasts requires morphological changes in cell shape and migration, which involve deformation of membranes (Swailes et al., 2006; Louis et al., 2008) and IRSp53 has been shown to produce membrane protrusion in animal cells (Suetsugu et al., 2006). It has been suggested that membrane protrusions such as filopodia play a critical role in myogenesis (Chen and Olson, 2001; Nowak et al., 2009). IRSp53 is an adaptor protein with an IMD (IRSp53 and missing in metastasis domain) at the N-terminus followed by a central Cdc42/Rac interactive binding (CRIB) domain and a SH3 domain at the C-terminus (Scita et al., 2008). IMD is also referred to as the I-BAR domain (Inverse-*B*IN-Amphiphysins-*R*VS) and it plays a role in F-actin bundling and filopodia formation (Yamagishi et al., 2004). It has been suggested that IRSp53 adopts an auto-inhibited conformation, due to the interaction between the N terminus of IRSp53

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(aa 1–178) and the central region (aa 180–317). Binding of Cdc42 to the CRIB domain of IRSp53 relieves the auto-inhibitory interaction and promotes the formation of filopodia (Krugmann et al., 2001). The SH3 domain of IRSp53 has been shown to bind to a number of proteins, including WAVE1, WAVE2, N-WASP and WIRE (Miki et al., 2000; Lim et al., 2008; Misra et al., 2010).

Cell–ECM adhesion is vital for many cellular processes such as proliferation, differentiation, cell shape changes, actin organization and migration (Enomoto et al., 1993; Boettiger et al., 1995; Velleman and McFarland, 2004). Cell–ECM adhesion is mediated mainly by integrins, a family of transmembrane heterodimeric glycoprotein receptors that physically link extracellular matrix (ECM) to the intracellular cytoskeleton at sites known as focal adhesions (FA) (Liu et al., 2011). Vinculin links the actin cytoskeleton to integrins and is often used as a marker for FA (Adams et al., 1998).

The purpose of the current study was to determine the role of IRSp53 in myogenic differentiation. We found that the expression of IRSp53 was down regulated during the differentiation of myoblast to myotubes. Knocking down the expression of IRSp53 in C2C12 cells enhanced the myogenic differentiation while over-expression of IRSp53 inhibited myogenic differentiation of C2C12 cells. The over-expression of IRSp53 caused a significant reduction in cell–fibronectin adhesion and inhibited the assembly of focal adhesions containing vinculin. The IMD domain of IRSp53 ($IRSp53^{IMD}$) is sufficient to induce filopodia, reduce cell–ECM adhesion and inhibit myogenic differentiation suggesting that the IMD domain of IRSp53 is responsible for negative regulation of myogenic differentiation.

2. Materials and methods

2.1. Cell culture and transfection

Mouse C2C12 cells were maintained in growth media, GM (DMEM/10% fetal bovine serum) at 37 °C in a 5% CO_2 humidified atmosphere. C2C12 cells were microporated using the Neon Transfection System (Invitrogen, CA, USA) according to manufacturer's instructions. Briefly, 5×10^6 cells in 100 μl of resuspension buffer were mixed with 10 μg of plasmid DNA and subjected to three 10 ms pulses at 1650 V. We were able to achieve 80–90% transfection efficiency with this method. Transfected cells were incubated for 36 h in GM before analysis or induced to differentiate by switching to differentiation medium (DM) (DMEM/2% horse serum).

2.2. Mouse Primary myoblasts isolation

Mouse Primary myoblasts were isolated from newborn normal mice (C57BL6) as described previously (Springer et al., 2002). The hind limb muscles were isolated, minced using scalpel and digested with 0.2% collagenase (Sigma, MO, USA) for 1 h. The cells were harvested by centrifugation and seeded in Ham F-10 media with 20% FBS and 2.5 ng/ml bFGF (basic Fibroblast Growth Factor). The cells were grown to confluence and switched to 2% horse serum (HS) containing DMEM for induction of differentiation.

2.3. DNA constructs

Plasmids expressing mouse specific shRNA (psh-IRSp53) and the corresponding scrambled shRNA (pscr-IRSp53) were constructed in pFIV (open Biosystems, CO, USA). Plasmids expressing IRSp53 (pIRSp53) and its mutants ($pIRSp53^{4A}$, $pIRSp53^{2A}$) have been described previously (Misra et al., 2010). Plasmid expressing $IRSp53^{IMD}$ (1–250 aa) and $IRSp53^{SH3}$ (367–521 aa) were generated in the same plasmid.

2.4. Gel electrophoresis and immunoblotting

Cells were lysed using RIPA lysis buffer, the resulting lysate was boiled in SDS-PAGE sample buffer for 5 min, and 30 μg of proteins were resolved on 10% SDS-PAGE gel and transferred onto nitrocellulose membrane. The membrane was probed with appropriate primary antibodies anti-MyHC (Bader et al., 1982) (Developmental Studies Hybridoma Bank, IA, USA), anti-MyoD (Becton, Dickinson and Company, USA), anti-IRSp53 (Santa Cruz Biotechnology, CA, USA) and developed using secondary antibody conjugated with horse radish peroxidase (Sigma–Aldrich, USA). All the experiments were carried out in triplicates and densitometry was performed with Image J analysis software (NIH). Band densities were measured as area under curve. Sample intensity was normalized to GAPDH intensity and Student's *t*-test was performed to determine statistical significance.

2.5. Measurement of fusion index

C2C12 cells undergoing differentiation were fixed and stained with DAPI and anti-MyHC (Bader et al., 1982). We captured six different images from random fields. The fusion index was calculated as the ratio of the nuclei in myotubes to the total number of nuclei in the field. The assays were done in triplicate and the error bars represent the standard deviation of 3 independent experiments.

2.6. Immunofluorescence

C2C12 grown on coverslips were probed with appropriate primary antibodies and alexa-488 secondary antibody while the actin cytoskeleton was visualized using alexa-568 Phalloidin (Misra et al., 2010). Fluorescence images were acquired using Olympus IX51 fitted with Cool $SNAP^{HQ}$ camera and analyzed using Metamorph software (Molecular Devices).

2.7. Cell binding and spreading assay

Human Fibronectin (Sigma) dissolved in sodium bicarbonate buffer was used to coat the wells of 96-well microtiter plates (Misra et al., 2007). C2C12 cells were labeled with Calcein AM (Invitrogen) and added to fibronectin coated wells (20,000 cells per well) and allowed to adhere for 30 min and the bound cells were quantified using a fluorescent plate reader after washing off the unbound cells. The assays were done in triplicate and the error bars represent the standard deviation of 3 independent experiments (Misra et al., 2007). Spreading assay was carried out by adding the cells to wells coated with fibronectin and imaging them at 10 min intervals. The mean surface area was calculated using MetaMorph software (Molecular Devices, CA, USA). The data is an average of 3 independent experiments with a total of 30 cells quantified for each experiment. Quantification of vinculin patches were carried out as described previously (Benoit et al., 2009). We counted the number of vinculin patches in 20 cells per transfection and from three independent experiments.

2.8. Filopodia measurement and analysis

Filopodia induction was analyzed as described (Misra et al., 2010). We analyzed 30 fluorescent cells per transfection (membrane projections between 8 and 20 μm) (Lim et al., 2008) and three independent transfections were performed for each construct. Values presented in bar charts represent mean ± S.D.