PDZRN3 (LNX3, SEMCAP3) is required for the differentiation of C2C12 myoblasts into myotubes

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Summary

PDZRN3 contains a RING-finger motif in its N-terminal region, two PDZ domains in its central region and a consensus-binding motif for PDZ domains at its C-terminus. It was identified in silico as a homolog of the protein known as LNX1 or SEMCAP1, which possesses ubiquitin ligase activity and binds the membrane protein Semaphorin 4C. However, PDZRN3 itself has not previously been characterized. We have now evaluated the properties and functions of PDZRN3. The PDZRN3 gene was shown to be expressed in various human tissues including the heart, skeletal muscle and liver and its expression in mouse skeletal muscle was developmentally regulated. Both the differentiation of C2C12 mouse skeletal myoblasts into myotubes and injury-induced muscle regeneration in vivo were found to be accompanied by up-regulation of PDZRN3. The differentiation-associated increase in the expression of PDZRN3 in C2C12 cells follows that of myogenin and precedes that of myosin heavy chain. Depletion of PDZRN3 by RNA interference inhibited the formation of myotubes as well as the associated up-regulation of myosin heavy chain in C2C12 cells. Our data suggest that PDZRN3 plays an essential role in the differentiation of myoblasts into myotubes by acting either downstream or independently of myogenin.

Key words: PDZRN3, Myogenic differentiation, Skeletal muscle, C2C12 cell

Introduction

PDZ domains were originally identified in the postsynaptic density protein PSD-95, the *Drosophila* tumor suppressor discs-large and the tight junctional protein ZO-1, but they are now known to be present singly or in multiple tandemly repeated copies in a diverse set of proteins (Hung and Sheng, 2002). These domains either bind to a consensus motif that is present at the C-terminus of target proteins or interact with internal peptide fragments of such proteins. They function as a protein-protein interaction module that mediates the clustering of signaling molecules and contributes to the organization of protein networks.

Among PDZ domain-containing proteins, members of the LNX (ligand of NUMB-protein X)/PDZRN (PDZ domain-containing RING finger) family contain an N-terminal RING finger and two or four PDZ domains (Katoh and Katoh, 2004). LNX1 (also known as PDZRN2 or SEMCAP1) and LNX2 (PDZRN1) contain four PDZ domains, whereas LNX3 (PDZRN3 or SEMCAP3) and LNX4 (PDZRN4 or SEMCAP3L) contain two PDZ domains.

LNX1 binds to NUMB (Dho et al., 1998), an intrinsic inhibitor of the conserved Notch signaling pathway that contributes to the control of cell fate and to signal integration during development (Artavanis-Tsakonas et al., 1999; Conboy and Rando, 2002). NUMB is asymmetrically distributed in dividing neural precursors and becomes segregated into one daughter cell, where it inhibits Notch signaling, thereby allowing the two daughter cells to adopt distinct fates (Zhong et al., 1996). LNX1 functions as an E3 ubiquitin ligase that mediates the ubiquitination and degradation of NUMB (Nie et al., 2002). It is therefore thought to augment Notch signaling by reducing the abundance of NUMB. By contrast, LNX3 and LNX4, which contain two PDZ domains instead of the four found in LNX1, have not been characterized at the protein level. Each of these two proteins also possesses a consensus-binding motif for class I PDZ domains at its C-terminus.

We have now examined the properties and functions of LNX3 (PDZRN3). We show that the PDZRN3 gene is expressed in a variety of organs and tissues including the heart, skeletal muscle and liver. In skeletal muscle, the expression of PDZRN3 was found to be developmentally regulated. Furthermore, the protein is essential for differentiation of myoblasts into myotubes.

Results

Molecular structure of PDZRN3

We performed a yeast two-hybrid screen of a human heart cDNA library with the three PDZ domains of rat PSD-95 as the bait to identify PDZ domain-binding proteins. One of the 17 positive clones obtained by screening a total of 5×10^5 clones was found to contain an insert of ~3 kb that includes a putative open reading frame of 1506 bp. The final three amino acids at the C-terminus of the protein encoded by the insert were found to be Thr-Thr-Val, which match the consensus-binding motif for class I PDZ domains. A search of the GenBank database revealed that the insert corresponded to the partial sequence of KIAA1095, which was isolated in a random cloning strategy (Kikuno et al., 1999). The predicted protein

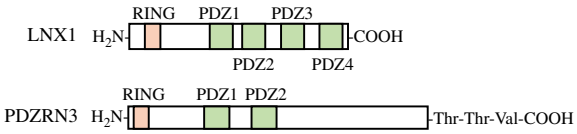

Fig. 1. Schematic representation of the PDZRN family proteins LNX1 (PDZRN2 or SEMCAP1) and PDZRN3 (LNX3 or SEMCAP3). LNX1 contains an N-terminal RING finger and four PDZ domains, whereas PDZRN3 contains an N-terminal RING finger, two PDZ domains and a consensus-binding motif for class I PDZ domains at its C-terminus. RING-finger and PDZ domains are shown as pink and green boxes, respectively.

has also been designated LNX3, SEMCAP3 or PDZRN3. It contains a RING-finger motif in the N-terminal region, two PDZ domains in the central region and the consensus-binding motif for class I PDZ domains at the C-terminus (Fig. 1). LNX3 and SEMCAP3 were deposited in GenBank as homologs of LNX1, an E3 ubiquitin ligase for NUMB (Nie et al., 2002) and of SEMCAP1, a semaphorin cytoplasmic domain-associated protein (Wang et al., 1999), respectively. The gene for this protein has been characterized by bioinformatics and was designated *PDZRN3* (PDZ domain-containing RING finger 3) (Katoh and Katoh, 2004). However, no functional characterization of the protein has been described previously. We use the name PDZRN3 for this protein in the present study.

Tissue distribution of PDZRN3 mRNA

We examined the expression of *PDZRN3* in various adult human tissues by northern blot analysis (Fig. 2). A transcript of ~5.5 kb was abundant in the heart, skeletal muscle and liver and was present in smaller amounts in the brain, colon, small intestine, placenta and lung. We also examined the abundance of PDZRN3 mRNA in mouse skeletal muscle at various times after birth by reverse transcription (RT) and polymerase chain reaction (PCR) analysis. The amount of PDZRN3 mRNA was relatively high until 14 days after birth but had decreased significantly by 21 days (Fig. 3), suggesting that PDZRN3 might be involved in muscle development.

Molecular characterization of PDZRN3

To characterize the properties and functions of PDZRN3 in skeletal muscle, we isolated a mouse PDZRN3 cDNA by PCR from a mouse skeletal muscle cDNA library. Immunoblot analysis revealed that the protein produced by this cDNA in transfected HeLa cells was about the same size (~150 kDa) as that of the endogenous protein in myotubes of the mouse skeletal muscle cell line C2C12 (Fig. 4A), confirming that the clone contains the entire coding region.

Given that PDZRN3 contains both PDZ domains and a C-terminal binding motif for class I PDZ domains, we examined whether the C-terminus binds to the PDZ domains both with a yeast two-hybrid assay and with an in vitro binding assay performed with a glutathione S-transferase (GST) fusion protein of the C-terminal region of PDZRN3 and histidine-tagged PDZ domains. No association of the C-terminus with either of the two PDZ domains was detected (data not shown), indicating that these regions of PDZRN3 do not mediate an intramolecular interaction.

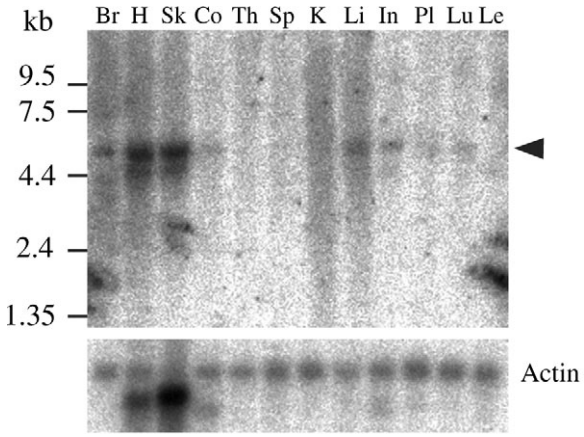

Fig. 2. Northern blot analysis of the tissue distribution of PZDRN3 mRNA. A nylon membrane containing fractionated polyadenylated RNA from adult human tissues was subjected to hybridization with a [^{32}P]-labeled human PDZRN3 cDNA probe. The arrowhead indicates a hybridizing 5.5 kb mRNA. The blot was also probed for β-actin mRNA as a loading control. The positions of molecular size standards are indicated on the left. Br, brain; H, heart; Sk, skeletal muscle; Co, colon; Th, thymus; Sp, spleen; K, kidney; Li, liver; In, small intestine; Pl, placenta; Lu, lung; Le, peripheral blood leukocytes.

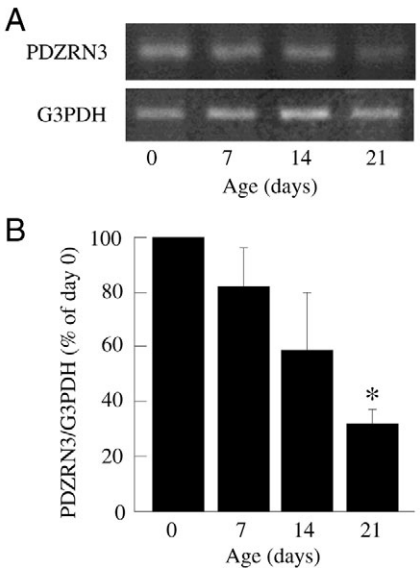

Fig. 3. Abundance of PDZRN3 mRNA in skeletal muscle during postnatal development. (A) Total RNA prepared from skeletal muscle of mice at the indicated ages was subjected to RT-PCR analysis of PDZRN3 and glyceraldehyde-3-phosphate dehydrogenase (G3PDH, control) mRNAs. (B) The amount of PDZRN3 mRNA was quantified by densitometric analysis of gels similar to that shown in (A), normalized by the amount of G3PDH mRNA and expressed as a percentage of the normalized value for day 0. Data are means ± s.e.m. from four independent experiments. *P<0.05 compared with the value for day 0.

PDZRN3 is a homolog of SEMCAP1, the PDZ domain of which binds to the C-terminus of the membrane protein Semaphorin 4C (Wang et al., 1999). We therefore examined whether PDZRN3 also interacts with Semaphorin 4C. We

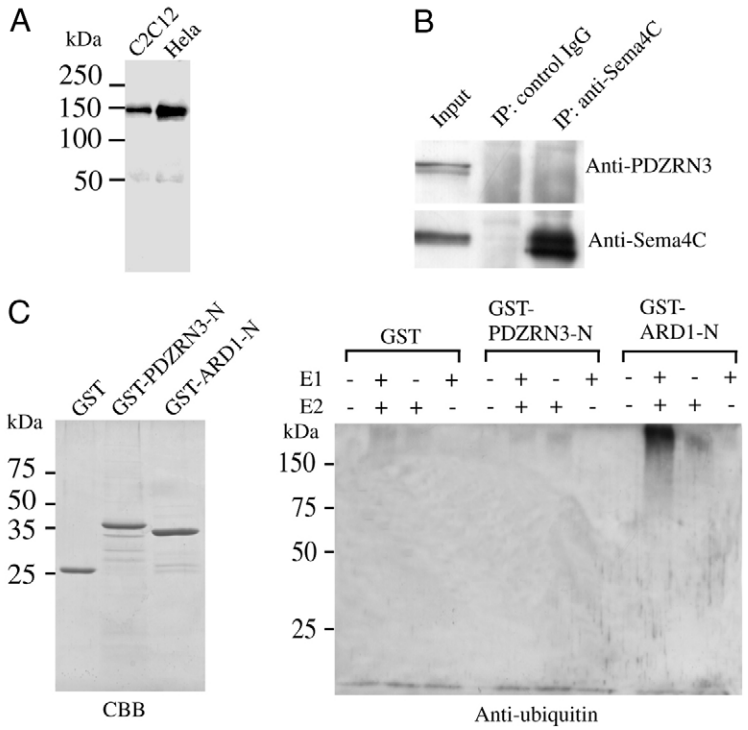

Fig. 4. Molecular characterization of PDZRN3. (A) Lysates of C2C12 myotubes as well as of HeLa cells infected with an adenovirus containing mouse PDZRN3 cDNA were subjected to immunoblot analysis with polyclonal antibodies to PDZRN3. The positions of molecular size standards are indicated on the left. (B) Lysates of C2C12 myotubes were subjected to immunoprecipitation (IP) with rabbit anti-Semaphorin 4C or control immunoglobulin G (IgG) and the resulting precipitates were subjected to immunoblot analysis with monoclonal antibodies to PDZRN3 or to Semaphorin 4C. Myotube lysates were also subjected directly to immunoblot analysis (Input). (C) GST or GST fusion proteins containing the RING-finger domain of PDZRN3 (GST-PDZRN3-N) or of ARD1 (GST-ARD1-N) were incubated with E1 or E2, as indicated, in a ubiquitination assay. The reaction mixtures were then subjected to immunoblot analysis with anti-ubiquitin (right panel). The purity of the GST and GST fusion proteins used for the ubiquitination assay was evaluated by SDS-PAGE and staining with Coomassie brilliant blue (CBB, left panel).

previously showed that C2C12 cells express Semaphorin 4C (Ko et al., 2005) and they also express PDZRN3 (Fig. 4A). Immunoblot analysis revealed that immunoprecipitates prepared from lysates of C2C12 myotubes with antibodies to Semaphorin 4C did not contain detectable amounts of PDZRN3 (Fig. 4B).

Given that the RING-finger domain of LNX1 (SEMCAP1) has been shown to possess E2-dependent E3 ubiquitin ligase activity (Nie et al., 2002), we also examined whether the RING finger of PDZRN3 exhibits such activity with an in vitro ubiquitination assay. Consistent with previous observations (Vichi et al., 2005), immunoblot analysis with antibodies to ubiquitin revealed E2-dependent E3 activity of a GST fusion protein containing the RING-finger domain of ADP-ribosylation factor domain protein 1 (ARD1, positive control) as a smear in the high molecular mass region of the membrane (Fig. 4C). By contrast, no such activity was apparent with a GST fusion protein containing the RING-finger domain of PDZRN3.

Up-regulation of PDZRN3 during myogenic differentiation of C2C12 cells

To explore further the functions of PDZRN3 in skeletal muscle, we examined its expression in the mouse C2C12 myoblast cell line by immunoblot analysis. The abundance of PDZRN3 in C2C12 myoblasts was relatively low, but it increased in association with the induction of myotube differentiation (Fig. 5). Replacement of growth medium with differentiation medium (DM) resulted in the formation of myotubes within 3 days and >80% of the cells had fused to form multinucleated myotubes by 5 days (Fig. 5A). Comparison with the expression profiles of differentiation markers of skeletal muscle revealed that the up-regulation of PDZRN3 followed that of myogenin and preceded that of myosin heavy chain (MHC) (Fig. 5B,C).

Inhibition of myotube formation by RNAi-mediated depletion of PDZRN3

To clarify the role of PDZRN3 in terminal myogenic differentiation, we examined the effects of its depletion by RNA interference (RNAi). Transfection of C2C12 myoblasts with a small interfering RNA (siRNA) specific for PDZRN3 mRNA resulted in marked inhibition of myotube formation and MHC expression induced by culture in DM (Fig. 6A). A control siRNA had no such effects. Immunoblot analysis confirmed that the PDZRN3 siRNA, but not the control siRNA, largely blocked the up-regulation of PDZRN3 and MHC induced by DM (Fig. 6B). It did not affect the up-regulation of MyoD (data not shown) and myogenin (Fig. 6B), however. Consistent with this latter finding, the number of myogenin-expressing cells did not differ between myotubes and PDZRN3-depleted myoblasts 5 days after exposure to DM (Fig. 7). Overexpression of PDZRN3 in C2C12 myoblasts with the use of an adenoviral vector had no marked effect on the DM-induced differentiation of myoblasts into myotubes (data not shown), even though PDZRN3 was found to be necessary for such differentiation (Fig. 6).

Up-regulation of PDZRN3 during injury-induced muscle regeneration in vivo

To determine whether PDZRN3 participates in skeletal muscle differentiation in vivo, we examined its expression in a mouse model of muscle regeneration (Cooper et al., 1999; Figueroa et al., 2003). Muscle damage was induced by intramuscular injection of cardiotoxin and regeneration was allowed to proceed normally (Fig. 8A), as described previously (Ko et al., 2005). Immunoblot analysis revealed that MyoD and myogenin were undetectable in uninjured muscle but were induced maximally at 3-5 days after injury, gradually returning to basal levels thereafter (Fig. 8B). Although PDZRN3 was expressed at a low level before injury, its abundance was also increased 3-5 days after injury. Immunohistofluorescence analysis of regenerating muscle 5 days after injury also revealed the expression of PDZRN3 (Fig. 8C). The abundance of PDZRN3 appeared higher in immature myotubes that expressed myogenin in their nuclei than in more mature

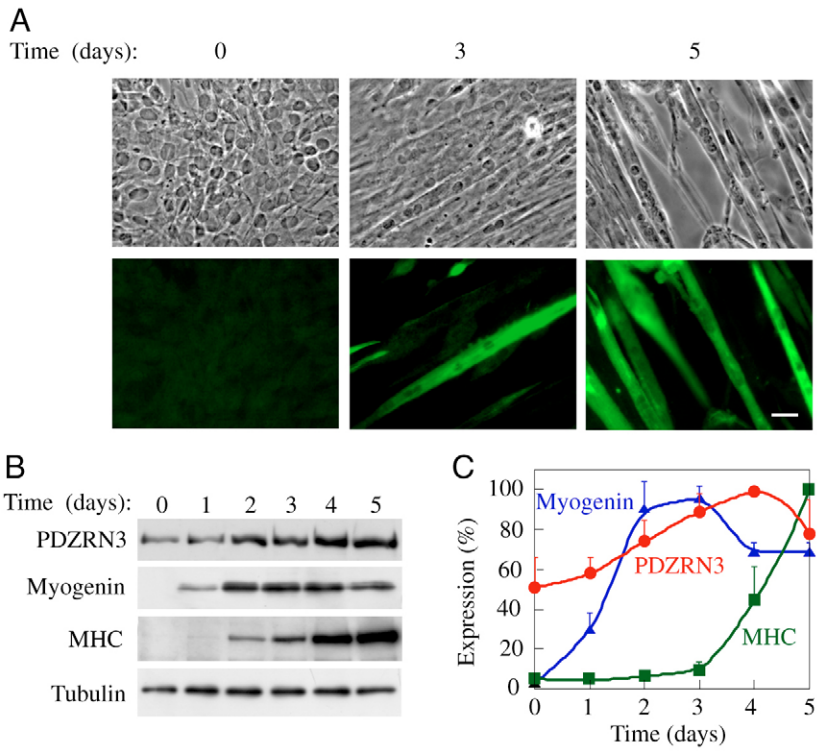

Fig. 5. Up-regulation of PDZRN3 during differentiation of C2C12 myoblasts into myotubes. (A) C2C12 myoblasts induced to differentiate by culture for the indicated times in DM were both examined by phase-contrast microscopy (upper panels) and subjected to immunofluorescence analysis with anti-MHC (lower panels). Bar, 25 μm. (B) C2C12 cell lysates prepared at the indicated times after induction of differentiation were subjected to immunoblot analysis with antibodies to the indicated proteins. (C) The amount of each protein was quantified by image analysis of blots similar to that shown in (B), normalized by the amount of α-tubulin and expressed as a percentage of the maximum normalized value in each experiment. Data are means ± s.e.m. from four independent experiments.

myotubes in which myogenin was not detected. These results thus suggested that PDZRN3 might contribute to terminal myogenic differentiation in vivo.

Discussion

Prior to our study, PDZRN3 had been analyzed in silico as a homolog of LNX1 (Katoh and Katoh, 2004) and had also been designated SEMCAP3 as a homolog of SEMCAP1, the latter of which was shown to bind the transmembrane protein Semaphorin 4C (Wang et al., 1999). However, no functional characterization of this protein had been described. We have now evaluated the properties and functions of PDZRN3. Whereas LNX1/SEMCAP1 possesses E3 ubiquitin ligase activity attributable to its RING-finger domain as well as Semaphorin 4C-binding activity attributable to its PDZ domain, we found that PDZRN3 exhibited neither activity. The PDZRN3 gene was shown to be expressed in various human tissues, including the heart, skeletal muscle and liver, and its expression was developmentally regulated in mouse skeletal muscle. In addition, we have shown that both the differentiation of C2C12 mouse skeletal myoblasts into myotubes and injury-induced muscle regeneration in vivo are accompanied by up-regulation of PDZRN3. The differentiation-associated increase in the expression of PDZRN3 in C2C12 cells follows that of the myogenic transcription factor myogenin and precedes that of MHC. Depletion of PDZRN3 by RNAi revealed that PDZRN3 plays an essential role in the differentiation of C2C12 myoblasts into myotubes. Given that the regeneration of skeletal muscle after injury occurs by a process that is highly similar to embryonic muscle differentiation (Snider and Tapscott, 2003), the up-regulation of PDZRN3 during injury-induced muscle regeneration suggests that PDZRN3 participates in myogenic differentiation not only in cultured cells but also in vivo.

Members of a family of conserved basic helix-loop-helix (bHLH) transcription factors that include MyoD, Myf5 and myogenin are key regulators of the specification and differentiation of muscle progenitors (Pownall et al., 2002). MyoD and Myf5 are expressed in proliferative myoblasts (Emerson, 1990) and are implicated in the establishment and maintenance of muscle progenitor lineages (Pownall et al., 2002), whereas myogenin is activated during myoblast differentiation (Wright et al., 1989) and regulates the transcriptional activation of muscle contractile protein genes (Lassar et al., 1991). Our observations that depletion of PDZRN3 by RNAi resulted in inhibition of myogenic differentiation but not of the expression of MyoD and myogenin indicate that PDZRN3 participates in signaling downstream of myogenin or independently of myogenin during differentiation of myoblasts into myotubes.

The RING-finger domain is a cysteine-rich motif that forms a cross-brace structure that is able to chelate two divalent zinc ions (Freemont et al., 1991). Some proteins that contain such a domain function as E3 ubiquitin ligases (Joazeiro et al., 1999; Lorick et al., 1999). Given that Notch signaling negatively regulates MyoD activity (Kuroda et al., 1999) and that LNX1 is an E3 ubiquitin ligase that mediates the ubiquitination and degradation of NUMB, an inhibitor of Notch signaling (Nie et al., 2002), it seemed possible that PDZRN3 might also function as an E3 for NUMB in the differentiation of myoblasts into myotubes. However, the RING finger of PDZRN3 was found not to manifest E2-dependent E3 ubiquitin ligase activity in an in vitro assay and depletion of PDZRN3 in C2C12 myoblasts had no substantial effect on the expression of MyoD or myogenin. PDZRN3 thus does not appear to function as an E3 for NUMB during myogenic differentiation.

In addition to myogenic transcription factors, various proteins including integrins (Schwander et al., 2003), cadherin (Goichberg et al., 2001), β-catenin (Martin et al., 2002), immunoglobulin superfamily members (Kang et al., 1998; Kang et al., 2002), small GTPases (Chen et al., 2003) and an inward rectifier K^+ channel (Konig et al., 2004) have been implicated in regulation of the differentiation of myoblasts into myotubes. We recently showed that up-regulation of

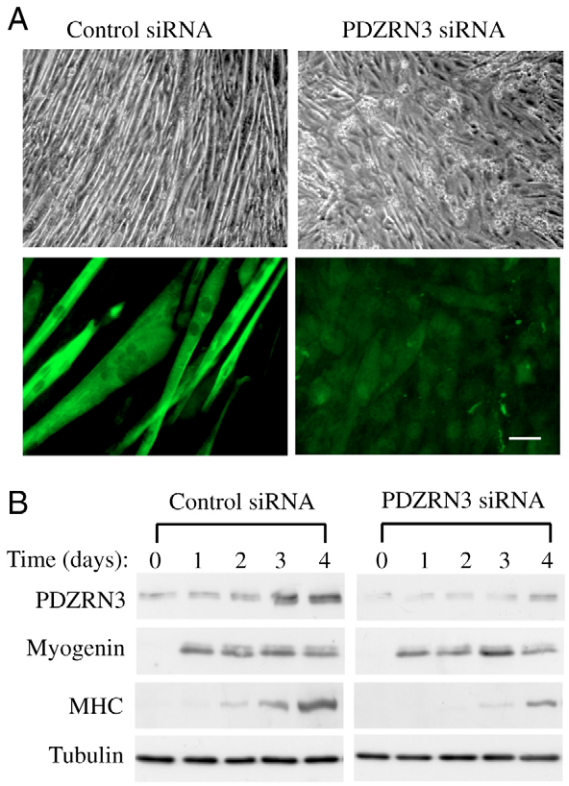

Fig. 6. Inhibition of myoblast differentiation by depletion of PDZRN3. (A) C2C12 cells were transfected with a control siRNA or an siRNA specific for PDZRN3 mRNA and were induced to differentiate by culture for 4 days in DM. The cells were then examined by phase-contrast microscopy (upper panels) and subjected to immunofluorescence analysis with anti-MHC (lower panels). Bar, 25 μm. (B) Lysates of cells treated as in (A) and cultured in DM for the indicated times were subjected to immunoblot analysis with antibodies to the indicated proteins.

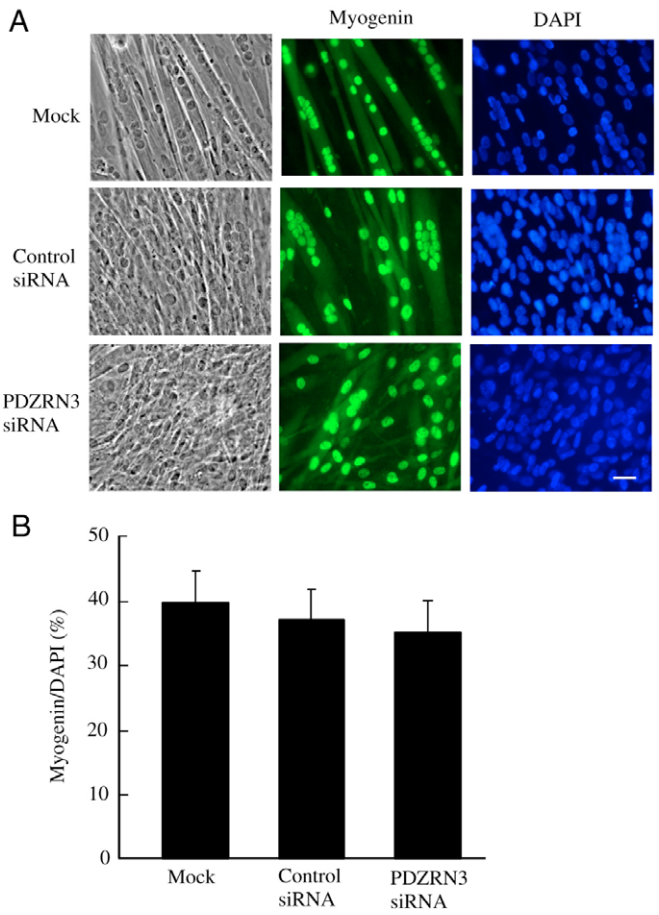

Fig. 7. Lack of effect of PDZRN3 depletion on expression of myogenin. (A) C2C12 cells were transfected or not (Mock) with a control siRNA or an siRNA specific for PDZRN3 mRNA and were induced to differentiate by culture for 5 days in DM. They were then examined by phase-contrast microscopy (left panels) and subjected to immunofluorescence analysis with anti-myogenin (middle panels). Nuclei were also stained with 4′,6-diamidino-2-phenylindole (DAPI, right panels). Bar, 25 μm. (B) The percentage of DAPI-stained nuclei that were positive for myogenin was determined in each of 10 randomly chosen fields (magnification, 200×) for cells treated as in (A). Data are means ± s.e.m. from a representative experiment.

Semaphorin 4C is also essential for myogenic differentiation (Ko et al., 2005). The role of Semaphorin 4C in myogenic differentiation differs from that of PDZRN3 in that the former is necessary for up-regulation of myogenin whereas the latter is not. We have also now shown that PDZRN3 does not possess Semaphorin 4C-binding activity, even though its homolog LNX1/SEMCAP1 does bind to Semaphorin 4C (Wang et al., 1999).

Although we have shown that PDZRN3 is essential for the differentiation of C2C12 myoblasts into myotubes in vitro and that PDZRN3 is up-regulated during muscle regeneration in vivo, it remains to be determined whether PDZRN3 is required for muscle differentiation and muscle regeneration in vivo. Some proteins, including MyoD (Dedieu et al., 2002) and p53 (Porrello et al., 2000), have been found to be essential for terminal myogenic differentiation in vitro, but no abnormalities of skeletal muscle have been detected in the corresponding knockout mice (Rudnicki et al., 1992; White et al., 2002). In the case of MyoD, this discrepancy has been attributed to functional redundancy with Myf5 (Rudnicki et al., 1993).

In addition to two PDZ domains in its central region, PDZRN3 possesses a consensus-binding motif for class I PDZ domains at its C-terminus. The C-terminus of PDZRN3 did not bind to the PDZ domains of this protein, however, suggesting that PDZRN3 may function as an adapter protein that interacts with other proteins through both its PDZ domains and its C-terminus. The observations that it is expressed in various tissues and that cell fusion does not occur in these tissues suggest that PDZRN3 contributes to different activities in different cell types in a manner dependent on its binding partners. Further characterization of the functions of PDZRN3 and its mechanisms of action will require identification of such binding proteins.

Materials and Methods

Antibodies

Polyclonal antibodies to PDZRN3 or to Semaphorin 4C were generated in rabbits with histidine-tagged C-terminal fragments of human PDZRN3 (amino acids 1034 to 1066) or of mouse Semaphorin 4C (amino acids 689 to 834) as antigens. The antibodies were purified by affinity chromatography with GST fusion proteins of the corresponding antigens. Monoclonal antibodies to PDZRN3 were prepared from a hybridoma produced by fusion of mouse myeloma cells and splenocytes from a

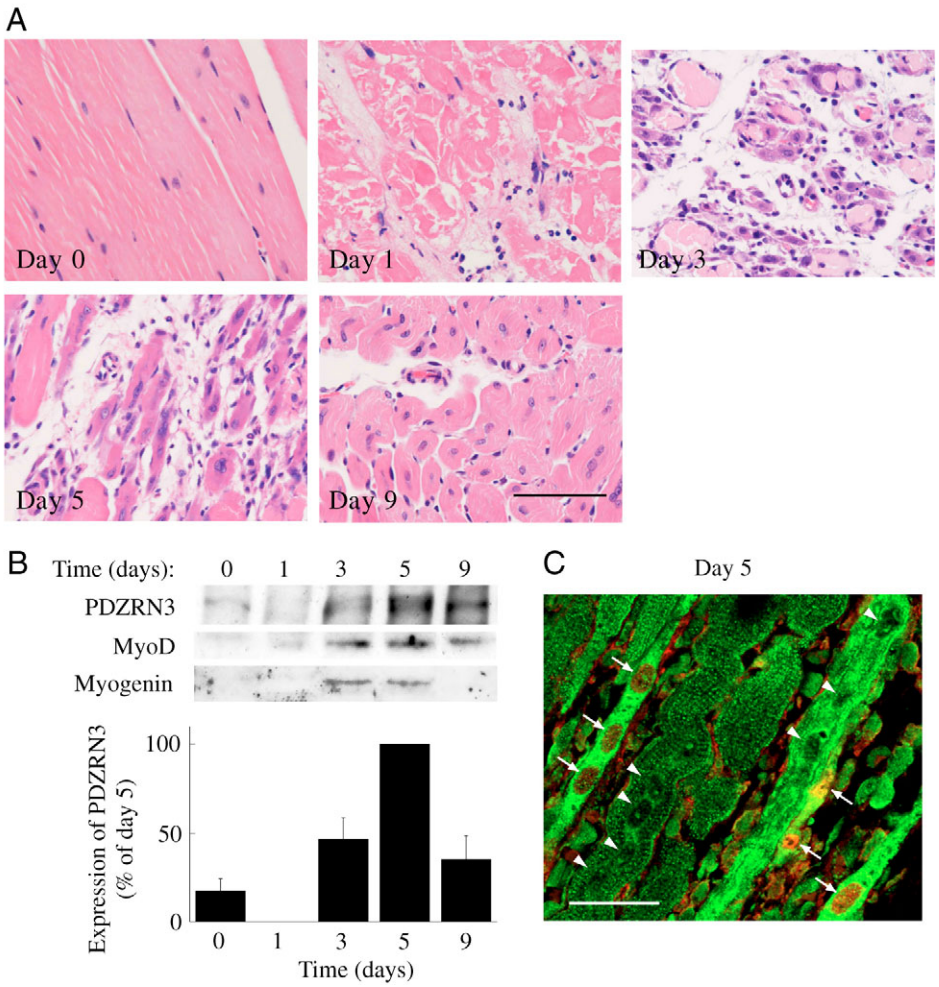

Fig. 8. Expression of PDZRN3 during regeneration of damaged mouse skeletal muscle. (A) Muscle injury and regeneration were induced by intramuscular injection of cardiotoxin in the hind limb and sections of muscle isolated before or 1, 3, 5, or 9 days after injury were stained with hematoxylin-eosin. Bar, 50 μm. (B) Homogenates of muscle isolated at the indicated times after injury were subjected to immunoblot analysis with antibodies to the indicated proteins. The abundance of PDZRN3 was quantified by image analysis of blots similar to that shown in the upper panel and expressed as a percentage of the value at 5 days after injury (lower panel); data are means ± s.e.m. from three independent experiments. (**C**) A section of muscle at 5 days after injury was double-stained with anti-PDZRN3 (green) and anti-myogenin (red). Myogenin-positive nuclei are indicated by arrows. Myogenin-negative nuclei in myotubes are indicated by arrowheads. Bar, 25 μm.

mouse injected with the histidine-tagged C-terminal fragment of human PDZRN3 used for injection of rabbits. Rabbit polyclonal antibodies to MyoD (sc-304) and to myogenin (sc-576) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal antibodies to MHC (M-4276), to α-tubulin (T-9026) and to ubiquitin (U-0508) were from Sigma (St Louis, MO), and those to myogenin (sc-12732) and to Semaphorin 4C (612487) were from Santa Cruz Biotechnology and BD Biosciences (San Jose, CA), respectively.

Yeast two-hybrid analysis

Yeast two-hybrid screening and assays were performed with a Matchmaker Gal4 Two-Hybrid System (Clontech, Mountain View, CA). The cDNA sequence corresponding to the region of rat PSD-95 containing its three PDZ domains (amino acids 65 to 400) was subcloned into pGBTK7 in-frame with the DNA sequence for the DNA binding domain of GAL4 and the resulting plasmid was introduced into the *Saccharomyces cerevisiae* AH109 reporter strain. The yeast cells were subsequently transformed with a human heart cDNA library (Clontech) and plated on medium-stringency selection plates that lacked His, Leu and Trp. The resulting colonies were replica-plated on plates lacking His, Leu and Trp, and a colony-lift assay was subsequently performed to detect expression of β-galactosidase. Plasmids from positive colonies were rescued and sequenced.

Northern blot analysis

A human 12-lane MTN blot membrane (Clontech) was used for northern blot analysis. The probe for PDZRN3 mRNA was prepared by PCR with a cDNA fragment corresponding to the first PDZ domain (amino acids 238 to 339) of KIAA1095 as the template, with a random primer labeling system (Rediprime II; Amersham, Piscataway, NJ) and in the presence of [α-^{32}P]dATP.

RT-PCR

Total RNA was extracted from mouse leg skeletal muscle at various stages after birth with the use of an SV total RNA isolation system (Promega, Madison, WI). Portions (100 ng) of the RNA were subjected to RT and the resulting cDNA was subjected to PCR with a protocol designed to maintain amplification in the exponential phase. The sequences of the PCR primers (sense and antisense, respectively) were 5′-CTGACTCTTGTCCTGCATCGGGACTC-3′ and 5′-ATGGGCTCCTTGGCTGTCTTGAAAGC-3′ for PDZRN3 and 5′-ACCACAGTCCATGCCATCAC-3′ and 5′-TCCACCACCCTGTTGCTGTA-3′ for G3PDH.

Cloning of mouse PDZRN3 cDNA

Mouse PDZRN3 cDNA was amplified by PCR from a mouse skeletal muscle cDNA library with primers (sense, 5′-CACCATGGCCCCACACTGGGCTGTCTGGC-3′; antisense, 5′-TCATACTGAAGACTCCTCTGGGTTG-3′) based on the sequence of mouse SEMCAP3A cDNA (GenBank accession no. AF127084) and was then cloned into pENTR/D-TOPO (Invitrogen, Calsbad, CA). The sequence of the cloned cDNA was verified by DNA sequencing. For adenovirus-mediated expression, the cDNA was transferred from the pENTR vector into pAd/CMV/V5-DEST (Invitrogen) and the resulting construct was introduced into 293A cells by transfection to produce the recombinant virus.

Cell culture

C2C12 cells were obtained from American Type Culture Collection (Manassas, VA) and were maintained under 5% CO_2 at 37°C in growth medium (GM) comprising Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% fetal bovine serum. Differentiation of myoblasts into myotubes was induced when the cells had achieved 90 to 95% confluence by switching the medium from GM to differentiation medium (DM) consisting of DMEM supplemented with 2% horse serum.

Ubiquitination assay

Bacterial expression vectors for the synthesis of GST fusion proteins containing the N-terminal region of mouse PDZRN3 (amino acids 1-256) or of human ARD1 (amino acids 1-110) were constructed by introducing the corresponding cDNAs into pDEST 15 (Invitrogen). The fusion proteins were purified from bacterial lysates with glutathione-Sepharose beads (Amersham). In the standard ubiquitination assay condition, the reaction mixture (90 μl) contained 0.3 μg of purified rabbit E1 (Biomol, Plymouth Meeting, PA), 1.5 μg of recombinant human UbcH6 (E2)

(Biomol), 15 μg of ubiquitin (Sigma), 4 mM ATP and 10 pmol of GST or the GST fusion protein containing the N-terminal region of PDZRN3 in ubiquitination buffer [0.1 mM dithiothreitol, 2 mM $MgCl_2$, 20 mM Tris-HCl (pH 7.4)]. The GST fusion protein containing the RING-finger domain in the N-terminal region of ARD1was used as a positive control (Vichi et al., 2005). After incubation at 30°C for 60 minutes, the reaction was terminated by the addition of SDS sample buffer (Laemmli, 1970) and the resulting mixture was subjected to SDS-polyacrylamide gel electrophoresis (PAGE) followed by immunoblot analysis with antibodies to ubiquitin.

Immunoprecipitation assay

Myotubes of C2C12 cells in a 10 cm dish were solubilized with 1% Triton X-100 in a solution containing 150 mM NaCl and 20 mM Tris-HCl (pH 7.5). After centrifugation to remove debris, the extract was mixed with 2 μg of rabbit polyclonal antibodies to Semaphorin 4C or control IgG and with 50 μl of protein G-Sepharose beads (Amersham). The mixture was incubated overnight at 4°C, after which the beads were washed five times and bound proteins were then solubilized in SDS sample buffer and subjected to SDS-PAGE followed by immunoblot analysis with mouse monoclonal antibodies to PDZRN3 or to Semaphorin 4C.

Immunoblot analysis

Skeletal muscle was weighed and then homogenized with a Polytron homogenizer in 5 volumes (per unit wet mass) of 0.3 M sucrose containing 5 mM Hepes-NaOH (pH 7.5). C2C12 cells cultured in 24-well plates were washed twice with phosphate-buffered saline and then lysed in 200 μl of a solution containing 150 mM NaCl, 2% SDS, 5 mM EDTA and 20 mM Tris-HCl (pH 7.5). The whole tissue homogenates and cell lysates were fractionated by SDS-PAGE and the separated proteins were transferred to a nitrocellulose membrane. The membrane was exposed consecutively to primary antibodies and horseradish peroxidase-conjugated secondary antibodies (Promega). Immune complexes were detected with enhanced chemiluminescence reagents (Amersham). Primary antibodies included mouse monoclonal antibodies to MHC, myogenin, or α-tubulin as well as rabbit polyclonal antibodies to MyoD, myogenin, or PDZRN3.

RNAi

Control and PDZRN3 mRNA-specific siRNAs were obtained in duplex form from Qiagen (Valencia, CA). The sequences targeted by the siRNAs were 5′-AATTCTCCGAACGTGTCACGT-3′ for the non-silencing control and 5′-AAGTCAGACAAGGATAGTTCA-3′ for PDZRN3. C2C12 cells in 24-well plates were transfected with siRNAs (1 μg per well) with the use of RNAiFect (Qiagen); the first transfection was performed in GM when the cells had achieved 50-60% confluence and the second and third transfections were performed in DM at intervals of 24 hours (when the cells were 70-80% and ~100% confluent, respectively).

Immunofluorescence analysis

C2C12 cells were grown on 15 mm cover glasses coated with 0.2% gelatin and were fixed and permeabilized for 5 minutes at –20°C with methanol. The cells were then incubated for 30 minutes at room temperature with 1% bovine serum albumin and exposed consecutively to mouse monoclonal anti-MHC or anti-myogenin and Alexa Fluor 488-conjugated goat antibodies to mouse IgG (Molecular Probes, Eugene, OR).

Muscle regeneration assay

Mice (BALB/c, 12 weeks old) were anesthetized by intraperitoneal injection of pentobarbital. Skeletal muscle was injured by intramuscular injection of 400 μl of 10 μM cardiotoxin (Latoxan, Valence, France) into hind limbs as described previously (Ko et al., 2005). Soleus and gastrocnemius muscles were examined before and at various times after injury with three animals per time point. For histological analysis, muscle was dissected, fixed with 4% paraformaldehyde, embedded in paraffin, sectioned and stained with hematoxylin-eosin. For immunohistofluorescence analysis, the sections were double-stained with rabbit polyclonal antibodies to PDZRN3 and mouse monoclonal antibodies to myogenin followed by Alexa Fluor 488-conjugated goat antibodies to rabbit IgG and Alexa Fluor 594-conjugated goat antibodies to mouse IgG. The images were recorded with a confocal laser-scanning microscope (Olympus CLSM GB-200). These experiments were approved by the Animal Ethics Committee of Yamaguchi University School of Medicine.

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