

## Analysis of a Shc Family Adaptor Protein, ShcD/Shc4, That Associates with Muscle-Specific Kinase<sup>∇</sup>

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**Shc family proteins serve as phosphotyrosine adaptor molecules in various receptor-mediated signaling pathways. In mammals, three distinct Shc genes have been described that encode proteins characterized by two phosphotyrosine-interaction modules, an amino-terminal phosphotyrosine binding (PTB) domain and a carboxy-terminal Src homology 2 domain. Here, we report the analysis of an uncharacterized fourth Shc family protein, ShcD/Shc4, that is expressed in adult brain and skeletal muscle. Consistent with this expression pattern, we find that ShcD can associate via its PTB domain with the phosphorylated muscle-specific kinase (MuSK) receptor tyrosine kinase and undergo tyrosine phosphorylation downstream of activated MuSK. Interestingly, additional sites of tyrosine phosphorylation, including a novel Grb2 binding site, are present on ShcD that are not found in other Shc family proteins. Activation of MuSK upon agrin binding at the neuromuscular junction (NMJ) induces clustering and tyrosine phosphorylation of acetylcholine receptors (AChRs) required for synaptic transmission. ShcD is coexpressed with MuSK in the postsynaptic region of the NMJ, and in cultured myotubes stimulated with agrin, expression of ShcD appears to be important for early tyrosine phosphorylation of the AChR. Thus, we have characterized a new member of the Shc family of docking proteins, which may mediate a specific aspect of signaling downstream of the MuSK receptor.**

Shc proteins comprise a family of adaptor molecules that function as molecular scaffolds in various signaling pathways, including those mediated by receptor tyrosine kinases (RTKs), cytokine receptors, extracellular matrix molecules, and oncogenic tyrosine kinases. Shc family proteins possess an amino-terminal phosphotyrosine (pTyr) binding (PTB) domain in addition to a carboxy-terminal Src homology 2 (SH2) domain, both of which can bind to phosphorylated tyrosine residues on cell-surface RTKs and other signaling proteins (41). Shc-related proteins are present in a wide array of vertebrates and invertebrates, suggesting that they are functionally important, and indeed mutations in both *Drosophila* and murine *Shc* genes can cause embryonic lethal phenotypes (20, 23).

Of the mammalian Shc proteins, ShcA (originally denoted Shc, for *Src* homologous and collagen) is the most well characterized (37). The PTB domain of ShcA binds pTyr-containing peptides within the consensus sequence Asn-Pro-X-pTyr (NPXpY), with additional selectivity for Ile or Val at the –5 position relative to the pTyr (13, 17, 54), whereas the SH2 domain preferentially recognizes pTyr-X-X-Φ sequences, where Φ is a bulky hydrophobic amino acid (47). Recruitment of ShcA to activated receptors via its PTB or SH2 domain

results in phosphorylation of ShcA on two tyrosine-based motifs in the central collagen homology 1 (CH1) region that serve as selective binding sites for the SH2 domain of the Grb2 adaptor (43), as well as other SH2-containing proteins, such as Grb7 (48) and SHIP (40). ShcA-Grb2 complexes can potentially activate Ras and its downstream effectors, in addition to phosphatidylinositol 3-kinase (16, 42). The adaptor function of ShcA is enhanced by the presence of multiple proline-rich sequences in the CH1 and amino-terminal collagen homology 2 (CH2) regions which mediate binding to Src homology 3 (SH3) domain-containing proteins, and the central CH1 region also possesses a motif that can recruit clathrin-associated adaptor components of receptor endocytosis complexes (34).

The human *ShcA* locus encodes three overlapping isoforms of 46, 52, and 66 kDa that are produced as a result of alternative mRNA splicing and differential translation initiation codon usage (37). In addition to ShcA (also named Shc1), two other mammalian Shc proteins have been described: ShcB (also known as Sli/SCK and Shc2) (18, 36) and ShcC (also known as Rai/N-Shc and Shc3) (30, 32, 36). Despite strong similarities in sequence, these Shc family proteins have distinct biological functions, likely due to differences in expression patterns. Murine ShcB and ShcC are primarily expressed in the nervous system, while ShcA is widely expressed, with the exclusion of the adult nervous system (4, 29, 37, 39). Studies of mutant mice lacking Shc proteins indicate that ShcB and ShcC have overlapping functions and are required for development and survival of certain neuronal populations (44). By contrast, mice lacking all ShcA isoforms die during embryogenesis with defects in cardiovascular development (20), while mice lacking

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only the 66-kDa isoform of ShcA display increased life span (25). Association with unique subsets of upstream receptors and downstream binding partners may further contribute to the biological specificity of Shc proteins (41).

Here, we have analyzed a fourth member of the Shc family of adaptor proteins, ShcD/Shc4. Mammalian ShcD is most closely related to ShcA; however, differences in expression of these two proteins suggest that they likely have nonredundant functions. In adult mice, ShcD appears to be primarily expressed in brain and skeletal muscle. Consistent with this expression pattern, we have found that ShcD can associate with *muscle-specific kinase* (MuSK), an RTK expressed at the skeletal neuromuscular junction (NMJ). On the postsynaptic muscle side of the NMJ, MuSK is stimulated by presynaptic motor neuron-derived agrin (53), which in turn leads to clustering and tyrosine phosphorylation of postsynaptic nicotinic acetylcholine receptors (AChRs) and remodeling of the neuromuscular synapse (10, 11). Mice lacking either agrin or MuSK fail to develop normal neuromuscular synapses and consequently die at birth due to an inability to move or breathe (5, 9). A principal physiological target for MuSK is a PTB domain-containing scaffold protein, Dok-7 (1, 35). We find that ShcD is also coexpressed with MuSK in the postsynaptic region of the NMJ; expression of ShcD appears to be involved in aspects of MuSK signaling which include regulation of early tyrosine phosphorylation of the AChR. ShcD is the only Shc family protein that detectably interacts with MuSK, indicating a degree of functional diversity among mammalian Shc docking proteins.

#### MATERIALS AND METHODS

**Plasmids.** Amino acid substitutions were carried out using PCR-based mutagenesis with an Expand High Fidelity PCR system (Roche) and were confirmed by DNA sequencing. Full-length cDNAs encoding mouse ShcA and ShcB as well as human ShcC ([hShcC] NCBI accession number BC026314) (ATCC) and ShcD (hShcD; BC033907) (Open Biosystems) were PCR amplified with or without a carboxy-terminal triple Flag epitope tag and cloned into pcDNA3 (Invitrogen). Full-length mouse ShcD (mShcD) cDNA was obtained by PCR amplification from an embryonic day 15.5 (E15.5) mouse embryo cDNA library and cloned as above. The PTB (residues 180 to 351) and SH2 (residues 524 to 603) domains of hShcD were cloned into pGEX-4T-1 (Amersham Biosciences) to generate glutathione *S*-transferase (GST) fusion proteins. Wild-type and mutant mouse MuSK cDNAs were cloned with an in-frame carboxy-terminal Myc epitope tag into pcDNA3.1 (Invitrogen).

For silencing of mShcD in C2C12 cells, target short hairpin RNA (shRNA) sequences corresponding to either bases 825 to 845 (ShcD<sub>1</sub>) or bases 1695 to 1715 (ShcD<sub>2</sub>) of the mouse *ShcD* gene were introduced into a human H1 RNA polymerase III promoter-based shRNA vector, pBINNS2. The pBINNS2 vector is derived from incorporation by PCR of the human H1 RNA polymerase III promoter (19) into the EcoRI and XhoI site of a self-inactivating murine stem cell virus (pMSCVpuro) plasmid modified through deletion of the 3' long terminal repeat. Point mutations were introduced into the target sequence of ShcD<sub>1</sub> to generate ShcD<sub>1</sub>x shRNA. A sequence corresponding to bases 1674 to 1696 in the mouse p66 *ShcA* gene was used to silence ShcA.

**Cell culture, transfection, and stimulation.** HEK 293T, COS-1, Phoenix, and C2C12 myoblasts (ATCC) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (HyClone), and C2C12 were induced to differentiate to myotubes in 2% fetal bovine serum-Dulbecco's modified Eagle's medium for 4 to 6 days. Transient transfection of HEK 293T and Phoenix cells was performed using polyethylenimine for 48 h. For generation of C2C12 knockdown lines, supernatant was first collected from Phoenix cells that had been transfected with retroviral shRNA vectors, and following centrifugation, the cleared supernatant was mixed with culture medium (1:1) in the presence of Polybrene (10 mg/ml; Sigma). Undifferentiated C2C12 cells were incubated for 10 h with virus-containing supernatant and incubated for an additional 10 h with fresh, diluted supernatant. Cells were then passaged into virus-free

culture medium for 24 h, and the protocol was repeated. C2C12 cells were infected a total of 10 rounds prior to differentiation. No selection drugs were added to the culture medium, and cells were treated as a pooled population to avoid clonal artifacts. For analysis of AChR phosphorylation in C2C12 cells, 1 nM recombinant agrin (R&D Systems) was added to the differentiation medium for 10 to 120 min prior to cell harvesting. Alternatively, for analysis of AChR clustering, myotubes were incubated with 0.1 nM recombinant agrin for 6 h at 37°C, washed twice with phosphate-buffered saline (PBS), incubated with 1 µg/ml fluorescence-conjugated  $\alpha$ -bungarotoxin ( $\alpha$ -BTX) (Alexa 594; Molecular Probes) for 1 h and fixed in 4% paraformaldehyde.

**Generation of ShcD antibodies.** The CH2 domain of mouse ShcD (residues 5 to 182) was cloned into pGEX-4T-1 as an in-frame fusion with GST to generate ShcD<sup>CH2</sup> antibodies. Alternatively, carboxy-terminal (CT) peptides specific to mShcD (QPIRKYDNTGLLPKK) or hShcD (QVVRKDNPNALLHSNK) were synthesized and used to generate mShcD<sup>CT</sup> or hShcD<sup>CT</sup> antibodies, respectively. All were used to raise polyclonal antisera in rabbits. Reactive sera were affinity purified using AminoLink Plus or Sulfolink columns (Pierce), according to the manufacturer's instructions. Animal husbandry was carried out in accordance with the Canadian Council on Animal Care standards.

**Peptide synthesis and association assays.** Peptides were synthesized on an AbiMed 431 synthesizer using standard Fastmoc techniques. Products were confirmed by mass spectrometric and amino acid analysis. Peptides used for antibody production were synthesized with an amino-terminal cysteine to allow coupling to keyhole limpet hemocyanin. Peptides used for binding assays were synthesized with an amino-terminal biotin group to facilitate recovery on streptavidin agarose beads (Pierce). Cell lysates were incubated with 5 µg of biotinylated peptide for 2 h at 4°C prior to recovery. The sequence of the MuSK peptide used is LLLDLRLHPNPM(p)YQRMPLLL.

**Lysate preparation and immunoblotting.** Lysates were prepared from cultured cells or adult (4 weeks) mouse tissues using PLC lysis buffer supplemented with fresh protease inhibitors (50 mM HEPES [pH 7.5], 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM MgCl<sub>2</sub>, 1 mM EGTA, 10 mM sodium pyrophosphate, 100 mM sodium fluoride, supplemented with 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml aprotinin, and 10 µg/ml leupeptin). Protein concentrations were determined using Bio-Rad protein assay reagent. Following addition of indicated antibodies, complexes precipitated with GST fusion protein (5 µg) or biotin-conjugated  $\alpha$ -BTX (5 µg) were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The following antibodies were obtained from commercial sources: monoclonal anti-Flag clone M2 (Sigma), monoclonal anti-Myc clone 9E10 (Invitrogen), monoclonal anti-pTyr clone 4G10 (Upstate Biotechnology Inc.), monoclonal anti-Grb2 (BD Bioscience), polyclonal anti-Grb2 (C-23) (Santa Cruz), monoclonal antitubulin clone DM1A (Sigma), monoclonal anti-myosin heavy chain clone MF20 (Developmental Studies Hybridoma Bank), and goat polyclonal anti-MuSK (N-19 and C-19, epitopes mapping near the N and C termini, respectively) (Santa Cruz). Rabbit polyclonal antibodies to MuSK, MuSK pY553, and MuSK pY574/755 have been described previously (15, 56). Rat monoclonal antibodies recognizing the AChR  $\beta$ -subunit (AChR- $\beta$ ; monoclonal antibody 124) were kindly provided by Jon Lindstrom (University of Pennsylvania, Philadelphia, PA). AChR- $\beta$  levels were quantified using QuantityOne software (Bio-Rad); levels of AChR- $\beta$  phosphorylation were normalized for relative expression of total AChR- $\beta$  in each lane.

**Immunohistochemistry.** Adult mouse gastrocnemius muscles were fixed in 1% paraformaldehyde at 4°C for 1 h, washed three times for 10 min each time in PBS, and cryoprotected in 30% sucrose-PBS overnight at 4°C prior to mounting in Tissue-Tek (Sakura Finetek USA, Torrance, CA). Denervated muscles were prepared by removing the sciatic nerve 4 days prior to tissue collection. To stain synaptic proteins, frozen sections (10 µm) of muscle were incubated for 3 h at room temperature in PBS containing 2% bovine serum albumin and the following antibodies: rabbit anti-MuSK 83033 (1:1,000) (56), rabbit antisynaptophysin (1:5) (Zymed, San Francisco, CA), or rabbit anti-ShcD CH2 (1:100). Prior to use, affinity-purified anti-ShcD CH2 was absorbed overnight at 4°C against a 100-fold excess of either ShcD CH2-GST or GST alone coupled to glutathione-coated beads. Sections were subsequently washed three times for 10 min each with PBS and then incubated with fluorescein isothiocyanate-conjugated anti-rabbit immunoglobulin G (1:200; Jackson ImmunoResearch, West Grove, PA) and Alexa 594-conjugated  $\alpha$ -BTX (1:1,000) (Invitrogen, San Diego, CA) in PBS containing 2% bovine serum albumin for 1 h at room temperature. Following washing as above, sections were mounted and imaged using a Zeiss (Oberkochen, Germany) LSM 510 confocal microscope.

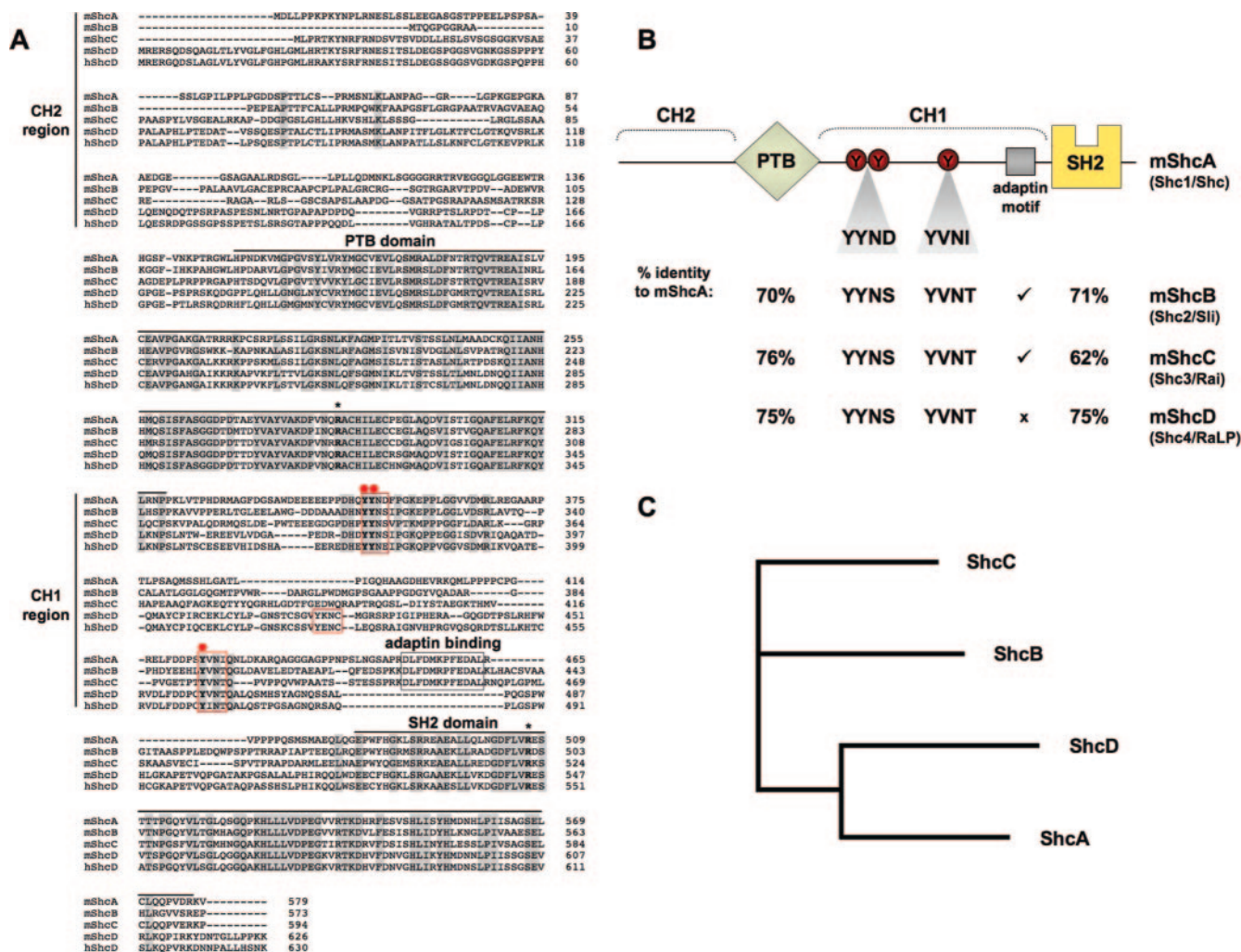


FIG. 1. Comparison of Shc family protein sequences. (A) Amino acid sequence alignment of murine ShcA (p66 isoform), ShcB, ShcC, ShcD, and hShcD, generated using T-Coffee, version 1.41, and shown in Clustal format (31). The PTB (as defined in reference 59) and SH2 domains are indicated, and the ShcA adaptin-binding motif that is found in ShcB and ShcC is boxed in black. Highlighted in bold are the conserved tyrosine-based Grb2 binding motifs (marked with red circles) and the key arginine residues in the PTB and SH2 domains that coordinate binding to pTyr (marked with asterisks). The three YXNX motifs in ShcD are boxed in red. (B) Modular domain structure of Shc family proteins from panel A, showing percent amino acid identity to ShcA in the PTB and SH2 domains. Red circles indicate the conserved tyrosine residues, and the gray box indicates the adaptin-binding motif in ShcA, which recruits plasma membrane adaptor proteins involved in clathrin-mediated endocytosis. Conservation of this motif in ShcB and ShcC is indicated by a check mark. (C) Phylogenetic relationship among Shc family proteins, indicating that ShcD is most closely related to ShcA.

RESULTS

**Identification of a fourth Shc family member, ShcD/Shc4.** A portion of an amino acid sequence similar to Shc was identified upon BLAST searches of both human and mouse sequence databases. The corresponding gene was predicted based on similarities in genomic organization with other Shc family members and overlap of partial cDNA sequences. Removal of intronic sequences allowed assembly of an open reading frame with a potential initiator methionine that would encode a human protein with 630 amino acids (626 amino acids in mouse) and a predicted molecular mass of approximately 69 kDa (Fig. 1A). This protein has a domain organization similar to the three previously characterized mammalian Shc family members, including an amino-terminal PTB domain and a carboxy-

terminal SH2 domain that flank a central CH1-like region; this CH1 sequence has three YXNX motifs which upon phosphorylation, could potentially form binding sites for the SH2 domain of Grb2 or related proteins (Fig. 1A and B). We therefore refer to this fourth Shc family member as ShcD; it has also been assigned the name RaLP for *Rai-like protein* (6) as well as Shc4. Comparison of the amino acid sequences of mammalian Shc family members indicates that ShcD is most highly related to ShcA (Fig. 1C). The highest conservation between all Shc family proteins is seen in regions corresponding to the PTB and SH2 domains, with more sequence divergence in the central CH1 and amino-terminal CH2 regions, and this also applies to ShcD (Fig. 1A and B). In the CH1 region, the adaptin-binding motif identified in ShcA is absent in ShcD,

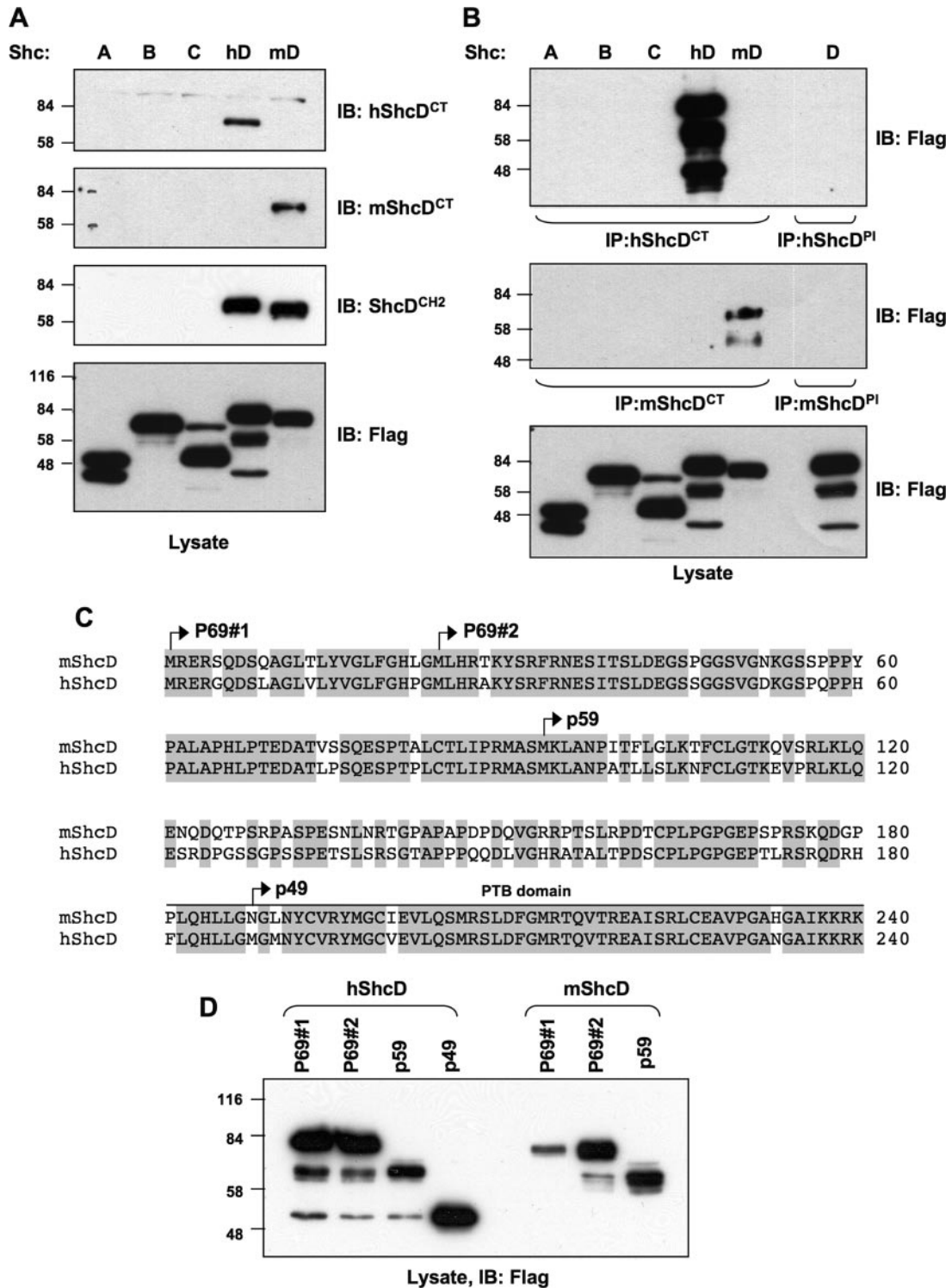


FIG. 2. Characterization of ShcD expression and ShcD-specific antibodies. (A) Flag-tagged cDNAs corresponding to mShcA, mShcB, and hShcC as well as hShcD and mShcD were transfected. Lysates were immunoblotted with multiple anti-ShcD antibodies or with anti-Flag antibodies. Carboxy-terminal peptide antibodies are specific to either human or mouse ShcD, whereas the CH2 domain GST fusion antibody recognizes both human and mouse ShcD. ShcD-specific antibodies do not cross-react with other Shc family proteins, including the p66 isoform of ShcA (not shown), and no signal was detected with preimmune serum (not shown). Transfection of ShcD results in the appearance of multiple Flag-reactive species. ShcB is expressed as a single isoform of 68 kDa, and ShcC is expressed as two isoforms of 64 and 52 kDa as a result of alternative initiation codon usage. (B) Parallel lysates were immunoprecipitated (IP) with ShcD<sup>CT</sup> antibodies. Preimmune (ShcD<sup>PI</sup>) serum for each antibody was used as a control to immunoprecipitate ShcD from either hShcD (hD)- or mShcD (mD)-transfected lysates, as appropriate (indicated as lane D). hShcD<sup>CT</sup> and mShcD<sup>CT</sup> are specific to human and mouse ShcD, respectively, and do not immunoprecipitate other Shc family proteins. (C) To determine whether the multiple ShcD Flag-reactive species were the result of alternative initiator codon usage, we engineered deletion constructs within the amino terminal CH2 region of hShcD and mShcD to allow codon initiation at methionine residues predicted to serve as start sites. The predicted molecular sizes (in kDa) are shown with a bent arrow above each potential start methionine residue. A methionine residue corresponding to p49 in hShcD is not conserved in mShcD. (D) ShcD deletions were transfected into HEK 293T cells, and lysates were immunoblotted with anti-Flag antibodies. Each truncation mutant comigrates with one of the shorter species produced from the full-length cDNA (p69#1). IB, immunoblot.

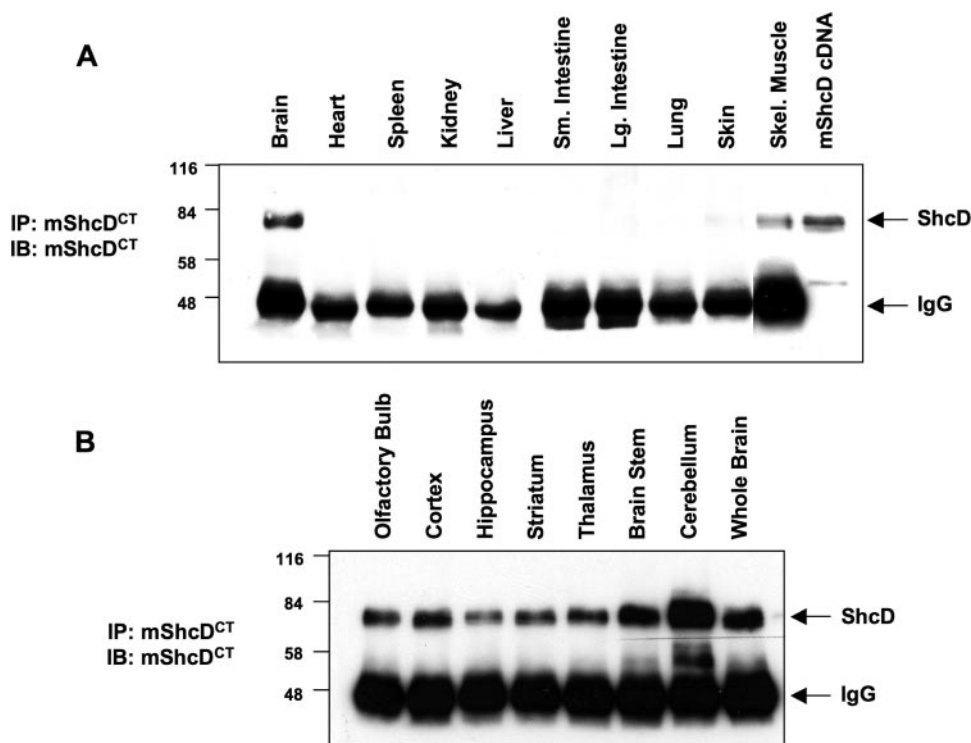


FIG. 3. Expression pattern of ShcD in adult mouse tissues. Antibodies specific to mShcD<sup>CT</sup> were used to immunoprecipitate (IP) ShcD from various mouse tissues. mShcD<sup>CT</sup> antibodies were also used for immunoblotting (IB). Lysate prepared from HEK 293T cells expressing mShcD cDNA was loaded as a positive control. ShcD expression is seen in brain and skeletal muscle. IgG, immunoglobulin G.

despite its high conservation in both ShcB and ShcC. Furthermore, the CH2 region is extended in ShcD, and it contains a number of unique proline-rich motifs that could serve as recognition sites for SH3 domain-containing proteins. This region of ShcD also possesses multiple potential initiator methionine residues, which could allow for translation of different protein isoforms of ShcD, as seen with ShcA and ShcC.

#### ShcD is primarily expressed in brain and skeletal muscle.

To investigate the signaling properties of ShcD, we raised antibodies against synthetic peptides corresponding to the carboxy-terminal region of either hShcD or mShcD (hShcD<sup>CT</sup> or mShcD<sup>CT</sup>, respectively). This carboxy-terminal sequence of ShcD differs from the corresponding sequence of other Shc proteins and should therefore result in the production of ShcD-specific antibodies, selective for either the human or murine proteins. A third ShcD antibody was raised against the CH2 domain of mShcD fused to GST (ShcD<sup>CH2</sup>). These antibodies were first used to detect the ShcD protein in lysates from HEK 293T cells transiently expressing either the hShcD or mShcD cDNAs with a carboxy-terminal Flag epitope tag. mShcA and mShcB as well as hShcC cDNAs were also expressed to investigate the specificity of the antibodies. Western blotting of transfected cell extracts detected a major protein with an apparent molecular mass of approximately 75 kDa, corresponding to either hShcD or mShcD (Fig. 2A). mShcD migrated slightly faster than hShcD, likely due to the absence of four amino acids in the CH1 region compared to hShcD. The ShcD antibodies were also used for immunoprecipitation from the same lysates; hShcD<sup>CT</sup> precipitated three major spe-

cies of approximately 50, 60, and 75 kDa that were reactive with Flag antibodies upon Western blotting, and mShcD<sup>CT</sup> precipitated two Flag-reactive species of approximately 60 and 75 kDa (Fig. 2B). These smaller protein products were also seen upon immunoblotting of transfected lysates with Flag antibodies (Fig. 2A) and with the ShcD antibodies in longer exposures (not shown). The molecular sizes of these smaller species suggest that they could arise as a consequence of alternate use of internal initiation codons. To pursue this possibility, we constructed a series of amino-terminal deletions in hShcD and mShcD to direct translation from various ATG (Met) sites in the CH2 region (Fig. 2C). These yielded truncated proteins that comigrated with the smaller polypeptide species encoded by the wild-type cDNA, denoted p69#1 (Fig. 2D). These findings suggest that ShcD can potentially be expressed as multiple protein isoforms that differ by the extent of their amino-terminal sequences.

To analyze the expression profile of ShcD in different tissues, lysates were prepared from various mouse tissues and equivalent amounts of lysate were immunoprecipitated with mShcD<sup>CT</sup> antibodies. Western blotting with the same antibodies specifically identified a 75-kDa protein in both brain and skeletal muscle but not in other tissues (Fig. 3A). This ShcD protein could also be detected in lysates prepared from E12.5 mouse embryos (not shown). ShcD appears to be widely expressed in the brain, as the 75-kDa product was detected by immunoblotting in olfactory bulb, cortex, hippocampus, striatum, thalamus, and brain stem; expression appeared marginally enhanced in the cerebellum (Fig. 3B). Interestingly, the

shorter isoforms of ShcD observed in transfected cells have not been detected in any tissues tested, suggesting that ShcD is primarily expressed as a single 75-kDa isoform *in vivo*.

**ShcD associates with MuSK.** The structural similarity of ShcD with other Shc family proteins suggests that it might associate with activated RTKs to mediate signal transduction events. The high degree of identity between the ShcD and ShcA PTB domains prompted us to compare their binding properties. To this end, we used the ShcD or ShcA PTB domain to probe an array of short peptides corresponding to more than 100 receptor PTB domain consensus-binding sites (46). Using this approach, we found a number of RTKs with the capacity to bind the ShcD PTB domain. Of interest, we identified the juxtamembrane NPXY motif in MuSK as a potential selective binding target for the PTB domain of ShcD but not ShcA, consistent with expression of ShcD in skeletal muscle.

To confirm the findings from the spot peptide array, synthetic peptides corresponding to the juxtamembrane region of MuSK were used to precipitate ShcA or ShcD from lysates of HEK 293T cells transiently expressing the full-length tagged cDNAs. The tyrosine phosphorylated MuSK juxtamembrane peptide specifically associated with ShcD but not ShcA, and the interaction with ShcD was dependent upon phosphorylation of the peptide (Fig. 4A). We next examined whether ShcD could interact with the full-length MuSK receptor in cells. Expression of MuSK in HEK 293T cells results in ligand-independent tyrosine phosphorylation of the receptor (Fig. 4B). Flag-tagged ShcD or ShcA was transiently coexpressed with MuSK in HEK 293T cells, and lysates from these cells were immunoprecipitated with anti-Flag antibodies. Under these conditions, ShcD associated with autophosphorylated MuSK and was tyrosine phosphorylated (Fig. 4B). By contrast, association of ShcA with MuSK and corresponding ShcA tyrosine phosphorylation were only detected at very low levels. Together, these results indicate that ShcD can selectively associate with the activated MuSK receptor and that ShcD may serve as a substrate for this RTK.

**The juxtamembrane region of MuSK is required for ShcD binding.** The NPXY motif in the juxtamembrane region of MuSK is functionally important, as MuSK mutants with substitutions of the asparagine or tyrosine residues in this motif fail to restore agrin-induced AChR clustering in MuSK-deficient myotubes (14, 15, 58). To investigate the contribution of these residues to ShcD binding, we introduced point mutations into MuSK so that either of these residues was altered to alanine (N550A and Y553A, respectively). We also generated a kinase-inactive form of MuSK (K611A) to explore the requirement for phosphorylation of the receptor. All mutant MuSK receptors were expressed at levels equivalent to wild-type MuSK in HEK 293T cells, and tyrosine phosphorylation of the NPXY mutants could be detected, albeit at lower levels than that seen in wild-type MuSK, whereas the K611A mutant was not phosphorylated on tyrosine (Fig. 4C). These observations contrast with those seen in MuSK-deficient myotubes where MuSK N550A and MuSK Y553(A/F) are not tyrosine phosphorylated in response to agrin (15, 58), and they support the hypothesis that a muscle-specific inhibitory protein binds to this region to repress catalytic activity (52). Alternatively, since Dok-7 is expressed selectively in muscle and enhances MuSK

phosphorylation upon binding to a low level of phosphorylated MuSK (35), it is possible that MuSK N550A and MuSK Y553(A/F) are not tyrosine phosphorylated substantially in muscle because they cannot recruit Dok-7. To further analyze phosphorylation of these mutants, we utilized a series of phospho-specific antibodies that recognize phosphorylation on the juxtamembrane tyrosine (pY553) or the activation loop tyrosines (pY754/755) of MuSK (15). While both MuSK N550A and MuSK Y553A appeared to be kinase active as determined by immunoblotting with pY754/755 antibodies (Fig. 4C), neither mutant was phosphorylated on the juxtamembrane tyrosine, suggesting that these mutants are phosphorylated primarily at tyrosine sites outside the juxtamembrane region. ShcD was then coexpressed with wild-type MuSK or MuSK mutants in HEK 293T cells. ShcD did not associate with kinase-inactive MuSK, indicating that the interaction between ShcD and MuSK is pTyr dependent (Fig. 4D). Moreover, mutations in the juxtamembrane NPXY sequence strongly reduced ShcD binding to MuSK and phosphorylation of ShcD (Fig. 4D). Taken together, these findings demonstrate that ShcD interacts with the phosphorylated juxtamembrane tyrosine residue on the MuSK receptor and that recruitment of ShcD to MuSK results in ShcD tyrosine phosphorylation.

**The PTB domain of ShcD mediates binding to MuSK.** Binding of Shc proteins to activated receptors can potentially occur via either the PTB or SH2 domains (24, 41), and peptide array data suggest that the ShcD PTB domain interacts with the phosphorylated NPXY juxtamembrane site. To determine which domain on ShcD is primarily responsible for binding to MuSK, we introduced substitutions in the PTB and SH2 domains of full-length ShcD, either alone or in tandem, which block the ability of each domain to bind pTyr-containing motifs (Fig. 1) (59). Wild-type or mutant forms of ShcD were coexpressed in HEK 293T cells with MuSK, and their ability to associate with MuSK and undergo tyrosine phosphorylation was examined. Surprisingly, disruption of either the PTB domain (PTB\*) or the SH2 domain (SH2\*) in full-length ShcD was not sufficient to disrupt the interaction between MuSK and ShcD, but mutation of the PTB and SH2 domains together (PTB\*/SH2\*) abolished both binding of ShcD to MuSK and its MuSK-dependent tyrosine phosphorylation (Fig. 5A). Similar results were also obtained when analyzing the interaction between ShcD and other RTKs (data not shown), indicating that this feature of ShcD is not unique to MuSK binding.

Results shown in Fig. 4D and 5A suggest that the SH2 domain of ShcD may act synergistically with the PTB domain to bind receptors such as MuSK. To compare the binding of these domains to the endogenous activated MuSK receptor, the isolated PTB and SH2 domains of ShcD were expressed as fusion proteins with GST and incubated with lysates from unstimulated or agrin-stimulated C2C12 myotubes. Figure 5B demonstrates that the isolated PTB domain of ShcD, but not the SH2 domain, specifically precipitated the phosphorylated MuSK receptor. Moreover, mutation of the predicted pTyr binding pocket of the ShcD PTB domain (PTB\*) (59) disrupted this interaction (Fig. 5B). Collectively, these experiments suggest that the PTB domain of ShcD is the primary element that mediates binding to MuSK but that the SH2 domain contributes cooperatively to this interaction in cells in the context of full-length ShcD.

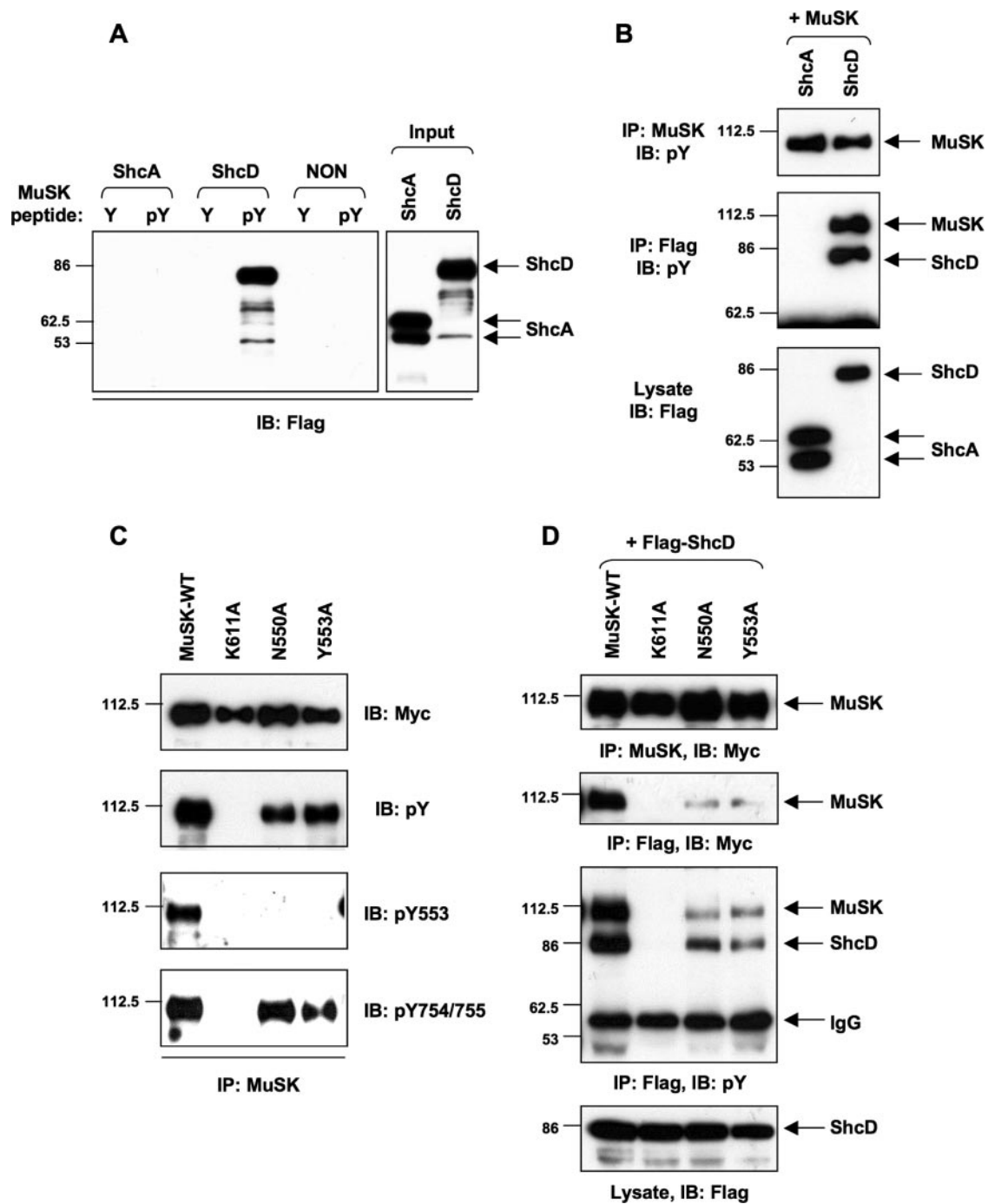


FIG. 4. Interaction and phosphorylation of ShcD and MuSK in HEK 293T cells. (A) Synthetic peptides corresponding to the juxtamembrane NPXY sequence of MuSK were incubated with lysates prepared from cells expressing Flag-tagged ShcA or ShcD. Nontransfected cells were used as a control. (B) Myc-tagged MuSK was coexpressed with Flag-tagged ShcA or ShcD, and lysates were immunoprecipitated with antibodies against either MuSK or Flag. Immunoblotting with anti-pTyr (pY) indicated that ShcD but not ShcA is tyrosine phosphorylated in the presence of activated MuSK and that ShcD binds activated MuSK. (C) Mutations in the kinase domain (K611A) and juxtamembrane NPXY sequence (N550A and Y553A) were introduced into MuSK to disrupt ShcD binding. Immunoblotting was performed using antibodies recognizing pan-pTyr (4G10), MuSK juxtamembrane tyrosine residue 553 (pY553) or MuSK activation loop tyrosine residues 754 and 755 (pY754/755). The K611A mutant showed complete loss of phosphorylation (panel two), whereas the juxtamembrane mutants N550A and Y553A remained catalytically active (bottom panel), despite the loss of phosphorylation in the NPXY motif (panel three). (D) MuSK mutants were coexpressed with ShcD, and lysates were immunoprecipitated with anti-MuSK or anti-Flag antibodies, followed by immunoblotting with anti-Myc or anti-pTyr. ShcD does not bind kinase-inactive MuSK, and mutations in the juxtamembrane NPXY sequence reduce ShcD phosphorylation and binding compared to wild-type MuSK controls. IB, immunoblot; IP, immunoprecipitation; IgG, immunoglobulin G; WT, wild type.

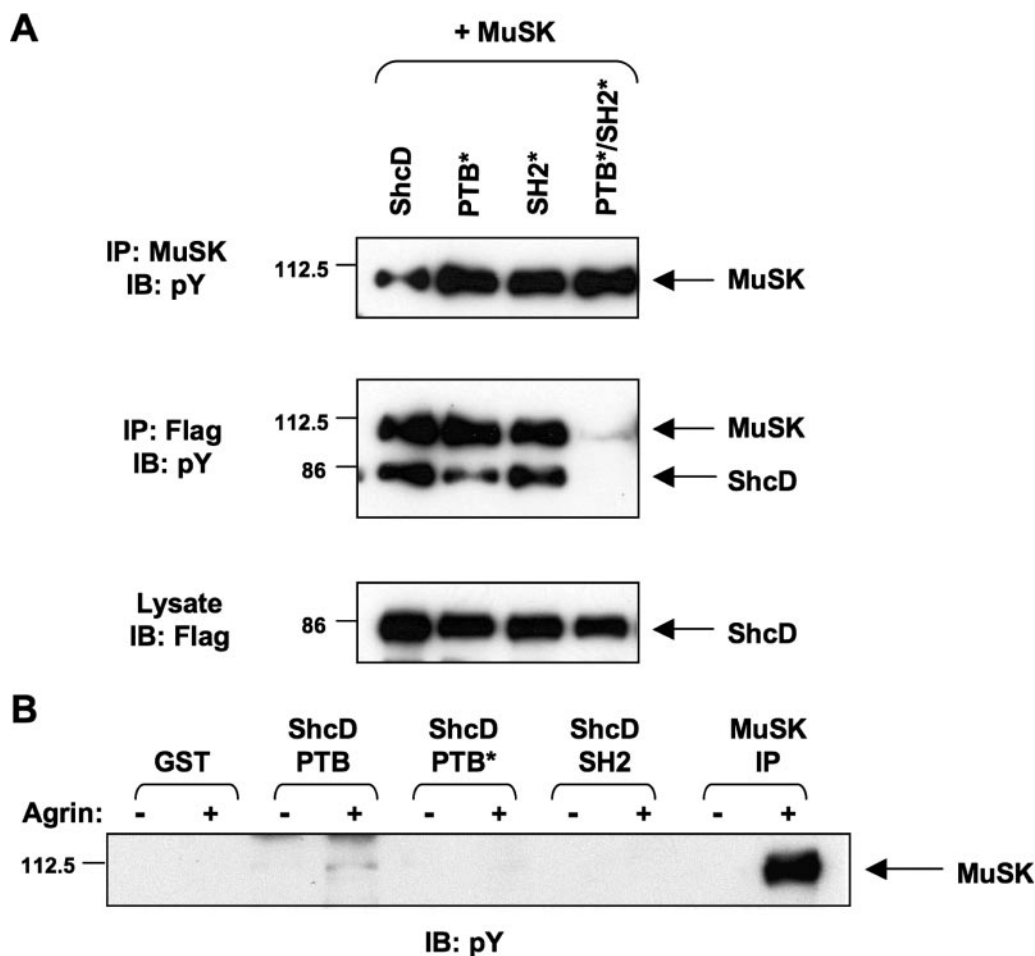


FIG. 5. The PTB domain of ShcD is sufficient to bind MuSK. (A) Mutations were introduced into the PTB (R315Q; PTB\*) and/or SH2 (R549K; SH2\*) domains of full-length ShcD to disrupt pTyr binding. Mutants were coexpressed with MuSK in HEK 293T cells, and lysates were immunoprecipitated (IP) with Flag antibodies, followed by immunoblotting (IB) with anti-pTyr antibodies. ShcD phosphorylation was markedly decreased with the PTB mutation alone, although mutation of both the PTB and SH2 domains in tandem was required to completely reduce ShcD phosphorylation and binding to MuSK. (B) GST fusion proteins of the isolated PTB, PTB\*, or SH2 domains of ShcD were incubated with lysates prepared from C2C12 cells that had been stimulated with 1 nM agrin (+) or left untreated (-). Parallel lysates were immunoprecipitated (IP) with anti-MuSK antibodies. Immunoblotting (IB) with anti-pTyr antibodies demonstrated that the isolated PTB domain, but not the PTB\* or SH2 domains of ShcD, could precipitate phosphorylated MuSK following agrin stimulation of C2C12 cells.

**Multiple tyrosine residues on ShcD function to recruit Grb2.** Vertebrate Shc proteins typically contain three CH1 pTyr sites, located in two conserved motifs [corresponding to YYN(S/E) and Y(V/I)NT in ShcD]. Interestingly, we have observed that an ShcD mutant with phenylalanine substitutions at these three conserved tyrosine residues (Y3F) can still undergo tyrosine phosphorylation (Fig. 6C). This finding is in contrast to an analogous ShcA mutant, and it suggests that ShcD has additional sites of tyrosine phosphorylation. In a stretch of amino acids bordered by the tyrosine residues conserved in all Shc proteins in the CH1 region, we identified three tyrosine residues conserved in mouse, rat, and human ShcD proteins that are not found in ShcA or other Shc proteins (Fig. 6A). A fourth additional tyrosine residue is also conserved in the CH2 region of ShcD (not shown). To determine whether these sites may contribute to the residual phosphorylation seen in the Y3F mutant, additional mutations of tyrosine to phenylalanine were introduced into the Y3F mutant of

ShcD (Fig. 6B). Coexpression of ShcD mutants with MuSK in HEK 293T cells demonstrated that substitution of Y424 in the Y3F background (Y4F) further reduced phosphorylation, and additional mutation of Y403 and Y413 (Y6F) completely abolished ShcD phosphorylation in the presence of MuSK (Fig. 6C). Importantly, all ShcD tyrosine-to-phenylalanine mutants retained the ability to associate with MuSK (Fig. 6C).

The sequences surrounding Y403 and Y413 do not conform to known binding sites for SH2 or PTB domain-containing proteins; however, Y424 is embedded within a consensus binding motif for the Grb2 SH2 domain (pYXNX). To determine whether this motif could serve to bind Grb2, we generated a synthetic peptide corresponding to the sequence surrounding Y424 (Fig. 6D). The phosphorylated Y424 peptide precipitated Grb2 from lysates of HEK 293T cells, whereas the unphosphorylated peptide did not (Fig. 6D). No additional binding partners were identified for this peptide following mass spectrometric analysis of precipitated lysates (data not shown).



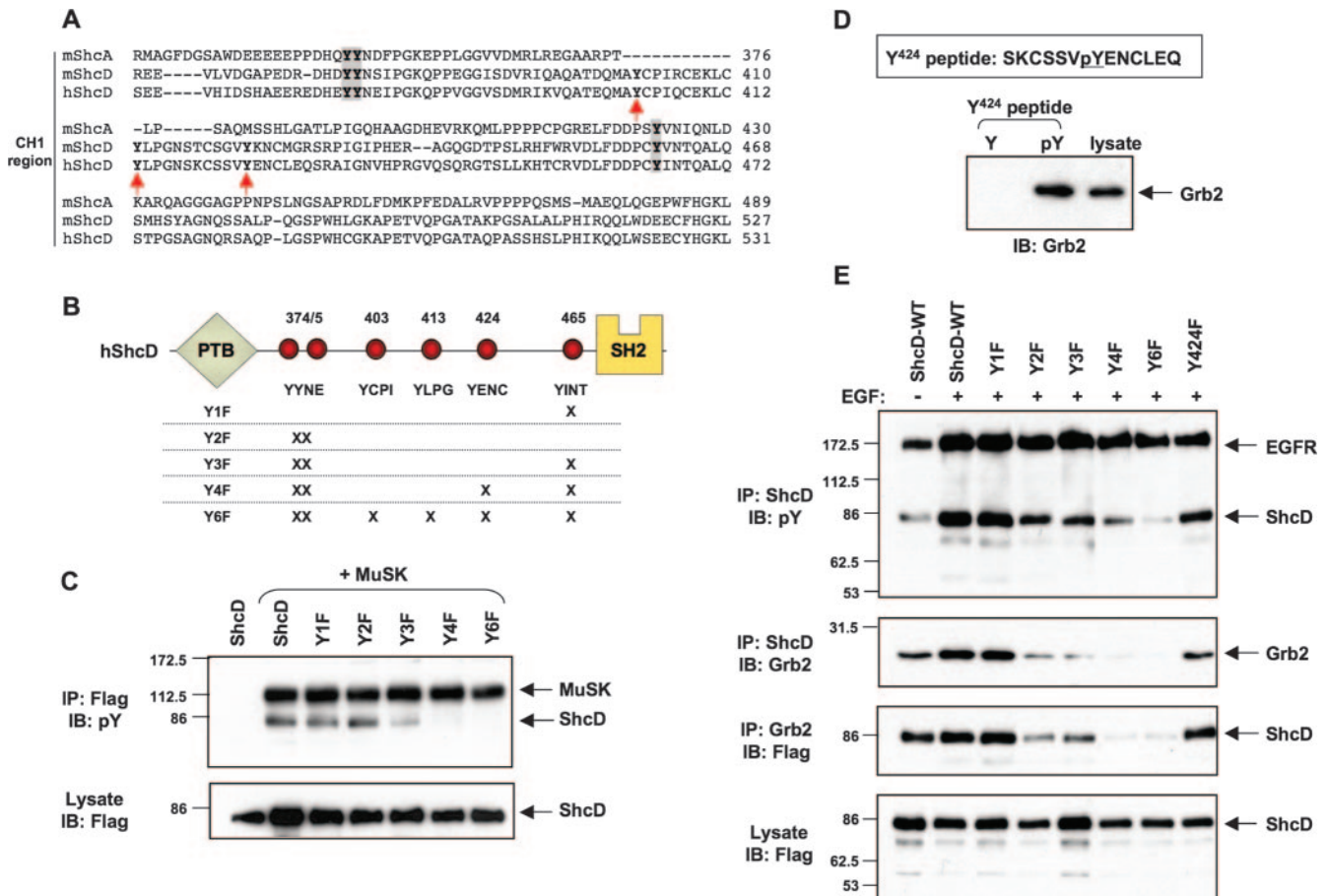


FIG. 6. Unique sites of tyrosine phosphorylation on ShcD. (A) Alignment of the central CH1 regions of mouse ShcA, ShcD, and hShcD. Conserved tyrosine residues are highlighted by gray boxes; those unique to ShcD are indicated with red arrows. (B) Relative positions of tyrosine residues in hShcD (red circles). Mutations of tyrosine (Y) to phenylalanine (F) were introduced at multiple positions, as indicated by X. (C) ShcD tyrosine-to-phenylalanine mutants were coexpressed with MuSK in HEK 293T cells. Lysates were immunoprecipitated with Flag antibodies, followed by immunoblotting with anti-pTyr. All mutants retained the ability to bind MuSK. The Y3F mutant lacking the three conserved Grb2 binding sites was still phosphorylated, while the Y6F mutant was no longer tyrosine phosphorylated. Phosphorylation of the Y4F mutant was decreased compared to the Y3F mutant, indicating that Y424 is likely to be phosphorylated in vivo. (D) Sequence of a synthetic phosphopeptide corresponding to Y424 in hShcD, which represents a putative Grb2 SH2 domain-binding site. The pTyr residue underlined was dephosphorylated as a control (represented as Y). Both peptides were incubated with lysates prepared from HEK 293T cells, and precipitates were immunoblotted with anti-Grb2 antibodies. (E) ShcD tyrosine-to-phenylalanine mutants were expressed in COS-1 cells, and cells were stimulated with 50 ng/ml EGF for 10 min or left unstimulated. Lysates were immunoprecipitated with anti-ShcD (mShcD<sup>CT</sup>) antibodies. Western blotting with anti-pTyr antibodies demonstrated that ShcD could coimmunoprecipitate the activated EGF receptor, and mutation of multiple YXN motifs on ShcD reduced ShcD phosphorylation in response to EGF stimulation. Immunoblotting with anti-Grb2 antibodies indicated reduced binding of the mutants to Grb2. Single mutation of the unique Grb2 binding site on ShcD (Y424) significantly decreased Grb2 binding as well as ShcD phosphorylation, and Grb2 binding to the Y4F mutant was virtually undetectable, thereby indicating that Y424 on ShcD is phosphorylated in vivo and binds Grb2. In a reciprocal experiment, lysates were immunoprecipitated with anti-Grb2 antibodies, followed by immunoblotting with anti-Flag antibodies, and comparable results were observed. Equivalent levels of ShcD mutant expression are seen in lysates immunoblotted with Flag antibodies. IP, immunoprecipitation; IB, immunoblot; WT, wild type.

To analyze whether the interaction between ShcD and Grb2 is induced in cells stimulated with growth factor, we expressed ShcD in COS-1 cells and stimulated these cells with epidermal growth factor (EGF), a mitogen that induces robust Shc phosphorylation. This resulted in enhanced phosphorylation of wild-type ShcD and increased binding to Grb2 (Fig. 6E). As observed with MuSK, mutation of one or all of the three conserved Shc CH1 tyrosine residues did not abolish tyrosine phosphorylation of ShcD, nor did it completely eliminate binding to Grb2 (Fig. 6E). However, further mutation of Y424 in the Y3F mutant (Y4F) strikingly reduced the interaction be-

tween ShcD and Grb2, even though the Y4F mutant was still detectably tyrosine phosphorylated (Fig. 6E). Interestingly, mutation of Y424 alone reduced both ShcD phosphorylation and the interaction between ShcD and Grb2 to an extent greater than that seen with mutation of Y465 in the YINT motif (the Y1F mutant, analogous to mouse ShcA Y313 in the YVNI sequence) (Fig. 6E). This suggests that Y424, which is unique to ShcD, is a relevant in vivo phosphorylation site that enhances recruitment of Grb2 to ShcD.

**Localization of ShcD and MuSK at neuromuscular synapses.** Expression of MuSK is restricted to the postsynaptic

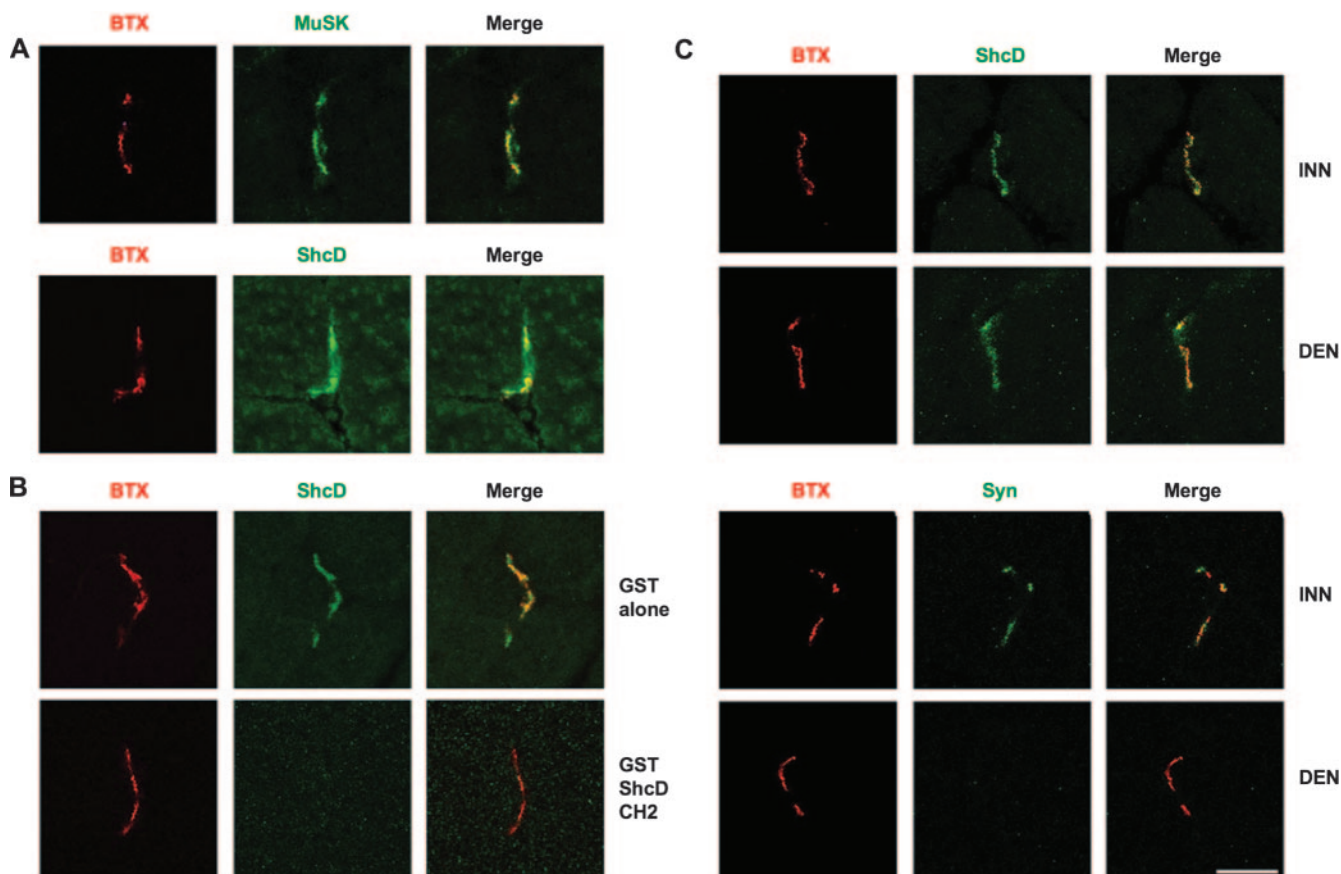


FIG. 7. ShcD colocalizes with MuSK at the NMJ (A) Sections from mouse gastrocnemius muscle costained with antibodies to MuSK or ShcD (both green) and  $\alpha$ -BTX (red) reveal overlapping expression at the NMJ. (B) To demonstrate specificity of the ShcD<sup>CH2</sup> antibodies in this approach, antibodies were preabsorbed with immunizing antigen (GST ShcD-CH2) or GST alone prior to staining. Staining is no longer detected when the antibody is preabsorbed by antigen. (C) Coexpression of ShcD with the AChR (as determined by  $\alpha$ -BTX) is seen at the NMJ in both innervated (INN) and denervated (DEN) gastrocnemius muscle sections, confirming postsynaptic localization of ShcD. Expression of the presynaptic marker synaptophysin (Syn) is no longer seen in the denervated muscle. Scale bar, 20  $\mu$ m.

membrane of the NMJ (53); to determine whether ShcD could serve as a binding partner for MuSK, we investigated the subcellular localization of ShcD in adult skeletal muscle. Frozen sections from mouse gastrocnemius muscle were stained with antibodies specific for ShcD or the extracellular domain of MuSK, in conjunction with  $\alpha$ -BTX, to highlight postsynaptic AChRs. We readily detected colocalization of ShcD with AChR clusters at the motor endplate, and the distribution of ShcD paralleled that of MuSK in the NMJ (Fig. 7A). The specificity of the ShcD antisera was confirmed by antigen competition; preabsorption with excess antigen (GST ShcD-CH2) but not GST alone abolished ShcD staining (Fig. 7B). To further investigate localization of ShcD to the postsynaptic membrane, we denervated mouse gastrocnemius muscles by resection of the sciatic nerve and again examined ShcD expression using immunofluorescence microscopy. ShcD expression persisted at denervated synaptic sites (where presynaptic nerve terminals had degenerated), and it remained colocalized with AChRs, similar to innervated muscle (Fig. 7C). Together these data demonstrate that ShcD is concentrated in the postsynaptic membrane of the NMJ, consistent with the potential role for ShcD in MuSK signaling.

**ShcD expression is not required for AChR clustering in response to agrin.** Signaling downstream of MuSK following agrin stimulation leads to clustering and tyrosine phosphorylation of AChRs, both of which are important for neurotransmitter sensitivity and synaptic transmission (3). Mutations in the juxtamembrane tyrosine residue of MuSK abrogate tyrosine phosphorylation of the  $\beta$ -subunit of the AChR and reduce AChR clustering (15, 58). To gain insight into the potential physiological involvement of ShcD in MuSK signaling, we utilized RNA-mediated interference (RNAi) to silence *ShcD* expression in the C2C12 mouse myoblast cell line. Two different shRNA molecules specific to mouse *ShcD* (ShcD<sub>1</sub> and ShcD<sub>2</sub>) were introduced into myoblasts, which were then differentiated to myotubes. A corresponding shRNA to ShcD<sub>1</sub> with two nucleotide point mutations that abrogate RNAi (ShcD<sub>1x</sub>) was utilized as a control, in addition to an shRNA specific to mouse *ShcA* or a plasmid control (vector). Lysates prepared from shRNA-infected myotubes demonstrated that the level of ShcD protein was reduced by roughly 90% in ShcD<sub>1</sub>- and ShcD<sub>2</sub>-expressing cells using this approach (Fig. 8A). Of note, ShcD-specific RNAi did not affect the level of ShcA expression or the ability of myoblasts to undergo fusion

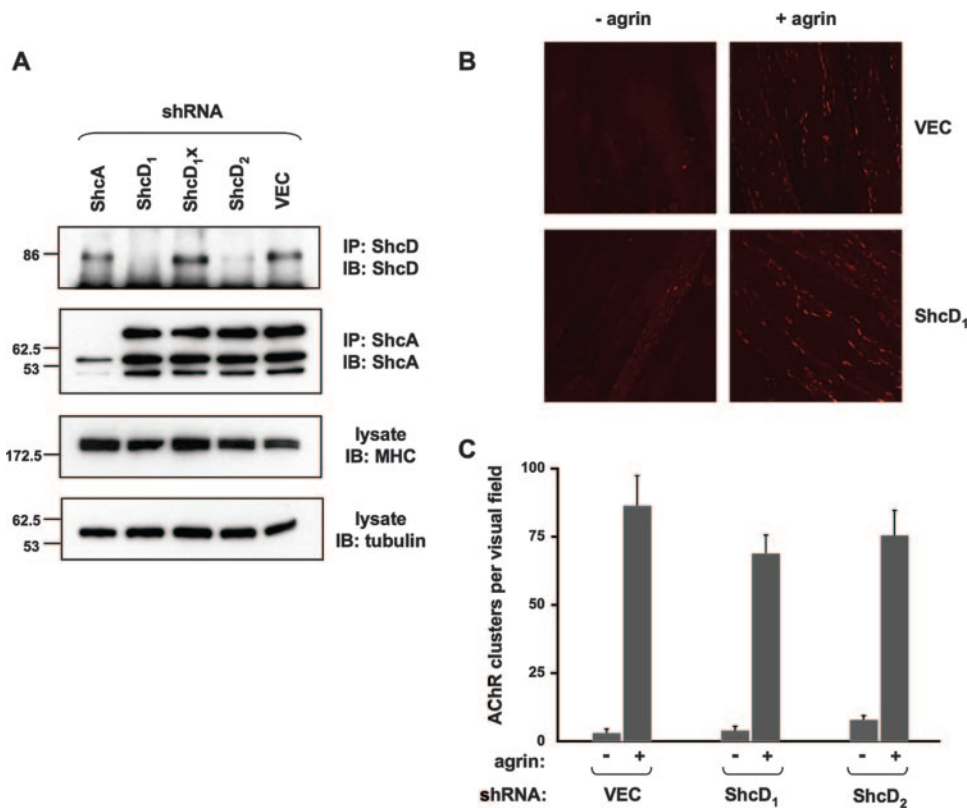


FIG. 8. Silencing of ShcD expression in C2C12 cells does not affect AChR clustering. (A) shRNA molecules specific to mShcA or mShcD were expressed in C2C12 cells using retroviral-mediated infection. Lysates obtained from pooled populations of infected cells were immunoprecipitated (IP) with anti-ShcA or anti-ShcD antibodies, followed by immunoblotting (IB) with ShcA or ShcD antibodies. Two distinct ShcD shRNA molecules (ShcD<sub>1</sub> and ShcD<sub>2</sub>) reduced ShcD expression but not ShcA expression, whereas the ShcA shRNA molecule (ShcA) only reduced ShcA expression. A mutant ShcD shRNA molecule (ShcD<sub>1,x</sub>) was no longer able to reduce ShcD expression. The vector alone did not affect levels of either ShcA or ShcD. Expression of myosin heavy chain (MHC) and tubulin in parallel lysates indicates equivalent levels of myoblast fusion and protein loading, respectively. (B) Myotubes expressing ShcD<sub>1</sub> shRNA or vector alone were stimulated with 0.1 nM agrin for 6 h (+agrin) or left untreated (-agrin) and incubated with fluorescent BTX for 1 h prior to fixation. (C) AChR clusters greater than 50 μm<sup>2</sup> were counted from at least 10 random visual fields using Volocity imaging software (Improvision, Inc.). Statistical analysis revealed no significant difference between vector-infected cells and ShcD shRNA lines, either with or without agrin stimulation (analysis of variance, *P* > 0.05). VEC, vector.

to form myotubes, as determined by immunoblotting with anti-ShcA or anti-myosin heavy chain antibodies, respectively (Fig. 8A). Using these cell lines, we examined whether reduced expression of ShcD had any effect on AChR clustering. Myotubes expressing ShcD shRNAs or vector alone were stimulated with agrin for 6 h or left unstimulated, and AChR clusters were visualized using α-BTX staining. Numerous AChR clusters were seen with ShcD<sub>1</sub> and ShcD<sub>2</sub> (not shown) shRNA following agrin stimulation (Fig. 8B), and quantitation revealed no statistical decrease in AChR clustering compared to vector-infected cells (Fig. 8C), thereby suggesting that ShcD may not be required for AChR aggregation.

**Early tyrosine phosphorylation of the AChR-β requires ShcD.**

To determine whether the AChR-β can become tyrosine phosphorylated in myotubes with reduced ShcD expression, we isolated AChRs using biotin-conjugated α-BTX from shRNA-infected myotubes that had been stimulated with agrin for 10 min. Both cell lines expressing ShcD shRNA molecules had reduced tyrosine phosphorylation of AChR-β compared to vector-infected cells (Fig. 9A), and quantitation revealed that this difference was approximately twofold (Fig. 9B). Previous reports have indicated that AChR-β phosphorylation is regulated in a tempo-

ral fashion, as distinct downstream kinases appear to mediate early and late phases of AChR tyrosine phosphorylation (26). To investigate whether ShcD may be required for one or both of these phases, we examined the kinetics of AChR-β tyrosine phosphorylation over the course of 120 min in ShcD<sub>1</sub>-expressing cells and compared this to control cells expressing the inactive RNAi counterpart, ShcD<sub>1,x</sub>. Myotubes infected with ShcD<sub>1</sub> or ShcD<sub>1,x</sub> shRNA molecules were stimulated with agrin, and AChR-β phosphorylation was examined. Consistent with our previous results, tyrosine phosphorylation of the AChR-β was strongly reduced in the ShcD knockdown cells following 10 min of agrin stimulation (Fig. 9C). Interestingly, however, beyond 30 min of agrin stimulation, phosphorylation of the AChR-β in cells with reduced ShcD expression was equivalent to that seen in cells expressing ShcD (Fig. 9C). These results indicate that ShcD expression contributes to the early but not late phases of agrin-induced tyrosine phosphorylation of the AChR-β.

**DISCUSSION**

Here we describe a previously uncharacterized member of the Shc family of docking proteins, which we have designated

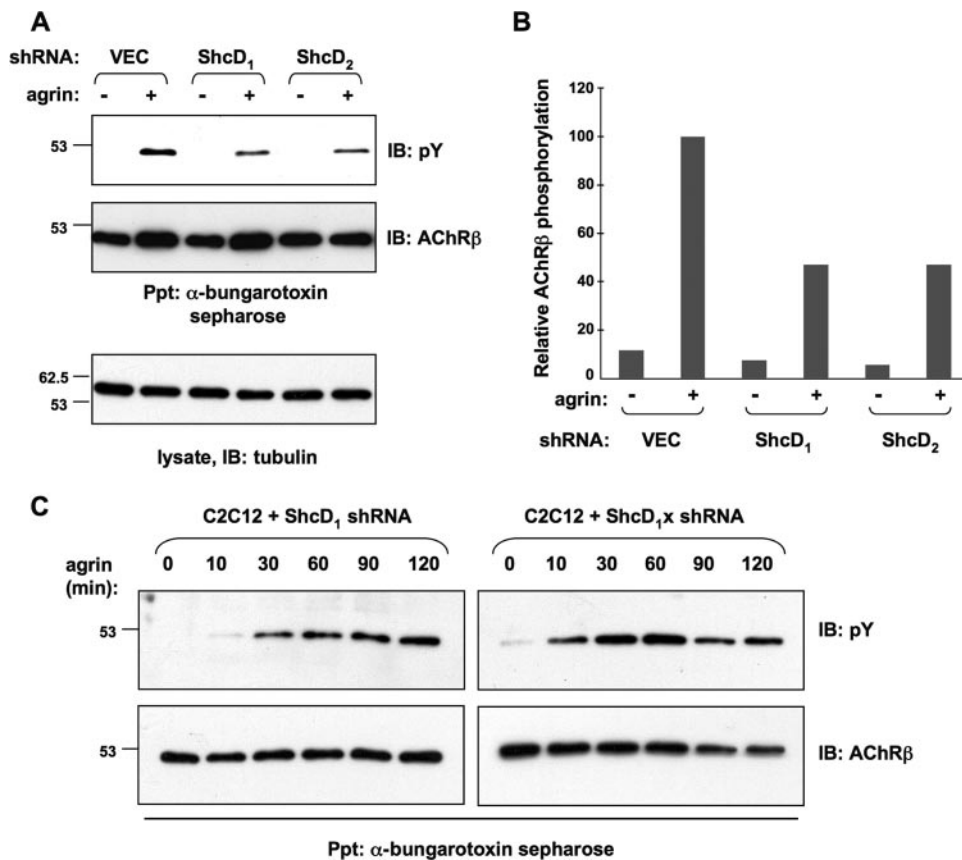


FIG. 9. ShcD mediates signaling events downstream of activated MuSK in C2C12 cells. (A) Myotubes expressing ShcD shRNA molecules were stimulated with 1 nM agrin for 10 min (+) or left untreated (-) prior to harvesting. Lysates were precipitated with  $\alpha$ -BTX Sepharose and immunoblotted with anti-pTyr or anti-AChR- $\beta$  antibodies. Parallel lysates were immunoblotted with antitubulin antibodies to demonstrate equal protein quantitation. Phosphorylation of the AChR- $\beta$  is reduced in stimulated C2C12 cells with reduced ShcD expression. (B) AChR- $\beta$  phosphorylation and expression levels in panel A were determined using QuantityOne software (Bio-Rad). Compared to vector-infected cells stimulated with agrin, phosphorylation of AChR- $\beta$  is reduced by approximately 50% in ShcD shRNA lines. (C) Time course of phosphorylation of the AChR- $\beta$  in C2C12 cells with altered ShcD expression. Cells expressing ShcD<sub>1</sub> or ShcD<sub>1x</sub> shRNA molecules were stimulated with 1 nM agrin for 10 to 120 min or left untreated (0), and lysates were processed as described in panel A. Delayed phosphorylation of the AChR- $\beta$  is seen in cells with reduced ShcD levels. VEC, vector; IB, immunoblot.

ShcD/Shc4. We show that ShcD can associate with the MuSK receptor and mediate aspects of signaling involved in NMJ development. We demonstrate that ShcD is present in skeletal muscle, consistent with a role in MuSK signaling, and it is coexpressed with MuSK at the postsynaptic region of the NMJ. Interestingly, ShcA is also expressed in skeletal muscle and in C2C12 myotubes though we have found that it does not associate with MuSK, consistent with previous reports (15, 46, 58). Instead, ShcA appears to function downstream of ErbB receptors in the induction of subsynaptic AChR subunit gene expression following neuregulin stimulation of muscle cells (57). ShcB and ShcC also do not appear to interact with MuSK (data not shown). It is interesting that the NPXY motif on MuSK represents a nonconventional Shc PTB domain binding site, as it contains a histidine residue at the -5 position (13, 17, 54). Therefore, despite similarities in their modular domain architecture and sequence, it appears as if ShcD is unique among Shc proteins in its ability to accommodate this divergent ligand. The ShcD PTB domain can also interact with a number of other RTKs and may consequently have a more

general role in tissues in which it is highly expressed, such as the brain.

Interaction between ShcD and MuSK occurs primarily through the NPXY motif in the juxtamembrane region of the receptor. Phosphorylation of the tyrosine residue in this motif (Y553) is important for interaction of the ShcD PTB domain with MuSK, and an asparagine residue at the -3 position (N550) is also required for optimal ShcD binding. Additional residues surrounding the NPXY motif further contribute to the binding specificity of the ShcD PTB domain (46). Interestingly, however, mutation of the NPXY motif is not sufficient to abrogate ShcD binding to MuSK, and mutation of both the PTB and SH2 domains of ShcD is required to completely eliminate the interaction, suggesting that multiple sites of ShcD binding may be present on the receptor. Tyrosine 576 in the kinase domain of MuSK is within an NXXY motif and, consistent with a role for this tyrosine residue in signaling, Y576 is phosphorylated *in vivo* (56), and its mutation reduces AChR clustering in MuSK-deficient myotubes (15). However, mutation of this residue does not reduce ShcD binding (data

not shown), suggesting that the PTB domain of ShcD does not associate significantly with Y576 on MuSK. Alternatively, the SH2 domain of ShcD may interact with phosphorylated tyrosine residues on MuSK. Although we have observed that the isolated SH2 domain of ShcD cannot precipitate MuSK from agrin-stimulated muscle cells, using a spot peptide array, we have found that one of the six phosphorylated tyrosine residues on MuSK, Y812, is bound by the isolated ShcD SH2 domain (46). Y812 is phosphorylated *in vitro* (56), but mutation of this residue does not affect agrin-stimulated clustering of AChRs (15). Y812 may serve to stabilize the interaction between ShcD and MuSK. It therefore seems likely that the PTB and SH2 domains of ShcD function cooperatively to mediate binding to the phosphorylated MuSK receptor.

Recruitment of ShcD to activated receptors results in its tyrosine phosphorylation and binding to Grb2. In addition to the two conserved motifs in the CH1 region, ShcD possesses an additional tyrosine residue in the CH1 region at position 424 that also mediates binding to Grb2. Distinct tyrosine kinases have different selectivity for the two conserved phosphorylation sites on mammalian Shc proteins (12, 33, 51, 55), and this effect may be amplified in ShcD with the presence of a third Grb2 binding site. It is worth noting that despite the conservation of the YYN and Y(V/I)N motifs in mammalian Shc proteins, ShcC binds weakly or not at all to Grb2 (28, 38), and it potentiates late but not early Ras activation, likely through its effect on phosphatidylinositol 3-kinase (38). Given the overlapping expression patterns of ShcC and ShcD in the nervous system, it is plausible that ShcD could participate in the early peak of Ras activation upon Grb2 recruitment to multiple YXN motifs. The unique signaling properties of ShcC are enhanced by the presence of additional sites of tyrosine phosphorylation in the CH1 region (28). One of these sites forms a YXXP motif and associates with the SH2 domain of the Crk adaptor protein (28). These novel sites of tyrosine phosphorylation are distinct from those identified on ShcD, suggesting that ShcC and ShcD may have evolved to couple with specific signaling adaptors downstream of neuronal RTKs.

Synaptogenesis at the NMJ requires agrin-dependent activation of MuSK signaling pathways that induce clustering and tyrosine phosphorylation of AChRs important for development of the postsynaptic muscle endplate. Numerous signaling proteins have been proposed to function downstream of MuSK, including Dishevelled (Dvl) (22), Abl (7), and 14-3-3 $\gamma$  (50), and membrane-associated guanylate kinase protein MAGI-1c (49) as well as a putative ariadne-like E3 ubiquitin ligase (2) can also associate with MuSK. Dvl and Abl are reported to affect AChR clustering (7, 22), while 14-3-3 $\gamma$  regulates synaptic gene expression at the NMJ (50). Of note, none of these proteins possesses a PTB domain for binding to the key NPXY juxtamembrane sequence on MuSK. Very recent experiments indicate that the PTB domain-containing adaptor protein Dok-7 associates with MuSK in a pTyr-dependent fashion, and this interaction enhances the catalytic activity of MuSK (35). Dok-7 expression is important for both clustering and tyrosine phosphorylation of the AChR, and mice deficient in Dok-7 display a similar phenotype as mice lacking agrin or MuSK (5, 9, 35). Human mutations in Dok-7 have been associated with congenital myasthenia syndromes, and these mutations result in impaired MuSK function (1). Together these

results demonstrate that Dok-7 is a major PTB-mediated effector of MuSK signaling. ShcD represents a second PTB domain-containing protein that may play a more minor role in MuSK signaling. Dok-7 may compete with ShcD for binding to the NPXY juxtamembrane sequence on MuSK, and this could explain our inability to coprecipitate MuSK and ShcD in C2C12 cells. Differential binding of Dok-7 and/or ShcD to MuSK may dictate whether AChRs undergo clustering or robust tyrosine phosphorylation. While reduced ShcD expression did not induce any defects in AChR clustering in C2C12 cells, ShcD appeared to be important for early tyrosine phosphorylation of the  $\beta$ -subunit of the AChR. Interestingly, a similar temporal effect on AChR phosphorylation is also observed following inhibition of Src family kinases (SFKs) (26). SFKs associate with and phosphorylate both MuSK and AChRs (8, 26, 27), and muscle cells derived from mice lacking Src and Fyn display unstable AChR clusters (45). ShcD could therefore contribute to the early Src-dependent phase of agrin-induced MuSK signaling, which regulates the stability of AChR clusters and proper maturation of the NMJ.

In summary, we have characterized a new member of the Shc family of docking proteins, ShcD/Shc4, thereby confirming that mammalian Shc proteins have expanded into a multiprotein family consisting of at least four members (21). ShcD may function as a scaffolding protein downstream of multiple RTKs, and the identification of ShcD as an additional component of the MuSK signaling platform indicates the complexity of signaling at the NMJ.

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#### ADDENDUM

While this article was under revision, Fagiani et al. reported the cloning and characterization of this same Shc family protein, which they have termed RaLP (6).

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