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The juxtamembrane region of MuSK has a critical role in agrin-mediated signaling

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MuSK is a receptor tyrosine kinase expressed selectively in skeletal muscle and localized to neuromuscular synapses. Agrin activates MuSK and stimulates phosphorylation and clustering of acetylcholine receptors (AChRs) at synaptic sites. We expressed wild-type or mutant MuSK in MuSK$^{-/-}$ myotubes and identified tyrosine residues in the MuSK cytoplasmic domain that are necessary for agrin-stimulated phosphorylation and clustering of AChRs. The activation loop tyrosines and the single juxtamembrane tyrosine were found to be essential for agrin-stimulated phosphorylation and clustering of AChRs. Further, we show that the juxtamembrane tyrosine, contained within an NPXY motif, is phosphorylated *in vivo* by agrin stimulation. We constructed chimeras containing extracellular and transmembrane domains from MuSK and cytoplasmic sequences from TrkA and found that inclusion of 13 amino acids from the MuSK juxtamembrane region, including the NPXY motif, is sufficient to convert a phosphorylated but inactive MuSK–TrkA chimera into a phosphorylated active chimera. These data suggest that phosphorylation of the MuSK NPXY site leads to recruitment of a phosphotyrosine-binding domain-containing protein that functions to stimulate phosphorylation and clustering of AChRs.

Keywords: acetylcholine receptors/agrin/MuSK/neuromuscular synapses/skeletal muscle

Introduction

Contact between the growth cone of a motor neuron and a developing skeletal muscle cell results in an exchange of signals between the neuron and muscle cell, resulting in the induction of a highly specialized postsynaptic membrane and a highly differentiated nerve terminal (Burden, 1998; Sanes and Lichtman, 1999). There is substantial evidence that agrin, an ~400 kDa protein that is expressed by motor neurons and concentrated in the synaptic basal lamina, has a critical role in inducing postsynaptic differentiation (McMahan, 1990). Importantly, agrin stimulates postsynaptic differentiation in muscle cells grown in cell culture, and mice lacking agrin fail to form neuromuscular synapses (Gautam *et al*., 1996; Ruegg and Bixby, 1998). Agrin induces postsynaptic differentiation by activating a receptor tyrosine kinase (RTK), termed MuSK, that is expressed selectively in skeletal muscle cells (Jennings *et al*., 1993; Ganju *et al*., 1995; Valenzuela *et al*., 1995; Glass *et al*., 1996). Although agrin does not bind MuSK directly, it stimulates the rapid phosphorylation of MuSK, which ultimately leads to the redistribution of previously unlocalized proteins, including acetylcholine receptors (AChRs), to synaptic sites (Glass *et al*., 1996). As expected for a component of an agrin receptor complex, MuSK is required for agrin to stimulate postsynaptic differentiation in muscle cells grown in cell culture, and mice lacking MuSK fail to form neuromuscular synapses (DeChiara *et al*., 1996).

The steps that follow MuSK activation and lead to clustering of postsynaptic proteins are not known. In addition to stimulating AChR clustering, agrin stimulates tyrosine phosphorylation of the AChR β-subunit (Wallace *et al*., 1991; Ferns *et al*., 1996). A downstream kinase appears to be essential for agrin-mediated signaling, since staurosporine blocks agrin-induced AChR clustering and phosphorylation without inhibiting MuSK phosphorylation (Wallace, 1994; Fuhrer *et al*., 1997). Clustering and phosphorylation of AChRs, however, appear to be independent, since β-subunit phosphorylation precedes but is not required for AChR clustering (Meyer and Wallace, 1998). These data suggest that MuSK either activates or recruits a kinase(s) that has a role in catalyzing tyrosine phosphorylation of AChRs and stimulating AChR clustering.

Pharmacological agents, including those that depolymerize microtubules, destabilize actin filaments, inhibit glycosylation, inhibit methylation, inhibit calmodulin, alter the cyclic nucleotide concentration or increase the activity of GTP-binding proteins, fail to affect agrin-induced AChR clustering (Wallace, 1988). Calcium is required for agrin to induce AChR clustering, but the precise role of calcium in agrin–MuSK signaling is not known (Wallace, 1988; Megeath and Fallon, 1998). Several lines of evidence support the idea that rapsyn, a 43 kDa peripheral membrane protein that is associated with AChRs, is a required intermediate on the pathway that couples MuSK activation to AChR clustering (Gautam *et al*., 1995). Nevertheless, it is unclear how MuSK activation leads to clustering of rapsyn.

Since RTKs initiate signaling by recruiting downstream components to the activated receptor, proteins that are immediately downstream of an activated RTK can be identified by first identifying sequences in the RTK that are necessary to activate downstream signaling (Schlessinger and Ullrich, 1992; Pawson, 1995). We expressed wild-type and mutant forms of MuSK in a MuSK$^{-/-}$ muscle cell line and identified sequences in MuSK that are necessary for agrin to induce phosphorylation and clustering of AChRs. We show that a juxtamembrane tyrosine, Y553, in MuSK is phosphorylated by agrin stimulation and that phosphorylation of this tyrosine residue is required for agrin-mediated signaling. We show

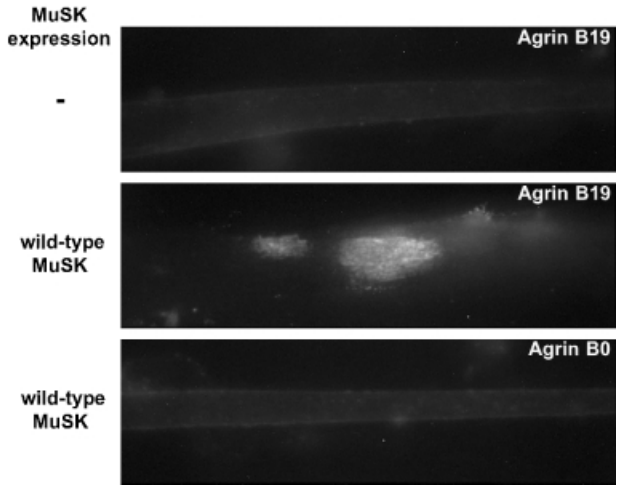


Fig. 1. Forced expression of wild-type MuSK in MuSK$^{-/-}$ myotubes restores agrin-mediated signaling. MuSK$^{-/-}$ myotubes and MuSK$^{-/-}$ myotubes infected with a retroviral vector encoding wild-type MuSK were treated with neural (A_4B_{19}) or non-neural (A_4B_0) agrin, and AChRs were labeled with Texas red-conjugated α-BGT.

that this tyrosine is within a consensus binding site for PTB domain-containing proteins, and our data support the idea that phosphorylation of this juxtamembrane tyrosine residue serves to recruit a PTB domain-containing protein to activated MuSK.

Results

Forced expression of MuSK in MuSK$^{-/-}$ myotubes restores agrin signaling

Ligand-stimulated activation of RTKs leads to phosphorylation of tyrosine residues in the activation loop of the kinase domain as well as phosphorylation of tyrosine residues outside the activation loop (Hubbard *et al*., 1994). These phosphotyrosine-containing sequences can serve as docking sites for signaling molecules that couple RTK activation to distinct downstream signaling pathways, leading to specific cellular responses (Schlessinger and Ullrich, 1992; Pawson, 1995). In order to learn more about how MuSK activation leads to clustering of synaptic proteins, we sought to identify tyrosine residues in MuSK that are required for agrin to stimulate clustering of AChRs.

We established an assay to measure MuSK function by generating MuSK-deficient muscle cell lines from embryos that were homozygous mutant for MuSK and that carried a temperature-sensitive large T oncogene (Jat *et al*., 1991). Cell lines could be maintained as proliferating myoblasts or induced to differentiate and form myotubes by controlling expression of large T. MuSK$^{-/-}$ myoblasts, like wild-type myoblasts, fuse and differentiate into multinucleated myotubes, and the morphology of wild-type and MuSK$^{-/-}$ myotubes is indistinguishable. Unlike myotubes from wild-type or MuSK heterozygous mice, MuSK$^{-/-}$ myotubes fail to cluster AChRs in response to agrin (Figure 1) (Glass *et al*., 1996). Myoblasts were infected with a recombinant retrovirus expressing wild-type MuSK, and transiently infected myoblasts were induced to differentiate into myotubes. Figure 1 shows that forced expression of wild-type MuSK restores agrin-stimulated AChR clustering in MuSK mutant myotubes. Like agrin-stimulated clustering of AChRs in wild-type myotubes, AChR clustering in the rescued myotubes is induced by neural (A_4B_{19}) and not non-neural (A_4B_0) agrin (Figure 1).

Identification of tyrosine residues required for MuSK function

The cytoplasmic domain of mammalian MuSK contains 17 tyrosine residues within the kinase domain and one tyrosine residue in the juxtamembrane region (Figure 2A) (Valenzuela *et al*., 1995). We expressed MuSK in insect cells using a baculovirus expression vector and mapped the tyrosines that are phosphorylated in MuSK in an *in vitro* kinase assay using a MALDI to assign the masses of ^{32}P-labeled tryptic peptides fractionated by HPLC (A.Watty, G.Neubauer, M.Dreger, M.Zimmer, M.Wilm and S.J.Burden, unpublished data). We found that the juxtamembrane tyrosine residue (Y553), two of the three tyrosine residues within the activation loop (Y754 and Y755) and two additional tyrosine residues within the kinase domain (Y576 and Y812) are phosphorylated in MuSK *in vitro*. To determine whether these tyrosine residues are important for MuSK signaling, we mutated each of these tyrosine residues and used the rescue assay to determine whether these mutated forms of MuSK could restore agrin-stimulated clustering of AChRs.

Figure 2 shows that the juxtamembrane tyrosine residue is essential for downstream signaling, since myotubes expressing MuSK Y553F fail to cluster AChRs in response to agrin (Figure 2B and C). Mutation of a single tyrosine within the activation loop (Y755) reduces AChR clustering by ~40%, whereas mutation of all three tyrosine residues (Y750, Y754 and Y755) within the activation loop abolishes AChR clustering (Figure 2C), indicating that MuSK kinase activity is required for agrin-stimulated AChR clustering. Other tyrosines within the kinase domain (Y576 and Y812) have little or no role in clustering AChRs, since myotubes expressing MuSK Y812F respond normally to agrin and myotubes expressing MuSK Y576F have 30% fewer AChR clusters than myotubes expressing wild-type MuSK (Figure 2C). Although we did not detect phosphorylation of MuSK Y831 *in vitro*, there is evidence that phosphorylation of this tyrosine residue is required for the function of certain RTKs (Ming *et al*., 1999). Figure 2 shows that Y831 is not required for MuSK function, since myotubes expressing MuSK Y831F cluster AChRs normally in response to agrin (Figure 2C). Because the level of MuSK expression from the retroviral vector is less than that of endogenous MuSK in wild-type cells, the ability of MuSK Y831F to restore agrin-mediated signaling is unlikely due to overexpression of defective MuSK.

Agrin stimulates phosphorylation of MuSK Y553

To determine whether Y553 is phosphorylated *in vivo* following agrin stimulation, we generated antibodies against a phosphopeptide sequence in MuSK containing phosphorylated Y553. We treated C2 myotubes with agrin, immunoprecipitated MuSK and probed Western blots with the Y553 phosphopeptide antibodies. Figure 3 shows that the Y553 phosphopeptide antibodies bind poorly to MuSK from untreated myotubes and intensely to MuSK from agrin-treated myotubes (Figure 3A). Antibody labeling is specific for the Y553 phosphopeptide, since the

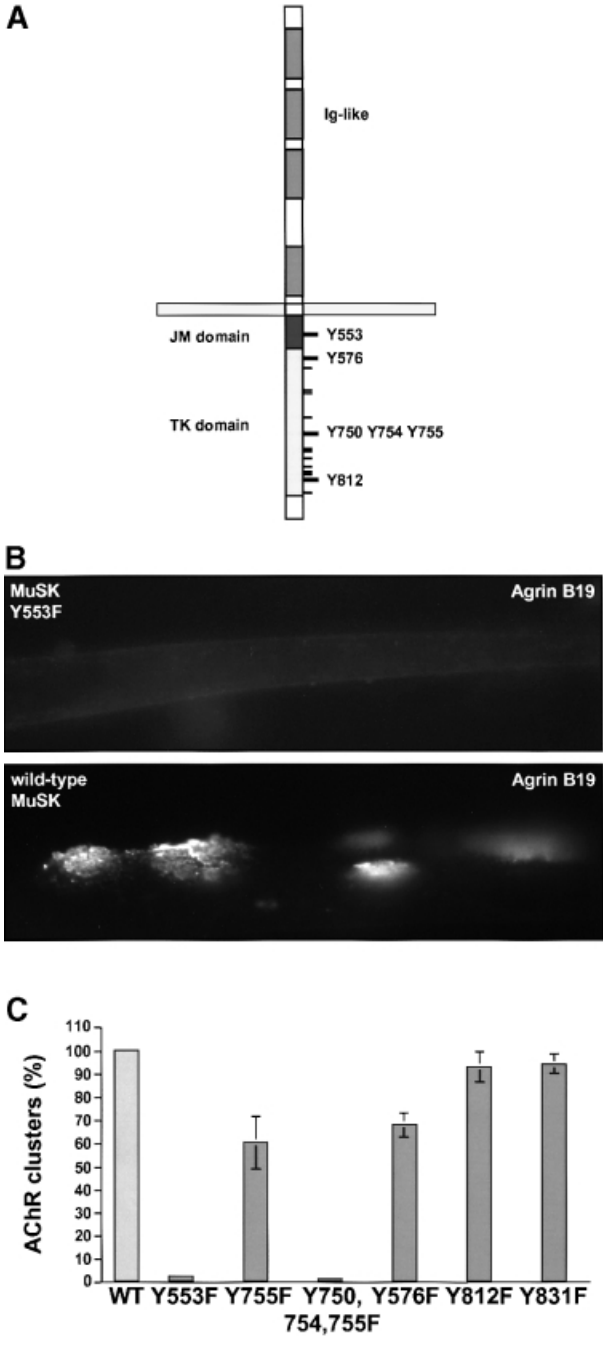


Fig. 2. Tyrosine residues within the activation loop and juxtamembrane region of MuSK are required for agrin-stimulated AChR clustering. (**A**) A cartoon showing the position of the single tyrosine residue in the juxtamembrane region of MuSK and the 17 tyrosine residues in the kinase domain of MuSK; the five tyrosine residues (Y553, Y576, Y754, Y755 and Y812) that are phosphorylated *in vitro* are indicated. (**B**) Neural (A_4B_{19}) agrin stimulates AChR clustering in MuSK$^{-/-}$ myotubes expressing wild-type MuSK but not MuSK Y553F. (**C**) The number of AChR clusters induced by agrin in MuSK$^{-/-}$ myotubes expressing wild-type or mutant MuSK. Mutation of the single juxtamembrane tyrosine (MuSK Y553F) or the three activation loop tyrosines (MuSK Y750,754,755F) results in a failure of neural agrin to stimulate AChR clustering. Mutation of a single tyrosine in the activation loop (Y755) or a tyrosine near the beginning of the kinase domain (Y576F) reduces the number of agrin-induced AChR clusters. Neural agrin induces a normal number (~10 per field) of AChR clusters in MuSK$^{-/-}$ myotubes expressing MuSK Y812F or MuSK Y831F.

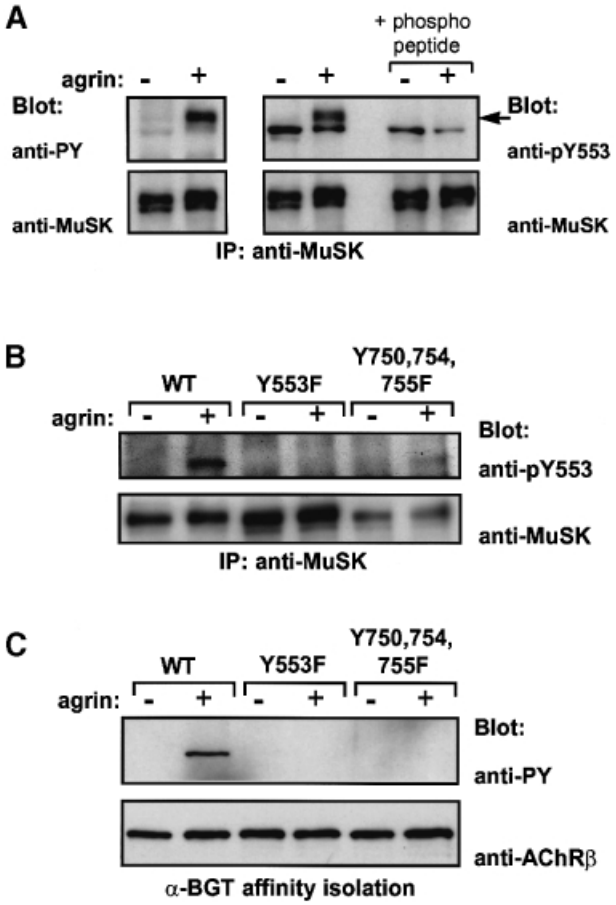


Fig. 3. Agrin stimulates phosphorylation of MuSK Y553. (**A**) MuSK was immunoprecipitated from C2 myotubes treated with neural agrin, and Western blots were probed either with antibodies to phosphotyrosine (anti-PY) or with antibodies to a Y553 phosphopeptide (anti-pY553). Antibodies to phosphotyrosine as well as antibodies to the Y553 phosphopeptide bind to MuSK (arrow) in muscle cells stimulated with neural agrin. Labeling with antibodies to the Y553 phosphopeptide is specific since labeling is blocked by pre-incubation of the antibodies with the phosphopeptide. Western blots were re-probed with antibodies to MuSK to ensure similar loading. (**B**) Antibodies to the Y553 phosphopeptide (anti-pY553) fail to bind MuSK Y553F and bind poorly to MuSK Y750,754,755F expressed in MuSK$^{-/-}$ myotubes. (**C**) Neural agrin stimulates tyrosine phosphorylation of the AChR β-subunit in MuSK$^{-/-}$ myotubes expressing wild-type MuSK but not MuSK Y553F or MuSK Y750,754,755F.

phosphorylated Y553 peptide competes for antibody binding (Figure 3A). Furthermore, TrkA contains a similar juxtamembrane sequence (Martin-Zanca *et al*., 1986), but phosphorylated TrkA protein, either expressed in baculovirus and phosphorylated in an *in vitro* kinase assay or isolated from nerve growth factor (NGF)-stimulated PC12 cells, is not recognized by the Y553 phosphopeptide antibodies (data not shown). To demonstrate further the specificity of the Y553 phosphopeptide antibodies, we treated MuSK$^{-/-}$ myotubes expressing MuSK Y553F with agrin, and probed Western blots of immunoprecipitated MuSK with the Y553 phosphopeptide antibodies. The Y553 phosphopeptide antibodies bind poorly to MuSK Y553F in untreated myotubes and agrin stimulation fails to increase antibody labeling (Figure 3B). Taken together, these data indicate that the Y553 phosphopeptide antibodies are specific for MuSK and that MuSK is phosphorylated *in vivo* at Y553 (Figure 3).

To determine whether mutation of the activation loop tyrosines results in a failure of agrin to stimulate phosphorylation of Y553, we treated MuSK$^{-/-}$ myotubes expressing MuSK Y750,754,755F with agrin, and we probed Western blots of immunoprecipitated MuSK Y750,754,755F with the Y553 phosphopeptide antibodies. Figure 3 shows that mutation of the activation loop tyrosines severely impairs but does not abolish phosphorylation of Y553 (Figure 3B). Although the activation loop tyrosines may have a separate role in MuSK function (Qian *et al*., 1998), it is possible that phosphorylation of these tyrosine residues is required to activate the kinase and phosphorylate Y553. These results demonstrate that agrin stimulation results in an increase in phosphorylation of Y553 and suggest that phosphorylation of Y553 is a critical step in MuSK function.

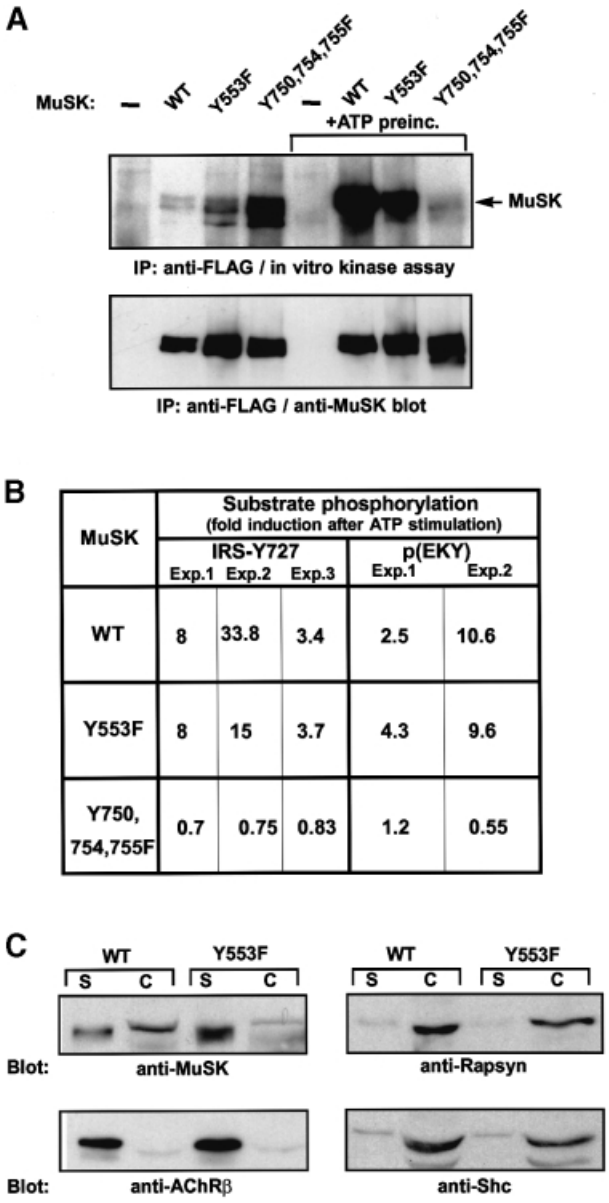
MuSK Y553 is required for agrin-stimulated AChR phosphorylation

Agrin stimulation of MuSK results in tyrosine phosphorylation of the AChR β-subunit (Wallace *et al*., 1991; Ferns *et al*., 1996). To determine whether the AChR β-subunit is tyrosine phosphorylated in myotubes expressing MuSK Y553F, we used α-bungarotoxin (α-BGT) to isolate AChRs from myotubes expressing wild-type or mutant MuSK and probed Western blots with antibodies to phosphotyrosine. Figure 3 shows that tyrosine phosphorylation of the AChR β-subunit is stimulated by agrin in myotubes expressing wild-type MuSK but not in myotubes expressing MuSK Y553F (Figure 3C). These results indicate that MuSK Y553 is required not only to cluster AChRs but also to stimulate AChR tyrosine phosphorylation.

MuSK Y553F is catalytically active and expressed on the cell surface

Although the failure of MuSK Y553 to rescue MuSK function may be due to a loss of Y553 phosphorylation, we considered the possibility that mutation of Y553 may have inactivated MuSK kinase activity. To determine whether mutation of Y553 reduces the kinase activity of MuSK, we expressed wild-type and mutant MuSK in insect cells and measured MuSK kinase activity in immunoprecipitates of MuSK from the baculovirus-infected cells. We measured the ability of MuSK to phosphorylate itself and to transphosphorylate a substrate peptide (Figure 4A and B). Wild-type MuSK and MuSK Y553F have low basal kinase activities that are stimulated substantially and similarly by ATP (Figure 4A and B). These results indicate that wild-type and Y553F mutant MuSK have similar kinase activities. MuSK Y750,754,755F has a higher basal kinase activity than wild-type MuSK, as expected for a destabilized activation loop (Hubbard *et al*., 1994), but this mutated kinase, unlike wild-type MuSK or Y553F MuSK, can not be stimulated further by ATP.

Because Y553 is embedded in an NPXY motif, which can function as an internalization signal in other membrane receptors (Chen *et al*., 1990), we considered the possibility that the failure of MuSK Y553 to rescue MuSK function might be due to a failure of MuSK to be expressed on the cell surface. We labeled cell surface proteins in intact cells expressing wild-type or MuSK Y553F with a



MuSK	Substrate phosphorylation (fold induction after ATP stimulation)				
	IRS-Y727			p(EKY)	
	Exp.1	Exp.2	Exp.3	Exp.1	Exp.2
WT	8	33.8	3.4	2.5	10.6
Y553F	8	15	3.7	4.3	9.6
Y750, 754,755F	0.7	0.75	0.83	1.2	0.55

Fig. 4. MuSK Y553F is expressed on the cell surface and is catalytically active *in vitro*. (**A**) *In vitro* kinase assays show that MuSK Y553F and wild-type MuSK are phosphorylated similarly *in vitro*, and stimulated by pre-incubation with ATP. In contrast, phosphorylation of MuSK Y750,754,755F is not stimulated by pre-incubation with ATP; the basal level of MuSK Y750,754,755F phosphorylation is increased probably due to destabilization of the activation loop. (**B**) *In vitro* kinase assays show that the IRS-Y727 and the p(EKY) peptides are phosphorylated similarly by MuSK Y553F and wild-type MuSK but poorly by MuSK Y750,754,755F. (**C**) Cell surface proteins in MuSK$^{-/-}$ myotubes expressing wild-type MuSK or MuSK Y553F were labeled with biotin-NHS. Proteins labeled with biotin and recovered with streptavidin were considered to be on the cell surface (S), whereas proteins that were not recovered with streptavidin were considered to be cytosolic (C). Western blots show that wild-type MuSK and MuSK Y553F are expressed on the cell surface. Control experiments show that the AChR β-subunit is expressed on the cell surface and that rapsyn and Shc are largely cytosolic.

membrane-impermeable, biotinylated *N*-hydroxysuccinimide ester (NHS-biotin), lysed the cells in detergent and isolated the biotin-labeled proteins with streptavidin. NHS-biotin selectively labels cell surface proteins, since membrane proteins that are exposed on the cell surface,

including AChRs, are labeled efficiently by NHS-biotin, whereas intracellular proteins, such as rapsyn and Shc, are labeled poorly by NHS-biotin (Figure 4C). Figure 4 demonstrates that MuSK Y553F, like wild-type MuSK, is expressed on the cell surface (Figure 4C). Thus, MuSK Y553F fails to restore MuSK function although the mutant protein is catalytically active and expressed on the cell surface.

Y553 is within an NPXY motif that can bind proteins with PTB domains

Because proteins containing phosphotyrosine-binding (PTB) domains bind phosphorylated NPXY motifs (Blaikie *et al*., 1994; Kavanaugh and Williams, 1994), we considered the possibility that a PTB domain-containing protein might bind the NPMY sequence in MuSK. In addition to the tyrosine residue, PTB domain-containing proteins require an asparagine at –3 and prefer a proline at –2, with respect to the tyrosine at +0 (van der Geer and Pawson, 1995). Therefore, we expressed MuSK N550A, P551A as well as Y553A in MuSK-deficient myotubes and determined whether the sequence requirements for agrin-stimulated signaling correspond to the sequence requirements for binding PTB domain-containing proteins. Figure 5 shows that myotubes expressing MuSK N550A and MuSK Y553A fail to cluster AChRs in response to agrin. Like wild-type MuSK and MuSK Y553F, MuSK N550A and MuSK Y553A are expressed on the cell surface (data not shown). Myotubes expressing MuSK P551A cluster AChRs in response to agrin, but the efficiency of agrin-stimulated AChR clustering in these myotubes is reduced (Figure 5A). Thus, there is a good correspondence between residues required for MuSK function and for binding PTB domain-containing proteins.

Neither Shc, IRS-1, IRS-2 nor FRS2 are activated in agrin-stimulated myotubes

Signaling proteins containing PTB domains become tyrosine phosphorylated following their recruitment to activated receptors (van der Geer and Pawson, 1995). To determine whether Shc (Pelicci *et al*., 1992), IRS-1/2 (Sun *et al*., 1991) or FRS2 (Kouhara *et al*., 1997) is tyrosine phosphorylated following agrin stimulation, we treated C2 myotubes with agrin, immunoprecipitated tyrosine-phosphorylated proteins with an antibody against phosphotyrosine and probed Western blots with antibodies against Shc, IRS-1, IRS-2 or FRS2. Agrin fails to stimulate tyrosine phosphorylation of Shc, IRS-1, IRS-2 or FRS2 (Figure 5B). In contrast, tyrosine phosphorylation of Shc and FRS2 is stimulated in PC12 cells treated with NGF, and tyrosine phosphorylation of IRS-1 and IRS-2 is stimulated in L6 myoblasts treated with insulin (Figure 5B). Thus, neither Shc, IRS-1, IRS-2 nor FRS2 is activated by agrin.

We used a similar assay to determine whether agrin activates kinases that are often stimulated by other RTKs. We treated C2 myotubes with agrin and probed Western blots with antibodies against phosphorylated Erk1/2, JNK, p38 or AKT. Figure 5 shows that agrin fails to activate these downstream kinases (Figure 5C). Likewise, agrin fails to stimulate phosphorylation of the p85 subunit of phosphatidylinositol 3-kinase (PI3-kinase; data not shown). Consistent with these data, neither PD 09857, a MEK1 inhibitor, nor wortmanin, an inhibitor of PI3-kinase, inhibits agrin-induced AChR clustering (data not shown).

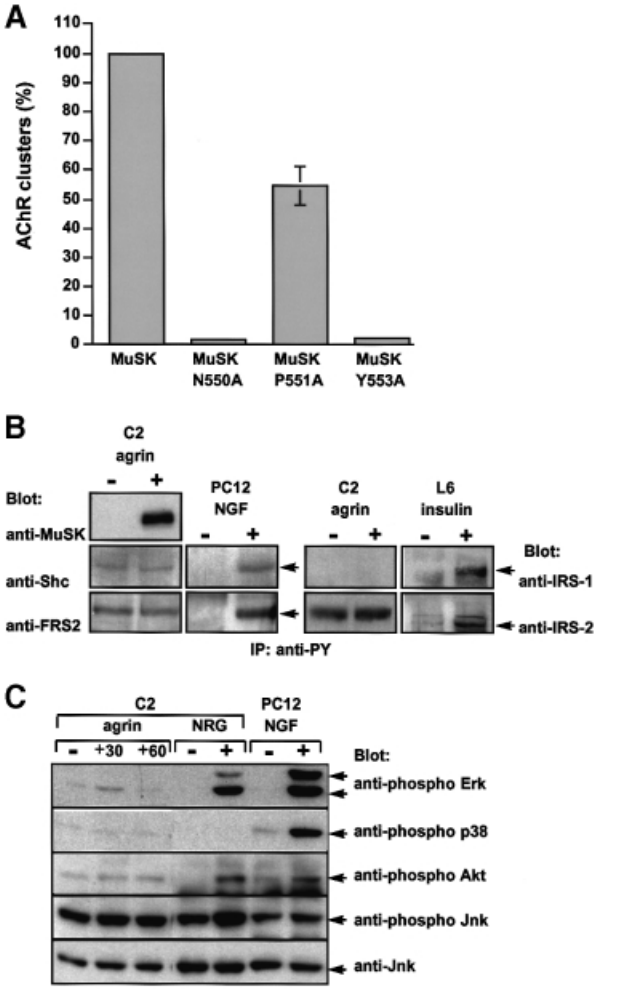


Fig. 5. The NPXY motif in MuSK is required for agrin-mediated signaling, but neural agrin fails to stimulate phosphorylation of Shc, IRS-1, IRS-2, FRS2 or components of MAP kinase or PI3-kinase signaling pathways. (**A**) Neural agrin fails to induce AChR clusters in MuSK$^{-/-}$ myotubes expressing MuSK N550A or MuSK Y553A and induces fewer AChR clusters in MuSK$^{-/-}$ myotubes expressing MuSK P551A. (**B**) Tyrosine-phosphorylated proteins were immunoprecipitated from C2 myotubes, PC12 cells or L6 myoblasts, and Western blots were probed with antibodies to Shc, IRS-1, IRS-2 or FRS2. Phosphorylation of Shc and FRS2 is stimulated in PC12 cells treated with NGF, and phosphorylation of IRS-1 and IRS-2 is stimulated in L6 myoblasts treated with insulin. (**C**) Western blots of lysates from C2 myotubes show that neural agrin fails to stimulate phosphorylation of p38, Akt or Jnk; agrin induces a modest increase in Erk1/2 phosphorylation. NGF stimulates phosphorylation of Erk 1/2, p38 and Akt in PC12 cells (Hempstead *et al*., 1992). Western blots were re-probed with antibodies to Jnk to ensure similar loading.

The juxtamembrane region of MuSK has a role in MuSK phosphorylation

Figure 6 shows that agrin fails to stimulate phosphorylation of MuSK N550A, MuSK Y553F and MuSK Y553A (Figure 6A), indicating that mutation of the NPXY motif is sufficient to impair phosphorylation of the entire kinase (Figure 6A). MuSK P551A is phosphorylated by agrin but less efficiently than wild-type MuSK (Figure 6B), and the reduction in phosphorylation parallels the reduction in AChR clustering (Figure 5A). Likewise, mutation of

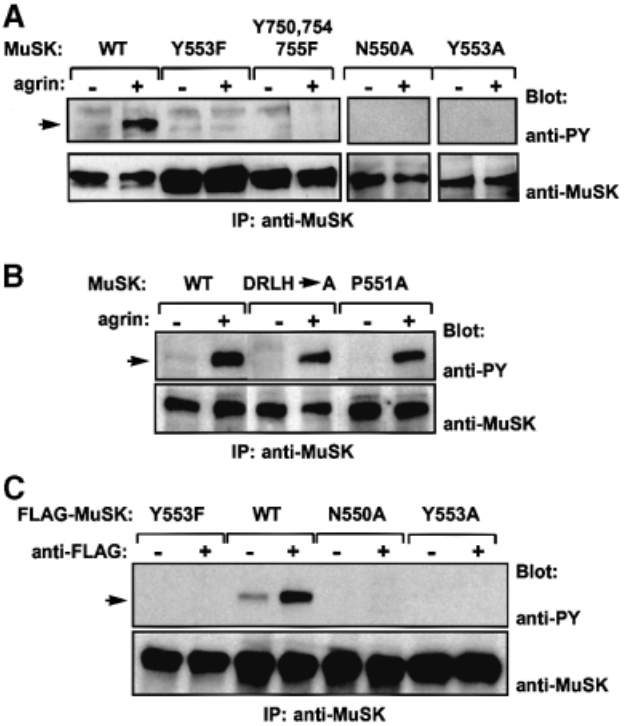


Fig. 6. The NPXY motif in MuSK is required for agrin-stimulated MuSK phosphorylation. (**A**) Neural agrin fails to stimulate phosphorylation of MuSK in MuSK$^{-/-}$ myotubes expressing MuSK Y553F, MuSK Y750,754,755F, MuSK N550A or MuSK Y553A. (**B**) Neural agrin stimulates MuSK phosphorylation in MuSK$^{-/-}$ myotubes expressing MuSK DRLH[545–548]AAAA or MuSK P551A. (**C**) Antibodies to a FLAG epitope tag, introduced in the extracellular region of MuSK, induce phosphorylation of wild-type MuSK but fail to induce phosphorylation of MuSK N550A and MuSK Y553A.

amino acid residues that flank the NPXY motif (MuSK DRLH[545–548]AAAA) and that have a role in binding PTB domain-containing proteins (van der Geer and Pawson, 1995) results in an ~2-fold reduction in the level of agrin-induced MuSK phosphorylation and AChR clustering (Figure 6B; data not shown). These results indicate that the asparagine and tyrosine residues within the NPXY motif have a critical role in activating the kinase domain of MuSK and/or preventing dephosphorylation of activated MuSK *in vivo*, whereas the proline residue and amino acids that are N-terminal to the NPXY motif are less crucial for MuSK phosphorylation and AChR clustering.

Current data support the idea that MuSK is not a direct receptor for agrin and that myotubes express an additional activity that is required for agrin to activate MuSK (Glass *et al*., 1996). These data have led to the suggestion that myotubes may express a protein that binds agrin, associates with MuSK and functions as a signaling complex with MuSK. Thus, it is possible that the juxtamembrane region of MuSK facilitates association between MuSK and this putative agrin-binding receptor and that mutation of the juxtamembrane region prevents this association and blocks agrin activation of MuSK. To bypass the requirement for an agrin receptor, we activated MuSK with antibodies against a FLAG epitope introduced into the extracellular domain of MuSK (Xie *et al*., 1997; Hopf and Hoch, 1998). Figure 6 shows that these antibodies induce phosphorylation of wild-type MuSK but not MuSK containing mutations in the NPXY motif (Figure 6C). Thus, the juxtamembrane NPXY motif is required for antibodies against MuSK, as well as agrin, to activate and/or maintain phosphorylation of MuSK. Since mutation of the NPXY motif does not reduce the *in vitro* kinase activity of MuSK (Figure 4A and B), these results suggest that the NPXY motif is required *in vivo* either to activate MuSK kinase activity or to recruit a protein that prevents a phosphatase from dephosphorylating MuSK. The following experiments provide evidence that the juxtamembrane region of MuSK has a role in clustering and phosphorylating AChRs that supersedes its role in regulating MuSK phosphorylation.

The juxtamembrane NPXY motif in MuSK has a critical role in clustering and phosphorylating AChRs

The cytoplasmic domains of MuSK and the Trk neurotrophin receptors are similar in sequence (Jennings *et al*., 1993). In particular, TrkA contains an NPXY motif in the juxtamembrane region that is critical for TrkA signaling (Stephens *et al*., 1994). The sequence flanking the NPXY motif in TrkA, however, conforms to a consensus binding site for Shc, and both Shc and FRS2 are recruited to this region of activated TrkA (Stephens *et al*., 1994; Meakin *et al*., 1999). To determine whether sequences in the TrkA cytoplasmic domain could substitute for sequences in the cytoplasmic domain in MuSK, we constructed MuSK–TrkA chimeras containing the extracellular and transmembrane domains from MuSK and cytoplasmic sequences from TrkA (Figure 7A). We expressed the MuSK–TrkA chimeras in MuSK$^{-/-}$ myotubes and asked whether the chimeras could restore agrin-mediated signaling. A chimera containing the extracellular and transmembrane domains of MuSK and the entire cytoplasmic domain of TrkA is phosphorylated by agrin stimulation (Figure 7B) but fails to induce clustering or phosphorylation of AChRs (Figure 7C and D). In contrast, the same MuSK–TrkA chimera, but including a substitution of 13 amino acids from the juxtamembrane domain of MuSK with the comparable region of TrkA, is phosphorylated by agrin stimulation (Figure 7B) and induces phosphorylation and clustering of AChRs (Figure 7C and D). Thus, 13 amino acids from the juxtamembrane region of MuSK, including the NPXY motif, are sufficient to convert a phosphorylated but inactive chimera into a phosphorylated and active chimera. These results are consistent with the idea that phosphorylation of the NPXY motif in MuSK leads to the recruitment of an adaptor protein(s) that is required for clustering and phosphorylating AChRs.

Discussion

Agrin stimulates the rapid phosphorylation of MuSK, leading to the redistribution of previously unlocalized proteins, including AChRs, to synaptic sites (DeChiara *et al*., 1996; Glass *et al*., 1996). The steps that follow MuSK activation and lead to clustering of postsynaptic proteins are not known. We expressed wild-type and mutant forms of MuSK in a MuSK$^{-/-}$ muscle cell line and found that agrin stimulates phosphorylation of a juxtamembrane tyrosine, Y553, in MuSK that is required for agrin to induce phosphorylation and clustering of

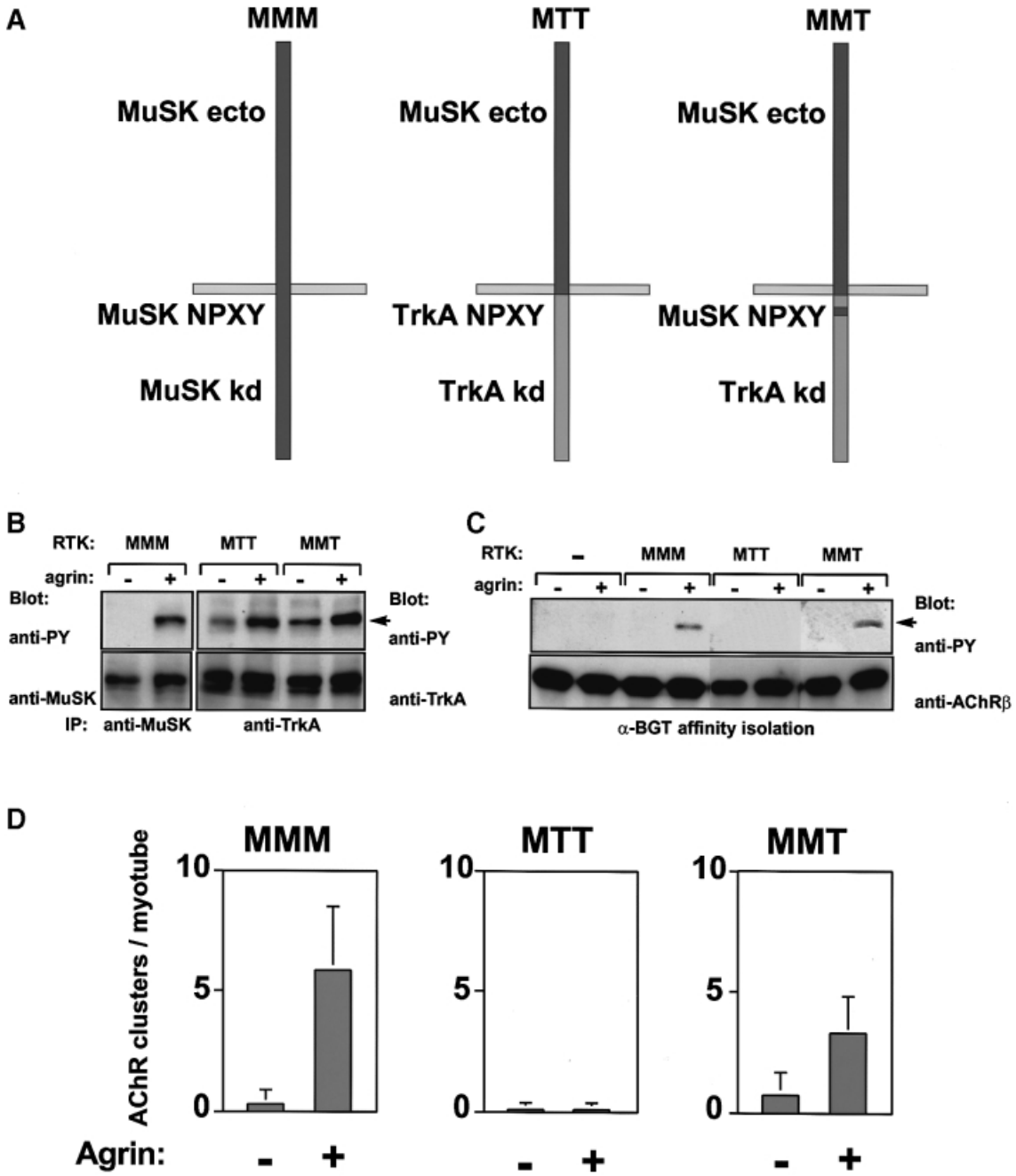


Fig. 7. The juxtamembrane region of MuSK has a critical role in agrin-mediated signaling. (**A**) The cartoon shows wild-type MuSK (MMM), a MuSK–TrkA chimera (MTT) containing the juxtamembrane (NPXY) and kinase domains (kd) of TrkA and a MuSK–TrkA chimera (MMT) containing 13 amino acids (MuSK NPXY) from the juxtamembrane domain of MuSK and the kinase domain of TrkA. (**B**) Neural agrin stimulates phosphorylation of MMM, MTT and MMT chimeras. MTT and MMT chimeras have a higher level of basal phosphorylation than wild-type MuSK. (**C**) Neural agrin stimulates phosphorylation of the AChR β-subunit in MuSK$^{-/-}$ myotubes expressing wild-type MuSK or the MMT chimera but not in MuSK$^{-/-}$ myotubes expressing the MTT chimera. (**D**) Neural agrin stimulates clustering of AChRs in MuSK$^{-/-}$ myotubes expressing wild-type MuSK or the MMT chimera but not in MuSK$^{-/-}$ myotubes expressing the MTT chimera ($p < 0.001$). The MMT chimera is ~2-fold less active than wild-type MuSK ($p < 0.001$). Although basal phosphorylation of the MTT and MMT chimeras is elevated similarly (B), only myotubes expressing the MMT chimera have an increased number of AChR clusters in the absence of agrin stimulation (D).

AChRs. This juxtamembrane tyrosine residue is within a consensus binding site for PTB domain-containing proteins, and our experiments suggest that phosphorylation of this tyrosine residue serves to recruit a PTB domain-containing protein to activated MuSK.

Experiments with MuSK–TrkA chimeras provide the most compelling data indicating a critical role for the MuSK juxtamembrane region in agrin-mediated signaling. Importantly, only 13 amino acids from the juxtamembrane region of MuSK are sufficient to convert a phosphorylated but inactive MuSK–TrkA chimera into a phosphorylated and active MuSK–TrkA chimera. Since these 13 amino acids include the MuSK NPXY motif, these data suggest that a PTB domain-containing protein is recruited to this site and functions to stimulate phosphorylation and clustering of AChRs even in the context of the juxtamembrane, kinase and C-terminal tail domains of TrkA.

Zhou *et al*. recently reported that the juxtamembrane

tyrosine in MuSK is required for agrin to stimulate MuSK phosphorylation and AChR clustering, and suggested that PTB domain-containing proteins are required for activation of MuSK's kinase activity (Zhou *et al*., 1999). We likewise found that the juxtamembrane tyrosine in MuSK is required for agrin to stimulate MuSK phosphorylation, and these results made it difficult for us to determine whether the failure to phosphorylate or cluster AChRs is due simply to reduced kinase activity or to an inability to recruit a PTB domain-containing adaptor protein. Our *in vitro* kinase experiments as well as experiments with the MuSK–TrkA chimeras provide evidence that the juxtamembrane region of MuSK has a role in phosphorylating and clustering AChRs that supersedes its role in regulating MuSK phosphorylation. Further, our experiments show that agrin stimulates phosphorylation of the juxtamembrane tyrosine in MuSK *in vivo* and that other tyrosines in the kinase domain of MuSK (Y576, Y754 and Y755) participate in agrin-mediated signaling.

The role of the juxtamembrane region of MuSK in activating the kinase activity of MuSK and/or maintaining MuSK phosphorylation *in vivo* is shared with the platelet-derived growth factor β (PDGFβ) and EphB2 receptors (Mori *et al*., 1993; Holland *et al*., 1997; Baxter *et al*., 1998; Zisch *et al*., 1998). Mutation of the Src-binding site in the juxtamembrane region of the PDGFβ receptor results in a substantial decrease in PDGF-stimulated receptor phosphorylation, largely due to a decrease in ligand-stimulated PDGFβ receptor kinase activity (Mori *et al*., 1993; Baxter *et al*., 1998). Because mutation of juxtamembrane tyrosines in MuSK and the PDGFβ receptor does not alter their ATP-stimulated kinase activities (Figure 4A and B) (Baxter *et al*., 1998), these juxtamembrane tyrosines are not required for kinase activity *per se*. The mechanisms by which juxtamembrane tyrosines regulate ligand-stimulated kinase activity *in vivo* are not known, but others have suggested that phosphorylation of the juxtamembrane tyrosines in the PDGFβ receptor is required to phosphorylate the proposed activation loop tyrosine, which leads to full activation of the kinase and further phosphorylation of the receptor and its associated proteins (Baxter *et al*., 1998). Mutation of a juxtamembrane tyrosine residue in the EphB2 receptor, involved in recruiting Src and Ras-GAP, also results in a decrease in ligand-stimulated receptor phosphorylation (Holland *et al*., 1997; Zisch *et al*., 1998). Our results suggest that the NPXY motif in MuSK is required *in vivo* either to activate MuSK kinase activity or to recruit a protein that prevents a phosphatase from dephosphorylating MuSK. Taken together, these results suggest that the juxtamembrane region of RTKs may have a more common role in regulating ligand-stimulated receptor phosphorylation than currently appreciated.

Because the asparagine residue within an NPXY motif is necessary to bind PTB domain-containing proteins (Trüb *et al*., 1995; Zhou *et al*., 1995; Eck *et al*., 1996), we reasoned that mutation of this asparagine residue in MuSK would prevent recruitment of a PTB domain-containing protein without disturbing MuSK phosphorylation. Our experiments, however, show that mutation of the asparagine residue results in a failure of agrin, as well as antibodies to MuSK, to stimulate MuSK phosphorylation. Since the asparagine in the NPXY motif is necessary to form a β-turn, it is possible that mutation of the asparagine results in structural changes that prevent the tyrosine within the NPXY motif from contacting the active site of the kinase.

Contacts between PTB domains and amino acids that are N-terminal to the NPXY motif have an important role in specifying recruitment of PTB domain-containing proteins (van der Geer and Pawson, 1995). For example, the PTB domain from Shc binds preferentially to a peptide with a hydrophobic residue at –5, and the PTB domain from IRS-1 binds preferentially to a peptide with a hydrophobic residue at –6, –7 and –8. Since MuSK (DRLHPNPMY) has histidine, asparagine and aspartate residues, rather than hydrophobic residues at positions –5, –7 and –8, the sequence adjacent to the juxtamembrane NPXY motif in MuSK does not conform to an Shc- or an IRS-1-binding site, and our experimental data indicate that neither Shc nor IRS-1 are recruited to activated MuSK. Likewise, agrin fails to stimulate phosphorylation of IRS-2 or FRS2, indicating that neither of these PTB domain-containing proteins is recruited to activated MuSK. IRS-3 is not expressed in skeletal muscle (Sciacchitano and Taylor, 1997), and IRS-4 is expressed at very low levels in skeletal muscle (Fantin *et al*., 1999). Further, because of sequence similarities in the IRS-1 and IRS-4 PTB domains (Lavan *et al*., 1997), it is likely that IRS-4, like IRS-1 and IRS-2, would fail to bind the MuSK juxtamembrane sequence. Because substitution of DRLH with alanine residues impairs but does not block MuSK–agrin signaling, a PTB domain-containing protein that would bind the juxtamembrane region of MuSK would be predicted to tolerate small hydrophobic residues N-terminal to the NPXY motif. Further studies will be required to identify the protein(s) that may be recruited to the juxtamembrane region of MuSK.

The tyrosine residues that are within the activation loop of MuSK have a critical role in agrin signaling. These results are consistent with the role of activation loop tyrosines in the insulin and fibroblast growth factor (FGF) receptors, where activation loop tyrosines are required for maximal ligand-stimulated kinase activity (Hubbard *et al*., 1998). These results indicate that MuSK kinase activity is required for agrin-mediated signaling and are consistent with experiments showing that a kinase-inactive form of MuSK can function as a dominant-negative inhibitor of agrin signaling (Glass *et al*., 1997). Mutation of Y576 reduces the number of AChR clusters that are induced by agrin, indicating that Y576 contributes to but is not essential for agrin signaling. Y576 is not conserved in TrkA, which may explain why the MuSK–TrkA chimera containing the juxtamembrane region from MuSK is ~2-fold less active than wild-type MuSK in clustering AChRs.

Agrin fails to stimulate well-characterized MAP kinase or PI3-kinase signaling pathways. Activated MuSK organizes and clusters synaptic membrane proteins and does not, like many RTKs, regulate gene transcription. For these reasons, it may be less surprising that MuSK activation fails to stimulate signaling pathways that commonly are associated with changes in gene expression, and these results suggest that MuSK may organize and assemble proteins in the postsynaptic membrane by distinctive signaling mechanisms.

Materials and methods

Cell culture

C2 myoblasts were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with glutamine, 4.5 mg/ml glucose, 15% fetal bovine serum (FBS), 0.5% chick embryo extract (Life Technologies) and gentamycin and shifted to fusion medium (DMEM, 2.5% horse serum and gentamycin) when 90% confluent. Bosc 23 cells (Pear *et al*., 1993) were grown in DMEM with 10% FBS, gentamycin and GPT selection reagent (Specialty Media Inc.). Sf9 and High Five cells were maintained in TMN-FH (Invitrogen) and EXCELL 400 (JRH Scientific), respectively.

MuSK$^{-/-}$ muscle cell lines

Limb muscles from E18 MuSK$^{-/-}$; H-2Kb-tsA58 E18 embryos were dissociated with 0.2% trypsin and 0.01% DNase. Cells were resuspended in growth medium (DMEM, glutamine, 4.5 mg/ml glucose, 10% FBS, 10% horse serum, 0.5% chick embryo extract, gentamycin), supplemented with 20 U/ml of recombinant mouse interferon-γ (IFN-γ; Life Technologies) and pre-plated for 20 min at 33°C. Non-adherent cells were transferred to a Matrigel-coated tissue culture dish (Falcon) and maintained in growth medium at 33°C/10% CO_2. Clones of cells were expanded under permissive conditions and assayed for their ability to differentiate into myotubes by removing IFN-γ and chick embryo extract and increasing the temperature to 39°C.

Expression constructs

Single amino acid substitutions in the rat MuSK cytoplasmic domain were generated by site-directed mutagenesis (Promega); the DRLH sequence in the juxtamembrane region of MuSK was replaced with alanines by PCR. MuSK cDNAs were ligated into the retroviral vectors pBabe/puro (Morgenstern and Land, 1990) and pLXSG (provided by Dr P.Lewis, Oregon Health Sciences University). A FLAG epitope was introduced into a 10 amino acid alternative insert in the extracellular domain of MuSK by PCR (Valenzuela *et al*., 1995). Wild-type and mutant FLAG-MuSK cDNAs were ligated into the pLXSG vector. In order to construct the MTT MuSK–TrkA chimera, a *Sal*I site was inserted into the MuSK cDNA by PCR. A *Sal*I fragment from a rat TrkA cDNA (Perez *et al*., 1995), encoding the entire cytoplasmic domain (amino acids 447–799) of TrkA, was ligated to the extracellular and transmembrane domains of MuSK through the *Sal*I site. To generate the MMT MuSK–TrkA chimera, *Pst*I and *Bsa*AI sites were introduced into the juxtamembrane region of the MTT chimera by PCR, and an oligonucleotide encoding amino acids 544–556 of MuSK was ligated into *Pst*I–*Bsa*AI sites. The MTT and MMT constructs were ligated into the pBabe/puro vector.

Retroviral infection of myoblasts

Bosc 23 cells were transfected with plasmids (Chen and Okayama, 1987), and virus-containing medium was collected 2 days post-transfection and used immediately for infection. The viral titer was estimated by measuring green fluorescent protein (GFP) expression, co-expressed from the LXSG retrovirus, by fluorescence activated cell sorting (FACS). Myoblasts, at 50% confluency, were incubated with virus-containing medium, supplemented with 2 μg/ml polybrene (Sigma), for 2 h at 33°C. For transient infection experiments, cells were switched to fusion medium when 90% confluent. Myoblasts infected with the pBabe/puro virus were selected in growth medium containing puromycin (2 ng/ml). Clones of cells were isolated and assayed for fusion.

AChR clustering assay

Constructs encoding neural (A_4B_{19}) or non-neural (A_4B_0) chick agrin were transiently expressed in COS cells (Tsim *et al*., 1992). The agrin-conditioned medium was collected, assayed for AChR clustering activity on C2 myotubes and used at the lowest concentration that stimulated maximal AChR clustering. Myotubes were stimulated with agrin for 7–16 h and incubated with Texas red-conjugated α-BGT to label AChRs. The cells were washed with phosphate-buffered saline (PBS), fixed in 1% formaldehyde and mounted under coverslips. The cells were viewed at 250× or 630× with a Zeiss Axioskop. We quantitated AChR clustering by counting the number of AChR clusters in 15 fields and calculated the number of AChR clusters per field. At least four independent experiments were performed for each MuSK mutant.

Immunoprecipitation and Western blotting

Cells were switched to DMEM without serum for several hours prior to stimulation with agrin, growth factors or antibodies to the FLAG epitope. Cells were treated with agrin or antibodies to the FLAG epitope for 30 min and with other growth factors for 10 min. Cells were rinsed with ice-cold PBS and extracted in lysis buffer (Fuhrer *et al*., 1997). Lysates were pre-cleared by centrifugation and incubated with antibodies for 4 h. The antibodies were captured with protein A–Sepharose beads, which subsequently were washed four times in lysis buffer. Bound proteins were eluted from the beads with SDS sample buffer, resolved by SDS–PAGE and transferred to Immobilon-P membranes (Millipore). Membranes were blocked in TBST (Tris-buffered saline, 0.05% Tween-20) containing 5% bovine serum albumin (for anti-phosphotyrosine blots) or skimmed milk and probed with antibodies. For peptide competition, 100 μM phosphopeptide was added to the blotting solution containing the anti-pY553 phosphopeptide antibodies for 30 min prior to incubation with the filter.

Biotinylation of surface proteins

Myotubes were washed with PBS supplemented with 0.1 mM $CaCl_2$/1 mM $MgCl_2$ (PBS/Ca/Mg) and incubated with 0.5 mg/ml EZ-link sulfo-NHS-LC-biotin (Pierce) in PBS/Ca/Mg at room temperature for 30 min. Cells were rinsed twice with PBS/Ca/Mg and incubated with DMEM at 39°C/10% CO_2. After 30 min, the cells were washed with ice-cold PBS and lysed in RIPA buffer [containing 0.5% SDS and supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 ng/ml aprotinin, 10 ng/ml leupeptin, 1 ng/ml pepstatin]. Lysates were pre-cleared by centrifugation, and biotin-labeled proteins were recovered by incubating with streptavidin–agarose for 4 h at 4°C, followed by four washes with RIPA buffer containing 0.1% SDS. Bound and unbound (10% of supernatant) proteins were resolved by SDS–PAGE and detected by Western blotting.

Baculovirus expression of MuSK

Wild-type MuSK, MuSK Y553F and MuSK Y750,754,755F, each containing a FLAG epitope, were subcloned into pAcSG2 (Pharmingen). Recombinant baculovirus was generated using the BaculoGold transfection kit as described by the manufacturer. Plaque-purified recombinant virus was amplified by repetitive infection of Sf9 cells. MuSK protein was isolated from High Five cells, which were infected with recombinant virus and lysed after 3 days.

In vitro kinase assays

Cells were lysed in 1% NP-40, 20 mM HEPES pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 10 ng/ml leupeptin, 10 ng/ml aprotinin and 1 ng/ml pepstatin. Proteins were collected by immunoprecipitation. The beads were washed three times with lysis buffer, once with kinase buffer (20 mM HEPES pH 7.4, 10 mM $MnCl_2$) and pre-incubated in kinase buffer containing 200 μM ATP at 30°C for 20 min. The beads were washed once with kinase buffer and then incubated in 30 μl of kinase buffer with 5 μCi of [γ-^{32}P]ATP (3000 Ci/mmol, NEN) and 0.1 mM vanadate at 30°C for 10 min. Proteins were resolved by SDS–PAGE, and radiolabeled proteins were visualized by autoradiography. For exogenous substrate phosphorylation, peptides [1 mM of a 13mer peptide, IRS-Y727, containing the 727 phosphorylation site in IRS-1, kindly provided by Dr J.Till, Skirball Institute, or 0.10 μM of a poly-glutamate/lysine/tyrosine peptide, p(EKY), Sigma], were added to the kinase reaction and incubated at 30°C for 30 min. The reaction was stopped by adding 45 μl of ice-cold 10% trichloroacetic acid (TCA), followed by centrifugation for 2 min. The supernatants were spotted on cation exchange filters (Whatman) and washed four times with 250 ml of ice-cold 0.5% phosphoric acid and once with acetone at room temperature. Filters were air-dried and counted in a β-counter.

Antibodies

Polyclonal antibodies to tyrosine-phosphorylated LHPNPMYQRMPLL, conjugated to keyhole limpet hemocyanin (KLH), were produced in rabbits and affinity purified (Research Genetics). Antibodies to the C-terminal sequence of MuSK were provided by Dr C.Fuhrer and were described previously (Fuhrer *et al*., 1997). The M2 anti-FLAG monoclonal antibody was purchased from Sigma. Streptavidin- and Texas red-coupled α-BGT were purchased from Molecular Probes, and monoclonal antibody 124 against the AChR β-subunit was a gift from Dr J.Lindstrom (University of Pennsylvania). The phosphotyrosine antibody mAb 4G10 and purified polyclonal antibodies to Shc, IRS-1 and IRS-2 were purchased from Upstate Biotechnology Inc. Polyclonal antiserum to FRS2 was provided by Dr J.Schlessinger (NYU Medical Center). Antibodies to rapsyn were produced in rabbits immunized with GST–mouse rapsyn and affinity purified on GST–rapsyn and

GST–pumC affinity columns. The phospho-specific antibodies to Erk, p38, Akt and Jnk were purchased from New England Biolabs, and the purified polyclonal antibodies to unphosphorylated Jnk and TrkA were purchased from Santa Cruz.

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