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Agrin Acts via a MuSK Receptor Complex

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Summary

Formation of the neuromuscular junction depends upon reciprocal inductive interactions between the developing nerve and muscle, resulting in the precise juxtaposition of a differentiated nerve terminal with a highly specialized patch on the muscle membrane, termed the motor endplate. Agrin is a nerve-derived factor that can induce molecular reorganizations at the motor endplate, but the mechanism of action of agrin remains poorly understood. MuSK is a receptor tyrosine kinase localized to the motor endplate, seemingly well positioned to receive a key nerve-derived signal. Mice lacking either agrin or MuSK have recently been generated and exhibit similarly profound defects in their neuromuscular junctions. Here we demonstrate that agrin acts via a receptor complex that includes MuSK as well as a myotube-specific accessory component.

Introduction

Signals from motor neurons to muscle, which regulate muscle contraction as well as other aspects of muscle function, are transmitted across a synaptic structure known as the neuromuscular junction (NMJ) (Hall and Sanes, 1993). Formation of this structure depends upon reciprocal inductive interactions between the developing nerve and muscle that result in the precise juxtaposition of a differentiated nerve terminal with a tiny and highly specialized patch on the muscle membrane, referred to as the motor endplate. This postsynaptic specialization is designed to optimize recognition of nerve-derived signals, and it markedly differs from the rest of the myofiber surface at both the ultrastructural and molecular levels. Molecularly, the postsynaptic surface is characterized by an extraordinarily dense clustering of receptors for nerve-derived signals. The most extensively studied of these are the receptors for the neurotransmitter acetylcholine (AChRs), which regulate the electrical activity and contractile state of the muscle. The postsynaptic membrane is also characterized by a unique molecular scaffold that appears responsible for maintaining the postsynaptic specialization by anchoring it to both the overlying basal lamina and the underlying cytoskeleton.

Nerve-derived signals appear to induce postsynaptic differentiation by at least two mechanisms (Hall and Sanes, 1993). First, these signals can induce redistribution of pre-existing molecules that are initially expressed throughout the surface of the myofiber. Second, nerve-derived signals appear able to induce localized transcription of certain genes only by the subsynaptic nuclei underlying the NMJ. Many questions still remain concerning the precise nature of the nerve-derived inductive signals. One key nerve-derived factor appears to be a protein, termed agrin, that was discovered for its ability to cause clustering of pre-existing AChRs on the surface of cultured myotubes (Godfrey et al., 1984; McMahan, 1990; Rupp et al., 1991; Tsim et al., 1992). Agrin has subsequently been shown to cocluster a number of synaptic components along with AChRs on the surface of cultured myotubes (Wallace, 1989). Evidence suggests that although agrin may play important organizing roles for the endplate, it cannot mediate all aspects of postsynaptic differentiation; for example, agrin is not thought to be responsible for inducing synapse-specific transcription of certain genes, but rather a factor alternatively referred to as ARIA or neuregulin is implicated in these inductions (Burden et al., 1995).

Agrin is a 200 kDa protein that can be subdivided into a number of distinct domains. The N-terminal half contains nine follistatin-like repeats that bear homology to Kazal-type protease inhibitor domains, while the C-terminal half contains four epidermal growth factor–like repeats and three regions homologous to the G domains of laminin (Rupp et al., 1991; Tsim et al., 1992). The clustering activity of agrin has been mapped to the C-terminal region; while the full-length form of agrin interacts tightly with the extracellular matrix, the C-terminal region can be made in soluble form and is referred to as c-agrin (Ruegg et al., 1992; Tsim et al., 1992; Ferns et al., 1993; Hoch et al., 1994). A number of different forms of agrin have been discovered, encoded by alternatively spliced transcripts (Ferns et al., 1992; Ruegg et al., 1992; Tsim et al., 1992; Hoch et al., 1993). The C-terminal region of agrin can contain variable insertions at two critical sites, referred to as the Y and Z sites, that can affect the clustering ability of the agrin (Ferns et al., 1992, 1993; Ruegg et al., 1992; Hoch et al., 1994); differential splicing results in transcripts encoding agrins with different combinations of these insertions. Importantly, expression of these different agrin forms are restricted to either muscle or nerve (Ruegg et al., 1992; Hoch et al., 1993). A neuronal-specific insertion at the Z position is absolutely required for the clustering ability of soluble c-agrin, and agrins with an eight amino acid insertion at this position are at least 10,000-fold more active than agrins lacking an insert at this position (Ruegg et al., 1992; Ferns et al., 1993). Muscle-derived agrins lack the insertion at the Z position, and thus are not potent clustering agents. A four amino acid insertion

at the Y position causes a modest additional increase in the activity of agrins having an insertion at the Z position, and the Y insertion also seems to be involved in the binding of agrin to heparin and proteoglycans (Ferns et al., 1993; Hoch et al., 1994).

A variety of data are consistent with the notion that the in vitro clustering actions of agrin reflect similar organizing roles for agrin during the in vivo formation of the NMJ (McMahan, 1990). Most important among these are the findings that the highly active forms of agrin are exclusively made by neurons and are deposited in the synaptic basal lamina (Ruegg et al., 1992; Hoch et al., 1993), and that antibodies to agrin block nerve-induced clustering of AChRs on cultured myotubes (Reist et al., 1992). The precise mechanism of action of agrin remains a mystery (Sealock and Froehner, 1994). Agrin induces intracellular tyrosine phosphorylations, including of the β-subunit of the AChR, and inhibitors of tyrosine phosphorylation block agrin-mediated clustering (Wallace et al., 1991; Qu and Huganir, 1994; Wallace, 1994, 1995; Ferns et al., 1996). Intriguing recent findings have revealed that agrin can directly bind to an extrinsic peripheral membrane protein, known as α-dystroglycan, via its laminin-like domains (Bowe et al., 1994; Campanelli et al., 1994; Gee et al., 1994; Sugiyama et al., 1994). α-dystroglycan is part of a protein complex, hereinafter referred to as the dystroglycan complex, that seems to provide for crucial anchoring between the extracellular basal lamina and the underlying cytoskeleton (Fallon and Hall, 1994). Extrasynaptically, the dystroglycan complex binds laminin in the extracellular matrix and associates with the actin scaffold via a spectrin-like protein known as dystrophin, whereas at the synapse agrin can substitute for laminin and utrophin replaces dystrophin (Fallon and Hall, 1994). In addition, the dystroglycan complex is apparently coupled to AChRs by a 43 kDa cytoplasmic protein known as rapsyn (Noakes et al., 1993; Apel et al., 1995).

Despite the findings that agrin can bind directly to α-dystroglycan and that the dystroglycan complex is coupled to AChRs via rapsyn, the role of α-dystroglycan as an agrin receptor remains unclear (Sealock and Froehner, 1994). Dystroglycan could be a required component of the functional agrin receptor that is responsible for initiating the signaling response, and might even be directly involved in activating signaling pathways. Although known components of the dystroglycan complex lack obvious signaling capabilities, it has been suggested, for example, that they might be associated with cytoplasmic tyrosine kinases. Alternatively, dystroglycan could play a more ancillary role, serving to concentrate agrin near a signaling-competent receptor or acting to couple such a receptor, via agrin, to some of its targets.

Transmembrane receptors with intrinsic tyrosine kinase activity, known as receptor tyrosine kinases (RTKs), play key roles in many biological responses (Schlessinger and Ullrich, 1992). Although such receptors are generally known for mediating mitogenic or survival responses to traditional growth factors or neurotrophic factors, it has been speculated that the organizing function of agrin might be mediated by an RTK, particularly since tyrosine phosphorylation seems to be required for agrin responses (Wallace et al., 1991; Qu and Huganir, 1994; Wallace, 1994, 1995; Ferns et al., 1996). We recently identified a receptor-like tyrosine kinase specific to the skeletal muscle lineage, which we termed MuSK—for muscle-specific kinase (Valenzuela et al., 1995). MuSK is expressed very early in the muscle lineage and becomes highly localized to the motor endplate as muscle matures; following denervation, MuSK once again becomes widely and highly expressed throughout the myofiber, as do AChRs (Valenzuela et al., 1995). The synapse-specific localization of MuSK suggested that it might play a critical role at the NMJ, and this was confirmed following the generation and characterization of mice homozygous for a *MuSK* gene disruption (DeChiara et al., 1996 [this issue of *Cell*]). Mice lacking MuSK die at birth, owing to an inability to breathe, and are generally immobile, resulting from the fact that these mice lack NMJs. Every aspect of NMJ formation appears to be blocked in these mice, with the apparent loss of both presynaptic differentiation and synapse-specific transcription, as well as of postsynaptic organization.

The defects in mice lacking MuSK appear much more profound than those that might have been predicted to result from disruption of the agrin signaling pathway, since agrin has mostly been characterized for its organizing actions on the postsynaptic side of the NMJ and was not thought to be required either for regulating presynaptic differentiation, or for inducing synapse-specific transcription. However, recent generation of mice lacking agrin revealed that NMJ formation in these mice is as profoundly disrupted as it is in mice lacking MuSK (Gautam et al., 1996 [this issue of *Cell*]); in both cases, it can be postulated that the effects on presynaptic differentiation and on synapse-specific transcription indirectly result from primary postsynaptic defects. Thus, the striking similarities between mice lacking agrin and mice lacking MuSK suggest that agrin, acting via the MuSK receptor, is required for initiating all aspects of NMJ formation. Here we demonstrate that agrin binds, and does indeed act via, a MuSK receptor complex.

Results

Agrin Fails to Induce AChR Clustering in Myotubes Lacking MuSK

The localization of MuSK to the NMJ, together with the absence of NMJs in mice lacking MuSK (*MuSK*$^{-}$/$^{-}$ mice), inspired us to ask whether MuSK is required for responsivity to agrin. To test this, we first isolated myoblasts from newborn *MuSK*$^{-}$/$^{-}$ or from control pups, attempted to differentiate them into myotubes in culture, and then assayed for their responsiveness to agrin. Myoblasts from both the control and *MuSK*$^{-}$/$^{-}$ mice were able to fuse and form long, twitching myotubes in culture. Together with the observation that skeletal muscle appears rather normal in *MuSK*$^{-}$/$^{-}$ mice, these findings indicate that MuSK is not critical for early muscle development and myoblast fusion. On the other hand, MuSK appeared to be absolutely required for AChR clustering in response to agrin. After stimulation with the most active form of c-agrin, containing both the 4 and 8 amino acid

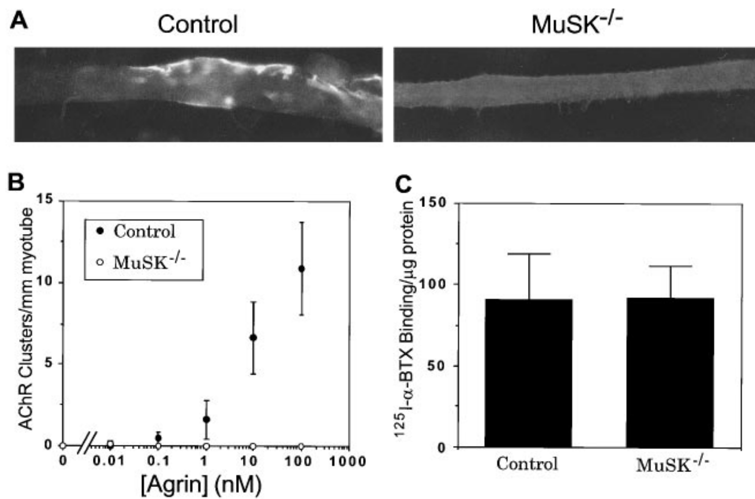

Figure 1. Agrin Induces AChR Clustering in Myotubes from Control but Not *MuSK*$^{-}$/$^{-}$ Mice

Myotubes derived from control and *MuSK*$^{-}$/$^{-}$ mice were treated overnight with varying concentrations of agrin$_{4,8}$, stained with rhodamine-conjugated α-bungarotoxin to label surface AChRs, and then either photographed at 64× magnification (shown here at ∼25×) under rhodamine optics ([A], challenge with 100 nM agrin depicted) or subjected to AChR cluster quantitation ([B], each point represents the mean ± SEM of 40 myotube segments) as described in the Experimental Procedures. Total AChRs on the myotubes before agrin treatment were determined by binding with [^{125}I]α-bungarotoxin ([C], each bar represents the mean ± SEM CPM bound per microgram of total cell protein (control, n = 6; *MuSK*$^{-}$/$^{-}$, n = 5) as described in Experimental Procedures.

insertions (c-agrin$_{4,8}$), AChR clusters were evident only in the myotubes from control mice (Figure 1A). While clusters were induced in normal myotubes with as little as 1 nM c-agrin$_{4,8}$, no clustering was observed in *MuSK*$^{-}$/$^{-}$ myotubes even after increasing the concentration of c-agrin$_{4,8}$ to as high as 100 nM (Figure 1B). Lack of detectable clustering was not due to the absence of AChRs, since myotubes from *MuSK*$^{-}$/$^{-}$ mice expressed similar numbers of AChR on their surface as did myotubes from control mice (Figure 1C). Thus, MuSK appears to be absolutely required for AChR clustering in response to agrin.

Agrin Induces Prominent and Rapid Tyrosine Phosphorylation of MuSK

The inability of agrin to induce AChR clustering in myotubes from *MuSK*$^{-}$/$^{-}$ mice demonstrates that MuSK is required for agrin responsiveness and is consistent with the possibility that MuSK serves as the functional agrin receptor. However, since clustering occurs over a period of hours, these results are also consistent with the possibility that MuSK acts much further downstream in the agrin signaling pathway. To begin to distinguish between these possibilities, we took advantage of the fact that RTKs become rapidly autophosphorylated on tyrosine upon challenge with their cognate ligand. We decided to assay four of the known forms of soluble agrin—which exhibit differing AChR clustering activities (Ferns et al., 1992, 1993; Ruegg et al., 1992; Hoch et al., 1994)—for their ability to induce phosphorylation of the MuSK receptor. Phosphorylation was assessed on the endogenous MuSK receptor that is highly expressed in myotube cultures, obtained by differentiating either the C2C12 mouse myoblast cell line (Valenzuela et al., 1995) or primary rat myoblasts. Strikingly, soluble agrins containing the 8 amino acid insert at position Z (c-agrin$_{4,8}$ and c-agrin$_{0,8}$), which are the forms capable of inducing AChR clustering, were also the forms that induced prominent tyrosine phosphorylation of MuSK (Figure 2A). The agrin most active in clustering (c-agrin$_{4,8}$) was also most active in inducing MuSK phosphorylation (Figure 2A). In contrast, the soluble agrins lacking the 8 amino acid

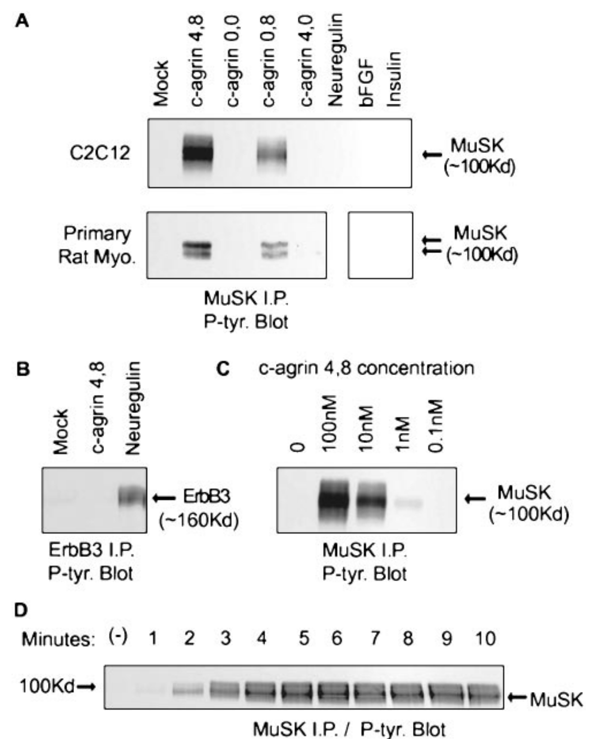

Figure 2. c-agrin$_{4,8}$ and c-agrin$_{0,8}$ Specifically Induce Rapid Tyrosine Phosphorylation of MuSK Receptors

C2C12 and primary rat myoblasts were differentiated into myotubes and stimulated with conditioned media from COS cells transfected with a plasmid control (Mock) or plasmids encoding the various forms of soluble agrin, with conditioned media containing neuregulin, or with purified bFGF or insulin, as labeled. Stimulations were for 10 min using 10 nM of the various factors, except as indicated in (C) and (D). Following factor challenges, the cells were lysed and subjected to immunoprecipitations (I. P.) for either the MuSK or ErbB3 receptors as indicated, then immunoblotted for phosphotyrosine levels. Only agrins containing the 8 amino acid insert at the Z position, but not other factors, could induce MuSK phosphorylation (A). Agrin could not induce phosphorylation of another muscle receptor, ErbB3 (B). MuSK phosphorylation occurred at low agrin concentrations (C) and very rapidly in response to agrin (D).

insert (c-agrin$_{4,0}$ and c-agrin$_{0,0}$), which cannot induce AChR clustering, also could not induce MuSK phosphorylation (Figure 2A).

The specificity of action of agrin was further explored by comparing its activity to growth factors known to have receptors on muscle. Of the several such factors tested, including insulin, fibroblast growth factor (FGF), and ARIA/neuregulin, only agrin could induce phosphorylation of MuSK (Figure 2A); since FGF also induces AChR clustering on myotubes (Peng et al., 1991), these results also indicate that MuSK phosphorylation is specific to agrin responses and not just to agents capable of inducing clustering. Furthermore, while such factors could be shown to induce phosphorylation of their own RTKs on myotubes (e.g., neuregulin induces phosphorylation of its cognate RTK, ErbB3, Figure 2B), agrin could only activate MuSK and not other RTKs (Figure 2B).

The activation of a RTK by its cognate ligand typically tends to occur rapidly, and we could demonstrate that agrin induces tyrosine phosphorylation of MuSK with kinetics similar to those seen for well-characterized RTK/ligand systems (e.g., Kaplan et al., 1991); induction was detectable by one minute, peaked within the first five minutes, and remained elevated for over an hour (Figure 2D and data not shown). The tyrosine phosphorylation of MuSK also occurred using agrin at concentrations similar to those noted for other ligands that act on RTKs (Ip et al., 1993), with phosphorylation detectable using 1 nM agrin (Figure 2C).

Together, the requirement of MuSK for agrin responsiveness, the ability of agrin to induce rapid and prominent MuSK phosphorylation, the specificity of agrin for MuSK as compared to other factors tested, and the precise correlation of agrin forms active in AChR clustering assays and in MuSK phosphorylation assays continue to support the notion that MuSK serves as the functional agrin receptor.

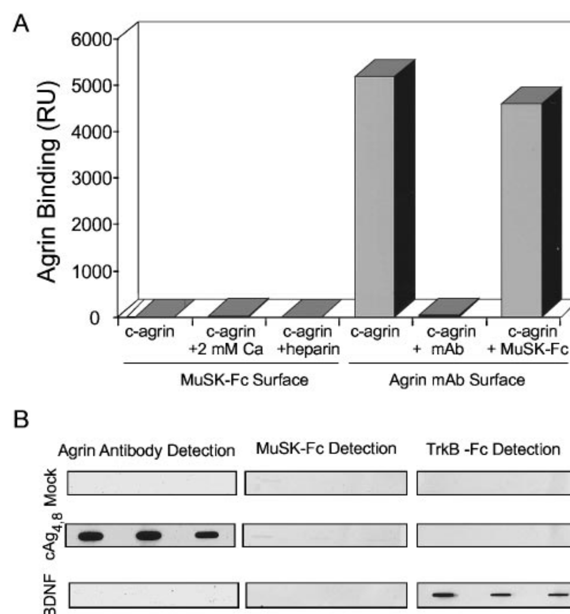

Figure 3. Agrin Can Not Detectably Bind to the Isolated Ectodomain of MuSK

Agrin was assayed for its binding to immobilized MuSK–Fc or to an immoblized agrin-specific monoclonal antibody (mAb), each coupled to a BIAcore sensorchip surface (A); bindings to the MuSK–Fc surface were also done in the presence 2 mM Ca^{2+} or heparin (0.01 mg/ml), as indicated, while bindings to the antibody surface were also competed with excess soluble monoclonal antibody or MuSK–Fc (each at 25 μg/ml), as indicated. Reciprocally, binding of soluble MuSK–Fc or monoclonal antibody to immobilized agrin was assayed by first binding conditioned media transfected with a plasmid control (Mock) or a plasmid encoding c-agrin$_{4,8}$ (cAg$_{4,8}$) to nitrocellulose, followed by detection using either the soluble MuSK–Fc or the agrin-specific monoclonal antibody, as indicated (B); TrkB–Fc detection of nitrocellulose-immobilized BDNF served as an additional control.

Agrin Does Not Directly Bind to an Isolated MuSK Ectodomain

If MuSK is indeed the functional agrin receptor, we would expect to be able to demonstrate binding of agrin to MuSK. In an attempt to demonstrate such binding, we first constructed an expression construct encoding a fusion protein between the ectodomain of rat MuSK and the Fc portion of human immunoglobulin G1 (designated MuSK–Fc), and then produced and purified the fusion protein. Similar receptor–Fc fusions have previously been used to characterize binding between RTKs and their ligands (e.g., Davis et al., 1994; Stitt et al., 1995). In a first approach, we used MuSK–Fc together with BIAcore biosensor technology (Fagerstam, 1991; Johnsson et al., 1991). The BIAcore technology allows for the direct and quantitative measure of binding of soluble ligands to receptors coupled onto a sensor chip. Recombinant MuSK–Fc was covalently coupled to a surface on the BIAcore sensor chip, and as a control, a monoclonal antibody specific for rat agrin was also coupled to a separate surface on the sensor chip; media containing c-agrin$_{4,8}$ was then passed over the two surfaces. While robust binding of the agrin to the antibody surface was easily detected, no binding of the agrin to the MuSK surface could be seen (Figure 3A). Furthermore, while binding to the antibody surface was specifically competable by excess soluble antibody added to the agrin-containing media, the binding was not competable by excess soluble MuSK–Fc (Figure 3A). Since agrin activity requires calcium (Bowe and Fallon, 1995), and because some heparin-binding factors require heparin to bind to their receptors (Goldfarb, 1990), we also attempted binding in the presence of calcium or heparin; in neither case was binding to the MuSK surface observed (Figure 3A).

Next, we tried to demonstrate binding of MuSK and agrin by attempting to use MuSK–Fc to detect agrin immobilized onto nitrocellulose. In contrast to our control experiments, in which immobilized brain-derived neurotrophic factor (BDNF) was easily detected by an Fc fusion of its cognate receptor (TrkB–Fc) (Figure 3D), and in which immobilized agrin was easily detected by the agrin-specific monoclonal antibody (Figure 3B), immobilized agrin could not be detected by MuSK–Fc (Figure 3C).

The negative binding results described above demonstrate that the isolated MuSK receptor is not sufficient to bind agrin. Thus, despite the plethora of functional

data indicating that agrin acts via MuSK, MuSK may not directly serve as a receptor for agrin. Alternatively, MuSK may require additional components or modifications that are required for it to bind and respond to agrin.

Agrin Activates MuSK in a Cell Context–Dependent Fashion: Requirement for a Myotube-Specific Accessory Component

Based on the results described above, we decided to consider the possibility that the agrin–MuSK interaction requires additional components. To further explore this possibility, we determined the cell context dependency for agrin activation of MuSK; we reasoned that if an accessory component were required, it might be specifically expressed only on cells normally responding to agrin. Thus, we ectopically expressed full-length cDNAs encoding rat, human, and chicken MuSK in fibroblasts and assayed for whether these MuSK receptors could be inducibly phosphorylated by agrin. When expressed in fibroblasts, none of the three species of MuSK could be phosphorylated in response to agrin (data not shown). While this data supported the possibility that MuSK requires an accesssory myotube-specific component to respond to agrin, it was also possible that our cDNAs encoded MuSK variants that could not respond to agrin; this was a particularly worrisome possibility since there are multiple differently spliced versions of the MuSK transcript (Valenzuela et al., 1995), we did not know which of the forms were normally agrin responsive, and our cDNAs only accounted for a subset of the variant forms. Thus, we decided to express our cDNAs in myoblasts so that we could verify that they could mediate responses to agrin when expressed in the right context. For this purpose we chose to express the chicken MuSK (D. J. G. et al., unpublished data) in the mouse C2C12 myoblast cell line, since the chicken MuSK could easily be distinguished from the endogenous mouse MuSK based on size and by using particular antibodies. When expressed in undifferentiated myoblasts, the chicken MuSK did not undergo phosphorylation in response to any isoforms of agrin (Figure 4, upper gel, lanes labeled Undif), just as it did not undergo phosphorylation in fibroblasts; undifferentiated C2C12 cells do not express appreciable amounts of endogenous MuSK (Figure 4, lower gel, lanes labeled Undif) (see also Valenzuela et al., 1995), so we could not compare activation of the endogenous mouse MuSK in myoblasts. Upon differentiation into myotubes, the introduced chicken MuSK was as effectively activated by agrin as was the endogenous mouse MuSK (Figure 4, upper gel, lanes labeled Diff); both introduced and endogenous MuSK had identical profiles of responsivity to the various forms of agrins, with activations mediated only by forms having the 8 amino acid insert at the Z position. Thus our cDNAs encode MuSK proteins that are perfectly competent to undergo agrin-induced phosphorylation, but they can only be activated by agrin in the context of a differentiated myotube, consistent with the notion that agrin activation of MuSK requires a myotube-specific accessory component that is not expressed in fibroblasts or undifferentiated myoblasts.

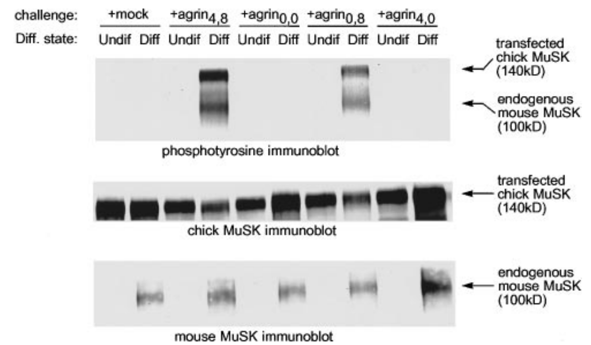

Figure 4. Agrin Can Only Induce MuSK Phosphorylation in the Context of a Differentiated Myotube: Evidence for a Myotube-Specific Accessory Component

Agrin-inducible phosphorylation of an introduced chick MuSK receptor was evaluated in a clone of C2C12 myoblasts stably transfected with a chick MuSK expression vector. The introduced chick MuSK is expressed regardless of whether this C2C12 clone is undifferentiated (Undif) or differentiated into myotubes (Dif) (middle gel), in contrast to the endogenous mouse MuSK, which is only expressed in differentiated cells (bottom gel). However, the chick MuSK can only be inducibly phosphorylated in response to agrin when it is assayed in differentiated myotubes (upper gel). The chick MuSK displays the same specificity for activation by the various agrin isoforms (each at 10 nM for 10 min) as does the endogenous mouse MuSK (compare transfected chick MuSK and endgenous mouse MuSK in upper gel).

A Receptor Complex Can Be Demonstrated between Agrin, MuSK and a Myotube-Specific Accessory Component(s)

Altogether, the above data indicate that agrin requires MuSK to mediate clustering and that agrin activates MuSK very rapidly, but that agrin does not directly bind to a purified MuSK ectodomain and can only activate MuSK in the context of a myotube. These findings are consistent with the possibility that MuSK is a requisite part of an agrin receptor complex, but that although MuSK provides a key signaling function for this complex, it requires another component(s) to bind to agrin. Similar types of receptor complexes have been described for other ligands. Perhaps some of the best characterized examples include the receptor complexes for ciliary neurotrophic factor (CNTF) and its cytokine relatives (Davis et al., 1993; Stahl and Yancopoulos, 1993). To interact with its two-signal transducing β receptor components, gp130 and LIFRβ, CNTF must first bind to its α receptor component, known as CNTFRα. CNTFRα serves no signaling role, and is in fact linked to the surface via a glycosylphosphatidylinositol linkage and thus has no cytoplasmic domain. The receptor complex for CNTF is built in step-wise fashion: CNTF first binds to CNTFRα; this initial complex can then bind to and recruit a single β component; finally, a complete complex forms that involves β component dimerization, which is required for signal initiation (Figure 5A). In the final complex, CNTF seems to make contacts with all three receptor components. Interestingly, receptor complexes for CNTF can be built in solution using just the soluble ectodomains of the various components. Furthermore, if just one of the receptor components is linked to the surface, a receptor complex can be built

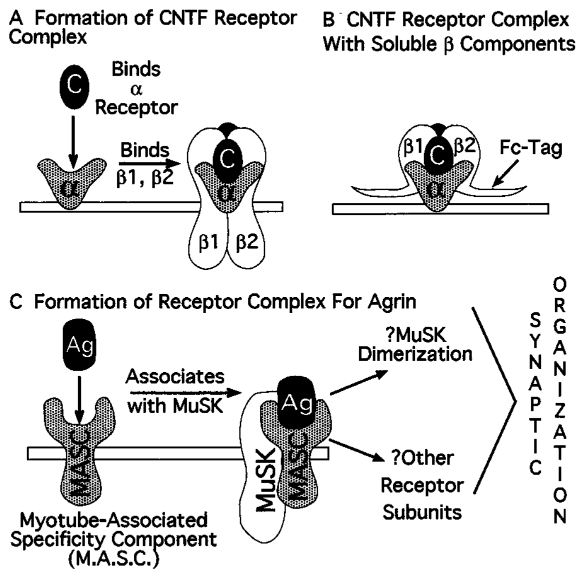

Figure 5. Relevant Models for the Agrin/MuSK Receptor Complex

(A) Schematic representation depicting the step-wise assembly of the multicomponent receptor complex for ciliary neurotrophic factor (CNTF); β1, gp130; β2, LIFRβ.

(B) Schematic depiction of the use of soluble β receptor components (Fc-tagged) to build a CNTF receptor complex attached to the cell surface via only one of its components, the nonsignaling α component; surface binding of the soluble β components can be detected using antibodies recognizing the Fc tag.

(C) Schematic representation of one of several possible models of the MuSK receptor complex for agrin, depicting requirement for a myotube-associated specificity component (MASC) and possible interactions to additional components that may be required for signaling or coupling to various effectors or substrates; these couplings may be mediated extracellularly (for example, via agrin binding to the dystroglycan complex) or intracellularly (for example, via interactions of SH2 domain–containing proteins to phosphorylated tyrosines on MuSK).

around it using soluble versions of the other components, but only in a CNTF-dependent fashion (Figure 5B).

If agrin binds to MuSK in a receptor complex, we reasoned that we might be able to manipulate this complex in much the same way the CNTF receptor complex can be manipulated. To explore the possibility that myotubes specifically express an accessory component(s) required for agrin to bind MuSK (Figure 5C), we decided to test whether we could specifically build a receptor complex on the surface of myotubes, but not on other cells, using agrin together with a soluble version of the MuSK receptor to complex to the putative accessory component(s) on the surface of myotubes. Confirming this possibility, we found that the binding of soluble MuSK–Fc to the surface of cells can be increased using agrin, but only on the surface of differentiated myotubes and not on the surface of fibroblasts or myoblasts (Figure 6A). These data demonstrate that complexes can form between agrin and MuSK, but only in the presence of a myotube-associated specificity component, MASC (as suggested in Figure 5C). Interestingly, although forms of c-agrin containing the 8 amino acid insert at the Z position are best able to promote agrin-dependent

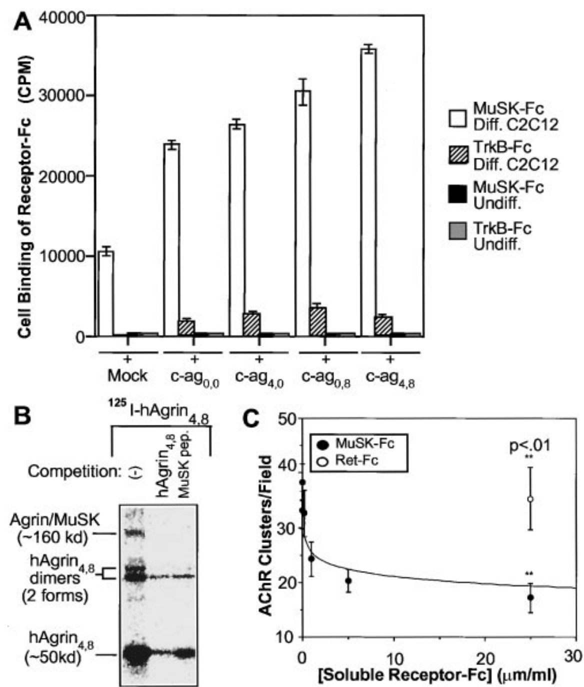

Figure 6. Evidence for an Agrin/MuSK Receptor Complex Utilizing a Myotube-Specific Accessory Component

(A) Formation of agrin/MuSK complexes on the surface of myotubes: undifferentiated (Undiff.) or myotube-differentiated (Diff.) C2C12 cells were assayed for their ability to bind either MuSK–Fc or a control receptor–Fc fusion (TrkB–Fc), in the absence or presence of various agrin isoforms (provided in conditioned media from transient COS transfections); specific binding of MuSK–Fc to the myotube surface, which is enhanced by exogenously provided agrin, is suggested to involve complexes analogous to those depicted in Figure 5B.

(B) Direct binding of agrin to MuSK is demonstrated by cross-linking analysis. Radiolabeled agrin (a recombinant C-terminal fragment of human agrin, termed $hAgrin_{4,8}$) at 1 nM was chemically cross-linked to the surface of myotubes. Following cross-linking, lysates were immunoprecipitated with a MuSK-specific antibody (left lane). The cross-linking was also done in the presence of excess (150 nM) unlabeled agrin (middle lane), while the immunoprecipitation was also done in the presence of excess peptide (corresponding to that used to generate the antibody) to block the MuSK precipitation; positions of the agrin/MuSK complex, as well as of various forms of unbound monomeric and dimeric agrin (see text), are indicated.

(C) Inhibition of agrin-induced AChR clustering by MuSK–Fc: agrin-induced AChR-clustering (using 10 nM $c\text{-}agrin_{4,8}$) was performed on C2C12 myotube cultures in the presence of varying concentrations of soluble MuSK–Fc or a control receptor–Fc fusion (Ret–Fc); the soluble MuSK–Fc specifically inhibits, presumably by forming inactive complexes on the cell surface with agrin and the myotube-specific accessory component.

MuSK complex formation, forms of c-agrin without this insert can also form these complexes. The ability of all the soluble forms to promote complex formation, including those lacking the 8 amino acid insert for activity, may be related to previous findings that matrix-bound forms of agrins lacking the Z insert can activate clustering (Ferns et al., 1992). Thus, although soluble agrins lacking inserts at the Z position do not seem capable of signaling, they may be able to form partial complexes, while matrix-associated forms of these

same agrins can proceed to form complete signaling-competent complexes. Interestingly, ligands for the EPH family of RTKs provide an example of ligands that bind but do not activate their receptors when presented in soluble form, but which can act as potent activators when bound to the cell surface (Davis et al., 1994); deliberate clustering of the soluble ligands can allow them to activate as well, suggesting that the role of surface-attachment is to allow for ligand-clustering (Davis et al., 1994).

In the absence of added agrin, the MuSK–Fc exhibited much higher binding to myotube surfaces than did several control receptor–Fc fusion proteins (Figure 6A, data shown for TrkB–Fc); the MuSK–Fc, however, displayed similar agrin-independent binding to both myoblast and fibroblasts as did control receptor–Fc proteins (Figure 6A and data not shown). Specific binding of MuSK–Fc to myotube surfaces, in the absence of exogenously provided agrin, may indicate that MuSK has an affinity for its myotube-specific accessory component in the absence of ligand. Alternatively, since myotubes make muscle forms of agrin (lacking the 8 amino acid insert at the Z position), the specific binding of MuSK–Fc to myotubes in the absence of added agrin could be explained by the formation of a complex between the added MuSK–Fc and endogenously expressed muscle agrin along with the accessory component; adding additional exogenous soluble agrin may simply allow for even more MuSK to be recruited into complexes with the myotube-specific accessory component. Although both myoblasts and myotubes make endogenous agrin, myoblasts seemingly cannot form complexes with added MuSK–Fc since they do not express the required accessory component.

To confirm that MuSK directly interacts with agrin as part of its receptor complex, we next demonstrated that radiolabeled agrin could be cross-linked to MuSK receptors on the surface of myotubes. Immunoprecipitations using a MuSK-specific antibody, from lysates of myotubes chemically cross-linked to radiolabeled recombinant human agrin (Valenzuela et al., unpublished data), contained complexes corresponding in size to agrin/MuSK complexes (Figure 6B). These agrin/MuSK complexes were not seen in the presence of excess unlabeled agrin, or if a peptide was used to block MuSK precipitation (Figure 6B). Additional radiolabeled species that immunoprecipitated with the MuSK antibody correspond to forms of agrin that are associated with, but not cross-linked to, MuSK, presumably due to the low efficiency of cross-linking (Figure 6B); low levels of additional agrin complexes, perhaps involving MASC, could also be detected in these immunoprecipitations.

Finally, if our findings that soluble MuSK could form complexes with its requisite myotube-specific accessory component are correct, then this soluble receptor should also act as an inhibitor of agrin-mediated responses by sequestering the accessory component and preventing it from interacting with the endogenously expressed, signaling-competent MuSK. Indeed, addition of increasing amounts of MuSK–Fc did inhibit agrin-mediated clustering of AChRs (Figure 6C) as well as agrin-induced MuSK phosphorylation (data not shown) in a dose-dependent manner, while control receptor–Fc proteins had no inhibitory effect.

Discussion

MuSK Is a Requisite Subunit of the Agrin Receptor Complex but Requires a Myotube-Specific Accessory Component

The localization of MuSK to the NMJ (Valenzuela et al., 1995), together with the observations that *MuSK*$^{-}$/$^{-}$ mice have profound NMJ defects similar to those seen in agrin-deficient mice (DeChiara et al., 1996), initially suggested that MuSK might serve as the functional receptor for nerve-derived agrin. In this manuscript we have provided data demonstrating that, although MuSK alone is not sufficient to mediate responses to agrin, it serves as a crucial component of the receptor complex for agrin (see Figure 5C). Our finding here that myotubes derived from *MuSK*$^{-}$/$^{-}$ mice do not undergo agrin-mediated AChR clustering provided the first evidence that MuSK was absolutely required for agrin responsiveness but, because clustering occurs over a period of hours, did not address whether MuSK was acting proximally or distally in the agrin-signaling pathway. Assays for induction of MuSK tyrosine phosphorylation yielded results consistent with the notion that MuSK acted proximally as the direct receptor for agrin, since agrin induced MuSK phosphorylation with the kinetics characteristic of ligands for RTKs; furthermore, agrin acted with a specificity profile and concentration dependence that would be expected if it were the MuSK ligand. However, we were able to demonstrate that agrin does not bind to the purified MuSK ectodomain. This finding led us to consider the possibility that in order for agrin to interact with MuSK, it requires an accessory component(s). Motivated by this possibility, we proceeded to demonstrate that agrin-induced MuSK phosphorylation depends on an accessory component expressed in differentiated myotubes but not in fibroblasts or undifferentiated myoblasts. Furthermore, we were able to form complexes between agrin and soluble versions of the MuSK ectodomain, but only in the presence of the accessory component(s) found on the surface of myotubes (see Figure 5C); agrin directly binds to MuSK in this receptor complex, as demonstrated by cross-linking analysis using radiolabeled agrin. Finally, as would be predicted from our observation that soluble MuSK could form complexes with the accessory component on the surface of myotubes, excess soluble MuSK could apparently sequester this accessory component and inhibit agrin responses. Altogether our data indicate that MuSK is a requisite subunit of a multicomponent receptor complex used by agrin, and that it requires a myotube-specific accessory component(s) to bind and mediate responses to agrin.

There are many other examples of ligands that use multicomponent receptor complexes (Stahl and Yancopoulos, 1993). Most cytokines, as noted above for CNTF and its relatives, use such receptor complexes. In these receptor complexes there are often components whose primary role is to bind ligand, whereas other subunits can both bind and signal. In the case of RTKs, there are also examples of multicomponent receptors. For example, FGFs need to bind heparin, usually presented on surface proteoglycans, in order to bind and activate their RTK subunits (Goldfarb, 1990). The Trk family of

RTKs, which bind to nerve growth factor (NGF) and the related neurotrophins, present a less well-understood example of RTKs that may interact with an accessory component known as p75 (Barbacid, 1993). In the case of ARIA/neuregulin, receptor complexes can include two different members of the ErbB family (Burden et al., 1995). In all these examples of multicomponent receptor complexes, signal initiation seems to depend on ligand-mediated dimerization of signal-transducing subunits that are included within the receptor complex; this dimerization can involve either homodimerization of identical subunits, or heterodimerization of related subunits.

The Myotube-Associated Specificity Component, MASC

Before considering potential candidates for the other component(s) of the agrin receptor complex, it is worth remembering that the signaling function of MuSK appears quite unusual as compared with other RTKs. While most RTKs are thought to mediate growth or survival responses, or rather simple differentiative events, MuSK seems instead to mediate a very complicated organizing function. The structure of the NMJ has already been shown to depend on complex molecular scaffolds that anchor components in place, by linking them to the extracellular matrix as well as to the cytoskeleton. Since they combine to initiate formation of the entire postsynaptic macrostructure, and also are contained within it, it seems likely that both agrin and MuSK will have many interactions with molecules at the NMJ, for both structural and signaling purposes. One can imagine that MuSK interacts with proteins that serve to link it to the molecular scaffold at the junction, both extracellularly and intracellularly; it may bind to other proteins that are direct substrates for its kinase activity, or that act merely as couplers of MuSK to its substrates. Thus, the myotube-specific accessory component(s) required for agrin to bind and activate MuSK may be difficult to distinguish from other (perhaps more abundant) molecules interacting with agrin and MuSK that serve other functions; even the accessory component(s) itself may be multifunctional, and serve not only to help bind agrin, but to couple this ligand/receptor system to other proteins as well.

Does α-dystroglycan correspond to the accessory component required for agrin to activate MuSK? Although it satisfies the criterion of being able to bind to forms of agrin both with and without the 8 amino acid insert at the Z position, several pieces of data strongly argue against α-dystroglycan as the sole accessory component. Most importantly, α-dystroglycan is widely expressed on many cell types, including on myoblasts and fibroblasts, so it is not myotube specific. In addition, recent findings that the dystroglycan complex does not cluster at the endplate in rapsyn-deficient mice (Gautam et al., 1995) suggests that MuSK activation can be uncoupled from dystroglycan coclustering, since several aspects of agrin/MuSK-mediated signaling (including MuSK clustering) remain intact in these mice. However, since AChRs do not cluster in rapsyn-deficient mice, the dystroglycan complex may well play a required role in coupling MuSK activation to AChR clustering, perhaps even via its shared binding to agrin; such a possibility would nicely explain the finding that the ability of agrin to bind dystroglycan as well as to induce AChR clustering are both calcium dependent. In addition, although dystroglycan may not correspond to the critical accessory component required for agrin to activate MuSK, it may still play a more passive ancillary role by concentrating agrin on the muscle surface and helping present it to the MuSK receptor complex. In any case, α-dystroglycan certainly appears to be the most abundant agrin receptor, and may well be obscuring lower levels of the requisite accessory receptor for MuSK. Since MuSK apparently does not bind agrin in the absence of the accessory component, previous biochemical approaches to isolate agrin-binding proteins (involving extraction and isolation of proteins from the cell surface) would not have been expected to have identified isolated MuSK as an agrin-binding protein.

There are other clues concerning the possible identity of accessory receptor components for agrin. Recent data implicate a synapse-specific carbohydrate in agrin responses (Martin and Sanes, 1995); enzymatic removal of this carbohydrate blocks agrin responsiveness, whereas lectin-mediated clustering of proteins with this carbohydrate, or enzymatic unmasking of more of this carbohydrate, potentiate or even mimic agrin. These data could be reconciled with the concept of a MuSK receptor complex if such a carbohydrate marked and was required for agrin recognition by the MuSK accessory component. Alternatively, it could be that such a carbohydrate is required on MuSK itself. Along these lines, it should be pointed out that a myotube-specific modification of MuSK, whether it involves carbohydrate or other modifications, may allow MuSK to directly bind agrin, and thus raises the possibility that modified MuSK itself might correspond to the myotube-specific "accessory" component; in this case, soluble recombinant MuSK would presumably be able to dimerize with the correctly modified MuSK—via agrin—on the myotube surface. A related scenario might involve the expression of a MuSK relative in myotubes, and the need for heterodimerization between MuSK and this relative—as can occur with the ErbBs—to create a functional agrin receptor. Finally, since agrin forms lacking the ability to bind heparin are still active, it seems unlikely that binding to a proteoglycan at the Y site would be critical for linking agrin to its accessory component.

Mechanism by Which MuSK Activation Triggers Postsynaptic Organization

Previous studies had implicated tyrosine phosphorylation in the agrin response (Wallace et al., 1991; Qu and Huganir, 1994; Wallace, 1994, 1995; Ferns et al., 1996). The finding that the critical signaling component of the agrin receptor complex is an RTK validates the notion that specific agrin-induced tyrosine phosphorylations initiate organization and formation of the NMJ. The only well-characterized agrin-induced tyrosine phosphorylation, other than that of MuSK, is of the AChR β component (Wallace et al., 1991; Qu and Huganir, 1994; Ferns et al., 1996). As would be expected, although phosphorylation of AChRb is relatively rapid and begins by 15 min after agrin addition, it trails MuSK phosphorylation. It is unknown whether AChRβ phosphorylation is required or contributes to AChR clustering, or whether it is

a direct substrate of MuSK. The critical MuSK-mediated phosphorylations may well involve proteins that are clustered or directly involved in building the molecular scaffold that maintains the postsynaptic specialization. It is easy to imagine that direct phosphorylation of key proteins may trigger complexes or aggregations to form—for example, AChRβ phosphorylation may trigger its association and coaggregation with rapsyn or other coupling proteins. Since MuSK appears to be much less abundant than many of the proteins that are clustered, it would presumably act catalytically on such structural targets. Alternatively, MuSK-mediated phosphorylations may not directly trigger clustering events, but rather MuSK may activate downstream enzymes or signaling pathways that in turn trigger clustering. These possibilities emphasize the need to identify direct substrates and signaling pathways activated by MuSK.

MuSK Signaling in Other Contexts: in the Adult, Atrophies and Dystrophies?

The complex type of organizational role played by MuSK at the NMJ distinguishes it from other growth factors that use RTKs. However, this unusual role does not preclude MuSK from having more traditional growth factor–like effects. Exploration of the signaling pathways activated by MuSK will certainly help address whether MuSK uses unique signaling pathways, or instead shares pathways with other RTKs. When it was realized that the Trk family of RTKs served as the receptors for nerve growth factor and its relatives, it was thought that the Trks might prove to have unusual substrates because they mediated neuronal survival and differentiation responses, as opposed to the conventional type of proliferative responses that had previously been attributed to RTKs. It turns out, instead, that the Trks activate much the same substrates as mitogenic RTKs, but that these activations are ultimately interpreted much differently within the context of a postmitotic neuron (Glass and Yancopoulos, 1993). In fact, ectopic expression of the Trks in proliferation-competent cells, such as fibroblasts, allows them to mediate conventional mitogenic responses indistinguishable from those of FGFs or epidermal growth factors (Glass et al., 1991). Thus, it will be important to determine whether the unusual actions of MuSK are a consequence of unique signaling capabilities or simply its unique site of expression.

Current studies of agrin and MuSK have almost completely focused on their roles during formation of the NMJ. In the entirely different context of an adult muscle, MuSK is dramatically upregulated in situations where the muscle is at risk for atrophy, including during forced immobilization (Valenzuela et al., 1995). It will certainly be of interest to determine what kinds of actions MuSK mediates in the context of an adult muscle, if these could be more along the trophic types more usual for an RTK, and if these actions would be of benefit during muscle atrophy or other muscle diseases. Along these lines, it is worth noting that MuSK, via agrin, now appears to be linked to the dytroglycan complex. Mutations in at least three of the components of this complex (dystrophin, emerin, and adhalin) account for different types of muscular dystrophies (Campbell, 1995). The mechanism by which problems in the dystroglycan complex lead to progressive muscle wasting are poorly understood, and efforts to understand this mechanism are primarily focused on problems with sarcolemmal stability. The association of an RTK with the dystroglycan complex would certainly raise the possibility that signaling defects could play an important contributory role in dystrophies. Interestingly, in humans, MuSK overlaps with the region reported to contain the Fukuyama muscular dystrophy mutation.

Experimental Procedures

Transient and Stable Transfections of Agrin- and MuSK-Encoding Plasmids into COS and C2C12 Cell Lines

Transient transfections using either previously described agrin constructs (Ferns et al., 1993) or empty vector controls, or stable transfections of a chick MuSK-expression construct (Glass et al., unpublished), were performed as described (Glass et al., 1991; Ip et al., 1992). Agrin concentrations in conditioned media derived from transient transfections were estimated by immunoblot comparisons with purified agrin of known concentration.

AChR-Clustering Assays on MuSK^{-}/$^{-}$ Myotubes and on C2C12 Myotubes

Primary myoblast cultures were established from hind limb musculature of newborn MuSK^{-}/$^{-}$ or littermate control pups. This tissue was treated sequentially with collagenase and trypsin, then plated onto plastic tissue culture dishes. After 1 hr, nonadherent cells (principally myoblasts) were removed and plated onto chamber slides coated with poly-D-lysine and fibronectin. Myoblast cultures were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 25% fetal calf serum, 10% horse serum, and 50 μg/ml gentamycin. To induce myotube formation, cultures were switched to a medium consisting of DMEM containing 5% horse serum, L-glutamine and gentamycin to which 20 μM cytosine arabinoside was added after 24 hr. After an additional 2-3 days, contractile myotubes had formed abundantly in cultures from both MuSK^{-}/$^{-}$ and control pups. C2C12 cells were maintained and caused to differentiate in a serum-poor medium as previously described (Ferns et al., 1993).

For agrin-mediated AChR clustering assays on primary myotubes, cultures on chamber slides were treated overnight with c-agrin$_{4,8}$ at 0.01-100 nM; for evaluating MuSK-Fc as an inhibitor of clustering, differentiated C2C12 cells, on chamber slides coated with fibronectin and poly-D-lysine, were pretreated with MuSK-Fc or a control receptor-body (at the indicated concentrations) for 1 hr at 37°C before addition of ~10 nM agrin$_{4,8}$ for overnight incubation. Following overnight treatments with agrin, the cells were next incubated in rhodamine-conjugated α-bungarotoxin (BGT) to label AChRs, then fixed and mounted for fluorescence microscopy. To quantify the extent of AChR clustering, we viewed randomly selected myotubes under fluorescein optics, then switched to rhodamine optics and counted the number of AChR clusters within a reticule grid aligned along the long axis of the myotube.

AChRs on the surface of cultured primary myotubes were quantitated by incubating live cultures with 25 mCi ^{125}I-α-BGT for 1 hr at room temperature, washing, and then lysing the cells in 0.1 N NaOH. The protein concentration in aliquots of each extract was determined using a BCA protein assay kit (Pierce), while the remainder of the extract was counted in a gamma counter.

Receptor Tyrosine Phosphorylation Assays

The ability of various agrins and growth factors to induce MuSK or ErbB3 tyrosine phosphorylation, for the indicated times and at the indicated concentrations, was evaluated in primary rat myoblasts and in either untransfected C2C12 myoblasts, or in C2C12 myoblasts stably transfected with a chick MuSK-expressing plasmid (Glass et al., unpublished). The cells were challenged at confluence in an undifferentiated state, or approximately 4-5 days after being

induced to differentiate into myotubes in serum-poor media. After challenge, the cells were lysed, the extracts subjected to immunoprecipitation with receptor-specific antibodies, and then immunoblotted with either receptor-specific or phosphotyrosine-specific antibodies, using methods previously described (Stitt et al., 1995). Polyclonal antibodies for MuSK were generated as follows: for rat MuSK, rabbits were immunized with a peptide corresponding to the carboxy-terminal 20 amino acids of the rat MuSK protein (Valenzuela et al. 1995; our nomenclature for this antibody is: 41101K); for chick MuSK, rabbits were immunized with a peptide corresponding to the first 19 amino acids of the chick MuSK cytoplasmic domain (Glass et al., unpublished; peptide: TLPSELLLDRLHPNPMYQ; our nomenclature for this antibody is 52307K). The specificity of the antibodies was determined on COS cell expressed MuSK proteins, by both immune-precipitation and Western analysis, comparing untransfected COS cell lysates to lysates from rat and chicken-MuSK transfected COS cells. 41101K immune precipitates and Westerns rodent MuSK, but does not recognize chicken MuSK. 52307 immune precipitates and Westerns chicken MuSK. Antibodies to ErbB3 were obtained from Santa Cruz Biotechnology, Inc.

Production and Purification of Soluble MuSK-Fc, TrkB-Fc, and Ret-Fc

Baculovirus expression vectors encoding MuSK-Fc, TrkB-Fc, and Ret-Fc produced fusion proteins in which the ectodomains of rat TrkB, rat Ret, or rat MuSK, respectively, were linked to a spacer with the sequence Gly-Pro-Gly, followed by the hinge, CH2, and CH3 regions of human IgG1, beginning with the residues Glu-Pro-Lys, as described (Davis et al., 1994). Baculovirus infections into Spodoptera frugiperda SF-21AE insect cells were performed by standard methods (Stitt et al., 1995). The soluble Fc-containing proteins were purified by protein A-Sepharose (Pharmacia) chromatography.

BIAcore Analysis

The binding of agrin to immobilized MuSK-Fc as compared to a monoclonal antibody specific for agrin was evaluated by use of BIAcore biosensor technology (Pharmacia Biosensor), using approaches previously described (Stitt et al., 1995). Heparin and $CaCl_2$ were supplied by Sigma Chemical Co. (St. Louis, MO) and used without further purification. The agrin-specific monoclonal antibody (clone AGR131 generated to rat agrin) was purchased from StressGen Biotechnologies Corp. (Victoria, BC, Canada).

Evaluation of Cell-Surface Complexes of Agrin and MuSK

Cultures of confluent C2C12 cells, either undifferentiated or differentiated in serum-poor media for four to five days as described above, were transferred to 4°C and incubated for 90 min with either MuSK-Fc or TrkB-Fc (at 5 mg/ml), each in the presence of the indicated mock or agrin-containing conditioned media (with 100 nM agrin). Agrin levels were determined by Western analysis of the conditioned media with a rat agrin antibody (131, from StressGen, Inc.), using a purified agrin control of known concentration. Following these incubations, the cells were washed four times with phosphate-buffered saline (PBS) containing calcium and magnesium, and then incubated for an additional hour with radio-iodinated goat anti-human IgG (NEN/Dupont; 1 mCi/ml in PBS) to detect surface-bound receptor-Fc. After four additional washes, cells were solubilized in 0.1 N NaOH, and bound radioactivity was determined. The assay is similar to that described elsewhere (Davis et al., 1994).

Cross-Linking of Radiolabeled Agrin to MuSK

Flg-tagged human agrin protein corresponding to the COOH-terminal 50 kDa of human $agrin_{4,8}$ (Valenzuela et al., unpublished data) was expressed in COS cells and purified by affinity and size-exclusion chromatography to >95% purity. Then, 20 μg were iodinated by a modification of the lactoperoxidase method described previously (DiStefano et al., 1992). Incorporation of ^{125}I was greater than 80%; [^{125}I]h-$agrin_{4,8}$-flg was separated from free ^{125}I on a 1×3 cm Sephadex G-25 column prior to use in cross-linking assays. Specific activity was $\sim$4000 cpm/fmol ($\sim$2400 Ci/mmol). Biological activity of [^{125}I]h-$agrin_{4,8}$-flg was monitored by tyrosine phosphorylation of MuSK in C2C12 myotubes and was found to be indistinguishable from its unlabeled counterpart. For cross-linking studies, 10 cm plates of differentiated C2C12 myotubes were incubated in 1 nM of [125I]$agrin_{4,8}$ in 1.5 ml of PBS containing 1% BSA and 1 mg/ml glucose in the presence or absence of 150-fold excess unlabeled $agrin_{4,8}$ for 75 min at 4°C. The cross-linking agent DSS (disuccinimidyl suberate) was added to a final concentration of 0.2 mM and the plates were incubated at room temperature for 30 min, washed 3 times with 50 mM Tris/150 mM NaCl (pH 7.5), lysed, and subjected to immunoprecipitation with MuSK-specific antibodies. For peptide competition, peptide antigen was included in the immunoprecipitation at a final concentration of 20 μg/ml. The samples were then electrophoresed; fixed and dried gels were exposed for autoradiography.

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