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KINASE- AND RAPSYN-INDEPENDENT ACTIVITIES OF THE MUSCLE-SPECIFIC KINASE (MuSK)

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Abstract—The muscle-specific receptor tyrosine kinase (MuSK) is co-localized with nicotinic acetylcholine receptors (AChRs) in the postsynaptic membrane of the skeletal neuromuscular junction, and is required for all known aspects of postsynaptic differentiation. Studies *in vitro* have shown that Z^+-agrin, a nerve-derived proteoglycan, activates MuSK's kinase activity to promote clustering of AChRs and MuSK itself with a cytoplasmic, receptor-associated protein, rapsyn. These studies, however, have used soluble forms of agrin, whereas agrin is cell- or matrix-attached *in vivo*. We show here that immobilized (particle- or cell-attached) agrin but not soluble agrin is able to aggregate MuSK in the absence of rapsyn and that this aggregation does not require MuSK's kinase activity but does require MuSK's cytoplasmic domain. Moreover, immobilized agrin can promote clustering of AChRs by a mechanism that requires MuSK and rapsyn but does not require MuSK's kinase activity. These results imply that rapsyn and signaling components activated by MuSK kinase may be dispensable for some early aspects of postsynaptic differentiation. © 2004 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: acetylcholine receptor, agrin, neuromuscular junction, postsynaptic.

Conventional chemical synapses require a high density of neurotransmitter receptors in the postsynaptic membrane in order to function properly. Accordingly, there is intense interest in understanding how receptor aggregates are formed, maintained, and remodeled at synaptic sites. These mechanisms have been studied in detail at the vertebrate skeletal neuromuscular junction, where the neurotransmitter is acetylcholine, and pentameric nicotinic acetylcholine receptors (AChRs) are present at a density of 10–20,000/μm^2 directly beneath the nerve terminal. Recently, two proteins have been identified that are not only colocalized with AChRs throughout synaptogenesis but also required for formation of these high-density aggregates: a transmembrane *mu*scle-specific receptor tyrosine *k*inase (MuSK), and a *r*eceptor *a*ssociated *p*rotein associated with the cytoplasmic face of the *syn*aptic membrane (rapsyn) (Sanes and Lichtman, 1999b, 2001). It is therefore important to understand how MuSK and rapsyn interact with each other and with AChRs.

Rapsyn is a 43 kDa myristylated protein that is present at a 1:1 stoichiometry with AChRs, and may bind directly to their cytoplasmic loops (Burden et al., 1983; Bartoli et al., 2001). Coexpression of rapsyn with AChRs in heterologous cells leads to aggregation of the receptors, which are diffusely distributed when expressed on their own (Froehner et al. 1990; Phillips et al., 1991). Conversely, no AChRs clusters form in muscles from mice with a targeted mutation of the *rapsyn* gene, or in myotubes cultured from these mutants, even though AChRs are present at approximately normal levels (Gautam et al., 1995). Thus, rapsyn is sufficient for AChR aggregation in non-muscle cells and necessary for AChR aggregation in myotubes.

Although AChRs and rapsyn cocluster in non-muscle cells, they do not aggregate in myotubes unless MuSK is also present: no AChRs clusters form in muscles from $MuSK^{-/-}$ mutant mice, or in myotubes cultured from the mutants (DeChiara et al., 1996; Zhou et al., 1999; Herbst and Burden, 2000). Thus, MuSK may promote interactions between AChRs and rapsyn or it may remove some muscle-specific constraint that inhibits the interaction. In addition, MuSK and rapsyn can co-cluster when co-expressed in heterologous cells (Gillespie et al., 1996; Apel et al., 1997), although this association is not required for MuSK-dependent AChR clustering (Zhou et al., 1999). Thus, MuSK can both promote AChR-rapsyn clustering and associate with AChR/rapsyn clusters.

A key to neural control of postsynaptic differentiation is activation of MuSK by a proteoglycan, agrin. Agrin was isolated by virtue of its ability to promote AChR clustering in myotubes, and this effect was subsequently shown to depend on inclusion of sequences encoded by alternatively-spliced exons called "Z" and to be mediated by MuSK. *In vivo*, motoneurons express Z^+-agrin but muscle cells do not; it is transported down motor axons and secreted into the synaptic cleft, where it associates with the basal lamina (McMahan, 1990). Consistent with the idea that Z^+-agrin is a nerve-derived organizer of synaptogenesis, mutant mice lacking all forms of agrin or lacking only the Z^+ forms exhibit identical dramatic defects in AChR clustering (Gautam et al., 1996; Burgess et al., 1999).

Together, these results have led to a model in which the MuSK's kinase activity provides a signal that promotes rapsyn-dependent postsynaptic differentiation. Yet, several results suggest that this view may be oversimplified. Although AChRs and several other postsyn-

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Abbreviations: AChR, acetylcholine receptor; BSA, bovine serum albumin; BTX, α-bungarotoxin; CHO, Chinese hamster ovary; DMEM, Dulbecco's Modified Eagle medium; E, embryonic day; GFP, green fluorescent protein; MuSK, muscle-specific kinase; PBS, phosphate-buffered saline; PI3-K, phosphatidylinositol-3-kinase.

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aptic components fail to aggregate in rapsyn$^{-/-}$ muscle, a subset of synaptic proteins, including MuSK, are clustered at motoneuron-muscle contact sites in rapsyn$^{-/-}$ mutant mice (Gautam et al., 1995; Apel et al., 1997). Moreover, a transcriptional specialization of synapse-associated myonuclei, which leads to selective expression of the AChR subunit gene, is abolished in MuSK$^{-/-}$ muscles but intact in rapsyn$^{-/-}$ muscles (Gautam et al., 1995, 1996, 1999; DeChiara et al., 1996). We have therefore suggested that the nerve can induce a rapsyn-independent, MuSK-containing primary scaffold that can organize some aspects of postsynaptic differentiation and serve as a nucleation site for rapsyn-dependent recruitment of AChRs (Apel et al., 1997; Zhou et al., 1999). Likewise, although structure-function studies using MuSK$^{-/-}$ myotubes have shown that MuSK kinase activity is indispensable for AChR clustering in response to soluble Z^{+}-agrin (Zhou et al., 1999; Herbst and Burden, 2000), no studies to date have assessed the requirement of kinase activity for formation of the primary scaffold. In fact, precedents exist for kinase-independent activities of receptor tyrosine kinases. Both kinase-dependent and -independent activities have been clearly demonstrated for eph kinases, which are concentrated at some central synapses (Birgbauer et al., 2000; Dalva et al., 2000; Kullander et al., 2001), and for the *C. elegans* ROR kinase, which is the closest nematode relative of MuSK (Forrester et al., 1999). Finally, some early MuSK-dependent steps in postsynaptic differentiation can occur in the absence of agrin, and may reflect basal (ligand-independent) activity or the existence of an alternative ligand (Lin et al., 2001; Yang et al., 2001).

In the present study, we used wild-type, mutant, and transfected myogenic cells to show that MuSK can aggregate in the absence of rapsyn and its own kinase activity. (Note that throughout this paper, we use the term "kinase-independent" to refer specifically to the kinase activity of MuSK itself and not to the activity of kinases that act downstream of MuSK or that may act on MuSK). We also demonstrate that agrin's ability to recruit MuSK depends on the form in which it is presented: soluble agrin promotes MuSK aggregates only when the MuSK is active and only in the presence of rapsyn, whereas immobilized or cell-attached agrin can aggregate MuSK in a rapsyn- and kinase-independent manner. Unexpectedly, immobilized agrin can also initiate MuSK-dependent AChR clustering without activating MuSK kinase. Given the likelihood that nerve-derived agrin is matrix-associated rather than soluble *in vivo*, these results provide new insights into early events in synaptogenesis.

EXPERIMENTAL PROCEDURES

Cell culture

Primary myoblasts were dissociated from limb muscles of embryonic day (E)18 rapsyn$^{+/+}$, rapsyn$^{+/-}$ and rapsyn$^{-/-}$ mice, plated on gelatin-coated glass coverslips, and cultured in Dulbecco's Modified Eagle medium (DMEM) containing 10% horse serum, 5% newborn calf serum, and penicillin/streptomycin. After 3 days in culture, cells were placed in DMEM containing 2% horse serum to promote myoblast fusion. Motoneurons were isolated from E5 chick embryos by the method of Henderson et al. (1995) and added to the myotubes 5 days after initiation of fusion. The cocultures were maintained in DMEM plus 2% horse serum for an additional 2 days.

Myogenic cell lines from wild-type (rapsyn$^{+/+}$; R12) and rapsyn$^{-/-}$ (R11) mice were generated by M. Gautam and obtained from C. Fuhrer (Fuhrer et al., 1999). They were cultured in DMEM containing 20% heat-inactivated fetal calf serum, 2 mM L-glutamine, antibiotics, Fungizone (Genentech, San Francisco, CA, USA), and 4 U/ml γ-interferon (R & D Systems, Inc., Minneapolis, MN). MuSK$^{-/-}$ cells were generated by and obtained from D. Glass (Regeneron, Tarrytown, NY, USA) (Sugiyama et al., 1997). They were cultured in DMEM containing 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, antibiotics, Fungizone, and 4 U/ml γ-interferon as described previously (Zhou et al., 1999). Cells were maintained at 33 °C to maintain cell division. To induce differentiation into multinucleated myotubes, cells were transferred to DMEM containing 2% horse serum plus antibiotics and Fungizone but no interferon and maintained at 37 °C.

Chinese hamster ovary (CHO) cells that stably express Z^{+} or Z^{-} forms of full-length agrin (a gift of Dr. Richard Scheller (Gibco Invitrogen, Carlsbad, CA, USA); Ferns et al., 1993) were cultured in DMEM containing 10% fetal calf serum, 400 μg/ml G418 (Invitrogen, Carlsbad, CA, USA), and antibiotics, then resuspended and added to myotube cultures. In some cases, CHO cells were stained live with 10 μM Cyto-Tracker Blue Reagent (Molecular Probes, Eugene, OR, USA) prior to coculturing with myotubes.

Myogenic cells were transfected as myoblasts with Fugene6 reagent (Roche Molecular Biochemicals, Indianapolis, IN, USA), usually 2 days before initiating fusion. Constructs were generated by standard recombinant DNA techniques using the MuSK mutants described by Zhou et al. (1999) as a starting point.

Coupling agrin to polystyrene latex beads

Recombinant approximately 50 kDa carboxy-terminal fragments of Z^{+}- or Z^{-}-agrin were purified as described previously (Burgess et al., 2002). Polystyrene latex beads (Polysciences, Warrington, PA, USA) were washed once with 100% ethanol and twice with 0.1 M borate buffer (pH 8.5), coated at room temperature with 100 μg/ml agrin or bovine serum albumin (BSA) and then blocked with 10 mg/ml BSA. The coated beads were resuspended in DMEM containing 2% horse serum and applied to myotubes. After an incubation period of 16–20 h, cultures were fixed and stained.

Immunohistochemistry

Cultures were fixed for 20 min at room temperature in 2% paraformaldehyde in phosphate-buffered saline (PBS). Nonspecific binding sites were blocked by overnight incubation in 4% BSA in PBS at 4 °C. Cultures were then incubated sequentially with primary antisera in 4% BSA in PBS (2 h at room temperature) followed by fluorescently labeled secondary antisera in PBS (1 hour at room temperature). AChRs were labeled with 0.1 μM rhodamine-conjugated α-bungarotoxin (BTX). Antisera to rapsyn, agrin and MuSK were described previously (Apel et al., 1997; Zhou et al., 1999), and anti-green fluorescent protein (GFP) was purchased from Calbiochem (San Diego, CA, USA). All results presented were obtained in at least two separate experiments, and except when specified reported clustering or co-clustering was observed at the majority of bead–cell or cell–cell contact sites.

RESULTS

Neurons induce rapsyn-independent MuSK aggregation in myotubes

Myotubes synthesize AChRs in rapsyn$^{-/-}$ mutant mice, but no AChR clusters form. In contrast, MuSK does aggre-

gate at sites of neurite-myotube contact in these mutants (Gautam et al., 1995; Apel et al., 1997). These results suggested that motor axons can organize a rapsyn-independent, MuSK-containing primary scaffold. However, we were not able to exclude other possibilities; for example MuSK clusters might form spontaneously and be preferentially innervated by ingrowing axons, or MuSK might be associated with the axon itself. We therefore began the present study by seeking direct evidence for nerve-dependent but rapsyn-independent clustering of MuSK in myotubes. To this end, we cocultured purified chick spinal motoneurons with myotubes formed from myogenic cell lines derived from rapsyn$^{+/+}$ and rapsyn$^{-/-}$ mice (Fuhrer et al., 1999). To enhance our ability to detect low levels of MuSK, and to be sure that we were assaying muscle-associated MuSK, we transfected the myoblasts with an expression vector encoding a GFP-MuSK fusion protein. Following transfection, myoblasts were fused to form myotubes, embryonic motoneurons were added, and neurites were visualized with an antibody to NCAM that recognizes avian but not murine antigen (Lemmon et al., 1982).

Consistent with previous results, some ovoid patches rich in AChR and MuSK formed in uninnervated rapsyn$^{+/+}$ myotubes, and additional clusters formed at sites where neurites contacted the myotubes (Fig. 1a,c, and data not shown). At sites where neurites contacted rapsyn$^{-/-}$ myotubes, in contrast, MuSK aggregated in many (although not all) instances (Fig. 1d′) but AChRs never did (Fig. 1b′). We believe that these clusters were nerve-induced for two reasons. First, they were elongated and precisely matched the contour of the neurite. Second, no clusters of AChRs or MuSK were ever observed on uninnervated rapsyn$^{-/-}$ myotubes (Fig. 1e′, and data not shown). To test whether the inability of rapsyn$^{-/-}$ myotubes to cluster AChRs was a direct consequence of rapsyn deficiency, we reintroduced rapsyn by transfection. AChRs and MuSK coclustered in the transfected myotubes (data not shown). Thus, motoneurons can induce formation of MuSK aggregates in the absence of rapsyn.

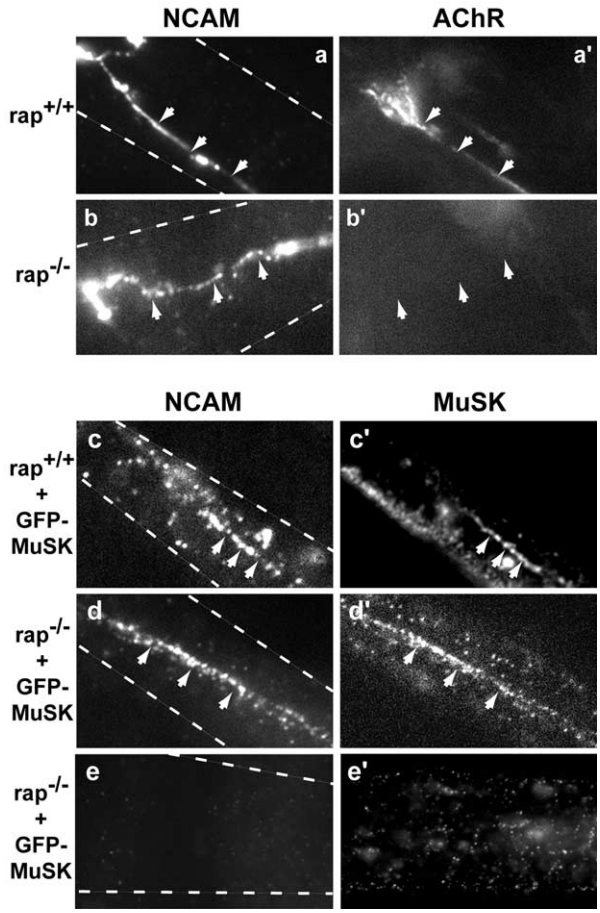

Fig. 1. Rapsyn-independent clustering of MuSK by motor neurites. Myogenic cell lines derived from wild type (rap$^{+/+}$; a, c) or rapsyn mutant (rap$^{-/-}$; b, d, e) mice were transfected with GFP-tagged MuSK then differentiated into myotubes, to which motoneurons were added. The motoneurons were visualized using avian-specific anti-NCAM (a–e), and myotubes were stained with BTX, to visualize AChRs (a′ and b′), or with anti-GFP, to visualize MuSK (c′, d′, and e′). Neurites cluster AChR and MuSK at sites of contact with myotubes expressing rapsyn; in contrast, they cluster MuSK but not AChRs at sites of contact with myotubes lacking rapsyn. In rapsyn$^{-/-}$ myotubes, MuSK is not clustered in neurite-free areas (e, e′). Dashed lines indicate borders of myotubes.

Immobilized agrin promotes rapsyn-independent aggregation of MuSK

What agents might motoneurons use to aggregate MuSK? An obvious candidate is agrin, based on its abilities to activate MuSK and to cluster AChRs. To test this possibility, we cocultured GFP-MuSK-expressing myogenic cells with CHO cells that expressed a membrane-tethered (SN) form of Z^{+}-agrin (Burgess et al., 2000). AChRs and MuSK coclustered at sites where Z^{+}-agrin-expressing CHO cells contacted rapsyn$^{+/+}$ myotubes (Fig. 2a). In contrast, MuSK regularly clustered at sites where Z^{+}-agrin-expressing CHO cells contacted rapsyn$^{-/-}$ myotubes, whereas AChR clusters were never detected at these sites (Fig. 2b). Similar results were obtained in primary myotubes, formed by fusion of myoblasts dissociated from rapsyn$^{+/-}$ and rapsyn$^{-/-}$ mice. In this case, we stained endogenous MuSK (Fig. 2c′ and d′). No clusters of AChRs or MuSK formed at sites where nontransfected CHO cells contacted rapsyn$^{+/-}$ or rapsyn$^{-/-}$ myotubes derived from either primary myoblasts or cell lines (data not shown). Thus, the ability of agrin to cluster MuSK is not secondary to its ability to cluster AChRs, and motoneurons may use agrin to cluster MuSK.

As noted in the introduction, MuSK is specifically activated by agrin isoforms that include peptides encoded by the alternatively spliced Z exons (Z^{+}-agrin). However, isoforms lacking these exons (Z^{-}-agrin) are active in some assays (Ferns et al., 1993), especially when it is substrate-associated (Jones et al., 1996). We therefore compared the ability of CHO cells expressing Z^{+}- and Z^{-}-agrin to cluster MuSK. Both CHO cell lines expressed agrin at similar levels as assessed by immunostaining with anti-agrin (data not shown). In contrast to results with cells expressing Z^{+}-agrin (Fig. 2), cells expressing Z^{-}-agrin were unable to cluster either AChR or MuSK, whether or not the myotubes expressed rapsyn (Fig. 3a and b).

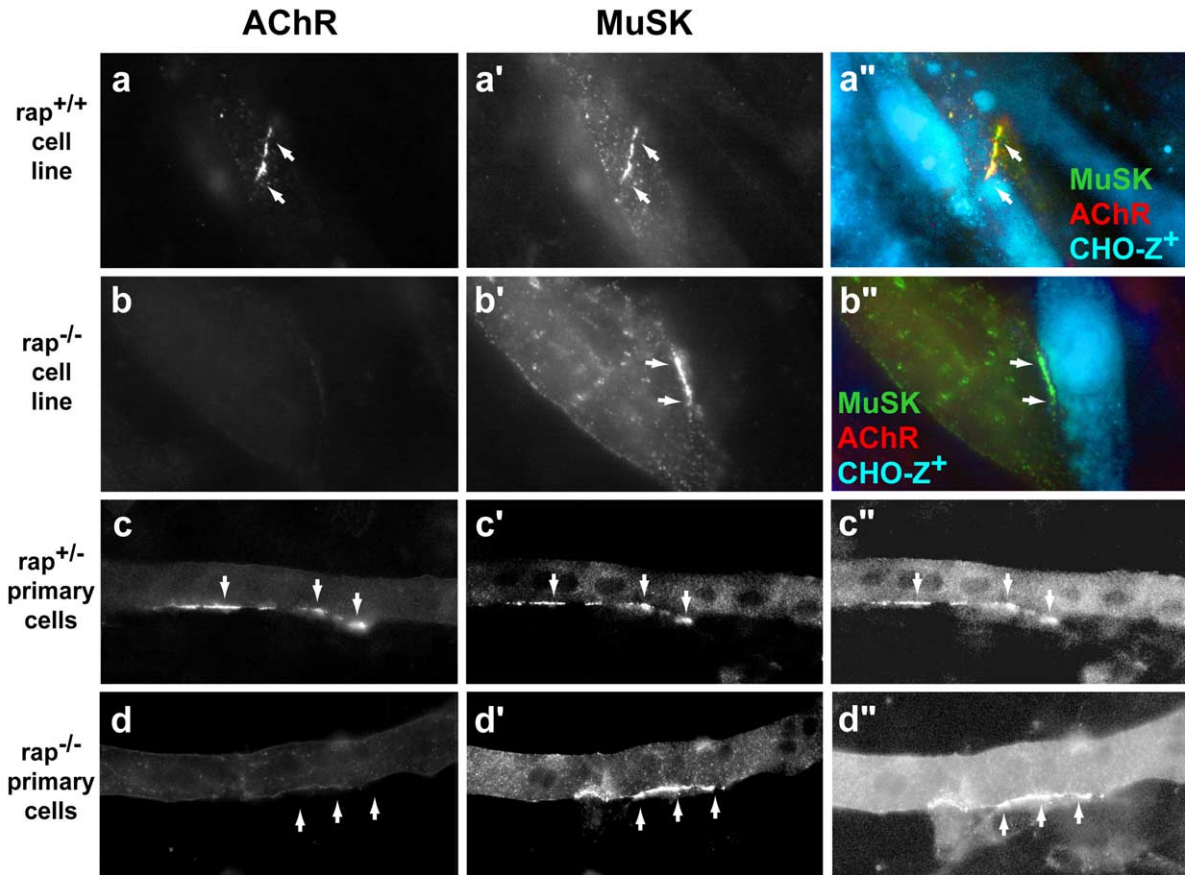

Fig. 2. Rapsyn-independent clustering of MuSK by cell-attached agrin. Myogenic cell lines and primary myoblasts from control ($rap^{+/+}$ or $rap^{+/-}$) and rapsyn mutant ($rap^{-/-}$) mice were cocultured with CHO cells expressing Z^+-agrin. Cell lines were transfected with GFP-MuSK as in Fig. 1. AChRs were labeled with BTX (a–d). MuSK was labeled with either anti-GFP (a′ and b′) or anti-MuSK (c′ and d′). CHO cells expressing Z^+-agrin were visualized either with Cyto-Tracker Blue (a″ and b″) or by background fluorescence seen with long exposures (c″ and d″). CHO cells expressing Z^+-agrin induce coclustering of AChRs (a and c) and MuSK (a′ and c′) in myotubes expressing rapsyn. These clusters occur only at sites of contact with Z^+-agrin-expressing CHO cells (a″ and c″). In myotubes lacking rapsyn, Z^+-agrin-expressing CHO cells cluster MuSK (b′ and d′) but not AChRs (b and d) at CHO cell-myotube contact sites (b″ and d″).

The ability of cell-associated agrin to cluster MuSK might depend on its association with endogenous components of CHO, or might reflect a requirement for immobilization and multimerization. To distinguish between these alternatives, we incubated $rapsyn^{+/-}$ and $rapsyn^{-/-}$ myotubes with polystyrene beads that had been coated with purified recombinant agrin fragments. The fragments comprised the carboxy-terminal approximately 50 kDa of agrin, either with or without the Z-exon-encoded peptide. The Z-containing fragment has been shown to activate MuSK and promote AChR clustering nearly as effectively as full-length agrin, but it lacks sites that bind to other receptors such as FGF, NCAM and at least some integrins (Gesemann et al., 1995; Burgess et al., 2002). Staining with anti-agrin showed that Z^+- and Z^--agrin were present at similar levels on the beads (Fig. 3h and data not shown). The Z^+-agrin-coated beads induced clusters of AChRs and MuSK at sites where they contacted control myotubes (Fig. 3h′ and data not shown), and clustered MuSK but not AChRs in $rapsyn^{-/-}$ myotubes (Fig. 3c and d). In contrast, beads coated with Z^--agrin clustered neither MuSK nor AChRs in control or $rapsyn^{-/-}$ myotubes (Fig. 3e and data not shown). These results show not only that purified agrin can cluster MuSK in the absence of rapsyn, but also that the small fragment capable of activating MuSK is also sufficient to cluster MuSK.

The ability of immobilized agrin to cluster MuSK led us to reexamine the ability of soluble agrin to cluster MuSK. We showed previously that soluble agrin was unable to cluster AChRs on $rapsyn^{-/-}$ myotubes (Gautam et al., 1995), but we had not considered the possibility that the agrin might have clustered MuSK. We therefore treated $rapsyn^{+/-}$ and $rapsyn^{-/-}$ myotubes with an approximately 50 kDa carboxy-terminal fragment of agrin containing the Z exon. As expected, this treatment was highly effective in promoting formation of AChR-MuSK coclusters on control myotubes (Fig. 3f). Agrin was completely ineffective, however, in clustering MuSK or AChRs on $rapsyn^{-/-}$ myotubes (Fig. 3g). Thus, while both soluble and also immobilized Z^+-agrin can cluster MuSK in the presence of rapsyn, only the latter can do so in its absence.

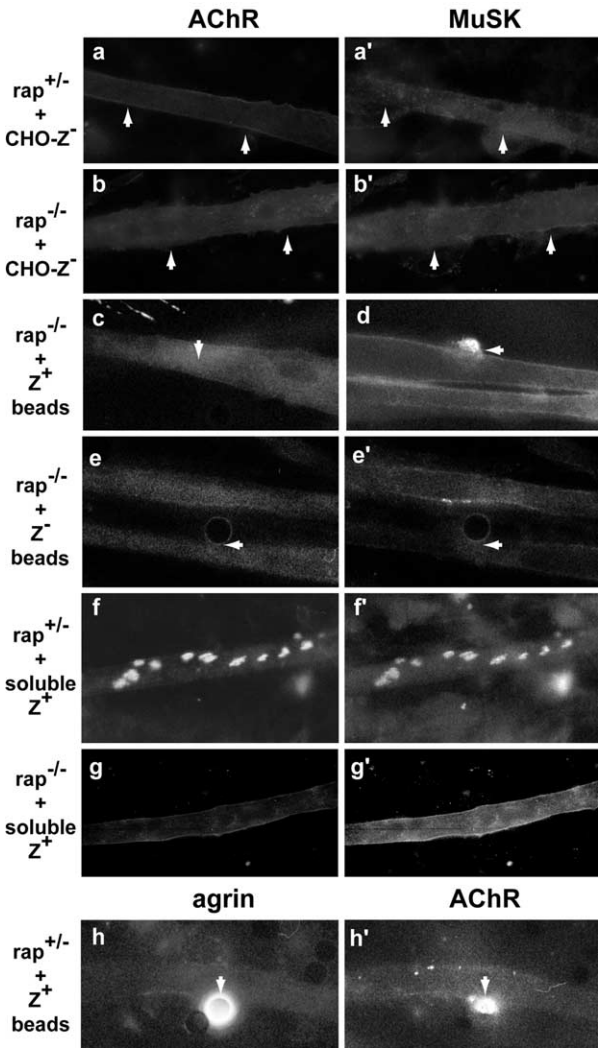

Fig. 3. Rapsyn-independent clustering of MuSK by agrin requires the Z inserts and immobilization. Primary myotubes of the indicated genotypes were cocultured with CHO cells expressing Z^-- grin (a and b), beads coated with Z^+-agrin (c, d, and h), beads coated with Z^-- agrin (e), or soluble Z^+-agrin (f and g). AChRs were labeled with BTX (a–g and h′), MuSK was labeled with anti-MuSK (a′–g′), and beads were labeled with anti-agrin (h). CHO cells expressing Z^--agrin cluster neither AChRs (a and b) nor MuSK (a′ and b′) whether or not rapsyn is expressed. Beads coated with Z^+-agrin cluster AChRs in myotubes that express rapsyn (h′). In myotubes that lack rapsyn, Z^+-agrin-coated beads cluster MuSK (d) but not AChRs (c). Z^--agrin-coated beads cluster neither AChRs (e) nor MuSK (e′). Soluble Z^+-agrin induces coclustering of AChRs (f) and MuSK (f′) in myotubes that express rapsyn, but not in myotubes that do not express rapsyn (g and g′). Arrows mark CHO cell-myotube contact sites in a and b and bead-myotube contact sites in c–e and h.

Kinase-independent aggregation of MuSK

Two results have shown that the kinase activity of MuSK is required for responsiveness to soluble agrin: a MuSK point mutant that lacks kinase activity ($MuSK^{K608A}$) inhibits agrin's ability to cluster AChRs when expressed in control myotubes (Glass et al., 1997), and fails to restore AChR clustering activity when expressed in $MuSK^{-/-}$ myotubes (Zhou et al., 1999; Herbst and Burden, 2000). Moreover, $MuSK^{K608A}$ does not aggregate in response to soluble agrin (Zhou et al., 1999). However, results presented above show that immobilized and soluble agrin cluster MuSK in different ways. We therefore considered the possibility that formation of MuSK clusters by immobilized agrin might not require kinase activity.

To test this possibility, we constructed a GFP-$MuSK^{K608A}$ derivative and introduced either it or the wild-type GFP-MuSK described above into $MuSK^{-/-}$ myotubes. Transfected cells were then cocultured with CHO cells expressing membrane associated full-length Z^+-agrin. As expected, GFP-MuSK formed many clusters following treatment with soluble agrin, and aggregated at sites where myotubes were contacted by Z^+-agrin-expressing CHO cells (Fig. 4a′–c′ and Zhou et al., 1999). In contrast, GFP-$MuSK^{K608A}$ formed no clusters in cells treated with soluble agrin but consistently aggregated at sites of contact with Z^+-agrin-expressing cells (Fig. 4d′, e′ and data not shown). The effect of the agrin-expressing cells was specific, as demonstrated by the inability of CHO cells that expressed Z^--agrin to cluster either GFP-MuSK or GFP-$MuSK^{K608A}$ (Fig. 4f′ and data not shown). Thus, MuSK aggregation in response to cell-attached agrin is kinase-independent.

To ask whether MuSK can aggregate in the absence of both kinase activity and rapsyn, we transfected GFP-$MuSK^{K608A}$ into the $rapsyn^{+/+}$ and $rapsyn^{-/-}$ cell lines. GFP-$MuSK^{K608A}$ almost always clustered at CHO cell-myotube contact sites in both genotypes (Fig. 4g′ and h′). Although wild-type MuSK was present in these cells, its levels were much lower than those of GFP-$MuSK^{K608A}$, as assessed by immunostaining with anti-MuSK antibodies, so if MuSK dimerization is required for activity (Xie et al., 1997; Till et al., 2002), most dimers would contain at least one inactive subunit and therefore be inactive. Moreover, we selectively monitored aggregation of mutant MuSK by using the GFP tag. Therefore, although we cannot exclude the possibility that low levels of kinase-active MuSK are required for rapsyn-independent aggregation to occur, we conclude that inactive MuSK can be aggregated in the absence of rapsyn.

Kinase-independent ability of MuSK to cluster AChRs

Although immobilized Z^+-agrin clustered GFP-$MuSK^{K608A}$ to a similar extent in $MuSK^{-/-}$ and $rapsyn^{-/-}$ cells, its effects on these two cell types differed in a critical and unexpected way: no AChRs were detectable at sites of high GFP-$MuSK^{K608A}$ density in $rapsyn^{-/-}$ myotubes (Fig. 4h), but AChRs colocalized to a significant degree with GFP-$MuSK^{K608A}$ in $MuSK^{-/-}$ myotubes (Fig. 4d and e). The density of AChRs was consistently lower in $MuSK^{-/-}$ myotubes transfected with GFP-$MuSK^{K608A}$ than in myotubes transfected with GFP-MuSK, consistent with the known effects of the K608A mutation (compare Figs. 4b,c with 4d,e). Thus, once $MuSK^{K608A}$ is clustered, it can promote limited clustering of AChRs in a rapsyn-dependent but kinase-independent manner.

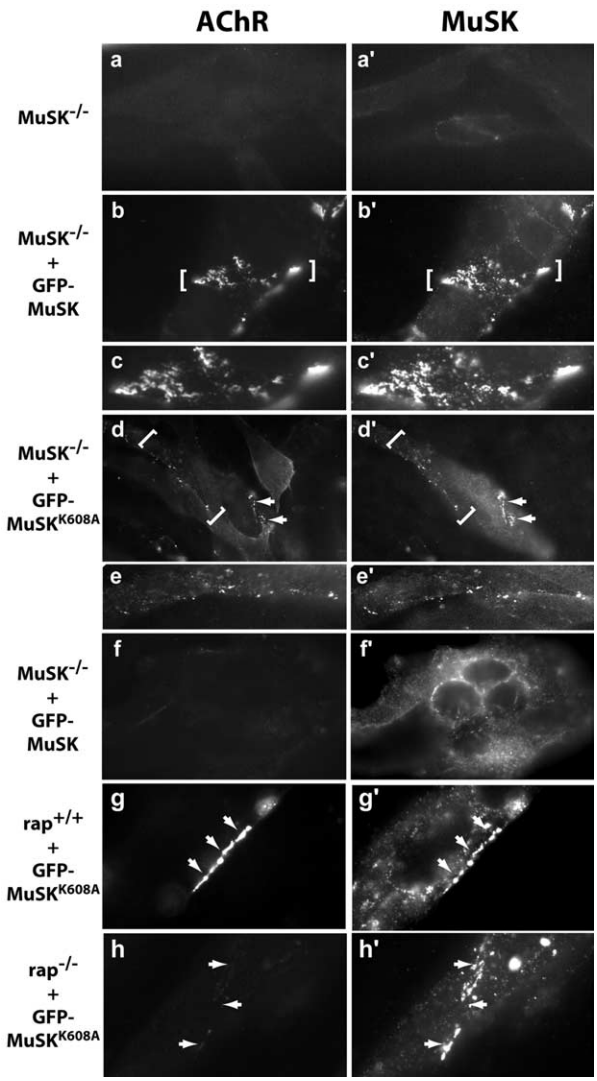

Fig. 4. Kinase-independent clustering of MuSK and AChRs by cell-attached agrin. Control ($rap^{+/+}$), $MuSK^{-/-}$, and $rapsyn^{-/-}$ myogenic cell lines were transfected with GFP-tagged active or kinase-dead MuSK ($MuSK^{K608A}$), then fused to form myotubes, which were cocultured with CHO cells expressing Z^{+}-agrin. AChRs and MuSK were labeled with BTX (a–h) and anti-GFP (a′–h′), respectively. In $MuSK^{-/-}$ myotubes, AChRs fail to cluster at sites of contact with CHO cells expressing Z^{+}-agrin (a), and MuSK itself is not detectable (a′). When MuSK is reintroduced into these myotubes, both AChRs (b and c) and MuSK (b′ and c′) cocluster at sites of contact with CHO cells expressing Z^{+}-agrin. Kinase-dead MuSK (d′ and e′) and AChRs (d and e) also cocluster at contact sites. Neither AChRs (f) nor MuSK (f′) cluster at sites of contact with CHO cells expressing Z^{-}-agrin. In control ($rap^{+/+}$) myotubes, both AChRs (g) and kinase-dead mutant MuSK (g′) cocluster at sites of contact with CHO cells expressing Z^{+}-agrin. In rapsyn-deficient myotubes, kinase-dead MuSK (h′) but not AChRs (h) cluster at these sites. Areas bracketed in b and d are shown at higher magnification in c and e, respectively.

Because these results were surprising, we performed a set of control experiments. First, we confirmed that soluble agrin was completely unable to promote clustering of AChRs via $MuSK^{K608A}$ (data not shown; see Zhou et al., 1999). Second, we showed that clustering of AChRs, like clustering of MuSK, was a specific response to Z^{+}-agrin, as neither wild-type CHO cells nor CHO cells expressing Z^{-}-agrin promoted AChR clustering (Fig. 4f and data not shown). Third, to test whether the AChR clusters were localized to CHO cell-myotube contact sites, we double-stained cultures with BTX plus an antibody to agrin. As shown in Fig. 5, the AChR aggregates corresponded precisely to sites at which agrin-rich processes contacted myotubes. Thus, MuSK is able to recruit AChRs to a limited extent by a kinase-independent mechanism.

Requirement of MuSK cytoplasmic domain for MuSK clustering

The cytoplasmic domain of MuSK bears several sites that might play roles in its aggregation by immobilized agrin. One is a putative binding site for PTB class proteins (550NPXY553), which has been shown to be essential for MuSK kinase activity and for MuSK's ability to promote AChR clustering in response to soluble agrin (Zhou et al., 1999; Herbst and Burden, 2000). Another is a carboxy-terminal tripeptide (VGV) that binds PDZ domain proteins, such as MAGI-1c and PICK1 (O'Brien et al., 1998; Torres et al., 1998; Xia et al., 1999; Strochlic et al., 2001). This site is inessential for activation of MuSK by agrin but was of interest because PDZ proteins have been implicated in forming kinase- and receptor-rich aggregates at neuron–neuron synapses (Garner et al., 2000; Sheng and Sala, 2001). Third is a putative phosphatidylinositol-3-kinase (PI3-K) binding site (Y831), which has been implicated in receptor clustering at inhibitory synapses (Kneussel and Betz, 2000).

To assess the requirement for PTB-binding proteins, we generated a GFP-$MuSK^{Y553F}$ mutant. To assess requirements for PDZ proteins and PI3-K, we mutated their binding sites in the context of a kinase-dead MuSK; that is, we generated GFP-$MuSK^{K608A,\Delta PDZ}$, and GFP-$MuSK^{K608A,Y831F}$ double mutants. Such double mutants were required because we knew that wild-type (kinase-active) MuSK would promote formation of AChR/rapsyn co-clusters, and associate with those clusters. In the presence of these large aggregates, a defect in the kinase-independent pathway would probably not be detected. In contrast, we wanted to know whether the kinase-independent pathway required association of MuSK with cytoplasmic proteins.

All three of these GFP-MuSK mutants were introduced into $MuSK^{-/-}$ myotubes, which were then cocultured with CHO cells expressing Z^{+}-agrin. In all cases, both MuSK and AChR clustered at sites of CHO cell-myotube contact (Fig. 6a,b, and b′); the incidence of co-clustering was not detectably different from that seen with GFP-$MuSK^{K608A}$. These results suggest that MuSK need not recruit PDZ proteins, PTB proteins or PI3-K in order to aggregate.

In view of these results, we asked whether the entire MuSK cytoplasmic domain was dispensable for MuSK aggregation. For this purpose, we generated a series of GFP-MuSK truncation mutants that lacked the carboxy terminal 20 (GFP-$MuSK^{K608A,\Delta C1}$) or 70 (GFP-$MuSK^{K608A,\Delta C2}$) amino acids of the cytoplasmic domain or the entire cytoplasmic domain (GFP-$MuSK^{\Delta cyto}$). The first

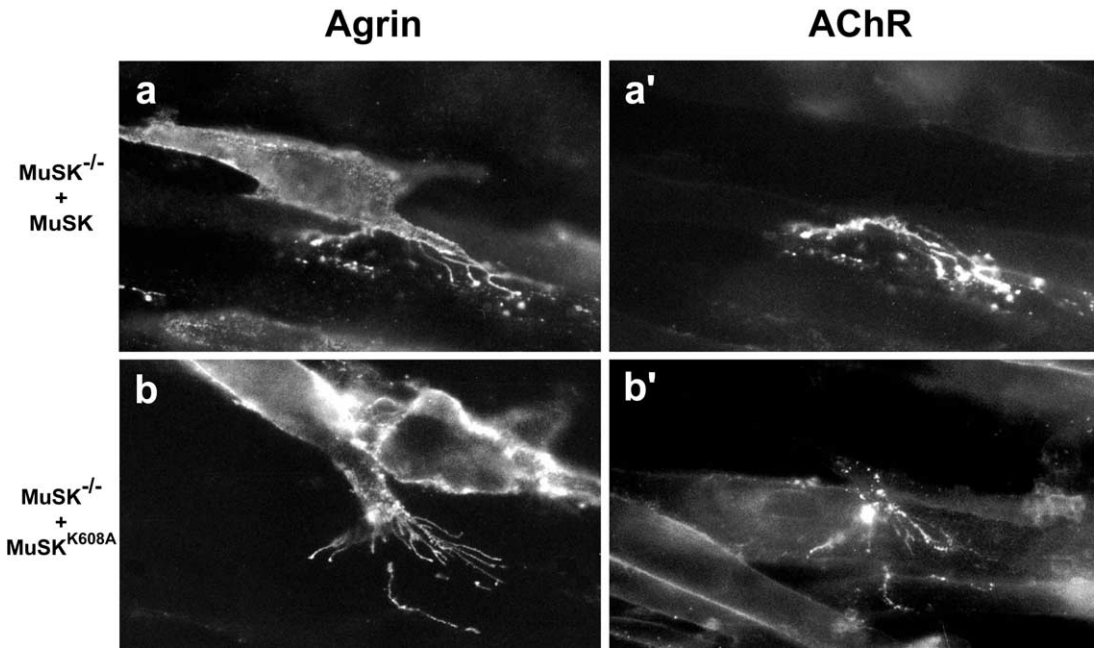

Fig. 5. Kinase-independent clustering of AChRs at sites of contact with Z^+-agrin. $MuSK^{-/-}$ myoblasts were transfected, fused, and cocultured with Z^+-agrin-expressing cells as in Fig. 4. Cultures were then labeled with anti-agrin (a and b) and BTX (a′ and b′). In the presence of either wild-type or kinase-dead MuSK, AChR clusters form precisely at sites of contact with agrin-rich processes, but the density of AChRs is higher in the presence of wild-type than mutant MuSK.

two of these mutants were also rendered kinase dead by inclusion of the K608A mutation for the reason presented above. All three mutants were transported to the cell surface in transfected $MuSK^{-/-}$ cells and all coclustered with rapsyn in cotransfected QT6 fibroblasts (data not shown; note that the cytoplasmic domain is dispensable for interaction of MuSK with rapsyn; Apel et al., 1997; Zhou et al., 1999). GFP-$MuSK^{K608A,\Delta C1}$ clustered at sites of contact with Z^+-agrin-expressing CHO cells and also promoted clustering of AChRs at these sites (Fig. 6a). In contrast, no clusters of GFP-$MuSK^{K608A,\Delta C2}$, GFP-$MuSK^{\Delta cyto}$, or AChRs were detectable (Fig. 6a and c). Taken together, these data demonstrate that the MuSK cytoplasmic domain is required for MuSK aggregation, and suggest the presence of critical sites in the carboxy-terminal third of the cytoplasmic domain.

DISCUSSION

Studies *in vivo* and *in vitro* have led to the view that agrin, acting either in soluble form or as if it were soluble, activates the kinase activity of MuSK to initiate a signal transduction cascade that leads rapsyn to cluster postsynaptic proteins, including AChRs (Sanes and Lichtman, 1999b, 2001). Here, we have used cultured muscle cells to test the effects of agrin on MuSK and AChRs. Although nothing we found throws the main features of the model into question, we are led to suggest that it is incomplete in significant respects. Our main results are: (1) neurites can induce clustering of MuSK in the absence of rapsyn, but induce clustering of AChRs only in the presence of rapsyn; (2) the ability of neurites to form this rapsyn-independent "primary scaffold" is mimicked by cell-attached agrin; (3) cell-attached agrin can also cluster MuSK in the absence of MuSK's kinase activity; (4) cell-attached agrin can cluster AChRs to a limited extent by a process that is MuSK-dependent but does not require MuSK's kinase activity; (5) agrin's ability to recruit MuSK depends on the form in which it is presented: whereas immobilized or cell-attached agrin can aggregate MuSK in a rapsyn- and kinase-independent manner, soluble agrin promotes MuSK aggregates only when the MuSK is active and only in the presence of rapsyn.

The different activities of soluble and immobilized agrin are relevant in relating results obtained from cultured myotubes to mechanisms of postsynaptic differentiation. In most studies of agrin *in vitro*, recombinant fragments have been applied in soluble form, and their effects on MuSK activation or AChR clustering assessed. It was natural to presume that nerve-derived agrin acts in the same way as recombinant agrin. Yet *in vivo*, it is likely that nerve-derived agrin is immobilized, either in the membrane or the extracellular matrix, and it is therefore noteworthy that immobilized but not soluble agrin can aggregate MuSK in the absence of kinase activity or rapsyn and even aggregate AChRs in the absence of kinase activity. We do not know whether agrin uses ancillary receptors or active signaling to cause this aggregation; the simplest possibility is that it acts by a direct binding or lectin-like mechanism. Whatever the mechanism, the implication is that agrin might be able to use a subset of the full complement of downstream signaling components to initiate some aspects of postsynaptic differentiation. That is, not only rapsyn but also sub-

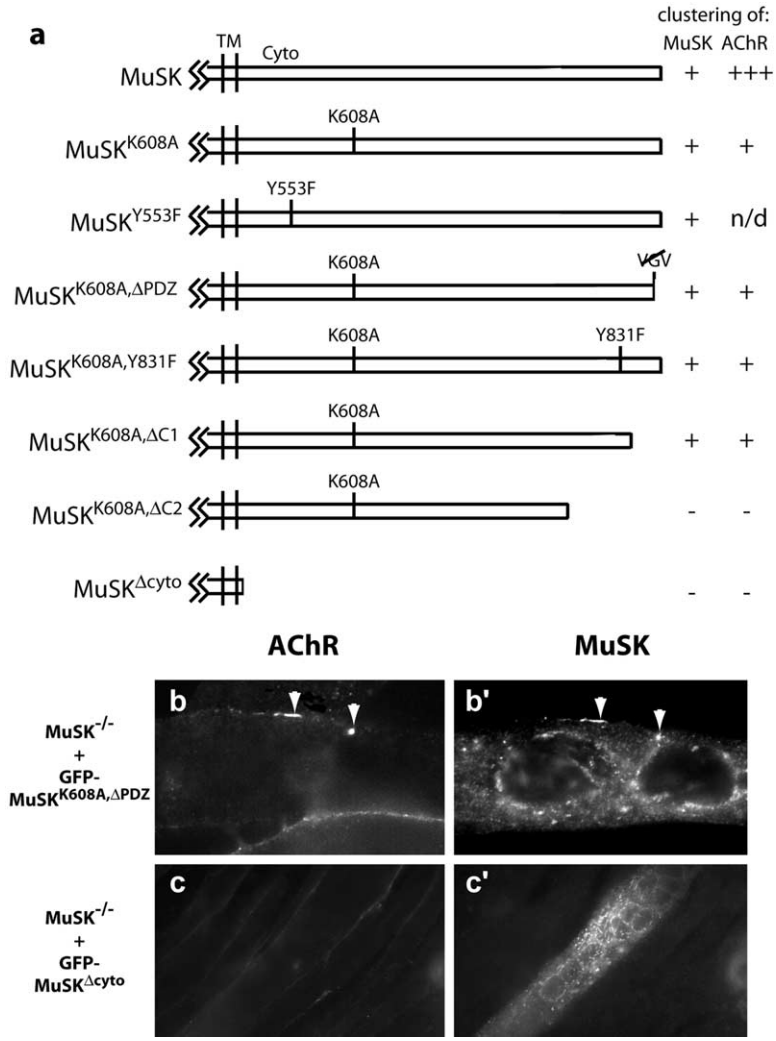

Fig. 6. Clustering of MuSK and AChRs requires the cytoplasmic domain of MuSK. GFP-tagged mutant MuSK constructs in a were expressed in MuSK$^{-/-}$ myotubes, which were then cocultured with Z^{+}-agrin-expressing cells. AChRs were labeled with BTX (b and c), and MuSK was labeled with anti-GFP (b′ and c′). In the context of kinase-dead MuSK, mutation of putative binding sites for PTB domain-containing proteins, PDZ domain-containing proteins, or phosphahdylinositol-3-kinase had no effect on clustering of either AChRs or MuSKs (a and b). In contrast, deletion of ≥70 amino acids of the cytoplasmic domain abrogated the ability of MuSK to cluster or to promote clustering of AChRs (a and c′).

strates of MuSK might be dispensable. Moreover, in that postsynaptic differentiation is more profoundly blocked in MuSK$^{-/-}$ mice than in agrin$^{-/-}$ mice (DeChiara et al., 1996; Gautam et al., 1996, 1999; Lin et al., 2001), it will be interesting to consider the possibility that some aspects of postsynaptic differentiation may depend on ligands that act primarily by aggregating rather than activating MuSK.

The existence of rapsyn-independent and -dependent aspects of postsynaptic differentiation supports the idea that MuSK is at the apex of several signaling pathways that together promote postsynaptic differentiation. Aggregation of AChRs and several cytoskeletal and membrane components clearly requires rapsyn. On the other hand, we have previously shown that aggregation of MuSK as well as clustering of at least two components of the synaptic cleft (acetylcholinesterase and laminin β2) occurs in the absence of rapsyn *in vivo* (Gautam et al., 1995; Apel et al., 1997). Now that some of these rapsyn-independent events can be mimicked *in vitro*, it should be possible to analyze their mechanisms. Moreover, transcriptional specialization of synapse-associated nuclei *in vivo* requires MuSK but not rapsyn (Gautam et al., 1995, 1995, 1999; DeChiara et al., 1996). In early studies, agrin did not appear to promote transcription *in vitro*, but recent studies have shown that under appropriate conditions such effects can be demonstrated (Jones et al., 1996; Gramolini et al., 1998; Meier et al., 1998). In these studies, the requirement for rapsyn has not yet been tested. Of particular interest is a recent study by Lacazette et al. (2003), who show that agrin can affect transcription by two separate signaling pathways; it is interesting to speculate that one is rapsyn-dependent and the other not.

The existence of distinct kinase-independent and -dependent mechanisms for AChR clustering is also of interest in considering a major difference between neuromuscular junctions and central synapses: receptor density is

much higher in the former. Current estimates for the density of receptors at inhibitory (GABAergic) and excitatory (glutamatergic) central synapses are in the range of 200–1200/μm^2 (Nusser et al., 1997; Nusser, 1999; Franks et al., 2002) whereas AChRs achieve a density of 10–20,000/μm^2 at the adult neuromuscular junction (Sanes and Lichtman, 1999b). This difference might imply that mechanisms for receptor accumulation at the neuromuscular junction differ fundamentally from those at central synapses, but we now speculate that receptor accumulation at all of these synapses may be more similar than has been believed. In all cases, a contact-mediated, kinase-independent scaffold achieves a moderate receptor density. At the neuromuscular junction, a MuSK kinase-dependent catalytic mechanism is critical for increasing the density of receptors and the size of the synapse. This need not be a temporally distinct stage, in that wild type MuSK can simultaneously mediate both mechanisms. Likewise, central synapses use kinase-dependent mechanisms to increase receptor density, but these processes are temporally distinct from those of early synaptogenesis, and are generally associated with synaptic plasticity (for example, long-term potentiation; Sanes and Lichtman, 1999a). From this perspective, the neuromuscular junction could be viewed as always being maximally potentiated.

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REFERENCES

Apel ED, Glass DJ, Moscoso LM, Yancopoulos GD, Sanes JR (1997) Rapsyn is required for MuSK signaling and recruits synaptic components to a MuSK-containing scaffold. Neuron 18:623–635.

Bartoli M, Ramarao MK, Cohen JB (2001) Interactions of the rapsyn RING-H2 domain with dystroglycan. J Biol Chem 276:24911–24917.

Birgbauer E, Cowan CA, Sretavan DW, Henkemeyer M (2000) Kinase independent function of EphB receptors in retinal axon pathfinding to the optic disc from dorsal but not ventral retina. Development 127:1231–1241.

Burden SJ, DePalma RL, Gottesman GS (1983) Crosslinking of proteins in acetylcholine receptor-rich membranes: association between the beta-subunit and the 43 kD subsynaptic protein. Cell 35:687–692.

Burgess RW, Nguyen QT, Son YJ, Lichtman JW, Sanes JR (1999) Alternatively spliced isoforms of nerve- and muscle-derived agrin: their roles at the neuromuscular junction. Neuron 23:33–44.

Burgess RW, Skarnes WC, Sanes JR (2000) Agrin isoforms with distinct amino termini: differential expression, localization, and function. J Cell Biol 151:41–52.

Burgess RW, Dickman DK, Nunez L, Glass DJ, Sanes JR (2002) Mapping sites responsible for interactions of agrin with neurons. J Neurochem 83:271–284.

Dalva MB, Takasu MA, Lin MZ, Shamah SM, Hu L, Gale NW, Greenberg ME (2000) EphB receptors interact with NMDA receptors and regulate excitatory synapse formation. Cell 103:945–956.

DeChiara TM, Bowen DC, Valenzuela DM, Simmons MV, Poueymirou WT, Thomas S, Kinetz E, Compton DL, Rojas E, Park JS, Smith C, DiStefano PS, Glass DJ, Burden SJ, Yancopoulos GD (1996) The receptor tyrosine kinase MuSK is required for neuromuscular junction formation in vivo. Cell 85:501–512.

Ferns MJ, Campanelli JT, Hoch W, Scheller RH, Hall Z (1993) The ability of agrin to cluster AChRs depends on alternative splicing and on cell surface proteoglycans. Neuron 11:491–502.

Forrester WC, Dell M, Perens E, Garriga G (1999) A *C. elegans* Ror receptor tyrosine kinase regulates cell motility and asymmetric cell division. Nature 400:881–885.

Franks KM, Bartol TM Jr, Sejnowski TJ (2002) A Monte Carlo model reveals independent signaling at central glutamatergic synapses. Biophys J 83:2333–2348.

Froehner SC, Luetje CW, Scotland PB, Patrick J (1990) The postsynaptic 43K protein clusters muscle nicotinic acetylcholine receptors in *Xenopus* oocytes. Neuron 5:403–410.

Fuhrer C, Gautam M, Sugiyama JE, Hall ZW (1999) Roles of rapsyn and agrin in interaction of postsynaptic proteins with acetylcholine receptors. J Neurosci 19:6405–6416.

Garner CC, Nash J, Huganir RL (2000) PDZ domains in synapse assembly and signalling. Trends Cell Biol 10:274–280.

Gautam M, DeChiara TM, Glass DJ, Yancopoulos GD, Sanes JR (1999) Distinct phenotypes of mutant mice lacking agrin, MuSK, or rapsyn. Brain Res Dev Brain Res 114:171–178.

Gautam M, Noakes PG, Moscoso L, Rupp F, Scheller RH, Merlie JP, Sanes JR (1996) Defective neuromuscular synaptogenesis in agrin-deficient mutant mice. Cell 85:525–535.

Gautam M, Noakes PG, Mudd J, Nichol M, Chu GC, Sanes JR, Merlie JP (1995) Failure of postsynaptic specialization to develop at neuromuscular junctions of rapsyn-deficient mice. Nature 377:232–236.

Gesemann M, Denzer AJ, Ruegg MA (1995) Acetylcholine receptor-aggregating activity of agrin isoforms and mapping of the active site. J Cell Biol 128:625–636.

Gillespie SK, Balasubramanian S, Fung ET, Huganir RL (1996) Rapsyn clusters and activates the synapse-specific receptor tyrosine kinase MuSK. Neuron 16:953–962.

Glass DJ, Apel ED, Shah S, Bowen DC, DeChiara TM, Stitt TN, Sanes JR, Yancopoulos GD (1997) Kinase domain of the muscle-specific receptor tyrosine kinase (MuSK) is sufficient for phosphorylation but not clustering of acetylcholine receptors: required role for the MuSK ectodomain? Proc Natl Acad Sci USA 94:8848–8853.

Gramolini AO, Burton EA, Tinsley JM, Ferns MJ, Cartaud A, Cartaud J, Davies KE, Lunde JA, Jasmin BJ (1998) Muscle and neural isoforms of agrin increase utrophin expression in cultured myotubes via a transcriptional regulatory mechanism. J Biol Chem 273:736–743.

Henderson CE, Bloch-Gallego E, Camu W (1995) Purified embryonic motoneurons. In: Nerve cell culture: a practical approach (Cohen J, Wilkin G, eds), pp 69–81. London: Oxford University Press.

Herbst R, Burden SJ (2000) The juxtamembrane region of MuSK has a critical role in agrin-mediated signaling. EMBO J 19:67–77.

Jones G, Herczeg A, Ruegg MA, Lichtsteiner M, Kroger S, Brenner HR (1996) Substrate-bound agrin induces expression of acetylcholine receptor epsilon-subunit gene in cultured mammalian muscle cells. Proc Natl Acad Sci USA 93:5985–5990.

Kneussel M, Betz H (2000) Clustering of inhibitory neurotransmitter receptors at developing postsynaptic sites: the membrane activation model. Trends Neurosci 23:429–435.

Kullander K, Mather NK, Diella F, Dottori M, Boyd AW, Klein R (2001) Kinase-dependent and kinase-independent functions of EphA4 receptors in major axon tract formation in vivo. Neuron 29:73–84.

Lacazette E, Le Calvez S, Gajendran N, Brenner HR (2003) A novel pathway for MuSK to induce key genes in neuromuscular synapse formation. J Cell Biol 161:727–736.

Lemmon V, Staros EB, Perry HE, Gottlieb DI (1982) A monoclonal antibody which binds to the surface of chick brain cells and myotubes: cell selectivity and properties of the antigen. Brain Res 255:349–360.

Lin W, Burgess RW, Dominguez B, Pfaff SL, Sanes JR, Lee KF (2001) Distinct roles of nerve and muscle in postsynaptic differentiation of the neuromuscular synapse. Nature 410:1057–1064.

McMahan UJ (1990) The agrin hypothesis. Cold Spring Harb Symp Quant Biol 55:407–418.

Meier T, Masciulli F, Moore C, Schoumacher F, Eppenberger U, Denzer AJ, Jones G, Brenner HR (1998) Agrin can mediate acetylcholine receptor gene expression in muscle by aggregation of muscle-derived neuregulins. J Cell Biol 141:715–726.

Nusser Z (1999) A new approach to estimate the number, density and variability of receptors at central synapses. Eur J Neurosci 11:745–752.

Nusser Z, Cull-Candy S, Farrant M (1997) Differences in synaptic GABA(A) receptor number underlie variation in GABA mini amplitude. Neuron 19:697–709.

O'Brien RJ, Lau LF, Huganir RL (1998) Molecular mechanisms of glutamate receptor clustering at excitatory synapses. Curr Opin Neurobiol 8:364–369.

Phillips WD, Kopta C, Blount P, Gardner PD, Steinbach JH, Merlie JP (1991) ACh receptor-rich membrane domains organized in fibroblasts by recombinant 43-kildalton protein. Science 251:568–570.

Sanes JR, Lichtman JW (1999a) Can molecules explain long-term potentiation? Nat Neurosci 2:597–604.

Sanes JR, Lichtman JW (1999b) Development of the vertebrate neuromuscular junction. Ann Rev Neurosci 22:389–442.

Sanes JR, Lichtman JW (2001) Induction, assembly, maturation and maintenance of a postsynaptic apparatus. Nat Rev Neurosci 2:791–805.

Sheng M, Sala C (2001) PDZ domains and the organization of supramolecular complexes. Annu Rev Neurosci 24:1–29.

Strochlic L, Cartaud A, Labas V, Hoch W, Rossier J, Cartaud J (2001) MAGI-1c: a synaptic MAGUK interacting with MuSK at the vertebrate neuromuscular junction. J Cell Biol 153:1127–1132.

Sugiyama JE, Glass DJ, Yancopoulos GD, Hall ZW (1997) Laminin-induced acetylcholine receptor clustering: an alternative pathway. J Cell Biol 139:181–191.

Till JH, Becerra M, Watty A, Lu Y, Ma Y, Neubert TA, Burden SJ, Hubbard SR (2002) Crystal structure of the MuSK tyrosine kinase: insights into receptor autoregulation. Structure (Camb) 10:1187–1196.

Torres R, Firestein BL, Dong H, Staudinger J, Olson EN, Huganir RL, Bredt DS, Gale NW, Yancopoulos GD (1998) PDZ proteins bind, cluster, and synaptically colocalize with Eph receptors and their ephrin ligands. Neuron 21:1453–1463.

Xia J, Zhang X, Staudinger J, Huganir RL (1999) Clustering of AMPA receptors by the synaptic PDZ domain-containing protein PICK1. Neuron 22:179–187.

Xie MH, Yuan J, Adams C, Gurney A (1997) Direct demonstration of MuSK involvement in acetylcholine receptor clustering through identification of agonist ScFv. Nat Biotechnol 15:768–771.

Yang X, Arber S, William C, Li L, Tanabe Y, Jessell TM, Birchmeier C, Burden SJ (2001) Patterning of muscle acetylcholine receptor gene expression in the absence of motor innervation. Neuron 30:399–410.

Zhou H, Glass DJ, Yancopoulos GD, Sanes JR (1999) Distinct domains of MuSK mediate its abilities to induce and to associate with postsynaptic specializations. J Cell Biol 146:1133–1146.

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