Association of muscle-specific kinase MuSK with the acetylcholine receptor in mammalian muscle

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During synaptogenesis at the neuromuscular junction, a neurally released factor, agrin, causes the clustering of acetylcholine receptors (AChRs) in the muscle membrane beneath the nerve terminal. Agrin acts through a specific receptor which is thought to have a receptor tyrosine kinase, MuSK, as one of its components. In agrin-treated muscle cells, both MuSK and the AChR become tyrosine phosphorylated. To determine how the activation of MuSK leads to AChR clustering, we have investigated their interaction in cultured C2 myotubes. Immunoprecipitation experiments showed that MuSK is associated with the AChR and that this association is increased by agrin treatment. Agrin also caused a transient activation of the AChR-associated MuSK, as demonstrated by MuSK phosphorylation. In agrin-treated myotubes, MuSK phosphorylation increased with the same time course as phosphorylation of the β subunit of the AChR, but declined more quickly. Although both herbimycin and staurosporine blocked agrin-induced AChR phosphorylation, only herbimycin inhibited the phosphorylation of MuSK. These results suggest that although agrin increases the amount of activated MuSK that is associated with the AChR, MuSK is not directly responsible for AChR phosphorylation but acts through other kinases.

Keywords: acetylcholine receptor/agrin/muscle-specific kinase MuSK/neuromuscular junction/tyrosine phosphorylation

Introduction

Differentiation of the synaptic apparatus at the neuromuscular junction occurs through the exchange of signals between nerve and muscle cells. One of the earliest signals is the release of agrin by nerve terminals (McMahan, 1990; Reist *et al.*, 1992). Agrin acts on a specific receptor in the muscle cell membrane to cause the clustering of acetylcholine receptors (AChRs) beneath the nerve terminal (Nastuk *et al.*, 1991). Due to alternative splicing, agrin exists in several isoforms that differ in AChR clustering activities and tissue distribution (Ferns *et al.*, 1992; Ruegg *et al.*, 1992). The most active forms are expressed exclusively in neurons, whereas the predominant agrin forms in muscle are much less effective in aggregating AChRs (Ferns *et al.*, 1993; Hoch *et al.*, 1993). In

addition to its action on the AChR, neural agrin causes other synaptic components to be concentrated in association with the AChR clusters (Wallace, 1989), suggesting that it is a general organizing factor for postsynaptic differentiation. In genetically altered recombinant mice that do not express agrin, differentiated synapses fail to form and few nerve-associated AChR clusters are seen on muscle fibers (Gautam *et al.*, 1996).

The muscle receptor for neurally released agrin has not been fully defined, but a trk-related receptor tyrosine kinase, MuSK, appears to be one of its components (Jennings *et al.*, 1993; Valenzuela *et al.*, 1995). Like the AChR, MuSK is localized in the synapse in mature muscles and its expression is increased extrasynaptically following denervation (Valenzuela *et al.*, 1995). Neural agrin induces tyrosine autophosphorylation of MuSK, and appears to bind a complex containing MuSK and one or more muscle-specific factors (Glass *et al.*, 1996). Most importantly, mice in which the MuSK gene has been disrupted have a phenotype that is similar to that seen in agrin-deficient mice, in that the muscle fibers lack differentiated synapses and nerve-associated AChR clusters (DeChiara *et al.*, 1996). In addition, cultured myotubes from the MuSK^{-/-} mice fail to cluster AChRs in response to agrin (Glass *et al.*, 1996). Another possible constituent of the receptor for agrin is α -dystroglycan, which is the predominant agrin-binding protein in muscle, and which is a component of the dystrophin–utrophin glycoprotein complex of skeletal muscle (Bowe *et al.*, 1994; Campanelli *et al.*, 1994; Gee *et al.*, 1994; Sugiyama *et al.*, 1994). Although α -dystroglycan binds to agrin, its functional role in agrin-mediated synaptic organization has remained unclear (Sealock and Froehner, 1994) because it binds to active neural as well as inactive muscle isoforms of agrin (Sugiyama *et al.*, 1994; Bowen *et al.*, 1996) and because short recombinant C-terminal fragments of neural agrin, which do not bind to α -dystroglycan, retain significant AChR clustering activity (Gesemann *et al.*, 1995, 1996; Hopf and Hoch, 1996).

In addition to the activation of MuSK, agrin induces tyrosine phosphorylation of the β subunit of the AChR (Wallace *et al.*, 1991; Qu and Haganir, 1994; Meier *et al.*, 1995; Ferns *et al.*, 1996). In mammalian muscle, this phosphorylation is transient, with a time course that precedes the time course of AChR cluster formation. Although not demonstrated to be required for AChR cluster formation in muscle cells, phosphorylation of the AChR β subunit is well correlated with the formation of AChR clusters under several conditions, suggesting that it may play a critical role (Ferns *et al.*, 1996; Meier *et al.*, 1996). In support of this idea, inhibitors that block tyrosine phosphorylation of the AChR also block AChR cluster formation. Thus, herbimycin, a specific inhibitor of tyrosine kinases, and staurosporine, a more general inhibitor

of protein kinases, block both agrin-induced AChR phosphorylation and AChR clustering (Wallace, 1994; Ferns *et al.*, 1996).

Agrin-induced tyrosine phosphorylation of the AChR could, in principle, occur by several mechanisms; agrin-activated MuSK could phosphorylate the AChR directly or it could act through other kinases. We have previously investigated AChR phosphorylation in mammalian muscle by src family kinases (Fuhrer and Hall, 1996). Our experiments showed that src binds to, and can phosphorylate, fusion proteins derived from the cytoplasmic loop of the AChR β subunit; the binding region on the β subunit contains a conserved tyrosine that is phosphorylated in the receptor isolated from the electric organ of *Torpedo* (Wagner *et al.*, 1991). Immunoprecipitation experiments showed that muscle AChR is associated with both src and fyn (Fuhrer and Hall, 1996), raising the possibility that src phosphorylates the AChR, and that fyn becomes bound as a result of this phosphorylation (Swope and Huganir, 1993, 1994). Another route leading to AChR phosphorylation might involve direct phosphorylation of the β subunit by MuSK. In support of this idea, the AChR β subunit is tyrosine phosphorylated when the AChR, MuSK and the postsynaptic 43 kDa protein, rapsyn, are co-expressed in transfected QT-6 quail fibroblasts (Gillespie *et al.*, 1996). As this phosphorylation requires MuSK kinase activity, the β subunit of the AChR could be a direct substrate for MuSK, as proposed by Gillespie *et al.* (1996); alternatively, MuSK activation could be an early step in the pathway leading to AChR phosphorylation. In this system, phosphorylation of the AChR β subunit depends on co-expression of both MuSK and rapsyn, raising the possibility that all three proteins are part of a complex involved in AChR clustering.

To clarify the relationship between MuSK and AChR phosphorylation, we have investigated the association between the two proteins in unstimulated and agrin-stimulated mammalian C2 muscle myotubes. We find that MuSK is associated with the AChR and that receptor-bound MuSK rapidly becomes tyrosine phosphorylated after addition of neural agrin. Block of tyrosine phosphorylation of the AChR β subunit by the kinase inhibitor staurosporine can be achieved without affecting MuSK activation, however, suggesting that another tyrosine kinase is responsible for agrin-induced phosphorylation of the AChR.

Results

MuSK is associated with the AChR in C2 myotubes

In our initial experiments, we sought to detect interaction between MuSK and the AChR in C2 myotubes using co-precipitation experiments. For this purpose, we used a polyclonal antiserum raised against the C-terminus of MuSK that specifically recognized mouse MuSK on immunoblots (Figure 1A and B) and in immunoprecipitations (see Figure 4C). As shown previously by Glass *et al.* (1996), MuSK migrated as a band, or sometimes a doublet, with an apparent molecular weight of ~110 kDa. To test for its association with the AChR, we first isolated the AChR from C2 myotube extracts with α -bungarotoxin covalently coupled to Sepharose beads. We then looked

Fig. 1. Association of MuSK with the AChR in C2 myotubes. (A and B) Characterization of MuSK antibodies. Extracts made from C2 myotubes or COS cells transfected with a mouse MuSK expression vector were analyzed by immunoblotting with pre-immune and immune sera as indicated. (C) Co-isolation of MuSK with AChRs. C2 extracts were incubated with α -bungarotoxin coupled to Sepharose beads (TS) to isolate the AChR. As controls, 10 μ M free toxin was added to compete for receptor binding to toxin-Sepharose (+T), or non-conjugated Sepharose beads were used (CS). AChR-associated MuSK was analyzed by SDS-PAGE and immunoblotting with MuSK-specific antibodies. A fraction (0.3%) of the initial lysate was loaded as a standard (L). For the lower panel, the blot was stripped and reprobed with mAb 124, which is reactive with the AChR β subunit. (D) Co-immunoprecipitation of the AChR with MuSK. C2 myotube extracts were incubated with the indicated amounts of MuSK antibodies, and the presence of AChRs in the immunoprecipitates was assessed by non-reducing SDS-PAGE and mAb 124 immunoblotting. Controls included a portion (0.5%) of the initial lysate (L) as well as an incubation of MuSK antibodies with blank lysis buffer (–lysate sample). (E) The AChR is not associated with the transferrin receptor. Toxin-Sepharose precipitations of the AChR were analyzed by immunoblotting using antibodies reactive with the transferrin receptor. L represents 0.3% of the initial lysate, F the efficiency of AChR recovery using toxin-Sepharose. After precipitation with toxin-beads, the indicated percentages of samples were analyzed by mAb 124 immunoblotting. For comparison, fractions of the initial lysate were loaded.

for the presence of MuSK by stripping the beads with SDS sample buffer, separating the proteins by SDS-PAGE and immunoblotting with the MuSK-specific antibody. The immunoblots show that MuSK was present on the beads (Figure 1C); its presence required the AChR, since when AChR precipitation was prevented by the addition

of excess α -bungarotoxin to the C2 extract prior to incubation with the beads, no MuSK was detected. MuSK was also not detected when unconjugated Sepharose beads were used as a control. In each case, the presence or absence of the AChR on the beads was confirmed by stripping the blots and reprobing with an antibody that recognizes the β subunit of the AChR. By comparing the content of MuSK in AChR isolates with the content in the initial lysate, we estimate that $0.29 \pm 0.02\%$ (mean $\pm$ SD, data from three experiments) of the total MuSK in the cell lysates is bound to the toxin–Sepharose beads. A similar comparison for the AChR (Figure 1F) shows that $14.9 \pm 4.7\%$ of the total AChR in C2 extracts can be recovered on the beads. Taken together, these values suggest that at least 2% of the total MuSK in C2 myotubes is associated with the AChR.

Several experiments were carried out to determine whether the co-precipitation of MuSK with the AChR represents a specific association rather than non-specific trapping of membrane proteins. First, the extent of MuSK co-precipitation using toxin–beads was constant and reproducible over several experiments. Second, the association of MuSK with the AChR isolated on toxin–beads was not significantly changed by cell lysis and washing under stringent conditions of high salt (0.5 M NaCl) and detergent (1% Triton X-100 and 0.5% deoxycholate; data not shown). Such conditions resulted in the loss of other proteins such as β -dystroglycan (C.Fuhrer, J.E.Sugiyama and Z.W.Hall, unpublished observations), a membrane component of the dystrophin–utrophin glycoprotein complex that is found at synapses (Matsumura *et al.*, 1992). Third, as a measure of non-specific binding of membrane proteins, we analyzed preparations of AChRs isolated by toxin–beads for the presence of the transferrin receptor, a prominent membrane protein found at the plasma membrane and in endosomal compartments (Ralston and Ploug, 1996). In an immunoblot analysis with specific monoclonal antibodies, no detectable transferrin receptor was seen in AChR precipitates (Figure 1E). Finally, we tested for association of MuSK with the AChR by immunoprecipitating MuSK and looking for the presence of the AChR by immunoblotting with the antiserum against the AChR β subunit. Although some non-specific binding to protein A–Sepharose occurred, the AChR was clearly detected in the MuSK immunoprecipitates (Figure 1D). Taken together, we believe that association of MuSK with the AChR is specific and that the two proteins form a stable complex which excludes other membrane proteins.

Agrin increases the association between MuSK and the AChR

We then used the co-precipitation assays to examine the effects of agrin on the association of MuSK with the AChR. C2 myotubes were incubated for increasing times with 5 nM of the soluble C-terminal fragment of either the most active, neural-specific (C-Ag_{12,4,8}) or the predominant muscle (C-Ag_{12,0,0}) isoform of agrin (Ferns *et al.*, 1993). We examined the association of MuSK and the AChR at three times after the beginning of the treatment: 40 and 90 min, when agrin-induced tyrosine phosphorylation of the AChR β subunit is near its peak, but few clusters are seen, and 16 h, when AChR phosphorylation has declined, but cluster formation is maximal (Ferns *et al.*, 1996). As

Fig. 2. Neural agrin leads to increased association of MuSK with the AChR. C2 myotubes were stimulated for the indicated times with 5 nM neural (4,8) or muscle (0,0) agrin. (A) Cell extracts were incubated with α -bungarotoxin–Sepharose beads to isolate AChRs, and receptor-associated MuSK was visualized by SDS–PAGE and MuSK immunoblotting. In a control sample, 10 μ M free toxin was added to block AChR binding to the beads (+T). To confirm that equal amounts of receptor are present in the appropriate lanes, the blot was stripped and reprobed with mAb 88B, which is specific for the γ and δ AChR subunits (lower panel). (B) Agrin-treated cells were lysed and subjected to immunoprecipitation with MuSK antiserum followed by immunoblotting with mAb 124. As controls, we omitted MuSK antibodies (–ab) or the lysate (–L). We also analyzed a fraction (0.3% in A, 0.1% in B) of the total C2 cellular extracts as a standard (L). (C) Quantitation of the effect of neural agrin on the amount of AChR-associated MuSK. Experiments as described in (A) were quantitated by densitometric scanning of films, and values of untreated cells were set to 100%. Data represent mean $\pm$ SD of four experiments. * Differs significantly from control, $P < 0.01$ (by ANOVA followed by Bonferroni's t -test).

shown by both α -bungarotoxin–Sepharose precipitation followed by MuSK immunoblotting and by MuSK immunoprecipitation followed by AChR Western blotting, agrin increases the interaction between MuSK and the AChR (Figure 2A and B). This effect is specific for neural, but not muscle, agrin and is seen after a 16 h treatment; quantitation shows that the increase is ~ 2.5 -fold and is highly significant (Figure 2C). Agrin's effects were most pronounced at a concentration of 5 nM; we observed smaller changes at lower amounts (0.2 nM; data not shown). In control experiments, the amounts of AChRs were the same in all lanes; hence agrin did not affect the level of the AChRs in C2 cells (Figure 2A, bottom; see also Jones *et al.*, 1996).

Agrin-induced activation of AChR-associated MuSK correlates with phosphorylation of the AChR β subunit

To determine whether AChR-associated MuSK is activated by agrin, we treated C2 myotubes for increasing times with 0.1 nM neural or muscle agrin. AChRs were then isolated with α -bungarotoxin–Sepharose beads and the associated proteins examined by immunoblotting with phosphotyrosine-specific antibodies (Figure 3). After 15 min of treatment with neural agrin, a doublet of immunoreactive bands was detected that migrated at ~ 110 kDa. Immunoreactivity remained elevated at 40 and

Fig. 3. Neural agrin causes a rapid and transient activation of AChR-associated MuSK. Cells were treated for increasing times with 0.1 nM neural (4,8) or muscle (0,0) agrin. AChRs were isolated with α -bungarotoxin–Sepharose precipitation and activation of associated MuSK was visualized by phosphotyrosine immunoblotting. A doublet of bands with the same molecular weight as MuSK (~110 kDa) becomes transiently tyrosine phosphorylated, with a peak at ~40 min. Controls included addition of 10 μ M free toxin (+T) and the use of non-conjugated control Sepharose (CS). The identity of the ~110 kDa doublet (MuSK) was confirmed by stripping and reprobing the blot with MuSK antibodies (lower panel).

90 min but, after 5 and 15 h, the tyrosine-phosphorylated bands were no longer detected. By stripping and reprobing the blot with MuSK-specific antibodies, we identified this doublet as AChR-associated MuSK. Phosphotyrosine-immunoreactive bands were not observed when free excess toxin was added to the C2 extracts prior to AChR isolation, when unconjugated Sepharose was used or when the cells were treated with muscle agrin.

We then used this system to compare three phosphorylation events induced by agrin: activation of the total cellular pool of MuSK, activation of AChR-associated MuSK and phosphorylation of the AChR β subunit. For these experiments, C2 myotubes were treated for increasing times with a range of neural and muscle agrin concentrations. Cell extracts were then split into two parts; one part was incubated with α -bungarotoxin–Sepharose beads to isolate AChRs, whereas the other part was subjected to immunoprecipitation with MuSK-specific antibodies. Both precipitations were analyzed by phosphotyrosine immunoblotting. We first examined the agrin concentration dependence of the phosphorylations. As shown in Figure 4A, 5 nM of neural agrin caused phosphorylation of AChR-associated MuSK, of the AChR β subunit and of total MuSK within as little as 5 min, whereas in each case muscle agrin had no effect. At lower concentrations of agrin, all three phosphorylation events were visible after 40 min, but not after 5 min, of treatment. A slight reduction at that time in the extent of phosphorylation of AChR-bound MuSK was accompanied by decreased phosphorylation of both the AChR β subunit and total MuSK. Finally, at the lowest concentration of agrin tested, none of the three phosphorylation events was detected. We then performed a time course of agrin treatment, using two concentrations of neural agrin. Activation of both AChR-associated MuSK and of total MuSK was maximal after 40 min of treatment with 5 or 0.1 nM neural agrin, and decreased to close to background levels after a 15 h incubation (Figure 4B). In contrast, phosphorylation of the AChR β subunit followed a similar initial time course, but showed a slower decline and was

Fig. 4. Dose dependence and time course of agrin-induced MuSK activation and AChR β subunit phosphorylation. (A and B) C2 myotubes were treated for increasing times with the indicated concentrations of neural or muscle agrin. After lysis, cell extracts were split into two parts; the first was incubated with α -bungarotoxin–Sepharose beads to isolate AChRs (top and middle panels), whereas the second part was subjected to immunoprecipitation with MuSK-specific antibodies (bottom panels). Both precipitates were analyzed by SDS–PAGE and phosphotyrosine immunoblotting. Protein bands were identified by their molecular weight and by stripping and reprobing blots with the appropriate antisera (not shown). Agrin-induced phosphorylation of AChR-associated MuSK, AChR β subunits and of the total cellular pool of MuSK correlate over a wide range of agrin concentrations. (C) Controls for the specificity of MuSK immunoprecipitation. Cells were stimulated for 15 min with 15 nM of neural agrin. Lysates were subjected to immunoprecipitation with pre-immune or immune anti-MuSK sera with or without the addition of an excess (2.5 mg/ml) of free immunizing MuSK peptide. Samples were analyzed by phosphotyrosine immunoblotting.

still visible after 15 h. We noted a difference in the time course of MuSK activation at 5 nM as compared with 0.1 nM agrin: the high concentration induced a rapid activation of MuSK within 5 min, whereas at the low concentration, activation occurred with slower kinetics, as it was only detected after 15 min and reached a peak after 40 min. Control experiments confirmed the specificity of immunoprecipitation with MuSK antibodies; thus no agrin-induced tyrosine phosphorylation of MuSK was seen when using the pre-immune serum or when adding an excess of free MuSK C-terminal immunizing peptide to MuSK immunoprecipitations (Figure 4C). In summary, the phosphorylation events induced by agrin are correlated: phosphorylation of AChR-associated MuSK shows a similar dependence on agrin concentration and initial time course as does phosphorylation of total MuSK and of the

Fig. 5. Effects of herbimycin and staurosporine on agrin-induced phosphorylation of MuSK and the AChR β subunit. C2 myotubes were pre-treated for 6 h with 1 μ M herbimycin (H), 10 nM staurosporine (S) or a DMSO carrier control (c). Cells were then incubated with the indicated amounts of agrin for 5 or 40 min, and cells extracts were analyzed by precipitation with α -bungarotoxin-Sepharose beads followed by phosphotyrosine immunoblotting (top and middle). In some cases, parallel dishes of C2 myotubes were treated with agrin (5 and 40 min with 1 and 0.1 nM of agrin, respectively), and analyzed by precipitation with MuSK antibodies followed by phosphotyrosine immunoblotting (bottom). Herbimycin blocks all phosphorylation events, whereas staurosporine leaves MuSK activation intact, but strongly reduces AChR β subunit phosphorylation.

AChR; dephosphorylation of both total and AChR-bound MuSK, however, occurs more rapidly than that of the AChR.

Staurosporine blocks agrin-induced AChR β subunit phosphorylation, but not activation of AChR-associated MuSK

To investigate further the relationship between MuSK activation and AChR phosphorylation, we used the tyrosine kinase inhibitors, herbimycin and staurosporine, that were shown previously to block agrin-induced AChR clustering and tyrosine phosphorylation of the AChR β subunit (Wallace, 1994; Ferns *et al.*, 1996). C2 myotubes were incubated with either 1 μ M herbimycin or 10 nM staurosporine for 6 h before adding agrin, conditions that have been shown to be optimal for inhibition of AChR clustering and phosphorylation. Cell extracts were then prepared and subjected to α -bungarotoxin-Sepharose or MuSK immunoprecipitation followed by phosphotyrosine immunoblotting. Under all conditions tested, herbimycin blocked activation of both AChR-bound and total MuSK as well as phosphorylation of AChR β subunits. In contrast, staurosporine had no effect on agrin-induced activation of MuSK, either for the total pool or for that associated with the AChR, but strongly decreased phosphorylation of the β subunit of the AChR, as previously reported (Figure 5). When cells were treated with increasing concentrations of staurosporine, ranging from 1 to 20 nM, agrin-stimulated phosphorylation of the AChR β subunit was progressively inhibited, but no effect was seen on activation of MuSK (Figure 6). Most dramatically, at 20 nM of staurosporine, agrin-induced AChR phosphorylation was completely abolished, whereas activation of neither total nor AChR-bound MuSK was significantly altered. These experiments show that staurosporine does not affect agrin-induced MuSK activation, but blocks phosphorylation of the β subunit of the AChR caused by agrin. We have thus found conditions that uncouple activation of MuSK from AChR phosphorylation, suggesting that MuSK does not directly

Fig. 6. Staurosporine does not interfere with agrin-induced MuSK activation, but blocks agrin-induced phosphorylation of the AChR β subunit. (A) Myotubes were pre-incubated for 5 h with the indicated concentrations of staurosporine or a carrier control (c) and then stimulated for 40 min with 1 nM neural agrin. Lysates were processed by α -bungarotoxin-Sepharose or MuSK antibody precipitation and analyzed by phosphotyrosine immunoblotting. Increasing the concentration of staurosporine up to 20 nM progressively reduces agrin-induced AChR β subunit phosphorylation without substantially altering MuSK phosphorylation. (B) Quantitation of staurosporine's effects by densitometric scanning. Data from four experiments as described in (A) were evaluated and are shown as mean $\pm$ SD. Phosphorylation signals from cells not treated with agrin were set to 0%. Agrin-induced control signals of cells incubated with carrier, lacking staurosporine, were set to 100%. All phosphorylation signals were normalized for the AChR contents of cells as visualized by reprobating blots containing toxin precipitates with mAb 88B.

phosphorylate the AChR β subunit. As we have reported previously that src can phosphorylate the AChR, and that AChRs isolated from C2 myotubes are associated with src-related kinases, we examined the effects of agrin on association of fyn and src with the AChR. As shown in Figure 7, treatment of cells with 2.5 nM of neural agrin did not change the relative amounts of either kinase bound to the AChR. In addition, neural agrin (0.1 or 2.5 nM) did not affect the overall level of tyrosine phosphorylation of src and fyn, as measured by kinase immunoprecipitation using specific antibodies, followed by phosphotyrosine immunoblotting.

Discussion

The principal result of this work is the demonstration that MuSK, the receptor tyrosine kinase that is thought to mediate the effects of agrin in clustering AChRs in muscle

Fig. 7. Agrin does not change the amount of fyn and src associated with the AChR, nor does it affect the overall phosphotyrosine level of total src and fyn. **(A)** C2 myotubes were incubated for up to 15 h with 2.5 nM of neural agrin. Cell lysates were analyzed by precipitation with α -bungarotoxin–Sepharose beads and immunoblotting with antibodies specific for fyn or src; 0.1% of the total lysate was included as a control (L). **(B)** C2 cells treated with neural or muscle agrin as indicated were subjected to immunoprecipitation with fyn- or src-specific antibodies followed by phosphotyrosine immunoblotting. Over the course of several experiments, the signals shown in (A) and (B) displayed some variation; however, no significant agrin effect was seen. The figure shows one representative experiment for each treatment (A and B).

cells, is closely associated with the AChR in C2 myotubes, even in the absence of neural agrin. Treatment of the myotubes with agrin results in tyrosine phosphorylation of the AChR-associated MuSK in a manner that is similar to agrin-induced tyrosine phosphorylation of the AChR β subunit. MuSK is unlikely to be directly responsible for phosphorylation of the AChR, however, as staurosporine, which blocks agrin-induced phosphorylation of the β subunit, does not affect MuSK activation. These results suggest that phosphorylation of the AChR is mediated by a kinase that is downstream from MuSK, and is possibly src or fyn (Fuhrer and Hall, 1996).

Association of MuSK with the AChR

A portion of the total pool of MuSK in C2 myotubes is associated with the AChR. As judged by several criteria, this association appears to be highly specific. For example, apart from other controls, the transferrin receptor, a prominent component of the surface membrane and of endocytic compartments (Ralston and Ploug, 1996), is not co-precipitated with the AChR. The continued association of MuSK, but not β -dystroglycan, with the AChR under conditions of higher stringency (C.Fuhrer, J.E.Sugiyama and Z.W.Hall, unpublished observations) further suggests that the interaction is specific and excludes other proteins, even transmembrane proteins that are concentrated in the postsynaptic membrane (Matsumura *et al.*, 1992). Our calculation that ~2% of the total MuSK can be co-isolated with the total AChR is likely to be a minimal estimate of this association, as all MuSK–AChR complexes may not survive extraction; the percentage of MuSK associated with the AChR in intact C2 myotubes may thus be substantially higher than 2%.

Our experiments do not demonstrate a direct interaction between MuSK and the AChR; thus the two proteins could be associated through one or more additional components. A protein that is likely to be part of the AChR–MuSK complex is rapsyn, the 43 kDa protein that is

Fig. 8. Tentative model of proteins interacting with the AChR in mammalian muscle. Arrows indicate putative steps in agrin's signaling pathway. For details, see text.

closely associated with the AChR in an ~1:1 stoichiometry in *Torpedo* synaptic membranes and muscle cells (LaRoche and Froehner, 1986, 1987). Rapsyn is essential both for AChR aggregation *in vitro* and for proper synapse formation and AChR clustering *in vivo* (Froehner *et al.*, 1990; Phillips *et al.*, 1991; Gautam *et al.*, 1995). It can be cross-linked to the AChR in *Torpedo* membranes (Burden *et al.*, 1983), and apparently can interact with each of the AChR subunits (Maimone and Merlie, 1993; Yu and Hall, 1994). In preliminary experiments, we have found that rapsyn and the AChR can be co-precipitated together from extracts of muscle cells (C.Fuhrer and Z.W.Hall, unpublished observations). When co-expressed in heterologous quail QT-6 fibroblasts, rapsyn regulates tyrosine phosphorylation of proteins that are localized at clusters of AChRs (Qu *et al.*, 1996). Furthermore, in this expression system, rapsyn clusters and activates MuSK, which leads to phosphorylation on tyrosine residues of many cellular proteins, including AChRs (Gillespie *et al.*, 1996). Although further experiments will be required to establish that all three components are present in the same complex, and to determine the nature of their specific interactions, MuSK could, in principle, interact with either the AChR, or rapsyn or both (Figure 8). Interestingly, a recent report by Apel *et al.* (1997) shows that rapsyn-induced clustering of MuSK in transfected fibroblasts that also express AChRs is mediated by the ectodomain of MuSK, suggesting the existence of a transmembrane intermediate between the two proteins. In combination with these observations, our data suggest that the AChR itself could be such a link between MuSK and rapsyn. Alternatively, other proteins may be part of the complex containing MuSK and the AChR, and MuSK could interact with them. Both src and fyn, for example, are associated

with the AChR in C2 myotubes (Fuhrer and Hall, 1996). Our experiments also imply that different regulatory mechanisms underly clustering of AChRs in transfected fibroblasts as opposed to myotubes (see also Qu *et al.*, 1996). In fibroblasts, both activation of MuSK and AChR clustering are induced by co-expression of rapsyn (Gillespie *et al.*, 1996); in muscle cells, despite the presence of all three proteins, neither of these processes occurs efficiently, unless neural agrin is added. Myotubes thus appear to contain additional regulatory pathways that link both MuSK activation and AChR aggregation to agrin.

Agrin-induced activation of AChR-associated MuSK

AChR-associated MuSK becomes phosphorylated in response to agrin, and this activation occurs in the same manner as activation of the total pool of MuSK. These results confirm and extend the previous observation (Glass *et al.*, 1996) that high concentrations (nanomolar range) of recombinant soluble agrin cause a rapid activation of MuSK. In addition, we have found that concentrations of agrin as low as 20 pM also activate MuSK, but with substantially slower kinetics. These findings contrast with those seen for other receptor tyrosine kinases. The trkA receptor, for example, shows maximal activation after 5 min, with a marked decline after only 15 min of treatment with nerve growth factor (NGF) (Kaplan *et al.*, 1991). The slow activation of MuSK could reflect complex interactions of agrin with a multi-component receptor. Alternatively, the delay may originate from the initial binding of agrin to heparin-containing components of the extracellular matrix or to α -dystroglycan, a prominent agrin-binding protein of muscle cells (Bowe *et al.*, 1994; Campanelli *et al.*, 1994; Gee *et al.*, 1994; Sugiyama *et al.*, 1994). Once bound to such proteins, agrin may then be presented to its functional receptor, including MuSK, and start its signaling cascade. The activation of MuSK is transient, even in the continued presence of agrin; MuSK phosphorylation thus precedes AChR cluster formation by several hours (Wallace, 1992; Ferns *et al.*, 1996), even at the lowest concentrations of agrin. This time course is consistent with the postulated role of MuSK early in the signaling pathway of agrin.

In addition to activating MuSK, agrin also increases the association of MuSK with the AChR, but with a time course that is significantly slower than MuSK activation and that is similar to the time course of AChR clustering. Our calculations show that this observed increase is ~2.5-fold; as not all of the newly formed AChR–MuSK complexes may withstand our extraction procedure, however, this increase may only be an indication of a more prominent agrin-induced association between the two proteins occurring in intact myotubes. Since MuSK phosphorylation is transient, the protein that becomes associated with the receptor after agrin treatment is presumably not tyrosine phosphorylated, and therefore not activated. The significance of this increased association is unknown. The effect is specific for neural agrin and presumably reflects an accumulation of MuSK in AChR clusters, similar to the localization of MuSK at the neuromuscular junction *in vivo* (Valenzuela *et al.*, 1995). In untreated C2 myotubes, a small number of spontaneous AChR clusters are usually observed; it is therefore possible

that MuSK, both in the absence of neural agrin and after agrin treatment, is associated preferentially with aggregated AChRs. In any case, as agrin causes additional muscle proteins such as rapsyn or acetylcholinesterase to accumulate in AChR aggregates (Wallace, 1989), MuSK may interact with other proteins and provide a structural link between the AChR and other components of the postsynaptic apparatus. In agreement with this notion, Apel *et al.* (1997) have suggested that rapsyn may interact with MuSK in multiple ways and that this complex could play both structural and signaling roles in agrin-induced specialization of the neuromuscular junction. By linking together postsynaptic proteins, AChR-bound MuSK may thus play a role in maintaining AChR clusters similar to that of focal adhesion kinase (FAK) in integrin signaling at focal adhesion sites (Clark and Brugge, 1995).

MuSK activation and phosphorylation of the β subunit of the AChR

Agrin causes tyrosine phosphorylation of the β subunit of the AChR (Wallace *et al.*, 1991; Qu and Haganir, 1994; Meier *et al.*, 1995; Ferns *et al.*, 1996); although the conserved tyrosine phosphorylation site of this subunit is not necessary for rapsyn-induced AChR clustering in heterologous cells (Yu and Hall, 1994; Qu *et al.*, 1996), the available evidence suggests that tyrosine phosphorylation of the AChR β subunit could be important for receptor aggregation in muscle cells. Our experiments reveal that the agrin-induced phosphorylation of MuSK, both total and AChR-associated, and of the AChR β subunit are closely correlated and occur with the same initial time course. The duration of the phosphorylation is different in the two cases, however, as phosphorylation of the β subunit lasts longer than MuSK activation. The difference in stability of the phosphorylated state may reflect the action of different phosphatases in the two cases. Phosphatase-mediated dephosphorylation of the AChR has been demonstrated in *Torpedo* electric organ (Mei and Haganir, 1991) and has been inferred from the action of phosphatase inhibitors in chick muscle (Wallace, 1995).

The similarity in time course of agrin-induced phosphorylation of the two proteins suggests that activated MuSK could be directly responsible for phosphorylation of the AChR, as has been proposed previously (Gillespie *et al.*, 1996). However, our experiments on the effect of staurosporine on phosphorylation of the two proteins suggests that this is not the case. Concentrations of staurosporine that completely blocked agrin-induced phosphorylation of the AChR β subunit failed to block the activation of MuSK, as measured by tyrosine phosphorylation. Although we did not measure MuSK activation directly, experiments in other systems suggest that activation of receptor tyrosine kinases requires tyrosine phosphorylation. Indeed, according to the currently accepted general model, receptor tyrosine kinases (RTKs) are activated by a mechanism in which the ligand induces receptor dimerization and a *trans*-phosphorylation of one dimeric partner by the other (Schlessinger and Ullrich, 1992; Heldin, 1995). With respect to the inhibitor, kinase inhibition by staurosporine is not completely understood, but appears to occur by a mechanism other than competition with the phosphoacceptor substrate (Ruegg and

Burgess, 1989; Ward and O'Brian, 1992; Meggio *et al.*, 1995). In addition, staurosporine has been shown to inhibit both ligand-induced RTK autophosphorylation and phosphorylation of direct substrates in several signaling systems. Examples include the insulin receptor and its substrate IRS-1 (Nishimura and Simpson, 1994), and the trkA receptor for NGF and its substrate phospholipase C- γ 1 (Vetter *et al.*, 1991; Ohmichi *et al.*, 1992). Thus, our observation that staurosporine selectively inhibits agrin-induced tyrosine phosphorylation of the AChR β subunit, but leaves autophosphorylation of MuSK intact, strongly implies that the β subunit is not a direct substrate for activated MuSK.

As MuSK is required for synapse formation *in vivo* and for agrin-induced AChR clustering in cultured myotubes (DeChiara *et al.*, 1996; Glass *et al.*, 1996), the differential inhibitory effects of herbimycin and staurosporine suggest that it acts early in the pathway, upstream of the events leading to AChR phosphorylation. MuSK activation, by itself, appears not to be sufficient to produce AChR clusters, as staurosporine leaves agrin-induced MuSK activation intact, but blocks receptor clustering (Ferns *et al.*, 1996). Further intracellular signaling steps, downstream of MuSK, thus seem to be necessary to cause AChR aggregation, and such steps might involve phosphorylation of the AChR itself and/or phosphorylation of additional muscle proteins. Apart from inhibiting serine and threonine kinases as well as some RTKs, staurosporine, similarly to herbimycin, is a potent inhibitor of cytosolic tyrosine kinases including members of the src family (Uehara *et al.*, 1989; Meggio *et al.*, 1995). Thus, although not directly responsible for agrin-induced AChR phosphorylation, MuSK could activate one or more intermediate kinases in the agrin signaling pathway. Using co-precipitation and GST fusion proteins, we have found previously that src is a major kinase responsible for phosphorylation of the AChR β subunit and that AChRs are associated with both src and fyn in C2 myotubes (Fuhrer and Hall, 1996). Our experiments reported here reveal neither a change in the association of src and fyn with the AChR nor a change in the overall tyrosine phosphorylation state of the kinases due to agrin. However, as only a minor fraction of the total cellular content of src and fyn is found in association with AChRs, agrin may very well cause an activation of those kinases that are bound to the AChR. As src-related kinases are subject to activating as well as inactivating tyrosine phosphorylations (Cooper and Howell, 1993), such an activation may not be detected by conventional phosphotyrosine immunoblotting. In addition, as is the case in cellular adhesion of fibroblasts, src-related kinases might also play roles in agrin's signaling pathway that are independent of their kinase activity but require their SH2 and SH3 domains (Kaplan *et al.*, 1995).

Thus, the findings reported in this study, coupled with those reported earlier, suggest a model in which agrin binds to a receptor complex containing MuSK and other components, including the AChR, src and fyn (Figure 8). Binding of agrin to the complex activates MuSK, which then activates src and/or fyn, resulting in phosphorylation of the β subunit of AChRs and possibly other proteins. Additionally, as we have no evidence for whether all proteins are part of a single complex, src or fyn might be

complexed to AChRs not associated with MuSK, and become activated by other complexes containing AChR-bound MuSK. In any case, activation of MuSK that is associated with the AChR is likely to be crucial for AChR cluster formation. The protein interactions regulated by subsequent tyrosine phosphorylation might then be the initial steps in assembly of a specific postsynaptic complex that concentrates AChRs under the nerve terminal and stabilizes them by association with the cytoskeleton.

Materials and methods

Cell culture

Cell culture reagents were purchased from Gibco BRL. All cell lines were grown at 37°C in 8% CO₂. COS cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/l D-glucose, 10% fetal bovine serum, 2 mM glutamine and penicillin/streptomycin. C2C12 mouse muscle cells were propagated as myoblasts on 10 or 15 cm plastic dishes (Nunc) in DMEM supplemented with 20% fetal bovine serum, 0.5% chick embryo extract, 2 mM glutamine and penicillin/streptomycin. After reaching 90% confluence, cells were shifted to fusion medium containing DMEM, 5% horse serum and 2 mM glutamine. Fusion of myoblasts to generate myotubes was evident after 1 day. By day 2, contracting myotubes were usually observed, and cells were used for experiments.

Expression of agrin constructs

To obtain soluble forms of agrin, the COOH-terminal half of agrin isoforms was expressed in transfected COS cells. Expression constructs encoding the most active, neural-specific isoform (C-Ag_{12,4,8}) or the predominant muscle isoform (C-Ag_{12,0,0}; Ferns *et al.*, 1993) were transiently expressed in COS cells by using an adenovirus-mediated DEAE-dextran method of transfection (Forsayeth and Garcia, 1994). After transfection, the medium was collected and replaced every day, for 3 days. The concentration of agrin in the medium was determined by immunoblotting using an agrin-specific antiserum and purified agrin of known concentration as a standard (Sugiyama *et al.*, 1994; Bowen *et al.*, 1996).

Preparation of mouse MuSK expression vector

First strand cDNA was synthesized from C2 mouse muscle total RNA using Superscript reverse transcriptase with oligo(dT) as the primer, as directed by the manufacturer (Gibco BRL). We obtained the coding region of mouse MuSK (−1 to +2607) by PCR amplification of C2 first strand cDNA using 5'-GGA ATT CAC CAT GAG AGA GCT CGT CAA CAT TCC-3' as the forward primer and 5'-GCT CTA GAT TAG ACA CCC ACC GTT CCC TCT G-3' as the reverse primer. The resulting DNA fragment was digested with *Eco*RI and *Xba*I, and cloned into the mammalian expression vector, pMT23 (a gift of the Genetics Institute, Cambridge, MA), for expression in COS cells.

Antibodies

The rabbit antiserum recognizing MuSK was made against a peptide corresponding to the C-terminal 20 amino acids of rat MuSK and was produced commercially by Research Genetics. We performed several specificity tests with this antiserum, such as immunoblotting with pre-immune serum (Figure 1A) and immunoprecipitations with an excess of free MuSK immunizing peptide (Figure 4C). To ascertain that the antibody recognizes mouse MuSK, the expression vector described above was transfected into COS cells, which were then analyzed by immunoblotting (Figure 1B). In all cases, MuSK was detected as a band, or sometimes a doublet, of ~110 kDa. Although useful for Western blotting and immunoprecipitation, the MuSK antibody did not stain MuSK in immunofluorescence experiments, neither at the neuromuscular junction of tissue sections prepared from adult rats, nor in agrin-treated C2 myotubes. The rat monoclonal antibody against the AChR β subunit, mAb 124, was a kind gift from Dr J.Lindstrom (University of Pennsylvania, Philadelphia, PA), and the mouse monoclonal antiserum, mAb 88B, reactive with the AChR γ and δ subunits, was generously provided by Dr S.C.Froehner (University of North Carolina, Chapel Hill, NC). A rat monoclonal antiserum recognizing the mouse transferrin receptor, R17 217 (Lesley *et al.*, 1989), was a generous gift from Dr J.Lesley (The Salk Institute, San Diego, CA). For phosphotyrosine immunoblotting, we used a mixture of two commercially available

mouse monoclonal antibodies, 4G10 (Upstate Biotech.) and PY20 (Transduction Labs). Antibodies recognizing src and fyn were as described previously (Fuhrer and Hall, 1996).

Co-immunoprecipitation assays

To assay association of MuSK with the AChR, untreated or agrin-treated C2 myotubes were rinsed on ice with phosphate-buffered saline (PBS) supplemented with 1 mM Na orthovanadate and 50 mM NaF and extracted at 4°C in lysis buffer [1% digitonin or NP-40, 50 mM NaCl, 30 mM triethanolamine, pH 7.5, 5 mM EGTA, 5 mM EDTA, 50 mM NaF, 2 mM Na orthovanadate, 10 mM *p*-nitrophenylphosphate, 50 μ M phenylarsine oxide, 1 mM benzamidine, 1 mM *N*-ethylmaleimide, 1 mM Na tetrathionate, 1 mM phenylmethylsulfonyl fluoride (PMSF), 25 μ g/ml aprotinin and 25 μ g/ml leupeptin]. Insoluble material, such as nuclei and cytoskeletal elements, was removed by centrifugation at 18 000 g for 5 min.

To precipitate the AChR, the cleared lysates were incubated with α -bungarotoxin coupled to Sepharose beads (Gu and Hall, 1988). As a control for specificity, we added an excess (10 μ M) of free uncoupled α -bungarotoxin to some lysates. Other control samples were precipitated with uncoupled Sepharose beads. Bound proteins were eluted by boiling the beads in SDS-PAGE sample buffer. The proteins were then separated by reducing SDS-PAGE and transferred to nitrocellulose membranes. To detect AChR-associated MuSK, the nitrocellulose was probed with the MuSK antibody, and immunoreactive bands were visualized using horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence (ECL; Amersham Corp.). In these experiments, the association of MuSK with the AChR was robust and was still observed under increasingly stringent conditions, including up to 0.5 M NaCl, 1% Triton X-100 and 0.5% deoxycholate in both lysis and wash buffers (data not shown). Some blots were stripped by incubating them for 20 min in 200 mM glycine, 0.1% Tween-20, pH 2.5; the blots were then reprobed with mAb 124 or mAb 88B to confirm that equal amounts of AChRs were present in all lanes. Quantitation of ECL immunoblotting data was performed by scanning the films with a computerized densitometer (LaCie Silverscanner IV) and using the NIH Image 1.54 software (National Institutes of Health).

To immunoprecipitate MuSK, cleared C2 myotube extracts were incubated with 0.5–2.5 μ l of MuSK antiserum, followed by protein A-Sepharose. Although we assume that MuSK antibodies do not precipitate all the MuSK expressed in C2 myotubes, this precipitation still reflects the total cellular pool of MuSK as opposed to a precipitation with toxin-beads that selects for AChR-bound MuSK. Furthermore, the efficiency of MuSK immunoprecipitation was constant and reproducible over the course of several experiments (see also Figure 6A, bottom panel). In control incubations, we left out either the MuSK antibody or the cellular lysate. To examine MuSK-bound AChRs, beads were processed as described above and proteins separated using non-reducing SDS-PAGE. MuSK-associated AChRs were then visualized by immunoblotting with mAb 124.

Tyrosine phosphorylation and tyrosine kinase inhibitors

To examine agrin-induced tyrosine phosphorylation of MuSK and the AChR, myotubes were treated with agrin and extracted in lysis buffer as described above. Cleared lysates were incubated either with α -bungarotoxin-Sepharose beads to isolate the AChR and receptor-associated MuSK or with MuSK antibodies to precipitate MuSK. Bead-precipitated proteins were eluted into SDS sample buffer, subjected to SDS-PAGE and analyzed by phosphotyrosine immunoblotting. In the experiments with kinase inhibitors, myotubes were pre-incubated for 5–6 h with 1–20 nM staurosporine (Sigma), 1 μ M herbimycin A (Gibco BRL) or carrier controls. Cells were then incubated with agrin, extracted and analyzed by AChR or MuSK precipitation followed by phosphotyrosine immunoblotting. Myotubes treated with inhibitors appeared healthy, as judged by their morphology and occasional spontaneous contractions. Quantitation of the effects of kinase inhibitors was done as follows. The relative phosphotyrosine contents of total MuSK, AChR-associated MuSK and the AChR β subunit were first quantitated by densitometric scanning of immunoblot exposures prepared from samples precipitated with MuSK antibodies or toxin-Sepharose. To correct for differences in the AChR content of cellular lysates, the values were normalized for the relative amounts of AChRs in toxin-Sepharose precipitates, as revealed by quantitation of stripped blots reprobed with mAb 88B. Normalized signals of agrin-treated cells not incubated with staurosporine were set to 100%, and signals of untreated cells were set to 0%. Signals of agrin-treated cells incubated with staurosporine were then calculated accordingly. Data were obtained from four independent experiments.

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