Src-Class Kinases Act within the Agrin/MuSK Pathway to Regulate Acetylcholine Receptor Phosphorylation, Cytoskeletal Anchoring, and Clustering

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Synaptogenesis at the neuromuscular junction requires agrin-induced stable localization of acetylcholine receptors (AChRs) at the endplate. The effects of agrin are transduced by the muscle-specific receptor tyrosine kinase (MuSK). This study provides evidence that Src-class protein tyrosine kinases mediate the effects of agrin-activated MuSK to regulate clustering and anchoring of AChRs in skeletal muscle. MuSK was complexed with both Src and Fyn in the C2 mouse muscle cell line. These associations were enhanced by agrin and by increasing protein tyrosine phosphorylation with pervanadate. Coupling between MuSK and the Src-class kinases in vivo appeared to be caused by a phosphotyrosine–SH2 domain interaction because binding of MuSK to the SH2 domains of Fyn and Src in vitro was specific, enhanced by phosphorylation, and dependent on MuSK autophosphorylation. In addition, Src and Fyn phosphorylated MuSK. AChR phosphorylation, stimulated by agrin or pervanadate, was inhibited by blocking Src-class kinases with PP1. Furthermore, agrin-induced clustering and cytoskeletal anchoring of AChRs was dependent on Src-family kinases. These data support the conclusion that Fyn and Src act downstream of MuSK to regulate the stable localization of AChRs at the neuromuscular endplate during agrin-induced synaptogenesis.

Key words: synaptogenesis; neuromuscular junction; Src; Fyn; skeletal muscle; cytoskeleton; phosphorylation; MuSK; agrin; PP1; Src homology 2 domain; pervanadate
cascade by which Src-family kinases may mediate synapticogenesis at the NMJ. The relationship between MuSK and Src-class kinases was explored. In addition, we examined whether the effects of agrin to stimulate AChR phosphorylation, cytoskeletal anchoring, and clustering are dependent on Src-class kinases.

**MATERIALS AND METHODS**

*Constricts.* The full-length coding sequence of Torpedo Fyn in pBK-CMVlac (pBK-CMV) was described previously (Mohamed and Swope, 1999). Src and the dominant negative Src in pCDNA3 (Biscardi et al., 1996) were gifts from Dr. R. Huganir (Johns Hopkins University). The rat monoclonal antibody (mAb) 148 against the AChR subunit, JH1358. AChR blots were stripped and reprobed with the supernatants were immunoprecipitated with antibodies specific for MuSK (GW002) or AChR (88b). Phosphorylation of MuSK was analyzed by Western blotting with a mixture of the anti-phosphotyrosine antibodies, 4G10 and PY99. Phosphorylation of AChR was detected with a mixture of the phosphorylation state-specific anti-AChR β subunit, JH1360, and anti-AChR δ subunit, JH1358. AChR blots were stripped and reprobed with AChR β subunit mAb 148.

**Binding of MuSK to SH2 fusion proteins.** SH2 domain fusion proteins were prepared as described previously (Swope and Huganir, 1994). In brief, the cDNAs for SH2 domains from Src-class kinases and Grb2 were inserted into a bacterial expression vector containing the sequence for glutathione S-transferase (GST). The SH2–GST fusion protein was grown in BL21 bacteria and purified on glutathione agarose (Sigma). The fusion protein still bound to the agarose was subsequently used as an affinity reagent. Transfected QT-6 cells expressing MuSK or MuSK[K686R] were solubilized in 2% SDS, 50 mM Tris, pH 7.4, 150 mM NaCl, 2 mM EDTA, 4 mM EGTA, 2 mM sodium orthovanadate (SDS-lys buffer) for 10 min at 22°C. The lysates were diluted to a final concentration of 0.4% SDS and 2% Triton X-100 at 1 mg/ml protein. Lysates were sonicated for 30 sec on ice and spun at 225,000 × g for 10 min at 4°C. Solubilized proteins after centrifugation were immunoprecipitated with the polyclonal anti-myc antibody (JH235). Immunoprecipitates were treated by Western blotting with a mixture of anti-phosphotyrosine monoclonal antibodies 4G10 and PY99. Phosphorylation of MuSK and AChR in C2 myotubes. C2 myotubes were preincubated for 1 hr in DMEM supplemented with 0.1% horse serum followed by pretreatment with or without 10 or 20 μM PPI for 1 hr. Myotubes were then incubated with or without 100 μM agrin or 30 μM pervanadate. Treated myotubes were rinsed twice with ice-cold PBS and fixed in 4% paraformaldehyde in ice for 10 min. Tritis, pH 7.4, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 2 mM EDTA, 4 mM EGTA, and 1 mM sodium vanadate (RIPA buffer) supplemented with the protease inhibitors, 0.1 mM phenylmethylsulfonylfluoride, 10 μg/ml pepstatin, 10 μg/ml chymostatin, 10 μg/ml antipain, and 10 μg/ml Trypsol. Lysates were centrifuged at 225,000 × g for 10 min, and the supernatants were immunoprecipitated with antibodies specific for MuSK (GW002) or AChR (88b). Phosphorylation of MuSK was analyzed by Western blotting with a mixture of the anti-phosphotyrosine antibodies, 4G10 and PY99. Phosphorylation of AChR was detected with a mixture of the phosphorylation state-specific anti-AChR β subunit, JH1360, and anti-AChR δ subunit, JH1358. AChR blots were stripped and reprobed with AChR β subunit mAb 148.

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Grade, Sigma) and pretreated with or without 10–20 μM PPI followed by treatment with or without 100 μM agrin. Treated myotubes were rinsed twice with ice-cold PBS and extracted on ice with 10 mM HEPES, pH 7.4, 50 mM NaCl, 300 mM sucrose, 1 mM MgCl2, and 1 mM Na-orthovanadate (Prives buffer) (Prives et al., 1982) containing 0.5% Triton X-100 and the protease inhibitors 0.1 mM phenylmethylsulfonylfluoride, 10 μg/ml pepstatin, 10 μg/ml chymostatin, 10 μg/ml antipain, and 10 μg/ml Trasylol. Treatment of cultured myotubes with Prives buffer rapidly extracts the plasma membrane and other membranous organelles but leaves the cytoskeleton intact (Prives et al., 1982). After the desired time, the buffer was removed, and the cells were rinsed once with Prives buffer with 0.1% Triton X-100. Residual cytoskeletal proteins were solubilized and scraped from the dish with SDS-PAGE sample buffer, sonicated for 30 sec, and spun at 225,000 × g for 10 min at 4°C. Solubilized cytoskeletal proteins were analyzed by SDS-PAGE and Western blotting using rat mAb 148, specific for the AChR subunit. For determining the effects of PPI on the amount of AChRs, sister C2 cultures were treated and processed as above, except the Prives buffer contained no Triton X-100.

In vitro kinase assays. C2 myotubes were washed two times in ice-cold PBS and then solubilized in RIPA buffer plus the protease inhibitors 0.1 mM phenylmethylsulfonylfluoride, 10 μg/ml leupeptin and 10 μg/ml Trasylol. Lysates were spun at 225,000 × g for 10 min at 4°C. Supernatants from each well were loaded onto 10% SDS-PAGE gels and divided into two portions. One-third of each supernatant was used for immunoprecipitation of Fyn, using mAb Fyn 15, Src using mAb Ab-1, or MuSK using GW002. The immunocomplexes were immobilized using protein G- or protein A-Sepharose and washed three times in RIPA buffer, then twice in kinase reaction buffer without ATP. The immunoprecipitates were used in an in vitro phosphorylation reaction with 20 μM ATP and 7.5 μg ac-dc acid-decarboxylase as described previously (Feder et al., 1990). The reactions were analyzed by Western blotting with a mixture of the anti-phosphotyrosine antibodies, 4G10 and PY99.

Treatments with PPI, agrin, or pervanadate. For biochemical analysis, C2 myotubes were preincubated in DMEM supplemented with 0.1% horse serum for 1 hr, then PPI, agrin, or pervanadate were added directly to the serum-free medium. For immunoprecipitation experiments, 2C myotubes were preincubated for 1 hr in DMEM-F12 medium supplemented with 0.1% RIA grade BSA before agrin or PPI treatments. QT6 cells were treated with pervanadate in growth medium containing Medium 199 (Life Technologies) supplemented with 1% DMSO, 5% FBS, 10% trypose phosphate broth, and 1% penicillin-streptomycin. For microscopic analysis of clustering, myotubes in DM were treated overnight with agrin. The concentrations of agrin, pervanadate, and PPI were 100 pm, 30 μM, and 10–20 μM, respectively.

Immunocytochemistry. Myotubes in DM were incubated at 37°C/8% CO2 for 1 hr with 80 mM tetramethylrhodamine-α-bungarotoxin or Texas Red-α-bungarotoxin (Molecular Probes, Eugene, OR). Cells at room temperature were washed with PBS, fixed for 15 min with 3% paraformaldehyde/4% sucrose, and then permeabilized with 0.1% Triton X-100 for 10 min. Myotubes expressing the AChR β subunit were identified by double labeling with the pan-Src kinase antibody, sv2, followed by FITC-goat-anti-rabbit secondary antibody (Molecular Probes). Cells were visualized by epifluorescence on a Nikon Eclipse TE300 inverted microscope using a 20× objective. Images were captured with a Magnafire digital camera and software. Cluster sizes were analyzed with Adobe Photoshop.

Immunoblotting. Proteins were resolved by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon, Millipore, Bedford, MA) by electrobloctting. The PVDF membranes were blocked in 5% nonfat milk in Tris-buffered saline (50 mM Tris, pH 7.4, 200 mM NaCl) containing 0.05% Tween 20. For phosphotyrosine immunoblotting, the PVDF membranes were blocked in 3–5% BSA. The dilutions for primary antibodies were as follows: anti-MuSK antibody (α-MuSK-ZH), anti-phosphotyrosine 4G10, anti-phosphotyrosine 1PY99, and anti-AChR β subunit mAb 148, each at 1:1000; anti-Fyn (Fyn3) and Src2 (Pan-Src), each at 1:500; anti-myc mouse monoclonal (9E10), anti-active state Src kinase-specific rabbit antibody (GW001), and anti-Src mouse monoclonal (GD11), each at 1:200. Horseradish peroxidase-conjugated secondary antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA) and were used at dilutions of 1:50,000. Horseradish peroxidase was detected using the enhanced chemiluminescence (ECL) Pierce, Rockford, IL) followed by exposure of BioMax MR-1 film (Eastman Kodak, Rochester, NY) or, for greater sensitivity, HyperFilm ECL (Amersham, Arlington Heights, IL). For some experiments, blots were stripped by incubation at 55°C for 30 min in 62.5 mM Tris, pH 6.8, 2% SDS, and 100 mM β-mercaptoethanol and reanalyzed by Western blotting.

Agrin production. Medium from COS-7 cells expressing a C-agrin 4.8 or transfected with empty plasmid pBK-CMV was collected and replaced each day for 2 d. The concentration of agrin in the collected medium was determined by Western blotting (Sugiyama et al., 1994) using agrin-specific polyclonal antiseraum (JH1037) and standard purified agrin of known concentration provided by Dr. J. E. Sugiyama (NIHDS, NIH). Fusion protein production. SH2 domain fusion proteins derived from Fyn, Src, and Grb2 as well as backbone GST protein were prepared as described previously (Swope and Huganir, 1993). Pervanadate preparation. Sodium pervanadate was prepared as described previously (Meier et al., 1995).

Quantification of Western analysis. Analysis of Western blots was performed by scanning exposed films in transmissometer mode and quantifying the digital data with a GS-710 Imaging Densitometer and Multianalyzet software (Bio-Rad, Hercules, CA). The integrated optical density (O.D.) for the area of each band, stated as volume or O.D. × area and here abbreviated as O.D., was determined using Multianalyzet software. To determine the linear range of Biomax film and HyperFilm ECL, serial dilutions of several proteins, including MuSK, Src, Fyn, and AChR, were prepared in PVDF, probed with an appropriate antibody, reacted with ECL reagent, and exposed to film. The O.D. versus dilution factor was plotted; O.D. values between 0.2 and 10 were linear for both Biomax film and HyperFilm ECL. Thus, quantification was performed using bands that were not saturated and had O.D. values within this range. All data for coimmunoprecipitation and phosphorylation experiments were normalized to random variability in the expression and immunoprecipitation of each specific protein. Each experiment was performed three times, unless indicated otherwise. Data are expressed as the mean ± SEM for n = 3. Except for Figures 8C and 9E, all figures represent the data of a single representative experiment.

Protein determinations. Protein concentrations were determined by the method of Lowry using BSA as a standard (Lowry et al., 1951).

RESULTS

Association of Src-class kinases with MuSK

To test for a relationship between Src-class kinases and MuSK, complex formation between these two types of kinases was examined initially. C2 myotube proteins were solubilized under mild conditions of 1% Triton lysis buffer to maintain protein–protein interactions and immunoprecipitated with MuSK-specific antibodies. When the precipitates were analyzed for Src by Western blotting, there was specific coimmunoprecipitation of Src with MuSK compared with empty beads (Fig. 1A, lane 2 vs 4). In a similar manner, Fyn was specifically coimmunoprecipitated with MuSK as demonstrated with a Fyn-specific antibody in Western analysis of the anti-MuSK immunoprecipitates and control beads (Fig. 1B, lane 2 vs 4). Activation of either receptor tyrosine kinases, such as the PDGF receptor, results in binding of Src-class kinases (Claesson-Welsh, 1994). In fact, the trkB receptor kinase, which is closely related to MuSK, binds Fyn (Iwasaki et al., 1998). Therefore, whether activation of MuSK by agrin enhanced association of MuSK with Src and Fyn was tested. Treatment of C2 cells for 1 hr with agrin increased complex formation between MuSK and both Fyn and Src (Fig. 1A,B, lane 1 vs 2). The percentage of each kinase complexed with MuSK was determined
by comparing the Src and Fyn signal in the MuSK immunoprecipitate with an aliquot of lysed cells. Densitometry scanning quantification, with normalization for recovery of MuSK, was performed to determine the percentage of Src and Fyn complexed with MuSK. In the absence of agrin, the percentage of total Src and Fyn precipitated with MuSK was 1.7 ± 0.2 and 2.10 ± 0.08%, respectively. After agrin treatment, the percentage of Src coimmunoprecipitated was increased to 3.26 ± 0.6% over nontreated cells (p < 0.05), whereas the percentage of Fyn was increased to 3.8 ± 0.3% (p < 0.025). For both kinases, an enhancement of association with MuSK in response to agrin occurred in every experiment. The average percentage increase in association of Fyn and Src with MuSK caused by agrin was 80 ± 20 and 110 ± 50%, respectively. These data indicated that Src and Fyn were in a complex with MuSK and that this association was stimulated by agrin.

The existence of this complex was further tested by the reciprocal experiment. Fyn and Src immunoprecipitates were isolated from C2 myotubes and analyzed for MuSK. MuSK was coimmunoprecipitated with both Fyn and Src but not precipitated by empty beads (Fig. 1D, lanes 2 vs 4). By comparing with an aliquot of lysate (Fig. 1D, lane 7), the percentage of MuSK complexed with Fyn and Src was calculated to be 1.6 ± 0.3 and 1.3 ± 0.3%, respectively. Agrin also affected the coimmunoprecipitation of MuSK with Fyn and Src (Fig. 1D, lane 1 vs 2 and lane 3 vs 4). After treatment of C2 cells with agrin, the percentage of MuSK precipitated with Fyn rose to 2.8 ± 0.6% (p < 0.05), and the percentage precipitated with Src rose to 2.0 ± 0.4% (p < 0.025). In three experiments, agrin enhanced association of MuSK with Fyn and Src, 80 ± 20 and 57 ± 5%, respectively. These data provided strong evidence that in C2 myotubes, Fyn and Src were complexed with MuSK under control conditions and that this association was stimulated further by agrin.

Because Fyn, Src, and MuSK are all protein tyrosine kinases, the effect of increasing phosphorylation on complex formation was examined. Treatment of C2 cells with pervanadate, an inhibitor of protein tyrosine phosphatases (Huyer et al., 1997), resulted in a dramatic increase in protein tyrosine phosphorylation, indicating constitutive phosphorylation and dephosphorylation in C2 myotubes (A. S. Mohamed and S. L. Swope, unpublished results). To examine the effect of phosphorylation on association of MuSK and Src-class kinases, myotubes were treated under control conditions or with pervanadate, and MuSK was immunoprecipitated from the lysates. Src-class kinase(s) was specifically coprecipitated with MuSK as demonstrated by analysis of the MuSK precipitates by Western blotting using a pan-Src-class kinase antibody (Fig. 2A, lane 2 vs 8). Coimmunoprecipitation of the Src-class kinase(s) with MuSK was enhanced by treatment with pervanadate (Fig. 2A, lane 1 vs 2). In two experiments, inhibition of protein tyrosine dephosphorylation increased the coimmunoprecipitation of Src-class kinases with MuSK from 1.35 ± 0.03 to 2.8 ± 0.1%, and in a third experiment the increase was from 3.8 to 7.3%. Thus, all three experiments showed an increase in complex formation between Src kinases and MuSK by pervanadate treatment, with an average increase of 117 ± 6%. The reverse analysis was also performed. Fyn and Src were immunoprecipitated from control and pervanadate-treated myotubes, and the precipitates were analyzed for MuSK. Pervanadate treatment increased coimmunoprecipitation of MuSK with Fyn (Fig. 2B, lane 1 vs 2). The percentage of MuSK associated with Fyn in control and pervanadate-treated cells was 1.4 ± 0.2 and 2.6 ± 0.4% (p < 0.025), respectively. Stimulation of complex formation by pervanadate occurred in every experiment, and the mean increase was 88 ± 9%. The association of MuSK with Src was also reproducibly increased by a pervanadate treatment (Fig. 2B, lane 3 vs 4). The percentage of MuSK precipitated with Src increased from 1.01 ± 0.06 to 2.4 ± 0.2% (p < 0.025), with an average increase of 140 ± 20%. These data suggested that in C2 cells, association of MuSK with Src and Fyn was being repressed by a constitutively active protein tyrosine phosphatase and was enhanced by increased phosphorylation when the phosphatase was inhibited.

Association between receptor kinases, such as the PDGF receptor, and Src-class kinases is mediated by a phosphotyrosine–SH2 domain interaction. Because association of MuSK and Src-class kinases was enhanced by increased tyrosine phosphorylation, it was predicted that MuSK might bind with the SH2 domains of Fyn and Src. To test this possibility, fusion protein affinity chromatography was used. SH2 domain-containing fusion
proteins derived from Fyn, Src, and Grb2 were incubated with myc-tagged MuSK solubilized from heterologous transfected QT-6 fibroblasts. MuSK bound to the Fyn and Src SH2 domain fusion proteins as detected by anti-myc Western analysis (Fig. 3A). The binding was specific in that MuSK did not bind to the backbone fusion protein or the Grb2 SH2 fusion protein. In addition, MuSK did not bind to the SH3 domains of Fyn or Src (J. R. Kraas and S. L. Swope, unpublished results). Because MuSK was solubilized under extremely stringent conditions of 2% SDS lysis buffer, which promotes dissociation of protein complexes, these data suggested that MuSK bound directly to the Fyn and Src SH2 domain fusion proteins. However, an indirect interaction cannot be ruled out.

The concentration-dependence for binding of MuSK to the Fyn and Src SH2 domains was analyzed. Increasing concentrations of Fyn and Src SH2 domain fusion proteins, 0.3–30 μg, were incubated with solubilized myc-tagged MuSK. MuSK bound slightly better to the Src SH2 domain (Fig. 3B). These data suggested that association of MuSK with Fyn and Src was mediated by binding of the Src-class kinases’ SH2 domains to the receptor tyrosine kinase and that MuSK bound with a slightly higher affinity to Src.

Receptor tyrosine kinases bind Src-class kinases via phosphotyrosine motifs on the receptors. In vivo, these tyrosines are phosphorylated by ligand-induced dimerization and auto/transphosphorylation of the receptor tyrosine kinases (Claesson-Welsh, 1994; Thomas and Brugge, 1997). Whether the binding of MuSK to the SH2 domain fusion protein of Src-class kinases was dependent on MuSK transphosphorylation was tested using a catalytically inactive MuSK construct containing a lysine to arginine point mutation (MuSK[K686R]) (Gillespie et al., 1996). First, whether MuSK autophosphorylation in the QT6 cells occurred was determined by testing whether MuSK[K686R] could be a substrate for wild-type MuSK. In fact, myc-tagged MuSK[K686R] was phosphorylated when coexpressed with non-tagged wild-type mouse MuSK, but not when expressed alone (data not shown). Thus, wild-type MuSK was capable of auto/transphosphorylation in this system.

The catalytically inactive MuSK[K686R] was used to test whether binding to the SH2 domain of Src was dependent on autophosphorylation. Heterologous cells expressing the wild type or MuSK[K686R] were treated without or with pervanadate to inhibit dephosphorylation and then solubilized. Lysates were in-
cubated with the Src SH2 domain fusion protein and binding of myc-tagged wild type or MuSK[K686R] was determined by anti-myc Western analysis. In the absence of pervanadate treatment, wild-type MuSK bound to the Src SH2 domain but MuSK[K686R] did not (Fig. 3C, lane 7 vs 8). This absence of binding was not caused by altered expression of the inactive MuSK because analysis of the lysates demonstrated that both wild type and MuSK[K686R] were expressed (Fig. 3C, lanes 11 and 12). Treatment of the cells expressing wild-type MuSK with pervanadate to block dephosphorylation caused an increase in the binding of wild-type MuSK to the Src SH2 domain fusion protein (Fig. 3C, lane 1 vs 7). However, after treatment with pervanadate, binding of MuSK[K686R] was still not detected (Fig. 3C, lane 2). Analysis of the lysates demonstrated that the expression levels of wild type and MuSK[K686R] were unaffected by pervanadate (Fig. 3C, lanes 5 and 6 vs 11 and 12). These data indicated that binding of the wild-type MuSK to the SH2 domain fusion protein was dependent on tyrosine phosphorylation of MuSK. In addition, the phosphorylation was attributable to autophosphorylation of MuSK because catalytically inactive MuSK was unable to bind to the SH2 domain fusion protein.

Phosphorylation of MuSK and the AChR by Src-class kinases

A mechanism for reciprocal regulation between receptor and Src-class kinases has been elucidated in other systems. Ligand-activated autophosphorylation of receptor tyrosine kinases results in binding of Src-class kinases via their SH2 domains. This binding leads to phosphorylation of the receptor kinase by Src-class kinases and thus creates binding sites on the receptor for an additional SH2 domain containing enzymes and adapter proteins (Claesson-Welsh, 1994). As described above and by others, MuSK does autophosphorylate (Gillespie et al., 1996). Whether MuSK could be a substrate for Src and Fyn was tested next. For this analysis, the myc-tagged MuSK[K686R] was coexpressed with Src and Fyn in a heterologous cell line. Because MuSK was not catalytically active, any MuSK phosphorylation could not be caused by autophosphorylation. In fact, when MuSK[K686R] was expressed alone and then immunoprecipitated with anti-myc antibody, no phosphorylation was observed, as demonstrated by phosphotyrosine Western blotting (Fig. 4A, lane 7); however, when coexpressed with Src, MuSK[K686R] was phosphorylated (Fig. 4A, lane 1). Some random variability in the isolation of MuSK occurred (Fig. 4B, lanes 1–8); however, changes in the amounts of MuSK precipitated could not account for the “all” or nothing phosphorylation of MuSK[K686R] in the presence or absence of Src, respectively (Fig. 4A,B, lane 1 vs 7). Isolation of MuSK was dependent on transfection with the MuSK[K686R] plasmid because MuSK could not be precipitated from control cells transfected with empty plasmid (Fig. 4B, lane 10). Thus, catalytically inactive MuSK could be phosphorylated upon coexpression with Src.

Phosphorylation of MuSK by Fyn was also tested. In this transfection system, the activity of expressed Fyn is low, but Fyn activity can be increased by rapsyn (Mohamed and Swope, 1999). In fact, phosphorylation of MuSK via Fyn was observed only after coexpression of rapsyn (Fig. 4A, lane 3 vs 4). In two of three experiments, phosphorylation of MuSK by Src was also potentiated by rapsyn. We tested whether the phosphorylation of MuSK was specific for Src-family kinases. Bik, a non-receptor kinase distinct from but related to the Src-family, mediated little or no phosphorylation of MuSK, demonstrating the specificity of the effect of Src and Fyn (Fig. 4A, lanes 5 and 6). These data supported the idea that MuSK can be phosphorylated by Src and Fyn and in addition suggested that MuSK is a substrate for these kinases in muscle.

Whether Fyn and Src phosphorylate MuSK in muscle cells was examined pharmacologically. PP1 is a highly selective Src-class kinase inhibitor. Depending on the specific Src family kinase and the cell type examined, PP1 has ID50 values of 10–100 nM in vitro and 0.5–50 μM in intact cells (Hanke et al., 1996; Liu et al., 1999). First, inhibition of Fyn and Src by PP1 in C2 myotubes was demonstrated. Activation of Src-class kinases results in auto/tansphosphorylation at Y416 (chicken Src numbering) within the catalytic domain (Thomas and Brugge, 1997). Thus, phosphorylation at this site can be used as a measure of activation. We developed an anti-Y416 phosphorylation state-specific antibody, GW001, to examine the activity of Src-class kinases by Western blotting, as described in Materials and Methods. The ability of PP1 to inhibit Src and Fyn activities in C2 cells was analyzed using the anti-PY416 Src antibodies. The Y416 phosphorylation of Fyn and Src immunoprecipitated from control C2 myotubes was low (Fig. 5A, lanes 1 and 5). Treatment of C2 myotubes with pervanadate increased the phosphorylation of Fyn and Src on Y416 (Fig. 5A, top panel, lane 1 vs 2 and lane 5 vs 6). Thus, pervanadate revealed constitutive activity and autophosphorylation of Fyn and Src. Pretreatment of myotubes with 10 or 20 μM PP1 inhibited the pervanadate-induced increase in Y416 phosphorylation on Fyn and Src (Fig. 5A, lane 2 vs 3 and 4 and lanes 6 vs 7 and 8). The changes in anti-PY416 Src signal could not be explained by alterations in the isolation of Fyn and Src (Fig. 5A, bottom panel). Upon normalization for the amount of kinase isolated, Fyn autophosphorylation/activity was found to be 75 ± 7 and 90 ± 2% with 10 and 20 μM PP1, respectively. Src activity was inhibited by 60 ± 10 and 60 ± 10% with 10 and 20 μM PP1, respectively. The higher sensitivity of Fyn to PP1, compared with Src, agreed with a previous report (Hanke et al., 1996). These data demonstrated that as measured by autophosphorylation, PP1 blocked the activity of Fyn and Src in C2 myotubes.

To reveal constitutive phosphorylation of MuSK by Src-class kinases, pervanadate and PP1 were also used. C2 myotubes were treated with pervanadate, and endogenous MuSK phosphorylation...
Src-class kinases phosphorylate and regulate AChRs of skeletal muscle. Phosphorylation of the AChR by Src and Fyn was also tested pharmacologically. Once again, C2 cells were treated with or without pervanadate and PP1. AChRs were immunoprecipitated and tyrosine phosphorylation was examined using phosphorylation state-specific anti-β and anti-δ subunit antibodies. AChR phosphorylation was low in control cells, whereas pervanadate dramatically increased receptor phosphorylation (Fig. 5C, lane 1 vs 2). When we corrected for recovery of AChRs (Fig. 5C, bottom panel), a 22- to 26-fold increase in phosphorylation of the β subunit and a 10- to 130-fold increase in phosphorylation of the δ subunit occurred. This phosphorylation appeared to be attributable, in part, to Src-class kinases, as demonstrated by inhibition with PP1 (Fig. 5C, lane 2 vs 3 and 4). Cells pretreated with 10 and 20 μM PP1 showed a 60 ± 7 and 70 ± 10% inhibition of pervanadate-induced β subunit phosphorylation, respectively. Inhibition of δ subunit phosphorylation was 54 ± 4 and 72 ± 7% by 10 and 20 μM PP1, respectively. Therefore, phosphorylation of AChRs by Src-class kinases occurred in C2 cells.

MuSK phosphorylation in C2 myotubes was inhibited by PP1 (Fig. 5B). These data suggested that Src-class kinases phosphorylated MuSK in muscle cells. However, because MuSK autophosphorylates, the effect of PP1 on MuSK might have been to inhibit autophosphorylation. Whether PP1 could block MuSK kinase activity in vitro was used to further test the conclusion that MuSK was phosphorylated by Src kinases in C2 cells. C2 myotubes were treated with or without pervanadate, MuSK was immunoprecipitated, and MuSK activity was analyzed in vitro using enolase as a substrate (Fig. 6A). Phosphorylation of both MuSK and enolase was detected by anti-phosphotyrosine immunoblotting. In control cells, no phosphorylation of MuSK or enolase was observed (Fig. 6A, top and bottom panels, lane 2). Pervanadate treatment caused a striking increase in both MuSK and enolase phosphorylation (Fig. 6A, top and bottom panels, lane 1). Phosphorylation of enolase occurred in vitro because it was dependent on ATP in the reaction (Fig. 6A, bottom panel, lane 3). In contrast, MuSK phosphorylation was independent of ATP in the in vitro reaction, demonstrating that MuSK phosphorylation occurred in the intact myotubes (Fig. 6A, top panel, lane 1 vs 3). As expected, inclusion of 1 or 5 μM PP1 in the in vitro reaction did not affect the in vitro phosphorylation of MuSK (Fig. 6A, top panel, lane 1 vs 4 and 6). In addition, enolase phosphorylation by MuSK was not affected by 1 or 5 μM PP1 added to the in vitro reaction (Fig. 6A, bottom panel, lane 1 vs 4 and 6). The phosphorylation of enolase in the presence of 10 and 20 μM PP1 was 96 ± 4 and 104 ± 5%, respectively, of the level in the absence of PP1. These data demonstrated that PP1 did not directly inhibit the enzymatic activity of MuSK.

In contrast, PP1 did block the enzymatic activity of Fyn and Src in vitro. Using the same paradigm, myotubes were treated with or without pervanadate, Fyn and Src were isolated, and in vitro phosphorylation of enolase in the presence or absence of PP1 was determined. Pervanadate treatment of the myotubes stimulated in vitro phosphorylation of enolase by both Fyn and Src (Fig. 6B, top panel, lane 1 vs 2 and lane 8 vs 9). The phosphorylation of enolase by Fyn and Src occurred in vitro because it was dependent on ATP in the reaction (Fig. 6B, lane 1 vs 3 and lane 8 vs 10). Fyn-mediated phosphorylation of enolase was inhibited by PP1 (Fig. 6B, lane 1 vs 4 and 6). Fyn activity was inhibited in vitro by 69 ± 5 and 96 ± 4% with 1 and 5 μM PP1, respectively. Similarly, Src activity was inhibited by 91 ± 1 and 100 ± 1% with 1 and 5 μM PP1, respectively (Fig. 6B, lane 8 vs 11 and 13). These data...
reduced by PP1 (Fig. 7). Agrin blocked agrin-stimulated MuSK phosphorylation by 40%. These data argue that phosphorylation of MuSK upon agrin treatment was attributable, in part, to Src-class kinases. 

**Figure 6.** Effect of inhibiting Src-class kinases on Fyn, Src, and MuSK kinase activities in vitro. A, C2 myotubes were treated with pervanadate at 30 μM (PV+) or vehicle (PV−) for 30 min. Myotubes were solubilized in RIPA buffer and centrifuged, and supernatants representing 30% of a 60 mm dish were immunoprecipitated with anti-MuSK antibody GW002 (MuSK). The kinase activity of immunoprecipitated MuSK was determined with an in vitro kinase assay using enolase as a substrate in the presence of 1 or 5 μM PP1 (PP1+) or vehicle (PP1−) and the presence (ATP+), or absence (ATP−), of ATP. For examining MuSK phosphorylation, kinase reactions were resolved by 7% SDS-PAGE and analyzed by Western blotting using anti-phosphotyrosine antibodies 4G10 and PY99 (α-PY) (top panel). Enolase was resolved by 8% SDS-PAGE and analyzed by Western blotting using anti-phosphotyrosine antibodies 4G10 and PY99 (α-PY) (bottom panel). Arrowheads indicate the position of MuSK (MuSK) and enolase (ENO). B, Supernatants from A representing 30% of a 60 mm dish were immunoprecipitated with anti-Fyn antibody Fyn 15 (Fyn) or anti-Src antibody Ab-1 (Src). The kinase activities of immunoprecipitated Fyn and Src were determined with an in vitro kinase assay using enolase as substrate as described in A. Enolase and Src-class kinase activities were resolved by 8% SDS-PAGE and analyzed by Western blotting using anti-phosphotyrosine antibodies 4G10 and PY99 (α-PY) (top panel). The blots were stripped and reanalyzed by Western blotting using the pan-Src kinase antibody SrcR (pan-SrcRks) (bottom panel). Arrowheads indicate the positions of enolase (ENO) and Src-class kinases (SrcKs).

**Figure 7.** Effect of inhibiting Src-class kinases on agrin-induced MuSK and AChR phosphorylation in vitro. A, C2 myotubes were treated with 10 or 20 μM PP1 (PP1+) or vehicle (PP1−) for 1 hr followed by treatment with 100 pm agrin (+) or conditioned medium vehicle (−) for 10 min. Myotubes were solubilized in RIPA buffer and centrifuged, and supernatants representing 40% of each 60 mm dish were immunoprecipitated with anti-MuSK antibody GW002 (MuSK). The immunoprecipitates were resolved by 7% SDS-PAGE and analyzed by Western blotting using a mixture of anti-phosphotyrosine antibodies 4G10 and PY99 (α-PY) (top panel). Supernatants representing 1% of each 60 mm dish were resolved by 7% SDS-PAGE and analyzed by Western blotting with anti-MuSK antibody MuSK-ZH (α-MuSK) (bottom panel). Arrowheads indicate the position of MuSK. B, Supernatants representing 40% of a 60 mm dish, as in A, were used for immunoprecipitation with anti-AChR antibody 88b (AChR). The immunoprecipitates were resolved by 8% SDS-PAGE and analyzed by Western blotting using a mixture of the phosphorylation state-specific anti-AChR β subunit (JH1360) and anti-AChR δ subunit (JH1358) antibodies (AChR) (top panel). The blot was stripped and reanalyzed by Western blotting using anti-AChR β subunit antibody mAb 148 (AChR) (bottom panel). Arrowheads indicate the positions of the AChR β and δ subunits.

demonstrated that the enzymatic activity of Src and Fyn kinases from C2 myotubes was inhibited by PP1, whereas the activity of MuSK was not. Furthermore, these data support the idea that phosphorylation of MuSK and the AChR in C2 myotubes was attributable to Src-class kinases (Fig. 5B,C).

Agrin activates MuSK and induces MuSK phosphorylation (Glass et al., 1996). Whether agrin-stimulated phosphorylation of MuSK was dependent on Src-class kinases was tested. For this experiment, C2 myotubes were treated with agrin, MuSK was immunoprecipitated, and phosphorylation was examined by Western blotting. Agrin induced MuSK phosphorylation (Fig. 7A, lane 1 vs 2). The effect of agrin on MuSK phosphorylation was reduced by PP1 (Fig. 7A, lane 2 vs 3 and 4). At 10 and 20 μM, PP1 blocked agrin-stimulated MuSK phosphorylation by 40 ± 10 and 69 ± 6%. These data argue that phosphorylation of MuSK upon agrin treatment was attributable, in part, to Src-class kinases.

Phosphorylation of the AChR in response to agrin does not occur in myotubes derived from a MuSK knock-out mouse (Glass et al., 1997). Thus, agrin-stimulated phosphorylation of the AChR is dependent on MuSK. Whether agrin-stimulated/MuSK-mediated AChR phosphorylation was dependent on Src-family kinases was examined. C2 myotubes were treated with agrin, AChRs were isolated, and phosphorylation was analyzed by Western blotting using the phosphorylation state-anti-β and anti-δ subunit antibodies. Agrin induced AChR phosphorylation (Fig. 7B, lane 1 vs 2). The agrin-stimulated phosphorylation of the AChR was inhibited by PP1 (Fig. 7B, lane 2 vs 3 and 4). The AChR β subunit phosphorylation was blocked by 51 ± 7 and 69.5 ± 7.0% with 10 and 20 μM PP1, respectively. The δ subunit phosphorylation was blocked by 60 ± 10 and 74 ± 8% with 10 and 20 μM PP1, respectively. These data indicated that agrin activated Src-class kinases, resulting in phosphorylation of the AChR in C2 myotubes.

**Cytoskeletal anchoring of the AChR by Src-class kinases**

Agrin stimulates AChR anchoring to the cytoskeleton in muscle cells (Wallace, 1992). Anchoring of AChRs is thought to be regulated by tyrosine phosphorylation (Wallace, 1992; Mohamed and Swope, 1999). Because Src-class kinases appeared to mediate the effect of agrin to stimulate AChR phosphorylation (Fig. 7B), a role for these kinases to regulate receptor anchoring was tested. The levels of anchored AChRs were determined by examining the resistance of the receptors to extraction with 0.5% Triton X-100, an approach previously demonstrated to be useful for analyzing interaction of AChRs with the cytoskeleton (Prives et al., 1982). For this experiment, myotubes were treated with agrin, agrin plus PP1, or vehicle. The cells were then extracted with 0.5% Triton X-100 (PV+).
X-100 over a time course of 0–20 min. The detergent-resistant cytoskeletal material remaining on the plate was eluted with SDS-PAGE sample buffer and analyzed for AChRs by Western blotting. With no extraction, the levels of total AChRs in the nonextractable fraction were enhanced by agrin, and the effect of agrin was significantly reversed by PP1 (Fig. 8C). These data indicated that Src-class kinases mediate at least part of the effect of agrin to induce cytoskeletal anchoring of the AChR in muscle cells. Furthermore, these results support the importance of Src-class kinases in the stabilization of AChRs at the endplate during formation of the NMJ.

**Maturation of AChR clustering by Src-class kinases**

Whether the effect of agrin to induce AChR clustering was mediated by Src-class kinases was also examined. Two approaches were used: inhibition of Src kinases with PP1 or expression of a dominant negative Src. C2 myotubes were treated with agrin in the presence or absence of PP1, and AChR clustering was analyzed using tetramethylrhodamine-α-bungarotoxin or Texas Red-α-bungarotoxin labeling and epifluorescence microscopy. Agrin-induced AChR clustering was still apparent in the presence of PP1 (Fig. 9, A vs B). However, the size of receptor aggregates was altered by inhibition of Src-class kinases. The size distribution of receptor clusters was analyzed by binning into categories of <15 μm, 15–25 μm, and >25 μm (Fig. 9E, +PP1 and −PP1). The most obvious effect was a loss of large clusters and a dramatic shifting of clusters to sizes <15 μm. The percentage of clusters >25 μm in agrin-treated cultures was 57 ± 3%, n = 5, whereas after inhibition of Src-class kinases the number was reduced to 4.0 ± 0.1%, n = 5 (Fig. 9E, −PP1 vs +PP1 clear bars). Conversely, the percentage of clusters <15 μm was 13 ± 3% in control cells and 86 ± 6%, n = 5, in PP1-treated cells (Fig. 9E, −PP1 vs +PP1, hatched bars). These data indicated that inhibition of Src-class kinases with PP1 blocked the formation of large AChR clusters in response to agrin.

The role of Src-class kinases in the maturation of large AChR clusters was corroborated using a dominant negative approach. C2 cells were transfected to express a dominant negative Src and treated with agrin, and AChR clustering was analyzed. Cells expressing the dominant negative Src were identified by staining with a pan-Src kinase antibody, src2, whereas AChRs were identified with Texas Red-α-bungarotoxin. Again, agrin-induced clustering occurred in cells expressing the dominant negative kinase; however, compared with control cultures transfected with empty plasmid, the size of receptor clusters was reduced by expression of dominant negative Src (Fig. 9, C vs D). In contrast, expression of GFP did not affect AChR clustering (data not shown). For control cells, 37 ± 2% of the clusters were 15–25 μm and 33 ± 3% were >25 μm, whereas 31 ± 4% of the clusters were <15 μm (see Fig. 9E, pBK). In contrast, in the cells expressing the dominant negative Src, 91 ± 2% of the clusters were <15 μm, whereas only 3 ± 1% were >25 μm (Fig. 9E, DN Src). These results provide strong evidence that Src-class kinases are critical for maturation of AChR clustering.

*Figure 8.* Effect of inhibiting Src-class kinases on agrin-induced AChR cytoskeletal anchoring. A, C2 myotubes were treated with 20 μM PP1 (PP1 +) or DMSO vehicle (PP1 −) for 1 h followed by treatment with 100 pm agrin (Agrin +) or conditioned medium vehicle (Agrin −) for 1 h as indicated. Treated myotubes were extracted with Prives buffer containing 0.5% Triton X-100 for the indicated times and rinsed once with Prives buffer containing 0.1% Triton X-100. The proteins remaining on the dish were extracted using SDS-PAGE sample buffer, sonicated, and centrifuged. Aliquots of the SDS-extracted proteins representing 10% of a 35 mm dish were resolved by 8% SDS-PAGE and analyzed by Western blotting using anti-AChR β subunit antibody mAb 148. B, Sister C2 cultures were treated with or without agrin, with or without PP1 as in A, and treated with Prives buffer without Triton X-100. Dishes were rinsed once with Prives buffer without Triton X-100. The proteins on the dish were extracted using SDS-PAGE sample buffer, sonicated, and centrifuged. SDS-extracted proteins representing 10% of 35 mm dishes were resolved by 8% SDS-PAGE and analyzed by Western blotting using anti-AChR β subunit antibody mAb 148. C, Four independent experiments as in A were analyzed. C2 myotubes were treated with agrin (†), agrin + 20 μM PP1 (■), or vehicle control (▲). The data were quantified by densitometric scanning and represent the mean ± SEM of the percentage of the total level of AChRs that remained on the dishes after 0.5% Triton X-100 extraction. In A and B, arrowheads indicate the position of the AChR β subunit.
DISCUSSION

It has been predicted that a protein tyrosine kinase downstream of MuSK directly phosphorylates the AChR (Burden, 1998; Sanes et al., 1998; Fuhrer et al., 1999; Sanes and Lichtman, 1999). In this study, MuSK was found to be complexed with the Src-class kinases Fyn and Src in the C2 mouse muscle cell line. These associations were enhanced by agrin and by increasing protein tyrosine phosphorylation. MuSK bound specifically to the SH2 domains of Fyn and Src, and this in vitro binding was dependent on autophosphorylation of MuSK. In addition, Fyn and Src phosphorylated MuSK in transfected cells. A highly selective inhibitor of Src-family kinases, PP1, blocked the enzymatic activity of Fyn and Src from C2 myotubes but did not affect the kinase activity of MuSK. With use of PP1, phosphorylation of MuSK and the AChR was demonstrated to be, in part, via Src-class kinases. In addition, Src-family kinases mediated agrin-induced anchoring of AChRs as well as maturation of AChR clustering. These data establish a structural and functional relationship for MuSK with Src and Fyn and provide evidence that Src-class kinases mediate the effects of agrin on the AChR.

Role of AChR phosphorylation in clustering and anchoring

The diffusion trap model proposes that after contact of the motor neuron with skeletal muscle, a “sticky zone” is created at the contact site where mobile AChRs become trapped (Edwards and Frisch, 1976). In support of this theory, nonsynaptic AChRs are able to migrate freely, whereas clustered receptors are less mobile in the membrane (Young and Poo, 1983; Podleski and Salpeter, 1988; Meier et al., 1995). Accumulation of AChRs at the nerve–muscle contact site is due to migration of receptors to the developing endplate (Anderson and Cohen, 1977). Furthermore, anchoring of AChRs as well as maturation of AChR clustering. These data establish a structural and functional relationship for MuSK with Src and Fyn and provide evidence that Src-class kinases mediate the effects of agrin on the AChR.

Figure 9. Effect of inhibiting Src-class kinases on agrin-induced AChR clustering. A, B, C2 myotubes were treated with 20 μM PP1 (A) or DMSO vehicle (B) for 1 hr followed by treatment with 100 pM agrin for 12 hr in the continued presence or absence of PP1. C, D, C2 myotubes were transfected with the dominant negative Src (C) or empty plasmid (D). After 55–60 hr, cells were treated for 12 hr with 100 pM agrin. For A–D, AChR clusters were visualized with Texas Red α-bungarotoxin, whereas for C and D, expression of the dominant negative Src was visualized by src staining, as described in Material and Methods. Scale bar, 20 μm. E, Cluster sizes were analyzed and binned into three categories: <15, 15–25, and >25 μm. The data represent the mean ± SEM, n = 5 experiments, for the effect of PP1 and n = 3 experiments for the effect of dominant negative Src. For agrin − PP1, agrin + PP1, agrin on pBK-CMV-transfected cells, and agrin on dominant negative Src-transfected cells, a total of 525, 518, 673, and 417 clusters, respectively, were analyzed. Note: The change in the receptor size distribution between the control transfected cells and the DMSO control for the PP1 experiment derives from the fact that low concentrations of DMSO appeared to promote agrin-induced clustering.
mathematical modeling demonstrates that free diffusion of AChRs is fast enough to account for receptor accumulation at the endplate during synapse formation (Edwards and Frisch, 1976; Chao et al., 1981; Young and Poo, 1983; Kuromi et al., 1985). Thus, trapping of receptors at the endplate is critical for synapticotogenes at the NMJ. The data presented here indicate that local activation of Src-class kinases may be a mechanism to create a sticky zone at the developing endplate.

Several lines of evidence support AChR tyrosine phosphorylation as a mechanism for receptor aggregation and stabilization within the cytoskeleton. Innervation induces AChR clustering and anchoring as well as receptor phosphorylation (Hall and Sanes, 1993; Qu and Huganir, 1994). Agrin also induces AChR phosphorylation and clustering (Godfrey et al., 1984; Wallace et al., 1991). Receptor phosphorylation in response to agrin precedes clustering, consistent with the idea that phosphorylation causes clustering (Wallace, 1992). Conditions that induce AChR phosphorylation, such as agrin treatment, cotransfection with Src-class kinases, and pervanadate treatment, also stimulate receptor anchoring (Wallace, 1992; Meier et al., 1995; Wallace, 1995; Mohamed and Swope, 1999). Furthermore, phosphorylation and anchoring of the AChR always occur with the same time course, suggesting a close functional relationship (Wallace, 1992, 1995). In addition, phosphorylated AChRs are more slowly extracted and hence are more tightly associated with the cytoskeleton than nonphosphorylated receptors (Meier et al., 1995). Conversely, protein tyrosine kinase inhibitors block AChR phosphorylation, clustering, and anchoring (Figs. 8, 9) (Wallace, 1994; Ferns et al., 1996; Mohamed and Swope, 1999). Of significance, a mutant AChR β subunit lacking the tyrosine phosphorylation site assembles with endogenous AChR subunits of a muscle cell line, producing a receptor that forms only half the normal number of agrin-induced clusters. Furthermore, mutation of the β subunit abolishes agrin-induced cytoskeletal anchoring of AChRs (Borges and Ferns, 2001). Thus, tyrosine phosphorylation of the AChR β subunit can regulate anchoring and clustering of AChRs. The results reported here reinforce this conclusion and support Src-class kinases in mediating agrin-induced phosphorylation of AChRs.

Cytoskeletal anchoring of AChRs appears to be reversible, involving two dynamic processes: actin polymerization and binding of AChRs to the cytoskeleton. Formation of clusters is associated with local actin polymerization. Furthermore, the newly formed clusters are sites for continued actin assembly. In fact, the force generated by actin polymerization is sufficient to move AChR clusters within the membrane (Dai et al., 2000). AChR anchoring is also dynamic via reversible binding with the cytoskeleton. AChRs appears to exist in either a nonphosphorylated and mobile state or a phosphorylated and anchored state (Meier et al., 1995). Two pieces of evidence indicate that these two forms of the receptor must exist in a dynamic equilibrium. First, AChR phosphorylation and cytoskeletal anchoring in response to agrin occur before clustering (Wallace, 1992). Second, shifting the equilibrium far into the phosphorylated-anchored form of the AChR, using either pervanadate or viral Src, blocks receptor clustering, indicating that phosphorylated receptors are immobilized before they can aggregate (Anthony et al., 1984; Meier et al., 1995; Wallace, 1995). Therefore, cytoskeletal polymerization and attachment of AChRs to the cytoskeleton must be dynamically regulated for proper postsynaptic localization of receptors. The results presented here suggest that reversible phosphorylation of AChRs via Src-class kinases may provide a dynamic mechanism for regulating receptor anchoring.

Role of additional phosphoproteins in synaptogenesis

The endplate consists of a complex matrix of proteins (Sanes and Lichtman, 1999). Tyrosine phosphorylation is necessary for synapticotogenesis at the NMJ; however, it is not known whether the AChR is the only important substrate. Several components of the postsynaptic complex, in addition to AChRs, are tyrosine phosphorylated. These proteins include rapsyn as well as α-dystrobrevin-1 and syntrophin of the dystrophin glycoprotein complex (Wagner et al., 1993; Wagner and Huganir, 1994; Qu et al., 1996; Balasubramanian and Huganir, 1999; Mohamed and Swope, 1999). Phosphorylation of these proteins may also be important for promoting the cytoskeletal anchoring or protein–protein interactions necessary for synapticotogenesis. Both rapsyn and α-dystrobrevin are crucial for normal synapticotogenesis at the NMJ; however, the structures of the NMJs in rapsyn and α-dystrobrevin knock-out mice are different. In rapsyn−/− mice, AChR clustering is completely absent in vivo as well as in response to agrin in culture (Gautam et al., 1995). Whether rapsyn phosphorylation regulates its critical role in synapse formation is an interesting question. In α-dystrobrevin−/− mice,
NMJs form but the AChRs have an abnormal, patchy distribution. In addition, agrin-induced formation of large AChR clusters is defective in myotubes derived from α-dystrobrevin−/− mice (Grady et al., 2000). Rapsyn and α-dystrobrevin-1 appear to have very different molecular functions in forming the NMJ, and both are phosphorylated. Hence, in addition to anchoring of AChRs, protein tyrosine phosphorylation, perhaps via Src-family kinases, may regulate the critical role of rapsyn in synaptogenesis as well as the function of α-dystrobrevin-1 in endplate maturation.

A molecular model for the role of Src-class kinases in synaptogenesis

The results presented here provide a clearer picture of how agrin, MuSK, rapsyn, and Src-class kinases might regulate synapse formation at the NMJ. A current model for agrin-stimulated signal transduction and regulation of AChRs at the NMJ is depicted in Figure 10. In this model, the AChR exists in an equilibrium between a mobile nonphosphorylated state and an anchored phosphorylated state (Fig. 10A,B). Both the mobile and anchored AChRs may be in a complex of proteins including MuSK, Src-class kinases, rapsyn, and α-dystroglycan of the dystrophin glycoprotein complex (Cohen et al., 1995; Fuhrer and Hall, 1996). The motoneuron, via released agrin, alters the equilibrium by stimulating local AChR phosphorylation and anchoring (Fig. 10A,B). Thus, the AChR/rapsyn/MuSK/Src-class kinases complex is recruited to nascent sites of endplate formation, which are thought to be marked by MuSK (Fuhrer and Hall, 1996; Apel et al., 1997). For this to occur, agrin activates MuSK via the postulated MuSK accessory specificity component (Glass et al., 1997). Stimulation of MuSK results in binding of Src or Fyn, or both, via their SH2 domains, to an autophosphorylation site(s) on MuSK (Figs. 1–3), which is a common mechanism for activation of Src-class kinases (Thomas and Brugge, 1997). Because these Src-family kinases have multiple roles in muscle cell function (Claycomb and Lanson, 1987; Castellani et al., 1995; Hirayama et al., 1997; Sanes and Lichtman, 1999), the pools of Src and Fyn activated by MuSK are small, as demonstrated recently (Mittaud et al., 2001). Activation of Src and Fyn by MuSK may be enhanced by rapsyn (Fig. 4) (Mohamed and Swope, 1999; Mittaud et al., 2001). Src and Fyn then phosphorylate MuSK (Figs. 4–7), rapsyn (Mohamed and Swope, 1999), and the AChR (Figs. 5, 7) (Swope and Huganir, 1993; Fuhrer and Hall, 1996; Mohamed and Swope, 1999). Thus, as the mobile receptors come near the nascent synapse, they are phosphorylated and trapped at the endplate via cytoskeletal anchoring, resulting in aggregation of receptors postnaptically (Figs. 8, 9) (Mohamed and Swope, 1999). Furthermore, phosphorylation of additional postnaptic elements, such as rapsyn and α-dystrobrevin-1, may regulate the initiation of aggregation and/or stabilization and condensation of protein complexes. This model incorporates results from numerous studies and provides interesting hypotheses for future testing.

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