Tyrosine phosphorylation of the β subunit of the acetylcholine receptor (AChR) has been postulated to play a role in AChR clustering during development of the neuromuscular junction. We have investigated the mechanism of this phosphorylation in mammalian C2 myotubes and report that the tyrosine kinase Src binds and phosphorylates glutathione S-transferase fusion proteins containing the N-terminal half of the cytoplasmic loop of the β subunit. No binding occurs to the related kinases Fyn or Yes or to the corresponding regions from the γ and δ subunits. Furthermore, AChRs affinity-isolated from C2 myotubes using α-bungarotoxin-Sepharose were specifically associated with Src and Fyn and had tyrosine-phosphorylated β subunits. We suggest that AChRs are initially phosphorylated by Src and subsequently bind Fyn in a phosphotyrosine-dependent manner. These interactions are likely to play an important role in construction of the specialized postsynaptic membrane during synaptogenesis.

Protein-tyrosine phosphorylation is a widely used mechanism for regulating cellular functions, particularly those involving growth or differentiation factors. Several protein-tyrosine kinases are highly expressed in brain (1–3) and are associated with synaptic structures (4), suggesting that they play a general role in synaptic function. At the neuromuscular junction and at its homologous synapse in the electric organ of Torpedo, tyrosine phosphorylation appears to be important for regulating both the function and the distribution of the nicotinic acetylcholine receptor (AChR) during development (5, 6).

The AChR is a ligand-gated ion channel with a pseudosymmetric pentameric structure consisting of four homologous subunits in the ratio α,β,γ,δ. Each subunit traverses the membrane four times, with a long, cytoplasmic loop between transmembrane domains 3 and 4 (7, 8). In the Torpedo AChR, a single conserved tyrosine residue in the cytoplasmic loop of each of the β, γ, and δ subunits is phosphorylated by a kinase activity in the postsynaptic membrane (9). In this tissue, two members of the Src family of tyrosine kinases, Fyn and Fyk, account for a substantial fraction of the total tyrosine kinase activity and have been shown in immunoprecipitation experiments to be associated with tyrosine-phosphorylated AChRs (10, 11). Phosphorylation of the AChR subunits is accompanied by an increase in the rate of rapid desensitization of the receptor by cholinergic ligands, a change that is also produced by phosphorylation of the receptor on serine residues (12, 13).

Tyrosine phosphorylation of the AChR appears to play an important role in synaptogenesis. At the mammalian neuromuscular junction, tyrosine phosphorylation in the postsynaptic membrane, possibly of the AChR, increases during the late, postnatal stage of synaptic maturation (14). Tyrosine phosphorylation of the AChR may also be related to one of the earliest steps in synapse formation, the clustering of AChRs in the postsynaptic membrane underlying the nerve terminal (5). Studies on the development of the chick neuromuscular synapse have shown that the earliest detectable AChR clusters in vivo contain phosphotyrosine and that in vitro AChR clustering and phosphotyrosine co-staining in chick myotubes depend on innervation by co-cultured neurons (15). Both in nerve-muscle cultures and in vivo, the aggregation of AChRs at the nascent neuromuscular junction is caused by agrin released from motor nerves (16, 17). The addition of agrin to cultured myotubes induces both widespread AChR cluster formation and tyrosine phosphorylation of the AChR (15, 18, 19). In mammalian muscle, this phosphorylation, which is specific for the β subunit of the receptor, reaches a peak in 1 h and precedes AChR clustering (20). The kinase inhibitors herbimycin A and staurosporine block both AChR phosphorylation and AChR clustering (20, 21). A receptor tyrosine kinase, MuSK, which is localized to synapses in adult muscle, appears to be part of the signaling receptor for agrin (22). Activation of MuSK also results in tyrosine phosphorylation of the AChR (23, 24).

The protein-tyrosine kinase or kinases that are responsible for phosphorylating the AChR in mammalian muscle cells are unknown. We report here experiments to identify the kinase(s) responsible for tyrosine phosphorylation of the AChR and to examine the interaction of Src family kinases with the AChR in mammalian muscle. Our results show that Src from C2 myotubes selectively binds to fusion proteins derived from the long, intracellular loop of the AChR β subunit; by immunodepletion and in vitro phosphorylation we find that bound Src phosphorylates the fusion proteins. We also show that AChRs isolated from C2 myotubes are tyrosine-phosphorylated on their β subunits and are associated with two members of the Src family, Fyn and Src. We describe a model for the interaction between the AChR and the Src family members and suggest that these kinases are likely to play a role in the construction of a postsynaptic...
aptic complex and immobilization of the AChR at developing synapses.

**EXPERIMENTAL PROCEDURES**

**Generation of GST Fusion Proteins**—Glutathione S-transferase fusion proteins containing segments of the long cytoplasmic loop of the AChR α-subunits were generated using cDNAs encoding the α, β, and δ subunits of the mouse muscle nicotinic AChR in the vector pSM (25) as templates. AChR segments were amplified using the polymerase chain reaction in combination with oligonucleotides that bordered the domains of interest and were designed to create a 5′ BamHI and a 3′ EcoRI restriction site. Amplified DNA segments were isolated, digested with BamHI and EcoRI, and ligated into GST-2T vectors (Pharmacia Biotech Inc.) cleaved with the same enzymes. Polymerase chain reaction-amplified DNA inserts were control sequenced using a commercially available dideoxy sequencing kit from U.S. Biochemical Corp. To analyze expression of Src, Fyn, and Yes antigens in total extracts, Src, Fyn, and Yes antibodies followed by protein A- or protein G-Sepharose. Precipitates were then analyzed by nonreducing SDS-PAGE and immunoblotting with src-CT, Src, Fyn, and Yes antibodies followed by protein A- or protein G-Sepharose. Precipitates were then analyzed by nonreducing SDS-PAGE and immunoblotting with src-CT, Src, Fyn, and Yes antibodies followed by protein A- or protein G-Sepharose. Alternatively, Src-related kinases were first immunoprecipitated with 1 μg of src-CT, Src, Fyn, and Yes antibodies followed by protein A- or protein G-Sepharose. Precipitates were then analyzed by nonreducing SDS-PAGE and immunoblotting with src-CT, Src, Fyn, and Yes antibodies followed by protein A- or protein G-Sepharose. Immunoprecipitates were then analyzed by nonreducing SDS-PAGE and immunoblotting with src-CT, Src, Fyn, and Yes antibodies followed by protein A- or protein G-Sepharose. The corresponding sequence of β-subunit amino acids 333–469; β9,9.10, β-subunit amino acids 333–455; βA,9.9, β-subunit amino acids 333–405; β9,9, β-subunit amino acids 349–405; β9.10, β-subunit amino acids 349–455; β10, β-subunit amino acids 406–455; γ9, γ-subunit amino acids 346–417; δ9, δ-subunit amino acids 353–420. The α-microinjection sequence consists of the 10δ9 α-subunit 9,9-9 Malpighian tubules. AChR segments were generated using cDNAs encoding the AChR α-subunits of the mouse muscle nicotinic AChR in the vector pSM (25) as templates. AChR segments were amplified using the polymerase chain reaction in combination with oligonucleotides that bordered the domains of interest and were designed to create a 5′ BamHI and a 3′ EcoRI restriction site. Amplified DNA segments were isolated, digested with BamHI and EcoRI, and ligated into GST-2T vectors (Pharmacia Biotech Inc.) cleaved with the same enzymes. Polymerase chain reaction-amplified DNA inserts were control sequenced using a commercially available dideoxy sequencing kit from U.S. Biochemical Corp. In E. coli Image 1.54 software (National Institutes of Health).
restricted to particular cell types, these kinases show a widespread tissue distribution and are thought to be involved in a variety of cellular signal transduction pathways (29, 30). Extracts of C2 myotube cultures were immunoblotted with an antisera, src-CT, that recognizes all three. Arrowheads indicate the position of Src-related kinases. Under reducing conditions, all three kinases show essentially the same mobility. Src-CT and Fyn antibodies also react with unknown kinases. Under nonreducing conditions, Yes displays a slightly slower mobility than Src or Fyn. All three kinases are recognized by src-CT antisera on immunoblots.

**Fusion Proteins Containing the β Subunit Cytoplasmic Loop**—We then constructed GST fusion proteins containing all or part of the cytoplasmic loop connecting transmembrane domains 3 and 4 of the β subunit of the mouse muscle AChR (Fig. 2). The gene encoding the β subunit contains two exons, 9 and 10, that together encode most of the loop (31). The fusion protein β9 covers exon 9, whereas the fusion proteins β10 and β9,10 contain the products of exon 10 and both exon 9 and exon 10, respectively (Fig. 2). The region encoded by exon 9, which is N-terminal, contains two tyrosine residues. One of these (Tyr-390), which is conserved between species, is phosphorylated in the AChR isolated from the Torpedo electric organ (9). Exon 10 encodes most of the C-terminal half of the loop which contains a third tyrosine as well as the amphipathic helix (32). As controls we made fusion proteins with sequences from the cytoplasmic loops of the AChR γ and δ subunits that correspond to the exon 9 region of the β subunit (γ9 and δ9 proteins). Whereas the γ region lacks tyrosine residues, the δ region contains two, one of which is homologous to Tyr-390 of the β subunit and is phosphorylated in the Torpedo AChR (9).

**The N-terminal Half of the AChR β Subunit Loop Specifically Interacts with Src**—To determine whether the β loop of the AChR interacts with members of the Src family, Nonidet P-40 extracts of C2 myotubes were incubated with purified GST β fusion proteins adhering to glutathione-Sepharose beads. Bound kinases were identified by elution of the beads with SDS buffer, followed by SDS-gel electrophoresis and immunoblotting. Experiments with src-CT showed that one or more Src family member(s) bound to fusion proteins containing the β subunit exon 9 region, but not to a fusion protein containing only β exon 10 (β10) nor to GST alone (Fig. 3A). Apparent differences in the extent of binding to Src kinases between β exon 9-containing fusion proteins (Fig. 3A) were not reproducible over the course of several experiments and may be related to variations in the folding efficiency of the different fusion proteins. Binding to the β exon 9 region was specific, however, as interactions with β10 or GST were not observed, even when large amounts (20 μg) of fusion proteins and long immunoblot exposure times were used. In control experiments, the ability of the AChR γ and δ subunits to bind to Src-related kinases was tested using the γ9 and δ9 fusion proteins (Fig. 3B). No binding was detected to the γ9 region, consistent with the absence of tyrosine residues. Surprisingly, the δ9 region, which contains...
two tyrosine residues, also failed to bind Src family kinases. Together, these findings indicate that the exon 9 region of the AChR β subunit specifically binds at least one kinase of the Src family.

To identify the specific kinase(s) involved, we analyzed the adsorptions with antibodies specific for Src, Fyn, or Yes. Each antibody was also tested against the original C2 myotube lysate to allow comparison of the results with different antisera. As shown in Fig. 4, βγ and βγ9,10 fusion proteins bound Src, but no binding was detected for Fyn or Yes. Binding capabilities of γ and δ regions homologous to the exon 9 region in β. Binding of Src-related kinase(s) is only observed for βγ fusion proteins but not for the homologous regions of γ or δ, even when 5 μg of fusion protein is used. A fraction of the initial cell lysate was analyzed as a standard (L). Lower molecular weight proteins in the lower panels represent degradation products of the GST fusion proteins. The arrowhead indicates Src-related kinases.

![Image](https://example.com/image1.png)

**Fig. 3.** Fusion proteins containing the exon 9 region of the β-cytoplasmic loop bind to a Src-related kinase(s). Nonidet P-40 lysates from C2 myotubes were incubated for 2 h at 4 °C with either 2 or 5 μg of the indicated GST fusion proteins attached to glutathione-Sepharose. Adsorptions were analyzed by reducing SDS-PAGE followed by immunoblotting using src-CT antiserum (upper panels). Nitrocellulose blots were stripped and reprobed with an antibody against glutathione S-transferase to confirm that equal amounts of GST fusion proteins were present in the relevant adsorptions (lower panels). A, binding of constructs containing portions of the β-cytoplasmic loop to Src-related kinase(s). Apparent differences in kinase binding efficiencies varied between experiments. No binding was seen to β10 and GST, B, binding capabilities of γ and δ regions homologous to the exon 9 region in β. Binding of Src-related kinase(s) is only observed for βγ fusion proteins but not for the homologous regions of γ or δ, even when 5 μg of fusion protein is used. A fraction of the initial cell lysate was analyzed as a standard (L). Lower molecular weight proteins in the lower panels represent degradation products of the GST fusion proteins. The arrowhead indicates Src-related kinases.

![Image](https://example.com/image2.png)

**Fig. 4.** The exon 9 region of the β-cytoplasmic loop specifically binds to the tyrosine kinase Src. C2 myotubes grown on 10-cm dishes were lysed and adsorbed to 5 μg of fusion proteins as described in the legend to Fig. 3. Kinases (arrowheads) binding to the various fusion proteins were analyzed by immunoblotting with src-CT and antibodies specific for Src, Fyn, and Yes. To normalize for different antibody affinities, a constant fraction (0.25%) of the initial lysate was included (L). βγ and βγ9,10 fusion proteins bound to Src, but no comparable binding was observed to Fyn and Yes. Densitometric quantitation of data revealed that 5 μg of βγ bound to and immobilized ~1.4% of Src initially present in the cell extracts. Using src-CT to immunoblot, this value was ~0.5%.

by Src—To determine whether associated Src is able to phosphorylate the β exon 9 region, we adsorbed C2 extracts with βγ fusion protein beads and subsequently incubated the washed and immobilized Src-βγ complex with ATP under phosphorylating conditions. Tyrosine phosphorylation was monitored by immunoblotting with antibodies against phosphotyrosine (Fig. 5). The βγ fusion protein showed a low degree of reactivity with this antiserum, even in the absence of incubation with ATP (Fig. 5A, lane 1). The same signal was seen with the γ9 fusion protein, which lacks tyrosine residues in its AChR portion, and presumably represents nonspecific cross-reactivity of phosphotyrosine antibodies with both bacterially expressed proteins. Upon incubation of the βγ fusion protein with C2 extracts and subsequently with ATP, two phosphorylated bands were observed, a prominent band of ~34 kDa and a minor band of ~60 kDa (Fig. 5A, lane 2). Longer exposures of immunoblots revealed no other bands. Based on their molecular weights and the results of stripping and reprobing the blots with the appropriate antibodies (data not shown), we identified the 34- and 60-kDa bands as the βγ fusion protein and Src, respectively. When C2 extracts were adsorbed with γ9 or with GST protein beads, followed by incubation with ATP, no specific phosphotyrosine staining was observed (Fig. 5A, lanes 16 and 18). Furthermore, no phosphorylation was detected using δ9 or β10 fusion proteins, but strong phosphorylation did occur when the βγ9,10 fusion protein was used (data not shown). Thus tyrosine phosphorylation is specific for the β exon 9 region of fusion proteins.

To analyze whether this phosphorylation is carried out specifically by bound Src, we immuno-depleted the C2 lysates with Src-specific antibodies prior to adsorption. Phosphorylation of the βγ fusion protein and Src was drastically reduced by depletion with either Src-specific antibodies or with src-CT but was unchanged when antibodies against Fyn or Yes were used.

**The Exon 9 Region of the AChR β Subunit Is Phosphorylated**
Interaction of AChRs with Src-related Kinases

**A**

| Depletion: µg ab | src-CT | src | fyn | yes | PAS | PGS |
|------------------|-------|-----|-----|-----|-----|-----|
| 1                | ++    | ++  | ++  | ++  | ++  | ++  |
| 5                | +     | +   | +   | +   | +   | +   |
| 10               | +     | +   | +   | +   | +   | +   |

**B**

Control Immunoblots of lysates

**C**

![Image](image)

**D**

![Image](image)

**Fig. 5. The exon 9 region of the β-cytoplasmic loop is tyrosine-phosphorylated by Src.** To determine whether bound Src phosphorylates the β exon 9 region, in vitro tyrosine phosphorylation of β9 adsorbrates and the effect of kinase depletion were analyzed. For kinase depletion, C2 myobute lysates were incubated with 1 or 5 µg of src-CT, Src, Fyn, or Yes antisera (lanes 5–12). Antibodies with bound kinases were removed by two rounds of precipitation with protein A- or protein G-Sepharose. In control experiments, incubation with antibodies was omitted (lanes 1–4, 13–18). Depleted and control lysates were then incubated with 5 µg of β9, γ9, or GST proteins as indicated. Adsorbates (except lanes 3, 15, and 17) were subjected to in vitro phosphorylation with ATP. A, tyrosine phosphorylation of fusion proteins and associated proteins was visualized by reducing SDS-PAGE and anti-phosphotyrosine immunoblotting. B, aliquots of control and depleted cell lysates were analyzed by immunoblotting with specific antibodies against Src, Fyn, and Yes (open arrowheads) to reveal the degree of kinase depletion prior to adsorption with fusion proteins. Phosphorylation and depletion assays show that β9 (but not γ9 or GST) and an interacting protein of ~60 kDa (Src; solid arrowheads) become tyrosine-phosphorylated and that Src, but not Fyn or Yes, accounts for most of this activity. Residual activity in Src-depleted lysates is somewhat higher than after kinase removal with src-CT. This difference may originate from src-CT antisera preferentially binding to the more exposed C terminus of kinase active Src. Lanes 1 and 3 and 2 and 4 are duplicates. PAS and PGS, protein A- and protein G-Sepharose, respectively. C, phosphorylation of β9 by purified Src. Isolated Src kinase was incubated for 3 h at 25°C with 5 µg of β9, GST, or no substrate under phosphorylating conditions including [γ-32P]ATP. Autoradiography reveals that β9 (~34 kDa) and Src (~60 kDa), but not GST, become phosphorylated efficiently. D, time and temperature dependence of Src-mediated β9 phosphorylation. Phosphorylated β9 protein bands were excited from SDS-polyacrylamide gels and quantitated by liquid scintillation counting. Phosphorylation reaches a plateau after about 5 h at 25 or 37°C. (Fig. 5A, lanes 5–12). Analysis of the depleted C2 lysates confirmed that each antibody removed a substantial fraction of its corresponding kinase without affecting the others (Fig. 5B). Src-CT removes all kinases, including Src, to an intermediate degree, yet depletes the phosphorylating activity slightly better than the Src-specific antibody. This discrepancy may be due to preferential depletion by src-CT of the enzymatically active form of Src in which its C terminus is not engaged in an intramolecular SH2 domain interaction but is exposed and therefore more accessible for antibody recognition (33). Together, these results show that the β exon 9 fusion protein is phosphorylated by bound Src, and, as Src family kinases are known to phosphorylate themselves, they suggest that phosphorylation of bound Src is due to autophosphorylation. To confirm the ability of Src to phosphorylate the β9 fusion protein, we incubated the fusion protein with purified Src kinase and [γ-32P]ATP. Phosphorylation of the β9 fusion protein, but not the parental GST protein, was observed (Fig. 5C). Phosphorylation was more efficient at 25 or 37°C than at 4°C (Fig. 5D); under optimal conditions, 24 h at 25°C, about 1% of the β9 fusion protein was phosphorylated.

**Src and Fyn Are Bound to the AChR in C2 Myotubes—**To determine whether Src interacts with the AChR in vivo as it does with the β subunit fusion proteins in vitro, we examined the AChR purified from muscle cells for associated Src family kinases. C2 myobute cultures were extracted with a mild detergent (1% digitonin or Nonidet P-40) and the AChRs precipitated using α-bungarotoxin conjugated to Sepharose beads. The beads were then stripped under denaturing conditions and the proteins analyzed by SDS-PAGE and immunoblotting. When immunoblots were probed with src-CT (Fig. 6A), a Src family kinase was observed. The kinase was not observed when uncoupled Sepharose beads were used as a control nor when binding of the AChR to the beads was blocked by preincubation of the receptor in the cell lysates with free toxin. Thus the presence of the Src family kinase on the beads was dependent on its association with the AChR. Accordingly, the Src kinase was seen on immunoblots only when the AChR was also present, as observed by stripping the blots and reprobing them with an antibody to the β subunit (Fig. 6C). In contrast, a contaminating src-CT reactive band of ~90 kDa and unknown identity was isolated under all conditions tested. Stripped immunoblots were also analyzed with antibodies to phosphorysotyrosine. Two bands were consistently seen (Fig. 6B), a 50-kDa band whose mobility was identical to the β subunit of the AChR (Fig. 6C) and a 60-kDa band with the same mobility as the Src-related kinase seen with src-CT (Fig. 6A). A third band of ~110 kDa (Fig. 6B) was not consistently observed. Tyrosine phosphorylation of certain proteins, such as the δ subunit of the AChR in chick, is reported to be sensitive to degradation during boiling in SDS loading buffer (15). However, we did not see additional phosphorysotyrosine bands specifically precipitated with α-bungarotoxin-Sepharose beads, even when unboiled samples were used and when immunoblots were analyzed with an alternative phosphorysotyrosine antiserum (data not shown).

The identity of the Src-related kinase(s) associated with the AChR was determined on immunoblots using the kinase-specific antibodies. As shown in Fig. 7, two kinases were specifically co-isolated with the AChR, Fyn and Src; no detectable Yes was seen. Under these conditions, the fraction of each kinase associated with the AChR was small: approximately 0.04% of the total Fyn and 0.01% of the total Src in the extract were recovered with the AChR from α-bungarotoxin-Sepharose. By comparing the amount of isolated AChR to the receptor content of the initial lysate, on the other hand, we estimate that we recover only about 4% of the total AChR in the extract (data not shown).
FIG. 6. Specific co-isolation of a Src-related kinase(s) with the AChR from C2 myotubes. C2 myotubes were lysed in a buffer containing 1% digitonin. AChRs were isolated by an incubation for 2 h at 4°C with α-bungarotoxin conjugated to Sepharose beads. As controls, we either added 10 μM excess free toxin to the lysate to compete for AChR binding to toxin-Sepharose, or we used nonconjugated Sepharose beads. A, analysis of co-isolated Src-related kinase(s) was performed by immunoblotting with src-CT. One or several kinases were isolated in a specific manner with toxin-Sepharose beads (solid arrowhead). The ~90-kDa src-CT-reactive, contaminating band represented nonspecific binding as it was found in both control and experimental samples. A fraction of the initial cell lysate was analyzed as a control (dotted arrowhead). Analysis of the ~90-kDa src-CT-reactive, contaminating band represented nonspecific binding as it was found in both control and experimental samples. B, the nitrocellulose membrane was stripped and reprobed with an antibody recognizing phosphotyrosine. C, a second reprobing of the same blot was performed with antibody m124 reactive with the AChR β subunit. The absence and presence of the β subunit (open arrowhead) confirms that the AChR is specifically isolated with toxin-Sepharose. In this experiment, the amount of the control lysate loaded (L) was too small for the β subunit to be visualized with m124 antisera. Analysis shows that one or several Src-related kinases are co-isolated with the AChR and that the kinase(s) and the β subunit of the AChR are tyrosine-phosphorylated. Similar results were obtained when using Nonidet P-40 instead of digitonin as the detergent.

shown). Therefore we calculate that in C2 myotubes about 1% of the total Fyn and about 0.25% of the total Src are associated with AChRs. Taken together these experiments show that the AChRs in C2 myotubes contain tyrosine-phosphorylated β subunits and are specifically associated with two tyrosine kinases of the Src family, Src and Fyn.

DISCUSSION

We have investigated the mechanism by which the AChR becomes tyrosine-phosphorylated in mammalian C2 myotubes and find that the tyrosine kinase Src associates with, and phosphorylates, the region encoded by exon 9 in the N-terminal half of the cytoplasmic loop of the β subunit of the AChR. In addition, AChRs isolated from C2 myotubes were specifically associated with two Src-related kinases, Src and Fyn, and the AChRs were found to have tyrosine-phosphorylated β subunits.

As judged by several criteria, binding of Src to GST fusion proteins containing the β exon 9 region was specific. First, binding of Src to this region occurred in a sequence-specific way. Binding was not observed to fusion proteins containing only the C-terminal half of the β loop, encoded by exon 10, nor to GST protein lacking AChR segments. Second, binding of β exon 9 fusion proteins specifically occurred to Src, but not to Fyn or Yes, two other members of the Src family. Third, the corresponding regions from the γ and δ subunits of the AChR, although highly homologous to the exon 9 region of β, did not show detectable binding to either Src, Fyn, or Yes. Fourth, and most importantly, the β exon 9 region was a substrate for both purified Src and for bound Src derived from muscle cell extracts. Moreover, as the phosphorylation activity was abolished by immunodepletion of C2 lysates with Src-specific antibodies, Src appears to be the major, and perhaps the only, tyrosine kinase in C2 myotubes that constitutively recognizes, binds, and phosphorylates initially unphosphorylated β subunits of the AChR. Src-mediated phosphorylation of the β subunit may involve Tyr-357 but is more likely to occur on Tyr-390; the corresponding tyrosine of the Torpedo AChR is phosphorylated and the flanking amino acids show features of consensus tyrosine phosphorylation sites (34). Detailed analysis reveals that the residues surrounding Tyr-390 resemble sites phosphorylated by receptor tyrosine kinases more closely than motifs preferred by cytosolic kinases (34). Our observation that Src phosphorylates the β9 region, however, still renders Tyr-390 a potential substrate for this kinase. Adsorption with fusion proteins followed by phosphorylation revealed that the β exon 9-homologous regions of δ and γ fail to associate with tyrosine-phosphorylating activity (data not shown), which correlates with the inability of Src family kinases to bind to these regions.

This specificity is notable, because the AChR isolated from postsynaptic membranes of the Torpedo electric organ can be tyrosine-phosphorylated in vitro at a high stoichiometry on its β, γ, and δ subunits (9) and because the critical β and δ tyrosine residues are conserved in the mouse AChR subunits. The selective binding of the unphosphorylated β subunit, but not the δ or γ subunits, to Src, suggests that this interaction may be relevant to AChR phosphorylation during synaptogenesis at the mammalian neuromuscular junction.

As binding of the β exon 9 region to Src is not phosphotyrosine-dependent, it does not appear to involve the SH2 domain of Src. Apart from the catalytic region, Src contains two other functional domains that could be responsible for binding to the β exon 9 segment, its unique N-terminal domain and its SH3 domain (29, 30, 33). The exon 9 region of β contains 10 prolines, whereas the corresponding γ and δ segments only show 5 and 4, respectively. However, since the proline residues in β are distributed throughout the exon 9 region and do not show the high proline density found in typical polyproline-SH3 domain interactions (35, 36), we assume that binding to β does not occur via the SH3 domain of Src. Binding may rather involve the unique domain of Src, as the unique domains of Src-related kinases have been shown to mediate constitutive binding to a
The number of transmembrane receptors (29). For example, the unique region of Lck is responsible for its association with CD4 (37), and binding to components of the T- and B-cell receptors has been shown to involve the unique domains of Fyn and Lyn (38, 39). Interestingly, to our knowledge, no constitutive interactions have been described between Src and transmembrane proteins lacking kinase activity, although Src is well known to bind via its SH2 domain to activated, phosphorylated receptor tyrosine kinases such as the platelet-derived growth factor receptor (40). Our observed binding of Src to the β subunit of the AChR may thus represent a class of Src interactions that is specific for differentiated post-mitotic cells such as myotubes. One possibility that we cannot exclude is that a third, ancillary protein mediates the binding of Src to the β exon 9 region. If such a protein is involved, however, it is not subject to tyrosine phosphorylation, as our assays did not identify any phosphorylated proteins other than Src and the fusion proteins.

None of the GST fusion proteins tested bound to Fyn or Yes, yet AChRs isolated from C2 myotubes by α-bungarotoxin-Sepharose are associated with Fyn as well as with Src. While we cannot exclude the possibility that association of the AChR with Fyn involves one or several domains of the receptor not covered by our fusion proteins, it seems most likely that this association involves an interaction between the tyrosine-phosphorylated AChR β subunit and the SH2 domain. This inference is based on the observations that in C2 myotubes the β subunit of the AChR is tyrosine-phosphorylated (Fig. 6) and that in the electric organ of Torpedo phosphorylated AChRs are associated with the SH2 domains of two kinases of the Src family, Fyn and Fyk (10, 11). In co-immunoprecipitation experiments, Fyn interacted both with phosphorylated δ and β subunits, whereas Fyk preferentially interacted with phosphorylated δ subunits. Expression of Fyk, a Src-related kinase with homologies to Fyn and Yes, has not been reported in organisms other than Torpedo. Furthermore, in our precipitates with α-bungarotoxin-Sepharose, we failed to detect tyrosine-phosphorylated proteins other than the AChR β subunit, Src, or Fyn. Together, these findings suggest that abundant tyrosine phosphorylation of the AChR δ subunit, as well as binding of Fyk to phosphorylated δ, may occur specifically in the electric organ of Torpedo but not in mammalian muscle. In mammalian C2 myotubes, on the other hand, our findings suggest a model in which newly synthesized, unphosphorylated AChRs are initially tyrosine-phosphorylated by Src on their β subunits. The phosphorylated β subunits may then interact with the SH2 domain of Fyn. Src may remain bound to the β subunits to yield a Fyn-Src-AChR complex or dissociate from the AChR prior to its association with Fyn.

What is the functional significance of the association of the AChR with Src and Fyn in C2 myotubes? The most likely hypothesis is that AChR phosphorylation is related to clustering and immobilization of the AChR in the myotube membrane. Phosphotyrosine labeling, as observed by immunofluorescence, is concentrated both at the mature synapse and at AChR clusters in developing myotubes (14, 15, 19). In addition, a variety of physiological and nonphysiological stimuli that result in AChR aggregation, including agrin, neuregulin, basic fibroblast growth factor, polymer beads, and electric fields, appear to act through mechanisms that involve protein-tyrosine kinase activation (18, 41–44). A number of postsynaptic proteins such as β-dystroglycan, syntrophin, paxillin, the 87-kDa protein, or dystrophin contain tyrosine phosphorylation consensus sites or have been shown to be tyrosine-phosphorylated in Torpedo electric organ (45–49). In our experiments with unstimulated C2 myotubes, small but significant fractions of the total kinases present in C2 cells are associated with the AChRs, ~1% of the total Fyn and ~0.25% of the total Src. Considering the high level of AChR expression in C2 myotubes, we thus expect only a small proportion of surface AChRs to be associated with Src and Fyn. C2 myotubes spontaneously form a small number of AChR clusters (27), and the phosphorylated AChRs and those associated with kinases could represent AChRs that are in the process of spontaneous cluster formation. Such a mechanism could involve phosphorylation of critical muscle proteins by AChR-associated Src and/or Fyn kinases and subsequent immobilization of phosphorylated AChRs by protein-protein recognition mediated by SH2 domain-phosphotyrosine interactions. In agreement with this idea, immunofluorescence studies have shown that C2 myotubes contain some domains composed of coextensive aggregates of phosphotyrosine and AChRs (19).

An important question raised by these experiments is whether Src and Fyn are part of the agrin signaling pathway. A number of experimental observations link tyrosine phosphorylation of the AChR with agrin-induced clustering. After treatment with agrin, tyrosine phosphorylation of the AChR occurs before AChR clustering; and inhibitors that block tyrosine phosphorylation of the AChR also block clustering (20, 21, 50). The agrin dose-response curve is the same for both events, and the two are correlated under several different conditions (20). Agrin has recently been shown to act via a receptor tyrosine kinase, MuSK, which apparently acts as part of a receptor complex involving other, unidentified proteins (23). Activation of MuSK has been shown to result in both autophosphorylation and tyrosine phosphorylation of the AChR (23, 24). Thus, although the intracellular signaling pathway by which agrin induces AChR clustering is unknown, it appears to involve activation of protein-tyrosine kinases and tyrosine phosphorylation, perhaps of the AChR itself.

A possible interpretation of the association of Fyn and Src with the AChR in unstimulated cells and its relevance for agrin's signaling pathway is suggested by consideration of other receptors. Several signaling receptors that lack intrinsic tyrosine kinase activities, such as cytokine (e.g., interleukin-2) and lymphocyte (e.g., T-cell and B-cell) receptors, are hetero-oligomeric transmembrane proteins that constitutively associate with nonreceptor tyrosine kinases of the Jak and Src family, respectively, before onset of signaling (51–53). Upon extracellular stimulation with ligand, the cytoplasmic kinases associated with the receptors phosphorylate them, thereby beginning the signaling cascade. In a similar way, activation of the agrin receptor could, through as yet undefined steps, result in phosphorylation of the AChR by bound Src or Fyn and in increased association of the AChR with Fyn. This may then lead to phosphorylation of other postsynaptic proteins and/or their association with the AChR. Alternatively, tyrosine phosphorylation of the AChR may be unrelated to the initial clustering events but rather to downstream events in the pathway regulating AChR aggregation. Accordingly, the initial clustering of AChRs, leading to formation of microclusters, could itself result in activation of receptor-associated Src or Fyn. Tyrosine phosphorylation of the AChR might thus be related to enlargement and stabilization of the clusters or to the recruitment of other proteins to the aggregates. In any case, the elucidation of the signaling pathways and the protein-protein interactions related to AChR clustering is an important area of future research. Our experiments indicate that Src and Fyn, associated with the AChR, are likely to play important roles in these pathways.

Acknowledgments—We are grateful to Dr. Stephen Hardy for helpful advice on the construction and expression of GST fusion proteins. We also thank Drs. Michael Ferns and Svetlana Sh trom for their help with
the preparation of α-bungarotoxin-Sepharose and Pam Schwartzberg for insights into signaling by Src-related kinases. Members of the Hall lab are acknowledged for critically reading the manuscript and Dr. Michael Ferns for helpful advice during the course of this study.

Note Added in Proof—Holmes et al. (Holmes, T. C., Fadool, D. A., Ben, R., and Levitan, I. B. (1996) Science, in press) have found that the potassium channel, hKv1.5, is also associated Src tyrosine kinase and that the channel is tyrosine-phosphorylated. Regulation of ionic channel function via interaction with Src may thus be a general phenomenon.

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