Phosphorylation and Cytoskeletal Anchoring of the Acetylcholine Receptor by Src Class Protein-tyrosine Kinases

ACTIVATION BY RAPSYN*

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Src class protein-tyrosine kinases bind to and phosphorylate the nicotinic acetylcholine receptor of skeletal muscle. This study provided evidence for the functional importance of Src kinases in regulating the nicotinic acetylcholine receptor at the neuromuscular junction. Three Src class kinases, Fyn, Fyk, and Src, each formed a complex with the endplate-specific cytoskeletal protein rapsyn. In addition, cellular phosphorylation by each kinase was stimulated by rapsyn in heterologous transfected cells. Several lines of evidence supported rapsyn as a substrate for Src kinases. Most importantly, rapsyn regulation of Fyn, Fyk, and Src resulted in phosphorylation of the nicotinic acetylcholine receptor β and δ subunits and anchoring of the receptor to the cytoskeleton. Both nicotinic acetylcholine receptor phosphorylation and cytoskeletal anchoring were blocked by the Src kinase-selective inhibitor herbimycin A. Rapsyn alone also induced a modest increase in nicotinic acetylcholine receptor phosphorylation and cytoskeletal translocation. However, inhibition by herbimycin A and a catalytically inactive dominant negative Src demonstrated that the effects of rapsyn were mediated by endogenous Src kinases. These data support the importance of Src class kinases for stabilization of the nicotinic acetylcholine receptor at the endplate during synaptic differentiation at the neuromuscular junction.

Synaptic transmission is a precise but dynamic function mediated by a unique subcellular structure containing specialized proteins. A fundamental question in neurobiology is what are the molecular mechanisms underlying synaptogenesis. Historically, the neuromuscular junction of skeletal muscle has provided a system in which to identify the specialized proteins, characterize their function, and begin to determine how the synapse is formed (for a review, see Ref. 1). A crucial process during synaptogenesis is the recruitment of postsynaptic elements to the developing endplate. Prior to innervation, the immature myotube expresses nicotinic acetylcholine receptors (AChR) that are diffusely distributed throughout the membrane. However, upon innervation, the receptors aggregate to very high concentrations at the site of nerve muscle contact and become anchored there (2). The signal transduction cascade by which the motor neuron induces AChR clustering on the myotube has been under intense investigation. Agrin has been identified as a neuronally derived extracellular signal for AChR clustering (3). The receptor for agrin appears to include the transmembrane muscle-specific kinase MuSK (4, 5). Thus, agrin and its receptor MuSK are important for initiating AChR clustering in response to the motor neuron.

Rapsyn is an intracellular peripheral membrane protein crucial for postsynaptic localization of the AChR. Rapsyn is expressed in a one-to-one ratio with the AChR (6), can be cross-linked to the receptor (7), and is precisely colocalized with the AChR from the earliest times of cluster formation (8). Expression of rapsyn with the AChR in nonmuscle cells is sufficient to induce receptor clustering (9, 52, 53). Furthermore, rapsyn is necessary for AChR clustering as demonstrated by targeted disruption of the Rapsyn gene; mice lacking rapsyn die soon after birth, and the cultured myotubes from these mice do not form either spontaneous or agrin-induced AChR clusters (10). Complementing its effect on clustering, several lines of evidence suggest that rapsyn forms a cytoskeletal anchor for the AChR. Extraction of rapsyn from the postsynaptic membrane increases the rotational mobility of the AChR (11). In addition, rapsyn is an actin-binding protein (12), and cotransfection of rapsyn with the AChR increases linkage of the receptor to the cytoskeleton (13). Recently, rapsyn has been shown to induce cocolustering of the AChR with dystroglycan, a component of the dystrophin glycoprotein complex that functions as a transmembrane link between the extracellular matrix and the intracellular cytoskeleton in muscle (14). In addition, rapsyn is in close association with dystroglycan as demonstrated by chemical cross-linking (15). Thus, rapsyn appears to mediate both AChR clustering and anchoring of the receptor to the cytoskeleton.

Protein tyrosine phosphorylation has been increasingly implicated as an important intracellular signaling mechanism for modulating synaptic transmission at the neuromuscular junction. Postsynaptically, protein tyrosine phosphorylation regulates the AChR in three independent ways. First, the AChR itself is phosphorylated on tyrosine residues and this phosphorylation regulates the kinetics of receptor desensitization (16).

1 The abbreviations used are: AChR, nicotinic acetylcholine receptor(s); Btk, Bruton’s tyrosine kinase; CT-Src antibody, C-terminal anti-pan-Src class kinase antibody; pBK-CMV, pBK-CMVΔlac; SH2 and SH3, Src homology 2 and 3, respectively; Src, c-Src; SrcK— the dominant negative inactive c-Src; MuSK, muscle-specific kinase; PAGE, polyacrylamide gel electrophoresis.
Second, a receptor protein-tyrosine kinase, ErbB, mediates synapse-specific transcription of the AChR (17). Third, phosphorylation of the AChR and/or other postsynaptic proteins on tyrosine residues appears to be important for receptor aggregation during synaptogenesis.

Numerous lines of evidence indicate that protein tyrosine phosphorylation is critical for AChR clustering and cytoskeletal anchoring at the endplate. The AChR of innervated myotubes is both phosphorylated and clustered, while receptor from noninnervated myotubes is neither phosphorylated nor clustered (18–20). Agrin stimulates AChR phosphorylation as well as clustering (19, 21). The agrin-induced phosphorylation precedes AChR clustering, and protein-tyrosine kinase inhibitors block both phosphorylation and clustering, consistent with a causal relationship (21–23). In addition, MuSK, a component of the agrin receptor, is a protein-tyrosine kinase (5). Cotransfection of MuSK with rapsyn activates MuSK, resulting in AChR phosphorylation (24), and MuSK has been shown to form a complex with AChR (25). Furthermore, mice with a disruption of the gene encoding MuSK do not form neuromuscular synapses (26). In addition to clustering, AChR stabilization at the endplate appears to involve tyrosine phosphorylation. Agrin-induced cytoskeletal anchoring of the receptor shows a close temporal correlation with phosphorylation, and increasing AChR phosphorylation with a phosphatase inhibitor results in cytoskeletal association (27–29). Furthermore, a kinase inhibitor that blocks agrin-induced AChR tyrosine phosphorylation also inhibits cytoskeletal anchoring of the receptor (22).

These studies support the importance of protein tyrosine phosphorylation in AChR localization to the endplate; however, the precise mechanism by which phosphorylation is involved is still unclear.

We have identified two Src class protein-tyrosine kinases, Fyn and Fyk, that may participate in the signal transduction cascade regulating the AChR (30, 31). Together, Fyn and Fyk comprise the predominant protein-tyrosine kinase activity in the AChR-enriched Torpedo californica electric organ postsynaptic membrane, a biochemical model of the neuromuscular junction endplate (30). Fyn and Fyk form a complex with the AChR and can directly phosphorylate the receptor in vitro (30). The association of the receptor with the two kinases is mediated by a high affinity binding of the tyrosine-phosphorylated AChR δ subunit to the Src homology 2 (SH2) domains of the kinases (31). Others have shown that in the C2C12 mouse muscle cell line, the AChR is associated with Fyn and c-Src (Src) and that an AChR β subunit fusion protein can be phosphorylated by Src (32). These results suggest that Src family protein-tyrosine kinases may phosphorylate and regulate the AChR in vivo. In both neuronal and nonneuronal systems, Src family kinases have been implicated in cytoskeletal function (33, 34). In this study, we have begun to investigate how Src class kinases fit into the signal transduction cascade by which the AChR is regulated. We have tested the ability of Fyn, Fyk, and Src to be regulated by rapsyn, to phosphorylate the AChR and rapsyn, and to induce anchoring of the receptor to the cytoskeleton (64).

EXPERIMENTAL PROCEDURES

Expression Constructs—The full-length coding sequence of Torpedo Fyn (30) was subcloned into the EcoRI and HindIII sites of the expression vector pBK-CMVlac (pBK-CMV) using polymerase chain reaction techniques. The full-length Torpedo Fyk containing the 5′- and 3′-untranslated sequences (30) was excised from the pBS vector and subcloned into the EcoRI site of pBK-CMV. The mouse rapsyn in pGWI-CMV was a gift from Dr. R. L. Huganir (Johns Hopkins University) (24). Src and the dominant negative c-Src (SrcK) in the expression vector pcDNA3 were gifts from Dr. S. Parsons of the University of Virginia (35). The Bruton’s tyrosine kinase (Btk) cDNA in the mammalian expression vector pCI5-2 was a gift from Dr. S. Desiderio (Johns Hopkins University) (36).

Antibodies—The anti-Fyn and anti-Fyk polyclonal antibodies have been described previously (30). Anti-rapsyn 1234A (37) and anti-AChR γ and δ subunits (88b) (38) antibodies were gifts from Dr. Stanley Steinbach (University of North Carolina). The anti-aphosphotyrosine monoclonal (JH233) (18), phosphorylation-specific anti-AChR β subunit (JH1360) (24), phosphorylation-specific anti-AChR δ subunit (JH1358) for which recognition of the AChR δ subunit was dependent on tyrosine phosphorylation (data not shown), and anti-AChR α subunit monoclonal (number 109) (24) antibodies were all generous gifts from R. L. Huganir. The anti-Btk antiserum 1280 was a gift from S. Desiderio. The anti-phosphotyrosine monoclonal antibody (4G10) (24) was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). The C-terminal anti-Src antibody that recognizes all Src kinases, SRC 2 (CT-Src); the N-terminal anti-Src antibody that recognizes only Src kinase, C-SRC N-16; and the monoclonal antiphosphotyrosine antibody (PY99) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Cell Culture and Transfection—The Q-F18 cell line expressing high levels of Torpedo fetal AChR (15). In each case, Dr. J. Huganir was maintained as described (39). Cultures at about 50% confluence were transfected using the CaPO4 precipitation method (40). Cells were transfected with 20 μg/100-mm dish of total plasmid DNA; 10 μg of each construct was used and supplemented, if necessary, with empty pCMV. Using the Green Lantern vector (Life Technologies, Inc.), transfection efficiency was determined to be ≥50%. In some experiments, cells were transfected in the presence of 1 μM herbimycin A, a SO carrier. The protein concentration per plate was not affected by expression of Src family kinases, rapsyn, or herbimycin A (data not shown).

Torpedo Electric Organ Tissue Preparation—Homogenate and postsynaptic membranes of Torpedo electric organ were prepared by methods we have previously described (30).

Immunoprecipitation and Coimmunoprecipitation—Transfected Q-F18 electric organ cultures of Torpedo electric organ fractions were solubilized in 50 mM Tris, pH 7.4, 150 mM NaCl, 2 mM EDTA, 1 mM EGTA, 1% Triton X-100, and 1 mM NaVO4 (lysis buffer) without or with 1% deoxycholate and 0.1% or 0.2% SDS for 20 min on ice (30). For analysis of in vivo rapsyn phosphorylation, Torpedo electric organ homogenate was solubilized in lysis buffer without Triton X-100 but containing 2% SDS, treated with or without boiling for 3 min, diluted in lysis buffer to a final concentration of 0.4% SDS and 2% Triton X-100 at 1 mg/ml protein and sonicated twice. Lysates were spun at 225,000 g for 10 min, and supernatant proteins were immunoprecipitated as described (30). Immunoprecipitates were analyzed for coprecipitated proteins by immunoblotting.

Immunoblotting—Proteins were resolved by SDS-PAGE and transferred to polyvinylidene difluoride membranes (Immobilon, Millipore Corp.) by electroblotting. The polyvinylidene fluoride membranes were blocked in Blotto (Pierce). The antibodies in Blotto were all diluted into acetylcholine-Sepharose affinity resin (41). In short, cells were homogenized in 62.5 mM Tris, pH 6.8, 2% SDS, and 100 mM EDTA, 10 mM EGTA, 10 mM sodium pyrophosphate, 0.1 mM phenylmethylsulfonyl fluoride, 0.1 mM leupeptin, 10 μg/ml pepstatin, 10 μg/ml chymostatin, 10 μg/ml antipain, 10 units/ml trasylol, 20 mM benzamidine, 1 mM sodium vanadate, and 1% Triton X-100. The lysates were centrifuged at 15,000 × g for 10 min, and the supernatants were diluted into an equal volume of the above buffer containing 100 mM NaF and 20 mM iodoacetamide and used for purification of AChR. In some
The samples were sonicated for 30 s and spun at 225,000 x g, and Triton X-100 pellets subsequently solubilized with SDS-PAGE sample buffer. Supernatants were boiled for 3 min. The supernatants representing the membranes protein were analyzed by the immune complex kinase assay as described (43). Excised from polyacrylamide gels and processed as described (30).

**Protein Determinations**—Protein concentrations were determined by the method of Lowry using bovine serum albumin as a standard (43).

**RESULTS**

**Regulation of Src Class Protein-tyrosine Kinases by Rapsyn**—A role for rapsyn in protein phosphorylation by Src family kinases was examined by testing whether Fyn, Fyk, and Src are regulated by rapsyn. First, association of rapsyn with Fyn and Fyk in *Torpedo* electric organ postsynaptic membranes was examined. After solubilization of postsynaptic membranes under mild detergent conditions of 1% Triton X-100, rapsyn was isolated by immunoprecipitation, and the precipitates were used in the presence of Src class kinases by Western blotting using an antibody that recognizes all members of the Src family. As shown in Fig. 1A, one or more Src class kinases was enriched in the rapsyn immunoprecipitate compared with the precipitation with empty protein G-Sepharose beads. Densitometry scanning analysis demonstrated that 0.4 ± 0.2% (mean ± S.E.; n = 4) of the postsynaptic Src class kinases were associated with rapsyn. Library screening as well as polymerase chain reaction analysis have indicated that Fyn and Fyk are the only Src class kinases expressed in *Torpedo* electric organ; Src itself does not appear to be expressed in this tissue. Thus, the data in Fig. 1A indicated that rapsyn formed a complex with Fyn and/or Fyk in *Torpedo* electric organ. Specific complex formation between Fyn and Fyk was tested using the complementary approach. Fyn and Fyk were immunoprecipitated from electric organ postsynaptic membrane proteins solubilized with 1% Triton X-100, and the precipitates were analyzed for rapsyn. Rapsyn was detected in both the Fyn and Fyk but not the preimmune immunoprecipitates (Fig. 1B). Densitometry scanning analysis demonstrated that 0.14 ± 0.05% of the postsynaptic rapsyn was associated with Fyn, while 0.46 ± 0.09% was associated with Fyk under these conditions (mean ± S.E.; n = 5). With higher stringency solubilization buffer containing deoxycholate and SDS, coprecipitation of rapsyn with Fyn and Fyk was reduced (Fig. 1B). Thus, rapsyn forms a specific but apparently low affinity complex with Fyn and Fyk in *Torpedo* electric organ postsynaptic membranes.

The specificity of complex formation between rapsyn and Src kinases was examined using the Q-F18 cell line as a heterologous transfection system. This system has been extensively utilized to dissect the molecular mechanism for rapsyn’s role at the neuromuscular junction (9, 13, 24, 44). When Fyn, Fyk, or Src was expressed with rapsyn in the Q-F18 cell line, rapsyn immunoprecipitated with each of the kinases (Fig. 1C). In contrast, rapsyn did not immunoprecipitate with coexpressed Btk, a nonreceptor protein-tyrosine kinase that contains SH2 and SH3 domains (45), thus demonstrating Src class kinase specificity for complex formation with rapsyn (Fig. 1C). These data demonstrated that all three Src class kinases that have been implicated in regulating the AChR, Fyn, Fyk, and Src, were able to form a specific complex with rapsyn.

**FIG. 1. Coimmunoprecipitation of rapsyn with Src class protein-tyrosine kinases.** A, *Torpedo* electric organ postsynaptic membrane proteins (1 mg) were solubilized at 1 mg/ml in lysis buffer and centrifuged for 10 min at 225,000 x g, and the supernatants were immunoprecipitated with anti-rapsyn (1234A) antibody (aRap) or empty protein G-Sepharose (POS) as described under "Experimental Procedures." The immunoprecipitates and 30 μg of solubilized postsynaptic membrane supernatant after centrifugation (PS) were resolved by 8% SDS-PAGE and analyzed by Western blotting using a pan-Src antibody (CT-Src) as described under "Experimental Procedures." The arrowhead indicates the position of rapsyn. B, *Torpedo* electric organ postsynaptic membrane proteins (500 μg) were solubilized at 1 mg/ml in lysis buffer without (Triton) or with (Triton/DOC/SDS) 1% deoxycholate plus 0.1% SDS and centrifuged for 10 min at 225,000 x g, and the supernatants were immunoprecipitated with anti-Fyn (aFyn), anti-Fyk (aFyk), or preimmune (Prei) serum as described under "Experimental Procedures." The immunoprecipitates and 1 μg of solubilized postsynaptic membrane supernatant after centrifugation (PS) were resolved by 10% SDS-PAGE and analyzed by Western blotting using an anti-rapsyn monoclonal antibody (1234A) as described under "Experimental Procedures." Molecular mass markers, in kilodaltons, are indicated to the left. The arrowhead indicates the position of rapsyn.

C, Q-F18 cells were transfected as described under "Experimental Procedures" with rapsyn pGW-CMV plus either Fyn pBK-CMV (Fyn + Rapsyn), Fyk pBK-CMV (Fyk + Rapsyn), Src pCDNA3 (Src + Rapsyn), or pCI-NS2-Btk (Btk + Rapsyn) as indicated. After 48 h, cells were scraped from the dish, solubilized in lysis buffer, and centrifuged at 225,000 x g for 10 min. Supernatants representing 750 μg of protein were immunoprecipitated with anti-Fyn (aFyn), anti-Fyk (aFyk), anti-Src (aSrc), anti-Btk (aBtk), or preimmune (Prei) serum as indicated. The immunoprecipitates and 30 μg of the lysate supernatants after centrifugation (Lys) were analyzed by 10% SDS-PAGE and Western blotting using an anti-rapsyn monoclonal antibody (1234A) as described under "Experimental Procedures." The arrowhead indicates the position of rapsyn.

2 S. L. Swope, unpublished data.
Association between Src family kinases and rapsyn may serve either a structural or regulatory function. A regulatory role for the interaction was tested by examining the effect of rapsyn on total cellular phosphorylation by Src kinases. Q-F18 cells were transfected with Fyn, Fyk, or rapsyn, all three kinases were reduced in the soluble compartment (Fig. 3A) and cytoskeletal (Fig. 3B) fractions. Upon coexpression of rapsyn, all three kinases were reduced in the soluble compartment (Fig. 3A) and increased in the cytoskeletal compartment (Fig. 3B). Quantification by densitometry scanning demonstrated that, compared with kinase expression alone, Fyn, Fyk, and Src levels in the soluble fraction were reduced to 88% ± 4%, 65% ± 9%, and 72% ± 7%, respectively, by coexpression of rapsyn (mean ± S.E., n = 7). In a complementary fashion, Fyn, Fyk, and Src levels in the cytoskeleton were increased to 210% ± 60%, 150% ± 30%, and 160% ± 30%, respectively, by coexpression of rapsyn (mean ± S.E., n = 6). These data demonstrated that, for each kinase, rapsyn induced a modest but significant increase in cytoskeletal association, apparently due to a translocation from the soluble compartment. Thus, the ability of rapsyn to preferentially activate phosphorylation by cytoskeleton-associated Fyn, Fyk, and Src as shown in Fig. 2 may reflect two mechanisms: activation of phosphorylation by kinases already present in the cytoskeleton and translocation of activated kinases to the cytoskeleton.

**Phosphorylation of Rapsyn**—The primary sequence of mouse rapsyn suggests a tyrosine phosphorylation motif surrounding Tyr^{261}GlulGlu-Thr-Glu-Leu-Tyr^{261}-Cys-Gly (46). This motif is conserved in the *Torpedo* and human forms of rapsyn, indicat-
Regulation of the AChR by Src Kinases

In vitro phosphorylation of rapsyn on tyrosine residues was also examined in Torpedo electric organ. Solubilized Torpedo electric organ postsynaptic membrane proteins were immunoprecipitated with anti-rapsyn, anti-Fyk, anti-Fyn, or preimmune serum and incubated under phosphorylating conditions with [γ-32P]ATP. Autophosphorylation of Fyn and Fyk was observed (Fig. 4B) as previously demonstrated (30). In addition, a predominant phosphorylated 43-kDa band was detected in the rapsyn immunoprecipitate (Fig. 4B). Phosphorylation of the 43-kDa band occurred on serine and tyrosine residues as demonstrated by phosphoamino acid analysis (Fig. 4C). These data are consistent with protein-tyrosine and serine kinases coimmunoprecipitating with rapsyn and phosphorylating the cytoskeletal protein in vitro. Since Fyn and Fyk coimmunoprecipitated with rapsyn (Fig. 1, A and B), these data suggest that the in vitro tyrosine phosphorylation could be mediated by Fyn and/or Fyk.

The ability of rapsyn to phosphorylate the AChR by Src kinases, the Q-F18 heterologous cell system was used. Rapsyn was expressed in Q-F18 cells with or without coexpression of Fyn, Fyk, or Src. The cellular proteins were solubilized under stringent conditions, 1% deoxylcholate and 0.2% SDS, in which rapsyn was not coimmunoprecipitated with Fyk (64), immunoprecipitated with anti-phosphotyrosine antibody, and analyzed by anti-rapsyn Western blotting. Rapsyn was immunoprecipitated by the anti-phosphotyrosine antibody after cotransfection with the kinases (Fig. 4D). The precipitation was specific, since rapsyn did not immunoprecipitate with preimmune serum. In addition, rapsyn was not immunoprecipitated with the anti-phosphotyrosine serum when expressed in the absence of kinases (Fig. 4D). These data demonstrated that rapsyn could be tyrosine-phosphorylated in an Src class kinase-dependent manner. The fact that Fyn, Fyk, and Src formed a complex with rapsyn, that rapsyn could be phosphorylated by a coimmunoprecipitating protein-tyrosine kinase, that rapsyn was phosphorylated on tyrosine residues in vivo, and that rapsyn was tyrosine-phosphorylated upon cotransfection with Fyn, Fyk, and Src strongly suggested that this cytoskeletal element is a substrate for Src class kinases.

**Phosphorylation and Cytoskeletal Anchoring of the AChR by Src Class Kinases**—The major objective of this study was to test for regulation of the AChR by Src family kinases. Since rapsyn activates cellular phosphorylation by Fyn, Fyk, and Src and rapsyn and Src kinases are important for cytoskeletal function, we have examined both phosphorylation and translocation of the AChR to the cytoskeleton in response to rapsyn and the kinases. To test for an effect on phosphorylation, Q-F18 cells expressing the AChR were transfected in order to express Fyn, Fyk, Src, or rapsyn alone or in combination. The AChR was affinity-purified, and the samples were analyzed for AChR content using α, γ, and δ subunit antibodies (Fig. 5B) and...
phosphorylation using phosphorylation state-specific anti-β and -δ subunit antibodies (Fig. 5A). Little or no AChR phosphorylation was detected in control cells or upon expression of Fyn, Fyk, or Src alone. However, upon coexpression of rapsyn with Fyn, Fyk, or Src, there was a dramatic increase in the phosphorylation of the AChR β and δ subunits. A third unidentified band that migrated at 55 kDa may have been the γ subunit, which is known to be tyrosine-phosphorylated in the C2C12 mouse muscle cell line (29). Rapsyn alone also increased the phosphorylation of the δ subunit (Fig. 5) and the β subunit as revealed by longer film exposure (data not shown). These data agreed with the ability of rapsyn to induce AChR β and δ subunit phosphorylation upon transient transfections in the QT-6 heterologous system (49). The stimulation of AChR phosphorylation was quantified by comparing densitometry scans of Western blots for the AChR protein and phosphorylation levels; band intensity for phosphorylation was divided by intensity for protein levels. Depending on the specific subunit and kinase analyzed, the effect of kinase plus rapsyn was 2–20-fold greater that the effect of rapsyn alone (Table I). These results indicated that all three kinases were capable of phosphorylating the AChR. In addition, Fyn-, Fyk-, and Src-mediated AChR phosphorylation occurred only upon expression of rapsyn. These data suggest that the ability of rapsyn to regulate Fyn, Fyk, and Src as shown in Fig. 2 resulted in phosphorylation of the AChR by these kinases.

To further test the significance of Src class kinases in AChR phosphorylation, the effect of herbimycin A, a protein-tyrosine kinase inhibitor selective for Src kinases (51), was examined. Herbimycin A inhibited Fyn-, Fyk-, and Src-mediated basal and rapsyn-regulated phosphorylation of total cellular proteins in both the soluble and cytoskeletal fractions (Fig. 2, B and C). Since herbimycin A blocked Src kinase-mediated phosphorylation of total cellular proteins, we used the inhibitor to test for...
the involvement of Src kinases in AChR phosphorylation. Herbimycin A inhibited the effect of rapsyn plus Fyn, Fyk, or Src on AChR phosphorylation (Fig. 5A). These data further support the importance of Src class kinases in AChR phosphorylation.

The physiological relevance of AChR phosphorylation by Src family kinases was examined by testing whether the phosphorylation was associated with anchoring of the receptor to the cytoskeleton. Q-F18 cells were transfected in order to express Fyn, Fyk, or Src on AChR phosphorylation (Fig. 5A). These data further support the importance of Src class kinases in AChR phosphorylation.

The positions of AChR α, β, γ, and δ subunits are as indicated.

TABLE I

| Subunit | Fyn + rapsyn | Fyk + rapsyn | Src + rapsyn |
|---------|--------------|--------------|--------------|
| δ       | 7 ± 1        | 1.9 ± 0.4    | 17 ± 4       |
| β       | 8 ± 3        | 2.9 ± 0.9    | 19 ± 6       |

Fig. 5. Tyrosine phosphorylation of the AChR β and δ subunit by Src class kinases. A, Q-F18 cells were transfected with empty pBK-CMV (pBK-CMV) or expression construct Fyn pBK-CMV (Fyn), Fyk pBK-CMV (Fyk), or Src pcDNA3 (Src) without or with rapsyn pGW-CMV (Rap) in the absence (−herbimycin) or presence (+herbimycin) of 1 μM herbimycin A as indicated. After 48 h, cells were solubilized, and the AChR was purified using acetylcholine-Sepharose affinity chromatography as described under “Experimental Procedures.” Aliquots of purified AChR representing approximately equal amounts of receptor, as determined by blot Western analysis with anti-AChR antibody (88b), were resolved by 8% SDS-PAGE and analyzed by Western blotting using a mixture of the phosphorylation-state-specific anti-AChR β subunit (JH1369) and anti-AChR δ subunit (JH1358) antibodies as described under “Experimental Procedures.” B, the blot from A was stripped and reanalyzed by Western blotting using a mixture of anti-AChR γ and δ (88b) and α subunit (109) antibodies as described under “Experimental Procedures.” The positions of AChR α, β, γ, and δ subunits are as indicated.

greater translocation of the receptor to the cytoskeleton compared with rapsyn alone. Thus, for Fyn, Fyk, or Src plus rapsyn the increase in AChR at the cytoskeleton over the effect of rapsyn alone was 1.9 ± 0.1, 1.7 ± 0.1, and 2.6 ± 0.2 (mean ± S.E.; n = 8), respectively, as determined by quantitative scanning densitometry. These data demonstrated that Fyn, Fyk, and Src could induce anchoring of the AChR to the cytoskeleton in a rapsyn-dependent manner. In addition, the rapsyn-stimulated effect of Src kinases to increase cytoskeletal anchoring of the receptor was essentially abolished by herbimycin A, suggesting that kinase activity was necessary for AChR translocation (Fig. 6A). These data indicated an important role for Src kinases in AChR anchoring to the cytoskeleton.

The ability of Src kinases to phosphorylate and translocate the AChR to the cytoskeleton suggested that phosphorylation of the receptor in the soluble fraction resulted in anchoring of the phosphorylated receptor to the cytoskeleton. To test this model, we examined whether the AChR that was translocated and anchored to the cytoskeleton in response to Src kinases was actually phosphorylated. The cytoskeletal fractions that had been examined for AChR protein as shown in Fig. 6A were reanalyzed for AChR tyrosine phosphorylation using the phosphorylation-specific AChR β subunit antibody. Detection of δ subunit phosphorylation by immunoblotting was precluded due to high background in the 65-kDa molecular mass range. Phosphorylated β AChR was detected in the cytoskeletal fraction of cells expressing Fyn, Fyk, or Src with rapsyn (Fig. 6B). No phosphorylated receptor was detected upon expression of the kinases alone. In addition, the appearance of the phosphorylated receptor in the cytoskeleton upon coexpression of Fyn, Fyk, and Src with rapsyn was blocked by herbimycin A (Fig. 6B). These data demonstrated that phosphorylated AChR was anchored to the cytoskeleton in cells expressing activated Src kinases. Since phosphorylated receptor was detected in the soluble fraction (Fig. 5), the model most consistent with these data is that phosphorylation of the AChR in the soluble fraction by Src kinases was followed by translocation and anchoring of the receptor to the cytoskeleton.
Regulation of the AChR by Src Kinases

This study has provided evidence that Src class protein-tyrosine kinases may be important for postsynaptic differentiation during synaptogenesis at the neuromuscular junction. Fyn, Fyk, and Src formed a complex with and were regulated by the synapse-specific cytoskeletal protein rapsyn. In addition, several lines of evidence indicated that, both in vivo and in vitro, rapsyn functioned as a substrate for Src kinases. Most importantly, regulation of Fyn, Fyk, and Src by rapsyn resulted in phosphorylation of the AChR β and δ subunits and anchoring of the receptor to the cytoskeleton. Qualitatively, all three kinases mediated the same functional effects: phosphorylation of total cellular proteins, rapsyn, and the AChR as well as anchoring of the AChR to the cytoskeleton. However, the order of efficacy was Src > Fyn > Fyk. Whether the differences in efficacy reflect biologically significant properties of the kinases or technical issues was not clear. However, the limited effects of Fyk on AChR phosphorylation in the soluble fraction (Fig. 5) may have resulted from rapsyn's regulation of this kinase occurring primarily in the cytoskeleton (Figs. 2 and 6). Both AChR phosphorylation and cytoskeletal anchoring were blocked by the Src kinase-selective protein-tyrosine kinase inhibitor herbimycin A, consistent with a causal relationship. Rapsyn alone also induced AChR phosphorylation and cytoskeletal translocation. However, inhibition by herbimycin A as well as a catalytically inactive dominant negative Src demonstrated that the effects of rapsyn on the AChR were mediated by endogenous Src kinases.

Accumulating evidence supports rapsyn as a crucial intracellular clustering factor for endplate components including rapsyn itself, the AChR, MuSK, and dystroglycan (8–10, 14, 24, 52, 53). Both the AChR and dystroglycan have been demonstrated to be in a complex with rapsyn (7, 15, 54). However, complex formation between rapsyn and Src kinases, as shown here, was the first evidence for association of this cytoskeletal protein with an enzymatic activity. Whether rapsyn and Src kinases associated via a direct binding or a complex of proteins was not determined. Affinity chromatography using Src kinase fusion proteins was inconclusive. Quantitation of the percentage of Src kinases and rapsyn complexed together demonstrated that 0.6% of rapsyn and 0.4% of Src class kinases of Torpedo electric organ postsynaptic membrane were associated. This level of rapsyn complexed with Src class kinases was similar to the 1% of RasGAP complexed with p56Lck (55) and 1% of RACK1 complexed with Src (56) in other systems. Since rapsyn, with the AChR, is the major protein in these postsynaptic membranes, these data suggested that more than one molecule of rapsyn was complexed with each Src kinase. In addition, since Fyn, Fyk, and Src associate with the AChR (30–32) and rapsyn (this report), complex formation between...
rapsyn and Src kinases may have been mediated by the AChR.

Further studies will be needed to determine the mechanism by which rapsyn stimulated phosphorylation by Src class kinases. It may be that rapsyn directly or indirectly regulates kinase enzymatic activity or, alternatively, brings the active kinases in contact with substrates. The mechanisms for activation of Src class kinases are diverse (for a review, see Ref. 57). Although Src kinases are enriched in the cytoskeleton, regulation of Fyn, Fyk, and Src by rapsyn would represent a novel "ligand" for activation. In contrast to receptor tyrosine kinases, activation of Src kinases has not been shown to be mediated by dimerization. Furthermore, rapsyn did not induce aggregation of Src kinases. Therefore, the ability of rapsyn to stimulate Fyn, Fyk, and Src was apparently not due to clustering of the kinases. However, the fact that the enzymatic activity regulated by rapsyn was a tyrosine phosphorylation was significant in light of the implicated role of protein-tyrosine kinases in synapse formation (18, 19, 21–23).

Rapsyn was found to be tyrosine-phosphorylated both in vivo and in vitro in this study. Others have also demonstrated rapsyn to be phosphorylated on tyrosine residues when transfected into QT-6 cells and on serine residues in an in vitro kinase assay using Torpedo membranes (49, 58). The results presented here support rapsyn as a substrate for Src family kinases. The relevance of rapsyn phosphorylation is not clear at this time. Rapsyn clusters do not contain phosphotyrosine in the absence of AChR, suggesting that rapsyn itself is not a predominant phosphotyrosine-containing protein in rapsyn clusters expressed in a heterologous system (49). However, the proposed serine (59) and tyrosine (this study) phosphorylation sites lie on either side of the C-terminal zinc finger domain of rapsyn, suggesting an interesting biological purpose. Mutation within this domain reduces zinc binding and AChR clustering; however, mutation of the putative serine phosphorylation site does not affect receptor clustering (60). Nonetheless, detection of in vivo phosphorylation of rapsyn purified from Torpedo electric organ supports the biological significance of this post-translational modification.

AChR phosphorylation by Src kinases in the Q-F18 heterologous system occurred on the β and δ subunits upon coexpression of rapsyn. The β, γ, and δ subunits of the AChR purified from Torpedo electric organ contain phosphotyrosine (50), and all three subunits are phosphorylated in vitro by Fyn and Fyk (30). Agrin stimulates tyrosine phosphorylation of only the β subunit in the C2C12 mouse muscle cell line (23, 29), while agrin or innervation in chick and innervation in rat induce phosphorylation of the β and δ subunits (18, 19). Phosphorylation of the β subunit is particularly significant, since this subunit is believed to anchor the receptor to the cytoskeleton via a direct binding to rapsyn (7). The ability of Src kinases to phosphorylate and anchor the AChR to the cytoskeleton, as shown here, was consistent with this hypothesis (Fig. 8). One functional significance of δ subunit phosphorylation may be to mediate binding of the AChR to Src kinases as demonstrated in Torpedo electric organ (31) (Fig. 8). Thus, AChR tyrosine phosphorylation may aid in stabilizing the receptor at the endplate as well as coaggregating structural and enzymatic elements important for synaptogenesis (Fig. 8).

Formation of the postsynaptic endplate involves AChR aggregation and anchoring to the cytoskeleton. Both processes, aggregation and cytoskeletal anchoring, have been proposed to be mediated by tyrosine phosphorylation of the AChR. Induction of AChR phosphorylation by agrin or a phosphatase inhibitor is associated with reduced receptor mobility; however, only agrin induces receptor clustering (29). Cytoskeletal association of the receptor shows a close temporal correlation with phosphorylation in response to both agrin and inhibition of phosphatase activity (27–29). In contrast, receptor clustering in response to agrin occurs many hours after phosphorylation and anchoring to the cytoskeleton (21, 27). In order for receptor aggregation to occur subsequent to anchoring, translocation to the cytoskeleton must be a dynamic process (Fig. 8). In support of an equilibrium between phosphorylated/anchored and dephosphorylated/mobile receptor being a requirement for aggregation, very high levels of phosphorylation induced by v-Src (61) or pervanadate (28) inhibit AChR clustering. In addition, myotubes contain very active phosphatases (28). Mature cluster formation may require phosphorylation and cytoskeletal anchoring of the AChR but also be dependent on other processes such as coaggregation of enzymes and structural components important for localized endplate formation. Expression of Fyn, Fyk, or Src alone in Q-F18 cells did not result in AChR clustering. In addition, although the dominant negative SrcK-did inhibit AChR phosphorylation (Fig. 7), it did not block agrin induced AChR clustering. These data indicate that phosphorylation by Src kinases did not mediate rapsyn’s effect to induce AChR clustering in transfected cells and are consistent with previous reports indicating that phosphorylation of the AChR is not required for rapsyn- or agrin-induced receptor clustering (49, 53). However, data presented here demonstrate that regulation of Src class kinases by rapsyn resulted in AChR phosphorylation and anchoring of the receptor to the cytoskeleton. The importance of protein-tyrosine kinases in anchoring of the AChR is supported by analysis of chick myotubes, where a kinase inhibitor blocks agrin-induced receptor tyrosine phosphorylation and cytoskeletal anchoring (22). In addition, as shown here, Src class kinases were complexed with the endplate-specific cytoskeletal element rapsyn. Furthermore, Fyn, Fyk, and Src associate with the AChR itself (30–32). Interaction of Fyn and Fyk with the AChR is mediated by the SH2 domains of the two kinases binding the tyrosine-phosphorylated δ subunit of the receptor (Fig. 8; Ref. 31). Since SH2 domains bind tyrosine-phosphorylated proteins, this domain may enable Src class kinases to aid in the coaggregation of other phosphotyrosine containing elements of the endplate. Thus, the ability of Src class kinases to phosphorylate the AChR, induce

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4 A. S. Mohamed and S. L. Swope, unpublished results.
receptor translocation to the cytoskeleton, and bind postsynaptic components, including the receptor and rapyn, demonstrated that these kinases fulfill functional roles important for formation and maturation of the endplate (Fig. 8).

Several reports implicate the transmembrane protein-tyrosine kinase MuSK as a receptor for agrin (5, 26). Activation of MuSK by agrin or rapyn results in AChR phosphorylation (24, 62). In addition, induction of AChR clustering by agrin is dependent on MuSK, and the AChR forms a complex with MuSK (5, 25, 62). Since MuSK is a protein-tyrosine kinase, activated MuSK may directly phosphorylate the AChR (24). Alternatively, MuSK may initiate a cascade that results in phosphorylation of the AChR by nonreceptor protein-tyrosine kinases such as Fyn, Fyk, or Src (Fig. 8). Consistent with this model, agrin activation of MuSK does not result in AChR phosphorylation in myotubes derived from a rapyn-deficient mouse (62). In addition, agrin-stimulated MuSK activity is not affected by the protein kinase inhibitor staurosporine, while AChR phosphorylation is inhibited (25). Therefore, MuSK activity is not sufficient for phosphorylation of the AChR, which appears to be dependent on a rapyn-mediated process. Since rapyn is a powerful intracellular clustering agent, aggregation of additional endplate components may be required for MuSK-mediated AChR phosphorylation. Alternatively, the rapyn-dependent process may be activation of Src class kinases (Fig. 8). Such a mechanism would be a novel signaling pathway between receptor and nonreceptor protein-tyrosine kinases. Classically, receptor tyrosine kinases such as the platelet-derived growth factor receptor activate Src family kinases via binding of the autophosphorylated growth factor receptor to the Src kinase SH2 domain (for a review see Ref. 57). We previously suggested that Src class kinases at the neuromuscular junction may be activated by receptor tyrosine kinases and thus mediate AChR clustering in response to growth factors such as fibroblast growth factors (30). However, the Trk receptor subfamily of tyrosine kinases, of which MuSK is a member, has not been clearly established as an activator of Src class kinases (63). Thus, MuSK and Src family kinases may act sequentially within a single pathway or, alternatively, in parallel to provide two mechanisms for phosphorylation and regulation of the AChR.

It has been proposed that AChR aggregation results from nerve-induced local activation of a protein-tyrosine kinase resulting in phosphorylation and accumulation of receptors in the vicinity of the activated kinase. MuSK is localized specifically at the endplate in skeletal muscle and thus is appropriately positioned to respond to nerve-derived signals (4). In contrast, Src kinases are ubiquitously expressed throughout the body. In *Torpedo* electric organ, Fyn and Fyk are expressed in both the postsynaptic and nonpostsynaptic membranes (30). Thus, Src family kinases are not specifically expressed at the endplate. However, being expressed at the postsynaptic membrane, Src kinases are in a position to mediate neuron-activated signal transduction mechanisms. Since rapyn is localized specifically at the endplate, the ability of rapyn to regulate Src kinases, resulting in AChR phosphorylation and cytoskeletal anchoring, supports Src class kinases as important players in the molecular mechanism underlying synaptogenesis.

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