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Rapsyn C-terminal domains mediate MuSK-induced phosphorylation of the muscle acetylcholine receptor

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Abstract

At the developing vertebrate neuromuscular junction, postsynaptic localization of the acetylcholine receptor (AChR) is regulated by agrin signaling via the muscle specific kinase (MuSK) and requires an intracellular scaffolding protein called rapsyn. In addition to its structural role, rapsyn is also necessary for agrin-induced tyrosine phosphorylation of the AChR, which regulates some aspects of receptor localization. Here, we have investigated the molecular mechanism by which rapsyn mediates AChR phosphorylation. In a heterologous COS cell system, we show that MuSK and rapsyn induced phosphorylation of β subunit tyrosine 390 (Y390) and δ subunit Y393, as in muscle cells. Mutation of β Y390 or δ Y393 did not inhibit MuSK/rapsyn-induced phosphorylation of the other subunit in COS cells, and mutation of β Y390 did not inhibit agrin-induced phosphorylation of the δ subunit in Sol8 muscle cells; thus, their phosphorylation occurs independently, downstream of MuSK activation. In COS cells, we further show that MuSK-induced phosphorylation of the β subunit was mediated by rapsyn, as MuSK plus rapsyn increased β Y390 phosphorylation more than rapsyn alone and MuSK alone had no effect. Intriguingly, MuSK also induced tyrosine phosphorylation of rapsyn itself. We then used deletion mutants to map the rapsyn domains responsible for activation of cytoplasmic tyrosine kinases that phosphorylate the AChR subunits. We found that rapsyn C-terminal domains (amino acids 212–412) are both necessary and sufficient for activation of tyrosine kinases and induction of cellular tyrosine phosphorylation. Moreover, deletion of the rapsyn RING domain (365–412) abolished MuSK-induced tyrosine phosphorylation of the AChR β subunit. Together, these findings suggest that rapsyn facilitates AChR phosphorylation by activating or localizing tyrosine kinases via its C-terminal domains.

Keywords

neuromuscular junction; synaptogenesis; agrin; postsynaptic membrane

At the developing neuromuscular junction in vertebrates, several nerve-derived signals combine to localize the acetylcholine receptor at postsynaptic sites (Sanes and Lichtman, 2001, Burden, 2002, Kummer et al., 2006). One essential factor is agrin, which signals via the MuSK receptor tyrosine kinase and induces and/or stabilizes clustering of the AChR in the postsynaptic membrane (reviewed in (Kummer et al., 2006). Interestingly, embryonic muscle

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is prepatterned and AChR clusters occur in the central region of the muscle prior to and even in the absence of neural innervation (Lin et al., 2001, Yang et al., 2001). However, upon innervation, agrin is required for stable aggregation of AChR at nerve-muscle contacts, counteracting an acetylcholine-driven dispersal of AChR that eliminates aneural aggregates (Lin et al., 2005, Misgeld et al., 2005). Indeed, in agrin and MuSK knockout mice, AChR clusters are largely eliminated by birth and the mice die due to an inability to move and breath (DeChiara et al., 1996, Gautam et al., 1996). Downstream of MuSK activation, an important mediator of AChR clustering is the intracellular, peripheral membrane protein, rapsyn, which associates with the AChR in the postsynaptic membrane in approximately 1:1 stoichiometry (Froehner, 1991). When expressed in heterologous cells, rapsyn self-aggregates and is sufficient to cluster, anchor and stabilize the AChR (Froehner et al., 1990, Phillips et al., 1991, Phillips et al., 1993, Phillips et al., 1997, Wang et al., 1999). Moreover, in rapsyn null mice, there is a complete absence of AChR clusters at developing synaptic sites (Gautam et al., 1995). Together, these findings suggest that rapsyn binds the receptor, clustering and anchoring it in the postsynaptic membrane.

Although rapsyn mediates AChR localization, it is unclear how this is regulated by agrin signaling in muscle cells. Potentially, protein interactions underlying localization could be regulated via posttranslational modifications of the AChR, rapsyn, or additional binding proteins. Consistent with the first possibility, agrin/MuSK signaling induces rapid tyrosine phosphorylation of the AChR β and δ subunits (Mittaud et al., 2001, Mohamed et al., 2001), mediated by an intervening cytoplasmic tyrosine kinase (Fuhrer et al., 1997), perhaps of the src and/or abl families (Mohamed and Swope, 1999, Finn et al., 2003). Phosphorylation correlates closely with reduced mobility and detergent extractability of the AChR (Meier et al., 1995, Borges and Ferns, 2001), suggesting that it regulates linkage to the cytoskeleton. In addition, it precedes AChR clustering (Ferns et al., 1996) and tyrosine kinase inhibitors that block phosphorylation also block clustering (Wallace et al., 1991, Ferns et al., 1996). Consistent with these findings, mutation of the tyrosine phosphorylation site in the β subunit abolishes agrin-induced cytoskeletal anchoring of mutant AChR and impairs its aggregation in muscle cells (Borges and Ferns, 2001). Moreover, mice with targeted mutations of the β subunit intracellular tyrosines have neuromuscular junctions that are simplified and reduced in size, with decreased density and total numbers of AChRs (Friese et al., 2007). Phosphorylation of the β subunit contributes to AChR localization, therefore, but it is unclear whether it does so by regulating rapsyn interaction (Fuhrer et al., 1999, Marangi et al., 2001, Moransard et al., 2003).

In addition to its structural role, rapsyn also functions in agrin signaling. Notably, agrin-induced phosphorylation of the AChR β and δ subunits is significantly decreased in rapsyn null myotubes (Apel et al., 1997, Mittaud et al., 2001), and rapsyn activates src family kinases in heterologous cells (Qu et al., 1996, Mohamed and Swope, 1999), resulting in tyrosine phosphorylation of multiple cellular proteins. Thus, rapsyn may facilitate MuSK-induced phosphorylation of the AChR by activating and/or localizing the relevant cytoplasmic tyrosine kinases. In this study, we have investigated how rapsyn mediates the functionally important tyrosine phosphorylation of the AChR and have mapped the COOH-terminal domains required for tyrosine kinase activation and receptor phosphorylation.

EXPERIMENTAL PROCEDURES

Expression Constructs

Rat MuSK that was myc-tagged at the N-terminus was expressed in the pRcRSV vector (provided by S. Burden, NYU). Mouse muscle nAChR subunits (α, β, ε, δ) were expressed using the pcDNA3 vector with a CMV promoter (Invitrogen; Carlsbad, CA). To epitope tag the β- subunit, a KpnI site was introduced at the C-terminal extracellular tail, and double

stranded oligonucleotides that coded for the hemagglutinin (HA) or 142 epitopes (Das and Lindstrom, 1991) were ligated into this site. To tag the δ- subunit, a ClaI site was introduced and the 142 epitope ligated into this site. Tyrosine 390 of the β subunit and 393 of the δ subunit were mutated to phenylalanines using polymerase chain reaction-based site-directed mutagenesis (Quickchange Kit, Stratagene; La Jolla, CA). Mouse rapsyn was expressed using the pcDNA3 vector. Rapsyn deletion mutants that were His6-tagged at the C-terminus were generated through PCR and ligated into the pMT23 vector; rapsyn C-terminal deletion constructs retained the N-terminal myristoylation sequence whereas C-terminal fragments lacked this sequence. All constructs were confirmed by sequencing.

Assaying Phosphorylation of AChR

COS cells were grown on 10 cm dishes in DMEM-HI containing 10% fetal bovine serum and penicillin-streptomycin. When the cells reached 85–90% confluency they were transfected overnight with 20μg of plasmid DNA encoding the AChR subunits (α, β, ε, δ) using the calcium phosphate method (Profection kit, Promega; Madison, WI). For these experiments, the β and δ subunits were tagged with HA and 142 epitopes, respectively. After 2 days of expression, the live cells were surface labeled with biotin-conjugated α-bungarotoxin (Molecular Probes; Eugene, OR) for 1 hour, rinsed, and then scraped off and pelleted in Ca^{++}/Mg^{++}-free PBS containing 1 mM sodium vanadate. Cells were then resuspended in buffer containing 1% Triton X-100 and 25 mM Tris-Glycine pH 7.5, 150 mM NaCl, 5 mM EDTA, 1 mM sodium vanadate, 50 mM sodium fluoride and the protease inhibitors: 1 mM bisbenzimidine, 1 mM sodium tetrathionate, and 1 mM PMSF. After extraction on ice for 10 minutes, the detergent soluble and insoluble fractions were separated by centrifugation at 16,000 g for 4 min. The insoluble pellet fraction was resuspended directly in 200 μl of SDS-PAGE loading buffer. The soluble lysates were incubated with streptavidin-agarose beads (Molecular Probes; Eugene, OR) for 1 hour to isolate AChR pre-labeled with biotin-conjugated α-bungarotoxin. MuSK was then immunoprecipitated with anti-MuSK polyclonal antibody (gift of J. Sugiyama and Z. Hall, UCSF and NIH) and protein G beads. In both cases, proteins isolated on the beads were eluted in 30 μl of 2x SDS-PAGE loading buffer.

Sol8 muscle cells were grown on 10 cm dishes and transfected with 142-tagged β subunit using the calcium phosphate method as previously described (Borges and Ferns, 2001). After treatment with agrin for 1 hr (200 pM C-Ag4,8), the cells were extracted as described above. AChR containing tagged β subunit was immunoprecipitated with mAb142 and then the remaining AChR was pulled down using biotin-conjugated α-bungarotoxin and streptavidin-agarose beads (Molecular Probes; Eugene, OR).

For immunoblotting, samples were electrophoretically separated on 10% SDS-PAGE gels, and transferred onto PVDF membranes. To detect phosphorylated AChR β and δ subunits, the proteins were probed with polyclonal antibodies JH-1360 and JH-1358, respectively (Mohamed and Swope, 1999) in buffer containing 4% Blotto, 20 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.5% NP40, and 0.1% Tween-20. The blots were then incubated with horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody (Amersham Corp.; Arlington Heights, IL) and bound antibody was visualized by chemiluminescence (ECL, Amersham Corp.). To identify the β and δ subunit phosphorylation bands, the immunoblots were reprobed for tagged β and δ subunits with monoclonal antibodies anti-HA (Roche; Indianapolis, IN) or mAb142 (Sigma-Aldrich; St. Louis, MO). The identity of the bands was also confirmed via mutations of β Y390 and δ Y393, which eliminate phosphorylation of the respective subunits. Blots were also reprobed for the α subunit using mAb210 (Babco; Berkley, CA) to compare levels of expression of the AChR. Rapsyn immunoblots were performed using monoclonal antibody mAb1234 (gift of S. Froehner, Univ. Washington), polyclonal antibody B5668

(generated against amino acids 133–153 of rapsyn) or anti-His6 for tagged versions of rapsyn. Reprobes for tagged MuSK were performed using anti-myc monoclonal antibody 9E10.

To quantify levels of tyrosine phosphorylation, we carried out densitometric analysis of the β and δ subunit bands in both soluble and pellet fractions using Sci-Scan 5000 Bioanalysis software (USB; Cleveland, OH). As only 20% of the pellet fraction was used for immunoblotting, these values were multiplied by 5 and added to the corresponding value for the soluble fraction in order to give total AChR subunit phosphorylation. The data was also normalized to the level of AChR, detected by immunoblotting for the tagged β subunit (anti-HA or 142). In order to average several independent transfection experiments, all values were expressed as a percentage of the maximal signal, which was obtained in COS cells co-expressing AChR, MuSK and rapsyn, or in Sol8 muscle cells treated with agrin.

Assaying total cellular phosphorylation

COS cells grown on 6 cm dishes were transfected overnight with 9 μg of plasmid DNA. After 1 day of expression, cells were rinsed, scraped off and pelleted in Ca^{++}/Mg^{++}-free PBS containing 1 mM sodium vanadate. The cells were then lysed in 100 μl of extraction buffer (1% Triton X-100, 25 mM Tris-Glycine pH 7.5, 150 mM NaCl, 5 mM EDTA, 1 mM sodium vanadate, 50 mM sodium fluoride, 1 mM bisbenzimidine, 1 mM sodium tetrathionate, and 1 mM PMSF), and after centrifugation, the residual pellets were resuspended in 100 μl of SDS loading buffer and boiled. To assay cellular protein tyrosine phosphorylation, 10 μl of the pellet fraction were separated on 10% acrylamide gels and immunoblotted with anti-phosphotyrosine antibodies 4G10 and PY20. The intensity of the phosphotyrosine signal was quantified using Sci-Scan 5000 Bioanalysis software (USB; Cleveland, OH) where we performed a line scan through all the bands in each lane, from the top of the separating gel to ~45 kD in order to avoid rapsyn. To average independent transfection experiments, we normalized the data in each experiment by subtracting the background in COS cells transfected with empty vector (ie. 0%) and expressing levels of cellular phosphorylation as a percentage of that in COS cells expressing wild type rapsyn (ie. 100%). This best controls for the variation in transfection efficiency (and resulting cellular phosphorylation) between experiments, by comparing rapsyn versus rapsyn plus MuSK, or rapsyn deletion mutants to full-length rapsyn. Phosphorylation of rapsyn was measured independently and rapsyn levels were assayed by blotting duplicate gels with mAb1234 or anti-His6. Phosphorylation of rapsyn was also confirmed by immunoprecipitating with polyclonal anti-rapsyn antibodies B5668 and B6766 (generated against amino acids 133–153 and 402–412 of rapsyn, respectively), and immunoblotting with anti-phosphotyrosine monoclonal antibodies 4G10 and PY20.

Assaying AChR extractability

COS cells growing in 6 well plates were transfected with AChR and rapsyn deletion mutants using Fugene (Roche). After 2 days of expression, the live cells were incubated with 10 nM I^{125}-BuTX for 1 hr to label surface AChR and rinsed 3x with PBS. Cells were then lysed in 200 μl of extraction buffer (as above) for 10 minutes on ice and the detergent soluble and insoluble fractions separated by centrifugation at 16,000 g for 4 min. Both lysate and pellet fractions were counted with a gamma counter to assay rapsyn's effect on detergent extractability of the AChR (ie. anchoring).

RESULTS

MuSK/rapsyn-induced phosphorylation of AChR β and δ subunits

Agrin/MuSK signaling in muscle cells induces a rapid tyrosine phosphorylation of the AChR, which contributes to its postsynaptic localization. Surprisingly, receptor phosphorylation is dependent on rapsyn, a scaffolding protein that clusters and anchors the AChR in the

postsynaptic membrane. To investigate the mechanism by which rapsyn mediates the functionally important phosphorylation of the receptor, we first recapitulated MuSK-induced AChR phosphorylation in a simplified, heterologous cell system. To do this, we transiently expressed the AChR subunits (α, β, ε, δ) in COS cells in combination with rapsyn and MuSK. To assay phosphorylation, surface AChR was labeled with biotin-conjugated α-BuTX, pulled down from cell lysates and immunoblotted with antibodies specific for the phosphorylated β (Y390) and δ (Y393) subunits (Wagner et al., 1991, Mohamed and Swope, 1999). AChR retained in the detergent-insoluble pellet fraction was also immunoblotted, as previous studies have shown that rapsyn anchors significant amounts of receptor to the cytoskeleton (Phillips et al., 1993, Mohamed and Swope, 1999). In agreement with previous work (Gillespie et al., 1996), we find that coexpression of MuSK and rapsyn significantly increased phosphorylation of the AChR β and δ subunits (Fig 1A,B). The MuSK/rapsyn-induced increase in phosphorylation was greatest for the β subunit, whereas basal phosphorylation was highest for the δ subunit; these findings in heterologous cells mirror previous observations in muscle cells (Meier et al., 1995, Mittaud et al., 2001). Moreover, most phosphorylated AChR was found in the pellet fraction, consistent with previous findings in both heterologous and muscle cells (Meier et al., 1995, Borges and Ferns, 2001).

Several studies have shown β and δ subunit phosphorylation to be closely linked, both temporally and in their sensitivity to kinase blockers (Mohamed and Swope, 1999, Mittaud et al., 2001). This could reflect parallel phosphorylation of the 2 sites by the same - or a similar - kinase. Alternatively, phosphorylation might be sequential, with phosphorylation at one site recruiting a kinase that then phosphorylates the second subunit. To distinguish these possibilities, we expressed AChR with β subunit Y390F or δ subunit Y393F mutations and tested the effect on MuSK/ rapsyn-induced phosphorylation of the other subunit (Fig 1A,B). We find that mutation of β Y390 did not block δ subunit phosphorylation, and similarly, mutation of δ Y393 did not abolish phosphorylation of the β subunit, suggesting that their phosphorylation is independent.

To confirm that AChR phosphorylation occurs via a similar mechanism in muscle cells, we transfected Sol8 myotubes with 142-tagged, wild type or Y390F β subunit, which we have shown previously to be incorporated into AChR that is expressed on the myotube surface (Borges and Ferns, 2001). After agrin treatment, the cells were extracted and AChR containing tagged β subunit was selectively immunoprecipitated using anti-142 antibodies and assayed for β and δ phosphorylation (Fig 2A,B). Consistent with our results in COS cells we find that β subunit Y390F mutation did not block δ phosphorylation in muscle cells (85 ± 6% of wild-type levels; mean ± SEM). We were unable to test the effect of δ Y393F mutation on β phosphorylation due to low expression of the introduced δ subunit. Together with the results from COS cells, however, these findings demonstrate that phosphorylation of the β and δ subunits is independent and occurs in parallel, downstream of MuSK activation.

Rapsyn facilitates MuSK-induced phosphorylation of the AChR β subunit

Agrin/MuSK -induced phosphorylation of the AChR is significantly impaired in rapsyn null muscle cells (Apel et al., 1997, Mittaud et al., 2001). To confirm that rapsyn is also required in our heterologous cell system, we expressed the AChR in combination with rapsyn and/or MuSK, and assayed phosphorylation as described above. As in muscle cells, basal phosphorylation of the δ subunit was higher than the β subunit. Rapsyn co-expression increased both β and δ subunit phosphorylation, whereas MuSK alone did not affect phosphorylation. Moreover, co-expression of rapsyn plus MuSK increased β phosphorylation above that with rapsyn or MuSK alone (Fig 3A,B; n=6; $p=0.002$ and <0.0001 respectively; Student's T-test). Thus, in agreement with previous work, we find that rapsyn facilitates MuSK-induced phosphorylation of the AChR β subunit (Gillespie et al., 1996).

Rapsyn C-terminus is necessary and sufficient for activation of cellular tyrosine kinases

Rapsyn likely mediates MuSK-induced phosphorylation of the AChR by activating and/or localizing cytoplasmic tyrosine kinases that phosphorylate the β and δ subunits. Indeed, rapsyn activates src family kinases in heterologous cells, resulting in increased tyrosine phosphorylation of multiple cellular proteins (Qu et al., 1996, Mohamed and Swope, 1999). Moreover, agrin specifically activates src-family kinases associated with the AChR in muscle cells and this occurs in a rapsyn-dependent manner (Mittaud et al., 2001). We examined, therefore, whether rapsyn's activation of kinases is enhanced by MuSK and which domains in rapsyn are responsible. First, we expressed rapsyn and/or MuSK in COS cells and after detergent extraction, assayed phosphorylation in the pellet fraction by immunoblotting with anti-phosphotyrosine antibodies 4G10 and PY20. As previously reported (Qu et al., 1996, Mohamed and Swope, 1999), rapsyn alone significantly increased cellular tyrosine phosphorylation compared to controls transfected with empty vector (by ~250%; Fig 4A,B). In contrast, MuSK alone did not increase phosphorylation significantly and MuSK coexpression with rapsyn did not increase overall cellular phosphorylation beyond the levels found with rapsyn alone (Fig 4A,B).

One interesting exception was that MuSK increased tyrosine phosphorylation of rapsyn itself. In immunoblots of the pellet fraction, we found ~350% increase in tyrosine phosphorylation of a 43 kD band corresponding to rapsyn (Fig 4A,C; arrowed band). This was confirmed in immunoblots of rapsyn deletion mutants, where a strong phosphotyrosine signal corresponded exactly to the different-sized rapsyn mutants (see Fig 5). In addition, we detected tyrosine phosphoryation of rapsyn that was specifically immunoprecipitated from the soluble fraction (Fig 4D), although interestingly, the level of tyrosine phosphorylation was significantly lower than in rapsyn anchored in the pellet fraction. The MuSK-induced phosphorylation of rapsyn suggests that its function might also be regulated by agrin-induced phosphorylation during synaptogenesis.

To then map the domains in rapsyn responsible for kinase activation (Fig 5A), we tested a series of deletion mutants for their ability to induce cellular tyrosine phosphorylation. Full-length wild type and His6-tagged rapsyn increased levels of overall cellular tyrosine phosphorylation by approximately 2.5 to 3 fold (Fig 5B). In contrast, 3 different rapsyn C-terminal deletion mutants failed to increase tyrosine phosphorylation above background levels, despite being expressed at levels equivalent to wild type rapsyn. Indeed, deletion of just the RING domain and following 10 amino acids (rapsyn 1–365) abolished kinase activation (Fig 5C).

We then tested whether the rapsyn C-terminus alone activated kinases and increased tyrosine phosphorylation. Strikingly, we found that 2 different rapsyn C-terminal fragments (rapsyn 158–412 and 212–412) both increased cellular tyrosine phosphorylation to levels similar to that seen with wild type rapsyn (Fig 5D,E). In an effort to map the responsible domains further, we tested 2 additional GFP-tagged C-terminal fragments (GFP-rapsyn 200–284 and 255–412); despite being highly expressed, neither increased cellular phosphorylation (data not shown). Together, these results demonstrate that the C-terminal half of rapsyn is sufficient for activation of cellular tyrosine kinases, and within this region, the RING domain and following 10 amino acids are necessary for activation.

Rapsyn C-terminus is required but not sufficient for AChR phosphorylation

Next, we investigated whether rapsyn's C-terminal domains mediate MuSK-induced phosphorylation of the AChR, focusing on the functionally important phosphorylation of the β subunit (Borges and Ferns, 2001, Friese et al., 2007). To do this, we co-expressed AChR and MuSK together with rapsyn C- and N-terminal deletion mutants and then assayed tyrosine

phosphorylation of the β subunit. Consistent with our previous results, we found that all 3 rapsyn C-terminal deletions abolished MuSK-induced β subunit phosphorylation (Fig 6A,B). Thus, the RING domain and following 10 amino acids is required for rapsyn's ability to mediate AChR tyrosine phosphorylation. The C-terminus of rapsyn was not sufficient for AChR phosphorylation, however, as β subunit phosphorylation was undetectable with either of the rapsyn C-terminal fragments (rapsyn 158–412 and 212–412; Fig 6C,D).

Loss of rapsyn-induced AChR phosphorylation could be due to deletion of kinase binding/activating domains or loss of association with the receptor. To address this, we immunostained transfected COS cells for the different deletion mutants and observed some membrane localization; however, rapsyn C-terminal deletion mutants failed to form well defined surface clusters and we were unable to assay colocalization with the AChR (data not shown). Instead, we assayed the ability of the rapsyn mutants to anchor surface AChR labeled with I^{125}-BuTX. When co-expressed with full-length rapsyn, ~57% of surface AChR was anchored and recovered in the pellet fraction, compared to ~9% when receptor was expressed alone (Fig 6E). Of the rapsyn mutants, rapsyn 1–365 anchored ~32% of surface receptor, but further deletion mutants did not anchor receptor consistently above control levels. This indicates that rapsyn 1–365 still targets to the plasma membrane and associates with surface AChR and that the lack of receptor phosphorylation likely stems from the deletion of the C-terminal kinase interaction domains.

Rapsyn C-terminal phosphorylation is not required for β subunit phosphorylation

Finally, we tested whether MuSK-induced phosphorylation of rapsyn (Fig 4) might regulate its ability to mediate AChR phosphorylation. While numerous conserved tyrosine and serine phosphorylation consensus sites are present in rapsyn we focused on those in the C-terminal domains implicated in kinase activation. Mutation of tyrosines 336 or 362, serines 338, 369 or 383, or serines 405 and 406 together did not noticeably inhibit rapsyn-mediated phosphorylation of the AChR β subunit (Fig 7).

DISCUSSION

In this study, we investigated how rapsyn mediates agrin-induced phosphorylation of the AChR, a signaling event that regulates postsynaptic localization (Borges and Ferns, 2001, Friese et al., 2007). We show that rapsyn mediates parallel – but independent – phosphorylation of the β and δ subunits and that this requires the C-terminal domains of rapsyn. Our deletion analysis shows that rapsyn 212–412 is sufficient for activation of cellular tyrosine kinases that phosphorylate the receptor subunits, and within this region, the RING domain and following 10 amino acids (365–412) are necessary for kinase activation and receptor phosphorylation. Thus, in addition to its structural role in localizing the AChR, rapsyn facilitates receptor phosphorylation by activating tyrosine kinases via its C-terminal domains.

Phosphorylation of AChR β and δ subunits

In muscle cells, agrin- or nerve-induced clustering of the receptor involves tyrosine phosphorylation of both the AChR β and δ subunits (Wallace et al., 1991, Meier et al., 1995, Ferns et al., 1996, Mittaud et al., 2001). Following MuSK activation, phosphorylation of each subunit occurs with a similar time-course and is inhibited by the same pharmacological agents (Mittaud et al., 2001, Mohamed et al., 2001). Moreover, both β and δ subunit phosphorylation are dependent on rapsyn, with phosphorylation levels being significantly decreased in rapsyn null myotubes (Mittaud et al., 2001). Basal levels of phosphorylation are higher for δ than β, however, and agrin-induced phosphorylation occurs with higher stoichiometry on β (Meier et al., 1995, Mittaud et al., 2001). In addition, several findings suggest that β and δ subunit phosphorylation may serve different functions. Mutation of the β subunit tyrosine

phosphorylation site (Y390) impairs agrin-induced anchoring and clustering of mutant AChR in muscle cells (Meyer and Wallace, 1998, Borges and Ferns, 2001). Moreover, a recent study shows that mice with targeted mutations of all 3 tyrosines in the β subunit loop have neuromuscular junctions that are simplified and reduced in size, with significantly decreased numbers of AChRs (Friese et al., 2007). Consistent with these findings, we have found that agrin induces clustering of chimeric proteins consisting of CD4 fused to the β subunit intracellular loop but not δ loop, and this is dependent on β Y390 phosphorylation (Ferns et al; submitted). This suggests that β subunit phosphorylation regulates protein interactions involved in receptor localization, whereas δ loop phosphorylation serves other functions. Indeed, tyrosine-phosphorylated δ subunit of Torpedo AChR binds the cytoplasmic tyrosine kinases fyn and fyk (Swope and Huganir, 1994), and the adaptor protein, Grb2, via their SH2 domains, consistent with a signaling role (Colledge and Froehner, 1997).

Agrin/MuSK-induced phosphorylation of the β and δ subunits also appears to be independent. Despite the higher basal phosphorylation of δ and its YxxL motif that could allow recruitment of adaptor proteins or kinases (Swope and Huganir, 1994, Fuhrer and Hall, 1996), we found that mutation of δ Y393 did not block MuSK/rapsyn-induced β phosphorylation in heterologous cells. Similarly, mutation of β Y390 did not abolish δ phosphorylation in heterologous or muscle cells. Thus, MuSK-induced phosphorylation of the two subunits is not sequential, with phosphorylation at one site recruiting a kinase that then phosphorylates the second subunit; rather, phosphorylation of the two subunits occurs in parallel.

MuSK-induced phosphorylation of the AChR β and δ subunits involves an intervening cytoplasmic tyrosine kinase (Fuhrer et al., 1997), whose activity and/or localization appears to be rapsyn-dependent (Mohamed and Swope, 1999, Mittaud et al., 2001). Indeed, agrin-induced phosphorylation of the AChR is significantly decreased in rapsyn null myotubes (Apel et al., 1997, Mittaud et al., 2001) and rapsyn activates cytoplasmic kinases in heterologous cells (Qu et al., 1996, Mohamed and Swope, 1999). By assaying a series of rapsyn deletion mutants, we show that the rapsyn C-terminus is necessary and sufficient for rapsyn's ability to activate kinases and increase cellular phosphorylation. Deletion of amino acids 366–412 abolished rapsyn-induced phosphorylation, and conversely, the C-terminal half of rapsyn (aa 212–412) was sufficient to induce phosphorylation. Together, this implicates a C-terminal region encompassing the Zn^{++}-binding RING-H2 domain (Froehner, 1991, Scotland et al., 1993, Bezakova and Bloch, 1998) and C-terminal 10 amino acids containing a consensus serine phosphorylation site (aa 406) (Froehner, 1991). The RING domain has been previously implicated in binding to β-dystroglycan (Bartoli et al., 2001) but no function has yet been ascribed to the extreme C-terminus (Han et al., 1999). These findings suggest that rapsyn facilitates AChR phosphorylation by regulating tyrosine kinases via its C-terminal domains, and consistent with this, we find that deletion of just the RING domain abolishes MuSK-induced phosphorylation of the AChR β subunit. We cannot discount, however, that rapsyn C-terminal domains also act to inhibit tyrosine phosphatases.

While our findings define the rapsyn domains required for kinase interaction, the identity of the cytoplasmic tyrosine kinase remains unclear. One candidate is src family kinases, which are associated with the AChR (Swope and Huganir, 1993, 1994, Fuhrer and Hall, 1996) and are specifically activated following agrin treatment of muscle cells (Mittaud et al., 2001). Src phosphorylates the β and δ subunits in heterologous cells and this is blocked, both by src dominant negative constructs and inhibitors (Mohamed and Swope, 1999). However, agrin induces normal phosphorylation (and clustering) of the AChR in src/fyn double knockout muscle cells (Smith et al., 2001), and studies using src family inhibitors have yielded differing results (Mohamed et al., 2001, Smith et al., 2001). A second candidate is abl/arg kinases, which are localized at the neuromuscular junction (Finn et al., 2003). Inhibitors of these kinases reduce AChR phosphorylation and clustering (Finn et al., 2003, Mittaud et al., 2004), but it remains

unclear whether they directly phosphorylate the receptor. A further possibility is that src and abl kinases combine to phosphorylate the AChR, with their activities being temporally segregated (Mittaud et al., 2004).

Also unclear is whether rapsyn activates or clusters cytoplasmic tyrosine kinases via its C-terminus. Consistent with activation, rapsyn associates with and activates src family kinases in heterologous cells, leading to phosphorylation of AChR and other endogenous cellular proteins (Mohamed and Swope, 1999). Similarly, rapsyn is required for agrin's activation of AChR-associated src family kinases in muscle cells (Mittaud et al., 2001). Rapsyn-induced clustering of kinases could also facilitate AChR phosphorylation, however we were unable to detect rapsyn-associated clusters of src or abl/arg kinases in heterologous cells (data not shown). In addition, no significant concentration of src or abl/arg family kinases has been reported in the postsynaptic membrane at early neuromuscular synapses; abl/arg kinases have been detected only postnatally (Finn et al., 2003). Thus, we favor the idea that rapsyn primarily activates kinases and the requisite kinases may interact with the rapsyn/AChR complex with relatively low affinity or stoichiometry.

Phosphorylation of rapsyn

In COS cells, we found that MuSK alone did not increase global cellular tyrosine phosphorylation and MuSK co-expression with rapsyn did not enhance phosphorylation beyond that induced by rapsyn alone. However, we did observe specific, MuSK-induced phosphorylation of the AChR (discussed above) and rapsyn. Intriguingly, tyrosine phosphorylation of rapsyn was increased ~3.5 fold by MuSK, suggesting that rapsyn may be a downstream target for phosphorylation in the agrin signaling pathway in muscle. Consistent with this, rapsyn contains several conserved tyrosine phosphorylation consensus sites and rapsyn purified from Torpedo electric organ is tyrosine phosphorylated to some degree (Qu et al., 1996, Balasubramanian and Huganir, 1999, Mohamed and Swope, 1999). The specific site (s) of phosphorylation is unclear as mutation of Y336 and Y362 did not abolish rapsyn phosphorylation (data not shown) or impair phosphorylation of the AChR. However, decreasing levels of phosphorylation were observed with progressively larger N- and C-terminal deletions (Fig 5B,D), suggesting that tyrosines in the central region of rapsyn may be the major sites of phosphorylation. Thus, although it remains to be confirmed that agrin induces rapsyn phosphorylation in muscle, this finding raises the possibility that post-translational modification of rapsyn contributes to AChR clustering and anchoring at the NMJ.

In summary, we find that MuSK-induced phosphorylation of the AChR β and δ subunits occurs in parallel, likely mediated by the same or similar kinases. Moreover, our findings suggest that rapsyn facilitates this phosphorylation by activating or localizing tyrosine kinases via its C-terminal domains. These findings imply a common mechanism for agrin-induced phosphorylation of the β and δ subunits, however they likely have distinct functions in AChR localization and function.

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ABBREVIATIONS

aa, amino acid; AChR, acetylcholine receptor; BuTX, α-bungarotoxin; C-terminal, carboxyl terminal; MuSK, muscle specific kinase; N-terminal, amino terminal.

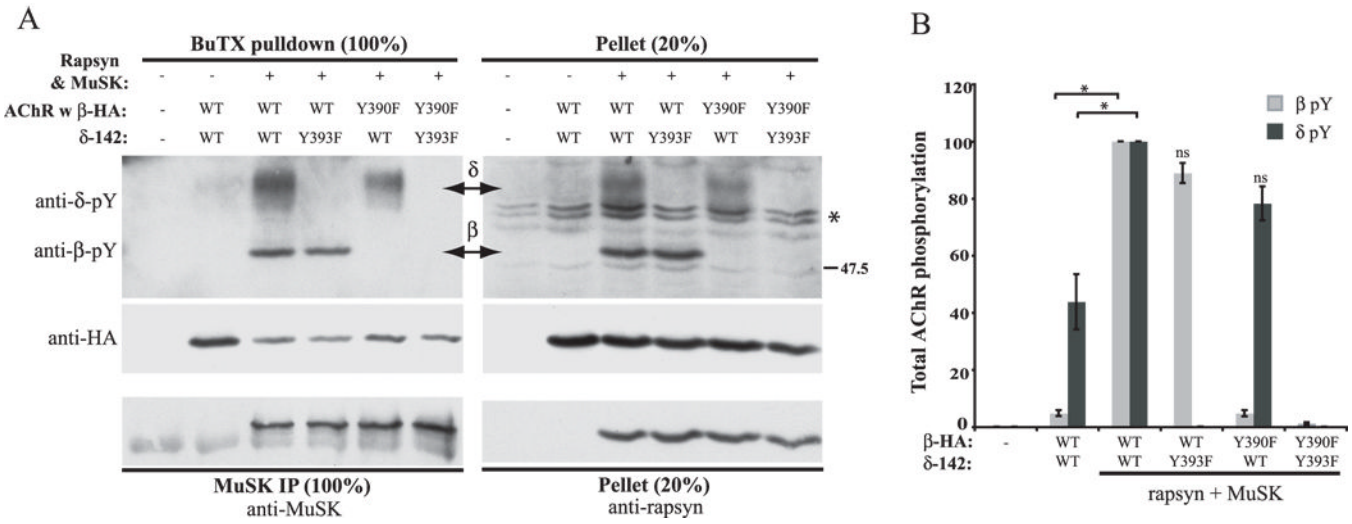

Figure 1. Phosphorylation of AChR β and δ subunits is not interdependent

AChR was transiently expressed in COS cells with either wild type or tyrosine-mutated β and δ subunits (β Y390F and δ Y393F). (A) After detergent extraction, we assayed MuSK/ rapsyn-induced phosphorylation of surface AChR isolated from the soluble lysate (100% of BuTX pulldown) and also AChR retained in the insoluble pellet (20% of pellet) by immunoblotting with β and δ subunit phospho-specific antibodies (JH-1360 and JH-1358, respectively). The blots were reprobed with anti-HA antibody to detect HA-tagged β subunit and levels of AChR expression. Arrows indicate the β and δ subunit phosphorylation bands and * marks a nonspecific band present in the pellet fraction. (B) Quantification of total AChR phosphorylation in both soluble and pellet fractions shows that rapsyn plus MuSK induced significant phosphorylation of the β and δ subunits ($p<0.00001$ and $p<0.05$, respectively; n=5, Student's T-test). Mutation of β subunit Y390 (Y390F) did not block δ subunit phosphorylation (78 ± 6% of wild type levels, mean ± SEM; n=5; ns, difference not significant; Student's T-test), nor did δ subunit Y393F mutation block β subunit phosphorylation (89 ± 3.5% of wild type levels; n=5, difference not significant). Thus, rapsyn/MuSK-induced phosphorylation of the two sites occurs independently.

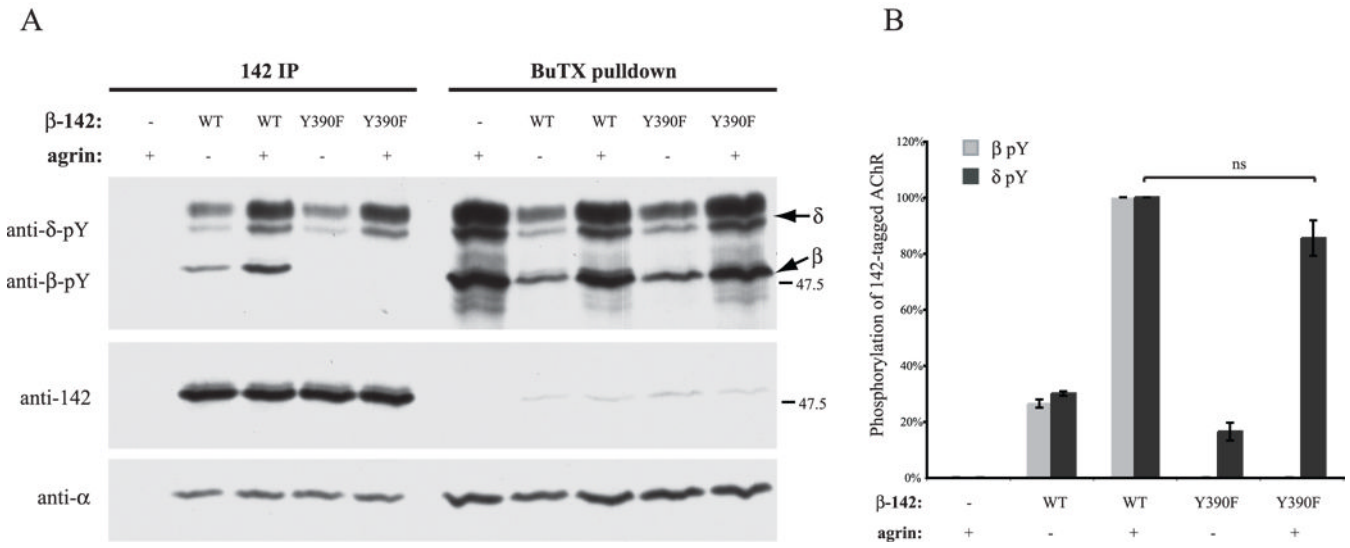

Figure 2. Phosphorylation of AChR δ subunit is not dependent on β subunit phosphorylation in muscle cells

(A,B) Sol 8 muscle cells were transfected with 142-tagged wild-type or Y390F β subunit. After detergent extraction, AChR containing tagged β subunit was immunoprecipitated with mAb142 (142 IP), followed by isolation of the remaining AChR with αBuTX (BuTX pulldown). Isolates were then immunoblotted with β and δ subunit phospho-specific antibodies (JH-1360 and JH-1358, respectively). The blots were reprobed with anti-142 antibody to detect tagged β subunit and with mAb 210 to determine AChR levels. For AChR containing tagged wild type β subunit (142 IP), agrin induced tyrosine phosphorylation of the β and δ subunits, similar to endogenous AChR (BuTX pulldown). In AChR containing Y390F β subunit, absence of β phosphorylation did not inhibit agrin-induced phosphorylation of the δ subunit (85 ± 6% of wild type level; n=3; ns, difference not significant, Student' T-test).

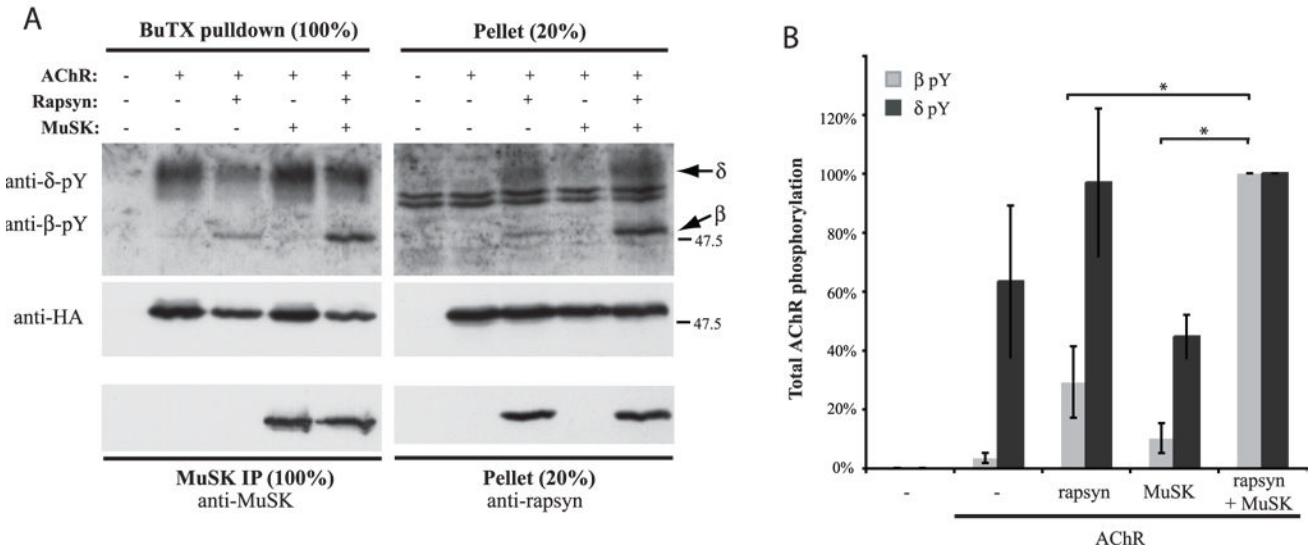

Figure 3. Rapsyn mediates MuSK-induced phosphorylation of AChR β subunit

(A) AChR was transiently expressed in COS cells alone or with rapsyn and/or MuSK. After detergent extraction, we assayed phosphorylation of surface AChR isolated from the soluble lysate (100% of BuTX pulldown) and also AChR retained in the insoluble pellet (20% of pellet) by immunoblotting with β and δ subunit phospho-specific antibodies (JH-1360 and JH-1358, respectively). Levels of AChR were determined by blotting for HA-tagged β subunit, and expression of MuSK and rapsyn confirmed by blotting with anti-MuSK and mAb1234 antibodies, respectively. (B) Quantification of total AChR phosphorylation (in both soluble and pellet fractions) shows that rapsyn co-expression increased β and δ subunit phosphorylation, whereas MuSK alone had no effect (n=3–6). Co-expression of both rapsyn and MuSK increased the levels of β subunit phosphorylation above that with rapsyn or MuSK alone ($p=0.002$ and $p<0.0001$, respectively; Student's T-test), indicating that rapsyn facilitates MuSK-induced β subunit phosphorylation.

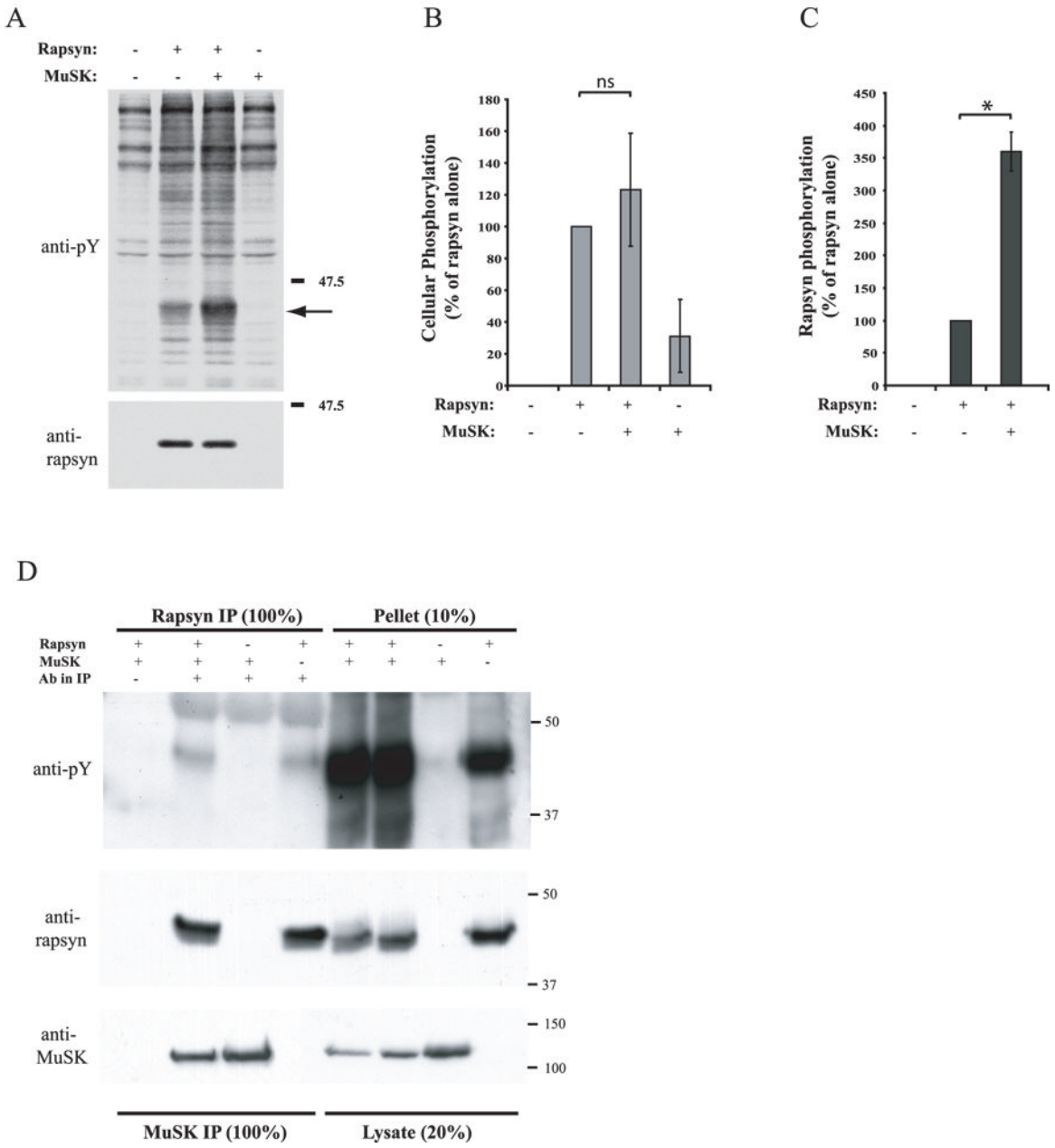

Figure 4. Rapsyn activates cellular tyrosine kinases independent of MuSK

(A) COS cells transfected with rapsyn and/or MuSK were extracted and the pellet fractions immunoblotted with monoclonal anti-phosphotyrosine antibodies, 4G10 and PY20 to assay levels of phosphorylation. Rapsyn expression increased tyrosine phosphorylation of multiple cellular proteins, indicating that it activates cytoplasmic tyrosine kinases. MuSK expression did not increase general cellular phosphorylation, either alone or in combination with rapsyn. However, it did increase phosphorylation of the 43 kD band corresponding to rapsyn (arrow). (B) Quantification of relative phosphotyrosine levels in the COS pellet fractions, expressed as a percentage of the level with rapsyn alone. MuSK expression did not increase phosphorylation above control levels or enhance the effect of rapsyn (123 ± 35% of rapsyn alone, mean ± SEM; n=3; difference not significant, Student's T-test). (C) MuSK co-expression significantly increased the tyrosine phosphorylation of rapsyn (360 ± 31% of rapsyn alone, mean ± SEM;

n=3; p=0.01, Student's Ttest). (D) COS cells transfected with rapsyn and/or MuSK were extracted and rapsyn was immunoprecipitated from cell lysates and immunoblotted along with the residual pellet with antiphosphotyrosine antibodies, 4G10 and PY20. Rapsyn immunoprecipitated from the soluble lysate is tyrosine-phosphorylated, although to a lesser extent than rapsyn anchored in the insoluble pellet. Reprobing with mAb1234 shows the relative levels of rapsyn in the soluble and insoluble fractions, and immunoblotting with mAb 9E10 confirms expression of myc-tagged MuSK.

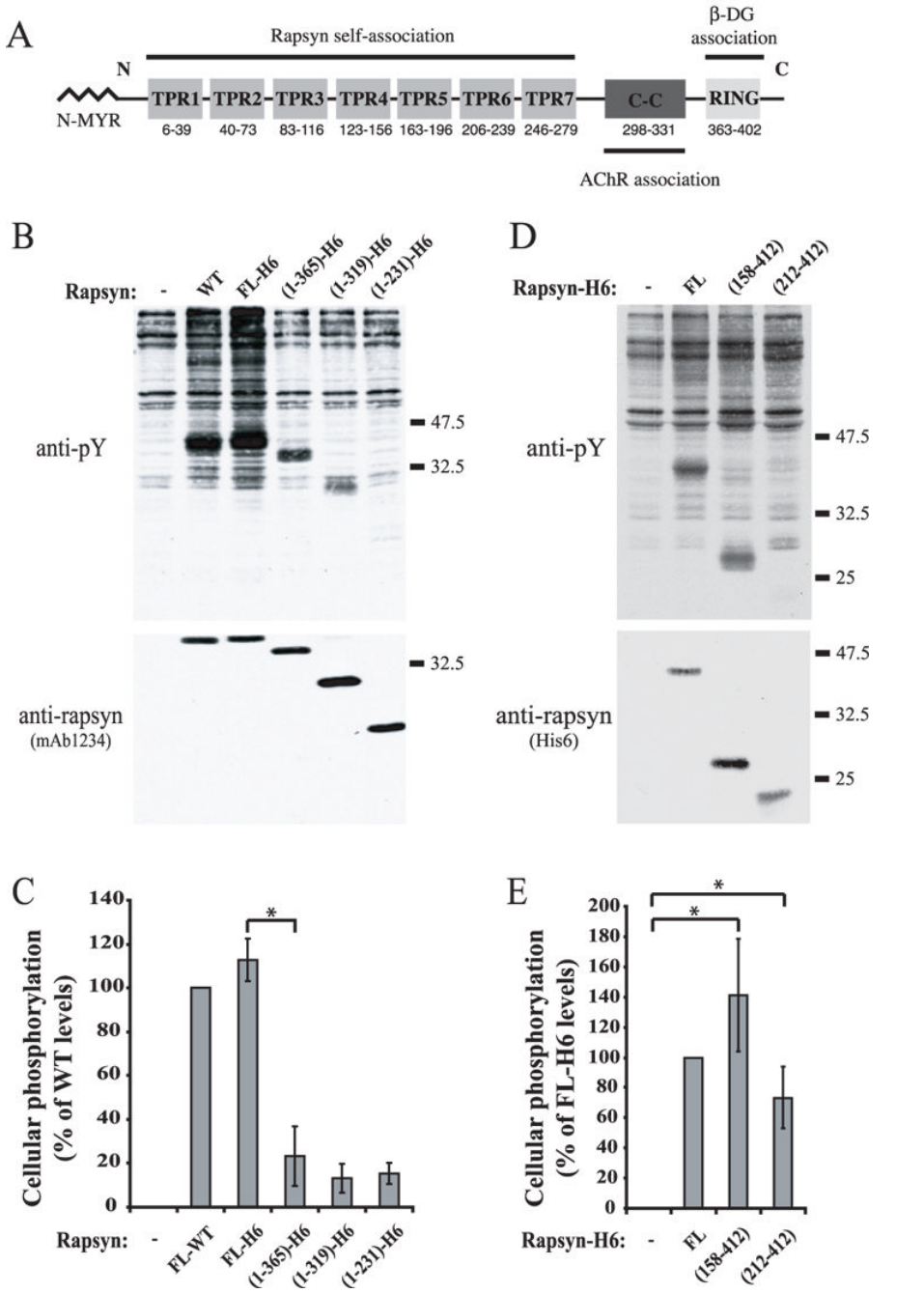

Figure 5. Rapsyn C-terminal domains are necessary and sufficient for kinase activation

(A) Schematic representation of rapsyn's putative domain structure. Rapsyn's tetratricopeptide repeats (TPRs) are implicated in self-aggregation (Ponting and Phillips, 1996, Ramarao and Cohen, 1998, Ramarao et al., 2001, Eckler et al., 2005), and the coiled-coil (C–C) and RING domains are responsible for rapsyn's interaction with AChR (Ramarao and Cohen, 1998) and β-dystroglycan (Bartoli et al., 2001), respectively. (B) COS cells transfected with rapsyn deletion mutants were extracted and the pellet fractions immunoblotted with monoclonal antibodies 4G10 and PY20. Duplicate immunoblots were probed for rapsyn to confirm similar levels of expression. Deletion of rapsyn's C-terminal domains decreased cellular phosphorylation to control levels. (C) Quantification showing that the levels of cellular

phosphorylation obtained with rapsyn 1–365, 1–319 and 1–231 were significantly less than that induced by full-length rapsyn (FL) (rapsyn 1–365: 20 ± 12% of rapsyn FL, respectively; mean ± SEM; n=3; $p<0.01$, Student's T-test), and none were significantly above control values or different from each other (ANOVA - Tukey test). (D) Expression of rapsyn C-terminal fragments was sufficient to increase cellular tyrosine phosphorylation. (E) Rapsyn 158–412 and 212–412 increased phosphorylation significantly above control (n=3; $p=0.02$ for each, Student's T-test) and to levels similar to that with full-length rapsyn (139 ± 38% and 73 ± 20% of rapsyn FL, respectively; mean ± SEM, differences not significant, ANOVA - Tukey test).

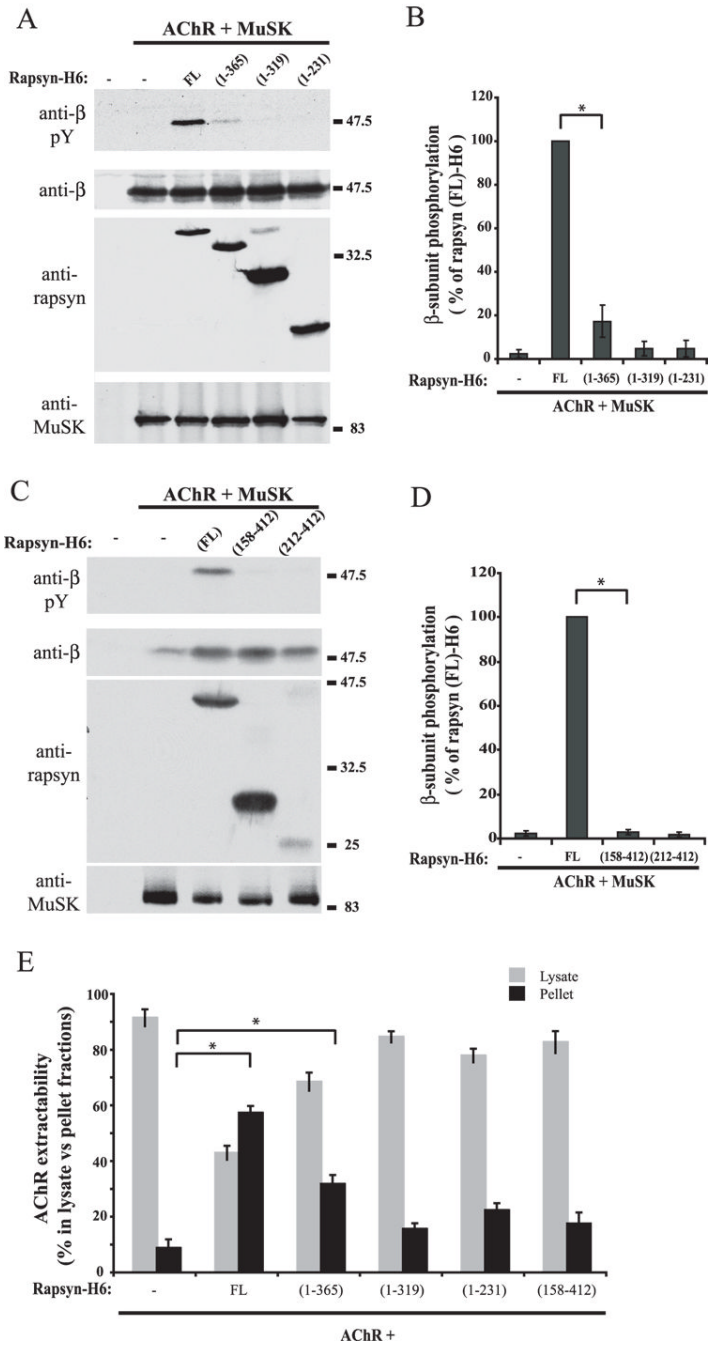

Figure 6. Rapsyn C-terminus is necessary but not sufficient for MuSK-induced AChR phosphorylation

(A) Surface AChR was isolated from COS cells also expressing MuSK and different rapsyn deletion mutants and immunoblotted with β Y390 phospho-specific antibody (JH-1360). Immunoblots were also probed for AChR β subunit (mAb124), rapsyn (His6), and MuSK (9E10) to determine their expression levels. Deletion of rapsyn C-terminal domains reduced β subunit phosphorylation to near control levels. (B) Quantification showing that the levels of β subunit phosphorylation obtained with rapsyn C-terminal deletion mutants were significantly less than that induced by full-length rapsyn (rap 1–365: 17 ± 7% of rapsyn FL, mean ± SEM; n=3; $p<0.01$ Student's T-test) and none were significantly above control values or different

from each other (ANOVA - Tukey test). (C and D) Levels of β subunit phosphorylation obtained with rapsyn 158–412 and 212–412 were significantly less than that induced by full-length rapsyn (3 ± 1% and 2 ± 1% of levels with rapsyn FL, respectively; mean ± SEM; n=5; $p<0.001$, Student's T-test) and neither were significantly above control values (ANOVA - Tukey test). (E) Percentage of I^{125}-αBuTX-labeled AChR in lysate versus pellet fractions following detergent extraction. When co-expressed with full-length rapsyn, 57 ± 2% of surface AChR was anchored in the insoluble pellet, compared to only 9 ± 3 % when receptor was expressed alone (mean ± SEM; $p<0.0001$; n=5, Student's T-test). Rapsyn 1–365 also anchored significant amounts of surface AChR (32 ± 3%; $p<0.001$; n=5, Student's T-test), but further deletion mutants did not anchor receptor consistently above control levels (ANOVA – Tukey test).

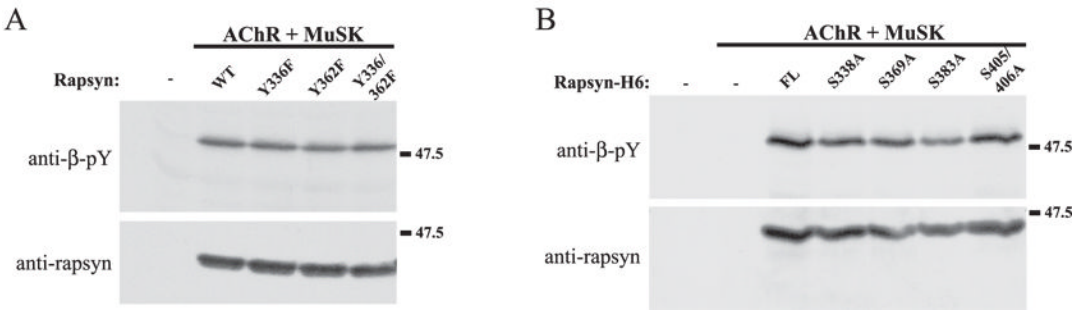

Figure 7. Rapsyn C-terminal phosphorylation is not required for β subunit phosphorylation

AChR was isolated from COS cells expressing MuSK and different rapsyn mutants and immunoblotted with phospho-specific antibody to β subunit Y390 (JH-1360). Mutation of potential tyrosine (Y336F, Y362F in A) or serine (S338A, S369A, S383A, S405/406A in B) phosphorylaton sites in rapsyn's C-terminus did not impair rapsyn-mediated β subunit phosphorylation.