Activation of Muscle-specific Receptor Tyrosine Kinase and Binding to Dystroglycan Are Regulated by Alternative mRNA Splicing of Agrin*\(^{\dagger}\)

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Agrin induces the aggregation of postsynaptic proteins at the neuromuscular junction (NMJ). This activity requires the receptor-tyrosine kinase MuSK. Agrin isoforms differ in short amino acid stretches at two sites, called A and B, that are localized in the two most C-terminal laminin G (LG) domains. Importantly, agrin isoforms greatly differ in their activities of inducing MuSK phosphorylation and of binding to α-dystroglycan. By using site-directed mutagenesis, we characterized the amino acids important for these activities of agrin. We find that the conserved tripeptide asparagine-glutamate-isoleucine in the eight-amino acid long insert at the B-site is necessary and sufficient for full MuSK phosphorylation activity. However, even if all eight amino acids were replaced by alanines, this agrin mutant still has significantly higher MuSK phosphorylation activity than the splice version lacking any insert. We also show that binding to α-dystroglycan requires at least two LG domains and that amino acid inserts at the A and the B splice sites negatively affect binding.

Agrin is a heparan sulfate proteoglycan best known for its function to induce and maintain postsynaptic specializations at the neuromuscular junction (NMJ).\(^{3}\) Agrin was purified from the electric organ of Torpedo californica based on its activity to induce the clustering of acetylcholine receptors (AChRs) on cultured muscle cells (1). This biological function and the fact that agrin-like immunoreactivity is high in motor neurons led to the hypothesis that agrin is the motor neuron-derived factor inducing the formation of postsynaptic structures in vivo (2). Indeed, agrin-deficient mice lack postsynaptic specializations and die at birth of respiratory failure (3, 4). Moreover, agrin alone is sufficient to induce postsynaptic structures in nerve-free regions of adult, fully innervated muscles in vivo (5–8). These data are strong evidence that agrin is both required and sufficient to generate functional postsynaptic structures in skeletal muscle (for reviews see Refs. 9 and 10). Recent evidence also suggests a role of agrin in the formation of the immunological synapse (11) and of synapses in the brain (12, 13). Moreover, a miniaturized form of agrin might be of therapeutic relevance for the treatment of a particular form of congenital muscular dystrophy (14–16).

At least three sites within the agrin gene undergo alternative mRNA splicing resulting in several protein isoforms (see Fig. 1A). Alternative usage of exons at the 5’-end of the gene results in one isoform that binds to the coiled-coil region of laminins via the N-terminal agrin (NtA) domain (17–19) and an another isoform that allows agrin to be incorporated into plasma membranes as a type II transmembrane protein via a transmembrane (TM) region (20, 21). The secreted, NtA form of agrin is expressed in non-neuronal cells and motor neurons and is required to immobilize agrin in the synaptic basal lamina at the NMJ, while the TM form is primarily expressed by neurons of the brain (20, 21). Two additional splice sites are located within laminin G (LG) domains LG2 and LG3. The splice site in LG2, called A in chick and y in rodents, can contain a four-amino acid long insert (A4) that encodes a heparin binding site (22–24). This insert is included in agrin transcripts expressed in neurons and glial cells whereas non-neuronal cells in the periphery, such as muscle, Schwann, and kidney cells, lack this insert (25–27). The second splice site, called B in chick and z in rodents, is encoded by two exons of 24 bp and 33 bp in length. Alternative usage of these exons results in protein isoforms with 0 (B0), 8 (B8), 11 (B11), or 19 (B19) amino acid long inserts. The presence of an insert is essential for AChR aggregation in vitro (25, 28, 29) and for the formation of functional NMJs in vivo (30).

The agrin postsynapse-inducing activity is mediated by the muscle-specific receptor-tyrosine kinase MuSK (31, 32). However, agrin does not bind to MuSK directly, suggesting that

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\(^{3}\) The abbreviations used are: NMJ, neuromuscular junction; AChR, acetylcholine receptors; LG, laminin G; PBS, phosphate-buffered saline; HRP, horseradish peroxidase; PDB, Protein Data Bank; MuSK, muscle-specific receptor tyrosine kinase.
Site-directed Mutagenesis of Agrin

MuSK is the signaling but not the binding component in the agrin receptor complex. Another function that involves the LG domains of agrin is the binding to α-dystroglycan, a peripheral membrane protein that is tightly associated with the transmembraneous β-dystroglycan and arises from a common precursor protein by posttranslational cleavage (33). Binding to α-dystroglycan has also been found in other LG domain-containing proteins such as laminin-α1 and -α2 (33–35), perlecan (35), and neurexins (36). Although initially postulated, binding of agrin to α-dystroglycan is not necessary for its AChR-aggregating activity (23). Several lines of evidence indicate that the binding of agrin to α-dystroglycan is also regulated by alternative mRNA splicing at the A and B sites. For example, a C-terminal fragment that includes the four-amino acid long insert at the A-site and the eight-amino acid long insert at the B-site (agrinA4B8) binds to α-dystroglycan with severalfold lower affinity than the corresponding fragment lacking amino acid inserts (agrinA0B0; Ref. 37). Thus, splicing at the A- and B-site is a critical determinant for several functions of agrin.

The molecular details that are important for the different functions of agrin are not known but structural insights obtained by x-ray crystallography and solution NMR of the LG3 domain from different agrin splice variants (38) have led to the following conclusions: 1) the overall fold of the agrin-LG3 domain is very similar to that of LG domains of other proteins (39–42); 2) the amino acid insert at the B-site is highly flexible indicative of an "induced-fit" mechanism of docking to its putative receptor; 3) the structure of different splice forms of the LG3 domain diverge already seven amino acids before and six amino acids after the B-site; and 4) depletion of Ca2+ from LG3B0 induces structural changes at the same amino acid residues where the different protein isoforms diverge. Ca2+ depletion also induces a high flexibility in LG3B8 and the most dynamic residues are those whose orientations differ between LG3B0 and LG3B8. These studies thus led us to speculate that amino acids involved in the agrin AChR-aggregating function might not only be found within the B-splice insert but also outside.

We now used site-directed mutagenesis of conspicuous amino acids to identify those involved in MuSK phosphorylation. We find that an agrin mutant where all amino acids of the eight-amino acid insert at the B-site were replaced by alanines is the tripeptide Asn-Glu-Ile with the strongest contribution of amino acids outside. Moreover, we find that an agrin mutant where all amino acids of the amino acids involved in the agrin AChR-aggregating function (23). Several lines of evidence indicate that the binding of agrin to α-dystroglycan is also regulated by alternative mRNA splicing at the A and B sites. For example, a C-terminal fragment that includes the four-amino acid long insert at the A-site and the eight-amino acid long insert at the B-site (agrinA4B8) binds to α-dystroglycan with severalfold lower affinity than the corresponding fragment lacking amino acid inserts (agrinA0B0; Ref. 37). Thus, splicing at the A- and B-site is a critical determinant for several functions of agrin.

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GAA GGC AGC TGG, and as_cAgrin_BamHI_G3 CAC GAG GAT CCC TTT GGC TGA GC. The PCR products were digested with NheI and BamHI and ligated into C45_A14B8 cut with NheI/BamHI. Sequencing was used to assure that all constructs were correct.

Mutant proteins were generated by site-directed mutagenesis of C45_A14B8 that was cloned into pBluescript KS+ (Stratagene). Mutations were introduced by forward and reverse primers with overlapping 5'-ends that encoded sequences on either side of the mutation (primer sequences are available on request). PCR was on the whole plasmid with long expansion polymerase kit (Roche Applied Science). Template DNA was removed by DpnI digestion and the new plasmid containing the mutation was transformed into DH10B. Mutations were confirmed by sequencing. Mutated constructs were subcloned into pCEP-Pu containing the BM-40 signal sequence and human γ1-Fc tag by BamHI/EcoRI digestion.

Quantification of Agrin—To determine the concentration of recombinant agrin, 100 μl of goat anti-human γ1 IgG (5 μg/ml, BIOSOURCE) in 50 mM sodium carbonate buffer (pH 9.6) was immobilized on a 96-well high binding microtiter plate (Costar) overnight at 4 °C. Agrin-containing media or purified agrin was incubated for 1 h followed by 3 washes with PBST (PBS + 0.05% Tween 20) and incubation of an HRP-conjugated goat anti-human γ1 IgG antibody (70 ng/ml; BIOSOURCE) for 3 h at room temperature. After four washes with PBST, the amount of agrin was measured by using McEvans solution, ABTS and H2O2 as an HRP substrate. Absorbance was measured on an ELISA reader at 405 nm at several time points. As a standard, affinity-purified C45_A14B8 at the concentration range between 0.1 and 100 ng/ml was used.

Solid Phase MutSK Phosphorylation Assay—C2C12 myotubes were grown in gelatin-coated 96-well plates (Falcon). Agrin stimulations were routinely done for 30 min with protein diluted in 0.44 mM KH2PO4, 1.34 mM Na2HPO4, 5.6 mM glucose, 137 mM NaCl, 1 mM MgCl2, 1.25 mM CaCl2, 25 mM HEPES, and 1 mg/ml bovine serum albumin, pH 7.4. Myotubes were subsequently rinsed and extracted in lysis buffer (20 mM HEPES, 1 mM EDTA, 1 mM EGTA, 150 mM NaCl, 1% Nonidet P40, phosphatase, and protease inhibitors) for 30 min at 4 °C. The extracted proteins were transferred to a 96-well high binding microtiter plate (Costar) that had previously been coated with 100 μl of the anti-phosphotyrosine antibody 4G10 (1 μg/ml, Upstate) in 50 mM sodium carbonate buffer, pH 9.6 and had been blocked with PBS containing 0.5% Tween-20 and 0.5% Top Block (Juro). Extracts of C2C12 myotubes were incubated in the microtiter plates for 5 h at 4 °C. Plates were washed four times with PBS containing 0.1% Tween 20 (PBST) followed by an overnight incubation of protein A-purified anti-MuSK IgG (194T; dilution 1:5,000) in PBS containing 0.1% Tween 20 and 0.1% Top Block (incubation buffer). Plates were rinsed 4 times with PBST and incubated for 4 h at 4 °C with 100 μl of an HRP-conjugated donkey anti-rabbit IgG (0.2 μg/ml; Jackson Immunoresearch). Subsequently, the plate was washed with PBST four times and once with PBS. For detection, QuantaBlue (Pierce) was mixed according to the manufacturer’s advice, and 100 μl of the solution was added to each well. The reaction was stopped after 30 min by QuantaBlue stop solution. Fluorescence was measured on an ELISA reader (Gemini) with the excitation wavelength of 325 nm and detection at 420 nm.

Purification of α-Dystroglycan—Purification was essentially done as described elsewhere (45). In brief, 10 g of frozen chicken breast were homogenized in 50 mM Tris- HCl, pH 7.4; 0.2 M NaCl (buffer A). After centrifugation (12,000 × g), the supernatant was filtered through a Whatman filter paper. The cleared solution was incubated at 4 °C with 10 ml of DEAE-Sephacel beads (Amersham Biosciences) that had been equilibrated in buffer A. After extensive washes with buffer A, bound proteins were eluted from the beads by 50 mM Tris- HCl, pH 7.4; 0.5 M NaCl (buffer B). Eluates were then incubated with 5 ml of WGA-Sepharose previously equilibrated in buffer B. After washing the resin 5 times with buffer B, elution from WGA-Sepharose was achieved by adding 300 mM N-acetylglucosamine. Eluted proteins were then dialyzed against 150 mM NaCl, 10 mM Tris- HCl, pH 7.4. The enrichment of α-dystroglycan was verified by transfer overlay assay as described below.

Solid Phase α-Dystroglycan Binding Assay—Enzyme-linked binding assays were performed with 100 μl of α-dystroglycan (5 μg/ml) in 50 mM sodium carbonate buffer pH 9.6, immobilized on a 96-well high binding microtiter plate by absorption overnight at 4 °C. After blocking with PBS containing 0.05% Tween-20, 1 mM CaCl2, 1 mM MgCl2 and 3% BSA (blocking buffer), wells were incubated with agrin proteins diluted in blocking buffer. Wells were then washed with blocking buffer and incubated with an HRP-conjugated goat anti-human γ1 IgG (70 ng/ml in blocking buffer). The amount of HRP was measured using McEvans solution and ABTS/H2O2 as substrate. Absorbance was measured on an ELISA reader at 405 nm. To avoid saturation of the signal, measurements were performed every 5 min.

Transfer Overlay Binding Assay—Purified chicken skeletal muscle α-dystroglycan was denatured with reducing SDS sample buffer, separated on a 3–12% SDS-PAGE (46), and transferred to nitrocellulose (47). Blots were blocked for 2 h with PBS containing 0.05% Tween-20, 1 mM CaCl2, 1 mM MgCl2 and 5% milk powder (blocking buffer). The membrane was cut into strips and incubated overnight at 4 °C with agrin diluted in blocking buffer. Nitrocellulose strips were washed three times with blocking buffer and subsequently incubated for 2 h with an HRP-conjugated goat anti-human γ1 IgG (1 μg/ml). Finally, the membrane strips were washed three times with blocking buffer and once with PBS containing 0.05% Tween-20, 1 mM CaCl2 and 1 mM MgCl2. Immunoreactive protein bands were visualized by the ECL detection method (Pierce).

Curve Fitting—Data were fitted using a single class of equivalent binding sites according to Equation 1,

\[ A_i = A_{sat} \times \left[ \frac{c}{K_d + c} \right] + A_0 \]

where \( A_i \) represents the absorbance measured at a particular concentration of the ligand, \( K_d \) the dissociation constant, \( c \) the concentration of the ligand, and \( A_{sat} \) and \( A_0 \) are the absorbances at saturation and in the absence of ligand, respectively. Data were normalized according the equation \((A_i - A_0)/(A_{sat} - A_0)\).

The software used was Prism GraphPad 4.0.

Modeling—The splice insert model was built based on the solution NMR structure of LG3_to (PDB code: 1Q56) together
with both calcium containing x-ray structures of LG3\textsubscript{B8} (PDB code: 1PZ8) and LG3\textsubscript{B11} (PDB code: 1PZ9). Residues missing in the splice insert were included manually. The lowest energy structure was subject to 200 cycles of unrestrained Powell minimization using CNS (48). Harmonic restraints were imposed on the protein atoms (3 kcal/mol Å\(^2\)) with increased weight (20 kcal/mol Å\(^2\)). The model of the LG2 domain and the EGF domain were generated using SWISS-MODEL. The tandem structure of LG2-EGF-LG3 was created on the basis of typical elbow angle described for Fv-fragments. To avoid sterical clashes between LG2-EGF and EGF-LG3, the same refinement protocol as described above was performed.

RESULTS

Constructs and Cellular Assays—
To facilitate purification of the recombinant protein from the medium of transfected HEK293 EBNA cells and to increase apparent binding affinities, all agrin fragments were expressed as fusion constructs with the Fc part of human \(\gamma\)H9253\textsubscript{1} (Fig. 1B). The first set of agrin mutants was derived from a construct encoding a 45-kDa, C-terminal fragment (called C45\textsubscript{A0/4B0/8/11}) that comprises LG2 and LG3 and one EGF-like (EG) domain. This fragment is known to be the smallest one that induces AChR aggregation with a half-maximal response (EC\textsubscript{50}) at picomolar concentration (29). To test the influence of individual splice inserts independent of the second site, we also generated constructs encoding only LG2 or LG3 (Fig. 1B). For competition experiments, we further used a miniaturized form of agrin A0B0 (N25C95 A0B0 or mini-agrin; 14). We mutated amino acid residues that were identical between fish and mammals (bold in Fig. 1C).

Because agrin-induced AChR aggregation requires MuSK phosphorylation, we first established a two-side enzyme-linked immu-
nosorbent assay (ELISA) that allowed us to determine reliable dose-response curves for the different constructs. The assay (Fig. 1D) is based on the use of the monoclonal antibody 4G10 (49), which recognizes tyrosine-phosphorylated proteins and an anti-MuSK antiserum that recognizes MuSK in immunohistochemistry and Western blot analysis (supplementary Fig. S1). The ELISA detects MuSK phosphorylation in cultured C2C12 myotubes after stimulation by 2 nM neural agrin (Fig. 1E; MT + agrin). A nonspecific signal was detected in non-treated myotubes (MT) or myoblasts incubated with 2 nM neural agrin (MB + agrin). This signal persisted even when no cell extract was added (w/o cells). The background signal was, however, lost upon omitting the anti-MuSK IgG (w/o 1Ab) or the coating of the plates with 4G10 (w/o coating). Similarly, when preimmune serum was used instead of the anti-MuSK antiserum, the high background signal became lower (data not shown). This indicates that this relatively high background originates from tyrosine-phosphorylated IgG in the primary antiserum. As the background was very stable and agrin-induced MuSK phosphorylation resulted in a consistent, significantly higher signal, the assay was still sensitive and reproducible enough to measure phosphorylation of MuSK in cultured C2C12 myotubes.

**Contribution of Amino Acids within the B-site to Agrin-induced MuSK Phosphorylation**—To determine the contribution of amino acids within the B-inserts to the agrin MuSK phosphorylation activity, we compared the potency of agrin splice variants in the MuSK phosphorylation assay. As shown in Fig. 2A, the EC$_{50}$ value was lowest with C45A$_{4B8}$ followed by C45A$_{4B11}$. No activity was detected with C45A$_{4B0}$ and C45A$_{0B0}$. Thus, MuSK phosphorylation activity of agrin splice variants follows the same order as their AChR aggregation (29). Whereas even 100 nM of C45A$_{4B0}$ did not induce any MuSK phosphorylation, the same concentration induced some AChR clusters (Ref. 29; see also supplementary Fig. S2).

To get insights into the molecular details of agrin-induced MuSK phosphorylation, we next made a series of mutants where conserved amino acids of the B8 insert were altered to alanine (Fig. 2B). First, we replaced all eight amino acids by alanines (mutant C45B$_{8-A}$.). As shown in Fig. 2C, the potency and efficacy of C45B$_{8-A}$ was much lower than that of wild-type C45A$_{4B8}$. In strong contrast to C45A$_{4B0}$, however, C45B$_{8-A}$ still induced MuSK phosphorylation at high concentration (Fig. 2C). These data show that the amino acid sequence of the B-site is important to reach full potency but that the spacing per se also contributes to the activity suggesting that amino acids that flank the B-site also participate in the activity (see below).

To determine the amino acid motif in the B insert that is responsible for the high potency of C45A$_{4B8}$, we next mutated conserved amino acids in the B8-insert (see Fig. 2B for a list of mutants). Surprisingly, mutation of single amino acids did not alter the potency or efficacy of agrin constructs at all (Fig. 2C and Table 1). Thus, we next examined the influence of double and triple mutations. A conspicuous sequence of the B insert is the conserved tripeptide motif Asn-Glu-Ile (NEI) because the first two amino acids could complex Ca$^{2+}$, which has been shown to be important for the agrin AChR clustering activity (50, 51). Indeed, double or triple mutations of the NEI motif affected the agrin activity strongly. Whereas the double mutant C45NEI$_{B8-AES}$ and the triple mutant C45NEI$_{B8-AAA}$ had a similarly low activity as the C45B$_{8-A}$ mutant (Fig. 2C), the double mutant C45NEI$_{B8-AAI}$ had an EC$_{50}$ that was between wild-type C45A$_{4B8}$ and the C45B$_{8-A}$ mutant. To see whether the NEI-motif was the only critical determinant of agrin activity, we also tested the construct C45HSNEIPA$_{B8-AAA}$, where only the NEI motif is maintained and all the remaining amino acids in C45HSNEIPA$_{B8-AAA}$ were mutated to alanine.

**FIGURE 2. Influence of amino acid inserts and their sequence of the B-site on MuSK phosphorylation.** A, agrin-induced phosphorylation of MuSK requires amino acid inserts at the B-site, and C45A$_{4B8}$ is more potent than C45A$_{4B11}$. B, schematic representation of mutations within the eight-amino acid insert at the B-site. C, dose response curves for MuSK phosphorylation of mutants shown in B. Mutation of all amino acids to alanine (C45B$_{8-A}$) lowers potency strongly but does not abrogate all of the activity. This difference is based on mutations in the tripeptide NEI as alteration of at least two amino acids within this peptide lowers the potency to a similar extent (e.g., C45NEI$_{B8-AES}$, C45NEI$_{B8-AAI}$, C45NEI$_{B8-AAA}$). Interestingly, mutation of a single amino acid does not affect potency (C45B$_{8-A}$, C45E$_{8-B}$, C45I$_{8-A}$) and preservation of only the NEI tripeptide is sufficient to regain full potency (C45HSNEIPA$_{B8-AAA}$). Each data point represents the mean ± S.E. of at least four replicates.

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the B insert are changed to alanines. Indeed C45 had a similar activity as C45A4B8 (Fig. 2C and Table 1). These results clearly indicate that the NEI tripeptide and in particular the flanking asparagine and isoleucine accounts for the entire gain in activity from C45B8–8A to wild-type C45A4B8.

**Role of Amino Acids Flanking the B-site in MuSK Phosphorylation**—Next, we performed site-directed mutagenesis on amino acids that flank the B-site. Amino acids were selected based on the difference between the structure of the inactive LG3B0 and active LG3B11 or LG3B8 (38). The differences begin at histidine of position 1778 (His1778), include the amino acid inserts at the A- or the B-site (37, 55). To test whether the fragment comprising only the two most C-terminal LG domains still binds to α-dystroglycan, we first measured the binding of agrin to α-dystroglycan in overlay transfer assays. While we could not observe binding of C45A4B8 all the other isoforms did bind (Fig. 4A). In solid phase binding assays, the binding affinity to α-dystroglycan was lowest with C45A4B11 (Fig. 4B) and highest with C45A0B8 (Fig. 4C) while C45A0B0 (Fig. 4D) and C45A0B8 (Fig. 4E) had an intermediate affinity. Finally, C45A4B1 had a similar binding affinity as C45A4B8 (Fig. 4F). These data show that the presence of amino acid inserts at the A- or the B-site negatively influence binding to α-dystroglycan (see also Table 2). For the B-site, this influence is not caused by the amino acid composition itself, because mutants with very little MuSK phosphorylation activity (e.g. C45B8–8A or C45N-B8-A-A) had a similar binding affinity to α-dystroglycan as C45A4B8 (data not shown).

To measure the influence of splicing at the A- and B-site directly, we also generated constructs encoding only one LG domain. As previous experiments suggested that two LG domains are required for strong binding to α-dystroglycan (35, 37), we force-dimerized LG2 and LG3 with human γ1-Fc. As shown in Fig. 5, we indeed detected significant binding to α-dystroglycan using LG3B0 (Fig. 5A) and LG2A0 (Fig. 5B) but the binding was lost in LG3B8 and LG2A (data not shown). Thus, force-dimerized LG domains LG2 and LG3 do bind to α-dystroglycan but only if they do not contain amino acid inserts at the A- or the B-site. When we measured MuSK phosphorylation activity of the constructs coding for single LG domains, we indeed detected significant activity in LG2A and LG3B0 but none in LG3B8 or LG2A (Fig. 5C and Table 1). Mutants C45N1779A and the double mutant C45N1779A/N1791A became even more potent than the wild-type protein (Fig. 5B and Table 1). It is, however, important to note that C45N1779A and C45N1779A/N1791A were expressed at much lower levels than all the other constructs (supplementary Fig. S2B), suggesting that the protein is less stable.

**Agrin Binding to α-Dystroglycan Is Modulated by Inserts at A- and B-Sites**—The binding region to α-dystroglycan also partially overlaps with the region necessary for AChR aggregation (52–54). Several lines of evidence suggest that the binding affinity of agrin to α-dystroglycan is regulated by amino acid inserts at the A- and the B-site (37, 55). To test whether the fragment comprising only the two most C-terminal LG domains still binds to α-dystroglycan, we first measured the binding of agrin to α-dystroglycan in overlay transfer assays. While we could not observe binding of C45A4B8 all the other isoforms did bind (Fig. 4A). In solid phase binding assays, the binding affinity to α-dystroglycan was lowest with C45A4B11 (Fig. 4B) and highest with C45A0B8 (Fig. 4C) while C45A0B0 (Fig. 4D) and C45A0B8 (Fig. 4E) had an intermediate affinity. Finally, C45A4B1 had a similar binding affinity as C45A4B8 (Fig. 4F). These data show that the presence of amino acid inserts at the A- or the B-site negatively influence binding to α-dystroglycan (see also Table 2). For the B-site, this influence is not caused by the amino acid composition itself, because mutants with very little MuSK phosphorylation activity (e.g. C45B8–8A or C45N-B8-A-A) had a similar binding affinity to α-dystroglycan as C45A4B8 (data not shown).

To measure the influence of splicing at the A- and B-site directly, we also generated constructs encoding only one LG domain. As previous experiments suggested that two LG domains are required for strong binding to α-dystroglycan (35, 37), we force-dimerized LG2 and LG3 with human γ1-Fc. As shown in Fig. 5, we indeed detected significant binding to α-dystroglycan using LG3B0 (Fig. 5A) and LG2A0 (Fig. 5B) but the binding was lost in LG3B8 and LG2A (data not shown). Thus, force-dimerized LG domains LG2 and LG3 do bind to α-dystroglycan but only if they do not contain amino acid inserts at the A- or the B-site. When we measured MuSK phosphorylation activity of the constructs coding for single LG domains, we indeed detected significant activity in LG2A and LG3B0 but none in LG3B8 or LG2A (Fig. 5C and Table 1). Mutants C45N1779A and the double mutant C45N1779A/N1791A became even more potent than the wild-type protein (Fig. 5B and Table 1). It is, however, important to note that C45N1779A and C45N1779A/N1791A were expressed at much lower levels than all the other constructs (supplementary Fig. S2B), suggesting that the protein is less stable.

**Agrin Binding to α-Dystroglycan Is Modulated by Inserts at A- and B-Sites**—The binding region to α-dystroglycan also partially overlaps with the region necessary for AChR aggregation (52–54). Several lines of evidence suggest that the binding affinity of agrin to α-dystroglycan is regulated by amino acid inserts at the A- and the B-site (37, 55). To test whether the fragment comprising only the two most C-terminal LG domains still binds to α-dystroglycan, we first measured the binding of agrin to α-dystroglycan in overlay transfer assays. While we could not observe binding of C45A4B8 all the other isoforms did bind (Fig. 4A). In solid phase binding assays, the binding affinity to α-dystroglycan was lowest with C45A4B11 (Fig. 4B) and highest with C45A0B8 (Fig. 4C) while C45A0B0 (Fig. 4D) and C45A0B8 (Fig. 4E) had an intermediate affinity. Finally, C45A4B1 had a similar binding affinity as C45A4B8 (Fig. 4F). These data show that the presence of amino acid inserts at the A- or the B-site negatively influence binding to α-dystroglycan (see also Table 2). For the B-site, this influence is not caused by the amino acid composition itself, because mutants with very little MuSK phosphorylation activity (e.g. C45B8–8A or C45N-B8-A-A) had a similar binding affinity to α-dystroglycan as C45A4B8 (data not shown).

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domains, only LG3B8 was active while no activity was seen for any other construct (Fig. 5C and Table 1). In this assay, the measured EC$_{50}$ of LG3B8 was very close to its EC$_{50}$ measured for AChR aggregation assays (29), and was $\sim$4 times higher than the EC$_{50}$ of C45A4B8. As the increased MuSK phosphorylation activity of C45A4B8 might be due to the binding of agrin to $\alpha$-dystroglycan, as was suggested earlier (23, 24, 56), we also determined MuSK phosphorylation of C45A0B8 that binds to $\alpha$-dystroglycan with high affinity (14). This construct was used at a 100-fold molar excess to assure saturation of $\alpha$-dystroglycan binding sites on C2C12 myotubes. Co-incubation increased the apparent EC$_{50}$ for MuSK phosphorylation of C45A4B8 by 140 times and that of C45A0B8 by 60 times (Fig. 6, A and B). Interestingly, the apparent EC$_{50}$ of C45A4B8 in the presence of cN25C95A0B0 was very similar to that of LG3B8, suggesting that binding of agrin to $\alpha$-dystroglycan contributes substantially to the potency in MuSK phosphorylation. In another experiment we also determined the effect of heparin on MuSK phosphorylation. For both, C45A4B8 and C45A0B8, heparin shifted the EC$_{50}$ 20- and 10-fold, respectively (Fig. 6, C and D). The finding that MuSK phosphorylation of C45A4B8 was more strongly inhibited by heparin than that of C45A0B8 is likely due to the KSRK sequence of the A insert, which has been shown to bind to heparin (22–24). In summary, these experiments strengthen the view that binding of agrin to $\alpha$-dystroglycan and/or heparan sulfate proteoglycans increases MuSK phosphorylation activity.

**DISCUSSION**

Agrin is a good example for the capability of alternative mRNA splicing to regulate the function of a protein because inclusion of an amino acid insert at the B-site is absolutely essential for the formation of postsynaptic structures both in vitro and in vivo (reviewed in Refs. 57 and 58). This activity is mediated by the receptor tyrosine kinase MuSK (31) and only agrin isoforms that contain amino acid inserts at the B-site can activate MuSK (32; see also Fig. 2B). In this work, we established a reliable assay to determine the degree of MuSK phosphorylation in cultured myotubes and have used this assay to identify $\alpha$-dystroglycan with increased affinity compared with C45A4B8 (Table 2). Indeed, EC$_{50}$ of C45A0B8 was $\sim$4 times lower than that of C45A4B8 (Fig. 5D). This result is consistent with the idea that $\alpha$-dystroglycan can play an auxiliary role in MuSK phosphorylation.

If $\alpha$-dystroglycan would have an auxiliary role, inhibition of this binding should increase the apparent EC$_{50}$ of agrin in MuSK phosphorylation assays. We therefore measured MuSK phosphorylation in the presence of the agrin fragment N25C95A0B0 (also called mini-agrin), which binds to $\alpha$-dystroglycan (14). As the increased MuSK phosphorylation activity of C45A4B8 might be due to the binding of agrin to $\alpha$-dystroglycan, as was suggested earlier (23, 24, 56), we also determined MuSK phosphorylation of C45A0B8 that binds to $\alpha$-dystroglycan with high affinity (14). This construct was used at a 100-fold molar excess to assure saturation of $\alpha$-dystroglycan binding sites on C2C12 myotubes. Co-incubation increased the apparent EC$_{50}$ for MuSK phosphorylation of C45A4B8 by 140 times and that of C45A0B8 by 60 times (Fig. 6, A and B). Interestingly, the apparent EC$_{50}$ of C45A4B8 in the presence of cN25C95A0B0 was very similar to that of LG3B8, suggesting that binding of agrin to $\alpha$-dystroglycan contributes substantially to the potency in MuSK phosphorylation. In another experiment we also determined the effect of heparin on MuSK phosphorylation. For both, C45A4B8 and C45A0B8, heparin shifted the EC$_{50}$ 20- and 10-fold, respectively (Fig. 6, C and D). The finding that MuSK phosphorylation of C45A4B8 was more strongly inhibited by heparin than that of C45A0B8 is likely due to the KSRK sequence of the A insert, which has been shown to bind to heparin (22–24). In summary, these experiments strengthen the view that binding of agrin to $\alpha$-dystroglycan and/or heparan sulfate proteoglycans increases MuSK phosphorylation activity.

**TABLE 2**

Influence of amino acid inserts at the A- and B-site on the binding of agrin to $\alpha$-dystroglycan

| Insert | Apparent Kd mean ± SEM |
|--------|------------------------|
| C45A4B8 | 90.4 ± 102 |
| C45A0B0 | 47.4 ± 6 |
| C45A4B0 | 104.9 ± 14 |
| C45A0B8 | 69.0 ± 7 |

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**FIGURE 4.** Binding of agrin to $\alpha$-dystroglycan is influenced by amino acid inserts at the A- and the B-site. A, transfer overlay assays on purified $\alpha$-dystroglycan that was separated on a 7.5% SDS-PAGE and blotted to nitrocellulose. Except for C45A4B8, clear binding was detected for all agrin isoforms. B–F, solid phase binding assays with different agrin constructs. The order of binding affinities was C45A0B0 $\ll$ C45A4B0 $\approx$ C45A4B8 (see also Table 2). Each value represents the mean $\pm$ S.E. of three replicates.
amino acids in agrin that contribute to MuSK phosphorylation. Our results show that both, the spacing and the tripeptide Asn-Glu-Ile (NEI) of the B insert contribute to the difference in activity. The following results led us to conclude this. First, mutation of all eight amino acids or of the NEI tripeptide alone lowers the potency to the same extent (80-fold; Fig. 2C and Table 1). Second, the construct C45A4B8 or C45A0B8 (see Fig. 5 A and B, binding of single LG domains to α-dystroglycan is only possible with agrin constructs that lack amino acid inserts at the A- or the B-site. C, LG3_b0 is capable of inducing MuSK phosphorylation but with significantly lower potency than C45_A4B8 or C45_A0B8 (see D). MuSK phosphorylation activity of C45_A0B8 which binds with higher affinity to α-dystroglycan than C45_A4B8 (Fig. 2C), increases as the spacing and the tripeptide Asn-Glu-Ile (NEI) from the B insert are replaced by alanines. This is underlined by the conservation of the tetrapeptide NEIP in all so far known agrin sequences.

Our finding that the mutant C45_B8–8A, in which all eight amino acids in B8 that flank the NEI tripeptide were mutated to Ala, regains full potency (Fig. 2C and Table 1).

Based on the x-ray and NMR structure of the LG3 domain (38), the splice insert forms an almost linearly arranged recognition surface in close spatial proximity to the insert-specific rim sheet (Sr) with a maximal distance of 11 Å between the side chains of splice insert residues (Fig. 7A). The side chains of the most critical Asn and Ile are both pointing to the exterior of the loop. The exposition to the solvent and the lack of any stabilizing contacts to neighboring residues or water molecules in the crystals may offer an explanation for their functional importance. Glutamate in the middle of the tripeptide is oriented to the inside of the loop and can form a salt bridge to Lys1786. We propose that MuSK phosphorylation is dependent on a combination of hydrophobic and ionic interactions where the recognition surface composed of a calcium binding site, the insert-induced rim sheet and the solvent-exposed splice insert could act as adaptor modules.

We also measured MuSK phosphorylation activity of the agrin_A4B11 splice variant, which has been shown to have a considerably lower AChR aggregation activity than agrin_A4B8 (28, 29). In the MuSK phosphorylation assay, C45_A4B11 is 10 times less potent than C45_A4B8 but it is still considerably more potent than C45_B8–8A (Table 1). Thus, the 11 amino acids of the B insert actively contribute to the agrin activity. The structural comparison of the B8 and B11 inserts suggests that the Leu-Asp-Tyr residues in the B11-insert could have a similar function like the NEI tripeptide in B8. In comparison to glutamate, the side chain of aspartate in B11 could also establish the insert-stabilizing salt bridge with Lys1786. The reduced activity in C45_A4B11 can be explained by replacement of residues in positions 1 and 3 (Asn to Leu and Ile to Tyr) that changes the electrostatic properties, hydrophobicity, and ligand accessibility.

It is also particularly intriguing that single point mutations within the B8 insert do not affect agrin MuSK phosphorylation activity. As revealed in our model and further supported by dynamic data from the solution NMR studies (38), the splice insert is highly flexible, which could allow it to adapt many different spatial orientations. In particular, the fact that mutations of a single amino acid in the NEI tripeptide do also not affect activity suggests an additional structure-stabilizing effect of proline in the NEI-B8-AA1 double mutant. This is underlined by the conservation of the tetrapeptide NEIP in all so far known agrin sequences.

Our finding that the mutant C45_B8–8A, in which all eight amino acids in the B insert are replaced by alanines retains MuSK phosphorylation activity suggests that the spatial arrangement of the amino acid residues in the region flanking the B-site is also important for agrin activity. The finding that insertion of an amino acid stretch at the B-site affects MuSK phosphorylation activity irrespective of its sequence also explains why peptides encoding the eight-amino acid long insert or cyclic peptides thereof neither induce nor inhibit phosphorylation of MuSK.4 Because the agrin splice versions that lack an amino acid insert at the B-site do not have any AChR aggregation and MuSK phosphorylation activity, the amino acids outside of the B-site that are involved in this difference must differ between the structure of LG3_b0 and LG3_b11. The x-ray and NMR structure showed that the accommodation of the B-site into the LG3 domain does not change the overall structure of the LG3 domain but influences six to seven amino acids in the proximity to the B-site (38). We therefore mutagenized all of those amino acids and found that mutation

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4 P. Scotton and Markus A. Ruegg, unpublished data.
of Asn\textsuperscript{1779} resulted in a 60-fold increase in potency (Fig. 3B). The structure of LG3 suggests a switcher function of Asn\textsuperscript{1779} (38) as it marks not only the point of spatial differentiation between LG3\textsubscript{B0} and LG3\textsubscript{B8/B11} but it also reorients itself in LG3\textsubscript{B8} upon calcium depletion. The side chain of Asn\textsuperscript{1779} is oriented to the inside of the hydrophobic cleft between concave and convex \(\beta\)-sheet of LG3 and forms stabilizing hydrogen bonding networks with Asn\textsuperscript{1791} and Asp\textsuperscript{1932} (data not shown). Therefore, lack of this side chain has destabilizing effects on the structure and increases the flexibility of the B-loop, which may result in a higher MuSK phosphorylation and AChR aggregation activity. Our results would also fit to the model proposed earlier (38) that the plasticity of the interaction interface optimizes the selectivity through induced fit binding. The low expression level of the Asn\textsuperscript{1779} mutant indeed strengthens this hypothesis.

We also show direct evidence that splicing at the A and B sites influences agrin binding to \(\alpha\)-dystroglycan (Figs. 4 and 5A and Table 2). Interestingly, the presence of an amino acid insert at the A- or the B-site negatively correlates with binding to \(\alpha\)-dystroglycan. One interpretation of this result is that the binding of agrin to \(\alpha\)-dystroglycan is inhibitory for agrin MuSK phosphorylation activity. This seems, however, not to be the case as agrin mutants that contain an insert at the B-site but have low MuSK phosphorylation activity (e.g. C45\textsubscript{B8–8A}) bound to \(\alpha\)-dystroglycan with the same affinity as the highly potent forms (e.g. C45\textsubscript{AABB}; data not shown). In the contrary, agrin constructs that bind to \(\alpha\)-dystroglycan strongly (e.g. C45\textsubscript{A0B8}) are slightly more potent in MuSK phosphorylation assays. Thus, increased binding of agrin to \(\alpha\)-dystroglycan supports agrin MuSK phosphorylation activity. Our data also support the view that the binding of agrin to \(\alpha\)-dystroglycan is mediated by domains that are in the proximity of the splice sites A and B. Moreover, binding requires at least two LG domains. We therefore propose a model that accommodates all these findings (Fig. 7B). Our model is reminiscent of the Fv-fragment of antibodies with the splice insert regions mimicking hypervariable loop segments of CDR. Our model would explain the low cooperativity observed between \(\alpha\)-dystroglycan binding and MuSK phosphorylation. However, the model is based entirely on the presentation of the A and the B inserts at the same surface of the tandem molecule. It cannot be excluded that the elbow angle, which was refined in the modeling protocol to 112° might be adaptable. This
Site-directed Mutagenesis of Agrin

In another series of experiments we analyzed whether the binding of agrin to α-dystroglycan per se is important for agrin’s MuSK phosphorylation activity. Agrin-induced MuSK phosphorylation could be inhibited by heparin or N25C95A0B0. In contrast, MuSK phosphorylation induced by LG3B8 cannot be inhibited by N25C95A0B0 or heparin (data not shown). Moreover, the potency of C45A4B8 to phosphorylate MuSK dropped in the presence of N25C95A0B0 to that of LG3B8. Thus, agrin binding to the myotube surface seems largely driven by its binding to α-dystroglycan and this increases its efficacy of activating MuSK. Similarly, the LG3B8 domain is much more efficient in the presence of N25C95A0B0 to that of LG3B8. Thus, agrin might increase the levels of utrophin, which has been shown to be capable of compensating the loss of dystrophin in mouse models for Duchenne muscular dystrophy (60).

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