Mapping of the laminin-binding site of the N-terminal agrin domain (NtA)

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Agrin is a key organizer of acetylcholine receptor (AChR) clustering at the neuromuscular junction. The binding of agrin to laminin is required for its localization to synaptic basal lamina and other basement membranes. The high-affinity interaction with the coiled-coil domain of laminin is mediated by the N-terminal domain of agrin. We have adopted a structurally guided site-directed mutagenesis approach to map the laminin-binding site of NtA. Mutations of L117 and V124 in the C-terminal helix 3 showed that they are crucial for binding. Both residues are located in helix 3 and face the groove between the β-barrel and the C-terminal helical segment of NtA. Remarkably, the distance between both residues matches a heptad repeat distance of two alpha-helical residues which are solvent exposed in the coiled-coil domain of laminin. A lower but significant contribution originates from R43 and a charged cluster (E23, E24 and R40) at the open face of the β-barrel structure. We propose that surface-exposed, conserved residues of the laminin γ1 chain interact with NtA via hydrophobic and ionic interactions.

Keywords: agrin/basal lamina/coiled coil/laminin/neuromuscular junction

Introduction

The neuromuscular junction (NMJ) is required for precise, rapid and orderly communication between motor neurons and muscle cells. It is composed of the pre- and postsynaptic specializations, separated by a synaptic cleft that is occupied by the synaptic basal lamina. Agrin, a key organizer for the induction of postsynaptic specializations at the NMJ (Ruegg and Bixby, 1998) is stably associated with the mature synaptic basal lamina (Reist et al., 1987). This local immobilization of agrin derived from motor neurons is believed to be important for the maintenance of postsynaptic structures (McMahan, 1990).

Agrin is a multi-domain heparan sulfate proteoglycan with an apparent mol. wt of 400–600 kDa on SDS–PAGE. The most N-terminal domain (NtA) binds to the basal membrane component laminin (Denzer et al., 1998). It is followed by nine follistatin-like domains (FS) that are homologous to the Kazal type of protease inhibitors (Pathy and Nikolics, 1993). The C-terminal half of agrin comprises three laminin G-like domains (LamG) possessing two sites, A/y and B/z, which are subject to alternate splicing. The AChR aggregation activity of agrin is strongly modulated by the presence of the insert at both sites (Burgess et al., 1999).

Laminins are ubiquitous components of the tight network of glycoproteins, collagen IV and proteoglycans in basement membranes (Yurchenco et al., 2002). Laminin molecules consist of three chains (α, β and γ) that are interlinked by an extended coiled-coil domain, forming the long arm of the cruciform-shaped heterotrimer (Beck et al., 1990; Timpl and Brown, 1994, 1996; Maurer and Engel, 1996). By a combination of electron microscopy (Denzer et al., 1998) and mutational analysis (Kammerer et al., 1999) the binding site of laminin to NtA was localized to the central region of the ~60-nm-long arm of laminin-1.

The agrin-binding site in laminin-1 maps to a sequence of 20 residues within the γ1 chain (Kammerer et al., 1999). Interestingly, a coiled-coil conformation of the binding site appears to be necessary for the interaction with agrin. Sequences of the chains exhibit the typical heptad repeat (abcdefg)n of coiled-coil structures in which residues in positions a and d are restricted to the core, while residues in other positions are usually of charged and polar nature and exposed to the surface (Cohen and Party, 1990). Based on spectroscopic data and hydrodynamic analysis (Beck et al., 1990), interruptions within the trimeric coiled coil of laminin in the region of the binding site are likely, but further predictions are hampered by the so far unknown correct register between all three chains.

In contrast, the X-ray structure of the NtA domain, which mediates high-affinity interaction of agrin with the coiled-coil domain of laminin-1, was solved to 1.6 Å resolution (Stetefeld et al., 2001). The structure revealed a β-barrel fold flanked by α-helices at both termini which are characterized by a high content of charged amino acids (Figure 1). The C-terminal α-helix of chicken NtA (helix 3) contains a seven-residue splice insert comprising residues E126–A132, with an as yet unknown function. Motor neurons in developing spinal cord contain agrin transcripts that include the splice insert. However, the majority of agrin mRNA in non-neuronal tissues is characterized by the absence of the seven-residue insert (Denzer et al., 1995; Tsen et al., 1995).

In the current study, we have mapped the laminin-binding site of agrin. Based on the high-resolution X-ray structure of NtA, we have constructed a number of point mutants and deletions and expressed them in conjunction with the first FS domain of agrin (NtA-FS) (Figure 1). The mutant proteins were tested for stability and binding to laminin-1. Solid-phase binding assays and competition assays with 125I-labeled NtA-FS were used to quantify the effect of the mutations on the binding to laminin-1. The results show that the interaction of laminin-1 with the
N-terminal domain of agrin is a protein–protein interaction of high affinity ($K_D \approx 5 \text{ nM}$), mediated by the C-terminal helix 3 as the primary binding site and charged residues at the open face of the β-barrel as an auxiliary site.

Results

Expression and characterization of agrin fragments and mutants

In previous studies, either (i) full-length agrin, (ii) a construct that encodes an agrin fragment comprising NtA and the first FS domain (NtA-FS) or (iii) a fragment that lacks the first 130 amino acids from the N-terminus (cΔN Agrin) was used to investigate the interaction between agrin and laminin (Denzer et al., 1995, 1997, 1998). These data demonstrated that the very N-terminal domain of agrin (NtA) is indispensable for binding of agrin to laminin. However, it remained elusive whether the binding site for laminin is located on NtA alone, or whether the FS domain also contributes. Therefore, the single NtA-core domain composed of amino acid residues 1–132 and the NtA-FS (for sequence see Figure 1A) were
Table 1. Effect of mutations of NtA-FS on its laminin-1 binding activity

<table>
<thead>
<tr>
<th>Mutant</th>
<th>IC_{50} (nM)^a</th>
<th>Loss of activity^b</th>
<th>Half-maximal binding^c</th>
<th>Relative binding^d</th>
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<tbody>
<tr>
<td>NtA-FS</td>
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<td>1</td>
<td>1</td>
<td>1</td>
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<tr>
<td>NtA</td>
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<td>1.4</td>
<td>1</td>
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<td>H33A</td>
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^a IC_{50} values were determined by a radioligand competition assay.
^b Loss of activity refers to the ratio IC_{50} mutant/IC_{50} NtA-FS.
^c The concentration (nM) required to achieve half-maximal binding determined by ELISA.
^d Relative binding affinities refer to the ratio of half-maximal binding of mutant/NtA-FS. Single point mutations with a loss of activity >10- to 14-fold are defined as significant, whereas the triple combination is defined as less significant.

Purity was checked by SDS–PAGE, and bands with an apparent mol. wt of 22 kDa, 27 kDa for NtA and 35 kDa for NtA-FS were observed (data not shown). The additional band observed in the case of NtA might reflect heterogeneity in glycosylation (Hohenester et al., 1999; see also Supplementary data available at The EMBO Journal Online). Proper folding was tested by resistance to limited trypsin digestion according to established methods (Fox et al., 1991; Pokutta et al., 1994) together with circular dichroism (CD) spectroscopy (Figure 2 C and D). All mutations exhibited a similar stability as wild-type NtA-FS, with the exception of both deletions within the C-terminal helix 3 (Figure 2 C). The cleavage site was located by N-terminal sequencing, after residue R111 for ΔHelix3-A and R115 for ΔHelix3-B, respectively. These data indicate that point mutations did not cause unfolding of NtA-FS but rather deletions at helix 3 result in an increased accessibility of trypsin to previously inaccessible sites. The various mutants and deletions were examined for their ability to bind immobilized laminin-1 in solid-phase binding assays and in competition assays with [125I]-labeled NtA-FS protein (Table I; Figure 2). The binding could be inhibited by low concentrations of ~5.0 nM NtA-FS (see Materials and methods). The two binding assays (ELISA and radioligand competition assay) showed similar results. This also held true for all other mutants investigated.

Mapping of the laminin-binding region of NtA by site-directed mutagenesis

The design of mutants was guided by the three-dimensional structure of NtA and commenced on the basis of potential sites proposed in this work (Stetefeld et al., 2001). An attractive possibility is an interaction of the α-helical coiled-coil region of laminin with one of the α-helices that start (helix 1) or terminate (helix 3) the NtA domain (Figure 1). However, deletion of the N-terminal α-helix 1 (Δ6–12) had no effect on laminin binding (Stetefeld et al., 2001). In contrast, a deletion of α-helix 3 (Δ114–132), or part of this helix (Δ118–132), led to an almost complete loss of binding (Table I; Figure 2). To analyze this primary binding site in more detail, 12 mutants of helix 3, which forms a spacer between NtA and the following FS domain, were produced (Figure 1 B and D). These included changes in charge (for E118, E119, E121, E125, E126, H127, R128 and K129) and in hydrophobicity (for L114, L117, C123 and V124). Only two point mutations, L117A and V124D, resulted in a dramatic loss of binding affinity. These results clearly indicate a predominant role of residues at the ‘barrel face’ of helix 3, in contrast to solvent-exposed charged residues at the ‘FS face’, which is likely to be the region where NtA is covalently attached with the follistatin domain (Figure 1 B and D).

In order to analyze the possible function of the splice insert, the binding affinity of ΔSplice NtA-FS (Δ126–132) was analyzed, and revealed only a moderate loss of activity in comparison with NtA-FS containing the seven-residue insert (Table I). This is consistent with the observation that mutations of residues within the splice insert (E126A, H127A, R128A and K129A) showed only a minor effect on binding strength.
Of all point mutations at the β-barrel core domain, only replacement of R43 facing the groove between helix 3 and the β-barrel (Figure 1B) showed a similar dramatic effect as the mutations of L117 and V124 in helix 3 (Figure 2). This second region of mutational analysis included a triple mutation of a charged cluster of residues E23, E24 and R40 at the open face that caused a 16- to 20-fold decrease in binding affinity. The charged cluster (E23, E24, K38, R40, D58 and K62) that had been proposed as a potential binding site on a structural basis (Søtgefæl et al., 2001) is closely spaced and in proximity to the groove between helix 3 and the core (Figures 1B, C and 3).

Finally, a mutation was designed to test for the possible effect of glycosylation on laminin binding. N106 is the only predicted N-glycosylation site in NtA (Denzler et al., 1995). Its substitution by alanine had only a very moderate effect on laminin binding (Table I), suggesting that N-glycosylation is not essential for this function. The finding is supported by enzymatic deglycosylation of NtA-FS, which led to a small increase in electrophoretic mobility in SDS–PAGE (data not shown), but to no significant decrease in the binding strength to laminin.

Discussion
The interaction between laminin and agrin is essential for synapse formation in the peripheral nervous system and, in particular, in muscle (Burgess et al., 2000). It may also
play a role in other sites in the extracellular matrix for stabilization and interactions of basement membranes (Sugiyama et al., 1997; Burkin et al., 2000). The agrin-binding site of laminin-1 was found to reside in a 20-residue-long region of the γ1 chain in the long arm domain that has a three-stranded α-helical coiled-coil conformation (Kammerer et al., 1999). The α1 and β1 chains of laminin-1 were shown to contribute to a lesser extent to binding, and the maintenance of a proper coiled-coil structure was a prerequisite for binding (Kammerer et al., 1999). It may be expected from these data that agrin binds to all laminins that contain a γ1 chain, with variations in affinity imposed by the other chains. Laminin-1 is an embryonic form of the laminins (Ekblom, 1981), whereas laminin-2 and laminin-4 are found at later stages of skeletal myogenesis. These three laminins contain the γ1 chain (Timpl and Brown, 1994) and this holds true for the majority of the 12 laminins known today (Colognato and Yurchenco, 2000). No binding is thus expected to laminin-5 with its γ2 chain, because of the non-conserved binding motif in γ2 (Figure 4). Furthermore, binding was shown to be strongest for laminin-4 followed by laminins-1 and -2 (Denzer et al., 1997). Differential tissue distributions of laminins may therefore regulate agrin binding.

To demonstrate that NtA alone mediates the agrin–laminin interaction, binding activities of NtA and NtA-FS were compared and revealed identical values for both proteins (Table I). Together with the N106A mutant, these experiments clearly demonstrate that the high-affinity protein–protein interaction (Kd ~ 5 nM) is mediated exclusively by NtA and without involvement of attached carbohydrate chains.

The interpretation of the mutagenesis data in the context of the crystal structure of chicken NtA strongly indicates a major primary (α-helix 3) and a secondary (lower part of the charged cluster and R43 at the open face) binding site for laminin (Figures 1 and 3). Whereas single point mutations of two residues at helix 3 (L117A and V124D) together with the single R43A mutant result in a strong decrease in binding (40- to 100-fold), a triple mutation of E23A E24A R40A shows only a moderate loss of binding (20-fold; Table I). The lower part of the charged cluster together with R43 forms a narrow, almost linear array of charged residues at the surface of the β-barrel core domain (Figure 1C). Whereas salt bridges can be formed between K38–E24 and K62–E23 (average hydrogen bond distance of 3.3 Å) to stabilize antiparallel β-strands S1 and S2 of the β-barrel core, neither R40 nor R43 is complexed by solvent molecules or involved in side-chain interactions. The crucial residues of helix 3 (L117 and V124) are located on one side of the C-terminal α-helix 3 (barrel face) pointing to a groove formed between the β-barrel core domain and helix 3 (Figures 1D and 3). The major role of L117 is emphasized by the unusual positioning towards the exterior without stabilization effects to neighboring residues, indicating a direct binding partner for laminin-1.

A comparison of laminin sequences of γ chains from mouse, rat and human within the mapped binding region for NtA shows that there are highly conserved for γ1, but rather divergent for γ2 chains (Figure 4). Previous studies have demonstrated that the γ2 chain of laminin is not effective in competition assays with laminin-1 (Kammerer et al., 1999). To determine potential sites of interaction with an intact heterotrimeric coiled coil, a laminin model based on trimeric GCN4 [Protein Data Bank (PDB) code 1GCM; Harbury et al., 1993] was docked to the globular NtA domain using the program FTDock (Gabb et al., 1997; Figure 5).
The binding region of the γ1 chain within the three-stranded coiled coil of laminin can be fitted well into the groove facing helix 3 of NtA (Figure 5B). Since the distance between L117 and V124 exactly matches the seven-residue interspace between A1305 and A1312 of the laminin γ1 chain (Figure 4), an interaction is a likely possibility. The interaction of both aliphatic and solvent-exposed residues of the γ1 chain with V124 and L117 places the nearby D1308 and E1315 in a position for possible ionic interactions with R43 and R40 from NtA, respectively (Figure 5A). Two features of the model of the complex are certainly correct, namely, the placement of the laminin-binding site into the groove and the importance of helix 3 of NtA for the interaction with the coiled-coil structure. Based on our data, we propose that laminin binding is dependent on a combination of hydrophobic and ionic interactions (Figure 5).

Despite the high content of charged residues (>40% of all amino acid residues), ionic interactions of α-helix 3 are not detectable either with the β-barrel core domain or with the adjacent N-terminal helix 1. As the primary laminin-binding site, helix 3 shows a remarkable potential of spatial flexibility, and a short linker arm (L109–L114) forms the connector between the β-barrel and helix 3 that would facilitate a hinge-like motion of the C-terminal helix as a rigid body (Figure 1D). As a consequence, helix 3 can be described as a spacer linking NtA and FS domains.

The detailed model shown in Figure 5 is suggestive but may be open to modifications. The model is based entirely on interactions with the α-helix formed by the γ1 chain. However, it can not be excluded that, for example, R43 in NtA interacts with a negatively charged residue in the α- or β-chains, which were shown to be of secondary importance in binding (Kammerer et al., 1999). This problem can only be approached with a three-dimensional structure of the relevant regions of the coiled-coil domain of laminin. Our model presented here was designed assuming that the coiled-coil structure of laminin conserves its native three-stranded conformation upon binding. It is known, however, that a coiled coil may adapt to a new environment by means of a conformational change. It has been shown that the heterodimeric complex of the coiled-coil domain of cFos and cJun binds the DNA duplex like a pair of forceps (Glover and Harrison, 1995). If this is also true for the agrin-binding site in laminin, an opening of the coiled-coil structure and replacement of one of the laminin chains by helix 3 might be a possible mechanism.

Finally, it should be mentioned that the structure of a complex between a globular protein domain and a fibrillar coiled-coil structure is of general importance. Only a few interactions of this type have been defined for which structural information exists (Mayr et al., 1996; Sibanda et al., 2001). The X-ray structure of the complex–SNARE complex has only recently been solved to atomic resolution (Chen et al., 2002). Complexin binds in an antiparallel α-helical conformation to synaptobrevin and syntaxin helices of the SNARE motif. Binding of complexin causes only minimal structural changes within the four parallel, highly twisted α-helices formed by the SNARE motif.

Thus, the ideal approach would be the co-crystallization of complexes of the two interacting domains. To achieve this aim, three-stranded laminin fragments containing the relevant regions of the α- and β-chains, in addition to the already characterized γ1 chain, have to be prepared. Work in this direction is in progress.

Materials and methods

Expression and purification of NtA and NtA-FS

The construct pC116 7Fe (Denzer et al., 1997) was used as a template to generate the cDNA construct encoding NtA and NtA-FS by PCR using Vent polymerase (New England Biolabs). Primers used for NtA were NtA 5'-GAATTCGCTAGCGAGATGACGACAAAGGATCCCGGAAAGGAGC and NtA 3'-GGATCCGGGCGGCCAGCAAGAAGCTTCCGATGTTCCT and were cloned into the expression vector C-His pCEP-Pu in-frame to the BM-40 signal peptide (Kohfeldt et al., 1997). This vector included the BM-40 UTR (including a Kozak sequence), the BM-40 signal peptide for proper secretion in the culture medium and a C-terminal His6 tag. Primers used for NtA-FS were NtA-FS 5'-GGTAACCGCTAGCGGAGAGCTCGGGCGGAGG and NtA-FS 3'-TGTGATGGATTCGACGAGCTCTTCGACCAGGG. The reverse primer was designed to code for a stretch of His6 tag followed by a stop codon, and cloned into pCEP-Pu vector.
energy docked structures were subject to 100 cycles of unrestrained Powell minimization using CNS (Brunger et al., 1998). Harmonic restraints were imposed on the protein atoms (3 kcal/mol Å²) with increased weight (20 kcal/mol Å²).

**Supplementary data**
Supplementary data are available at The EMBO Journal Online.

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**References**


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