

Electron microscopic structure of agrin and mapping of its binding site in laminin-1

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Agrin is a large, multidomain heparan sulfate proteoglycan that is associated with basement membranes of several tissues. Particular splice variants of agrin are essential for the formation of synaptic structures at the neuromuscular junction. The binding of agrin to laminin appears to be required for its localization to synaptic basal lamina and other basement membranes. Here, electron microscopy was used to determine the structure of agrin and to localize its binding site in laminin-1. Agrin appears as an ~95 nm long particle that consists of a globular, N-terminal laminin-binding domain, a central rod predominantly formed by the follistatin-like domains and three globular, C-terminal laminin G-like domains. In a few cases, heparan sulfate glycosaminoglycan chains were seen emerging from the central portion of the core protein. Moreover, we show that agrin binds to the central region of the three-stranded, coiled-coil oligomerization domain in the long arm of laminin-1, which mediates subunit assembly of the native laminin molecule. In summary, our data show for the first time a protein-protein interaction of the extracellular matrix that involves a coiled-coil domain, and they assign a novel role to this domain of laminin-1. Based on this, we propose that agrin associates with basal lamina in a polarized way.
Keywords: coiled-coil/electron microscopy/extracellular matrix/heparan sulfate proteoglycan/synapse

Introduction

Basement membranes are extracellular, sheet-like matrices that underlie endothelial and epithelial cells, or surround muscle fibers, fat cells and peripheral nerves (Vracko, 1974). Several lines of evidence indicate that basement membranes are formed by the self-assembly of the two main constituents, the laminins and collagen IV (for review, see Yurchenco and O'Rear, 1994). Linkage of these two independent networks via nidogen/entactin gives rise to a primary scaffold to which other extracellular matrix (ECM) molecules bind (for review, see Timpl and Brown, 1996). The distinct molecular composition of basement membranes is thought to be important for

intercellular interactions determining cell differentiation and fate. These processes are mediated by the specific interaction of molecules of the ECM with cell surface receptors.

The synaptic basal lamina of the neuromuscular junction (NMJ) is a particularly well studied example of a basement membrane with an instructive role during embryonic development (reviewed in Hall and Sanes, 1993). This basal lamina contains molecules secreted from both the presynaptic motor neuron and the postsynaptic muscle fiber and, unlike extrasynaptic muscle basement membrane, includes molecules that direct the formation of synaptic specializations (Sanes *et al.*, 1978; Burden *et al.*, 1979). One component of synaptic basal lamina is the heparan sulfate proteoglycan (HSPG) agrin. Upon addition of agrin to cultured myotubes, molecules such as the acetylcholine receptor (AChR), acetylcholinesterase and $\beta 2$ chain-containing laminin become aggregated (Nitkin *et al.*, 1987; Wallace, 1989) as they are *in vivo*. Mice deficient in agrin lack synaptic specializations at the NMJ and die at birth probably due to their inability to breathe (Gautam *et al.*, 1996). In addition, when agrin is artificially deposited in the muscle basement membrane at ectopic sites, it induces the formation of postsynaptic specializations that are indistinguishable from those found at the innervated site (Cohen *et al.*, 1997; Jones *et al.*, 1997; Meier *et al.*, 1997; Rimer *et al.*, 1997). These findings show that agrin is the key molecule for induction of synaptic specializations at the NMJ.

Agrin is composed of multiple domains homologous to domains found in other ECM components (Rupp *et al.*, 1991; Smith *et al.*, 1992; Tsim *et al.*, 1992; Figure 1A). The most N-terminal region, called the NtA domain, has so far been described only in agrin and has been shown to be necessary and sufficient for binding to the laminins (Denzer *et al.*, 1995, 1997). The NtA domain is followed by nine follistatin-like (FS) domains that are homologous to the Kazal-type of protease inhibitors (Pathy and Nikolics, 1993). The C-terminal half of agrin, which is sufficient to induce aggregation of synaptic molecules on cultured myotubes (Nitkin *et al.*, 1987; Tsim *et al.*, 1992), comprises four epidermal growth factor-like domains (EG) and three laminin G-like domains (LG). The AChR aggregation activity of agrin depends on inserts at two sites, termed A and B in chicken, that are subject to alternative mRNA splicing. On cultured myotubes and when expressed at ectopic sites *in vivo*, only the agrin isoforms containing inserts at both sites are active in inducing AChR aggregation and in activating a signaling cascade (Ruegg *et al.*, 1992; Ferns *et al.*, 1993; Gesemann *et al.*, 1995; Glass *et al.*, 1996; Meier *et al.*, 1997). The non-AChR-aggregating isoform of agrin, however, binds with high affinity to α -dystroglycan (Sugiyama *et al.*, 1994; Gesemann *et al.*, 1996, 1998), a peripheral mem-

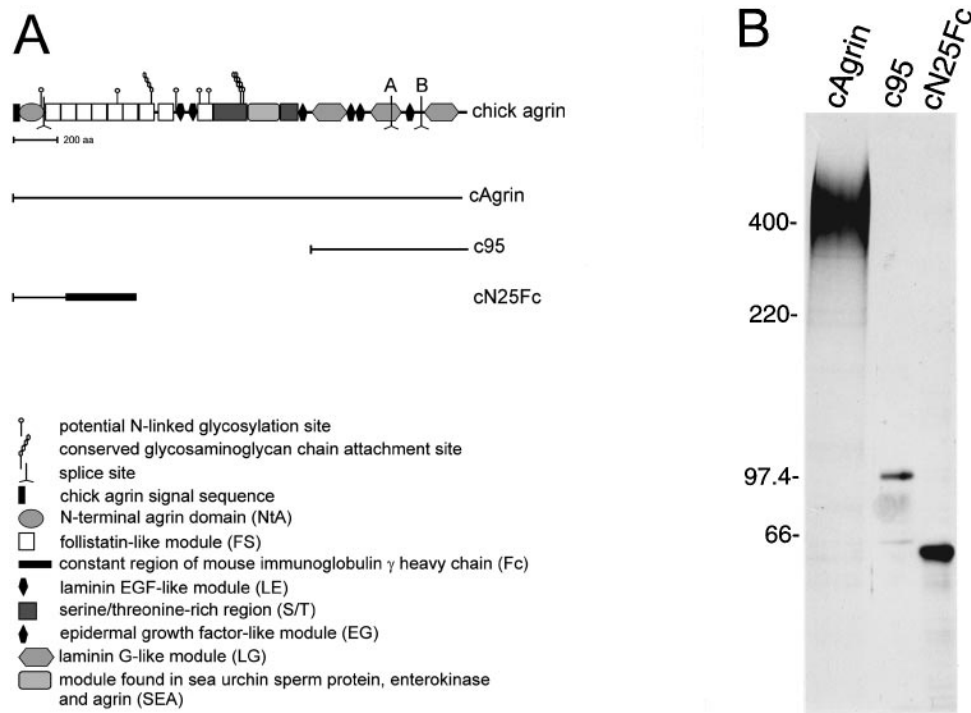


Fig. 1. Structure and SDS-PAGE analysis of chick agrin constructs. **(A)** Schematic diagram of the structural domains of chick agrin and representation of the regions covered by the agrin fragments used in this study. Symbols and designations of structural domains are according to Bork and Bairoch (1995). **(B)** Purified agrin proteins after separation by 3–12% SDS-PAGE and visualization by silver staining. cAgrin purified by anion exchange column using Mono Q-Sepharose is shown. The molecular mass of standard proteins is given in kDa.

brane protein, which is part of a protein complex that is important for linking the basement membrane to the underlying cytoskeleton (for review, see Henry and Campbell, 1996).

To exert its synaptogenic function *in vivo*, agrin must be stably incorporated into developing synaptic basal lamina and remain firmly attached to this structure throughout adulthood (McMahan, 1990). Agrin-like immunoreactivity has also been observed in many basement membranes of non-neuronal and non-muscle tissue (for reviews, see McMahan *et al.*, 1992; Denzer *et al.*, 1996), suggesting that agrin also binds to molecules of the ECM in these tissues. There is now strong evidence that the laminins mediate binding of agrin to synaptic basal lamina and non-synaptic basement membrane (Denzer *et al.*, 1997). The binding of agrin to laminin-1 is of high affinity ($K_d \sim 5$ nM) and is mediated by the most N-terminal, NtA domain of agrin (Denzer *et al.*, 1997).

In the current study, we report an analysis of the structure of agrin as visualized by electron microscopy (EM). Our results depict agrin as a thin, extended particle of ~ 95 nm length, with a globular N-terminus and a cluster of three globules at the C-terminus, suggesting that the NtA domain forms one globule and that the LG domains in the C-terminal half of the molecule form the cluster of three globules. The central portion of agrin comprising the FS domains forms a rod-like structure. In addition, we have mapped the agrin-binding site of laminin-1 to the central region of the three-stranded, coiled-coil domain in the long arm. In summary, our results indicate that agrin domains involved in protein-protein interactions form globular structures and that the central part of agrin may serve as a spacer-like region. In addition, the finding

that agrin binds to the three-stranded, coiled-coil domain of laminin-1 suggests that this region is not only important for oligomerization but that it also serves as a region involved in the formation of a basement membrane.

Results

Purification of recombinant agrin and agrin fragments

Full-length agrin and fragments thereof used in our studies were all derived from stably transfected 293 HEK cells and from transiently transfected COS-7 cells. Full-length agrin (cAgrin; Figure 1A) was purified either by immunaffinity chromatography using the monoclonal anti-agrin antibody 5B1 (Reist *et al.*, 1987) or from serum-free medium by anion exchange chromatography on Mono Q-Sepharose. The fragment c95 (Figure 1A) was purified by the 5B1 immunaffinity column while cN25Fc (Figure 1A), which is a chimeric construct between the NtA and the first FS domain of agrin and the constant region of a mouse IgG (Denzer *et al.*, 1997), was purified using protein A-Sepharose. SDS-PAGE of purified recombinant proteins revealed mainly a single band (Figure 1B). As documented earlier, cAgrin migrated as a broad band with an average M_r of 400 kDa. This is due to the attachment of heparan sulfate glycosaminoglycan side chains (HS-GAG) to the 225 kDa core protein (Denzer *et al.*, 1995; Tsen *et al.*, 1995). c95 and cN25Fc had apparent M_r s of 95 and 65 kDa, which is in accordance with their predicted size (Gesemann *et al.*, 1995; Denzer *et al.*, 1997).

Shape of agrin

Electron micrographs of rotary-shadowed full-length chick agrin revealed particles with a diverse appearance (Figure

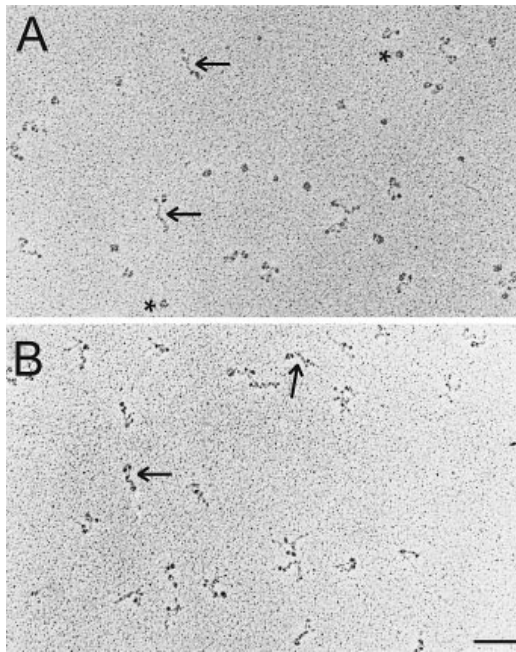


Fig. 2. Recombinant full-length chick agrin appears as an elongated strand-like particle. (A) Representative fields of rotary-shadowed cAgrin purified by affinity chromatography with the monoclonal anti-agrin antibody 5B1 and (B) by an anion exchange column using Mono Q-Sepharose. The arrows indicate rod-like particles assigned to agrin, while asterisks in (A) show presumed antibody 5B1 leaked from the affinity column. Bar: 100 nm.

2). To identify the structure of agrin, we compared agrin purified by an immunoaffinity column (Figure 2A) with agrin isolated by anion exchange chromatography (Figure 2B). Rod-like particles were observed in both preparations and were therefore assigned to agrin (arrows in Figure 2). The diverse shape of the particles may reflect a high flexibility of the central region and/or an interaction between internal domains. As the length of tangled-shaped particles could not be determined accurately, we measured 53 extended structures. The average total length of such selected particles was 95 ± 15 nm. Small globular particles seen in the cAgrin preparation purified by the mAb 5B1 affinity column (asterisks in Figure 2A) may be due to antibody leakage from the affinity column, because such particles were not observed in the preparation purified by anion exchange chromatography (Figure 2B). These globular particles often contained three substructures arranged in a Y-shaped fashion, which is the common appearance of IgGs by EM (Tschopp *et al.*, 1980).

Examination of agrin preparations at higher magnification revealed that the majority of the particles contained a cluster of three globules of about equal size (Figure 3A and B). These three globules are reminiscent of the structure of the C-terminal globules in laminin-1 and of the E8 proteolytic fragment (Beck *et al.*, 1990). To verify that the three globules originate from the C-terminal half of agrin, which contains the three LG domains separated by EG domains, we looked at the structure of the c95 agrin fragment. As shown in Figure 3C, c95 was indeed substructured into three globules, suggesting that each represents one of the LG domains. Frequently, another globular structure was apparent at the other end of the rod of full-length agrin (Figure 3A and B), and con-

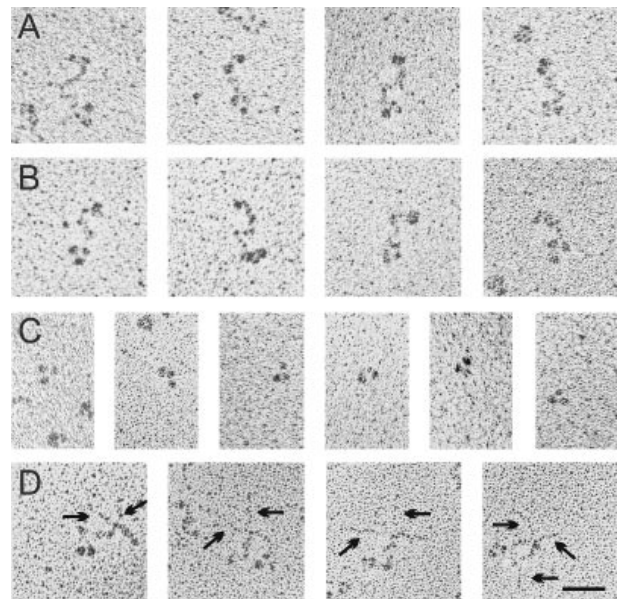


Fig. 3. The elongated rod-like particle of full-length agrin is substructured at both ends. Electron micrographs of selected particles of cAgrin after rotary shadowing (A, B and D) display the domain organization of the molecule. The average length of extended particles, like those depicted in (A) and (B), is 95 ± 15 nm ($N = 53$). Arrows in (D) indicate presumed glycosaminoglycan side chains. (C) Electron micrographs of rotary-shadowed, C-terminal agrin fragment, c95. Bar: 50 nm.

sequently may represent the NtA domain. This N-terminal globule seemed sometimes to be split into two globular structures (Figure 3A). In a few cases, the particles of full-length agrin contained ~ 40 nm long, thin strands emerging from a central region of their rods (arrows in Figure 3D). As agrin is an HSPG and the conserved HS-GAG attachment sites are located near the center of agrin (Figure 1A), these strands most likely represent HS-GAG chains.

Localization of the agrin-binding site in laminin-1

Laminin-1 is a heterotrimeric glycoprotein consisting of three different laminin chains, $\alpha 1$, $\beta 1$ and $\gamma 1$ (Figure 4A; Burgeson *et al.*, 1994). To map the agrin-binding site in laminin-1, we measured binding of the agrin fragment cN25Fc, which has been shown to be sufficient for binding (Denzer *et al.*, 1997), to native laminin-1 and to the proteolytic fragments depicted in Figure 4A. Laminin-1 and its proteolytic fragments were immobilized on microtiter plates and incubated with 5 nM iodinated cN25Fc. As reported earlier, cN25Fc bound to laminin-1 (Figure 4B) but, of all the laminin fragments used, binding was only seen to the C8-9, but not to E1, E8 or E3 fragments (Figure 4B). This finding restricts the site of interaction between agrin and laminin-1 to the N-terminal half of the coiled-coil domain of laminin-1.

As the apparent binding of cN25Fc to native laminin-1 was higher than to the C8-9 fragment (Figure 4B) and a similar difference was observed when [125 I]cAgrin was used (data not shown), we examined next whether this was due to distinct binding affinities by measuring dose-response curves. As shown in Figure 5, the binding of [125 I]cAgrin to laminin-1 and to the C8-9 fragment followed the same dose-response curve and maximal binding

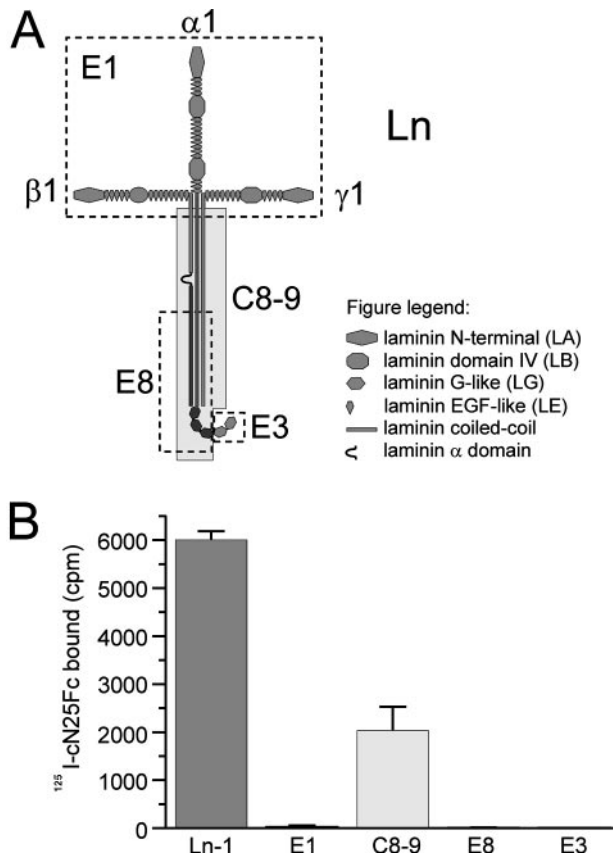


Fig. 4. The N-terminal agrin fragment binds to the three-stranded, coiled-coil domain of laminin-1. (A) Structural organization of laminin-1 (Ln) and of proteolytic fragments derived from laminin-1 by elastase treatment (E1, E3 and E8; dotted boxes) and by cathepsin G treatment (C8-9; shaded box) are shown. (B) Solid-phase radioligand-binding assay demonstrating the binding of the N-terminal agrin fragment to laminin-1 and to the fragments depicted in (A). Laminin-1 and its fragments were immobilized on microtiter plates and subsequently incubated with 5 nM [125 I]cN25Fc. Each value is the mean \pm SD of three measurements and represents binding after subtraction of the counts to bovine serum albumin-coated wells (61 ± 7 c.p.m.).

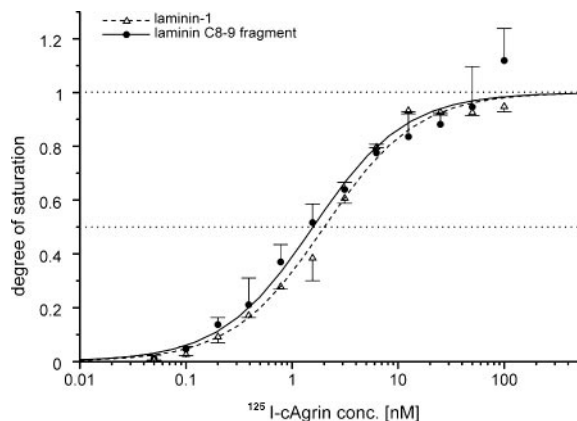


Fig. 5. Binding of agrin to the laminin C8-9 fragment is of high affinity. Dose-response curves for the binding of iodinated cAgrin to laminin-1 and to the C8-9 fragment. The binding curve shown is the result of one representative experiment. Each data point represents the mean \pm SD of three measurements with the corresponding background counts (bovine serum albumin-coated wells) subtracted. The K_d values for the binding to laminin-1 and to C8-9 in this particular experiment were 2.0 and 1.6 nM, respectively. Note that the K_d values varied between independent dose-response curves (K_d values: laminin-1, 6.5–2.0 nM; C8-9 fragment, 4.2–1.6 nM).

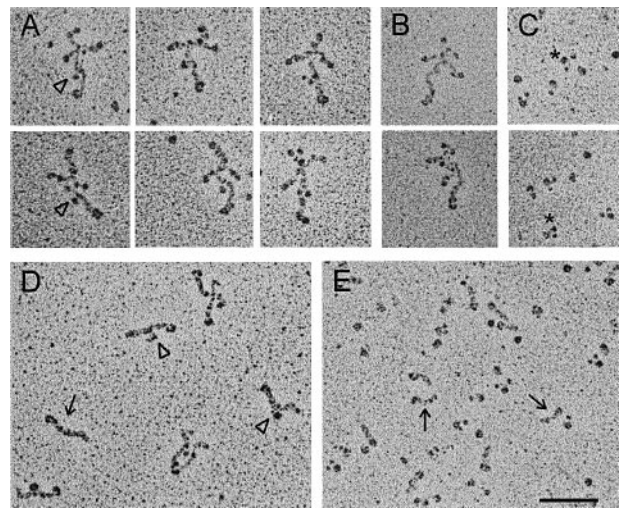


Fig. 6. The N-terminal agrin fragment binds to the central region of the long arm of laminin-1. Electron micrographs of rotary-shadowed laminin-1, the C8-9 and E8 laminin fragments and the N-terminal agrin fragment cN25Fc. (A) Recombinant cN25Fc (open arrowheads) binds to a single site in the central region of the long arm of laminin-1. No globular particle is seen in rotary-shadowed laminin-1 (B) while recombinant cN25Fc appears after rotary shadowing (C) either as a globular particle or as a globule from which two globular units emerge (asterisks). (D) The C8-9 fragment of laminin-1 (arrow) frequently is associated with cN25Fc (open arrowheads). (E) No association of cN25Fc is observed with the E8 fragment (arrows). Bar: 100 nm.

was reached at similar concentrations, indicating similar binding affinities. This is also exemplified in the dissociation constants, which were 2.0 nM for the binding of cAgrin to laminin-1 and 1.6 nM for the C8-9 fragment. The difference in the apparent binding shown in Figure 4B is therefore most likely a result of unequal coating of laminin-1 and the C8-9 fragment to the microtiter plates.

To map the agrin-binding site in the coiled-coil domain of laminin-1 more precisely, the complex between intact laminin-1 and cN25Fc was visualized by EM after rotary shadowing. When cN25Fc and laminin-1 were mixed in a 1:1 ratio, many complexes were observed between laminin-1 molecules and the agrin fragment (Figure 6A). cN25Fc, which displayed a globular structure, was positioned at a single site in the central region of the long arm of laminin-1 (open arrowheads in Figure 6A) and such a structure was not observed when the cN25Fc fragment was omitted (Figure 6B). To ensure that the globular structure was indeed the cN25Fc fragment, we also looked at the structure of this fragment. As shown in Figure 6C, the isolated particle had either a globular shape or was substructured into three globules (asterisks in Figure 6C), which were arranged in a Y-shaped fashion similar to the electron microscopic appearance of IgG (Tschopp *et al.*, 1980). Because cN25Fc forms dimers (Denzer *et al.*, 1997), one of the globules most probably accounts for the Fc region and the other two for the NtA domains of agrin. Consistent with the results from the binding assays, cN25Fc was also bound to the C8-9 (Figure 6D) but not to the E8 fragment of laminin-1 (Figure 6E).

In 56 electron micrographs of complexes between laminin-1 and cN25Fc, the agrin fragment was bound 33.5 ± 0.7 nm from the N-terminal end of the long arm

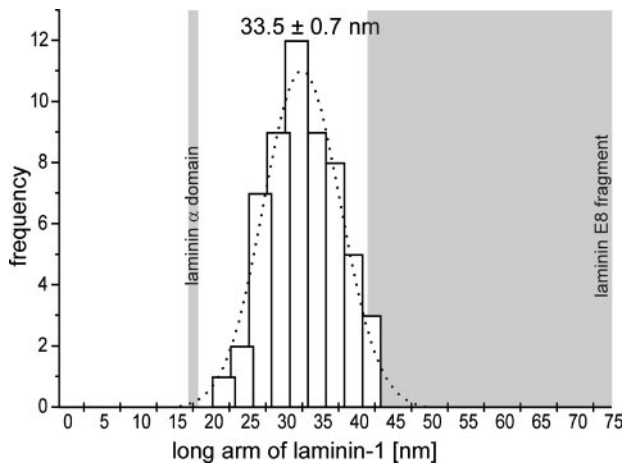


Fig. 7. Histogram plot of the agrin-binding site in the long arm of laminin-1. The position of the agrin-binding site relative to the 76 ± 1 nm ($N = 42$) long coiled-coil domain is shown. Shaded areas indicate positions of the α -domain as calculated by Beck *et al.* (1990) and of the E8 fragment as determined by Hunter *et al.* (1992) in the long arm of laminin-1.

of laminin-1 (Figure 7). The total length of the long arm in our laminin-1 preparation was 76 ± 1 nm ($N = 42$), which is in good agreement with the published data (Engel *et al.*, 1981). As indicated in Figure 7, the agrin-binding site apparently does not overlap with the predicted position of the laminin α -domain and overlaps little with the N-terminus of the E8 fragment.

The finding that agrin binds to a central region in the coiled-coil domain of laminin-1 raises the possibility that more than one laminin chain is involved in the interaction. We therefore measured whether agrin binds to individual chains of laminin-1 using a transfer overlay assay in which the laminin chains were separated on SDS-PAGE under reducing conditions, transferred to nitrocellulose and subsequently incubated with cAgrin. Some binding of cAgrin, which was visualized using anti-agrin antibodies, was observed to the 400 kDa band of the α 1 chain and the 220 kDa band of the β 1 and γ 1 chains (Figure 8A, lane 1). Because 100 nM cN25Fc did not significantly reduce the binding to either laminin chain (Figure 8A, lane 2), this binding is not mediated by the NtA domain. No laminin-like immunoreactivity was observed if the nitrocellulose had not been incubated with cAgrin (Figure 8A, lane 3), demonstrating the specificity of the anti-agrin antibodies. As a positive control, we performed the experiment the other way around, i.e. cAgrin was run on SDS-PAGE, transferred to nitrocellulose and overlaid with laminin-1 (Figure 8B). In this case, laminin-1 bound to cAgrin (Figure 8B, lane 1) and the binding was inhibited by the presence of 100 nM cN25Fc (Figure 8B, lane 2). Again, no cross-reactivity of the anti-laminin-1 antiserum with cAgrin was observed (Figure 8B, lane 4). These results show that binding of agrin to laminin-1 is not mediated by denatured, individual chains and thus suggest that binding may require a native coiled-coil conformation.

To test whether our failure to detect binding to β 1 chain may be due to the unfolding of the α -domain, which is a 30 amino acid long, cysteine-rich loop (Figures 5A and 7), under the reducing conditions used in the SDS-PAGE, we measured binding of cAgrin to reduced and alkylated laminin-1 and C8-9 fragment. This treatment selectively

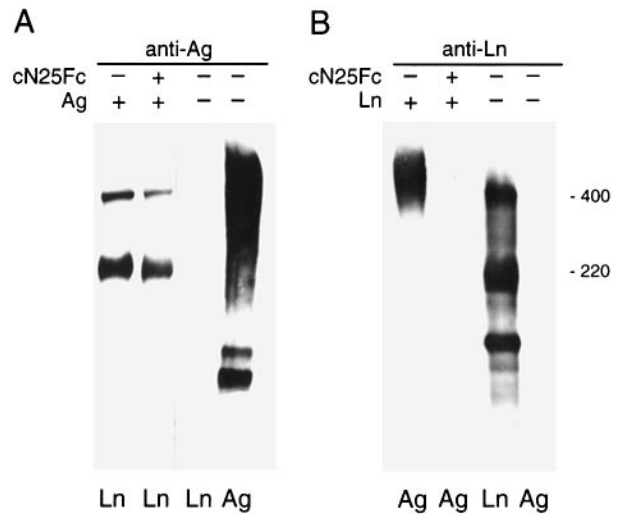


Fig. 8. Agrin does not bind to individual chains of laminin-1. Laminin-1 (indicated as Ln at the bottom of each lane) or recombinant full-length chick agrin (indicated as Ag at the bottom of each lane) were separated on 3–12% SDS-PAGE under reducing conditions, transferred to nitrocellulose and overlaid with cAgrin (Ag at the top of the lane) or laminin-1 (Ln at the top of the lane). Bound proteins were detected with anti-agrin antiserum (A) or anti-laminin-1 antibodies (B). Five nM of cAgrin bound to the α 1 chain (400 kDa) and the β 1/ γ 1 chains (220 kDa) of laminin-1 (A, lane 1). This binding is not mediated by the NtA domain of agrin as 100 nM of cN25Fc did not inhibit binding (A, lane 2). The binding of cAgrin to the α 1 and the β 1/ γ 1 chains was also not due to cross-reactivity of the anti-agrin antiserum with laminin-1 (A, lane 3). The anti-agrin antiserum is specific for agrin and recognizes recombinant agrin (A, lane 4). In contrast to agrin, laminin-1 can bind to cAgrin in the transfer overlay assay (B). The binding of 5 nM of laminin-1 (B, lane 1) is inhibited by 100 nM cN25Fc (B, lane 2) and is therefore mediated by the NtA domain of agrin. Anti-laminin-1 antibodies are specific (B, lane 3) and do not cross-react with cAgrin (B, lane 4).

destroys the structure of the α - and other cysteine-rich domains but leaves the coiled-coil domain intact (Antonsson *et al.*, 1995; Kammerer *et al.*, 1995). Specific binding of cAgrin to either laminin-1 or C8-9 was still observed (data not shown), suggesting that the α -domain is not required for the interaction.

Discussion

Structure of agrin

In this study, we show that agrin forms a thin, 95 nm long particle with one globule at the N-terminus and a cluster of three globules at the C-terminus (Figure 9). The globule at the N-terminus of the particle (Figure 9, region 1) most probably reflects the NtA domain. This follows from the appearance of this domain in the cN25Fc fragment (Figure 6, asterisk) and its position in the agrin sequence. Sometimes, there was a second N-terminal globule visible, which may represent a thickening of the N-terminal part of the rod.

The three globules at the C-terminus of agrin (Figure 9, region 3–5) are most likely formed by its three LG domains. This conclusion is based on the observation that the LG domains in laminin have a similar appearance in the EM (Beck *et al.*, 1990). While the LG domains of laminin are more clearly visible after negative staining, the C-terminal globules of agrin were seen readily by rotary shadowing. This difference may be due to the

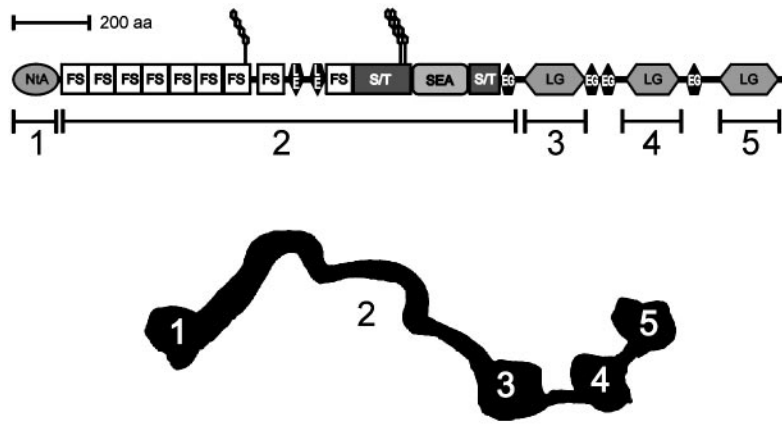


Fig. 9. Alignment of the domains of agrin to the structure of agrin. The schematic representation of the domain organization of agrin and the structure of a selected electron micrograph of agrin are shown. For symbols and designations of individual domains, see Figure 1A.

insertion of one or two EG domains between individual LG domains of agrin while the LG domains of laminin are tandemly repeated. The sequence of the C-terminal part of perlecan, another proteoglycan of basement membranes, is very similar to that of agrin (Noonan *et al.*, 1991). In agreement with our studies, the EM structure of this region is very similar to the C-terminal part of agrin (R.Timpl, personal communication).

The assignment of the globular structures to the NtA and the LG domains suggests that the central rod of ~85 nm length (Figure 9, region 2) is formed by the FS domains, the two laminin EG-like (LE) domains and the two serine/threonine-rich (S/T) regions, which are linked by a module found in sea urchin sperm protein, enterokinase and agrin (SEA; Bork and Patthy, 1995). This interpretation is based on the structure and the size of homologous domains in other ECM molecules.

The crystallized FS domain in BM-40 and the EM structure of agrin presented here allowed Hohenester *et al.* (1997) to design a model that assumes a linear arrangement of repeated FS domains. Their model predicts that a pair of FS domains form a 7 nm long, rod-like structure stiffened by interactions between adjacent FS domains. As agrin contains four such pairs, this part of agrin would be 28 nm in length. LE domains, which follow the repeated FS domains in agrin, also form a rod-like structure, as determined by EM of the LE domains of laminin-1 (Engel *et al.*, 1981; Engel, 1989; Beck *et al.*, 1990; Gerl *et al.*, 1991). Three consecutive LE domains of the laminin γ 1 chain form a rigid, rod-like structure of 7.6 nm (Stetefeld *et al.*, 1996). The two LE domains and the adjacent FS domain of agrin would hence elongate the rod by another 8.5 nm.

The two ST-rich regions, which are separated by the SEA domain, may also adopt a rod-like shape as ST-rich regions in mucin or in α -dystroglycan are extensively *O*-linked-glycosylated and appear as an elongated, flexible structure (Jentoft, 1990; Brancaccio *et al.*, 1995). Three hundred and sixty five amino acids constitute the ST-rich regions and the SEA domain of agrin. To predict how much they contribute to the length of the rod, we assumed that all 365 amino acids are arranged in an α -helical conformation, which is known to be a very extended structure. Each amino acid elongates the α -helix by ~0.15 nm, which makes the rod formed by the 365 amino

acids 55 nm long. According to this interpretation, the entire rod of agrin would be 91.5 nm long (28 nm + 8.5 nm + 55 nm), which is similar to the measured 85 nm.

The agrin-binding site in the coiled-coil domain of laminin-1

We show here that agrin binds near the center of the coiled-coil oligomerization domain in the long arm of laminin-1. This region is formed by all three laminin chains (for review, see Kammerer, 1997), and consequently the binding may involve one, two or all three chains.

Although our experiments do not discriminate conclusively between these possibilities, they allow the exclusion of certain interactions. Carbohydrates of laminin-1 are unlikely to be involved because such an interaction should be preserved after SDS-PAGE and transfer of the protein to nitrocellulose (Figure 8), as is the case, for example, for the binding of agrin to α -dystroglycan (Gee *et al.*, 1994; Sugiyama *et al.*, 1994; Deyst *et al.*, 1995; Gesemann *et al.*, 1996). For the same reason, a small linear peptide in the laminin chains is also unlikely. We also could not detect significant binding of agrin to recombinant fragments containing the α -domain of the laminin β 1 chain (unpublished data). This, together with the experiment where we selectively destroyed the structure of the α -domain by reduction and alkylation of its six cysteines, makes it unlikely that agrin binds to the α -domain.

As laminin isoforms differ in their chain composition (for review, see Timpl, 1996) and agrin binds to several laminin isoforms (Denzer *et al.*, 1997), it could well be that the affinity of the interaction is regulated by the laminin chain composition and this may be a mechanism for the formation of basement membranes *in vivo*. For example, during kidney development, individual, maturing basement membranes alter their laminin chain composition progressively (Miner *et al.*, 1997), and this tissue has been shown to contain agrin (Noakes *et al.*, 1995; Gesemann *et al.*, 1997).

Laminin is a glycoprotein of basement membranes with multiple binding partners. Most of the binding sites map to regions distinct from the coiled-coil oligomerization domain. The α 6 β 1 integrin appears to be the only binding partner that binds to the LG domains and the coiled-coil regions of the E8 fragment of laminin-1 in a concerted

fashion (Deutzmann *et al.*, 1990). The $\alpha 1\beta 1$ integrin of chicken has been reported to bind to a similar site in mouse laminin-1 (Lallier *et al.*, 1994). However, no such binding was observed with $\alpha 1\beta 1$ integrin of rat (Colognato-Pyke *et al.*, 1995). Our data demonstrate that a particular region in the center of the coiled-coil domain of laminin-1 binds to agrin without the involvement of other laminin domains. This is the first example of a protein interaction of an ECM molecule mediated by a coiled-coil domain. Hence, the coiled-coil domain of laminin-1 may not only be essential for the oligomerization of individual domains but may also play an important role in the formation of basement membranes like the synaptic basal lamina. To our knowledge, the only other example where binding to a coiled-coil domain has been demonstrated is the binding of the β -importin subunit to p62, which both are members of the nuclear pore complex (Percipalle *et al.*, 1997).

Agrin and organization of basement membranes

Agrin has been shown to be required and sufficient to organize synaptic structures at the NMJ (McMahan, 1990; Gautam *et al.*, 1996; Cohen *et al.*, 1997; Jones *et al.*, 1997; Meier *et al.*, 1997; Rimer *et al.*, 1997). Based on our current study, we can now propose a model for how agrin influences the formation of synaptic specializations.

Results obtained from many *in vitro* studies have shown that the C-terminus of agrin interacts with the postsynaptic muscle fiber. Interestingly, the last LG domain of agrin, if it contains the eight amino acid long insert at the B-site, is sufficient to induce AChR aggregation (Gesemann *et al.*, 1995). The binding of agrin to the synaptic basal lamina is conferred by the NtA domain (Denzer *et al.*, 1995, 1997; this study). Our EM data now show that these two domains are separated from each other by ~90 nm. The average distance between the presynaptic nerve terminal and the postsynaptic muscle fiber is ~50 nm. Hence, the length of agrin is sufficient to span the entire synaptic basal lamina, and we propose that motor neuron-derived agrin associates with synaptic basal lamina in a polarized way, having its N-terminus incorporated into synaptic basal lamina near the nerve terminal and its C-terminus at the muscle cell surface.

Several lines of evidence suggest that such a polarized orientation of agrin is also likely in basement membranes other than the synaptic basal lamina. The C-terminal LG domains of agrin bind to cell surfaces via α -dystroglycan not only in muscle, but in a variety of non-muscle tissues (Gesemann *et al.*, 1998). The linkage of basement membranes and all surfaces mediated by agrin may therefore also be important outside of the NMJ.

Materials and methods

Expression constructs

Constructs pc95, pcAgrin and pcN25Fc are described elsewhere (Denzer *et al.*, 1995, 1997; Gesemann *et al.*, 1995). Note that pcAgrin and pcN25Fc always carried a sequence encoding a seven amino acid insert at the splice site flanking the NtA domain of agrin (see Figure 1A; Denzer *et al.*, 1995). At the other two splice sites of chick agrin, called A and B (Ruegg *et al.*, 1992; Thomas *et al.*, 1993), no inserts were present.

Purification of agrin and of laminin-1, generation of laminin-1 fragments

Transfection of COS-7 cells (Gluzman, 1981) was carried out as described by Gesemann *et al.* (1995). The recombinant proteins cAgrin and c95

were obtained from conditioned medium of stably transfected HEK 293 cells (Graham *et al.*, 1977; Gesemann *et al.*, 1995; Denzer *et al.*, 1997) and cN25Fc from conditioned medium of transiently transfected COS cells. Purification of cAgrin by an anion exchange column using Mono Q-Sepharose and of cN25Fc by protein A-Sepharose was conducted as described in Denzer *et al.* (1997). Affinity purification of c95 and cAgrin using monoclonal anti-agrin antibody 5B1 (Reist *et al.*, 1987) was performed according to Gesemann *et al.* (1995). Mouse laminin-1 was purified from mouse Engelbreth-Holm-Swarm sarcoma as described (Timpl *et al.*, 1979). The elastase fragments E1, E3 and E8, and the cathepsin fragment C8-9 of mouse laminin-1 were generated and purified as described elsewhere (Ott *et al.*, 1982; Timpl *et al.*, 1987; Bruch *et al.*, 1989).

Solid-phase radioligand-binding assay

Iodinations of cAgrin and cN25Fc were performed as described in Gesemann *et al.* (1996). Laminin-1 and fragments thereof were diluted to 20 $\mu\text{g/ml}$ with 50 mM sodium bicarbonate, pH 9.6, immobilized in microtiter plates (Becton Dickinson) and incubated with iodinated agrin as described in Denzer *et al.* (1997). Dose-response curves were fitted by non-linear regression analysis using the equation $y = [(x/K_d)/(1 + x/K_d)]P$ to calculate the equilibrium dissociation constant (K_d) values of cAgrin to laminin-1 and to the laminin fragment C8-9. This equation assumes a single class of equivalent and independent binding sites, where y represents counts per min (c.p.m.), x the concentration of cAgrin and P c.p.m. at saturation. Accordingly, y/P represents the degree of saturation. Solubilization of coated protein with SDS sample buffer and analysis on SDS-PAGE followed by silver staining was used to confirm efficient coating of proteins onto the microtiter plates.

Electron microscopy

Samples of agrin (10–20 $\mu\text{g/ml}$), diluted in 0.2 M ammonium bicarbonate, were mixed 1:1 (v/v) with 80% (v/v) glycerol. Shortly after addition of the glycerol, the mixture was sprayed onto freshly cleaved mica. The mica chips were dried at $<10^{-5}$ Torr for at least 2 h. Rotary shadowing with platinum/carbon at an angle of 9°, carbon shadowing at 90°, replica formation and EM followed earlier protocols (Engel, 1994). Complexes between cN25Fc and laminin or laminin fragments (0.25 μM) were incubated for 2 h or longer at 4°C, if necessary diluted in 0.2 M ammonium bicarbonate, mixed with glycerol and further processed as described above. For negative staining, agrin (5 $\mu\text{g/ml}$) diluted in 0.2 M ammonium bicarbonate was adsorbed to a glow-discharged formvar/carbon support and stained with 2% uranyl formate (Engel, 1994). Molecular measurements on micrographs were fitted by single Gaussian curves using the Marquardt algorithm.

Transfer overlay assay

Laminin-1 (1.5 μg) or cAgrin (1 μg) were treated with reducing SDS sample buffer, separated by SDS-PAGE (Laemmli, 1970) and transferred to nitrocellulose (Towbin *et al.*, 1979). Blots were blocked with phosphate-buffered saline (PBS) containing 3% milk powder for 1 h and subsequently incubated with cAgrin or laminin-1 in blocking solution for 2 h. In the inhibition experiments, 100 nM of cN25Fc were included during the entire incubation. The blots were then washed three times with blocking solution, and incubated either with antiserum raised against cAN15Agrin (Denzer *et al.*, 1995) or antiserum 143 directed against murine EHS tumor laminin–nidogen complex (Aeschlimann and Paulsson, 1991) in blocking solution. Immunoreactivity was visualized by the ECL method (Amersham, Buckinghamshire, UK).

Alkylation of laminin-1 and the C8-9 fragment

An aliquot of each sample was incubated with 10 mM dithiothreitol for 1 h at 37°C to reduce the cysteine SH groups. The incubation was continued for another hour after the addition of *N*-ethylmaleimide to a concentration of 20 mM. The alkylated proteins were dialyzed against PBS and analyzed by SDS-PAGE under non-reducing conditions.

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