Integrins mediate adhesion to agrin and modulate agrin signaling

Paul T. Martin1,2 and Joshua R. Sanes1,*

1Department of Anatomy and Neurobiology, Washington University School of Medicine, 660 S. Euclid Avenue, St Louis, MO 63110, USA
2Department of Neurosciences, University of California, San Diego, School of Medicine, 9500 Gilman Drive, La Jolla, CA 92093-0691, USA

*Author for correspondence (e-mail: sanesj@thalamus.wustl.edu)

SUMMARY

Agrin, a basal lamina-associated proteoglycan, is a crucial nerve-derived organizer of postsynaptic differentiation at the skeletal neuromuscular junction. Because integrins serve as cellular receptors for many basal lamina components, we asked whether agrin interacts with integrins. Agrin-induced aggregation of acetylcholine receptors on cultured myotubes was completely blocked by antibodies to the β1 integrin subunit and partially blocked by antibodies to the αv subunit. Agrin-induced clustering was also inhibited by antisense oligonucleotides to αv and a peptide that blocks the αv binding site. Non-muscle cells that expressed αv and β1 integrin subunits adhered to immobilized agrin, and this adhesion was blocked by anti-αv and anti-β1 antibodies. Integrin αvβ1-negative cells that did not adhere to agrin were rendered adherent by introduction of αv. Together, these results implicate integrins, including αvβ1, as components or modulators of agrin’s signal transduction pathway.

Key words: acetylcholine receptors, muscle, neuromuscular junction, synapse formation

INTRODUCTION

During synapse formation at the vertebrate skeletal neuromuscular junction, the motor axon induces the formation of postsynaptic specializations in the muscle fiber (Hall and Sanes, 1993). A crucial signaling molecule in this process is agrin, a heparan sulfate proteoglycan (Tsen et al., 1995; Denzer et al., 1995) that is synthesized and secreted by motoneurons and incorporated into the basal lamina of the synaptic cleft (McMahan, 1990; Reist et al., 1992). Application of agrin to cultured myotubes stimulates formation of specialized domains that contain high density aggregates of neurotransmitter receptors (acetylcholine receptors or AChRs), associated with numerous cytoskeletal, membrane and extracellular matrix components that are postsynaptic-specific in vivo (Denzer et al., 1996). Conversely, agrin-induced AChR clustering and other aspects of postsynaptic differentiation are grossly deficient in mutant mice lacking agrin (Gautam et al., 1996). Agrin is also expressed by other central and peripheral neurons and may play roles in synapse formation in the brain (Bowe and Fallon, 1995).

In view of its central role in synaptogenesis, it is important to identify the receptors that mediate agrin’s effects. A critical component of the agrin receptor is a muscle-specific receptor tyrosine kinase called MuSK. MuSK is selectively expressed in skeletal muscle and concentrated, along with AChRs, in the postsynaptic membrane (Valenzuela et al., 1995; Apel et al., 1997). Mutant mice lacking MuSK fail to form neuromuscular junctions, showing a phenotype similar to (but more severe than) that of agrin mutants (DeChiara et al., 1996). Moreover, MuSK-deficient myotubes are unresponsive to agrin, a dominant negative MuSK mutant inhibits agrin-induced AChR clustering, and chemical cross-linkers can attach agrin to MuSK on the myotube surface (Glass et al., 1996, 1997). However, agrin does not bind to soluble forms of MuSK or to MuSK expressed in non-muscle cells (Glass et al., 1996). It is therefore likely that there are additional receptors in muscle that link agrin to MuSK and/or act in parallel to MuSK.

Attractive candidates for the role of co-receptor are the integrins, membrane proteins that serve as signal transducing receptors for numerous extracellular matrix proteins in both neural and nonneural tissues. Integrins are heterodimers of α and β subunits; 16 α and 8 β subunits have been identified so far in vertebrates. Both α and β subunits span the membranes; their extracellular domains cooperate to bind ligands and their intracellular domains transmit signals to the cell’s interior. Heterodimers differ in what ligands they bind and in what signals they transmit, so the repertoire of integrins that a cell expresses is a major determinant of how it responds to the environment (Hynes, 1992; Sonnerberg, 1993; Schwartz et al., 1995).

Several lines of evidence raised the possibility that integrins might play a role in agrin signaling. First, integrins have been shown to transduce many of the developmentally important signals that components of the extracellular matrix provide (Hynes, 1992, 1996). Second, integrins mediate most well-characterized developmental effects of laminin, a basal lamina protein that shares several regions of homology with agrin (Rupp et al., 1991; Ruegg et al., 1992; Mercurio, 1995; Delwel...
and Sonnenberg, 1996; Engvall and Wewer, 1996). Third, integrins are abundant in both developing and adult muscle. At least eight alpha subunits (\(\alpha_1\), \(\alpha_3\)-\(\alpha_7\), \(\alpha_9\) and \(\alpha_v\)) and three beta subunits (\(\beta_1\), \(\beta_3\) and \(\beta_5\)) are expressed in embryonic muscle (Bronner-Fraser et al., 1992; Duband et al., 1992; Lakonishok et al., 1992; Rosen et al., 1992; Bao et al., 1993; Enomoto et al., 1993; George-Weinstein et al., 1993; Palmer et al., 1993; Hirsch et al., 1994; McDonald et al., 1995; Gullberg et al., 1995a; Blaschuk et al., 1997) and at least four of these – \(\alpha_3\), \(\alpha_7\), \(\alpha_v\) and \(\beta_1\) – are present in the postsynaptic membrane at the adult neuromuscular junction (Bozyczko et al., 1989; Martin et al., 1996), as might be expected for an agrin receptor. Finally, recent studies have shown that integrins can interact, both directly and indirectly, with other receptor systems including some that, like MuSK, are receptor tyrosine kinases (Schwartz et al., 1995; Dedhar and Hannyen, 1996; Miyamoto et al., 1996; Sastry and Horwitz, 1996). Based on these parallels, we undertook to test the possibility that integrins are involved in agrin’s signal transduction pathway.

**MATERIALS AND METHODS**

**Reagents**

Sources of antibodies to integrins were as follows: JG22 (anti-chick \(\beta_1\); Greve and Gottlieb, 1982) and H1B4 (anti-chick \(\alpha_7\); Bao et al., 1993) were obtained from Developmental Hybridoma Bank (Iowa City, IA). O26 (anti-rat \(\alpha_v\); Song et al., 1992) was a gift from S. Kauflman (U. Illinois). TASC (anti-chick \(\beta_1\); Neugebauer and Reichardt, 1991), Chav (anti-chick \(\alpha_v\); Neugebauer et al., 1991) and Alf (anti-rat \(\alpha_3\); De Freitas et al., 1995) were gifts from L. Reichardt, Chicago (UCSF). L230 (anti-human \(\alpha_v\); Houghton et al., 1982), P5D2 (anti-human \(\alpha_3\); Carter et al., 1991) and 7G2 (anti-human \(\beta_3\); Gresham et al., 1989) were gifts from S. Blystone and E. Brown (Washington University). P4C10 (anti-human \(\beta_1\); Carter et al., 1990), P1B5 (anti-human \(\alpha_1\); Wayner et al., 1988) and GoH3 (anti-human and mouse \(\alpha_6\); Sonnenberg et al., 1987) were purchased from GibcoBRL (Gaithersburg, MD). Antibodies were partially purified from hybridoma-conditioned medium by precipitation in 50% ammonium sulfate, followed by extensive dialysis in phosphate-buffered saline (PBS). Antibody concentrations were estimated by SDS-gel electrophoresis, using purified immunoglobulin as a standard.

**Antisense oligonucleotides** with thio (S) diester linkages were obtained from Oligos Etc., (Portland, OR). Sequences used were:

- 5'GGCCA TGGGCGGGGCGGTGGG; \(\alpha_7\)
- 5'CAACGCTCTC; \(\alpha_7\)
- 5'AGCCATGCGGGGCGGCGGC; and reversed \(\alpha_v\) (control): 5'CGCGCCGGCCGGCGCTACCA; Peptides (GRGDSP and GRGESP) were purchased from GibcoBRL.

**Cell culture**

Primary cultures of chicken muscle were prepared from embryonic day (E)11 pectoral muscles. Cleansed dissected muscle was incubated in 0.2% trypsin for 30 minutes, triturated and cultured in Dulbecco’s Modified Eagles Media (DMEM) with 10% horse serum and 5% chick embryo extract. 3-5 days prior to addition of agrin, cells were transferred to media lacking chick embryo extract. Primary cultures of rat muscle were prepared from leg muscles of neonate pups. Dissected tissue was minced and trypsinized for 30-40 minutes in 0.2% trypsin, then triturated and cultured in DMEM plus 20% fetal calf serum (FCS). Fusion was induced by transfer to DMEM with 2% horse serum. C2 myogenic cells (ATCC, Bethesda MD) were cultured as myoblasts in DMEM with 20% fetal calf serum. Cells were grown to confluence then induced to form myotubes by changing the growth medium to DMEM with 2% horse serum. QT6 cells (ATCC, Bethesda, MD) were grown in Earl’s 199 medium with 10% tryptose phosphate broth, 5% FCS, and 1% DMSO. K562 cells (provided by S. Blystone and E. Brown, Washington University) were grown in Isocove’s modified Eagles media (IMEM) with 10% FCS. Cells were transfected with human \(\alpha_v\) and \(\beta_3\) cDNAs as described by Blystone et al. (1994). NCI-H69 cells (ATCC) were grown in RPMI with 10% FCS. 293 cells (ATCC) were grown in DMEM with 10% FCS.

All media contained 50 U/ml penicillin, and 50 µg/ml streptomycin, and all cells were incubated at 37°C in a 5% CO\(_2\) atmosphere.

**ACHR clustering**

ACHR clustering was induced with a recombinant 95 kDa C-terminal agrin fragment of the x=12, y=4, z=8 form, prepared as described by Ferns et al. (1993) and generously provided by J. Campanelli and R. Scheller (Stanford University). The agrin was electrotopographically pure, and was always added at 10 ng/ml, except for the experiment shown in Fig. 2B, where it was added at 5 ng/ml. Clustering was assayed by adding 50 nM rhodamine-\(\alpha\)-bungarotoxin (Molecular Probes, Eugene, OR) to live cells for 1 hour. Cultures were then washed in PBS, fixed in 1% paraformaldehyde, mounted in para-phenylenediamine/glycerol and viewed with an epifluorescence microscope. Clustering was quantitated as detailed by Martin and Sanes (1995).

For experiments using antibodies or peptides, the blocking reagent was added to myotubes for one hour, then agrin was added in the continued presence of blocking reagent for an additional 4-5 hours. For experiments using antisense oligonucleotides, myotubes were washed once each with PBS and OPTI-MEM serum-free medium (GibcoBRL). Nucleotides were then added at 0.2 or 2 µM in OPTI-MEM containing 6.6 µg/ml Lipofectin (GibcoBRL). Following incubation for 4 hours, cells were washed once each with OPTI-MEM and PBS, then incubated in fresh low serum media for 24 hours. Agrin was then added for 18 hours, after which ACHR clustering was assayed as above.

**Cell adhesion**

For use in adhesion assays, cells were detached from culture dishes without enzymes by trituration in Ca\(^2+\)-, Mg\(^2+\)-free saline. To prepare substrata for adhesion assays, culture dishes were coated with nitrocellulose as described by Lagenaur and Lemmon (1987), then 2 µl drops of the agrin fragment described above (20 µg/ml) or of laminin-1 (20 µg/ml; GibcoBRL) were added. After 30 minutes incubation, dishes were washed with PBS and then incubated with 10 mg/ml BSA to block residual binding sites on the nitrocellulose. Cells were added (40-50,000 in 0.4-0.5 ml of saline containing Mg\(^2+\) and Ca\(^2+\)), except where noted, and incubated for 60-90 minutes at 37°C, with agitation every 15 minutes. Nonadherent cells were then washed off, and adherent cells were fixed in 2% paraformaldehyde and counted. Cells were counted on one-quarter of a substratum drop in each well; this corresponds to approximately 10 microscope fields. In assays on 293 cells, 20 µg/ml heparin (Sigma) was included in the buffer to reduce non-specific adhesion.

**RESULTS**

**\(\beta_1\) integrins modulate agrin signaling**

All of the eight integrin \(\alpha\) chains known to be expressed in muscle (see Introduction) form heterodimers with the \(\beta_1\) subunit, and some dimerize only with \(\beta_1\) (Sonnerberg, 1993). We therefore began our study by asking whether JG22 (Greve and Gottlieb, 1982), an antibody that blocks all \(\beta_1\) integrins, affected agrin function in muscle. To this end, we assayed the ability of recombinant agrin to stimulate formation of high density ACHR clusters in cultured myotubes. This assay was
used to isolate agrin (McMahan, 1990) and reproduces a crucial aspect of agrin-dependent synaptogenesis in vivo (Gautum et al., 1996).

Small numbers of AChR clusters formed spontaneously on chick myotubes, but 5- to 10-fold more clusters appeared within 5 hours following application of 10 nM agrin (Fig. 1A,C). Addition of JG22 inhibited agrin-induced clustering, but not agrin-independent ‘spontaneous’ clustering (Fig. 1B,D). Inhibition was dose dependent and control antibodies were ineffective (Fig. 2A). Thus, agrin function requires β1 integrins.

The inhibition of agrin’s effects by JG22 suggested that integrins are required specifically for agrin signaling, but it was also possible that blockade of integrin perturbed myotube stability more generally, and thereby led indirectly to an inability to form AChR clusters. For example, adhesion of muscle cells to substrata is integrin-mediated (Neff et al., 1982) and myotubes detached from the gelatin-coated culture dishes at high JG22 stability more generally, and thereby led indirectly to an inability also possible that blockade of integrin perturbed myotube integrins are required specifically for agrin signaling, but it was a3, blocking antibodies to synapses. on fibers known to be competent to receive regeneration (J. R. S., unpublished observations)

v subunits are upregulated during muscle a7 and the agrin-responsive a1 integrins. We focused on the a3, a6, a7 and aα chains, for the following reasons: (a) All four chains are present in embryonic muscle at the time when synapses are forming (see Introduction). (b) All four bind laminin (Delwel and Sonnenberg, 1996), which contains several regions of homology to agrin (Rupp et al., 1991; Ruegg et al., 1992; Engvall and Wewer, 1996). (c) The aα3, a7 and aαv subunits are present in the postsynaptic membrane at adult neuromuscular junctions (Martin et al., 1996). (d) The aα3 and aαv subunits are upregulated during muscle regeneration (J. R. S., unpublished observations) on fibers known to be competent to receive synapses.

Cultured myotubes were treated with blocking antibodies to aα3, aα6, aα7 or aαv integrins. In each case, myotubes were cultured from the species for which antibodies were available: rat for aα3 and aα7, chick for aαv, and mouse for aα6. Antibodies to the aα3, aα6 and aα7 subunits had no significant effect, but anti-integrin aαv inhibited agrin-induced AChR clustering by ~40% (Fig. 3A). This result suggests that aαvβ1 is one, but perhaps not the only, integrin that modulates agrin signaling.

In light of the incomplete blockade by anti-aαv, we used two additional methods to assess the role of this subunit in agrin-dependent signaling. First, we tested a blocking peptide that contains the sequence RGD, which is a crucial determinant of the binding site in ligands of a subset of integrins, including aα integrins (Rooslahti, 1996). Although agrin does not contain an RGD sequence, it has been shown that aαv integrins bind both RGD-containing and non-RGD-containing ligands, and that RGD peptides inhibit interactions of aα integrins with both types of ligands (Vogel et al., 1993; Piali et al., 1995; Buckley et al., 1996; Sakamoto et al., 1996). GRGDSP inhibited agrin-induced AChR clustering in mouse myotubes by ~40%, whereas an inactive peptide, GRGESP, had no effect (Fig. 3B). Second, we attenuated integrin expression with antisense oligonucleotides complementary to the translation initiation sites of the integrin aα6, aα7 and aαv subunits. The oligonucleotides were introduced into C2 myotubes, then agrin was added and AChR clustering assayed. Anti-aα7 and control oligonucleotides had no effect on clustering at concentrations up to 2 μM, and anti-aα6 oligonucleotides enhanced the effect of agrin slightly (Fig. 3C and data not shown). In contrast, the oligonucleotide complementary to the aαv sequence completely inhibited agrin-induced AChR clustering at a concentration of 0.2 μM (Fig. 3C). Thus, three types of reagents directed against aα integrins – antibodies, peptides, and antisense oligonucleotides – all inhibited agrin-dependent AChR clustering, strongly implicating these integrins in agrin’s signaling mechanism.

**Integrin aαvβ1 mediates cell adhesion to agrin**

Results presented so far suggest that integrins are agrin receptors. However, it was also possible that integrins bound some distinct factor in the culture medium or on the substratum that was required for agrin to exert its effects. We therefore conducted short-term cell adhesion assays in saline to seek a direct interaction of agrin with integrins. Myotubes are poorly suited for adhesion assays, but we found that cells of the better

![Fig. 1. Effects of agrin, anti-integrin β1 or both on AChR clustering. Cultured chick myotubes were treated with anti-integrin β1 (JG22, 100 μg/ml) and/or agrin (10 ng/ml), then labelled with rhodamine-α-bungarotoxin to visualize AChR clusters. (A) No treatment; small numbers of clusters form spontaneously. (B) Anti-integrin β1 did not affect spontaneous AChR clustering. (C) Agrin induced clustering. (D) Anti-integrin β1 inhibited agrin-induced clustering. Bar is 50 μm.](image-url)
suited quail QT-6 fibroblast line expressed both αv and β1 integrin subunits (Galileo et al., 1992 and data not shown). Accordingly, we asked whether QT-6 cells adhered to substrata coated with the purified, recombinant agrin fragment that we had used to induce AChR clustering. In fact, QT-6 cells adhered to agrin-coated substrata in a magnesium-dependent manner (Fig. 4A). This dependence on magnesium is characteristic of integrin-mediated adhesion, because binding of ligands to integrin requires a divalent cation (Hynes, 1992). Adhesion was completely inhibited by JG22, the anti-β1 antibody that blocked agrin-dependent AChR clustering in chick myotubes, and partially inhibited by Chav, the anti-αv antibody that partially blocked clustering (Figs 2A, 3A, 4A). Adhesion was also partially blocked by the GRGDSP peptide, but was unaffected by control antibodies or peptides (Fig. 4A). Thus, integrins containing αv and/or β1 subunits are likely to bind directly to agrin.

In muscle cells, the integrin αv subunit forms heterodimers not only with β1 but also with other β subunits including β3 and β5 (Rajaraman, 1993; Gullberg et al., 1995b; Blaschuk et al., 1997). To ask whether αvβ1 heterodimers interact with

Fig. 2. β1 integrins modulate agrin-induced AChR clustering. (A) Effects of JG22, a blocking antibody to integrin β1, on AChR clustering in the presence (open bars) or absence (dark bars) of agrin. A similarly prepared antibody to an intracellular epitope was used as a control. (B) Effects of TASC (50 µg/ml), an activating antibody to integrin β1, on AChR clustering in the presence (open bars) or absence (dark bars) of 5 ng/ml agrin. TASC potentiates the effects of agrin. Bars show mean ± s.e.m. of values from 3-6 (A) or 5 (B) experiments.

Fig. 3. αv integrins modulate agrin-induced AChR clustering. Individual integrin subunits were blocked with antibodies (A), peptides (B) or oligonucleotides (C). (A) Blocking antibodies to the integrin α3, α6, α7 or αv subunits were added to agrin-treated myotubes from the appropriate species and their ability to block AChR clustering was assessed. Anti-α3 (Ralph) was added to rat myotubes at 200 µg/ml, anti-α6 (GoH3) to mouse myotubes at 100 µg/ml, anti-α7 (O26) to rat myotubes at 250 µg/ml and anti-αv (Chav) to chick myotubes at 200 µg/ml. Only anti-αv blocked clustering. (B) GRGDSP (80 µg/ml), which blocks the ligand binding site of αv, inhibited agrin-induced AChR clustering in C2 cells. The control peptide, GRGESP, was without effect. (C) Antisense oligonucleotides complementary to integrin α6, α7 and αv mRNAs were added at 200 nM to mouse C2 cells. The reverse of the αv sequence was used as a control. Only the antisense αv sequence inhibited agrin-induced clustering. Antisense α7 and control oligonucleotides were also without effect and α6 oligonucleotide was slightly stimulatory, at 2 µM. Bars show mean ± s.e.m. of values from 4-5 (A), 4 (B), or 5-6 (C) independent experiments.
Integrins as agrin receptors

We also tested the human NCI-H69 cell line, which expresses the laminin-binding α3β1 and α6β1 heterodimers, but does not express detectable αv (Elices et al., 1991). These cells adhered to laminin, as expected, but did not adhere to agrin (data not shown). Thus, of three laminin-binding β1 integrins tested – α3β1, α6β1, and αvβ1 – only αvβ1 mediated adhesion to agrin.

Forced expression of integrin αv renders cells adhesive to agrin

As an additional test of the ability of αv integrins to mediate adhesion to agrin, we asked whether addition of αv to a cell’s repertoire of integrin subunits could endow it with the ability to bind agrin. For this experiment, we used human K562 cell, which express the α5 and β1 subunits but not αv, α1, α3, α4, α6 or β3 (Blystone et al., 1994, 1995). These cells did not adhere to agrin under our assay conditions (Fig. 5A). In initial
We have provided four lines of evidence that agrin interacts with integrins First, agrin-induced AChR clustering on avian and mammalian myotubes is inhibited by reagents (antibodies, peptides, and antisense oligonucleotides) that interfere with αv or β1 integrin activity or expression. Second, an antibody that activates β1 potentiates the effect of agrin on AChR clustering. Third, two cell lines (quail fibroblasts and human epithelial cells) that express αv and β1 integrins bind to an immobilized agrin fragment, and this adhesion is inhibited by antibodies and peptides that bind αv or β1. Fourth, cells that do not adhere to agrin and do not express αv can be rendered adherent by forcing expression of αvβ3 on their surface. Together, these results suggest that αv and β1 integrins, probably including αvβ1, can serve as agrin receptors.

That integrins might be agrin receptors is not unexpected, given the known abilities of integrins to bind many components of basement membranes (e.g. laminins, collagens, entactin, tenascin and fibronectin) and to transmit numerous signals from the extracellular matrix to the cell’s interior (Hynes, 1992; Venstrom and Reichardt, 1993; Schwartz et al. 1995). To our knowledge, however, the present report provides the first experimental support for this proposition. Ma et al. (1993; see also Bowe and Fallon, 1995) noted that RGD peptides, which inhibit many but not all integrin-based interactions, do not affect the binding of agrin to cells. However, the binding being assayed was to α-dystroglycan (discussed below), and interactions with integrins might not have been detected under the assay conditions used. Moreover, at least one investigator found that RGD inhibited agrin-induced AChR clustering in chick myotubes by 31% (Table 1 in Wallace, 1988), but did not comment on this datum in the text. Thus, the impression may have arisen that integrins do not bind agrin, but we know of no data inconsistent with this possibility.

Agrin has previously been shown to interact, directly or indirectly, with several components of the myotube surface, including α-dystroglycan (Gee et al., 1994; Bowe et al., 1994; Sugiyama et al., 1994; Campanelli et al., 1994), the neural cell adhesion molecule (N-CAM; Cole and Halfter, 1996), heparin/heparan sulfates (Campanelli et al., 1996; O’Toole et al., 1996), N-acetylgalactosamine-terminated glycoconjugates (Martin and Sanes, 1995), the heparin-binding growth associated molecule HB-GAM/pleitrophin (Daggett et al., 1996) and MuSK (Glass et al., 1996). We now add integrins to this list.

In evaluating this multiplicity of potential receptors, lessons learned from a more intensively studied protein, laminin, may be informative. Both laminin and agrin are multidomain proteins of the basal lamina that provide critical developmental signals to neurons and muscles. Moreover, although the two proteins show no global similarity in primary sequence or predicted secondary structure, they share several discrete regions of significant homology. Most important in the present context, it has been clearly demonstrated that laminin binds to multiple receptors, including integrins, α-dystroglycan, cell adhesion molecules, heparan sulfate proteoglycans and other glycoconjugates (Mercurio, 1995; Mecham and Hinck, 1996).

One striking parallel is the presence of G repeats in both agrin and laminin α chains. All five of the known laminin α chains (laminins are heterotrimer of α, β, and γ chains) contain a large C-terminal globular (G) domain, composed of five to 200-amino-acid-long repeats, called G1-G5 (Sasaki et al., 1988; Engvall and Wewer, 1996; Miner et al., 1997). Much (but by no means all) of the bioactivity of laminin heterotrimmers resides in the G domain, which has been called the “business end” of the molecule (Engvall and Wewer, 1996). Isolated G domains mimic several effects of laminin, including promotion of neurite outgrowth and myoblast adhesion (Edgar et al., 1984; Yurchenco et al., 1993). Binding sites for heparin/heparan sulfate, α-dystroglycan and several integrins have been mapped to distinct G repeats, and much has been learned about the ways in which these receptors mediate laminin’s effects (Mercurio, 1995). Three similar repeats (G1-G3), separated from each other by EGF-like repeats, form much of the C-terminal half (~95 kDa) of agrin, which is fully active in AChR clustering (Ferns et al., 1993; Denzer et al., 1996). Deletion of the most N-terminal G repeat, G1, yields a ~50 kDa fragment that is about half as active as the 95 kDa fragment. Deletion of G2 yields a ~20 kDa fragment, which is ~1% as active as the 95 kDa fragment (Hoch et al., 1994; Gesseman et al., 1995). These results suggest that, although G3 is crucial for activity, G1 and G2 are also important. In fact, α-dystroglycan and heparin bind to distinct but overlapping sites within G2 (Gesseman et al., 1996; Campanelli et al., 1996; O’Toule et al., 1996; Hopf and Hoch, 1996). Thus, for agrin as for laminin, distinct G repeats may bind to multiple receptors to generate a signal.

Second, α-dystroglycan and integrins are both crucial laminin receptors, but they play different roles. α-Dystroglycan was isolated independently as a laminin-binding protein from brain and as a component of the dystrophin-glycoprotein complex that links the cytoskeleton to the basal lamina in muscle cells. The precise function of α-dystroglycan remains undefined, but available evidence favors the idea that it is primarily important for assembly and maintenance of the basal lamina (Henry and Campbell, 1996). Likewise, although it was originally suspected that α-dystroglycan might act as a signaling receptor for agrin (Gee et al., 1994; Bowe et al., 1994; Campenelli et al., 1994), attempts to block agrin function with antibodies to α-dystroglycan have given inconsistent results (Gee et al., 1994; Campenelli et al., 1994; Sugiyama et
al., 1994), and alternatively spliced forms of agrin that do not cluster AChRs bind at least as tightly to α-dystroglycan as fully active forms (Sugiyama et al., 1994; Gesseman et al., 1996; Hofp and Hoch, 1996). Thus, for agrin as for laminin, α-dystroglycan may be more involved in assembly and stabilization than in signaling. In contrast, there is abundant evidence that integrins are major signaling receptors for laminins, and the same may be the case for agrin.

Third, laminins signal through multiple integrins. At least 10 integrin dimers (αβ1, α2β1, α3β1, α6β1, α6β4, α7β1, α9β1, αβ3, α1β1β3 and αββ8), bind to various domains on laminins, (Delwel and Sonnenberg, 1996). Individual cells sometimes express several laminin-binding integrins, providing for cooperativity, specificity and diversity in signaling functions (Mercurio, 1995). Likewise, although our strongest evidence implicates αβ1 integrin as an agrin receptor, blockade of β1 consistently inhibited agrin activity (clustering or adhesion) more completely than blockade of αβ. Moreover, αβ1 and αβ3, both of which are expressed by muscle (Gullberg et al., 1995a,b; Blaschuk et al., 1997), can mediate adhesion to agrin. It is therefore possible that agrin, like laminin, makes use of multiple integrins, including β1 integrins that do not contain αv and αv integrins that do not contain β1.

Finally, laminins are potent promoters of neurite outgrowth and these effects are largely integrin-mediated (Venstrom and Reichardt, 1993). Intramuscular nerve branching patterns are greatly perturbed in agrin-deficient mutant mice (Gautam et al., 1997; Chang et al., 1997). For laminins, integrin-mediated outgrowth correlates with integrin-mediated adhesion. The integrin-dependent adhesion to agrin described here therefore raises the possibility that agrin’s effects on neurons, like its effect on muscle, involve integrins.

A major question that remains is the relationship of integrins to the best candidate agrin receptor, the tyrosine kinase MuSK (see Introduction). Recent studies in several systems have revealed previously unsuspected relationships between integrins and tyrosine kinases (Schwartz et al., 1995; Dedhar and Hannigan, 1996; Miyamoto et al., 1996), and provide precedents for a MuSK-integrin interaction. One possibility is that integrin and MuSK interact physically. The observation that MuSK alone cannot bind agrin is consistent with this idea; MASCl, the hypothesized binding subunit of the MuSK receptor complex (Glass et al., 1996), might be an integrin. Second, integrins could act downstream of MuSK. The observations that spontaneous (agrin-independent) clustering requires MuSK (Glass et al., 1996; DiChiara et al., 1996) but not integrin (this study) favors this possibility. Third, integrins and MuSK might provide parallel signaling pathways for agrin, with the MuSK pathway being essential and the integrin pathway facilitatory. In other systems, growth factors and adhesive ligands play synergistic roles in developmental processes (Sastry and Horwitz, 1997); synaptic differentiation might require agrin to exert both roles but via different pathways. The availability of mutant mice lacking MuSK (DeChiara et al., 1996; Apel et al., 1997) and several integrin subunits (Hynes, 1996) should facilitate tests of these and other possibilities.

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