Agrin and laminin induce acetylcholine receptor clustering by convergent, Rho GTPase-dependent signaling pathways

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Accepted 1 December 2006
Journal of Cell Science 120, 868-875 Published by The Company of Biologists 2007
doi:10.1242/jcs.03367

Summary

During neuromuscular junction formation, extracellular matrix-mediated signals cause muscle surface acetylcholine receptors (AChRs) to aggregate at synaptic sites. Two extracellular matrix proteins, agrin and laminin, have each been shown to initiate signaling pathways that culminate in AChR clustering in cultured muscle cells. Here we present evidence that laminin-induced AChR clustering is mediated by the activation of the Rho GTPases Cdc42, Rac and Rho. Clustering in response to laminin is blocked by the dominant negative mutants Cdc42N17, RacN17 and RhoN19, as well as by the Rho inhibitor C3 transferase. Moreover, laminin-induced AChR clustering is impaired by the Rho kinase inhibitor Y-27632. Agrin-induced AChR clustering has previously been shown to require activation of Cdc42, Rac and Rho. Therefore, although agrin and laminin use distinct transmembrane receptors to initiate AChR clustering, their signaling pathways converge at the level of Rho GTPase activation.

Key words: Neuromuscular junction, Acetylcholine receptors, Laminin, Agrin, Rho GTPases

Introduction

The formation of specialized contacts between cells is known to involve the integration of cues from different signaling pathways. A well-characterized example is the vertebrate neuromuscular junction (NMJ), where innervation induces the anatomical and biochemical specialization of the small patch of muscle surface immediately under the motor nerve ending. A prominent aspect of this specialization that is crucial for synapse function is the concentration of high densities of nicotinic acetylcholine receptors (AChR) in the postsynaptic membrane (Sanes and Lichtman, 1999; Sanes and Lichtman, 2001). There is evidence that multiple incoming signals are integrated to produce the clustering of AChR and associated components at these sites (Burden, 1998; Sanes and Lichtman, 2001). The most extensively studied of these signals is the neurally derived isoform of the extracellular matrix (ECM) proteoglycan agrin that is synthesized by motor neurons and secreted into the synaptic cleft (Colledge and Froehner, 1998; Hoch, 1999; McMahan et al., 1992). Agrin initiates its effects by binding to a surface complex comprised of a muscle-specific receptor tyrosine kinase (MuSK) and other as yet unidentified muscle surface components (Apel et al., 1997; DeChiara et al., 1996; Glass et al., 1996). The ECM glycoprotein, laminin, is also known to play a role in postsynaptic membrane organization including AChR clustering (Colognato and Yurchenco, 2000; Patton et al., 1997; Vogel et al., 1983). Laminin-induced AChR clustering, however, probably uses a different transmembrane signaling pathway than agrin. Soluble laminin has been reported to transduce AChR clustering through the α7β1 integrin and α-dystroglycan, and this process might not directly involve MuSK activation (Burkin et al., 1998; Jacobson et al., 2001; Montanaro et al., 1998; Sugiyama et al., 1997).

There is now considerable evidence that the laminin- and agrin-induced signaling pathways that direct AChR clustering are synergistic, so that simultaneous addition of laminin and agrin produces a more extensive AChR clustering response than that observed with either factor alone (Burkin et al., 2000; Denzer et al., 1997; Sugiyama et al., 1997). Although the signaling systems that induce AChR clustering in response to agrin and laminin have remained largely undefined, recent findings have suggested that formation of stable AChR clusters by both laminin and agrin require rapsyn, tyrosine phosphorylation of AChR β and δ subunits and activation of Src-related kinases (Borges and Ferns, 2001; Ferns et al., 1996; Marangi et al., 2001; Marangi et al., 2002; Mittaud et al., 2001; Mohamed et al., 2001; Smith et al., 2001). A mechanism through which agrin and laminin couple these components to direct the lateral movement of AChR on the surface of postsynaptic muscle cells into nascent synapses, however, remains elusive.

Focal changes in the peripheral cytoskeleton are thought to underlie the aggregation of AChR in postsynaptic membranes at NMJs (Dai et al., 2000; Hoch et al., 1994; Phillips, 1995). The monomeric G proteins Cdc42, Rac and Rho have been shown to integrate multiple extracellular signals and link these signaling events to dynamic changes in actin cytoskeleton organization (Bishop and Hall, 2000; Hall, 1998; Ridley, 2001; Takai et al., 2001). We have previously shown that agrin triggers the activation of Cdc42, Rac and Rho and that this

activation is crucial for agrin-induced AChR clustering (Weston et al., 2003; Weston et al., 2000). In the present study we show for the first time that AChR clustering induced by laminin is dependent on activation of monomeric G proteins. These findings provide a model for a mechanism in which the agrin and laminin signaling cascades converge at the level of Rho GTPase activation and direct the cytoskeletal reorganization that underlies AChR aggregation.

Results

As we have previously reported (Weston et al., 2003), agrin-induced AChR clustering on the myotube surface occurs in two sequential stages: the initial Rac-dependent coalescence of diffuse receptors into small aggregates termed microclusters (2-5 μm diameter), and the subsequent Rho-dependent aggregation of microclusters to form full-sized clusters (15-20 μm diameter). Although soluble laminin-induced clusters appear to have a slightly increased receptor density (Lee et al., 2002), they closely resemble agrin-induced clusters in shape and dimensions. Soluble laminin-induced AChR clustering further resembles agrin-induced clustering in its formation through microcluster intermediates (not shown). We, therefore, were interested in asking whether laminin-induced AChR clustering was dependent on the activation of Rho GTPases.

Laminin-induced AChR clustering requires Rac and Cdc42 activation

To determine whether Cdc42 and Rac participate in laminin-induced AChR clustering, C2 myoblasts were transfected with dominant interfering mutants of Rac (RacN17) and Cdc42 (Cdc42N17). The effects of these mutants on AChR surface distribution in laminin-treated and -untreated myotubes were examined by fluorescence microscopy. In contrast to laminin-induced AChR clustering seen in cells transfected with vector only (Fig. 1Aa,b), myotubes expressing RacN17 (Fig. 1Ac,d) or Cdc42N17 (Fig. 1Ae,f) did not display AChR clustering after laminin treatment. Quantitative comparison of the number of AChR clusters on the surface of myotubes expressing either RacN17 or Cdc42N17 mutants versus control myotubes showed a greater than 90% inhibition of laminin-induced AChR clustering by the dominant interfering mutants of both Rac and Cdc42 (Fig. 1B). These findings indicate that Rac and Cdc42 activation is required for laminin-induced AChR clustering.

Laminin activates Rac and Cdc42 in myotubes

To determine whether laminin activates Rac and Cdc42 in muscle cells, differentiated myotube cultures were treated with laminin (10 nM) and Rho GTPase activities were measured by affinity precipitation assays for cellular GTP-bound forms of Rac and Cdc42. Activation of Rac and Cdc42 was monitored by binding to GST-PBD (Sander et al., 1998). As can be seen in Fig. 2, these measurements show that laminin activates Rac (A) and Cdc42 (B), similar to the effect of agrin in differentiated muscle cells.

We have previously shown that agrin activates the c-Jun amino-terminal kinase (JNK) pathway (Weston et al., 2000), an effector pathway of Rac and Cdc42 that regulates multiple cellular processes including gene transcription (Coso et al., 1995; Gupta et al., 1996; Minden et al., 1995). To determine whether laminin also activates the JNK pathway in these cells,

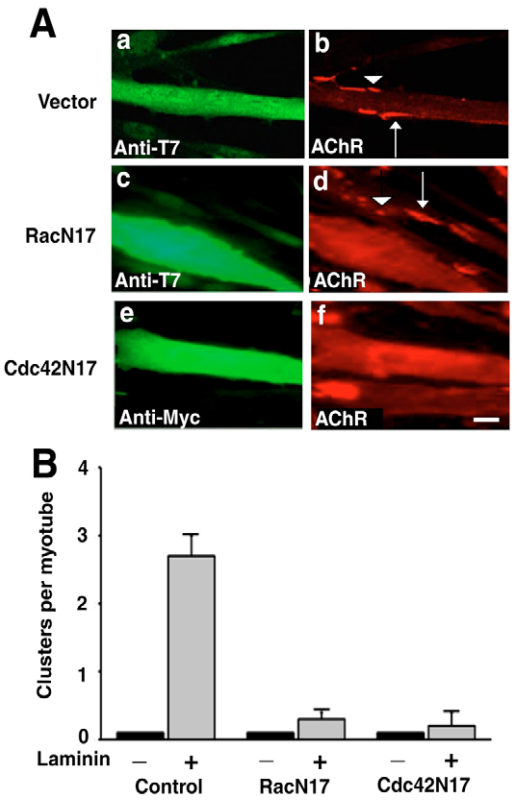

Fig. 1. Dominant interfering Rac and Cdc42 block the ability of laminin to cluster AChR. (A) In order to assess the contribution of Rac and Cdc42 activation to laminin-induced AChR clustering, vector (a,b), T7-tagged RacN17 (c,d) or myc-tagged Cdc42N17 (e,f) were transfected into C2 muscle cell cultures. Three days after transfection, the effects of AChR surface distribution in laminin-treated and -untreated myotubes were examined by fluorescence microscopy of cultures surface-labeled with TMR-Bgt. Those myotubes expressing the transfected constructs were identified using indirect immunofluorescence with anti-T7 or anti-myc antibodies, and FITC-labeled secondary antibody (a,c,e). Myotubes expressing RacN17 or Cdc42N17 did not display microclusters (arrowheads) or clusters (arrows) of AChR after laminin treatment (b,d,f); however, adjacent, non-transfected cells were able to cluster AChRs in response to laminin. (B) Quantitative comparison of the number of AChR clusters on the surface of transfected myotubes expressing the Rac and Cdc42 mutants versus vector-transfected myotubes clearly documented the blocking effect of the dominant interfering RacN17 and Cdc42N17 on laminin-induced AChR aggregation (error bars represent ±s.e.m., n=40 cells from five or more separate platings).

phosphorylation of c-Jun was measured in laminin-treated and -untreated cells. As shown in Fig. 3A, strong phosphorylation of c-Jun was observed in C2 myotubes after treatment with laminin. The time course for laminin activation of JNK was similar to that of agrin activation with phosphorylation of c-Jun reaching maximal levels at 15 minutes and returning to baseline by 60 minutes after laminin treatment (Fig. 3B). To verify that stimulation of JNK by laminin reflected the activation of Rac or Cdc42 rather than another upstream activator of JNK, similar assays were performed using cells transfected with dominant negative mutants of Rac and Cdc42. Fig. 4 shows that when C2 cells transfected with FLAG-

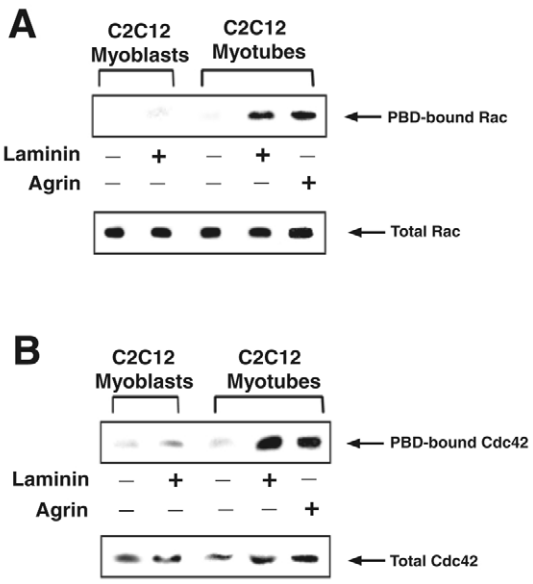

Fig. 2. Laminin activates Rac and Cdc42 in C2 myotubes. Laminin-induced activation of endogenous Rac (A) and Cdc42 (B) in C2 myotubes was measured using GST-PBD and western blotting with anti-Rac or anti-Cdc42 antibodies. Laminin activates Rac and Cdc42 similarly to agrin-induced Rac and Cdc42 activation in differentiated muscle cells.

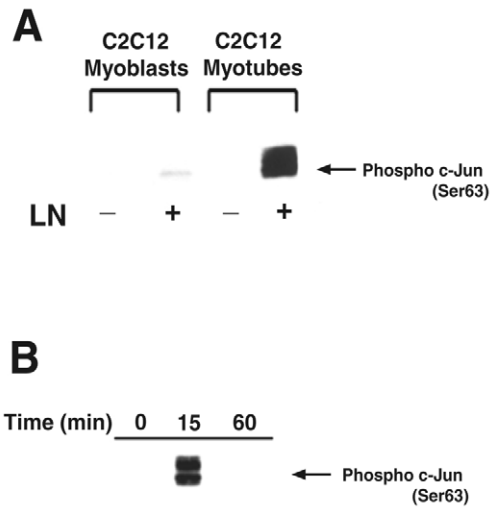

Fig. 3. Laminin activates JNK in C2 myotubes. (A) JNK activation by laminin was observed in differentiated C2 myotubes but not in undifferentiated myoblasts. (B) The time course of laminin stimulation of c-Jun phosphorylation in myotubes shows a transient response to laminin that results in c-Jun phosphorylation that reaches a maximum within 15 minutes and subsequently declines to baseline levels by 60 minutes.

epitope-tagged JNK were treated with laminin there was a sizeable increase in c-Jun phosphorylation. By contrast, laminin did not activate JNK in cells cotransfected with the dominant negative RacN17 (Fig. 4A). Conversely, expression of constitutively active Rac (RacV12) in laminin-untreated myotubes resulted in elevated c-Jun phosphorylation. These findings confirm that the increase in phosphorylated c-Jun seen after laminin treatment is a measure of Rac activity, extending the finding that laminin activates Rac-mediated signaling. To determine the influence of Cdc42 on laminin-induced JNK activation, measurements of JNK activation were performed as above using FLAG-tagged JNK-transfected C2 muscle cultures coexpressing myc epitope-tagged dominant negative Cdc42N17 or constitutively activated Cdc42V12. As shown in Fig. 4B, the Cdc42 mutants had parallel effects to the analogous Rac mutants. Dominant negative Cdc42 was found to block laminin activation of JNK, whereas constitutively activated Cdc42 produced markedly elevated c-Jun phosphorylation. Together, these findings provide strong evidence that laminin treatment of intact myotubes induces activation of Rac and Cdc42.

Rho activation is required for laminin-induced AChR clustering

To investigate whether laminin-induced AChR clustering is Rho-dependent, a dominant negative mutant of Rho (RhoN19) was microinjected into differentiated myotubes. After overnight incubation in the absence or presence of laminin, surface AChRs on myotubes were visualized. Dominant negative Rho was found to impair the ability of laminin to cluster AChR (Fig. 5Ad) compared with myotubes injected with vector and treated with laminin under identical conditions (Fig. 5Ab). As shown in Fig. 5B, there is a decrease in the number of laminin-induced AChR clusters and AChR microclusters present in the myotubes expressing RhoN19. This effect is similar to the effect of RhoN19 on agrin-induced clusters, although with agrin there is an increase in microclusters (Weston et al., 2003). To confirm that laminin-induced AChR clustering is mediated by Rho activation, the effect of exposure of myotubes to the Rho inhibitor C3 transferase (C3) was monitored. As shown in Fig. 6, C3 was found to block AChR cluster formation when added to differentiated muscle cell cultures prior to laminin treatment. Laminin-induced clustering was blocked by more than 50% in the presence of C3, similar to the effect on agrin-induced clustering.

To determine whether endogenous Rho is activated upon laminin treatment in muscle cultures, an affinity precipitation assay based on the selective binding of activated Rho to the Rhotekin Rho-binding domain (TRBD) was used (Ren et al., 1999). As can be seen in Fig. 7, laminin activates Rho in C2 myotubes, similar to the effect seen with agrin. In addition, the time course for Rho activation by laminin was similar to that of agrin with maximal, sustained activation occurring at 30 minutes (data not shown).

The effector pathway by which laminin induces AChR clustering was investigated using a pharmacological inhibitor of the Rho kinase (ROCK), Y-27632 (Ishizaki et al., 2000; Uehata et al., 1997). We observed that Y-27632 blocked laminin-induced AChR clustering by more than 40% in C2 myotubes (Fig. 8), indicating that a significant portion of the laminin signal responsible for AChR clustering requires ROCK activation. Y-27632 blocked agrin-induced clusters by ~80%, suggesting that possibly a greater proportion of the agrin signal is transduced through the Rho kinase effector pathway.

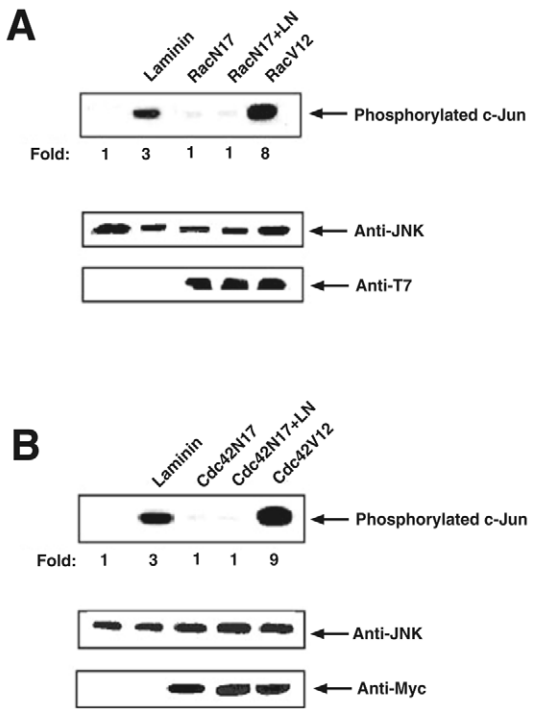

Fig. 4. Laminin activation of JNK is dependent on Rac and Cdc42 activation. (A) C2 cells were transfected with FLAG epitope-tagged JNK alone or in combination with expression plasmids encoding T7-epitope-tagged RacN17 or RacV12. Myotubes were treated for 15 minutes with laminin (10 nM), where specified, and the transfected JNK was immunoprecipitated from cell lysates with anti-FLAG antibody. The immunopurified JNK was incubated with [γ-^{32}P]ATP and GST-c-Jun as a substrate. Levels of transfected Rac and JNK expression were determined by western blotting with anti-T7 and anti-JNK1 primary antibodies, respectively. GST-c-Jun phosphorylation was visualized by autoradiography. As quantitated by a PhosphorImager, cells transfected with JNK alone showed a threefold increase in c-Jun phosphorylation when treated with laminin. By contrast, cells that were cotransfected with JNK plus RacN17 showed no JNK activation when treated with laminin. (B) C2 cells were cotransfected with FLAG epitope-tagged JNK and myc epitope-tagged Cdc42N17 or Cdc42V12. Immunoprecipitated JNK was incubated with [γ-^{32}P]ATP and GST-c-Jun. As quantitated by PhosphorImager, the threefold increase in phosphorylation induced by laminin was eliminated by the dominant negative Cdc42 mutant. Constitutively active mutants of Rac (RacV12) and Cdc42 (Cdc42V12) serve as positive controls for JNK activation. The fold-increases in activity in this figure were consistent over at least five separate experiments.

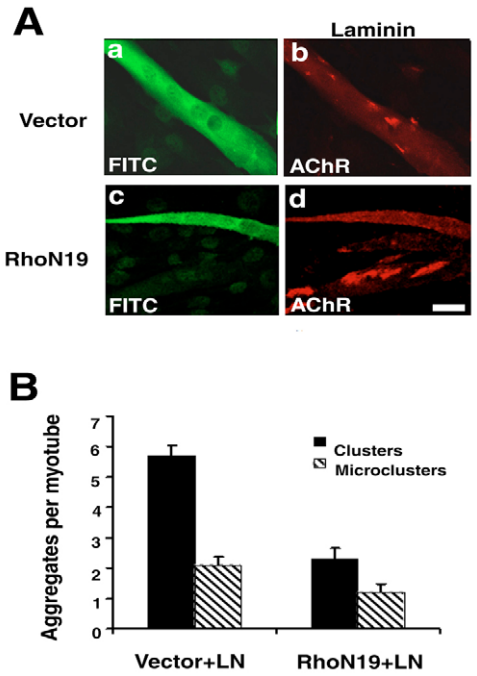

Fig. 5. Dominant interfering Rho inhibits the ability of laminin to cluster AChR. (A) In order to assess the contribution of Rho activation to laminin-induced AChR clustering, differentiated muscle cells were microinjected with RhoN19 or vector. After microinjection, the cells were treated with laminin (10 nM) overnight. One day after injection, the effects of AChR surface distribution in RhoN19-expressing (c,d) versus vector-expressing cells (a,b) treated with laminin were examined by confocal microscopy of cultures surface-labeled with TMR-Bgt. Those myotubes injected with RhoN19 were identified with co-injected FITC-goat anti-mouse antibody (a,c). Myotubes expressing RhoN19 did not display full-sized AChR clusters after treatment with laminin (d), compared with those myotubes injected with vector only (b). Scale bar, 10 μm. (B) Quantitative comparison of the number of AChR clusters on the surface of myotubes expressing the Rho mutant versus control myotubes clearly documents the inhibiting effect of the dominant interfering RhoN19 on AChR cluster (black bars) and microcluster (grey bars) formation in response to laminin (10 nM) treatment (error bars represent ±s.e.m.; n=40 cells from five or more separate platings).

Laminin matrix signaling-induced topologically complex AChR cluster formation requires active Rho

It was recently reported that, in contrast to structurally simple ovoid AChR clusters induced by soluble forms of agrin and laminin, substrate laminin elicits a more extensive clustering response that appears to closely approximate the events in the muscle cell during NMJ formation (Kummer et al., 2004). We have investigated whether our findings of a dependence on Rho GTPase activation with soluble laminin-induced AChR clusters extends to substrate laminin cluster formation. We find that blocking Rho activity impairs the formation of substrate laminin-induced complex AChR clusters. C2 cultures were transfected with green fluorescent protein (GFP)-RhoN19 to identify myotubes expressing dominant negative Rho and plated on laminin-coated coverslips. As can be seen in Fig. 9A, substrate laminin-induced AChR complex clustering was abolished in myotubes expressing RhoN19. By contrast, nontransfected myotubes in these cultures display the characteristic substrate laminin-induced AChR clusters. Quantification of the effects of RhoN19 shows that the blocking effect extends to all stages of substrate laminin-induced AChR clustering (Fig. 9B).

Discussion

Agrin and laminin each initiate transmembrane signaling pathways that induce AChR clustering in cultured muscle cells. In vivo these pathways are likely to have central roles in linking ECM-derived signals to the assembly of postsynaptic membranes at the NMJ. Here we show that the signaling

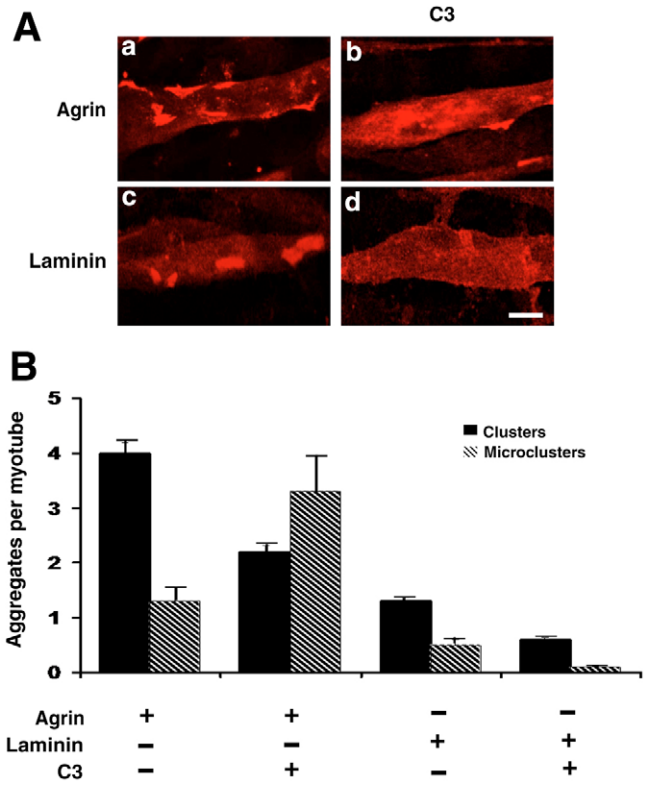

Fig. 6. C3 transferase impairs laminin-induced AChR clustering. (A) To confirm the results found with RhoN19 on laminin-induced AChR clustering, C3 transferase was added to C2 myotubes 3 hours prior to overnight laminin (10 nM) treatment. The effects on AChR surface distribution were examined by confocal microscopy of cultures surface-labeled with TMR-Bgt. C3 impairs the ability of agrin and laminin to form AChR clusters (b,d) compared with cells that were not treated with C3 (a,c). (B) Quantitative analysis of the effect of C3 on clustering of AChR clearly shows that inactivation of Rho impairs the formation of AChR clusters in response to laminin (10 nM) and agrin (5 nM) (error bars represent ±s.e.m.; *n*=100 cells from five or more separate platings).

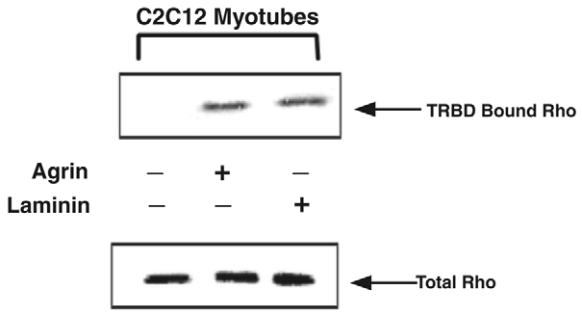

Fig. 7. Laminin stimulates increased binding of Rho to the Rho-binding domain of Rhotekin in C2 myotubes. Laminin-induced Rho activation in nontransfected C2 myotubes was measured by the increase of endogenous Rho bound to GST-TRBD. Cultures were treated with laminin (10 nM) for 15 minutes, and lysates were incubated with GST-TRBD and western blotted with antibody to Rho. Laminin caused increased association of Rho with GST-TRBD in myotubes that is similar to that activation of Rho seen with agrin.

pathways that regulate AChR clustering in response to laminin and agrin converge at the level of activation of the Rho GTPases. Our studies present several novel findings. First we show that laminin activates Cdc42, Rac and Rho in C2 cells and that this activation is required for soluble laminin-induced AChR clustering. Next, our data demonstrate that Rho GTPase activation is required for the formation of substrate laminin-induced complex AChR clusters. Thus, although some aspects of soluble and substrate laminin signaling have been reported to be different, including AChR cluster morphology and the requirement of MuSK (Kummer et al., 2004), our findings show that activation of the Rho GTPases is a common step in both experimental models.

Although most of the data presented here suggest that laminin-induced AChR clustering proceeds through a similar pathway to agrin-induced clustering with regard to the Rho GTPases, several key distinctions between laminin and agrin signaling are observed. We have previously shown that AChR cluster formation in response to agrin proceeds first through the Rac/Cdc42-dependent formation of AChR microclusters (2-5 μm diameter), then through the Rho-dependent condensation of these microclusters into full-sized clusters (15-20 μm diameter) (Weston et al., 2003; Weston et al., 2000). In the case of laminin, we find that when Rho activation is blocked either by expression of dominant negative Rho or by C3 transferase the formation of both full-sized AChR clusters and microclusters is impaired. This finding is in contrast to the effect of Rho blockade in agrin signaling, which results in a decrease in full-sized AChR cluster formation, but an increase in the number of AChR microclusters (Weston et al., 2003). We have shown that when the agrin signal is blocked at the level of Rho, Cdc42 and Rac signaling remains intact. Microaggregates of AChRs continue to form, however, these microclusters are unable to coalesce to larger full-sized clusters without Rho activation (Weston et al., 2003). These data suggest that the ability of agrin to activate Rac and Cd42 is not dependent on Rho activation. Based on our findings that Rho inactivation by RhoN19 or C3 transferase blocks both microcluster and full-sized cluster formation in response to laminin, activation of Rac and Cdc42 by laminin probably requires Rho activation.

Another difference between the agrin and laminin signaling pathways leading to AChR clustering that we have observed is that the ROCK inhibitor Y-27632 impairs laminin-induced AChR clustering by ~40%, whereas it blocks agrin-induced clustering by ~80%, suggesting that laminin-induced AChR clustering may use different effectors of Rho than agrin. Signaling downstream from Rho is mediated by two main effectors, ROCK and mDia1. ROCK has been shown to activate myosin, to cross-link straight anti-parallel actin filaments and to induce focal adhesion formation (Ishizaki et al., 1997; Kimura et al., 1996). mDia1, however, facilitates actin nucleation and polymerization and induces long, straight actin filaments (Higashida et al., 2004; Li and Higgs, 2003). In addition, although ROCK and mDia1 have been shown to have distinct actions, they also can work cooperatively to induce cytoskeletal reorganization (Nakano et al., 1999). A second piece of evidence to suggest that agrin and laminin signal AChR cluster formation through different combinations

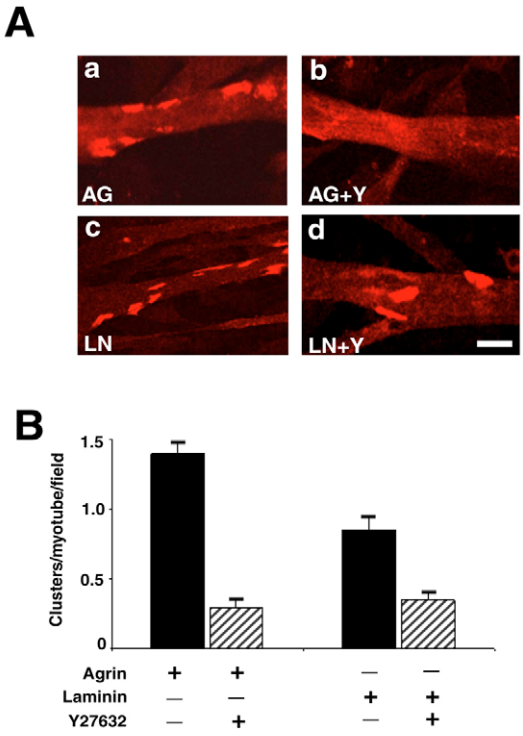

Fig. 8. Inhibition of ROCK impairs laminin-induced AChR clustering. To determine the contribution of ROCK to AChR clustering, the ROCK inhibitor Y27632 was added to differentiated C2 muscle cell cultures at a concentration of 20 μM. One day after treatment, the effect on laminin-induced AChR surface distribution in Y27632-treated and -untreated myotubes was quantitated and compared with the effect of ROCK inhibition on agrin-induced clustering. Inhibition of ROCK activation impaired agrin-induced AChR clustering by more than 80% (b), but only impaired laminin-induced AChR clusters by approximately 40% (d) compared with untreated cells (a,c) (error bars represent ±s.e.m.; *n*=100 cells from five or more separate platings).

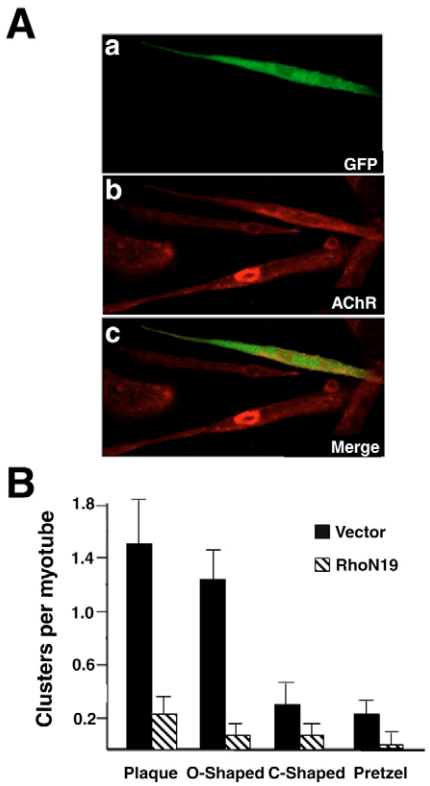

Fig. 9. Inhibition of laminin-induced complex AChR clustering by RhoN19. (A) To determine whether laminin-induced complex AChR cluster formation is dependent on activation of the Rho GTPases, as are soluble laminin-induced AChR clusters, C2 myotubes transfected with GFP-tagged RhoN19 were replated on laminin-coated coverslips. Cultures were surface-labeled with TMR-Bgt and examined at day 4 post-replating by confocal microscopy. Myotubes expressing RhoN19 (a) did not display AChR clusters (b) compared with nontransfected adjacent cells that formed complex clusters. (B) Quantitative comparison of the number of AChR clusters on the surface of transfected myotubes expressing the Rho mutant (hatched bars) versus control myotubes (black bars) clearly documents the inhibiting effect of the dominant interfering RhoN19 on complex AChR cluster formation in response to substrate laminin at all stages of development (error bars represent ±s.e.m.; *n*=40 cells from five separate platings).

of effectors is the finding that although agrin and laminin both activate Rho within 30 minutes, laminin requires at least 12 hours to form AChR clusters whereas agrin can form clusters within 4 hours. The precise downstream effectors of Rho used in the laminin and agrin signaling pathways remain to be determined, but we suggest that laminin and agrin probably have different signaling pathways downstream of the Rho GTPases.

It has been well established that there is crosstalk between Cdc42, Rac and Rho, their upstream activators and their downstream effectors (Bishop and Hall, 2000; Burridge and Wennerberg, 2004; Ridley, 2001; Rottner et al., 1999; Sander et al., 1999). Several previous findings have identified molecules that may play roles in the Rho GTPase signaling pathways that direct AChR clustering, including the finding that Dishevelled interacts with MuSK and with the Rac/Cdc42 effector, p-21 activated kinase (PAK) (Luo et al., 2002). Abl kinases have been shown to phosphorylate MuSK and are required for AChR clustering (Finn et al., 2003). These kinases may signal to the Rho GTPases through the guanine nucleotide exchange factor Trio, an upstream activator of Rac and Rho, that has been shown to be downstream of Abl (Finn et al., 2003; Lanier and Gertler, 2000; Liebl et al., 2000). Future studies should be aimed at identifying the activators and effectors of Cdc42, Rac and Rho that direct AChR clustering and how they influence each other to regulate both when and where AChR clusters form in muscle cells in response to agrin and laminin.

Materials and Methods

Reagents

Expression plasmids encoding wild-type Rac (RacWT), dominant negative Rac (RacN17), constitutively active Rac (RacV12), wild-type Cdc42 (Cdc42WT), dominant negative Cdc42 (Cdc42N17), constitutively active Cdc42 (Cdc42V12), wild-type Rho (RhoWT), dominant negative Rho (RhoN19) and constitutively active Rho (RhoV14) as well as C3 transferase protein were generously provided by D. Bar-Sagi (Stony Brook University, Stony Brook, NY). The GFP-RhoN19 plasmid was provided by M. Frohman and G. Du (Stony Brook University). A plasmid encoding GST fused to the Cdc42/Rac (p-21)-binding domain (GST-PBD) was a gift from J. Brugge (Harvard University, Boston, MA). The plasmid encoding GST fused to the Rho-binding domain (GST-TRBD) from the Rho effector protein

Rhotekin was a gift from M. Schwartz (University of Virginia, Charlottesville, VA). The p160 ROCK inhibitor Y-27632 was purchased from Sigma-Aldrich. Laminin-1 was generously provided by H. Kleinman (NIDCR, Bethesda, MD). cDNA encoding the C-terminal half of agrin was kindly provided by M. Ferns (University of California at Davis, Davis, CA). Soluble recombinant neural agrin was prepared from COS-7 cells transfected with cDNA encoding the C-terminal half of agrin (Ferns et al., 1993), and agrin concentrations were determined by comparison with purified agrin using western blotting.

Cell culture

C2 mouse muscle cells were plated on 12 mm diameter glass coverslips in 35 mm culture dishes for microscopy experiments or in 100 mm culture dishes for biochemical determinations and affinity precipitation assays. The cells were cultured in growth medium consisting of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 5% calf serum and 100 μg/ml penicillin and 100 μg/ml streptomycin (GIBCO) at 37°C with air/5% CO_2. To stimulate muscle differentiation, the growth medium was replaced with differentiation medium consisting of DMEM containing 2% horse serum (GIBCO) and 100 μg/ml each of penicillin and streptomycin two days after plating. Under these conditions the majority of C2 myoblasts fused to form multinucleated myotubes during the subsequent 3 days. To induce clustering of AChRs, cultures were treated with 10 nM soluble laminin or 5 nM soluble agrin, where specified. For the substrate laminin experiments, laminin-coated coverslips were prepared by coating each coverslip with 200 μl of 2 μg/ml polyornithine (Sigma-Aldrich) in distilled water for 30 minutes and allowing them to air dry. Next, a 20 μg/ml solution of laminin in DMEM (Invitrogen) was incubated over polyornithine-coated dishes for 3 hours at 37°C. Differentiated C2 myotubes were then replated onto glass coverslips coated with laminin.

Transfection and microinjection

For experiments utilizing transient transfections, one day post-plating C2 myoblast cultures were transfected with the indicated plasmids at a final concentration of 5 μg of DNA per ml, using Lipofectamine reagent and Plus reagent (Invitrogen). Following 3 hours of incubation, the transfection medium was replaced with differentiation medium for 3 days. For experiments involving substrate laminin, myoblasts cultured on 100 mm tissue culture dishes were transfected one day post-plating with GFP-RhoN19 plasmid at a final concentration of 5 μg of DNA/ml using Lipofectamine reagent. The transfection medium was replaced with differentiation medium for 2-3 days prior to replating the differentiated myotubes on substrate laminin.

For microinjection, after 3 days in differentiation medium, myotubes cultured on gridded coverslips were transferred to DMEM containing 20 μM 2,3-butanedione monoxime to prevent contraction during insertion of microneedles. During a 15-30-minute period a solution containing the indicated plasmid in microinjection buffer [50 mM HEPES (pH 7.2), 100 mM KCl, 5 mM Na_2HPO_4] was microinjected into the cytoplasm of multinucleated myotubes. The cultures were subsequently shifted back to differentiation medium and incubated at 37°C for a further 0.5-1 days. Where indicated, myotubes were treated with agrin or laminin at the concentrations specified during this interval.

Fluorescence microscopy

Cells plated on glass coverslips and subsequently either transfected or microinjected with cDNAs were labeled for 1 hour with 10 nM TMR-α-bungarotoxin (TMR-Bgt, Molecular Probes) in DMEM with 1 mg/ml bovine serum albumin (BSA) for 1 hour at 37°C, rinsed with Dulbecco's PBS and fixed in 3.7% formaldehyde-PBS for 30 minutes in order to visualize surface distribution of AChR. After fixation, cells were permeabilized in 0.2% Triton X-100/PBS at room temperature for 5 minutes, blocked with 10 mg/ml BSA-PBS for 3 minutes, incubated with anti-T7 antibody (Novagen) for 1 hour at 37°C, rinsed with PBS and stained by incubating with an FITC-conjugated goat anti-mouse antibody (Santa Cruz Biotechnology) for 1 hour at 37°C. Cultures that were microinjected with protein were co-injected with FITC goat anti-mouse antibody to identify the injected myotubes. Coverslips were mounted on slides using Aqua-Mount (Lerner Laboratories). Images were acquired using a Nikon PCM 2000 laser-scanning immunofluorescence confocal microscope and associated imaging system, and processed using Adobe Photoshop.

Rho and Rac activity assays

C2 myotubes were treated with 10 nM soluble laminin or 5 nM agrin for 15 minutes and then rinsed with ice-cold Tris-buffered saline (TBS) supplemented with 1 mM $MgCl_2$ and 0.5 mM $CaCl_2$. Cells were then lysed by incubation for 5 minutes on ice with either lysis buffer A [50 mM Tris-HCl, pH 7.4, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1 % sodium dodecyl sulfate (SDS), 500 mM NaCl, 10 mM $MgCl_2$, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM phenylmethylsulphonyl fluoride (PMSF)] or lysis buffer B (50 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 1% NP-40, 10% glycerol, 100 mM NaCl, 1 mM benzamidine, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 μg/ml aprotinin, 0.5% sodium deoxycholate), were centrifuged for 5 minutes at 21,000 ***g*** at 4°C, and the supernatants were used as cell lysates.

To measure Rho activation, an affinity precipitation method was used (Ren et al., 1999; Ren and Schwartz, 2000) in which cell lysates prepared with lysis buffer A were incubated with GST fused to the Rho-binding domain from the effector protein Rhotekin (GST-TRBD) bound to glutathione-coupled sepharose beads for 45 minutes at 4°C. The beads were washed four times with wash buffer (50 mM Tris, pH 7.2, containing 1% Triton X-100, 150 mM NaCl, 10 mM $MgCl_2$, 10 μg/ml each of leupeptin and aprotinin, and 0.1 mM PMSF). Bound Rho proteins were eluted with sample buffer (Laemmli, 1970) and detected by western blotting using a monoclonal antibody against RhoA (Santa Cruz Biotechnology). Western blots were developed using goat anti-mouse antibody coupled to horseradish peroxidase (HRP) and visualized with the enhanced chemiluminescence (ECL) detection system (Pierce Biotech).

In a similar manner, Rac activation was measured by affinity precipitation of cellular GTP-bound forms of Rac (Sander et al., 1998). In this case, cell lysates were prepared with lysis buffer B and incubated with GST fused to the Cdc42/Rac (p-21)-binding domain of PAK bound to glutathione-coupled sepharose beads (GST-PBD) for 30 minutes at 4°C. The fusion protein beads with bound proteins were then washed three times in an excess of lysis buffer, eluted in sample buffer and analyzed by western blotting with a mouse monoclonal antibody against Rac1 (Transduction Labs) or a monoclonal antibody against Cdc42 (9E10) (Santa Cruz Biotechnology). Blots were developed using sheep anti-mouse coupled to HRP and visualized by ECL. Each of the western blots shown here is representative of the findings of at least five separate experiments.

Kinase assays

To assay for JNK activation, C2 myotubes were treated with 10 nM laminin in DMEM at 37°C for the specified intervals. JNK was pulled down using a GST-c-Jun fusion protein (Gupta et al., 1996). The cells were washed with ice-cold PBS, lysed with 1.0 ml of lysis buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin, and 1 mM PMSF) and incubated with 2 μg of GST-c-Jun fusion protein beads (New England Biolabs) at 4°C overnight. The beads were washed twice each with lysis buffer and kinase buffer (25 mM Tris, pH 7.5, 5 mM β-glycerophosphate, 2 mM dithiothreitol (DTT), 0.1 mM Na_3VO_4, and 1 μg/ml leupeptin). The JNK activity present in the immunoprecipitate was determined by resuspension in 50 μl of kinase buffer supplemented with 100 μM adenosine 5′-triphosphate (ATP). After 30 minutes at 30°C, the reactions were terminated using 3× sample buffer. The proteins were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on 10% acrylamide gels and then transferred to nitrocellulose for western blotting. The membranes were immunoblotted with anti-phospho-c-Jun (Ser63) antibody (New England Biolabs). Immunocomplexes were visualized by ECL, and signals were quantitated using a BioRad imaging densitometer with Molecular Analyst software.

For the JNK assays using Rac- and Cdc42-transfected cells, C2C12 cells were cotransfected using Lipofectamine reagent with FLAG epitope-tagged JNK1 and either RacV12, RacN17, Cdc42V12 or Cdc42N17, and, after 2 days in differentiation medium, were treated with 10 nM laminin in DMEM at 37°C for varying amounts of time. The cells were then harvested and the transfected JNK was immunoprecipitated as described (Nimnual et al., 1998). The immunocomplexes were incubated in 50 μl of kinase buffer containing 10 μCi of [γ-^{32}P]ATP (7000 Ci/mmol) (ICN Biomedicals) and 3 μg GST-c-Jun as a substrate. After 30 minutes at 30°C, the reactions were terminated with 3× sample buffer. After fractionation by SDS-PAGE, GST-c-Jun phosphorylation was measured by autoradiography and quantitated using a Phosphorimager. Background levels of transfected Rac or Cdc42 and JNK expression were determined by western blotting and incubation with anti-T7 (Novagen), anti-myc or anti-JNK1 (Santa Cruz Biotechnology) primary antibodies. Immunocomplexes were visualized by ECL.

Rho pathway inhibitors

Differentiated C2 cells were treated with soluble C3 exotransferase from *Clostridium botulinum* (Aktories et al., 1989; Sekine et al., 1989; Wilde and Aktories, 2001) or the p160 ROCK inhibitor Y27632 (Ishizaki et al., 2000; Uehata et al., 1997) at varying time points and were monitored for AChR cluster formation in response to laminin or agrin. C3-transferase was applied to C2 myotubes at a concentration of 50 μg/ml and preincubated for 2 hours at 37°C prior to laminin or agrin treatment. The cultures were then incubated for an additional 8 hours in the presence of both C3-transferase and agrin or laminin followed by TMR-Bgt staining to visualize AChR clusters. For Y-27632, myotubes were treated with Y-27632 at a concentration of 20 μM and the cells were incubated at 37°C for 2 hours prior to addition of laminin. The effect of Y-27632 on agrin-induced AChR clustering was measured by labeling AChR clusters with TMR-Bgt.

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