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Involvement of p120 Catenin in Myopodial Assembly and Nerve–Muscle Synapse Formation

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ABSTRACT: **At developing neuromuscular junctions (NMJs), muscles initially contact motor axons by microprocesses, or myopodia, which are induced by nerves and nerve-secreted agrin, but it is unclear how myopodia are assembled and how they influence synaptic differentiation at the NMJ. Here, we report that treatment of cultured muscle cells with agrin transiently depleted p120 catenin (p120ctn) from cadherin junctions *in situ*, and increased the tyrosine phosphorylation and decreased the cadherin-association of p120ctn in cell extracts. Whereas ectopic expression of wild-type p120ctn in muscle generated myopodia in the absence of agrin, expression of a specific dominant-negative mutant form of p120ctn, which blocks filopodial assembly in nonmuscle cells, suppressed nerve- and agrin-induction of myopodia. Significantly, approaching neurites triggered reduced acetylcholine receptor (AChR) clustering along the edges of muscle cells expressing mutant p120ctn than of control cells, although the ability of the mutant cells to cluster AChRs was itself normal. Our results indicate a novel role of p120ctn in agrin-induced myopodial assembly and suggest that myopodia increase muscle–nerve contacts and muscle's access to neural agrin to promote NMJ formation.** © 2006 Wiley Periodicals, Inc. J Neurobiol 66: 1511–1527, 2006

Keywords: **NMJ; agrin; p120 catenin; myopodia; AChR**

INTRODUCTION

At vertebrate neuromuscular junctions (NMJs), acetylcholine (ACh) released by motor nerve termini activates muscle ACh receptors (AChRs) to trigger muscle contraction. At mature NMJs, AChRs are highly concentrated, to a density of $\sim$10,000/μm^2 (Fertuck and Salpeter, 1976), which ensures a muscle response to every nerve stimulation. A major goal of studies on NMJ development is therefore to uncover the molecular details of synaptic AChR clustering. Currently, it is widely accepted that AChR clustering in muscle is triggered by the activation of the receptor tyrosine kinase MuSK (muscle-specific kinase), which is stimulated by a nerve-secreted factor named agrin, and that it is mediated by the AChR-binding protein rapsyn and the assembly of a cortical actin scaffold in muscle (Glass and Yancopoulos, 1997; Sanes and Lichtman, 1999; Madhavan and Peng, 2003). During embryonic development, AChR clusters form in the central regions of muscle fibers before innervation in a MuSK- and rapsyn-dependent manner (Lin et al., 2001; Yang et al., 2001) and, during innervation, nerve-secreted agrin and ACh promote synaptic aggregation of AChRs and the dispersal of aneural AChR clusters (Lin et al., 2005; Misgeld et al., 2005; Madhavan and Peng, 2005a).

Compared with the process of AChR clustering, less is known about how nerve-muscle contacts are first established during NMJ formation. Recent stud-

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ies, however, have revealed that nerves induce filopodia-like microprocesses in cultured rodent muscle cells that make close contacts with innervating motor axons (Uhm et al., 2001). Such microprocesses, which have also been observed in embryonic mouse muscle (Misgeld et al., 2002), were first identified at NMJs in Drosophila and referred to as myopodia (Ritzenthaler et al., 2000). Interestingly, in rodent muscle cultures, myopodia are induced by the AChR-clustering factor agrin (Uhm et al., 2001), but how agrin signaling in muscle leads to myopodial assembly and how muscle–nerve interactions mediated by myopodia affect functional synaptic differentiation at the NMJ have remained unknown.

Interactions between cells are mediated to a large extent by specialized proteins called cell adhesion molecules. Important among this class of molecules are the transmembrane proteins named cadherins that associate with each other extracellularly to promote cell–cell adhesion (Yagi and Takeichi, 2000). Intracellularly, cadherins bind to cytoplasmic proteins called catenins that stabilize the cadherin junctions by providing links to the cortical actin cytoskeleton (Gumbiner, 2000). The catenins, in particular β-catenin and p120 catenin (p120ctn), also transduce signals that effect cytoskeletal and transcriptional changes (Peifer and Polakis, 2000; Anastasiadis and Reynolds, 2001). In the central nervous system, cadherins and catenins influence the connectivity and plasticity of synapses (Bruses, 2000; Yagi and Takeichi, 2000; Goda, 2002) and in the peripheral nervous system they are expressed by motor nerves and muscle (Cifuentes-Diaz et al., 1998; Padilla et al., 1998; Marthiens et al., 2002). In light of these observations, we investigated potential involvement of cadherin complex proteins in myopodial induction and NMJ development using biochemical, molecular, and cell biological assays on nerve and muscle primary cultures and cell lines. Our results indicate a novel role of muscle p120ctn in mediating agrin-dependent myopodial assembly and further suggest that myopodia not only increase muscle–nerve interactions but also enhance muscle's access to nerve-secreted agrin and thereby promote NMJ formation.

METHODS

Reagents

The agrin used in experiments described here was obtained from the conditioned medium of HEK293 cells transiently transfecting with a plasmid encoding full-length chick neural agrin (Daggett et al., 1996; Madhavan et al., 2005b). For immunolabeling p120ctn in *Xenopus* muscle cultures, we used the rabbit polyclonal antibody pAbF1 (Reynolds et al., 1996), and for labeling cadherin in these cells, we used an anti-EP-cadherin antiserum (a gift from Dr. Benjamin Geiger, Weizmann Institute of Science, Rehovot, Israel). Commercially available reagents used in this study were as follows: FITC-conjugated *Vicia villosa* isolectin B4 (VVAB4) and rhodamine-conjugated concanavalin A (con A) (Vector Laboratories, Burlingame, CA); monoclonal antiphosphotyrosine antibody mAb4G10 (Upstate Biotechnology, Lake Placid, NY); monoclonal antibodies against the tyrosine phosphatase Shp2, p120ctn, and β-/γ-catenins (BD Transduction Laboratories, San Diego, CA); monoclonal anti-β-dystroglycan (β-DG) antibody (Novo Castra Laboratories, New Castle, UK); rabbit polyclonal pan-cadherin antibody (Zymed Laboratories, South San Francisco, CA); rabbit polyclonal anticalcineurin antibody (Santa Cruz Biotechnology, Santa Cruz, CA); rhodamine-conjugated α-bungarotoxin (R-BTX) and phalloidin (Molecular Probes, Eugene, OR); FITC-conjugated anti-mouse and anti-rabbit secondary antibodies (Organo-Teknika-Cappel, Durham, NC, or Zymed Laboratories); horse radish peroxidase (HRP)-conjugated secondary antibodies (Jackson Immuno Research Laboratories, West Grove, PA); Protein A/G agarose (Santa Cruz Biotechnology); and Triton X-100 (Pierce Chemical Company, Rockford, IL).

Ectopic Expression of p120ctn

The p120ctn proteins used in ectopic expression studies were generated with carboxy-terminal green fluorescent protein (GFP) tags. GFP sequences were spliced onto the cDNA of either wild-type mouse p120ctn (a gift from Dr. Keith Burridge, Univ. of North Carolina at Chapel Hill) (Reynolds et al., 1992) or onto cDNAs of two mutant p120ctn isoforms, one lacking rho GTPase-regulating sequences (Δ-p120ctn) (Anastasiadis et al., 2000) and the other missing eight src tyrosine kinase phosphorylation sites (8YF-p120ctn) (Mariner et al., 2001). The resulting constructs or a GFP construct were subcloned into the pCS2+ vector for synthesizing mRNAs. The pCS2+ vectors containing these inserts were linearized and mRNAs were produced from them using SP6 polymerase with the mMESSAGE mMACHINE kit (Ambion, Austin, TX); mRNAs were injected into 2–4 cell stage *Xenopus* embryos, using a Drummond Nanojet oocyte injector (Drummond Scientific Co., Broomall, PA).

Xenopus Cell Cultures, Agrin-Treatment, and Labeling Experiments

All *in situ* labeling and ectopic expression experiments used primary cultures of *Xenopus* nerve and muscle cells, following procedures described previously (Peng et al., 1991). Briefly, myotomal muscle cells were cultured from wild-type or p120ctn/GFP-expressing *Xenopus* embryos at stage 20–22 and plated on glass cover slips coated with entactin-collagen IV-laminin (E-C-L) substrate (Upstate Biotechnol-

ogy). For nerve–muscle cocultures, spinal nerves were seeded onto muscle cells (plated 3–5 days earlier) and after 1 day, the cells were labeled with R-BTX and examined in the living state. Agrin-treatment was on muscle cells prelabeled with R-BTX or on unlabeled cells that were stained with FITC-VVAB4 or rhodamine-Con A before fixation; following 95% ethanol fixation, cells were labeled with rhodamine-phalloidin or with various primary antibodies and then secondary antibodies conjugated to fluorophores.

In ectopic expression studies of p120ctn/GFP, muscle cells were either treated with agrin or cocultured with nerves and then examined for myopodia, green fluorescence, and R-BTX labeling. For each culture preparation, the percentage of fluorescent cells with myopodia was calculated from experiments with duplicate samples, and each of the mRNAs was used in multiple (6 or more) separate injections and culture preparations; for counting, the cells scored varied no more than by a factor of $\sim$2 to 3 in their overall fluorescence intensity and this allowed us to compare cells with similar levels of exogenous proteins. In nerve–muscle cocultures, regions of muscle cells near neurites were identified and myopodia and AChR microclusters present within them were counted; from this, the myopodial and AChR cluster densities were calculated. For quantification, data were pooled from experiments and statistical analyses (*t* tests) were carried out using SPSS software.

Images of cells were captured using a Leitz Orthoplan or an Olympus IX70 Microscope with attached Hamamatsu ORCA II cooled-CCD or ORCA ER cameras controlled by Metamorph software (Universal Imaging, West Chester, PA), and composite figures were generated using Adobe Photoshop (San Jose, CA).

C2 Myotubes, Extract Preparation, Immunoprecipitation, and Immunoblotting

Cultured C2 mouse myotubes used for biochemical experiments were prepared as follows: myoblasts were grown to confluence in 6–10 cm culture dishes with Dulbecco's modified Eagle medium (DMEM) containing 20% fetal bovine serum (growth medium) and, to generate myotubes, the growth medium was replaced with DMEM containing 2% horse serum (differentiation medium); all assays used cells that had been maintained in the differentiation medium for 4–5 days. Myotubes were treated without or with agrin for different times at 37°C in the presence of a low concentration (10 μM) of Na-pervanadate, included to reduce tyrosine phosphatase activity; pervanadate (labeled ''P'' in figure legend where present) was prepared as described (Dai and Peng, 1998). After rinsing with phosphate-buffered saline, cells were scraped into a TX-100 extraction buffer (100 m*M* Tris, pH 7.4, 150 m*M* NaCl, 5 m*M* EDTA, 1% TX-100, and 1 m*M* pervanadate) and incubated on ice for 30 min with frequent mixing. Lysates clarified by centrifugation were either mixed with an SDS-sample buffer or used in immunoprecipitation experiments.

For immunoprecipitations, 1–2 μg antibodies were added to 0.55 or 0.95 mL extracts obtained from 6 or 10 cm dishes. Protein A/G agarose slurry (25–50 μL) was added and samples were mixed at room temp for 2 h. The agarose beads were spun down, washed with Tris buffered saline containing 0.1% TX-100, and eluted with SDS-sample buffer. For immunoprecipitations with monoclonal antibodies against p120ctn and β-catenin, an anti-Shp2 monoclonal antibody was used in parallel as a control, and for those with the pan-cadherin rabbit polyclonal antibody, an anti-calcineurin polyclonal antibody was used. Association between p120ctn or β-catenin and cadherin was analyzed by immunoprecipitations with the catenin antibodies because the anticadherin antibody used here was raised against conserved cytoplasmic sequences of cadherin; using it we were unable to cocapture either p120ctn or β-catenin with cadherin from cell extracts. Immunoprecipitates were resolved by electrophoresis (8% polyacrylamide gels) and transferred to PVDF membranes (Immobilon P; Millipore Corporation, Bedford, MA), which were blocked overnight with 5% nonfat milk in Tris buffered saline containing 0.1% Tween-20 (Milk-TBST). Primary antibodies used for immunoblotting were diluted to $\sim$0.1 μg/mL in Milk-TBST and protein bands bound by these antibodies were detected using HRP-conjugated secondary antibodies and enhanced chemiluminescence (West Pico; Pierce).

RESULTS

Nerve- and Agrin-Induction of Myopodia

To study myopodial assembly during NMJ formation, we used live cocultures of spinal nerves and myotomal muscle cells obtained from *Xenopus* embryos. In these cultures, myopodia developed along the edges of muscle cells approached by neurites [Fig. 1(A–C)], ranged in length from one to several micrometer, and often directly contacted neuritic processes. Labeling of these cultures with R-BTX revealed the presence of AChR microclusters in muscle close to or even within the myopodia [Fig. 1(C,D)], indicating that nerves induced postsynaptic differentiation at these sites.

Because the time at which growing neurites reach muscle cells in culture cannot be controlled, we treated muscle cells with agrin and labeled them with the antiphosphotyrosine antibody mAb4G10 and the lectin Con A, which allowed myopodia to be readily visualized [Fig. 2(A,B)]; separately, staining of these cells with fluorescent-phalloidin confirmed that myopodia were F-actin-containing protrusions (R.M. and H.B.P., unpublished observations). Our initial experiments showed that myopodia were robustly induced along the muscle cell periphery 2–4 h after the addition of agrin but to a lesser extent at later times, in accordance with previous results (Uhm et al., 2001); therefore, in experiments described below, myopodia

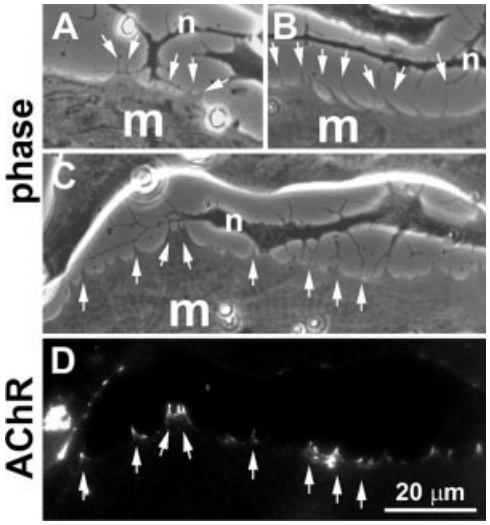

Figure 1 Myopodia in *Xenopus* nerve–muscle cocultures. Cocultures of spinal neurons and myotomal muscle cells derived from *Xenopus* embryos were examined for myopodia by phase-contrast microscopy (A–C). Myopodia (arrows) were detected along the edges of muscle cells (''m'') close to nerves (''n'') or neuritic processes. Clustering of muscle AChRs was visualized using rhodamine-conjugated α-bungarotoxin (R-BTX) (D).

were examined in cells treated with agrin for 2 h unless specified otherwise. We focused on the muscle periphery because in these cultures myopodia forming on the topside of cells could not be clearly seen, and we classified a cell as one with myopodia if it possessed at least two separate myopodia; because the sizes of individual muscle cells in our primary cultures varied significantly and because the formation of myopodia was transient, determining the percentage of cells with myopodia was a reliable way to estimate agrin's effect on the cells. Our results showed that myopodia were detected in only $\sim$10% of quiescent muscle cells along the edges (three experiments, $n = 405$), whereas they were present in $>$50% of the cells after agrin-treatment ($n = 385$), demonstrating that agrin markedly enhanced myopodial assembly in our *Xenopus* muscle cultures, much as in rodent muscle cultures (Uhm et al., 2001).

After agrin-treatment microclusters of AChRs, ranging in diameter from $\sim$1 to 3 μm, also developed in the muscle cells as expected [Fig. 2(C–H)], and, intriguingly, these clusters preferentially localized near myopodia (thin arrows) and occasionally were detectable within them, and areas of cells lacking AChR microclusters were mostly devoid of myopodia (thick arrows). We analyzed data pooled from six experiments and found that along the edges of muscle cells, 83.3% of the myopodia detected ($n = 736$) were within 2.5 μm of agrin-induced AChR microclusters and 80.1% of the AChR microclusters identified ($n = 744$) were within 2.5 μm from myopodia. When regions along muscle edges were selected randomly and all the myopodia and AChR microclusters in them were counted, increased myopodial formation was found to strongly correlate with enhanced AChR clustering (panel I). These observations raise the possibility that common effectors participate in the signaling pathways initiated by agrin that lead to the formation of myopodia and AChR clusters, but the reason for the close spatial relationship between myopodia and AChR clusters remains unknown.

Myopodia and Muscle Cell Cadherin Junctions

Myopodia that developed in our muscle cultures resembled filopodia that, in other cells, initiate adhesive contacts with neighboring cells. This led us to characterize myopodia by labeling with lectins and antibodies against cell surface and adhesion molecules. Agrin generated myopodia along muscle cell edges and contacts between them and neighboring muscle cells [Fig. 3(A–C)] were intensely stained by the *N*-acetylgalactosamine-specific lectin *Vicia villosa* agglutinin B4 (VVAB4); other muscle–muscle junctions were also strongly labeled by this lectin but the general muscle sarcolemma, contacts between muscle membrane and the E-C-L cover slip coating, and regions of myopodia that did not touch neighboring cells were not [Fig. 3(A–C)]. Labeling by VVAB4 was specific: it was abolished in the presence of excess nonfluorescent VVAB4; another *N*-acetylgalactosamine-binding lectin, soybean agglutinin, gave VVAB4-like staining; and preferential staining of myopodial or muscle contacts was not observed with the mannose-binding lectin, Con A (data not shown). These results suggested that VVAB4 receptor-enriched adhesion complexes are recruited to myopodia-muscle contacts, consistent with the soybean agglutinin staining previously demonstrated at newly formed *Xenopus* myotomal cell junctions (Chow and Poo, 1982).

Upon testing a panel of antibodies, we next found that an antibody against *Xenopus* EP-cadherin, like VVAB4, strongly labeled cell–cell junctions in quiescent muscle [Fig. 3(D,E)]. Similar labeling of muscle junctions was also observed with an antibody against the cadherin complex component p120ctn (see below). The staining obtained with these antibodies was not detected when the fluorescent secondary antibody was used with unrelated primary antibodies or

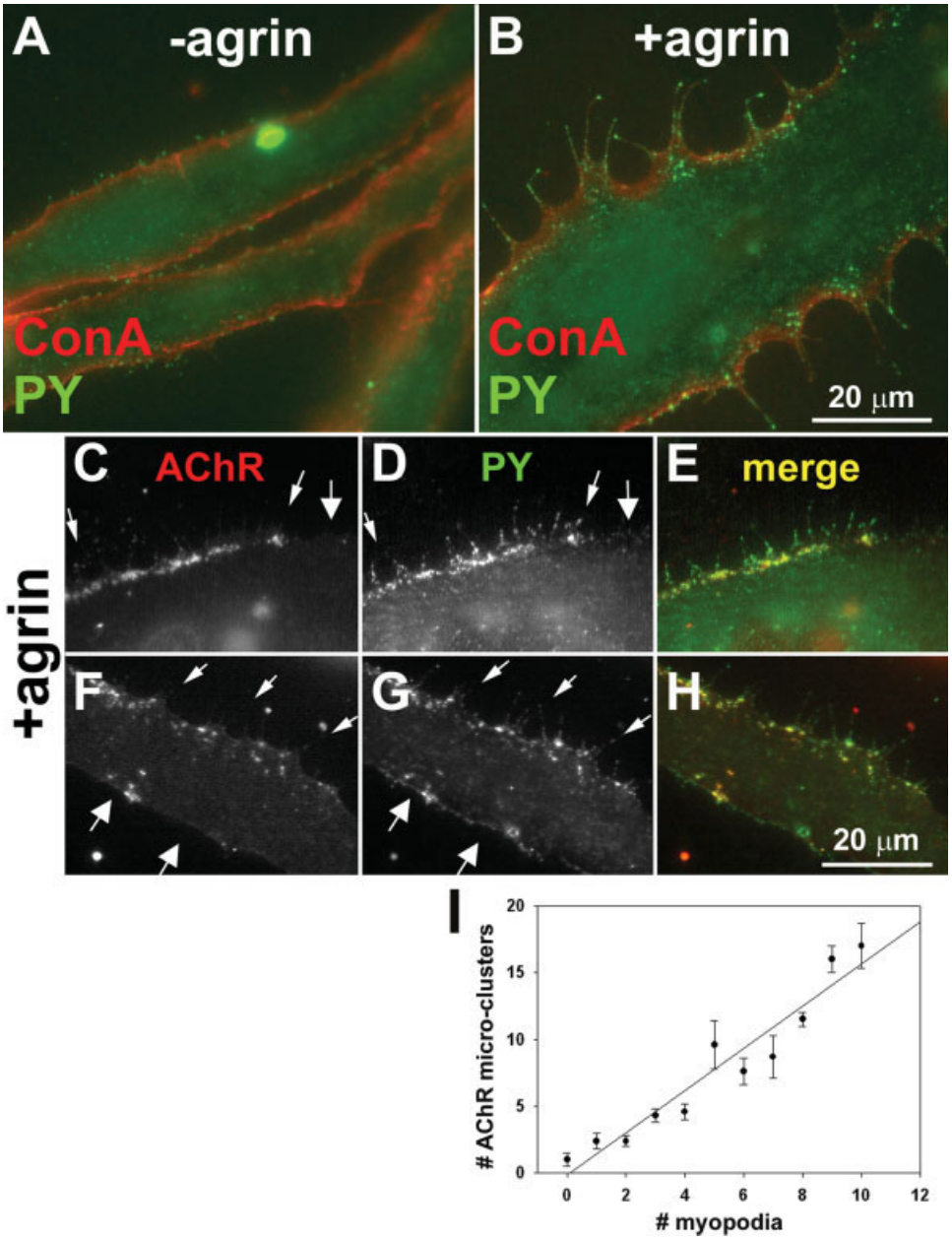

Figure 2 Agrin induction of myopodia and AChR microclusters. *Xenopus* muscle cells were treated without (A) or with (B) agrin for 2 h and stained with rhodamine-conjugated con A and a monoclonal antibody against phosphotyrosine (mAb4G10), followed by FITC-conjugated anti-mouse secondary antibody. In quiescent *Xenopus* muscle cells, myopodia were seldom detected along the edges (A), but they were observed in agrin-treated cells (B). In the agrin-treated cells, AChR microclusters (C, F) were found preferentially localized near myopodia, visualized by anti-phosphotyrosine antibody labeling (D, G; thin arrows), and regions of cells lacking AChR clusters were mostly devoid of myopodia (thick arrows). Both AChR microclusters and myopodia were strongly labeled by the antiphosphotyrosine antibody (merged images; E, H). (I) This graph shows the correlation between agrin-induced myopodial formation and AChR clustering. 40×10 μm^2 regions were randomly selected along the edges of agrin-treated muscle cells (at least 2 per cell) and all myopodia and AChR microclusters within them were counted. A total of 66 regions were examined which contained 0–14 myopodia ($n = 313$) and 0–27 AChR microclusters ($n = 464$); linear regression, $r^2 = 0.92$.

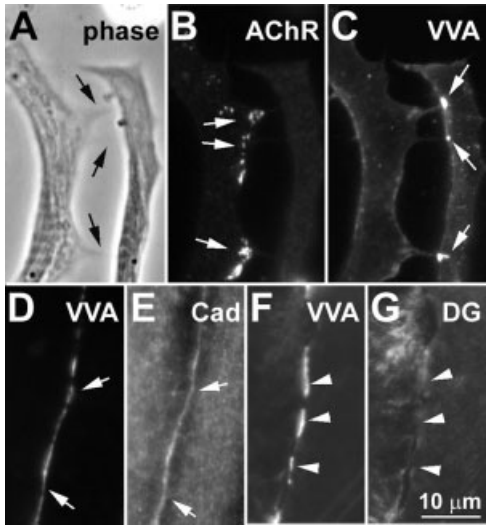

Figure 3 VVAB4 labeling of myopodial and muscle junctions. R-BTX-labeled *Xenopus* muscle cells were treated with agrin (2 h) and stained with FITC-conjugated VVAB4 (A–C). Agrin induced AChR microclusters and myopodia and, in this example myopodia from the muscle cell on the left contacted the cell on the right. Contacts between the myopodia and the neighboring muscle were strongly labeled by VVAB4 (C, arrows) but the general muscle membrane and regions of myopodia not touching the neighboring cell were not. VVAB4 stained all cell–cell junctions between quiescent muscle cells (D, F), and these sites were strongly labeled by an antibody against EP-cadherin (E, arrows) but not β-dystroglycan (G, arrowheads).

applied alone (data not shown). Moreover, a monoclonal antibody against β-dystroglycan, a subunit of the dystroglycan complex that can bind VVAB4 in vertebrate tissues (McDearmon et al., 2001), labeled the sarcolemma but did not preferentially stain muscle–muscle contacts [Fig. 3(F,G)]. These results supported the conclusion that muscle junctions labeled by VVAB4 contained cadherin complexes.

Effect of Agrin Signaling on Muscle p120ctn

Cadherin complex proteins promote the development of cell junctions and also actively participate in intracellular signaling. Of particular interest to us was p120ctn, which, in addition to regulating the adhesiveness of cadherin complexes, induces motile processes by modulating the activity of rho-family small GTPases: when dissociated from cadherin, p120ctn can generate ''dendritic'' protrusions in cells by inhibiting rho A and activating rac and cdc42 through guanine nucleotide exchange factors (GEFs) (Anastasiadis and Reynolds, 2001). We asked whether agrin signaling affects p120ctn by examining the cellular distribution of p120ctn relative to cadherin, focusing on cell–cell junctions where cadherin and p120ctn proteins were most clearly concentrated. Our results showed that p120ctn was enriched at >90% of the cell junctions of quiescent muscle [Fig. 4(A)] (n = 179 cells, >3 contacts/cell) but that it was depleted from nearly 80% of such sites after 1 h agrin-treatment [Fig. 4(B)] (n = 171). During agrin exposure, the muscle cells remained in physical contact and the anti-EP-cadherin antibody labeled the junctions between them [Fig. 4(C)]. Furthermore, loss of p120ctn from muscle contacts was reversible as junctional labeling for p120ctn was detected in cells treated with agrin for 4 h [Fig. 4(D)] or overnight (data not shown). Lastly, agrin addition redistributed p120ctn in muscle cells but not in nonmuscle cells derived from *Xenopus* embryos [Fig. 4(E,F)]. In above experiments, labeling with R-BTx demonstrated that agrin also stimulated muscle AChR clustering (data not shown).

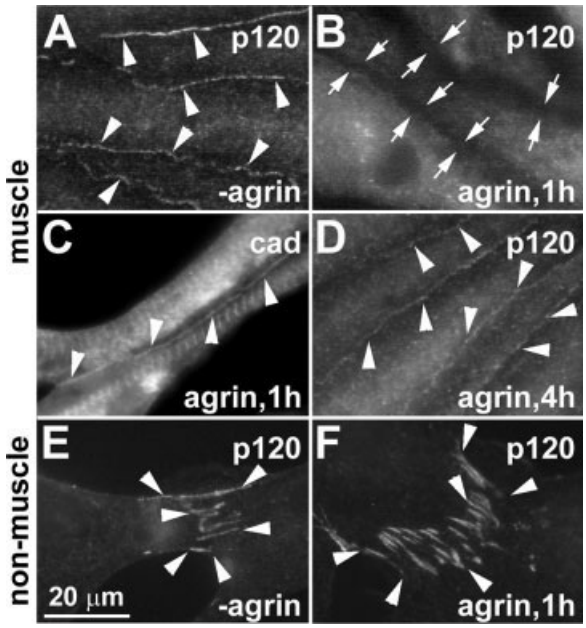

Figure 4 Localization and agrin-induced redistribution of p120ctn *in situ*. Labeling of *Xenopus* muscle (A–D) by anti-p120ctn and anti-EP-cadherin antibodies was examined in the presence or absence of agrin. In untreated cells, p120ctn was concentrated at cell–cell contacts (A; arrowheads), but it was depleted from these sites in cells exposed to agrin for 1 h (B; arrows). EP-cadherin was concentrated at muscle–muscle junctions (arrowheads) after 1 h agrin-treatment (C), and so was p120ctn in muscle cells exposed to agrin for 4 h (D). Agrin-treatment did not affect the junctional localization of p120ctn in nonmuscle cells (E, F; arrowheads).

Because binding to cadherin is critical for the localization of p120ctn at cell junctions (Thoreson et al., 2000), agrin-induced redistribution of p120ctn in muscle suggested that agrin signaling regulates the association between p120ctn and cadherin. We sought independent support for this by carrying out biochemical studies with cultured C2 mouse myotubes. These cells were chosen because the *Xenopus* muscle primary cultures, although well suited for *in situ* studies, provided insufficient material for biochemical assays. C2 myotubes were treated with or without agrin, and cell extracts prepared from them were immunoblotted with different antibodies. Agrin-treatment for 1 h did not change the amounts of p120ctn, cadherin, Shp2 [Fig. 5(A)], or β- and γ-catenin (not shown) in myotube extracts, making it unlikely that agrin rapidly alters the expression of these proteins. Addition of agrin, however, significantly increased the tyrosine phosphorylation of p120ctn in C2 myotubes, as shown by staining of p120ctn immunoprecipitated from these extracts [Fig. 5(B); top] by the antiphosphotyrosine antibody (bottom; lane 4 vs. 2). Anti-p120ctn and the control anti-Shp2 mouse monoclonal antibodies specifically captured their respective targets (top) and increased tyrosine phosphorylation of Shp2 was not detected after agrin-treatment (bottom). Under identical experimental conditions, agrin induced tyrosine phosphorylation and activation of its functional receptor MuSK (Madhavan et al., 2005b).

Tyrosine phosphorylation of p120ctn was characterized further using p120ctn immunoprecipitated from extracts of myotubes treated with agrin for different times [Fig. 5(C)]. Equal amounts of p120ctn were loaded (upper blot) and staining with antiphosphotyrosine antibody (middle blot) demonstrated that maximal p120ctn tyrosine phosphorylation occurred after 1 h agrin-treatment. Significantly, staining with the anticadherin antibody (lower blot) showed that the amount of cadherin associated with p120ctn was reduced at this time point compared with shorter or longer times (bottom; relative band intensity graph). Using pooled band intensity data from four separate experiments, we found that 1 h agrin-treatment nearly halved the amount of cadherin present in p120ctn immunoprecipitates relative to controls [Fig. 5(D)]. Other assays confirmed that cadherin specifically co-immunoprecipitated with p120ctn, as cadherin was not detected in immunoprecipitates of control proteins such as Shp2 [Fig. 5(E)], and that agrin-treatment did not significantly influence the co-immunoprecipitation of cadherin with either β-catenin [Fig. 5(E), top blot] or γ-catenin (bottom blot).

The above *in situ* and biochemical results showing that agrin increases muscle p120ctn's tyrosine phosphorylation and decreases its cadherin-association provided first evidence that muscle p120ctn is a downstream target of agrin signaling. These findings raised the possibility that ''cadherin-free'' p120ctn generated by agrin signaling mediates myopodial assembly, which was tested as described below.

Involvement of p120ctn in the Formation of Myopodia

To examine p120ctn's role in myopodial formation, we expressed exogenous p120ctn in *Xenopus* muscle cells to elevate the cadherin-free levels of p120ctn in cells. For this, we once again used primary muscle cell cultures, derived from *Xenopus* embryos injected with mRNAs encoding wild-type p120ctn tagged with GFP, or GFP alone as a control. In muscle cells expressing p120ctn-GFP, green fluorescence was observed at the muscle membrane and in the cell interior [Fig. 6(A-E)], and these cells frequently contained large, elongated protrusions (often several tens of μm in length) resembling ''dendritic'' structures [Fig. 6(A)] and the smaller myopodia [Fig. 6(B)] (see below for quantification). Moreover, p120ctn-GFP was concentrated at myopodial and muscle and muscle-muscle contacts [Fig. 6(C)], indicating recruitment of cadherin complexes to newly formed myopodial junctions. In contrast to these findings, few dendritic structures or myopodia were detected in cells expressing GFP only and GFP was not recruited to cell–cell contacts [Fig. 6(F)]. In muscle cells expressing p120ctn-GFP, spontaneously occurring AChR clusters appeared normal as did AChR clusters induced by overnight agrin-treatment [Fig. 6(D,E)], much as in cells expressing GFP only (not shown).

The above results showing that over-expression of p120ctn was sufficient for generating myopodia led us to test if p120ctn mediates agrin-induction of myopodia using dominant-negative interference assays. Here, we also asked what if any role p120ctn tyrosine phosphorylation plays in myopodial assembly. We ectopically expressed two different mutant forms of p120ctn in muscle cells: in one of the mutant proteins, Δ-p120ctn, sequences important for regulating rho GTPase were deleted and this protein blocks the formation of motile processes in nonmuscle cells (Anastasiadis et al., 2000); in the other mutant protein, 8YF-p120ctn, eight phosphorylation sites used by src tyrosine kinase were eliminated by point mutations (Mariner et al., 2001). Muscle cells were cultured from *Xenopus* embryos injected with mRNAs

encoding wild-type or mutant p120ctn proteins tagged with GFP, or GFP alone, and these were examined in the living state following treatment with or without agrin for 2 h. Myopodia were detected in few muscle cells expressing GFP before agrin-treatment [Fig. 7(A)] but after agrin-treatment they were observed in >60% of these cells (panel B; see panel I for quantification). In p120ctn-GFP-expressing muscle cells [Fig. 7(C,D)], myopodia were present in the absence of agrin-stimulation to a similar extent as in agrin-treated GFP-expressing cells, and addition of agrin further increased the fraction of cells with myopodia. In striking contrast, normal myopodia were rarely detected in muscle cells expressing Δ-p120ctn-GFP and agrin failed to induce myopodia in these cells [Fig. 7(E,F)]; even counting minute (~ 1 μm) membrane protrusions as myopodia, few Δ-p120ctn-GFP cells were found to contain such structures

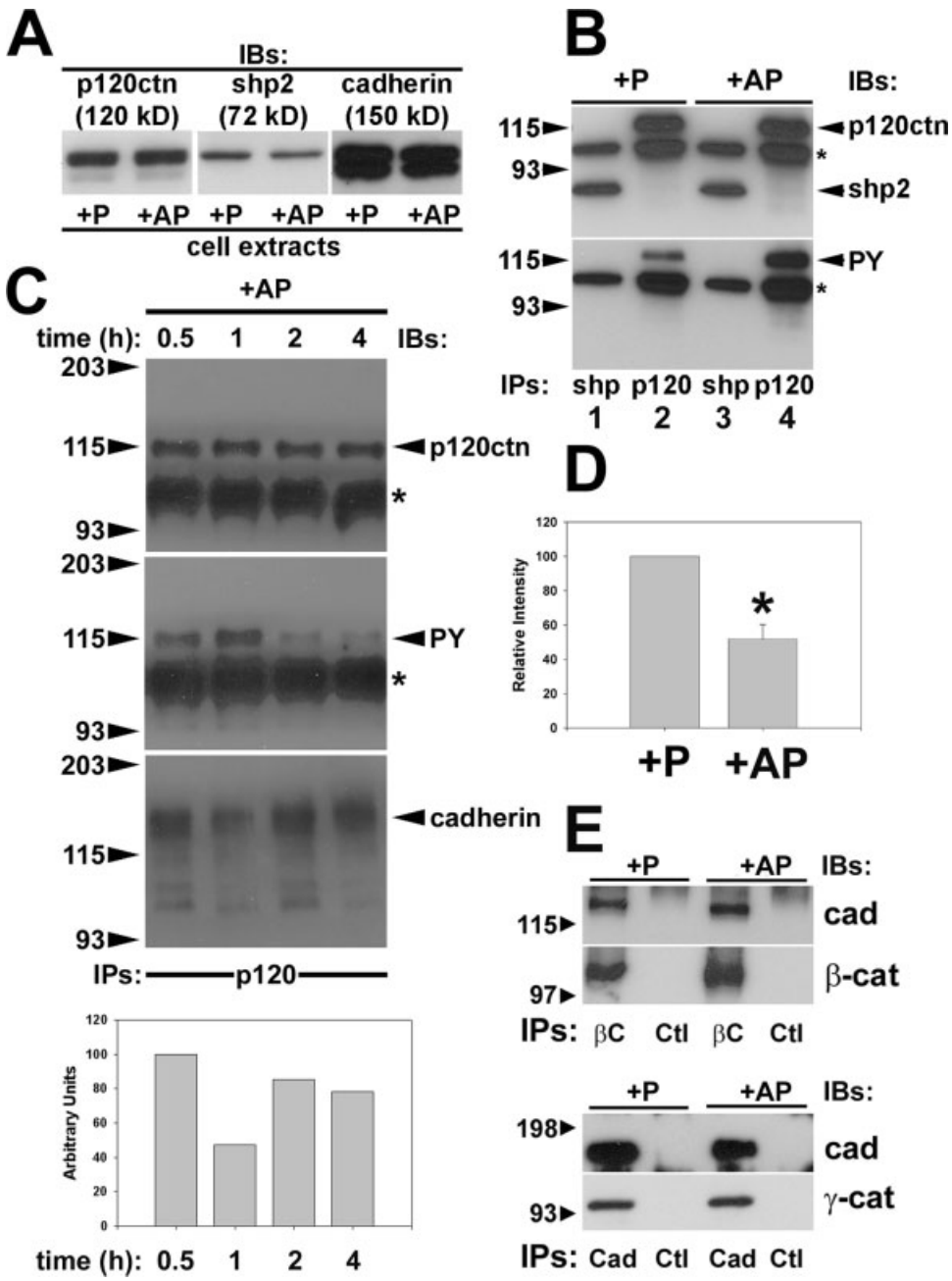

Figure 5

(panel I). Lastly, myopodia were present in quiescent muscle cells expressing 8YF-p120ctn-GFP, but in these cells agrin-treatment did not enhance myopodial formation [Fig. 7(G,H)]. Data pooled and quantified from multiple mRNA injections and culture preparations for GFP and wild-type and mutant p120ctn proteins are presented in Figure 7(I). Taken together, these results support the conclusions that agrin increases src-dependent tyrosine phosphorylation of p120ctn to enhance cadherin-free levels of p120ctn and that a p120ctn-dependent signaling pathway, possibly involving rho GTPases, mediates agrin-induction of myopodia.

Myopodia and Nerve-Muscle Synapse Formation

What is the role of p120ctn-dependent myopodial assembly in NMJ development? To address this question, we investigated how p120ctn signaling affects nerve-induction of myopodia and how myopodia influence synaptic differentiation in nerve–muscle cocultures. Spinal neurons derived from normal *Xenopus* embryos were cultured with muscle cells derived from embryos injected with GFP, wild-type p120ctn-GFP or Δ-p120ctn-GFP; the p120ctn deletion-mutant was chosen because it effectively blocked agrin-induction of myopodia (see Fig. 7). In live cocultures, we identified edges of muscle cells that were approached by growing neurites or neuritic processes (to within ~ 10 μm) but not stably contacted by them and we classified these sites as ''partial'' nerve–muscle contacts. We also identified those edges of muscle cells with which neurites made stable physical contacts and classified them as regions of ''overlap''. To evaluate NMJ formation at these two types of nerve–muscle contact, we examined AChR clustering occurring at these sites. The results of these studies are described below.

At partial nerve–muscle contacts, several myopodia were detected in muscle cells expressing wild-type p120ctn-GFP [Fig. 8(A,B)], or GFP only (not shown), but few myopodia were observed at such sites in muscle cells expressing Δ-p120ctn-GFP (panels C-D). As myopodia form transiently, we assessed levels of myopodial assembly by counting the total number of nerve-induced myopodia along multiple contacts in muscle cells expressing GFP or GFP-tagged p120tn proteins and then calculating the number of myopodia present per 10 μm of contact. Once again, for the mutant p120ctn cells, small membrane protrusions were included because these cells lacked longer myopodia. Several batches of nerve–muscle cocultures (>6) were prepared for each mus-

Figure 5 Effect of agrin on the tyrosine phosphorylation and cadherin-association of p120ctn in cultured mouse myotubes. (A) C2 mouse myotube cultures were treated without (''+P''; all panels) or with agrin (''+AP''; all panels) for 1 h, and Triton X-100 extracts prepared from them were examined by immunoblotting (''IBs''; all panels), using antibodies against p120ctn, Shp2, and cadherin; agrin-treatment did not affect the total levels of these proteins in muscle extracts. (B) Immunoprecipitations (''IPs''; all panels) were carried out using monoclonal antibodies against Shp2 (''shp'') or p120ctn (''p120'') from extracts of myotubes treated with or without agrin (1 h). IP samples were immunoblotted with anti-Shp2 and anti-p120ctn antibodies (top) or with antiphosphotyrosine antibody (bottom). Anti-Shp2 and anti-p120ctn antibodies specifically captured their target proteins and the anti-phosphotyrosine antibody more strongly stained p120ctn captured from agrin-treated cells compared with that from untreated cells (lane 4 vs. 2). In this and following panels, positions of MW markers are indicated on the left; asterisks on the right denote a nonspecific mouse secondary antibody-stained band. (C) Mouse myotubes were treated with agrin for different times (0.5–4 h) and p120ctn was immunoprecipitated from their extracts. After loading equal amounts of p120ctn (anti-p120ctn staining; top blot), blots were stained with antibodies against phosphotyrosine and cadherin. Agrin-treatment increased the tyrosine phosphorylation of p120ctn up to 1 h, which then decreased (middle blot). Cadherin coprecipitated with p120ctn (bottom blot), and the association between cadherin and p120ctn was diminished by 1 h agrin-treatment (bottom blot and relative band intensity graph). (D) Agrin-treatment reduced cadherin association with p120ctn. Immunoblotting with the anticadherin antibody was carried out on equally loaded p120ctn IP samples from untreated and agrin-treated (1 h) myotube extracts. The cadherin band staining intensities were measured and normalized relative to that obtained in IP samples from untreated myotube extracts. The coprecipitation of cadherin with p120ctn was significantly reduced by agrin-treatment (4 experiments; $p < 0.002$). (E) Cadherin coprecipitated with β-catenin (top) and γ-catenin (bottom) from myotube extracts, and this was not significantly affected by agrin-treatment. As controls in the IPs, for the anti-β-catenin antibody (top) a monoclonal anti-Shp2 antibody was used, whereas for the anti-cadherin antibody (bottom) a rabbit polyclonal antibody against calcineurin was used.

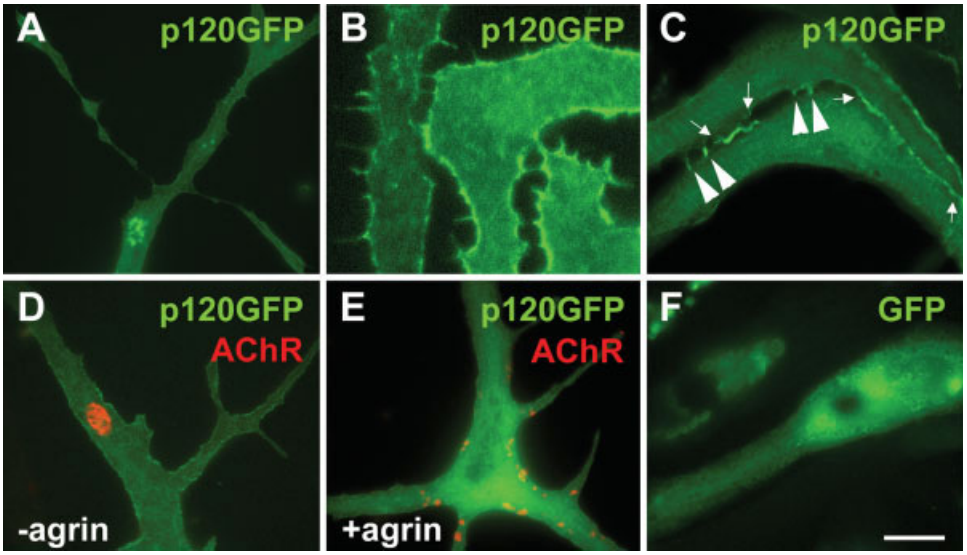

Figure 6 Ectopic p120ctn expression and myopodial formation in *Xenopus* muscle cells. Muscle cells were cultured from *Xenopus* embryos injected with mRNAs encoding a p120ctn-GFP fusion protein (p120GFP) (A–E) or GFP only (F). In p120ctn-GFP-expressing cells, elongated, ''dendritic'' structures (A) and myopodia (B) were present, and exogenous p120ctn-GFP was recruited to muscle–muscle (arrows) and myopodia-muscle (arrowheads) contacts (C). As shown by merged images in panels (D) and (C), respectively, in these cells, AChR hot spots and agrin-induced AChR clusters appeared normal. GFP expressed in muscle cells was not recruited to cell contacts, and it did not induce dendritic structures or myopodia (F). Scale bar = 20 μm in panels (A, D–F), 6.67 μm in panel (B), and 10 μm in panel (C).

cle type, and in each of these cultures numerous nerve–muscle contacts were examined (total: 50 partial contacts for wild-type and 55 contacts for mutant p120ctn cells, >1 mm in overall length in each case). These data showed that neurites generated >3-fold fewer myopodia in muscle cells expressing Δ-p120ctn-GFP compared with those expressing wild-type p120ctn-GFP or GFP (Table 1), suggesting that myopodial induction by neurites, like that by agrin, depends on a p120ctn-signaling pathway, possibly involving rho GTPases.

Myopodia from rodent muscle cells make close contacts with motor axons in cultures (Uhm et al., 2001) but whether this promotes synaptic differentiation is not known. In nerve–muscle cocultures, AChR clustering occurs in muscle near innervation sites in response to the agrin deposited by neurites along their tracks (Cohen et al., 1995; Peng et al., 2003). In our cocultures, we found that myopodia extended toward neurites and that AChR clusters were closely associated with them (see Fig. 1); therefore, we examined AChR clustering at partial contacts between neurites and muscle cells expressing wild-type or mutant p120ctn to investigate how the presence of absence of myopodia affects postsynaptic differentiation at incipient NMJs. Whereas AChR microclusters were readily found near nerve-contact edges of wild-type p120ctn-expressing muscle cells [Fig. 8, panels (E–G,K–M)], and GFP-expressing muscle cells (not shown), fewer AChR clusters were detected at such sites in cells expressing mutant p120ctn (panels H-J, N-P). Data pooled from all identified partial nerve–muscle contacts showed that the AChR microcluster density in mutant p120ctn-expressing muscle cells was significantly decreased with respect to control cells (Table 1). However, at sites where neurites directly and stably contacted wild-type or mutant p120ctn-expressing muscle cells (''overlap''), AChR clustering was induced to similar extents [Fig. 8(Q–S, T–V)], which is consistent with our results showing that ectopic expression of wild-type or mutant p120ctn in muscle did not influence clustering of AChRs *per se* occurring either spontaneously or in response to bath-applied agrin (Fig. 6; R.M. and H.B.P., unpublished observations). On the basis of these findings, we propose that signaling by p120ctn in muscle generates myopodia, which promote NMJ formation by increasing muscle's access to agrin deposited by nearby neurites, in addition to facilitating contacts between muscle and nerve.

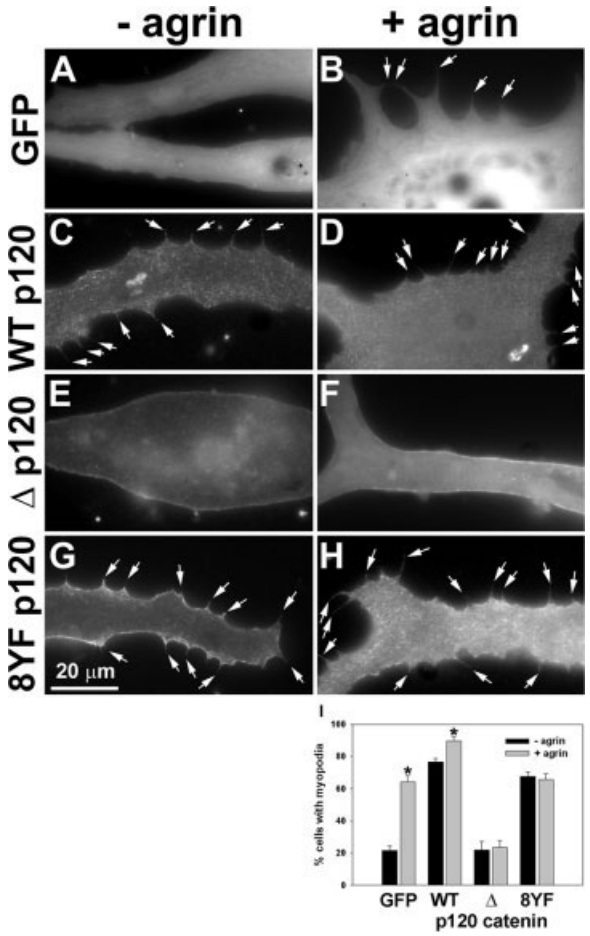

Figure 7 Ectopic expression of wild-type and mutant p120ctn proteins and agrin-induced myopodial assembly. To directly address the role of p120ctn in agrin-induced formation of myopodia, muscle cells cultured from *Xenopus* embryos injected with mRNAs encoding GFP or GFP-tagged wild-type and mutant p120ctn proteins were examined without (A, C, E, G) or with (B, D, F, H) agrin-treatment (2 h). Muscle cells and myopodia (arrows) were visualized by GFP fluorescence, and percentages of fluorescent cells with myopodia were determined (I). Agrin induced myopodia in GFP expressing cells (A, B) to a level that could be achieved by wild-type p120ctn (WT p120) expression alone (C); agrin-treatment increased the fraction of p120ctn-expressing cells with myopodia (D). Expression of the p120ctn deletion mutant protein (Δ p120) did not induce myopodia (E) and suppressed agrin's ability to generate myopodia (F). Myopodia were generated by over-expression of a p120ctn mutant protein lacking src phosphorylation sites (8YF p120) (G), but here agrin-treatment did not increase myopodial formation (H). Data were pooled from experiments carried out on 4–6 separate culture preparations, using mRNA-injected embryos (I); number of cells examined for GFP = 347; WT p120 = 526; Δ p120 = 408; and 8YF p120 = 301. Asterisks indicate *t* test *p* values below 0.005.

DISCUSSION

In this study, we investigated early muscle–nerve interactions that lead to NMJ formation, focusing on myopodia used by muscle to contact motor axons (Uhm et al., 2001). We have identified a novel function of p120ctn in myopodial induction by agrin and have proposed that myopodia not only promote muscle's interactions with nerve but also enhance muscle's ability to detect nerve-secreted agrin. These findings provide novel insights into agrin signaling and the postsynaptic changes that occur during the establishment of NMJs.

Myopodial Assembly and p120ctn

Agrin-activated MuSK triggers a signaling cascade in muscle involving multiple effectors (Sanes and Lichtman, 2001) to generate AChR clusters in an actin polymerization- and rho GTPase-dependent manner (Dai et al., 2000; Weston et al., 2000, 2003). Nerve-secreted agrin also stimulates muscle to extend myopodia (Uhm et al., 2001), but how this occurs was previously unknown. The findings of this study indicate involvement of p120ctn in mediating myopodial assembly.

Members of the p120ctn family—p120ctn, δ-catenin, ARVCF, and p0071—are multifunctional proteins that affect cell adhesion and cytoskeletal and nuclear signaling (Anastasiadis et al., 2000; Anastasiadis and Reynolds, 2001). The p120ctn protein, which was identified as a major src substrate in cells (Reynolds et al., 1989), associates with cadherins and modulates their function and surface expression (Reynolds and Carnahan, 2004). Whereas p120ctn insufficiency disrupts cadherin junctions (Ireton et al., 2002), p120ctn over-expression generates a ''dendritic'' phenotype in fibroblasts (Reynolds et al., 1996; Noren et al., 2000) and increases cell motility (Grosheva et al., 2001). The latter effects of p120ctn depend on its regulation of rho GTPases after dissociation from cadherin (Noren et al., 2000; Anastasiadis and Reynolds, 2001); rho GTPases are effector proteins that reorganize the actin cytoskeleton to generate filopodia, lamellipodia, and stress fibers in a variety of cell types (Etienne-Manneville and Hall, 2002). We have shown that agrin enhanced cadherin-free levels of p120ctn in muscle and that over-expressing wild-type p120ctn in muscle generated myopodia. Significantly, a dominant-negative mutant form of p120ctn that poorly inhibits rho GTPase (Anastasiadis et al., 2000) did not generate myopodia when expressed in muscle and blocked myopodial induction by nerve and agrin.

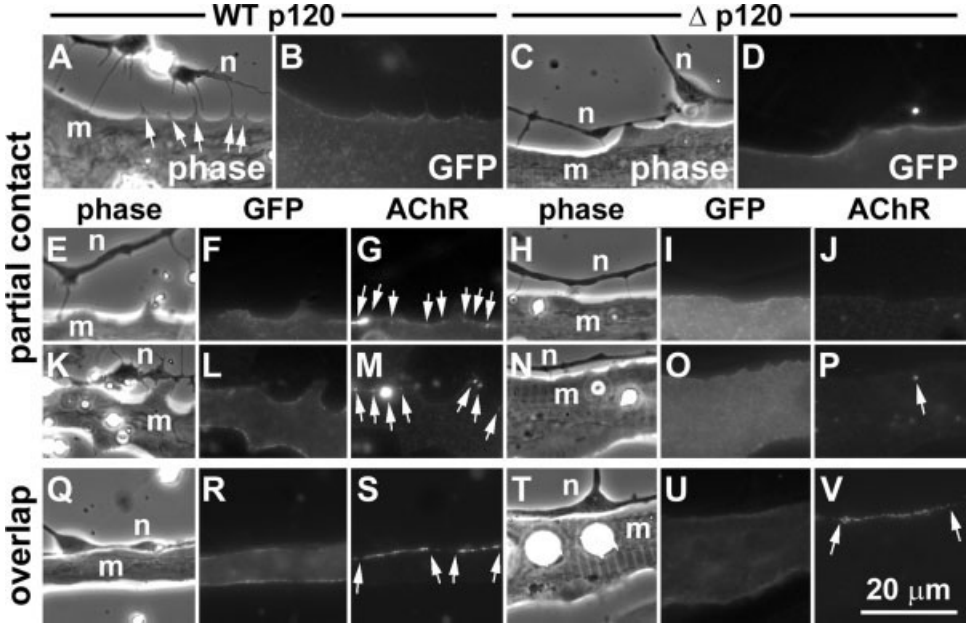

Figure 8 Ectopic expression of wild-type and mutant p120ctn proteins and nerve-induction of myopodia and AChR clusters. *Xenopus* muscle cells expressing exogenous wild-type p120ctn (left) or the deletion-mutant p120ctn (right) were cocultured for one day with spinal nerves. In live cultures, edges of muscle cells close to neuritic processes, classified as partial contacts, were examined for myopodia. At such sites, myopodia were readily detected in cells expressing wild-type p120ctn (A, B) (arrows) but few were present in cells expressing mutant p120ctn (C, D). Next, by R-BTX labeling, numerous AChR microclusters (arrows) were found in wild-type p120ctn-expressing cells (E–G; K–M) but fewer clusters were detected in the mutant cells (H–J; N–P). However, where neurites directly contacted muscle expressing wild-type (Q–S) or mutant p120ctn (T–V), AChR clusters were induced to similar levels. Myopodial and AChR microcluster densities at partial neurite-muscle contacts are presented in Table 1.

These results suggest that rho GTPase regulation by p120ctn is important for the formation of myopodia.

Intriguingly, neither the wild-type nor the deletion mutant of p120ctn expressed in muscle affected agrin-induced AChR clustering, although previous studies have shown that rho GTPases are important for this process and the maturation of AChR clusters (Weston et al., 2000, 2003). Involvement of different rho family proteins in the formation of myopodia and AChR clusters and/or the regulation of these GTPases by multiple upstream proteins at different stages of agrin stimulation of muscle may explain our observation of p120ctn effects on agrin-induction of myopodia but not AChR clusters. Development and use in future studies of probes that allow visualization of rho GTPase activity in live muscle cells should lead to a better understanding of when, where and at what levels these proteins signal during the formation of myopodia and AChR clusters and by which molecules they are regulated at the NMJ.

Agrin-activated MuSK signals through src family tyrosine kinases in muscle (Mittaud et al., 2001; Mohamed et al., 2001). We found that agrin enhanced myopodial induction in muscle cells expressing exogenous wild-type p120ctn but not a mutant p120ctn lacking src phosphorylation sites in its NH_2-terminal

Table 1 Myopodia and AChR Microclusters at Nerve–Muscle Contacts

Muscle Cell Expression	Myopodia (per 10 μm)	AChR Clusters (per 10 μm)
GFP	1.81 ± 0.16	1.9 ± 0.38
WT p120	2.37 ± 0.19	1.52 ± 0.19
Δ-p120	0.69 ± 0.09**	0.98 ± 0.19*

Myopodia and AChR microclusters that developed at partial contacts between neurites and muscle cells expressing GFP, wild-type p120ctn-GFP (WT p120), and the p120ctn deletion mutant (Δ-p120) were counted. Pooled data were used to calculate the average number of myopodia and AChR microclusters per 10 μm of nerve–muscle contact, both of which were significantly reduced in Δ-p120 muscle cells relative to GFP or WT p120ctn cells.

** $p < 0.0001$.

* $p < 0.04$.

domain (Mariner et al., 2001). One explanation for these results is that agrin stimulates src phosphorylation of exogenous wild-type p120ctn, but not of phospho-mutant p120ctn, to further elevate cadherin-free levels of wild-type p120ctn, whereas another explanation is that wild-type p120ctn's ability to induce cytoskeletal changes is directly affected by its tyrosine phosphorylation (Cozzolino et al., 2003). We favor the former view because, in the absence of agrin-stimulation, over-expressed phospho-mutant p120ctn was able to induce myopodia nearly as well as wild-type p120ctn.

How tyrosine phosphorylation affects p120ctn's binding to cadherin has been studied extensively but a clear consensus is lacking: on the one hand, tyrosine phosphorylation of p120ctn appears to require its interaction with cadherin and tyrosine phosphorylated p120ctn associates with cadherin complexes (Kinch et al., 1995; Cozzolino et al., 2000; Ozawa and Ohkubo, 2001), but, on the other hand, full-length p120ctn appears to regulate cadherin-mediated adhesion less well than p120ctn devoid of the NH_2-terminal domain containing tyrosine phosphorylation sites (Aono et al., 1999; Ozawa and Ohkubo, 2001; Ireton et al., 2002; Cozzolino et al., 2003). Additionally, p120ctn possessing or lacking the NH_2-terminal phosphorylation domain differentially associates with different cadherins, suggesting that tyrosine phosphorylation positively or negatively influences p120ctn's interaction with cadherins in an isoform-dependent manner (Seidel et al., 2004). In our experiments using muscle cells, increased tyrosine phosphorylation of p120ctn correlated with a decrease in its interaction with cadherin. What seems to be clear is that protein interactions and functions of p120ctn are tightly controlled by its tyrosine phosphorylation state: in addition to being a major tyrosine kinase substrate in cells, p120ctn has been shown to associate with three different tyrosine phosphatases, Shp-1 (Keilhack et al., 2000), RPTPμ (Zondag et al., 2000), and DEP1 (Holsinger et al., 2002).

Catenins and Postsynaptic Signaling

Cadherin complex proteins have been localized in motor nerves and muscle (Cifuentes-Diaz et al., 1998) but only a few studies have addressed catenin functions in postsynaptic differentiation at the NMJ. These studies examined pathways that affect β-catenin downstream from wnt and found direct or indirect influences on AChR clustering: adenomatous polyposis coli, a β-catenin-binding protein, participated in AChR aggregation (Wang et al., 2003), lithium, an inhibitor of glycogen synthase kinase that destabilizes cytosolic β-catenin, blocked AChR clustering (Sharma and Wallace, 2003), and wnt, which promotes β-catenin stability and nuclear signaling, regulated MuSK gene expression (Kim et al., 2003). More recently, δ-catenin and its binding-partner kaiso have been reported to activate the transcription of the AChR-clustering protein rapsyn in muscle cells (Rodova et al., 2004).

In the central nervous system, cadherins and catenins promote the development of proper synaptic connections, establish trans-synaptic adhesion complexes, interact with and affect the targeting of synapse-associated molecules, and signal within synapses (Bruses, 2000; Yagi and Takeichi, 2000; Goda, 2002). In hippocampal neurons, β-catenin redistributes between dendritic spines and shafts in a tyrosine phosphorylation- and depolarization-dependent manner to affect the morphology of synaptic structures (Murase et al., 2002), and dominant-negative inhibition of cadherin function induces loss of β-catenin from dendritic spines and produces filopodia-like spines (Togashi et al., 2002). The distribution of p120ctn during rat brain development indicates roles for it in cadherin-based adhesion and cytoskeletal signaling (Chauvet et al., 2003) and, in chick ciliary neurons, association of p120ctn and β-catenin with N-cadherin changes as synapses mature (Rubio et al., 2005); this latter finding is particularly intriguing because in our studies also the interaction between p120ctn and cadherin changed during synaptogenic signaling. Lastly, δ-catenin associates with the cytoskeleton and synaptic components in hippocampal neurons (Jones et al., 2002) and its over-expression enhances the formation of dendritic structures and the density of spines (Kim et al., 2002). Taken together with these observations, our p120ctn results from muscle further underscore the importance of catenins in regulating cytoskeletal dynamics during synapse establishment and modification.

Myopodial Adhesion and Function

Unlike in the CNS where pre- and post-synaptic partners intimately contact each other, at mature NMJs nerve and muscle are separated by a basal lamina (Sanes and Lichtman, 1999). However, nerve and muscle come into direct contact during innervation (Cohen and Weldon, 1980; Nakajima et al., 1980) and in nerve-muscle cultures the gap between myopodial and axonal membranes is small enough (Uhm et al., 2001) to allow trans-synaptic adhesion complexes to develop. We found that myopodial contacts with neighboring muscle cells were strongly labeled

by the lectin VVAB4, which also labels embryonic and mature NMJs (Martin, 2002), and exogenous p120ctn became enriched at myopodial and muscle contacts. Because cadherin recruits p120ctn to cell junctions (Thoreson et al., 2000), our results suggest that VVAB4-receptor- and cadherin-based adhesion complexes rapidly develop at new contacts made by myopodia with other muscle cells. Cadherins and catenins are present in the pre- and the post-synaptic compartments of the NMJ (Cifuentes-Diaz et al., 1998; Marthiens et al., 2002) and the expression of cadherin isoforms M, N, E, six, and 11 in muscle and/or motor nerve has been demonstrated (Cifuentes-Diaz et al., 1998). Whether or not adhesion complexes containing VVAB4-receptors and cadherin complexes also develop between nerve and muscle and what specific isoforms of cadherin and other adhesion molecules help establish initial nerve–muscle junctions are questions for future studies.

Myopodia were first described at Drosophila NMJs where they closely contacted neurons, intermingled with neuronal processes, and became clustered as synapses matured; myopodia may thus help ''match'' the synaptic partners at this NMJ (Ritzenthaler et al., 2000; Ritzenthaler and Chiba, 2001). In two other studies, myopodia have been found at rodent NMJs: myopodia were shown to be generated in response to nerve-secreted agrin (Uhm et al., 2001), and increased myopodial formation was demonstrated in embryonic muscle fibers of mice that developed defective NMJs because of a lack of ACh secretion from the nerve terminal (Misgeld et al., 2002). In these earlier studies, however, the functional role of myopodia in the establishment of vertebrate NMJs was not directly examined. Our finding that myopodia did not develop in response to nerve/agrin in muscle cells expressing the p120ctn deletion mutant protein allowed us to begin to address this issue.

In mutant p120ctn-expressing muscle cells, AChR clustering was reduced along edges approached by neurites compared to similar sites in control cells, although the mutant cells were capable of clustering AChRs normally. Based on these findings, we conclude that the mutant cells, containing few myopodia, detected lesser neurite-secreted agrin and developed fewer AChR clusters: because neurite-secreted agrin becomes bound to the extracellular matrix or to the substrate in cultures and AChR clusters develop where muscle cells contact this immobilized agrin (Cohen et al., 1995), a region of muscle with myopodia may better access nearby agrin than a region without myopodia. A similar scenario could also apply *in vivo* where agrin, a heparan sulfate proteoglycan, is anchored to the synaptic basal lamina. Thus, myopodia may function akin to filopodia, processes that help cells sample their local environment for molecular cues (Wood and Martin, 2002). Interestingly, AChR clusters associated closely with myopodia in our muscle cultures and in embryonic mouse muscle (Misgeld et al., 2002), suggesting that MuSK and other effectors of agrin are also present near or within myopodia.

In conclusion, autoactivation of MuSK, which produces AChR clusters in central regions of embryonic muscle fibers before innervation (Lin et al., 2001; Yang et al., 2001), may initially trigger myopodial assembly at modest levels, and neural agrin detected by these myopodia may further locally generate myopodia and AChR clusters to promote muscle–nerve interaction, reciprocal signaling, and synaptic differentiation. The involvement of agrin/MuSK in initiating myopodial assembly and AChR clustering may explain why these specializations develop near each other, as shown in this study, although downstream from MuSK activation at least two separate signaling pathways-one involving p120ctn for the formation of myopodia and another not involving p120ctn in the generation of AChR clusters-are likely stimulated. Future studies on myopodia at NMJs should yield insights into the functions of motile processes not only at synapses but also more generally in facilitating cell–cell communication. In this regard, two recent studies examining motile processes in other cell types are of significant interest: In the *Drosophila* central nervous system, microprocesses from midline glial cells (''gliopodia''), which developed in a rac-dependent manner, together with neuronal processes are thought to generate a ''filopodial-web'' that increases physical interaction and communication between glia and neurons (Vasenkova et al., 2005). And, in cultured A431 and HeLa cells, quantum dot-coupled epidermal growth factor (EGF) bound to and activated Erb1 receptors localized in filopodia, which were then transported to the base of filopodia and endocytosed; here again, the function assigned for the filopodia was to extend a cell's reach for its stimulant (Lidke et al., 2005).

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