Dual regulation of neuronal morphogenesis by a δ-catenin–cortactin complex and Rho

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δ-Catenin is a neuronal protein that contains 10 Armadillo motifs and binds to the juxtamembrane segment of classical cadherins. We report that δ-catenin interacts with cortactin in a tyrosine phosphorylation–dependent manner. This interaction occurs within a region of the δ-catenin sequence that is also essential for the neurite elongation effects. Src family kinases can phosphorylate δ-catenin and bind to δ-catenin through its polyproline tract. Under conditions when tyrosine phosphorylation is reduced, δ-catenin binds to cortactin and cells extend unbranched primary processes. Conversely, increasing tyrosine phosphorylation disrupts the δ-catenin–cortactin complex. When RhoA is inhibited, δ-catenin enhances the effects of Rho inhibition on branching. We conclude that δ-catenin contributes to setting a balance between neurite elongation and branching in the elaboration of a complex dendritic tree.

Introduction

Process elaboration involves a coordinated and complex repertoire of cellular systems. The capacity to elaborate processes has achieved its maximal complexity in neurons, which utilize the neuronal arbor to increase the number of synaptic contacts, set the frequency at which impulses spread, and establish regionally distinct functional outputs (Larkum et al., 1999). Many extracellular molecules, which affect neurite morphology, have been identified including secreted factors, plasma membrane-bound proteins, and proteins bound to the extracellular matrix. However, the intracellular pathways that control even simple neurite patterning, such as elongation versus branching, are poorly understood.

Small GTPases, which play a major role in actin organization, have been shown to be involved in the regulation of neuronal cell morphology including neuritogenesis. The role of RhoA in process formation was first demonstrated in studies using the Rho-specific ADP-ribosyltransferase C3 toxin (Clostridium botulinum C3 Rho-ADP-ribosylating exoenzyme), which could induce neurites in PC12 cells even without NGF treatment (Nishiki et al., 1990). Many additional studies have shown that RhoA is involved in the regulation of neurite outgrowth (Tigyi and Miledi, 1992; Jalink et al., 1994; Kozma et al., 1997; Zipkin et al., 1997; Lehmann et al., 1999; Sebok et al., 1999; Nakayama et al., 2000). Studies of neurite branching often focus on axons; however, dendritic branching is morphologically distinct from axonal branching with regard to tapering and branching patterns (Desmond and Levy, 1984). A role for RhoA in dendritic branching has been suggested. RhoA activation leads to a reduction in dendritic branching (Nakayama et al., 2000), and RhoA inhibition enhances branching (Neumann et al., 2002). Mediators of RhoA, such as Rho kinase, support these opposite effects on branching (Katoh et al., 1998). Likewise, Cip1/WAF1 inhibits Rho kinase and promotes neurite outgrowth and branching in hippocampal neurons (Tanaka et al., 2002). A distinct branch of the Rho GTPase family, the Rnd proteins, control rearrangements of the actin cytoskeleton and changes in adhesion (Nobes et al., 1998). Rapostlin, an effector for Rnd2, can induce neurite branching when expressed in NGF-treated PC12 cells (Fujita et al., 2002). A genetic screen in Drosophila identified several genes that control aspects of dendrite development including dendritic outgrowth, branching, and routing (Gao et al., 1999).

Because neuronal process elaboration must involve adhesive changes concomitant with extension into the neuropil, it is not surprising that adhesion molecules will be represented in this molecular toolbox. The p120tm family, which consists of a family of proteins with 10 Armadillo (Arm)* repeats characteristically spaced, are increasingly recognized for their dual roles in regulating adhesion and process elaboration.

*Abbreviations used in this paper: Arm, Armadillo; BDNF, brain-derived neurotrophic factor.
Coordinating these two functions is very likely a key role for the cadherin juxtamembrane sequence where all of these family members bind. δ-Catenin is a neuronal specific member (Ho et al., 2000) of this protein family, which also includes p120catenin, ARVCF, and p0071. Like p120catenin, δ-catenin can radically change cell morphology when overexpressed in fibroblasts (Reynolds et al., 1996; Kim et al., 2002).

The implementation of process elaboration requires reorganization of the actin cytoskeleton, and indeed linkages between the adherens junction and the actin cytoskeleton are well recognized. β-Catenin binds both to the COOH terminus of classical cadherins and to α-catenin, which binds directly or indirectly to actin (Yamada and Geiger, 1997). A second linkage through the p120catenin family of proteins can be surmised based on functional studies, but the actual components or pathway is unknown. Potentially, most informative is the extension of data derived for p120catenin to δ-catenin. Like δ-catenin, p120catenin induces a "branching phenotype" (Reynolds et al., 1996; Kim et al., 2002). An interaction between p120catenin and Rho1 and an accumulation of Rho1 in adherens junctions occurs in Drosophila (Magie et al., 2002). p120catenin inhibits Rho (Anastasiadis et al., 2000; Noren et al., 2000), and cadherin binding to p120catenin functions as a regulator of adhesion through Rho GTPases (Anastasiadis and Reynolds, 2001).

Cortactin is a linker protein to the actin cytoskeleton, which is well suited to couple tyrosine kinase signaling between membrane proteins and the cytoskeleton (Weed and Parsons, 2001). A centrally positioned series of repeats in cortactin bind and cross-link actin filaments in a tyrosine phosphorylation–dependent manner (Wu and Parsons, 1993; Huang et al., 1997). Through binding to the Arp2/3 complex, cortactin provides a site for actin filament nucleation (Weed et al., 2000; Uruno et al., 2001). Additionally, Rho GTPases can determine cortactin association with the actin system and contractile regulation in endothelial cells (Weed and Parsons, 1993; Huang et al., 1997). Through binding to RhoA pathway together with expression of δ-catenin further enhances the extension of secondary neurites. δ-Catenin appears positioned to regulate neurite growth through two separate pathways that balance primary process extension with branch formation.

Results

δ-Catenin induces neurites

A prominent effect of δ-catenin on cells is process elaboration. PC12 cells treated with NGF (100 ng/ml) at the time of GFP–δ-catenin transfection extended unbranched primary processes 36 h later (Fig. 1 A). When transfected with GFP alone, treatment of PC12 cells with NGF for the relatively short time of 36 h induced very short processes in a small percentage of the cells, whereas most of the cells remained rounded (Fig. 1 A). About 20% of GFP–δ-catenin–transfected cells elaborated processes, which were longer than one cell body in length, whereas none of the cells transfected with GFP alone had processes that extended one cell body or more. Due to the variation in expression levels in the transient transfections and the general inhibition of process elaboration by acute transfections, we repeated these experiments in a PC12 cell line that stably expresses δ-catenin (δ-PC12 cells). These cells extended long relatively unbranched processes after a 36-h treatment with NGF (Fig. 1 B). Not surprisingly the δ-PC12 cells displayed more uniform morphological changes. Comparing NGF-treated δ-PC12 cells with NGF-treated wild-type PC12 cells, we found that the mean number of primary processes increased from 2.7 ± 0.14 to 3.5 ± 0.21 (P < 0.005), and the total summed length of all processes increased from 12.80 ± 1.05 to 20.5 ± 1.7 μm (P < 0.001) (Fig. 1 B). Both NGF-treated δ-PC12 cells and NGF-treated wild-type PC12 cells had very few secondary processes, and their numbers did not statistically differ (Fig. 1 B).

Transfection of δ-catenin into cultured hippocampal neurons at 9 d in vitro also alters cell morphology (Fig. 2 A). Interestingly, the induced growth was restricted to dendrites even though transfected δ-catenin extended through the entire neuron as detected by the GFP label. The outgrowths induced by δ-catenin were thin processes of uniform caliber that emerged from the distal dendritic tree and varied greatly in length (Fig. 2, A and C). These processes created a sense of exaggerated filopodial protrusions, and like filopodia they
were labeled for actin but not for tubulin or MAP2 (Fig. 2 B). Both the length and number of protrusions in the most distal dendrites of δ-catenin–transfected neurons increased when compared with GFP-transfected cells (Fig. 2 C). For δ-catenin–transfected neurons, the mean length of the protrusions was 3.32 ± 0.37 μm, and there were 10.5 ± 0.72 protrusions over a 25-μm dendritic segment. For GFP-transfected neurons, the mean length of the protrusions was 1.67 ± 0.17 μm, and there were 5.16 ± 0.87 protrusions over a 25-μm dendritic segment. These differences were highly significant (P < 0.001) (Fig. 2 C). Staining of GFP and GFP–δ-catenin–transfected neurons for actin showed the extension of the signal into all the protrusions (unpublished data).

δ-Catenin deletion constructs (Fig. 3 A) were used to define a sequence essential for process elaboration. δ-Catenin construct ΔC205 (numbers refer to the number of aa deleted from the COOH terminus) completely failed to induce morphological changes when transfected into PC12 cells or primary neurons (Fig. 3 B). However, ΔC99 induced effects similar to those observed for GFP–full-length δ-catenin (Fig. 3 B) in both cell types. The ΔC332 deletion extended into the Arm repeat domain, and therefore, this construct did not localize to the plasma membrane, as occurs with full-length, transfected δ-catenin and endogenous δ-catenin, and did not induce morphological changes (unpublished data). The failure of this construct to induce processes is presumably due to its incorrect localization.
However, ΔC205, which includes all of the Arm repeats but deletes nearly the entire COOH terminus beyond the repeats, did localize in membrane patches similar to full-length, transfected δ-catenin and to a construct with only the Arm repeats (unpublished data) but was unable to induce any of the morphological changes described for the full-length protein (Fig. 3 B). Together, these deletion constructs suggested a region of 105 aa in the COOH terminal region of δ-catenin that was essential for process elaboration.

δ-Catenin is linked to cortactin
Immunostaining with δ-catenin antibodies demonstrated that the endogenous protein is coexpressive with filamentous actin (Fig. 4 A). This association was most obvious in neuronal growth cones. Among the candidate proteins which actin (Fig. 4 A). This association was most obvious in neuronal growth cones. Among the candidate proteins which actin (Fig. 4 A). This association was most obvious in neuronal growth cones. Among the candidate proteins which coimmunoprecipitated with polyclonal antibody (Fig. 4 C). A FLAG antibody also could link δ-catenin with cortactin (unpublished data) but was unable to induce any of the morphological changes described for the full-length protein (Fig. 3 B). Together, these deletion constructs suggested a region of 105 aa in the COOH terminal region of δ-catenin that was essential for process elaboration.

Tyrosine phosphorylation of δ-catenin
The tyrosine phosphorylation state of δ-catenin in δ-PC12 cells and primary neurons differed. δ-Catenin was tyrosine phosphorylated in the δ-PC12 cells (Fig. 6 A) and NGF did not alter the phosphotyrosine immunoreactivity (unpublished data). In contrast, tyrosine phosphorylation was not detected on δ-catenin in the primary neurons (Fig. 6 B).
The tyrosine phosphorylation of many proteins can be increased by pretreatment with H$_2$O$_2$ (200 μM) and orthovanadate (1 mM), which inhibit phosphatases. In primary neurons, H$_2$O$_2$ induced the tyrosine phosphorylation of δ-catenin, and H$_2$O$_2$ plus orthovanadate further increased the tyrosine phosphorylation (Fig. 6 B). The tyrosine phosphorylation of cortactin also increased after H$_2$O$_2$ treatment as reported (Li et al., 2000).

Inspection of the δ-catenin sequence indicates multiple consensus sites for tyrosine phosphorylation within the regions that flank the Arm repeats. In the sequence which is COOH terminal to the last Arm repeat there are 17 tyro-
sines. Mutation of various combinations of these tyrosines to phenylalanine including the single tyrosine at position 1,041, which is conserved in ARVCF and p120ctn, did not alter the intensity of the signal detected by a phosphotyrosine antibody in cultures transfected with mutant and wild-type constructs at equal transfection efficiencies (unpublished data). It is likely that the COOH terminus contains multiple tyrosine phosphorylation sites.

δ-Catenin is a Src family substrate

To determine the family of tyrosine kinases responsible for phosphorylating δ-catenin, δ-PC12 cells were treated with several tyrosine kinase inhibitors. We found that a treatment with PP2 (25 μM), a Fyn/Lck/Hck tyrosine kinase inhibitor (Fig. 7 A), was able to abolish endogenous δ-catenin phosphorylation in δ-PC12 cells. Nonreceptor tyrosine kinases are good candidates because p120ctn is a known Src substrate (Kanner et al., 1991; Reynolds et al., 1992) and δ-catenin is an in vitro substrate for Abl (Lu et al., 2002). In δ-PC12 cells, the basal level of tyrosine phosphorylation on δ-catenin can be completely inhibited by PP2 (Fig. 7 A). In primary cultured neurons, endogenous δ-catenin is only minimally tyrosine phosphorylated, but after H2O2 treatment, which can induce tyrosine phosphorylation of δ-catenin (Fig. 6 B), 25 μM PP2 completely inhibited δ-catenin tyrosine phosphorylation as detected by a phosphotyrosine antibody (Fig. 7 A). When Fyn was transfected into δ-PC12 cells, δ-catenin phosphorylation increased (Fig. 7 B), but endogenous phosphorylation was not abolished when a dominant-negative form of Fyn (Fyn K299M) was transfected, raising the possibility that other members of the Src family phosphorylate δ-catenin. Several δ-catenin deletion constructs were tested for H2O2-induced tyrosine phosphorylation, and the specific constructs on which tyrosine phosphorylation was induced by H2O2 matched the constructs on which Fyn transfection induced tyrosine phosphorylation (unpublished data). In addition to serving as a substrate for a Src family tyrosine kinase, a Fyn–SH3–GST construct and a Lck–SH3–GST construct pulled down δ-catenin in transfected COS1 cells and in neurons (Fig. 7 C). Neither Fyn–SH3 nor Lck–SH3 pulled down FLδΔP6, a δ-catenin construct which lacked the polyproline tract (Fig. 7 C). Together, this data suggest that a Src family nonreceptor tyrosine kinase binds to δ-catenin through its polyproline tract and phosphorylates δ-catenin.

Tyrosine phosphorylation regulates the δ-catenin–cortactin interaction

In contrast to primary neurons, the δ-catenin–cortactin complex was not observed in δ-PC12 cells under basal conditions presumably due to the difference in the basal tyrosine phosphorylation state between these cells and primary neurons. To test the hypothesis that tyrosine phosphorylation regulates the δ-catenin–cortactin interaction, we determined whether PP2 treatment, which can abolish tyrosine phosphorylation of δ-catenin in δ-PC12 cells, could restore the interaction. Indeed, when δ-PC12 cells (pretreated with 100 ng/ml NGF for 36 h) were exposed to PP2 (25 μM) for 1 h the formation of the δ-catenin–cortactin complex was detected (Fig. 6 D).

We next asked whether the activation of tyrosine phosphorylation in primary neurons could induce the dissociation of the δ-catenin–cortactin complex. When primary neurons were treated with H2O2 for 5 min or with H2O2...
The interaction between cortactin and δ-catenin was abolished (Fig. 6B), whereas the δ-catenin–N-cadherin interaction was preserved. This observation suggested that the δ-catenin–cortactin interaction depends on the tyrosine phosphorylation state.
of the cell and possibly on the tyrosine phosphorylation state of δ-catenin and/or cortactin. Interestingly, treatment of the hippocampal neurons with brain-derived neurotrophic factor (BDNF) (50 ng/ml) for 60 min also induced δ-catenin phosphotyrosine immunoreactivity and dissociation of the δ-catenin–cortactin complex (Fig. 6 C). Thus, two different cell types, primary neurons and δ-PC12 cells, with different basal levels of δ-catenin tyrosine phosphorylation have consistent δ-catenin–cortactin complex association or dissociation in response to increasing or decreasing their state of tyrosine phosphorylation.

**Primary process elongation versus branching**

The inhibition of δ-catenin tyrosine phosphorylation in NGF-treated δ-PC12 cells exposed to PP2 (25 μM for 1 h) enhanced the morphological changes induced by δ-catenin (Fig. 8). The total length of the processes increased from 15.1 ± 1.2 to 24.9 ± 2.4 μm (P < 0.0001), and the number of primary processes increased from 3 ± 0.26 to 5.1 ± 0.37 (P < 0.0001), but there was no change in the number of branches or secondary processes (Fig. 8 B). Wild-type PC12 cells, which have negligible if any δ-catenin, were unaffected by PP2 (unpublished data).

The pattern of δ-catenin process elaboration could be qualitatively changed when PC12 cells, treated with NGF for 36 h, also expressed the C3 exotoxin, a specific Rho GTPase inhibitor derived from *C. difficile*, which inhibits Rho by ADP ribosylation (Boquet, 1999). δ-Catenin greatly enhanced the elaboration of branches induced by Rho inhibition (Fig. 9, A and B). In PC12 cells cotransfected with GFP and C3 versus GFP-transfected PC12 cells, there was a two-fold increase in the number of primary processes and a 3.8-fold increase in the number of secondary processes. δ-PC12 cells cotransfected with C3 and GFP compared with δ-PC12 cells transfected with GFP showed a twofold increase in the number of primary processes and 9.2-fold increase in the number of secondary processes. When compared, the relative increase of secondary processes in δ-PC12 cells compared with PC12 cells was significant (Fig. 9 B). Because the secondary branches were usually quite short, the increased number of secondary branches did not significantly change the total summed length of the neurites. Thus, the inhibition of Rho GTPase with C3 changed the effect of δ-catenin on cell morphology from primary process extension to branch formation. In contrast, coexpression of a dominant-active Rho (V14-RhoA mutant) completely inhibited any δ-catenin-induced process formation (Fig. 9 A). The effect of C3 operates through Rho kinase as determined by the observation that the Rho kinase inhibitor Y27632 had a nearly identical effect as C3 in enhancing δ-catenin–induced branch formation (Fig. 9 B). Coexpression of the dominant-negative N17 mutants of Rac1 or Cdc42 or the dominant-active V12 mutants of Rac1 or Cdc42 with δ-catenin in PC12 cells had minimal morphological effects beyond the effects of δ-catenin alone (unpublished data). Inhibition of Rho in conjunction with δ-catenin greatly enhanced branching, whereas inhibition of tyrosine phosphorylation enhanced primary process extension. The independence of these growth pathways was supported by the finding that treatment of δ-PC12 cells with Y27632 did not induce the formation of δ-catenin–cortactin complexes despite the competency of these cells to elaborate branched processes (unpublished data).

The augmentation of branching by δ-catenin through Rho inhibition in contrast to the effects of inhibiting tyrosine kinases was even more apparent in the primary neurons (Fig. 9 C). C3 exotoxin had minimal effects on primary cultured neurons taken from embryonic day 18 rat hippocampus, but when cotransfected with δ-catenin C3 markedly exaggerated growth (Fig. 9 C). A profusion of new branches occurred over the entire dendritic tree rather than being confined to the more distal dendritic tree as occurred with δ-catenin expression alone. The increased branching may be due to de novo formation of branches or extension of preexisting filopodia. The length of the protrusions in the distal dendrites was significantly increased in δ-catenin–C3–transfected neurons (5.6 ± 0.7 μm) compared with δ-catenin–transfected alone (3.2 ± 0.37 μm) (P < 0.005). The number of protrusions in a 25-μm dendritic segment was 13.8 ± 0.65 in δ-catenin–C3–transfected cells compared with 10.5 ± 0.62 in δ-catenin–transfected cells (P = 0.006). In GFP-transfected control cells, the average length of the protrusions was 1.67 ± 0.17 μm, and C3 transfection did not cause a significant change (1.47 ± 0.11 μm). GFP-transfected cells and GFP–C3–transfected cells show an average of approximately five protrusions per 25-μm dendritic segment (5.16 ± 0.87 and 5 ± 0.7, respectively). In the case of RhoV14, we observed a simplification of the dendritic tree as described previously (Nakayama et al., 2000). Transfection of δ-catenin together with Rho V14 caused a significant decrease in the length of the protrusions (1.3 ± 0.12 μm) compared with δ-catenin–transfected cells (P < 0.001). δ-Catenin–RhoV14–cotransfected cells had an average of (6 ± 0.65) protrusions in a 25-μm dendritic segment, which differed significantly from cells transfected with δ-catenin alone (P < 0.001).

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**Figure 8.** Inhibition of Src family kinases enhances δ-catenin–induced primary process elongation. (A) δ-PC12 cells were treated with 100 ng/ml NGF for 36 h and then left untreated (a) or treated with PP2 for 1 h (b). Cells were labeled with a mAb δ-catenin. Bar, 5 μm. (B) Quantification of neurite length and number of primary and secondary processes before and after PP2 treatment. No secondary processes were observed in the absence or presence of PP2.
Dendrites of GFP–RhoV14-transfected neurons were completely retracted. Unlike Rho, coexpression of the dominant-negative N17 mutants of Rac1 or Cdc42 or the dominant-active V12 mutants of Rac1 or Cdc42 with δ-catenin in cultured hippocampal neurons had minimal additional morphological effects (unpublished data). The independence of the Rho inhibition and the tyrosine kinase inhibition growth pathways was supported by the finding that treatment of the primary neurons with LPA (10 μM for 10 min or 1 h), which activates RhoA (Tigyi et al., 1996), did not dissociate the δ-catenin–cortactin complex (unpublished data).

**Discussion**

**Tyrosine phosphorylation of δ-catenin–cortactin complex by an Src family kinase**

δ-Catenin is capable of inducing process elongation and branching, both of which require a reorganization of the actin cytoskeleton. The ability of δ-catenin to induce these processes is dependent upon its interaction with the tyrosine kinase Src. The δ-catenin–cortactin complex is a crucial mediator in these processes, and its tyrosine phosphorylation by Src family kinases is essential for the induction of process elongation and branching.
tin cytoskeleton. A sequence near the COOH terminus of δ-catenin just carboxy to the Arm repeats is required for these morphologic changes. This same region of δ-catenin binds to cortactin, and the data presented here suggested that the interaction is responsible for primary process extension. In support of this mechanism is the finding that constructs which lack the ability to bind cortactin cannot induce processes when transfected into PC12 cells or into primary neurons (Fig. 3). Thus, a functional property of δ-catenin maps to the same region as that of cortactin binding, making it likely that cortactin binding is required for some of the morphologic changes induced by δ-catenin.

The interaction between δ-catenin and cortactin is mediated by tyrosine phosphorylation as suggested by dissociation of the complex with increased Src family tyrosine kinase activity. Both δ-catenin and cortactin can be extensively tyrosine phosphorylated. Indeed, many proteins that are tyrosine phosphorylated by Src kinase family members are involved in actin organization including ezrin-radixin-moesin proteins, p120ctn, β-catenin, tensin, focal adhesion kinase, Crk-associated substrate, and actin filament-associated protein (Kanner et al., 1990). Moreover, cortactin directly and specifically binds endothelial cell myosin light chain kinase, and the interaction can be regulated by Src-mediated tyrosine phosphorylation of either protein (Dudek et al., 2002). Tyrosine phosphorylation of the potassium channel Kv1.2 also regulates its binding to cortactin (Hattan et al., 2002).

Positioned at a membrane proximate site, δ-catenin can influence dynamic cortical actin assembly through its recruitment of cortactin (Weed et al., 2000) in a phosphorylation-dependent manner. One potential role of the δ-catenin–cortactin interaction is to modulate the effects of cortactin on Arp2/3 actin polymerization. The Arp2/3 complex, comprising seven polypeptides, regulates both the formation and structure of actin networks directly (May, 2001). By dramatically increasing the nucleation rate, the Arp2/3 complex generates the large number of new filaments needed for actin network formation and helps create the branched network by cross-linking the slow growing pointed end of one filament to the side of another. However, Arp2/3 complex–mediated cross-links are relatively labile, and cortactin may stabilize Arp2/3 complex–mediated branches (Weaver et al., 2001). The role of cortactin and Arp2/3 complex in modulating the neuronal cytoskeleton in favor of neurite outgrowth is poorly understood, but δ-catenin could serve to localize protrusions to sites of neuronal activity. Both proteins also interact with postsynaptic scaffolding proteins: SHANK in the case of cortactin (Naisbitt et al., 1999) and S-SCAM in the case of δ-catenin (Ide et al., 1999). Multiple possibly redundant pathways leading to neurite extension converge around the Src kinase–mediated cortactin interactions. These include N-syndecan, a neurite outgrowth receptor that can bind the nonreceptor tyrosine kinase Src and its substrate, cortactin (Kinnunen et al., 1998), and the neural Wiskott-Aldrich syndrome protein

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Figure 10. **δ-Catenin and primary process extension versus branching.** Two different pathways regulate the effects of δ-catenin on process elaboration. Extracellular signals such as neurotrophins acting through regulation of Src family kinases can lead to δ-catenin–cortactin complex formation. The complex becomes competent to participate in primary process elongation by recruiting the Arp2/3 complex. Rho inhibition can be amplified by δ-catenin, which leads to branching.
(N-Wasp), which plays an essential role in neurite extension in PC12 cells and rat hippocampal primary culture cells (Banzai et al., 2000). N-Wasp and cortactin can bind simultaneously to the Arp2/3 complex and activate actin assembly (Weaver et al., 2002).

Modulation of neurite complexity

A balance between process elongation and branching is critical for the cell to achieve its ultimate shape. These two morphologic phenotypes may operate by distinct but not necessarily autonomous signaling pathways. Extensively branched processes regulated by Rho or primary process extension with minimal secondary branching regulated by Src are both induced in association with δ-catenin expression (Fig. 10). When Rho is inhibited, processes tend to branch, and when Src family tyrosine kinase activity is reduced, processes elongate without branching (Fig. 10). The ability of δ-catenin to enhance the effect of C3 could imply that C3 incompletely inhibits Rho-related proteins and δ-catenin allows further inhibition (indeed p120


totally inhibits RhoA), or δ-catenin affects related pathways similar to the ability of p120


to increase the activity of endogenous Cdc42 and Rac1 (Noren et al., 2000). A signaling node where these effects might be coordinated is through p190RhoGAP. The activity of some regulators of Rho such as p190RhoGAP can be modified by Src family phosphorylation (Arthur et al., 2000; Brouns et al., 2001). Stimuli that activate Src might modulate both pathways. An increase in tyrosine kinase as induced by H2O2 would have convergent inhibitory effects on process elaboration by both relieving the inhibition on Rho activity and dissociating the cortactin–δ-catenin complex. Under more physiologic circumstances, these effects might be regulated by neurotrophins, which can abolish Rho activation when bound to p75 receptor (Dailey and Smith, 1996) and, in the case of BDNF, activate Fyn kinase (Narisawa-Saito et al., 1999), and monoclonal δ-catenin antibody was from Transduction Laboratories, Inc. Polyclonal anticortactin antibody was from Santa Cruz Biotechnology, Inc. Monoclonal anti-MAP2 was from Chemicon, Inc., monoclonal antitubulin was from Sigma-Aldrich. Monoclonal anti-HA antibody was from Santa Cruz Biotechnology, Inc., and Alexa 594 goat anti-rabbit IgG, Alexa 488 goat anti-mouse IgG, and Phalloidin 594 were from Molecular Probes, Inc.

Plasmids

Cloning of full-length δ-catenin in pEGFP vector (CLONTECH Laboratories, Inc.) was described previously (Lu et al., 1999). Several δ-catenin deletion constructs in pEGFP were generated by restriction digests: ΔC205 and ΔC332 using the Nrl and Stul sites in δ-catenin, respectively. ΔC99 construct was generated by PCR using the QuickChange® Site-Directed Mutagenesis kit (Stratagene) for introducing two-stop codons right after aa number 1,148. FL ΔP6 lacking the polyproline track (219–224), was generated using the QuickChange® Site-Directed Mutagenesis kit (Stratagene).

The fusion proteins for GST–SH3 domain of mouse Fyn (85–139) and Lck (54–120) were from Santa Cruz Biotechnology, Inc. GST–mouse full-length cortactin and GST–CT–cortactin (aa 287–509) were provided by Sheila Thomas (Fred Hutchinson Cancer Research Center, Seattle, WA). Mouse cortactin–Flag construct in pCDNA3 was provided by J.T. Parsons (University of Virginia Health Sciences Center, Charlottesville, VA). Human Fyn and dominant-negative Fyn K299M constructs in pcDNA5 vector were provided by Marilyn D. Resh (Memorial Sloan Kettering Cancer Center, New York, NY). Plasmids corresponding to RhoV14 and C3 were provided by Jeff Settlemann (Massachusetts General Hospital, Charlestown, MA).

Immunoprecipitation and pull-down assays

For immunoprecipitation, cells were lysed in NP-40 buffer (10 mM Tris, pH 7.4, 1 mM EDTA, 1 mM EGTA, 50 mM NaCl, and 0.5% NP-40), incubated at 4°C for 30 min, and spun at 36,000 rpm for 30 min. Supernatants were precleared with protein G beads and normal mouse IgG for 30 min at 4°C. After preclearing, lysates were incubated with antibody or normal mouse IgG for 2 h. Protein G beads were added for an additional 2 h. Complexes were washed in NP-40 buffer three times, eluted in 2 × SDS buffer, and loaded in SDS-polyacrylamide gels. For δ-catenin immunoprecipitation, both monoclonal and polyclonal antibodies were used. For immunofluorescence and blotting, monoclonal δ-catenin antibody was used. GST–cortactin and GST–CT–cortactin in Escherichia coli and purified in glutathione-sepharose beads (Amersham Biosciences) by standard methods. Cell lysates in NP-40 buffer were incubated with GST fusion proteins bound to glutathione sepharose beads at 4°C for 30 min and washed three times in NP-40 buffer. Beads were resuspended in 2 × SDS buffer and loaded for SDS-PAGE.

Morphometry

The length of a particular process was measured by tracing a line from the cell body to the end of the process with MetaMorph software. In the PC12 cells, primary processes were defined as those emerging from the cell body. Secondary processes emerged from primary processes and were longer than 1.5 μm. Three independent experiments were quantified in all cases.

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Antibodies and other chemicals

An affinity-purified antibody (Ab62) raised against a δ-catenin peptide corresponding to aa 434–530 was described previously (Lu et al., 1999), and monoclonal δ-catenin antibody was from Transduction Laboratories, Inc. Polyclonal anticortactin antibody was from Santa Cruz Biotechnology, Inc. Monoclonal anti-MAP2 was from Chemicon, Inc., monoclonal antitubulin was from Sigma-Aldrich. Monoclonal anti-HA antibody was from Santa Cruz Biotechnology, Inc. Normal mouse IgG was from Santa Cruz Biotechnology, Inc. and Alexa 594 goat anti-rabbit IgG, Alexa 488 goat anti-mouse IgG, and Phalloidin 594 were from Molecular Probes, Inc. PP2 (a Src family tyrosine kinase inhibitor with specificity for Fyn, Lck, and Hick) and BDNF were purchased from Calbiochem and used at the concentrations indicated in Results. NGF was purchased from Roche, Inc.

Materials and methods

Cell culture and transfection

A stable δ-PC12 cell line was described previously (Lu et al., 2002), and PC12 cells were obtained from American Type Culture Collection. PC12 cell transfections used Lipofectamine 2000 (Invitrogen, Inc.). For the establishment of hippocampal cultures, embryos were removed from embryonic day 18 Sprague Dawley rats, and cultures were established as described (Rook et al., 2000). Primary hippocampal neurons were transiently transfected using Lipotectamine 2000. The media of cultures varying from 7 to 9 d in vitro was changed to DME containing 1 mM sodium sulyrate and 10 mM MgCl2, and cultures were incubated for 30 min at 37°C/5% CO2. During this incubation, 3 μl of lipotectamine was mixed with 150 μl of serum-free medium and added to a mixture of 150 μl serum-free medium plus 3 μg DNA. Precipitates were allowed to form during 20 min and were added drop wise above 25-mm coverslips. Cells were incubated at 37°C/5% CO2 during 2 h and returned to conditioned Neurobasal medium.

Immunofluorescence

For immunostaining, neurons cultured on coverslips were washed once in PBS and fixed with 4% PFA in PBS for 15 min RT, washed twice in PBS, and then permeabilized for 5 min in PBS containing 0.2% Triton X-100.
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