p120ctn associates with microtubules: inverse relationship between microtubule binding and Rho GTPase regulation

Clemens M. Franz and Anne J. Ridley

Ludwig Institute for Cancer Research, Royal Free and University College School of Medicine, 91 Riding House Street, London W1W 7BS, U.K and Department of Biochemistry and Molecular Biology, University College London, Gower Street, London WC1E 6BT, U.K.

Corresponding author: Prof Anne Ridley, Ludwig Institute for Cancer Research, 91 Riding House Street, London W1W 7BS, UK, Telephone + 44 (0) 20 7878 4033. Fax + 44 (0) 20 7878 4040. Email anne@ludwig.ucl.ac.uk

Running title: p120ctn associates with microtubules
Summary

p120ctn, an Armadillo protein and component of the cadherin adhesion complex, has recently been found to induce a dendritic morphology by regulating Rho family GTPases. We have identified specific serines within the Arm repeat domain which when mutated to alanine promote p120ctn association with interphase microtubules, leading to microtubule reorganization and stabilization. The mutant p120ctn also localized to the mitotic spindle and centrosomes. In contrast to wild-type p120ctn, the microtubule-associated p120ctn mutant did not activate Rac1 and did not induce a dendritic morphology. In addition, we show that a basic motif within the p120ctn Arm repeat domain known to be required for the inhibition of RhoA is also required for binding to microtubules. We therefore propose that binding of p120ctn to microtubules is inversely related to its ability to regulate Rho GTPases.
Introduction

p120 catenin (p120ctn) is a member of the armadillo family of proteins and localizes to intercellular junctions and the nucleus (1). In epithelial cells p120ctn localizes to adherens junctions by binding to the juxtamembrane domain of E-cadherin (2-6). In contrast to β- and γ-catenin, which act via α-catenin to link the E-cadherin complex to actin filaments, p120ctn does not appear to be involved in anchoring the E-cadherin complex to the actin cytoskeleton. Recently, p120ctn has been shown to have a supporting (7) but not essential role in Drosophila E-cadherin-mediated adhesion (7,8). It is nevertheless believed that p120ctn is important for proper E-cadherin-mediated adhesion in mammalian cells, although whether it acts positively (9,10) or negatively (11,12) to regulate E-cadherin clustering is not clear. p120ctn-binding may also have a stabilizing effect on the E-cadherin protein (13), possibly by preventing its ubiquitination and proteasomal degradation (14).

p120ctn was originally identified as a Src tyrosine kinase substrate (15) and the Src phosphorylation sites have recently been mapped (16). Tyrosine phosphorylation has been suggested to increase the affinity of p120ctn for cadherins (17,18) but the exact influence of tyrosine phosphorylation on p120ctn is unknown. p120ctn is also phosphorylated on a number of serine/threonine residues (12,19-22). The serine/threonine phosphorylation state of p120ctn correlates with its intracellular localization. Membrane-associated, E-cadherin–bound p120ctn is highly phosphorylated, whereas cytoplasmic p120ctn shows much reduced phosphorylation levels (10), suggesting that p120ctn is phosphorylated by membrane-associated kinases. Serine
phosphorylation events within the N-terminus of p120ctn have been suggested to regulate E-cadherin clustering (12).

In E-cadherin-deficient cancer cell lines, p120ctn is frequently observed in the nucleus (23) where it may interact with the transcription factor Kaiso (24). Matrilysin/MMP7, a matrix metalloproteinase, has been implicated as a target gene for Kaiso (25), but the significance of the p120ctn/Kaiso interaction remains to be established.

In addition to its role in cadherin-mediated adhesion, p120ctn has recently been found to be a regulator of Rho family GTPases (26), key players in coordinating a variety of cellular processes, including cell polarity, cell migration and cell adhesion (27). Overexpression of p120ctn in fibroblasts induces a so-called “dendritic morphology”, characterized by the extension of branching dendrite-like protrusions (5). The induction of a dendritic morphology by p120ctn involves inhibition of RhoA (28) and activation of Rac and Cdc42 (29,30). p120ctn might inhibit RhoA via a direct interaction (28), whereas the activation of Rac and Cdc42 has been suggested to occur via binding of the guanine nucleotide exchange factor Vav2 (30). The Arm repeat domain of p120ctn is required for the interaction with E-cadherin (4). There is evidence that the Arm repeat domain is also involved in the inhibition of RhoA (28), and cadherin-binding and Rho GTPase regulation have been proposed to be mutually exclusive events (30).

We report here that p120ctn can associate with microtubules and that this requires a basic motif in the Arm repeat domain. Our results with different p120ctn mutants suggest that binding of p120ctn to microtubules is inversely related to its ability to regulate Rho GTPases.
Experimental procedures

Cell culture MDA-MB-231 and Cos-7 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% bovine fetal calf serum (FCS), penicillin (100 IU/ml) and streptomycin (100 µg/ml). For NIH 3T3 fibroblasts, FCS was substituted with donor calf serum (DCS). Cells were grown in a humidified atmosphere containing 10% CO₂ (COS-7, NIH 3T3) or 5% CO₂ (MDA-MB-231) and passaged every 3 to 4 days or before reaching confluency.

Antibodies The following antibodies were used: mouse anti-VSV glycoprotein clone P5D4, mouse monoclonal anti-β-tubulin TUB 2.1, mouse monoclonal anti-acetylated tubulin clone 6-11B-1, rabbit polyclonal anti-γ-tubulin (all Sigma), mouse monoclonal anti-p120ctn (Transduction Laboratories), rabbit anti-Rac1 (Upstate Biotechnology), fluorescein isothiocyanate (FITC)-conjugated donkey anti-mouse IgG, Cy5-conjugated goat anti-mouse IgG (both Jackson Immunoresearch), TRITC-conjugated goat anti-rabbit IgG (Southern Biotechnology Associates), and sheep HRP-conjugated anti-mouse IgG (Amersham Pharmacia).

Transfection For electroporation, Cos-7 cells were grown in 15-cm tissue culture dishes to 95% confluency and split 1:2 the day before transfection. Plasmid DNA (10 µg) was electroporated into 2x10^7 cells in electroporation buffer (120 mM KCl, 10 mM K₂PO₄/KH₂PO₄ pH 7.6, 2 mM MgCl₂, 25 mM Heps pH 7.6, 0.5% Ficoll 400) using a Biorad Gene pulser at 250 V, 960 mF. Subsequently, cells were diluted 1:100 in growth medium and 0.5 ml of this dilution was added per 13-mm coverslip. For Lipofectamine 2000
(Invitrogen) transfections, 5 x 10^4 MDA-MB-231 cells were seeded per coverslip in 4-well plates the day before transfection and transfections were carried out according to the manufacturer’s protocol. Polyfect (Qiagen) was used to transfect NIH 3T3 cells. Cells were seeded at 2 to 4 x 10^4 cells per coverslip in 4-well plates the day before transfection and the transfection performed following the manufacturer’s instructions. Cells were left to express for 16 to 24 h before being lysed or fixed.

**Plasmid construction** To introduce a VSV-tag at the C-terminus of p120ctn, p120ctn-GFP (Noren et al., 2000) was digested with BsmBI and EcoRI to yield a 2.4-kb fragment. Using primers p120VSVF (5’ CGC-TGA-GAA-CTT-AGA-AGC-TGC 3’) and p120VSVR (5’GCT-AGC-TAG-CAA-TCT-TCT-GCA-TCA-AGG-GTG-CTC-C 3’), the C-terminal end of p120ctn was amplified and the KpnI restriction site was replaced with an NheI site by PCR, then digested with BsmBI and NheI to produce a fragment containing the last 424 bases of p120ctn-1A, followed by the NheI site. The EcoRI/BsmBI and BsmBI/NheI fragments of p120ctn were then ligated in tandem into EcoRI/NheI-digested pW5 (pcDNA3, into which a VSV-tag, preceded by an NheI site, had been incorporated, kindly provided by Dr G.Cory). Changes of p120ctn amino acids 538/539 and 587 and deletion of amino acids 622-628 were performed using the QuickChange Site-Directed Mutagenesis Kit (Stratagene). The correct sequence of all constructs was verified by sequencing.

**Rac pulldown assay** NIH 3T3 cells were seeded at 5 x 10^5 cells in 10-cm tissue culture plates. After 24 h cells were transfected with pcDNA3 vector (control), p120ctn-VSV or
538/539/587AAA-p120ctn-VSV using Polyfect transfection reagent. After expression for 24 h, cells were washed with ice-cold phosphate-buffered saline (PBS), lysed in 0.5 ml of 1 x Rac pulldown buffer ((RPB) 25 mM Hepes pH 7.5, 150 mM NaCl, 1% NP-40, 10 mM MgCl₂, 1 mM EDTA, 2% glycerol, 10 µg/ml leupeptin, 10 µg/ml aprotinin, 1 mM NaF, 1 mM Na₃VO₄) and immediately centrifuged for 3 min at 13000 g. Soluble supernatant (50 µl) was kept to determine total Rac levels in the cell lysate; the rest of the supernatant was incubated with 10 µl of PAK-1-PBD agarose beads (Upstate Biotechnology) for 1 h. For in vitro GTPγS/GDP loading of lysates as positive and negative controls, 20 µl 0.5 M EDTA, pH 8.8, and either GTPγS (final concentration 100 µM) or GDP (final concentration 1 mM) was added to 0.5 ml of lysate. After loading for 15 min at 30°C, nucleotide exchange was stopped by adding MgCl₂ to a final concentration of 60 mM and Rac-GTP was precipitated by incubation of the loaded lysates with 10 µl of PAK-1-PBD agarose beads at 4°C for 30 min. All beads were washed 3 times in RPB and resuspended in SDS sample buffer. Protein concentrations of the lysates were determined by Bradford protein assay and volumes containing equal amounts of proteins were loaded onto 10% polyacrylamide gels. Following SDS-PAGE and western blotting, pulldown samples and lysate samples were probed with antibodies against Rac1. Membranes carrying the lysate samples were stripped and reprobed with antibodies to the VSV epitope to determine expression levels of the transfected p120ctn. Band intensities were quantitated using Bio-Rad’s Quantity One software.

**Immunohistochemistry and image collection** Cells were fixed in 4% paraformaldehyde/PBS for 20 min and permeabilized with 0.2% Triton X-100/PBS for 5
min. The cells were subsequently incubated for 1 h with primary antibodies (anti-VSV 1:1000, anti-β-tubulin 1:200, anti-γ-tubulin 1:200, diluted in 0.5% BSA/PBS) followed by incubation with secondary antibodies (FITC-conjugated anti-mouse 1:100, TRITC-conjugated anti-rabbit 1:100, Cy5-conjugated anti-mouse 1:250) or with 1 µg/ml TRITC-phalloidin for 45 min and finally mounted in Moviol (Calbiochem). Confocal laser scanning microscopy was carried out with an LSM 510 (Zeiss, Welwyn Garden City, UK) mounted over an affinity-corrected Axioplan microscope (Zeiss) using a x40 1.3 NA oil immersion objective.
Results

Specific serine to alanine point mutations in the Arm domain promote association of p120ctn with interphase microtubules

We have recently identified several novel serine phosphorylation sites in p120ctn ([32] and unpublished data). As part of these studies, a number of candidate serine residues in the N- and C-terminus and in the Arm repeat domain of p120ctn were mutated to alanine residues. We did not detect any serine phosphorylation sites within the Arm repeat domain of p120ctn. However, expression of the constructs in Cos-7 cells showed that two p120ctn mutants carrying serine to alanine mutations within the Arm repeat domains 4 and 5 (SS538/539AA and S587A) localized along interphase microtubules in approximately 20% of transfected cells, whereas wild-type p120ctn did not localize to microtubules in these cells (Figure 1A and 1C). When the two mutations (SS538/539AA and S587A) were combined in one protein (SSS538/539/587AAA or AAA-p120ctn) the percentage of transfected Cos-7 cells showing p120ctn/microtubule colocalization increased to approximately 30% (Figure 1A and 1C). Proteins containing the single mutations S538A or S539A only rarely localized along microtubules (data not shown). Localization of AAA-p120ctn, but not wild-type p120ctn, along microtubules was also observed in the fibroblast cell lines, Swiss 3T3 cells (data not shown) and NIH 3T3 cells (see Figure 4A). In these cells, AAA-p120ctn was associated with microtubules in more than 80% of transfected cells. The association of p120ctn with microtubules did not appear to depend primarily on the expression levels of the mutants. Cells expressing similar amounts of AAA-p120ctn (as judged by comparing fluorescence intensities of neighbouring cells) showed either near-complete p120ctn/microtubule colocalization or
diffuse cytoplasmic and sometimes nuclear distribution of p120ctn. In some Cos-7 cells showing a low level of AAA-p120ctn expression, the staining for p120ctn along microtubules was not evenly distributed but was concentrated in distinctive puncta (Figure 1B). The association of AAA-p120ctn with microtubules is likely to be indirect, as it did not co-immunoprecipitate with α- or β-tubulin nor was it enriched in a detergent-insoluble microtubule-containing cell fraction (data not shown).

AAA- p120ctn and endogenous p120ctn localize to centrosomes and the mitotic spindle during mitosis
Association of AAA-p120ctn with tubulin-containing structures was not restricted to interphase microtubules. In Cos-7 cells, AAA-p120ctn localized to both the mitotic spindle and to centrosomes during mitosis (Figure 2A). Most of the exogenous p120ctn appeared to be associated with the mitotic spindle but a fraction of mutant p120ctn showed a punctate localization around the circumference of the cell. In contrast to the clear localization to the two mitotic centrosomes, AAA-p120ctn could never be detected at the single interphase centrosome, suggesting that the association of p120ctn with centrosomes occurs exclusively during mitosis. Consistent with this observation, a fraction of endogenous p120ctn was detected at centrosomes or the pericentrosomal region in the breast cancer cell line MDA-MB-231 cells during mitosis (Figure 2B). The recruitment of endogenous p120ctn to the centrosomes in these cells suggests a physiological role for the interaction of p120ctn with tubulin-containing structures during cell division. During interphase, no clearly defined centrosomes could be identified in these cells by γ-tubulin staining. It was therefore not possible to establish whether the
p120ctn/centrosome association also occurs in interphase. Localization of endogenous p120ctn to centrosomes was not observed in Cos-7 or NIH 3T3 cells, possibly because the expression level of p120ctn in these cells is too low to allow detection of a centrosome-associated pool.

*Association of p120ctn with microtubules leads to their stabilization*

p120ctn association with microtubules changed their morphology: they formed long, thick and curly bundles and were in some cases arranged in circles around the nucleus (Figure 1A). This is in contrast with typical interphase microtubules in fibroblasts, which originate from the MTOC and extend their (+) ends towards the plasma membrane. Microtubule bundling into large multi-filament structures has been linked to microtubule stabilization (33). During their maturation from unstable, highly dynamic to stabilized microtubules, the α-tubulin subunit of the tubulin dimer undergoes a series of modifications (34,35), such as acetylation of a C-terminal lysine. An anti-acetylated α-tubulin antibody was used to confirm that p120ctn binding to microtubules had a strong stabilizing effect on microtubules in MDA-MB-231 cells (Figure 3). Only a low level of acetylated tubulin was detected in untransfected cells or in cells transfected with GFP alone (Figure 3A’), whereas the thick microtubule cables in the AAA-p120ctn-GFP expressing cells stained very strongly for acetylated tubulin (Figure 3B’). Expression of wild-type p120ctn did not increase the level of acetylated α-tubulin in the body of the cell. Microtubules within the p120ctn-driven cellular extensions, however, showed strong staining for acetylated tubulin (Figure 3C’), suggesting that wild-type p120ctn may stabilize microtubules exclusively in these extensions.
**AAA-p120ctn does not induce a dendritic morphology or activate Rac1**

Overexpression of wild-type p120ctn is known to induce extension of protrusions (dendritic morphology) (5). In contrast, AAA-p120ctn did not stimulate branching in Cos-7 cells (Figure 1A). Rather, cells expressing AAA-p120ctn appeared to be more spread than untransfected cells. The p120ctn-induced dendritic morphology is especially prominent in NIH 3T3 fibroblasts (5), and this cell line was therefore chosen to quantitate the ability of wild-type and AAA-p120ctn to induce dendritic extensions. In preliminary experiments VSV-tagged p120ctn was found to be more potent in eliciting a dendritic morphology than p120ctn-GFP, although p120ctn-VSV and p120ctn-GFP showed the same intracellular distribution (data not shown). Consequently, wild-type p120ctn-VSV and AAA-p120ctn-VSV constructs were used to quantitate morphological responses.

Overexpression of wild-type p120ctn-VSV induced a dendritic morphology in more than 70% of transfected cells (Figure 4B). The cell body was highly constricted around the nucleus and cells showed a number of long protrusions, some of which extended for more than twenty times the cell body. The ends of the extensions often showed extensive arborisation reminiscent of dendritic spines. In contrast, AAA-p120ctn-VSV caused branching in less then 10% of transfected cells, those extensions that formed were short, and the cells appeared spread out (Figure 4A).

Overexpression of p120ctn in fibroblasts activates Rac and Cdc42 (29,30). Active Rac1 and Cdc42 are required for the p120ctn-induced branching: coexpressing dominant-negative versions of Rac1 or Cdc42 with p120ctn efficiently blocks branching (29,30). To determine whether the reason for the failure of AAA-p120ctn to induce
branching is the loss of its ability to activate Rac1, Rac pulldown experiments were performed (36). NIH 3T3 cells were transiently transfected with either wild-type p120ctn-VSV, AAA-p120ctn-VSV or with empty vector as a control. In a parallel transfection of p120ctn-GFP, the transfection efficiency was estimated to be between 50 and 70%. The pulldown experiments showed that wild-type p120ctn increased the levels of active Rac1 in the cells more than 2-fold (Figure 5A and B). AAA-p120ctn, however, did not raise active Rac1 levels significantly above the levels obtained when vector alone was transfected. Because of the level of transfection efficiency, the true level of Rac1 activation in response to wild-type p120ctn expression is underestimated in the pulldown experiments, which measure active Rac1 levels averaged over both transfected and untransfected cells. However, the difference in Rac1 activity between wild-type p120ctn and AAA-p120ctn-transfected cells was statistically highly significant (P<0.01). These results suggest that mutant, microtubule-associated p120ctn fails to induce branching because it is unable to activate Rac1.

Deletion of a basic motif in the Arm repeat domain of AAA-p120ctn prevents microtubule association and induction of the dendritic morphology

Association of AAA-p120ctn with microtubules coincided with the inability of this mutant to induce a dendritic morphology. To clarify further the relationship between microtubule binding and the dendritic morphology, we attempted to identify a region in p120ctn required for both functions. A cluster of lysines between p120ctn amino acids 622 to 628 (KKGKGGK), situated on a looped-out structure within Arm repeat 6 (1), has been shown to be required for p120ctn to induce a dendritic morphology in NIH 3T3
cells (28). In agreement with this finding, wild-type p120ctn-GFP missing the basic motif between amino acids 622 to 628 (ΔK-p120ctn-GFP) did not induce a dendritic morphology in Cos-7 cells (Figure 6). To test if this basic motif is also involved in microtubule binding, amino acids 622 to 628 were deleted in the microtubule-targeted protein AAA-p120ctn-GFP (ΔK-AAA-p120ctn-GFP). Deletion of the basic motif completely prevented localization of AAA-p120ctn to microtubules in Cos-7 cells (Figure 6). Substitution of lysines 622/623 with isoleucines also blocked microtubule association, whereas p120ctn carrying isoleucines at positions 627/628 associated with microtubules to the same degree as AAA-p120ctn, showing that lysines 627/628 are not required for microtubule binding (data not shown). Therefore, parts of the basic motif are involved in both the interaction of p120ctn with microtubules and the induction of the Rho GTPase-dependent dendritic morphology.
Discussion

p120ctn is known to associate with adherens junctions, regulate the activity of Rho GTPases and under some conditions translocate to the nucleus, but its association with microtubules has not previously been reported. We report here that p120ctn localization to microtubules is promoted by mutating three serines to alanines in the Arm repeat domain, and is also dependent on a lysine-rich motif. Binding of p120ctn to microtubules and its ability to activate Rac and induce a dendritic morphology are mutually exclusive.

The serines 538/539 and 587 mutated to alanines in AAA-p120ctn are highly conserved in p120ctn family members. In other Arm repeats of p120ctn, the consensus residue at the equivalent position is either serine or alanine (1), indicating that alanine residues at these positions are compatible with Arm repeat folding. The increased association of AAA-p120ctn with microtubules may reflect a structural change in the Arm repeat domain so that a microtubule-binding motif, such as the KKGKGKK motif between amino acids 622 and 628, is now more exposed. Alternatively, it is possible that the serines are phosphorylation sites, and that their phosphorylation reduces microtubule binding. Introduction of alanines at these sites would then prevent phosphorylation and microtubule dissociation. However, so far we have no evidence that these serines are phosphorylated, either from mass spectrometry (32) or from use of phospho-specific antibodies (unpublished data).

The observations that AAA-p120ctn localizes to the mitotic spindle and centrosomes and that endogenous p120ctn can localize to the pericentrosomal region during mitosis suggests that it might regulate microtubule organization around the centrosome. Drosophila melanogaster APC2 and armadillo have been shown to be
required for anchoring the mitotic spindle to cortical actin (37,38). AAA-p120ctn showed some punctate localization around the cell cortex during mitosis and could similarly be involved in anchoring astral spindle microtubules to the cortex. Interestingly, p120ctn is the only known component of the E-cadherin complex to undergo a change in its phosphorylation state during mitosis (39), consistent with it playing a role in spindle organization.

AAA-p120ctn occasionally showed a punctate localization along microtubules, similar to that observed for proteins or vesicles that interact with microtubules via motor proteins such as kinesins or dyneins. It is therefore possible that p120ctn is transported actively along microtubules. In agreement with our results, p120ctn has recently been reported to localize to perinuclear microtubules in cadherin-deficient cell lines and this has been suggested to involve association with kinesin (31). Other Arm repeat proteins have been shown to bind to motor proteins: β-catenin interacts with the motor protein dynein (40), whereas APC travels along microtubules by binding to kinesin superfamily proteins (41).

The inability of AAA-p120ctn to induce a dendritic morphology correlated with its lack of effect on Rac1 activity, suggesting that microtubule association and Rac1 activation are inversely related functions of p120ctn. p120ctn has been proposed to regulate Rho GTPases in at least two different ways: it inhibits RhoA via a direct interaction (28) and it activates Rac and Cdc42 via the exchange factor Vav2 (30). Both the activation of Rac/Cdc42 and the inhibition of RhoA are required for p120ctn to induce the dendritic morphology (28-30). However, expression of constitutively active Rac1 or Cdc42 alone is not sufficient to induce the dendritic morphology, possibly
because they need to cycle between active and inactive forms to stimulate extension (42). In contrast, inhibition of RhoA or its target ROCK is sufficient to induce neurite outgrowth and branching (43,44). Given that the activity levels of Rac and RhoA are usually inversely related, that increased Rac activity leads to decreased RhoA activity (45,46), and inhibition of RhoA signalling can induce an increase in Rac activity (47), p120ctn could affect the activities of both Rho and Rac by directly affecting the activity of only one. Interestingly, the basic motif KKGKGKK between amino acids 622 and 628, which we found to be required for p120ctn to associate with microtubules, is also required for the inhibitory effect of p120ctn on LPA-induced RhoA activity (28). Because p120ctn uses the same motif to bind to microtubules and to inhibit RhoA, microtubule association could lead to RhoA displacement and termination of the inhibitory effect on RhoA, and consequently to a decrease in Rac1 activity. Alternatively, microtubule binding could prevent Vav2 interaction and Rac activation and thereby indirectly prevent the decrease in RhoA activity.

A number of microtubule-associated proteins (MAPs), such as MAP-1B (48) and the yeast protein CBF5 (49), interact with microtubules via tubulin-binding motifs containing characteristic repeats of double lysines, which resemble the KKGKGKK motif of p120ctn. This basic motif is highly conserved among the p120ctn family members (1) and has been proposed to be a nuclear localization signal (50). We observed nuclear localization of ∆K-mutants in some transfected cells, though the nuclear localization of ∆K-mutants was much reduced compared to wild-type or AAA-p120ctn (unpublished data). The association of p120ctn with the microtubule network has recently been suggested to counteract nuclear import of p120ctn (31). In contrast, our data indicates
that p120ctn mutants which are unable to associate with microtubules show decreased nuclear localization. The precise functional link between microtubule-binding and nuclear translocation of p120ctn remains to be determined.

The association of p120ctn with microtubules leads to their remodelling into thick, curly bundles and to their stabilization as indicated by the increase in α-tubulin acetylation. Similarly, microtubules within p120ctn-driven extensions show a high degree of acetylation compared to microtubules in the cell body. It is therefore possible that p120ctn normally interacts with and stabilizes microtubules exclusively in protrusions. Interestingly, extension of neurites, like the p120ctn-induced dendritic morphology, requires Rac and Cdc42 activation and RhoA inhibition as well as microtubule polymerisation (51,52). In addition, microtubules become stabilized during neurite outgrowth, correlating with an increase in their acetylation (53-55). The combined abilities of p120ctn to stabilize microtubules, regulate Rho GTPases and induce a dendritic phenotype make it a prime candidate for playing an important role in promoting neurite outgrowth. In support of this idea, p120ctn localizes to growth cones and to dendritic spines in cultured hippocampal neurons and its expression is increased during rat brain development (56) and to neurites containing acetylated tubulin in NGF-treated PC12 neuronal cells (our unpublished data). NPRAP/δ-catenin, a closely related neuronal member of the p120ctn family, has recently been shown to enhance dendritic morphogenesis in primary hippocampal neurons and to induce dendrite-like processes in 3T3 fibroblasts, activities which required the stabilization of microtubules (57,58). Similarly, overexpression of p120ctn induces extensive dendritic extensions in PC12 cells (our unpublished data). The dendritic morphology observed in p120ctn-
overexpressing fibroblasts could therefore reflect a physiological function for p120ctn in neurons, where it would promote neurite outgrowth by stabilizing microtubules and regulating Rho GTPases.
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Figure Legends

FIG. 1. **Association of mutant p120ctn with interphase microtubules.** *A*, Cos-7 cells were electroporated with wild-type p120ctn-GFP (WT), 538/539AA-p120ctn-GFP (538/539AA), 587A-p120ctn-GFP (587A), or 538/539AAA-p120ctn-GFP (538/539/587AAA) plasmids and fixed after expression for 24 h. Cells were then stained with antibodies to β-tubulin and confocal images were collected, detecting the GFP-proteins by direct fluorescence (A-D) and β-tubulin by indirect immunofluorescence (A’-D’). Bar, 10 µm. *B*, A higher magnification image of a well-spread Cos-7 cell expressing low levels of 538/539/587AAA-p120ctn-GFP (AAA), showing a punctate localization of mutant p120ctn along microtubules. Bar, 5 µm. *C*, Cos-7 cells were transfected and processed for immunofluorescence analysis in (A). Images of at least ten random fields of view per coverslip were collected and the percentage of transfected cells displaying p120ctn/microtubule colocalization for the different p120ctn serine to alanine mutants determined. Results are the average (±S.D.) of three independent experiments (538/539AA = 22.7±6.5%, 587A = 19.5±5.1%, 538/539/587AAA = 31.9±10.1%). *D*, Diagram of the Arm repeat domain (amino acids 352 to 824) of p120ctn. Asterisks indicate the position of the alanine mutations introduced at residues 538/539 and 587 near the end of Arm repeat 4 and 5, respectively. Arm repeats 4, 6 and 9 contain inserted sequences (1), indicated by white bars. A basic motif between amino acids 622 and 628 is part of the insertion within Arm repeat 6 and was deleted to generate ΔK-p120ctn mutants (see FIG. 6).
FIG. 2. **Association of p120ctn with the mitotic spindle and centrosomes.**  

* A, Cos-7 cells were transfected with 538/539/587AAA-p120ctn-VSV (AAA) and fixed after expression for 24 h. Metaphase cells were identified by their condensed chromosomes, detected with Hoechst staining (not visible in the confocal image). Centrosomes were visualized by staining with antibodies against γ-tubulin and p120ctn by anti-VSV antibodies.  

* B, MDA-MB-231 cells were fixed and stained for endogenous p120ctn and γ-tubulin. Mitotic cells were identified by their spherical, contracted morphology and by Hoechst staining. Images were collected at the plane of the centrosomes. Bar, 10 µm.

FIG. 3. **Association of microtubules with AAA-p120ctn stabilizes microtubules.**  

MDA-MB-231 cells were transfected with a GFP expression vector (A, A’, A’’) 538/539/587AAA-p120ctn-GFP (AAA) (B, B’, B’’) or wild-type p120ctn-GFP (wt) (C, C’, C’’) and fixed after 24 h. Stable microtubules were visualized by staining for acetylated tubulin (Ac-Tub) (A’, B’, C’) and GFP proteins were visualized by direct fluorescence (A, B, C). To show the outline of cells, actin filaments were stained with TRITC-phalloidin (F-actin) (A’’, B’’, C’’). Bar, 10 µm.

FIG. 4. **AAA-p120ctn does not cause branching in NIH 3T3 cells.**  

* A, NIH 3T3 cells were seeded at 2.5 x 10⁴ cells per coverslip, grown for 24 h and then transfected with 0.8 µg wild-type p120ctn-VSV or AAA-p120ctn-VSV. Cells were fixed after 16 h and stained with anti-VSV antibodies. Representative images of cells expressing wild-type (WT) or AAA-p120ctn (AAA) are shown.  

* B, To quantitate p120ctn-
induced branching, images of at least ten random fields of view per coverslip were collected. Transfected cells were counted as showing branching if they showed two or more cellular extensions each at least three times the diameter of the nucleus. Results are the average (±S.D.) of three independent experiments (p<0.001).

FIG. 5. **Wild-type p120ctn but not AAA-p120ctn activates Rac1.** A, NIH 3T3 cells (1 x 10⁶) were seeded in 10-cm dishes and after 24 h transfected with 10 µg of empty vector (vec), wild-type p120ctn-VSV (wt) or AAA-p120ctn-VSV (AAA) plasmids. After expression for 16 h, cells were lysed, 30 µl of the soluble fraction retained to determine total Rac1 levels and Rac-GTP precipitated on PAK1-RBD beads from the remainder of the soluble fraction. As positive and negative controls, lysates from cells expressing empty vector were loaded with 100 µM GTPγS or 1 mM GDP. Total Rac1 levels in the lysates (“lysate”), as well as active Rac1 levels (“pulldown”) were determined by western blotting using a Rac1-specific antibody. Lysate blots were reprobed with antibodies against the VSV-epitope to show equal expression levels of the p120ctn-VSV proteins. B, Total and active Rac1 levels were quantitated by densitometry. Active Rac1 level in cells expressing the different constructs were normalized for total Rac1 levels and averaged (±S.D.) over three independent experiments (p<0.009).

FIG. 6. **A basic motif is required for the association of p120ctn with microtubules.** The basic motif KKGKGGKK between amino acids 622 to 628 in p120ctn (see FIG. 1C) was deleted in wild-type p120ctn-GFP (ΔK-p120ctn-GFP) and AAA-p120ctn-GFP (ΔK-
AAA-p120ctn-GFP). Cos-7 cells were transfected by electroporation with wild-type p120ctn-GFP (WT), AAA-p120ctn-GFP (AAA), ΔK-p120ctn-GFP (ΔK) or ΔK-AAA-p120ctn (ΔK-AAA) and fixed after 16 h. The p120ctn constructs were detected by direct immunofluorescence (A-D), and microtubules were visualized by staining with antibodies against β-tubulin (A’-D’). Bar, 10 µm.
Figure 1
Figure 2

A

AAA

γ-tubulin

B

p120ctn

γ-tubulin
Figure 3
Figure 4

A

WT

AAA

B

Percentage of transfected cells displaying dendritic phenotype

|       | WT         | AAA       |
|-------|------------|-----------|
| Value | 70         | 10        |

***
Figure 5

A

| Pulldown | GTPγS | GDP |
|----------|-------|-----|
| Lysate   | vec   | WT  | AAA |

anti-Rac

anti-Rac

anti-VSV

B

![Graph showing relative Rac activity](image)

- vector
- WT
- AAA

Relative Rac activity

0.5
1.0
1.5
2.0
2.5
3.0

**
Figure 6