p120 Catenin Regulates the Actin Cytoskeleton via Rho Family GTPases

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Abstract. Cadherins are calcium-dependent adhesion molecules responsible for the establishment of tight cell–cell contacts. p120 catenin (p120ctn) binds to the cytoplasmic domain of cadherins in the juxtamembrane region, which has been implicated in regulating cell motility. It has previously been shown that overexpression of p120ctn induces a dendritic morphology in fibroblasts (Reynolds, A.B., J. Daniel, Y. Mo, J. Wu, and Z. Zhang. 1996. *Exp. Cell Res.* 225:328–337.). We show here that this phenotype is suppressed by coexpression of cadherin constructs that contain the juxtamembrane region, but not by constructs lacking this domain. Overexpression of p120ctn disrupts stress fibers and focal adhesions and results in a decrease in RhoA activity. The p120ctn-induced phenotype is blocked by dominant negative Cdc42 and Rac1 and by constitutively active Rho-kinase, but is enhanced by dominant negative RhoA. p120ctn overexpression increased the activity of endogenous Cdc42 and Rac1. Exploring how p120ctn may regulate Rho family GTPases, we find that p120ctn binds the Rho family exchange factor Vav2. The behavior of p120ctn suggests that it is a vehicle for cross-talk between cell–cell junctions and the motile machinery of cells. We propose a model in which p120ctn can shuttle between a cadherin-bound state and a cytoplasmic pool in which it can interact with regulators of Rho family GTPases. Factors that perturb cell–cell junctions, such that the cytoplasmic pool of p120ctn is increased, are predicted to decrease RhoA activity but to elevate active Rac1 and Cdc42, thereby promoting cell migration.

Key words: cadherin • Rac • guanine nucleotide exchange factor • Vav2 • migration

Introduction

Cell–cell adhesion is critical to many aspects of multicellular existence, including morphogenesis, tissue integrity, cell–cell communication, normal cell growth, and differentiation (Takeichi, 1995; Vleminckx and Kemler, 1999; Gumbiner, 2000). Disrupted cell–cell adhesion is often observed in cancer cells and correlates with tumor invasion (Birchmeier and Behrens, 1994). Cadherins are a major class of homophilic cell adhesion molecules that mediate Ca^{+2}-dependent, cell–cell interactions (Takeichi, 1991; Gumbiner, 1996). These abundant adhesion molecules are responsible for the prominent adherens junctions found in epithelial tissues. At these cell–cell contacts, cadherins provide adhesion between adjacent cells, as well as sites for attachment of the actin cytoskeleton (Takeichi, 1995; Yap et al., 1997; Adams and Nelson, 1998). In addition, cadherin engagement has been implicated in signal transduction (Barth et al., 1997; Steinberg and McNutt, 1999). Attachment to the cytoskeleton is mediated through the binding to several cytoplasmic proteins, called catenins. β-catenin (armadillo) or γ-catenin (plakoglobin) bind directly to the distal region of the cadherin cytoplasmic tail (Aberle et al., 1994; Hulsken et al., 1994; Funayama et al., 1995; Jou et al., 1995) and interact with α-catenin, which associates directly or indirectly with actin filaments (Herrenkneft et al., 1991; Knudsen et al., 1995; Rimm et al., 1995; Niest et al., 1997).

Cell–cell adhesion is often inhibitory to cell migration, a phenomenon that has been referred to as "contact inhibition" (Abercrombie, 1979). In some situations, cadherins have been implicated in the suppression of migration, and loss of E-cadherin from epithelial tumors has been correlated with the acquisition of an invasive phenotype. Reexpression of E-cadherin has been found to suppress invasion of many tumor cells (Frixen et al., 1991; Vleminckx et al., 1991). Originally, the effects of E-cadherin on cell invasion and migration were interpreted as being due to the adhesive properties of E-cadherin, providing a mechanical restraint that impedes cell migration (Birchmeier and Behrens, 1994; Ben-Ze'ev and Geiger, 1998). Because at-

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tachment to the cytoskeleton via β- and α-catenin had been implicated in the development of strong adhesion, it was also anticipated that the β-catenin–binding site of the cytoplasmic domain would be critical in these inhibitory effects. However, a study aimed at analyzing functional sites within the cytoplasmic domain of E-cadherin identified the juxtamembrane region as the domain affecting motility and invasion (Chen et al., 1997). Similarly, the juxtamembrane region of N-cadherin was also found to be critical for the inhibition of neurite outgrowth by a dominant negative N-cadherin construct (Riehl et al., 1996). The juxtamembrane region has also been implicated in both positive and negative regulation of adhesion (Ozawa and Kemler, 1998; Yap et al., 1998; Ohkubo and Ozawa, 1999; Aono et al., 1999; Thoreson et al., 2000). Three proteins have been identified that bind to the highly conserved cadherin juxtamembrane region, p120 catenin (p120ctn[1]; Lampugnani et al., 1997; Yap et al., 1998; Ohkubo and Ozawa, 1999; Thoreson et al., 2000), δ-catenin/NPRAP/neurojungin (Paffenholz and Franke, 1997; Zhou et al., 1997; Lu et al., 1999), and ARVCF (Sirotkin et al., 1997; Mariner et al., 2000). Whereas p120ctn is widely distributed (Daniel and Reynolds, 1997), δ-catenin/NRAP/neurojungin expression is restricted to cells of the nervous system (Paffenholz and Franke, 1997; Zhou et al., 1997). ARVCF is ubiquitously expressed, but at a much lower level than p120ctn (Mariner et al., 2000). The interaction of p120ctn with the juxtamembrane region of cadherins, together with its relative prominence in non-neuronal cells raises the possibility that p120ctn participates in the inhibitory action of cadherins on cell migration.

p120ctn belongs to the Armadillo family of proteins that includes β- and γ-catenins (Reynolds et al., 1992; Peifer et al., 1994) and is the founding member of an emerging subfamily of Armadillo-domain proteins that includes δ-catenin/NRAP/neurojungin, ARVCF, p0071, and plakophilin 1, 2, and 3 (reviewed in Anastasiadis and Reynolds, 2000). p120ctn was originally discovered as a tyrosine-phosphorylated protein in Src-transformed cells (Reynolds et al., 1989), and it has been shown to be tyrosine phosphorylated in cells transformed by Ras (Kinch et al., 1995) and stimulated by various growth factors (Downing and Reynolds, 1990; Kanner et al., 1991). Recently, p120ctn was shown to bind the transcription factor, Kaiso (Daniel and Reynolds, 1999), and in some situations it has been detected within the nucleus (van Hengel et al., 1999). Overexpression of p120ctn in fibroblasts results in a striking morphological phenotype characterized by long dendritic processes and filopodial extensions (Reynolds et al., 1996).

The interaction of p120ctn with the juxtamembrane region of cadherins, which has been implicated in the regulation of cell migration and invasion, together with the dramatic morphological change in cells overexpressing p120ctn, has led us to investigate the effects of p120ctn on the actin cytoskeleton. In particular, we have been interested in whether p120ctn impacts the Rho family of low molecular mass GTP-binding proteins, thereby regulating the organization of the actin cytoskeleton. Here we show that the dendritic phenotype induced by p120ctn overexpression is inhibited by dominant negative forms of Cdc42 or Rac1 and by a constitutively active RhoA effector, Rho-kinase. Overexpression of p120ctn stimulates Cdc42 and Rac1 activation, but diminishes RhoA activity. We demonstrate that p120ctn interacts with the Rho family guanine nucleotide exchange factor (GEF) Vav2, which is required for the p120ctn-induced cytoskeletal changes. Additionally, we demonstrate that the p120ctn dendritic phenotype is suppressed by expression of cadherin constructs containing the juxtamembrane region. Expression of cadherins that lack this p120ctn-binding site fail to suppress the phenotype. Our results suggest that p120ctn can exist either bound to cadherins, where it is unable to affect the structure of the actin cytoskeleton, or in a cytoplasmic pool, where it can interact with Vav2 and possibly other regulators of Rho family activity. The distribution of p120ctn between cadherin-bound and cytoplasmic pools provides one mechanism for cross-talk between cadherin-mediated cell–cell junctions and the motile machinery of cells.

Materials and Methods

Cell Culture

CHO cells were grown in DME supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 1% nonessential amino acids (Sigma-Aldrich). NIH3T3 cells were cultured in DME containing 10% bovine calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. HEK293 and MDCK cells were grown in DME supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. CHO and NIH3T3 cells were grown in 10% CO_2 at 37°C and HEK293 and MDCK cells were grown in 5% CO_2 at 37°C.

Generation of Mammalian Expression Plasmids

To construct a p120-GFP fusion protein, a cDNA fragment of 336 bp encoding the COOH terminus of p120ctn was generated by PCR using the plasmid pRcCMVp120 86a (Reynolds et al., 1992) as a template. pRcCMVp120 86a was kindly provided by Dr. Al Reynolds (Vanderbilt University School of Medicine, Nashville, Tennessee). Using primers 5′-GGC-TAT-AAG-GAG-CTT-CGG-AAG-3′ and 5′-AGA-GGG-TAC-CTT-AAT-CTT-CTG-CAT-CAA-GGG-TGC-TCC-3′, the stop codon was eliminated and replaced by a KpnI site. The 2.6-kb EcoRI-XbaI fragment of pRcCMVp120 86a encoding the remaining NH_2-terminal portion of p120ctn was ligated in tandem with the XbaI- and KpnI-restricted PCR product into the EcoRI and KpnI sites of pEGFP-N1 (CLONTECH Laboratories Inc.). The resulting plasmid pEGFP-p120 was sequenced in both directions to verify the structure of the fusion protein as well as the absence of secondary mutations.

Vav2 green fluorescent protein constructs were engineered as described (Liu and Burridge, 2000). N17Rac1, N17Cdc42 and N19RhoA were gifts from Dr. Marc Symons (Onyx Pharmaceuticals, Richmond, CA). Constitutively active Rho-kinase was a gift from Dr. K. Kaibuchi (Nara Institute of Science and Technology, Nara, Japan). C-cadherin constructs were a generous gift from Dr. Barry Gumbiner and Dr. Carien Niessen (Memorial Sloan-Kettering Cancer Center, New York, NY; Yap et al., 1998). In summary, the cadherin construct, CT, contains a truncation of the predicted cytoplasmic domain replaced by a myc tag. The CT669 construct contains a replacement of the COOH-terminal 56 amino acids (starting at residue 669) with a 6X-myc tag. The CT-CAT construct contains a substitution of the predicted cytoplasmic sequence from the transmembrane-cytoplasmic junction to residue 670 by a 5X-myc tag.

Transient Transfection

Cells were transfected using Lipofectamine Plus reagents (GIBCO BRL) according to manufacturer's directions. For cotransfection experiments

[1]*Abbreviations used in this paper:* DN, dominant negative; GAP, GTPase activating proteins; GEF, guanine nucleotide exchange factor; GFP, green fluorescent protein; p120ctn, p120 catenin.

cells were transfected with a 1:1 ratio of the various DNA constructs. Cells were used for various assays at 20–24 h post-transfection.

Immunofluorescence

Cells were plated on glass coverslips the day before transfection at 5×10^6 cells per 60-mm tissue culture dish. Cells were fixed in 3.7% formaldehyde in PBS for 15 min, permeabilized with 0.5% Triton X-100 in TBS (50 mM Tris-HCl, 150 mM NaCl) for 3 min, washed in TBS, blocked for 30 min in TBS containing 1% BSA and 10% goat serum, and stained with appropriate antibodies. Cells were incubated with primary antibodies for 1 h, washed, and incubated with secondary antibody for 1 h. p120ctn monoclonal antibody (Transduction Laboratories) was used at 1:500 dilution, 9E10 (anti-myc antibody) monoclonal antibody (Sigma-Aldrich) was used at 1:1,000, C-cadherin polyclonal antibody was used at 1:1,000 (kindly provided by Dr. Barry Gumbiner and Dr. Carien Niessen, Memorial Sloan-Kettering Cancer Center, New York, NY), rhodamine-conjugated phalloidin (Molecular Probes, Eugene, OR) was used to visualize actin at 1:1,000 dilution, fluorescein (FITC)-conjugated goat anti–mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) was used at 1:100 and rhodamine-conjugated anti–mouse antibody (Chemicon) was used at 1:40. Images were obtained on an Axiophot microscope (ZEISS), using a MicroMAX 5 MHz cooled CCD camera (Princeton Instrument) and Metamorph Image software (Universal Imaging Corp.).

Migration Assay

To examine the role of p120ctn in cellular migration, we performed migration assays using a Transwell® cell culture chamber containing polycarbonate membrane inserts with 8-μm pores (Corning Costar Corp.). The undersides of the membranes were coated with fibronectin (10 μg/ml) in DME for 1 h at 37°C, then blocked for 0.5 h in 2% BSA in DME. Membranes were washed in PBS and 0.5 ml of DME was added to the lower chamber. Transfected CHO cells expressing either GFP or p120-GFP (10^6 cells/chamber) were allowed to migrate at 37°C for 2 h. Membranes were washed in PBS for 5 min, fixed in 3.7% formaldehyde in PBS for 10 min, permeabilized for 3 min in 0.5% Triton X-100 in PBS, washed for 5 min in PBS, and subsequently membranes were stained with DAPI (Sigma-Aldrich). Migrating cells were detected by DAPI staining or by GFP fluorescence.

Immunoprecipitations

Cells were lysed in TBS containing 1% Triton X-100, 2 mM EDTA, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM PMSF, 10 mM NaF, and 0.5 mM sodium orthovanadate. Lysates were clarified by centrifugation for 10 min in an Eppendorf microfuge at 14,000 rpm. The supernatants were first incubated with protein A–Sepharose beads (Chemicon International) for 45–60 min at 4°C to reduce nonspecific binding. Beads were pelleted and the supernatants were incubated with appropriate antibodies for 1 h at 4°C. Immunocomplexes were captured with protein A–Sepharose beads for 1 h with rotation. Beads were pelleted by centrifugation and washed four times with lysis buffer. Immunoprecipitated proteins were released from the beads by boiling in sample buffer and electophoresed on 10 or 15% polyacrylamide gels (Laemmli, 1970). Proteins were electrophoretically transferred to nitrocellulose or PVDF, blocked with 5% dried milk protein in TBS containing 0.1% Tween-20 and probed with appropriate antibodies as previously described (Chrzanowska-Wodnicka and Burridge, 1994). Blots were probed with anti-p120ctn monoclonal antibody (Transduction Labs) at 1:1,000, anti-Vav2 rabbit antiserum raised against the COOH terminus of Vav2 (Liu and Burridge, 2000) at 1:20,000, anti-Sos1 monoclonal antibody at 1:250 (Transduction Laboratories), anti–E-cadherin monoclonal antibody (C20820) at 1:2,500 (Transduction Laboratories), anti-p190RhoGAP monoclonal antibody at 1:1,000 (Transduction Laboratories), anti-C3G rabbit polyclonal antibody at 1:250 (Santa Cruz) and anti-GFP rabbit polyclonal antibody (CLONTECH Laboratories Inc.) at 1:100. Blots were developed for chemiluminescence using SuperSignal Substrate (Pierce Chemical Co.).

Subcellular Fractionation

Subcellular fractionation was essentially performed as described by Thoreson et al. (2000). In brief, cells were washed three times with PBS, and allowed to swell for 15 min in hypotonic buffer (10 mM Tris-HCl, pH 7.4, 1 mM $MgCl_2$, 1 mM EDTA, 1.5 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 mM NaF, and 0.5 mM sodium orthovanadate). Cells were then scraped and homogenized using 10 strokes with a loose Dounce homogenizer and 10 strokes with a tight Dounce homogenizer. 5 M NaCl was added to a final concentration of 0.15 M and nuclei were removed by centrifugation for 3 s at 14,000 rpm. The supernatant was then fractionated by ultracentrifugation for 30 min at 100,000 *g* at 4°C. The pellet was washed two times with PBS. Both the pellet and supernatant were reconstituted to an equal volume containing a final concentration of 1× Laemmli sample buffer. Fractions were analyzed by SDS-PAGE and Western blotting.

Rac1, Cdc42, and RhoA Activity Assays

The Rac1 and Cdc42 assays were performed as described (Bagrodia et al., 1998). GTP-bound Rac1 and Cdc42 were affinity precipitated using the Rac1/Cdc42–binding domain of PAK (PBD). Bound proteins were resolved on 15 or 17.5% SDS-PAGE and immunoblotted using anti-Rac1 (1:1,000) and anti-Cdc42 antibodies (1:250; Transduction Labs). The PBD was a gift from R. Cerione and S. Bagrodia (Cornell University, Ithaca, NY; Bagrodia et al., 1995). Densitometric analysis of films was performed using the Metamorph Image system (Universal Imaging). The relative amounts of active Rac1 or Cdc42 were determined by measuring the amount of Rac1 or Cdc42 sedimented by the GST-PBD relative to the total amount of Rac1 or RhoA in the whole cell lysates.

Measurement of GTP-bound RhoA was performed as described previously (Ren et al., 1999) using the RhoA-binding domain of Rhotekin expressed as a GST-fusion protein. The cDNA of the RhoA-binding domain (RBD) of Rhotekin comprising of amino acids 7–89 was cloned into the pGEX-2T vector and expressed as a GST fusion protein (kindly provided by Dr. L. Petch, University of North Carolina at Chapel Hill, NC).

PhosphorImager Analysis

For quantitation of RhoA levels, Western blots were probed with ECF Western blotting kit according to manufacturer's instructions (Amersham Pharmacia Biotech). Samples were quantified by chemifluorescence analysis using a Molecular Dynamics Storm imaging system. Values were then normalized for protein concentration and for amount of total RhoA in the whole cell lysates.

Results

Overexpression of p120ctn Induces a Loss of Stress Fibers and Focal Adhesions

Reynolds et al. (1996) have shown a striking morphological change in fibroblasts overexpressing p120ctn. These cells are characterized by long branching processes reminiscent of the arborized dendritic extensions in neurons. Since the formation of neuronal dendritic extensions is dependent on the remodeling of the actin cytoskeleton by small GTPases of the Rho family (Jalink et al., 1994; Kozma et al., 1997; Lamoureux et al., 1997; van Leeuwen et al., 1997), this phenotype suggests that p120ctn plays a role in actin reorganization. To directly monitor the influence of p120ctn on the structure of the actin cytoskeleton, we have generated a construct in which p120ctn has been fused to green fluorescent protein (GFP). Overexpression of both p120-GFP and p120ctn in NIH3T3 cells generates a phenotype (Fig. 1 A) similar to the one described by Reynolds et al. (1996). Analyzing the organization of actin in p120-GFP overexpressing cells that remain well spread reveals a loss of stress fibers in transfected cells (Fig. 1 B). The loss of stress fibers is accompanied by a strong reduction in the number and size of focal adhesions (Fig. 1 C). In contrast, control transfections with GFP did not affect the organization of actin or focal adhesions (Fig. 1, B and C). Overexpression of p120ctn or p120-GFP in a variety of cell types, including CHO and Rat1 fibroblasts, confirms that the decreased stress fibers and focal adhesions are not cell type

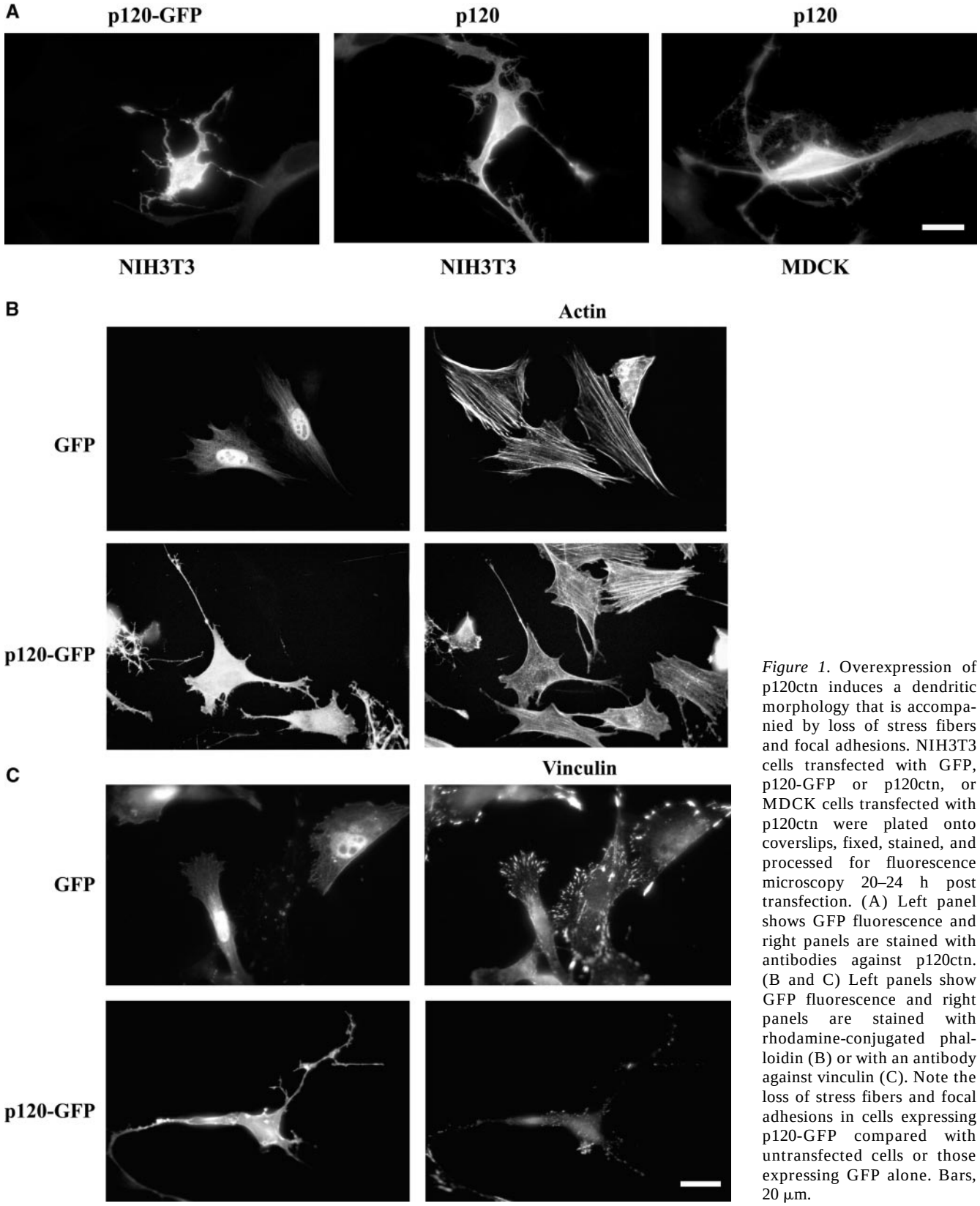


Figure 1. Overexpression of p120ctn induces a dendritic morphology that is accompanied by loss of stress fibers and focal adhesions. NIH3T3 cells transfected with GFP, p120-GFP or p120ctn, or MDCK cells transfected with p120ctn were plated onto coverslips, fixed, stained, and processed for fluorescence microscopy 20–24 h post transfection. (A) Left panel shows GFP fluorescence and right panels are stained with antibodies against p120ctn. (B and C) Left panels show GFP fluorescence and right panels are stained with rhodamine-conjugated phalloidin (B) or with an antibody against vinculin (C). Note the loss of stress fibers and focal adhesions in cells expressing p120-GFP compared with untransfected cells or those expressing GFP alone. Bars, 20 μm.

specific (data not shown). Moreover, the morphological effects of p120ctn overexpression are not limited to fibroblasts as p120ctn induces a dendritic phenotype in epithelial cells such as MDCK (Fig. 1 A, right), HEK293 and Hela cells (data not shown).

Cadherin Expression Suppresses the p120ctn-induced Phenotype

To examine whether the p120ctn-induced dendritic phenotype can be influenced by cadherins, we coexpressed

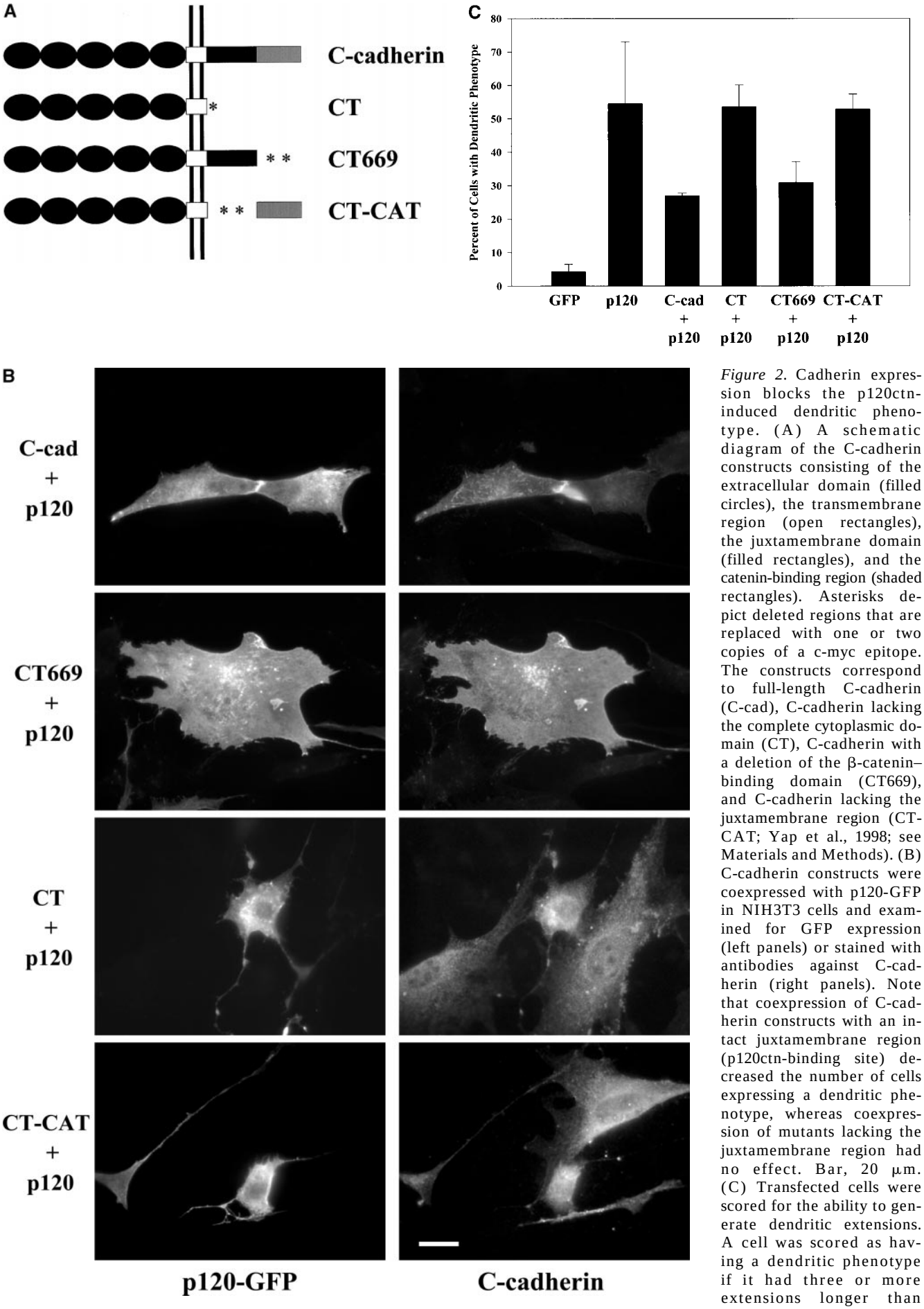


Figure 2. Cadherin expression blocks the p120ctn-induced dendritic phenotype. (A) A schematic diagram of the C-cadherin constructs consisting of the extracellular domain (filled circles), the transmembrane region (open rectangles), the juxtamembrane domain (filled rectangles), and the catenin-binding region (shaded rectangles). Asterisks depict deleted regions that are replaced with one or two copies of a c-myc epitope. The constructs correspond to full-length C-cadherin (C-cad), C-cadherin lacking the complete cytoplasmic domain (CT), C-cadherin with a deletion of the β-catenin–binding domain (CT669), and C-cadherin lacking the juxtamembrane region (CT-CAT; Yap et al., 1998; see Materials and Methods). (B) C-cadherin constructs were coexpressed with p120-GFP in NIH3T3 cells and examined for GFP expression (left panels) or stained with antibodies against C-cadherin (right panels). Note that coexpression of C-cadherin constructs with an intact juxtamembrane region (p120ctn-binding site) decreased the number of cells expressing a dendritic phenotype, whereas coexpression of mutants lacking the juxtamembrane region had no effect. Bar, 20 μm. (C) Transfected cells were scored for the ability to generate dendritic extensions. A cell was scored as having a dendritic phenotype if it had three or more extensions longer than the cell body. At least 100 cells coexpressing C-cadherin constructs and p120-GFP were counted for each experiment. Data are the means ± SD of four independent experiments.

C-cadherin constructs with p120-GFP in NIH3T3 cells. These constructs (illustrated in Fig. 2 A) correspond to wildtype C-cadherin (C-cad), a truncated mutant lacking the cytoplasmic domain (CT), a deletion mutant lacking the β-catenin–binding site (CT669), and a deletion mutant lacking the juxtamembrane region (p120ctn-binding site; CT-CAT; Yap et al., 1998). Expression of C-cad in NIH3T3 cells causes cells to adopt a less fibroblastic morphology characterized by the presence of stable cell–cell contacts. Coexpression of C-cad and p120-GFP suppresses the dendritic phenotype and results in colocalization of p120-GFP with C-cad at cell–cell junctions (Fig. 2 B, top, and C). Immunoprecipitation analysis revealed that C-cadherin associates with transfected p120-GFP (data not shown).

Coexpression of CT669 also decreases the number of cells exhibiting the p120ctn-induced dendritic phenotype (Fig. 2, B and C). We noted that cells expressing high levels of C-cad or CT669 generally resulted in more efficient suppression of the dendritic phenotype induced by p120ctn. The level of expression of the cadherin constructs was quite variable and this variability probably accounts for the overall suppression of the dendritic phenotype being ~50% (Fig. 2 C). In contrast, coexpression of C-cadherin mutants lacking an intact p120ctn-binding site (CT and CT-CAT) had no inhibitory effect (Fig. 2, B and C). These observations indicate that expression of cadherin constructs containing the juxtamembrane region suppresses the p120ctn-induced phenotype. We noted that many cells expressing both p120ctn and C-cad or the CT669 construct did not exhibit the dendritic phenotype even in the absence of stable cell–cell contacts. This observation suggests that elevating the level of cadherins competent to bind p120ctn is sufficient to suppress the dendritic morphology and that assembly of cell–cell junctions is not necessary for this effect.

To determine if p120ctn overexpression elevates the cytosolic pool of p120ctn, we performed a detergent-free subcellular fractionation as described by Thoreson et al. (2000). This assay employs a hypotonic lysis buffer to fractionate the cells into cytosolic (C) and membrane (M) fractions. We compared p120-GFP-transfected cells to untransfected NIH3T3 cells at a confluency where the majority of cells are making cell–cell contacts. In untransfected cells, p120ctn fractionates to both the cytosol and membrane fractions, whereas in p120-GFP-transfected cells, more p120ctn was detected in the cytosol (Fig. 3). Since only a fraction of cells (<50%) are transfected, we calculated that the level of cytosolic p120ctn is increased ~2–2.5-fold in transfected cells compared with untransfected cells. Together with the suppression of the phenotype by cadherin overexpression, these results suggest that the cytosolic level of p120ctn is critical in promoting the dendritic phenotype.

Inhibition of the p120ctn-induced Phenotype by Dominant Negative Rac1, Dominant Negative Cdc42 and Constitutively Active Rho-Kinase

The dendritic morphology, as well as the loss of stress fibers and focal adhesions induced by p120ctn overexpression, led us to examine if p120ctn is able to regulate Rho family GTPases. To determine whether the p120ctn-mediated morphological changes are dependent on the activity

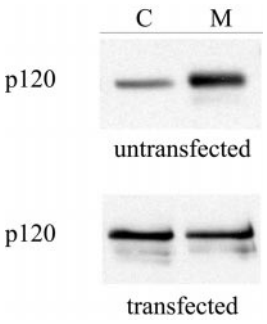


Figure 3. p120ctn overexpression increases the cytosolic pool of p120ctn. The relative distribution of p120ctn in cytosolic (C) and membrane (M) fractions was compared in both untransfected and p120-GFP-transfected NIH3T3 cells. Fractions were analyzed by SDS-PAGE and blotted with antibodies against p120ctn.

of Rho family GTPases, we coexpressed c-myc–tagged dominant negative forms of either Rac1 or Cdc42 with p120-GFP in NIH 3T3 cells. Coexpression of N17Rac1 blocks the p120ctn effects (Fig. 4 A), thereby decreasing the number of cells exhibiting the dendritic phenotype (Fig. 4 B). Similarly, coexpression of N17Cdc42 suppresses the p120ctn-induced phenotype (Fig. 4, A and B). Equivalent results were obtained in Hela cells (data not shown). In contrast, coexpression of a dominant negative form of RhoA, N19RhoA, enhances the dendritic phenotype, resulting both in an increase in cells exhibiting the dendritic phenotype and an increase in length and branching of extensions (data not shown).

Because overexpression of p120-GFP disrupts stress fibers and focal adhesions, we wanted to examine if this could be prevented by activation of the RhoA pathway. Rho-kinase is a downstream effector of RhoA that regulates contractility by phosphorylating, and thus inhibiting myosin phosphatase (Kimura et al., 1996). Coexpression of a constitutively active form of Rho-kinase suppresses the p120ctn-induced dendritic phenotype (Fig. 4, A and B). The effects of the dominant negative constructs, together with the observation that overexpression of p120-GFP induces a loss of stress fibers and focal adhesions, suggest that p120ctn might be a negative regulator of RhoA activity and a positive regulator of Rac1 and Cdc42 activity.

p120ctn Stimulates Rac1 and Cdc42 Activation but Decreases RhoA Activation

To confirm that p120ctn overexpression indeed leads to the activation of Rac1 and Cdc42, we performed an affinity precipitation assay to measure the amount of GTP-Rac1 and GTP-Cdc42 in lysates of transfected cells (Bagrodia et al., 1998; Benard et al., 1999). This assay makes use of the highly specific interaction of the GTP-bound forms of Cdc42 or Rac1 with p21-activated kinase (PAK), a downstream effector of these GTPases (Bagrodia et al., 1995; Manser et al., 1994). Using a GST-fusion protein containing the Cdc42- and Rac1-binding domain of PAK (PBD), activated Cdc42 and Rac1 can be isolated. After overnight recovery, transfected CHO cells expressing either GFP or p120-GFP were lysed and assayed for active Rac1 and Cdc42. Overexpression of p120-GFP induces a 1.9 ± 0.5-fold activation of Rac1 and a 3.1 ± 0.7-fold activation of Cdc42 (Fig. 5, A and B). Immunoblots of the cell lysates show that the levels of endogenous Rac1 and Cdc42 were identical in transfected cells. Cell lysates were also blotted with antibodies against GFP and p120ctn to analyze expression levels of these proteins in transfected cells (Fig. 5

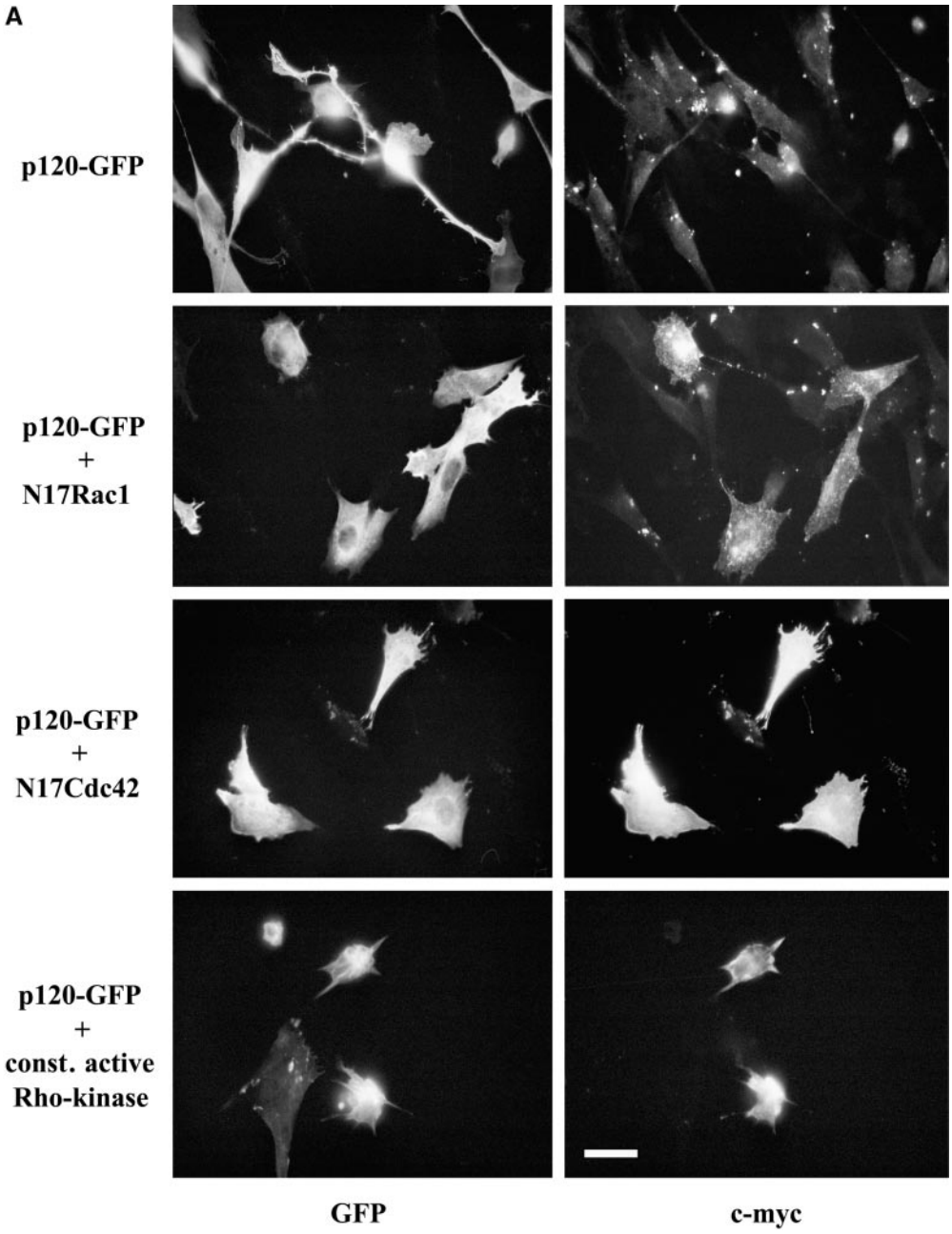
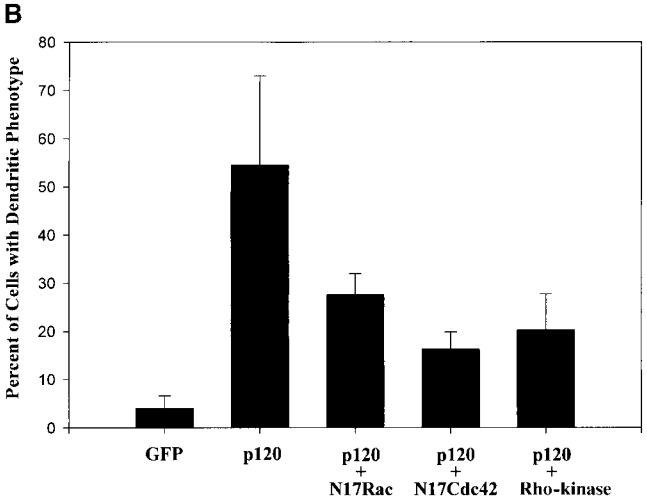


Figure 4. The p120ctn-induced dendritic phenotype is suppressed by coexpressed N17Rac1, N17Cdc42 and constitutively active Rho kinase. NIH3T3 cells were plated on coverslips and transfected with p120-GFP alone, p120-GFP and N17-Rac1, p120-GFP and N17-Cdc42, or p120-GFP and Rho-kinase. 20–24 h post transfection, cells were processed for fluorescence microscopy as described in Materials and Methods. Left panels show GFP expression and right panels are stained for c-myc to reveal the cells expressing N17Rac1, N17 Cdc42 and Rho-kinase. Bar, 20 μm. (B) Transfected cells were scored and compared for their ability to generate dendritic extensions (see Fig. 2 C). Data are the means ± SD of four separate experiments.



C). The expression level of p120-GFP is typically two- to threefold higher than that of endogenous p120ctn.

We also examined whether the observed loss of stress fibers and focal adhesions in cells overexpressing p120-GFP is a direct consequence of decreased RhoA activity. We performed an affinity precipitation assay similar to the one described above using a GST fusion protein containing the RhoA-binding domain of Rhotekin, a known target of RhoA (Ren et al., 1999). The activity of RhoA was measured in CHO cells transfected with either GFP or p120-GFP. As shown in Fig. 6 A, overexpression of p120-GFP decreases RhoA activity by ~45%. This reduction in Rho activity is derived from measuring the activity in the entire population and suggests that for transfected cells the level of reduction of RhoA activity is 90% or greater (assuming ~50% transfection efficiency).

Serum starvation of cells results in a decrease in RhoA

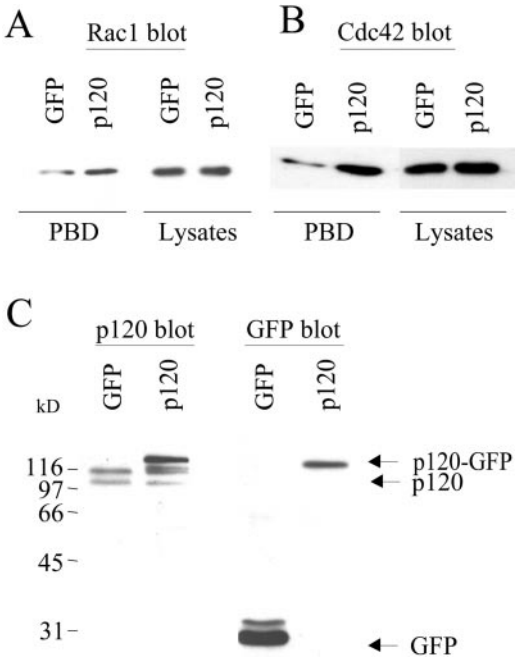


Figure 5. p120ctn induces Rac1 and Cdc42 activation. The level of active GTP-bound Rac1 (A) or GTP-bound Cdc42 (B) was measured in CHO cells transfected with either GFP or p120-GFP. Rac1 and Cdc42 activity assays were performed as described in Materials and Methods using a GST fusion protein derived from PAK (PBD) that selectively binds GTP-bound Rac1 and Cdc42. GST-PBD precipitations were immunoblotted with monoclonal antibodies recognizing Rac1 or Cdc42. Cell lysates were blotted with these antibodies to compare total levels of endogenous Rac1 and Cdc42 in transfected cells. In this representative experiment, p120-GFP induces a 2.4-fold activation of Rac1, the mean of four separate experiments being 1.9 ± 0.5. p120-GFP stimulates a 3.0-fold activation of Cdc42 in the representative experiment shown, the mean of three separate experiments being 3.1 ± 0.7. (C) The level of expression of p120-GFP and GFP in transfected cells was compared by blotting whole cell lysates with antibodies against p120ctn (left) or GFP (right). Endogenous p120ctn was detected as two bands, and p120-GFP was detected migrating above these. p120-GFP expression is usually two- to threefold higher than that of endogenous p120ctn.

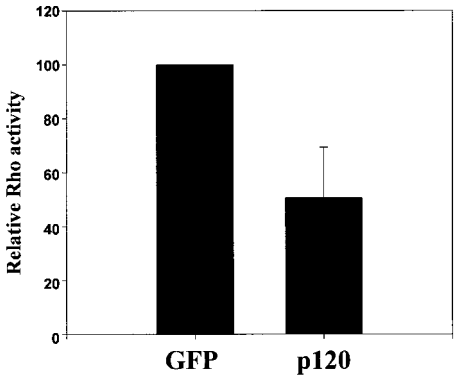


Figure 6. p120ctn decreases RhoA activity. The amount of GTP-bound RhoA was measured using a GST fusion protein containing the RhoA-binding domain of Rhotekin (RBD) that selectively binds only GTP-bound RhoA. GST-RBD precipitations were probed with a monoclonal antibody against RhoA. The amount of active RhoA was measured in CHO cells expressing either GFP or p120-GFP. Relative RhoA activity was determined using a phosphorimager as described in Materials and Methods. Data are the means ± SEM of three independent experiments.

activity, which correlates with the disruption of stress fibers and focal adhesions (Ren et al., 1999). We wanted to determine the relevance of the p120ctn-induced inhibition of RhoA by comparing it to the RhoA inhibition resulting from serum starvation. Activated RhoA was measured in CHO cells either kept in medium containing 10% serum or starved for 18 h in the presence of 0.5% serum. Under these conditions of serum starvation, there was a ~75% decrease in RhoA activity (data not shown). The decrease in RhoA activity brought about by overexpression of p120-GFP is in the same range as or even greater than the decrease resulting from serum starvation.

Overexpression of p120ctn Stimulates Cell Migration

Given that p120ctn stimulates Rac and Cdc42 activity and downregulates RhoA activity, we wanted to see if these biochemical effects correlated with biological responses that are known to be regulated by the Rho family GTPases. Rac activity is necessary for cellular migration; Cdc42 activity regulates the polarity of migratory cells; whereas excessive RhoA activity inhibits migration (Nobes and Hall, 1999). To examine the role of p120ctn in cellular migration, we overexpressed p120-GFP in CHO cells. These cells show changes in activity of Rho family GTPases (see above) but they develop a less arborized morphology compared with NIH3T3 cells. CHO cells were transfected with GFP or p120-GFP and plated in the absence of serum on transwell filters, the lower surface of which had been coated with fibronectin. Overexpression of p120ctn results in a twofold increase in cellular migration towards fibronectin ($P < 0.001$; Fig. 7). Migration was not studied in cell types in which p120ctn induced a pronounced dendritic phenotype. However, observing the development of the dendritic morphology by video microscopy revealed that it resulted from active motility leading to localized outgrowth (data not shown).

p120ctn Binds Vav2, a Guanine Nucleotide Exchange Factor for Rho Family GTPases

We sought to determine the mechanism by which p120ctn could increase the activity of Rac1 and Cdc42. The level of GTP-bound to Rho family GTPases is regulated by GEFs and GTPase activating proteins (GAPs). We examined the potential association of GEFs or GAPs with endogenous p120ctn from HEK293 cells that express higher levels of endogenous p120ctn compared with fibroblast cell lines. p120ctn was immunoprecipitated from HEK293 cells and immunoprecipitates were analyzed by blotting with antibodies against known exchange factors such as Sos1, C3G, and Vav2 and against p190RhoGAP, a RhoA-specific GAP (Fig. 8 A). Our results indicate that Vav2, a ubiquitously expressed GEF, selectively associates with p120ctn, but not Sos1, C3G, and p190RhoGAP. Control immunoprecipitations did not sediment Vav2 (Fig. 8 A, bottom).

The reverse experiment was also performed. Vav2 was

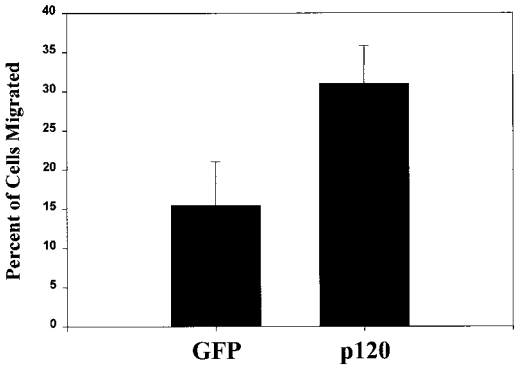
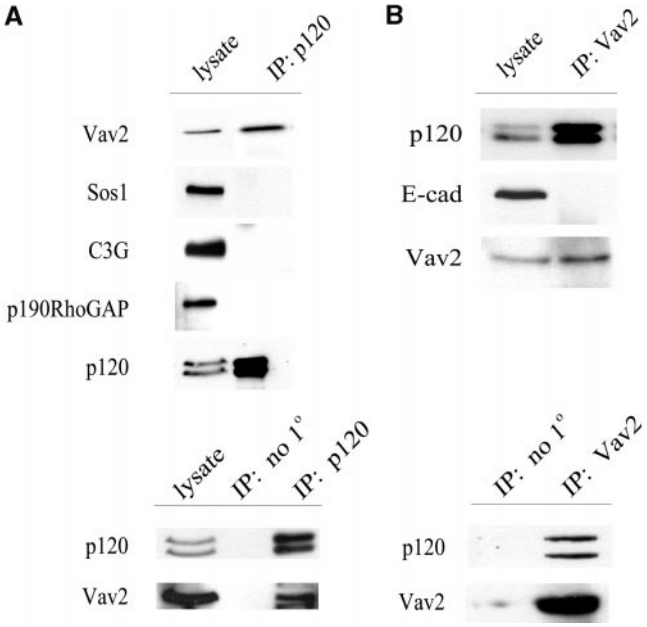


Figure 7. Overexpression of p120ctn stimulates cell migration. Migration assays were performed on CHO cells transfected with GFP or p120-GFP. Cells were plated in serum-free medium on a transwell membrane, coated on the underside with fibronectin (10 μg/ml). Cells migrating through the membrane were identified using fluorescence microscopy and counted. The graph represents the means of three separate experiments ± SEM ($P < 0.001$).



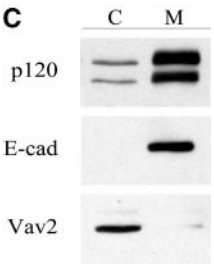


Figure 8. p120ctn associates with the guanine nucleotide exchange factor Vav2. (A) Endogenous p120ctn was immunoprecipitated from confluent HEK293 cells and electrophoresed adjacent to a HEK293 lysate. After transfer to nitrocellulose, it was probed with antibodies against the GEFs Vav2, Sos1, and C3G. p120ctn immunoprecipitates were also probed with antibodies against p190-RhoGAP. Blots were reprobed for total amount of p120ctn in lysates and in immunoprecipitations. (Lower panel) p120ctn immunoprecipitations and control immunoprecipitations using rabbit anti–mouse IgG were blotted for associated Vav2 and reprobed for the amount of p120ctn in lysates and immunoprecipitates. (B) Endogenous Vav2 was immunoprecipitated from HEK293 cells and probed with antibodies against p120ctn and E-cadherin. Vav2 was immunoprecipitated from HEK293 cell lysates and the immunoprecipitates probed with anti-p120ctn antibody or anti-Vav2 antibody (bottom). Lane 1 is a control immunoprecipitation in which protein A–Sepharose beads were used alone. (C) Detergent-free subcellular fractionation was performed to reveal the subcellular distribution of endogenous p120ctn, E-cadherin, and Vav2 in HEK293 cells (see Materials and Methods). Cytosolic (C) and membrane (M) fractions were analyzed by SDS-PAGE and blotted with antibodies against p120ctn, E-cadherin, and Vav2.

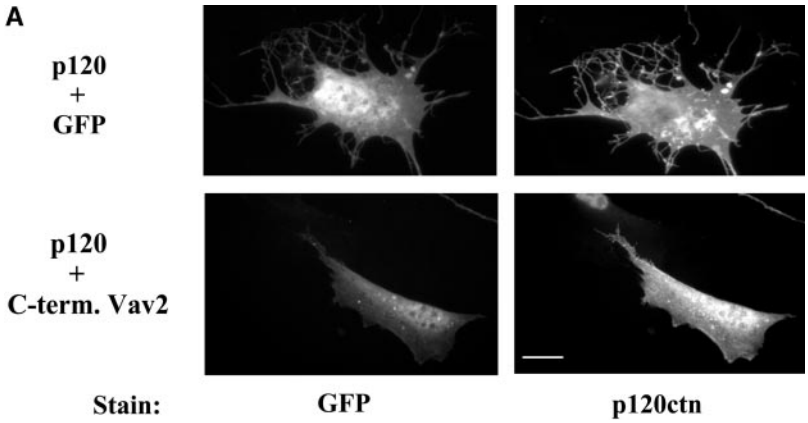
immunoprecipitated from HEK293 cells and the immunoprecipitates blotted for p120ctn. This revealed p120ctn sedimenting with Vav2 (Fig. 8 B). p120ctn was not detected in control immunoprecipitations (Fig. 8 B, bottom). Vav2 immunoprecipitates were also probed for association with E-cadherin. We were not able to detect endogenous E-cadherin in Vav2 immunoprecipitates suggesting that the p120ctn bound to Vav2 is in the soluble pool and is not associated with E-cadherin (Fig. 8 B). To explore this further, we examined the distribution of Vav2, p120ctn, and E-cadherin into cytosolic and membrane fractions in HEK293 cells. This analysis revealed that Vav2 is almost exclusively localized in the cytosol, whereas E-cadherin is found only in the membrane fraction (Fig. 8 C). Endogenous p120ctn was detected in both the cytosol and membrane fractions. In these experiments the amount of p120ctn that was found in the membrane fraction relative to the cytosol was greater than that found in a similar analysis of NIH3T3 cells (see Fig. 3), probably reflecting the increased expression of cadherins in these cells which are epithelial in origin.

The p120ctn-induced Dendritic Phenotype Is Suppressed by Coexpression of the COOH Terminus of Vav2

The COOH terminus of Vav2 consists of an SH2 domain flanked by two SH3 domains (Schuebel et al., 1998). The equivalent region in the hematopoietic GEF, Vav1, has been implicated in mediating association of the exchange factor with other signaling components. Point mutations in the SH2 domain of Vav1 impair its transforming activity (Katzav, 1993). Since Vav2 is a close homologue of Vav1, these observations suggest that expression of its COOH terminus may interfere with Vav2 signaling, and act as a dominant negative construct. To examine the role of Vav2 on the p120ctn-induced dendritic phenotype, we expressed in cells the COOH terminus of Vav2, containing the SH2 and SH3 domains, fused to GFP. Coexpression of this construct blocked the p120ctn-induced phenotype, whereas coexpression of GFP alone had no effect (Fig. 9, A and B).

Discussion

In this study we have found that p120ctn, a protein that binds to the cytoplasmic domain of cadherins, regulates the activity of several Rho family members and that p120ctn interacts with Vav2, a Rho family GEF. Our current interest in p120ctn was stimulated because previous work revealed that the juxtamembrane region of cadherins, to which p120ctn binds, can inhibit cell migration and invasion (Chen et al., 1997), is important for axonal outgrowth



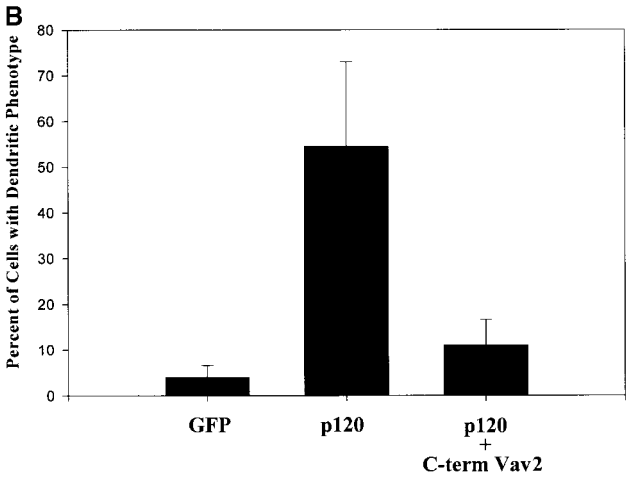


Figure 9. The p120ctn-induced dendritic phenotype is suppressed by coexpression of the COOH terminus of Vav2. NIH3T3 cells were plated on coverslips and transfected with p120ctn + GFP (top panel) or p120ctn + COOH terminus of Vav2 fused to GFP (C-term Vav2) (bottom). Left panels show GFP fluorescence of cells transfected with GFP or C-term Vav2-GFP and right panels are stained with anti-p120ctn antibodies. (B) Transfected cells were scored for the dendritic phenotype (see Fig. 2 C) and compared. Data are the means ± SD of four separate experiments.

(Riehl et al., 1996), and regulates adhesion (Ozawa and Kemler, 1998; Yap et al., 1998; Aono et al., 1999; Ohkubo and Ozawa, 1999; Thoreson et al., 2000). In these earlier studies, it was not obvious how the juxtamembrane region of cadherins might signal to the actin cytoskeleton and thereby affect migration. The discovery that p120ctn binds to the juxtamembrane region of cadherins (Lampugnani et al., 1997; Yap et al., 1998; Ohkubo and Ozawa, 1999; Thoreson et al., 2000) raised the possibility that p120ctn may participate in this signaling pathway. The striking dendritic phenotype, induced by overexpression of p120ctn in fibroblasts (Reynolds et al., 1996), further indicated that p120ctn has major effects on the organization of the actin cytoskeleton.

Our results show that p120ctn affects the actin cytoskeleton in at least two ways. First, we have found that overexpression of p120ctn results in disassembly of stress fibers and focal adhesions. The decrease in focal adhesions and stress fibers may contribute to increased migration because these structures are often antagonistic to migration (Herman et al., 1981; Ridley et al., 1995; Nobes and Hall, 1999). Consistent with loss of stress fibers and focal adhesions, we found that p120ctn overexpression results in decreased RhoA activity. Moreover, the dendritic phenotype induced by p120ctn is enhanced by dominant negative RhoA, but inhibited by constitutively active Rho-kinase, a downstream effector of RhoA that has been implicated in the assembly of stress fibers and focal adhesions (Kimura et al., 1996; Burridge et al., 1997). How might p120ctn decrease RhoA activity? The level of GTP in Rho family members is regulated by GAPs and GEFs. In principle, p120ctn could be stimulating GAP activity or inhibiting GEF activity. So far, we have not detected significant Rho GAP activity in p120ctn immunoprecipitates, nor do we have evidence that p120ctn inhibits a Rho GEF.

The second way in which p120ctn overexpression affects the actin cytoskeleton is by activating Cdc42 and Rac1, which are associated with enhanced cell motility (Ridley et al., 1995; Keely et al., 1997; Nobes and Hall, 1999). We observed that CHO cells in which p120ctn had been overexpressed revealed enhanced migration and this correlated with a less extreme phenotype in these cells. It seemed unlikely that fibroblasts, like NIH3T3 cells, exhibiting the highly arborized morphology induced by p120ctn expression, would reveal net translocation and we did not attempt to measure their locomotion. However, direct observation of cells overexpressing p120ctn indicated motile activity at localized regions of the cell periphery and that this generated the dendritic morphology (data not shown). Dominant negative forms of Cdc42 and Rac1 inhibited the development of this morphology.

The increase in Cdc42 and Rac1 activity induced by p120ctn overexpression led us to look for GEFs that might interact with p120ctn. We have found that p120ctn forms a complex with Vav2, a GEF that has activity for RhoA, RhoG, Cdc42, and Rac1 (Schuebel et al., 1998; Abe et al., 2000). Stimulation of Vav2 activity would account for increased Cdc42 and Rac1 activity, but activation of RhoA is not seen in cells expressing p120ctn. The potential stimulation of RhoA activity by Vav2 may be counteracted by a Rho GAP activity associated with p120ctn. Recent results from Sander et al. (1999) indicate that elevated Rac1 activity can suppress RhoA activity through some as yet undetermined mechanism. This may also contribute to decreased RhoA activity in cells overexpressing p120ctn.

How p120ctn generates the dendritic phenotype is not clear. There are similarities to neurite outgrowth, not only in the morphology of the cells, but also in the involvement of Rho family proteins. Neurite outgrowth is promoted by activation of both Cdc42 and Rac1 (Kozma et al., 1997; Lamoureux et al., 1997; van Leeuwen et al., 1997), and inhibited by activation of RhoA (Jalink et al., 1994). A similar situation is seen for the dendritic morphology induced by p120ctn. It is striking that both for neurite outgrowth (Kozma et al., 1997; van Leeuwen et al., 1997) and the de-

velopment of the p120ctn dendritic phenotype, agents that inhibit RhoA, thereby inhibiting myosin activity, promote an extended morphology. In contrast, activation of RhoA or Rho-kinase, which enhance myosin activity, inhibit neurite outgrowth and the p120ctn-induced phenotype (Jalink et al., 1994; Postma et al., 1996; Hirose et al., 1998). Although activation of Cdc42 and Rac1, combined with inhibition of RhoA, contribute to the dendritic phenotype, it seems unlikely that these activities alone are sufficient to generate this morphology. We suspect that other signaling pathways must also be regulated by p120ctn and participate in generating this striking phenotype.

Several studies have demonstrated that the development and maintenance of cadherin-based adherens junctions requires active Rac1, RhoA, and Cdc42 (Braga et al., 1997, 1999; Zhong et al., 1997; Kuroda et al., 1997, 1998; Takaishi et al., 1997; Jou and Nelson, 1998; Sander et al., 1999; Kaibuchi et al., 1999; Fukata et al., 1999). How these Rho family GTPases regulate and maintain junctional integrity is not fully understood. Our demonstration that a junctional protein, p120ctn, can affect the activity of RhoA, Rac1, and Cdc42 raises the possibility that p120ctn contributes to junction assembly and maintenance through its effects on these regulatory components. However, our data indicate that p120ctn affects Rho family GTPases when it is free in the cytoplasm and not when it is associated with cadherins.

Previous work has indicated that p120ctn exists in two pools, one fraction being bound to cadherins and a second fraction existing as a soluble, cytoplasmic pool (Shibamoto et al., 1995; Staddon et al., 1995; Kinch et al., 1995; Papkoff, 1997; Ozawa and Kemler, 1998; Thoreson et al., 2000). Expression of full-length cadherins, or cadherin constructs containing the juxtamembrane region responsible for binding p120ctn, suppress the p120ctn-induced dendritic phenotype. The phenotype, however, is unaffected by cadherin constructs that lack the juxtamembrane region, and which consequently are deficient in p120ctn binding. In addition, we have found that overexpression of p120ctn increases the cytosolic pool of p120ctn relative to the fraction associated with the membrane. Together, these observations lead to the conclusion that when p120ctn is bound to cadherins it is unable to induce the dendritic phenotype. A model is suggested by these findings (Fig. 10). We envisage the cytoplasmic pool of p120ctn being complexed with factors, one of which is Vav2, that are responsible for the dendritic phenotype. Under normal situations, the cytoplasmic pool of p120ctn is small (Thoreson et al., 2000), but the level of this pool will be affected by events that influence the binding of p120ctn to cadherins and by the amount of cadherins that are present. We observed that overexpression of cadherins suppresses the dendritic phenotype even in the absence of junction formation. This indicates that isolated cadherins are sufficient to bind p120ctn and that cadherin-mediated junctions are not required. However, it is known that when epithelia develop cadherin-based adherens junctions, this increases the half-life of cadherins on the cell surface (McCrea and Gumbiner, 1991; Shore and Nelson, 1991). Consequently, because junction formation stabilizes cadherins and increases their expression level, it is predicted that junction formation will shift the distribution of p120ctn from the soluble pool to the cadherin-bound state.

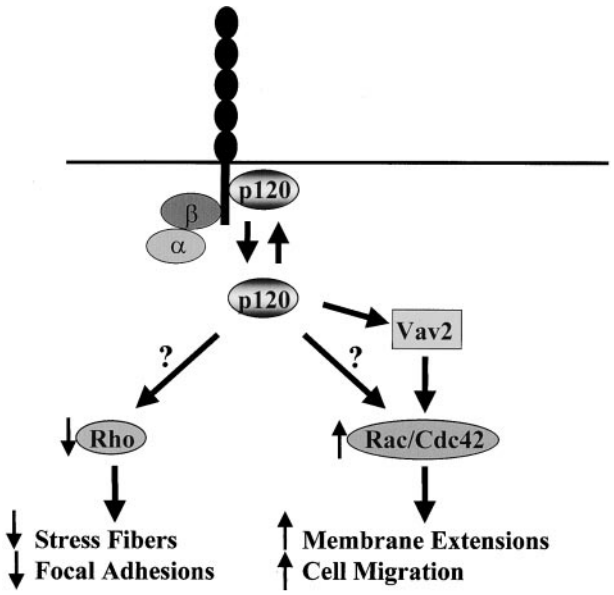


Figure 10. Scheme for how p120ctn may regulate the activity of RhoA, Rac1, and Cdc42. p120ctn exists in an equilibrium between two states, bound to the juxtamembrane region of cadherins or free in the cytoplasm. When it is not associated with cadherins, p120ctn deceases RhoA activity and increases the activity of Cdc42 and Rac1. One possible means by which p120ctn may activate Cdc42 and Rac1 is via association with the exchange factor Vav2.

It should be noted that the cadherin we used to suppress the p120ctn-induced phenotype was C-cadherin, an amphibian ortholog of E-cadherin. The juxtamembrane region of cadherins is highly conserved, but it has been suggested that the interaction of p120ctn may be of different affinity for different cadherins (Navarro et al., 1998). In this respect, it is pertinent that expression of N-cadherin in breast epithelial cells promotes cell migration (Nieman et al., 1999; Hazan et al., 2000). It will be interesting to determine the state of p120ctn in this situation and whether the size of its cytoplasmic pool is affected by N-cadherin expression or whether other factors are contributing to the mechanism by which N-cadherin promotes migration in these cells.

We became interested in p120ctn because of its interaction with the juxtamembrane region of cadherins and because this juxtamembrane region has been implicated in regulating cell migration. Many cells exhibit the phenomenon of contact inhibition of movement, that is their migratory activity is inhibited or diminished by contact with other cells. How this occurs has not been determined. The previous observations on the role of the juxtamembrane region of cadherins, combined with our current observations on p120ctn, suggest that the distribution of p120ctn between the cadherin-bound state and the cytoplasmic pool may be important in contact inhibition. Many tumors with increased invasiveness reveal a loss of E-cadherin expression (Birchmeier and Behrens, 1994). Our model (Fig. 10) predicts that loss of E-cadherin or perturbations of the

cell–cell junctions will increase the pool of p120ctn, which, in turn, will stimulate increased Cdc42 and Rac1 activity and decreased RhoA activity. The net effect will be to elevate the migratory activity of the cells. Restoration of E-cadherin to normal levels has been found to inhibit invasion (Frixen et al., 1991), and under these circumstances the increased expression of E-cadherin should sequester p120ctn, thereby diminishing its effects on Rho family proteins. Similarly, when cells are migrating in the absence of contact with neighbors, we anticipate elevated levels of p120ctn in the cytoplasmic pool. Cell–cell contact results in the development of adherens junctions and, as noted earlier, this increases the level of cadherin expression. Consequently, cell–cell adhesion will shift the balance between the two populations of p120ctn, from the cytoplasmic pool to the cadherin-bound pool. The phenomenon of contact inhibition is complex and probably involves the activation or suppression of multiple signaling pathways. However, our results indicate that p120ctn is one component that contributes to the cross-talk between cell–cell junctions and the motility of cells. Previous studies have shown that p120ctn is a target for both tyrosine phosphorylation and serine/threonine phosphorylation in response to various signaling pathways (Reynolds et al., 1989; Downing and Reynolds 1990; Kanner et al., 1991; Kinch et al., 1995; Ratcliffe et al., 1997; Aono et al., 1999; Ohkubo and Ozawa, 1999; Thoreson et al., 2000). Many of these signaling pathways affect both the integrity of cell–cell junctions and the motility of cells. It will be important in the future to explore how the various modifications of p120ctn regulate its interactions and thus its function.

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