A Novel Interaction between Kinesin and p120 Modulates p120 Localization and Function*§

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p120-catenin exists in a membrane-associated cadherin-bound pool, a cytosolic pool that affects Rho GTPases, and a nuclear pool that is thought to associate with the methylation-relevant transcriptional repressor Kaiso. We show here that cytoplasmic p120 can also associate both directly and indirectly with the microtubule network, and that p120 traffics along microtubules toward their plus ends. The direct binding required most of the armadillo repeats and was mutually exclusive for interaction with E-cadherin. Perturbing the p120-microtubule interaction with nocodazole or taxol markedly affected both the tubulin interaction and the balance between cytoplasmic and nuclear p120. The indirect binding occurred via a novel interaction between a segment of the p120 N-terminal domain and conventional kinesin heavy chains. Selective uncoupling of the p120-kinesin interaction by overexpression of the respective p120 and kinesin-binding fragments promoted nuclear p120 accumulation. In addition, expression of full-length kinesin reduced the nuclear accumulation of p120 and blocked the branching phenotype associated with p120 overexpression. Taken together, the data suggest that kinesin affects both the targeting and activity of p120 at several cellular locations.

Cadherins are cell-cell adhesion proteins that have critical roles in development, morphogenesis, and cancer (1–3). Their extracellular domains interact with one another in homophilic fashion, whereas their intracellular domains associate with cytoplasmic proteins termed catenins. α- and β-catenins constitute a physical linkage between the so-called catenin binding domain of cadherins and the actin cytoskeleton (3). In contrast, p120-catenin (hereafter p120) binds to the juxtamembrane domain and does not directly associate with the cytoskeleton (4). Several lines of evidence suggest that p120 is regulated by phosphorylation and acts as a switch that modulates cadherin adhesiveness (4).

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Epithelial cadherin (E-cadherin), an main adhesion molecule in epithelial tissues, is frequently down-regulated or lost altogether during the transition to metastatic cancer (2, 3). In E-cadherin-deficient cells, p120 is stranded in the cytoplasm where its role is uncertain. When overexpressed in normal cells, p120 saturates available cadherin-binding sites and floods the cytoplasm. High level p120 overexpression induces a dramatic branching morphology that has been attributed to inhibition of RhoA (5) and is independent of cadherin function. Low level overexpression promotes cell motility, which may involve the activation of Rac1 (6, 7). Thus, cytoplasmic pools of p120 affect cell morphology and motility through modulation of Rho-GTPases. These observations have led to the hypothesis that cytoplasmic pools of p120 in cadherin-deficient carcinomas may contribute to the metastatic phenotype (7, 8). An enhanced nuclear pool of p120 in cadherin-deficient cells (9, 10) could also contribute to these effects. Thus, like many other Arm repeat proteins, p120 appears to have roles at many locations, including cell-cell junctions, the cytoplasm, and the nucleus. Mechanisms associated with the trafficking of p120 to and from these various locations have not been examined.

Microtubules are polarized structures; during interphase they radiate through the cytoplasm with a minus end attached to the centrosome and a plus end oriented toward the cell periphery. Free microtubules undergo dynamic instability, switching between growing and shrinking phases or pausing. Microtubules that stay in pause for longer periods do not show tubulin turnover and are referred to as stable microtubules. In addition to contributing to structural rigidity, microtubules are utilized by specific molecular motors to transport proteins, vesicles, and organelles within a cell. Conventional kinesin is one of these molecular motors that is ubiquitously expressed and uses the energy of ATP hydrolysis to transport cargoes toward the plus ends of microtubules (11). It is a heterotetramer of two kinesin heavy chains (KHC) and two light chains (KLC) that function together to promote cargo movement.

Here we have identified several novel functional interactions between p120 and microtubules. One mode of interaction is likely direct and was competed by cadherin overexpression. Additionally, p120 associated with microtubules indirectly via a novel interaction with conventional kinesins, which promoted...
the movement of p120 toward the plus ends of microtubules. We have mapped the reciprocal binding sites on these proteins, and we used the interacting fragments to selectively interfere with p120-kinesin binding and function. The data suggest that kinesin affects both the targeting and activity of p120 at several cellular locations. These observations reveal novel physical and functional relationships between microtubules, p120, and kinesin that are likely to impact on the role of p120 in cell adhesion and tumor progression.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Transfections**—All cell lines were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 1% l-glutamine, 100 units/ml penicillin, and 100 mg/ml streptomycin (Invitrogen). For transient transfections, cells were plated onto glass coverslips in 6-well plates, incubated overnight, and transfected with a total of 4 μg of various expression plasmids per well, using the transfection reagent cytofectene (Bio-Rad) according to the manufacturer’s directions. In all experiments, 2 μg of p120 expression vectors were co-transfected with either 2 μg of control vector or 2 μg of cadherin cytoplasmic domain constructs, 1 μg of KHC together with 1 μg of control vector, 1 μg of KHC together with 1 μg of KLC, or 1 μg of KHC together with 1 μg of N1 or N3. For immunoprecipitation and mapping studies, 293 cells in 100-mm dishes were transiently transfected with 16 μg of DNA using the calcium phosphate method.

**Immunoprecipitations**—Immunoprecipitations were performed as described previously (12). All antibodies were used at 5 μg/500-μl samples for immunoprecipitations. For Western blotting, the 9E10 anti-Myc and 12CA5 anti-HA mAbs were used at 1 μg/ml final concentration. Similarly, the p120 mAbs 6H11 and 8D11 were used at 1 μg/ml, whereas pp120 (Transduction Laboratories) was used at 0.25 μg/ml. The anti-kinesin mAb H2 (Chemicon Inc.) was used at 1:1,000 final dilution. Goat anti-mouse IgG-horseradish peroxidase was used as the secondary antibody at 1:20,000.

**Immunofluorescence**—Immunofluorescence localization procedures have been described in detail (13). Briefly, 24 h post-transfection, cells were fixed in 3% paraformaldehyde for 30 min, permeabilized in 0.2% Triton X-100 for 5 min, and incubated with appropriate primary and secondary antibodies for 30 min at room temperature. Primary antibodies were the following: FlaSH (14) p120 rabbit polyclonal antibody used at 0.5 μg/ml; 12F4, 6H11, and 8D11 p120 filamentous localization antibodies (14) used at 1 μg/ml; 9E10 anti-Myc tag and 12CA5 anti-HA tag murine monoclonal antibodies used at 1 μg/ml; anti-kinesin heavy chain (clone H2) murine monoclonal antibody used at 1:1,000 (Chemicon Inc.); anti-α-tubulin mouse monoclonal antibody (Sigma T5168) and anti-α-tubulin rat monoclonal antibody (1864; Chemicon Inc.) used at 1:2,000 and 0.5 μg/ml, respectively. The secondary antibodies used were highly cross-absorbed goat anti-mouse Alexa 488 (green; Molecular Probes) or 596 (red), goat anti-rabbit Alexa 488 (green) or 596 (red), and goat anti-rat Alexa 596 (red) used at 1:600. Coverslips were mounted on glass slides using Poly/Mount (Polysciences Inc.) and visualized under a Nikon Optiphot 2 fluorescent microscope using a ×60 planApo Nikon objective. Photos were acquired with the Metamorph program using a CoolSnap Fx CCD camera and compiled in Photoshop and Powerpoint.
Yeast Two-hybrid—The Hollenberg mouse embryo cDNA library (E10.5) was used to screen for p120-interacting proteins. Yeast twohybrid screens were performed as described in detail elsewhere (15, 16). p120 interacted with a cDNA construct of KIF5C (GenBank accession number AF067180), containing amino acids 654–757 and inserted into the NotI restriction site of the pVP16 vector.

Expression and Purification of Recombinant Proteins—GST-p120 was produced in E. coli as described previously (5). GST-p120 on glutathione beads (Amersham Bioco.) was subjected to digestion with Factor Xa (8 units for 1 h at 22 °C) in 50 mM Tris-HCl (pH 8.0) containing 100 mM NaCl and 1 mM CaCl₂. Beads were removed by centrifugation, and the supernatants were incubated for 2 h with an excess of glutathione beads to remove uncut p120-GST. Recombinant p120 was then concentrated in general tubulin buffer (Cytoskeleton Inc.) using Amicon filters and used immediately in microtubule-associated protein spin-down assays.

Microtubule-associated Protein Spin-down Assays—293 cells were lysed in 0.5 ml of PEM buffer (80 mM Pipes, pH 6.9, 1 mM EGTA, 1 mM MgCl₂) supplemented with 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 2 μg/ml aprotinin, 1 mM sodium orthovanadate, 5 mM NaF. Cell lysates were ultracentrifuged at 100,000 × g for 40 min at 4 °C to remove any microtubules. Clarified lysate was then used for microtubule spin-down assays using a commercially available kit (BK029) from Cytoskeleton Inc. Microtubules were assembled from highly purified tubulin protein (>99% pure; Cytoskeleton Inc.). Briefly, 2.5 μl of buffer containing 50% glycerol was added to 20-μl aliquots of tubulin (100 μg of protein dissolved in PEM buffer containing 1 mM GTP), prior to incubation at 35 °C for 20 min. Newly formed microtubules were then stabilized by the addition of 180 μl of PEM buffer containing 20 μM Taxol (final microtubule concentration approximately equivalent to 10 nm). Microtubule binding reactions were performed by adding 20 μl of clarified cell lysate to 20 μl of microtubules in the presence of 10 μl of PEM containing 20 μM Taxol. Controls included microtubule-associated protein 2 (MAP2) used as a positive control, BSA as a negative control, as well as the lack of microtubules to test for nonspecific precipitation. After 30 min of incubation at room temperature, samples were added on top of a 100-μl cushion buffer supplemented with Taxol and ultracentrifuged at 100,000 × g for 40 min at room temperature. Protein in the pellets and the supernatants was denatured and solubilized by boiling in 1× LSB buffer, prior to SDS-PAGE. After SDS-PAGE, protein was visualized by Coomassie staining (MAP2 and BSA controls), or in the case of p120 it was visualized after transfer to nitrocellulose membranes and Western blotting with the anti-p120 mAb 5D11 at 1 μg/ml. The assay was essentially identical when recombinant p120 was used, with the exception that instead of cell lysate samples were loaded with ~1 μg of purified, recombinant p120.

Constrasts—The Rc-CMV-murine p120ctn isoforms 1A, 3A, 4A, and 1A-HA (epitope tagged) have been described previously (21). Similarly, the individual armadillo repeat deletion mutants, as well as p120-ΔC1 (missing amino acids 831–932), have been described previously (12), with the difference that fragments with each deletion were subcloned into Rc-CMV-p120–1A and verified by sequencing. The C-terminal p120 mutant ΔC2 was generated in Rc-CMV-p120–1A by PCR-mediated mutagenesis and contains 895 N-terminal amino acids of p120 1A. pGEX-3x-p120 (5), EGFP-p120 (7), pCAN-ΔCYTO (5), pcDNA3-KHC-Myc, pcDNA3-KLC-HA (19), N1-, and N3-p120 (20) have been described previously. The Rc-CMV-ΔArm) mutant, which lacks amino acids 392–821 of p120-1A, is identical to the ΔR3-11-HA p120 mutant described before (18). Finally, KIF-HA was generated by subcloning the p120-interacting KIF5C fragment from the pVP16 vector into pcDNA3-HA (16) using NotI digestion and confirming the sequence by sequencing. The identity of all other constructs was also verified by sequencing.

Live Cell Imaging—Goldfish fibroblasts (line CAR) were cultured and transfected as described (21). Cy3 tubulin was kindly provided by Fedor Severin (Dresden, Germany) and microinjected using a Leitz Micromanipulator M and Eppendorf Microinjector 5242. Video microscopy was performed as described in detail elsewhere (21).

Supplemental Material—One figure and three movies are included as Supplemental Material. The figure shows the rapid and reversible association of p120 with dynamic microtubules. The first two movies show the co-localization and temporal association of p120-GFP with dynamic microtubules, respectively, and the third movie shows p120-GFP sliding along microtubule tracks.
RESULTS

p120 Co-localizes with Microtubules—Ectopically expressed p120 (murine isoform 1A) exhibited a prominent filamentous staining pattern in 28% of transfected MDCK cells (see Fig. 1A), which co-localized with microtubules but not other elements of the cytoskeleton (not shown). The same staining pattern was observed after immunostaining with several different p120 antibodies, and with an epitope tag antibody (mAb12CA5) after expression of HA-tagged p120. Similar observations were made in several different cell lines, including NIH3T3, COS7, and MCF7 cells (not shown). Treatment of MDCK cells with the microtubule-depolymerizing agent nocodazole (10 μM, 90 min) disrupted the p120 filamentous staining (Fig. 1B). In addition, although most of the endogenous cytoplasmic p120 in cadherin-
deficient cancer cells exhibited a diffuse staining pattern, co-localization of p120 with microtubules could be clearly observed in well spread cells (Fig. 1C). These data suggest that p120 interacts directly or indirectly with microtubules.

To examine further the behavior of p120 on microtubules, we expressed p120-GFP in fish fibroblasts (CAR line) and observed its localization in real time by live cell imaging. p120-GFP was frequently observed along microtubules decorated with Cy3-tubulin (see Supplemental Material, movie 1). Interestingly, cytosolic p120 bound rapidly to newly polymerized, dynamic microtubules (see Supplemental Material, movie 2). Interestingly, cytosolic p120 bound rapidly to newly polymerized, dynamic microtubules (see Supplemental Material, movie 2). This association seems to be a regulated process as p120 can be seen to rapidly associate and dissociate with dynamic microtubules (Supplemental Material, Fig. 1 and movie 2). In agreement with this hypothesis, treatment of p120-overexpressing MDCK cells with staurosporine (50 nM), a general PKC inhibitor, significantly increased the number of cells exhibiting filamentous p120 staining (~82% of p120-overexpressing cells), whereas dimethyl formamide used as a vehicle control had no effect.

**p120 Associates with Polymerized Tubulin in Vitro**—To validate biochemically the interaction of p120 with microtubules, we attempted to co-precipitate ectopically expressed p120 from 293 cell lysates using a highly purified preparation of polymerized microtubules (Fig. 2A). Under these conditions, p120 and microtubules efficiently co-precipitated, but we could not distinguish between direct and indirect binding. Therefore, the experiment was repeated using purified recombinant p120. Fig. 2B shows that even highly purified preparations of p120 and microtubules co-precipitated, suggesting that p120 can bind microtubules directly.

The Binding of p120 to Cadherins or Microtubules Is Mutually Exclusive—Preliminary structure-function analysis demonstrated that co-localization with microtubules required extensive regions of p120, including the alternatively translated N-terminal region between p120 ATG start sites 3 and 4 (amino acids 103–324) and armadillo repeats 3, 5, and 7–10 (immuno-fluorescence data not shown). To determine whether overlapping elements within the armadillo repeats mediate mutually exclusive binding interactions with either E-cadherin or microtubules, we co-expressed p120 with the cadherin cytoplasmic domain ΔCYTO (Fig. 3). ΔCYTO expression strongly inhibited p120 co-localization with tubulin, suggesting that p120 cannot bind directly to tubulin and E-cadherin at the same time. As control, the experiment was repeated with a p120 mutant lacking armadillo repeat 1 (p120ΔA1), which cannot bind to cadherins. ΔCYTO did not block the microtubule localization of the p120ΔA1 mutant.

The plus-ended movement of p120 along microtubules suggested that p120 interacts with microtubule-associated motor proteins. The first evidence that this may be the case came from a yeast two-hybrid screen for p120-interacting proteins, where a portion of KIF5C (a conventional kinesin motor protein) was shown to interact with a p120 construct in the pBTM bait vector. KIF5C is a brain-specific member of the kinesin heavy chain family that mediates plus-ended movement when associated in a heterotetrameric complex with kinesin light chain (KLC). The yeast two-hybrid interaction between KIF5C (266.6 arbitrary units of β-galactosidase activity) was as strong as that with VE-cadherin (228.4 units), a known p120 partner. Meanwhile, the interaction between an empty vector and KIF5C was not detectable (0.0 units). To validate this potential interaction, p120 was co-immunoprecipitated from 293 cell ly-

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sates with a tagged version of the KIF5C fragment isolated previously in the yeast two-hybrid screen (KIF-HA containing amino acids 654–757 of KIF5C; Fig. 4B-1). When the cytosolic pool of p120 was increased by ectopic p120 expression in 293 cells, endogenous kinesin heavy chain co-precipitated with p120 (Fig. 4B-2), and p120 was co-immunoprecipitated with endogenous kinesin (Fig. 4B-3). In all cases, these proteins were not present in immunoprecipitates generated with control mAbs. Finally, ectopic p120 co-precipitated with overexpressed full-length kinesin heavy chain isoform 5B (KHC; kinesin; see Fig. 4B-4), an isoform found in most cells. Interestingly, ectopically expressed p120 co-precipitated with kinesin even in lysates of cells pretreated with nocodazole (10 μM, 90 min; data not shown), indicating that the interaction is not mediated by intact microtubules. These data strongly argue that p120 interacts with kinesin in cells.

In further support of this interaction, we observed co-localization at cell-cell contacts of endogenous p120 and kinesin in confluent MDCK epithelial cell cultures (Fig. 4C). In addition, co-localization of p120 and kinesin in cytoplasmic dots, especially around the nucleus, was evident in NIH3T3 fibroblasts (Fig. 4C) and sparsely plated MDCK cells (not shown). These data suggest that p120 may be delivered to sites of cell-cell contact by kinesin-mediated trafficking.

Kinesin Associates with the Alternatively Spliced p120 N Terminus—To map the kinesin interaction site on p120, we performed co-immunoprecipitation assays with an extensive panel of naturally occurring p120 isoforms and targeted deletion mutants (Fig. 5A). Interaction with kinesin did not require the p120 Arm repeat domain or C terminus (Fig. 5B). In contrast, the N-terminal end is required because the naturally occurring p120 isoform 4, which lacks the N-terminal 323 amino acids due to alternative splicing, does not bind kinesin (Fig. 5B). p120 isoform 3 (lacks 102 N-terminal amino acids) co-precipitated weakly with kinesin, indicating that the interaction occurs, in part, through sequences located between ATG start sites 3 and 4 (which correspond to the start of isoforms 3 and 4, respectively). However, in repeated experiments, the co-immunoprecipitation with p120 isoform 1 was significantly stronger than that with isoform 3, suggesting that amino acids 1–102 of p120, although not completely essential for interaction, confer a considerably higher affinity for kinesin. In agreement with this conclusion, an N-terminal fragment of p120 lacking the initial 102 amino acids (N3; amino acids 103–323)
FIG. 6. A, microtubule polymerization affects the nuclear accumulation of p120. Cadherin-deficient MDA-MB-231 cells were treated overnight with 3 μM nocodazole, 50 nM taxol, or DMSO as a control. Nocodazole treatment caused the depolymerization of the microtubule network and a pronounced nuclear accumulation of endogenous p120, witnessed after staining cells with the anti-p120 mAb 12F4. In contrast, taxol treatment caused stabilization of microtubules and promoted p120 nuclear exclusion. To quantify this effect we scored cells (six fields >80 cells per condition) as negative (−), if they exhibited clear nuclear exclusion of p120 (like the taxol panel), or as positive (+), if they exhibited visible nuclear p120 accumulation (like the nocodazole panel). Cells with intermediate phenotypes were not scored. Filled bars correspond to the percent of cells exhibiting nuclear exclusion in DMSO, nocodazole, and taxol-treated groups, whereas the open bars represent cells with nuclear accumulation.

B

C

Association of p120 with Microtubules and Kinesin

Figu. 6. A, microtubule polymerization affects the nuclear accumulation of p120. Cadherin-deficient MDA-MB-231 cells were treated overnight with 3 μM nocodazole, 50 nM taxol, or DMSO as a control. Nocodazole treatment caused the depolymerization of the microtubule network and a pronounced nuclear accumulation of endogenous p120, witnessed after staining cells with the anti-p120 mAb 12F4. In contrast, taxol treatment caused stabilization of microtubules and promoted p120 nuclear exclusion. To quantify this effect we scored cells (six fields >80 cells per condition) as negative (−), if they exhibited clear nuclear exclusion of p120 (like the taxol panel), or as positive (+), if they exhibited visible nuclear p120 accumulation (like the nocodazole panel). Cells with intermediate phenotypes were not scored. Filled bars correspond to the percent of cells exhibiting nuclear exclusion in DMSO, nocodazole, and taxol-treated groups, whereas the open bars represent cells with nuclear accumulation.

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associated weakly with kinesin, when compared with the full-length N-terminal p120 construct (N1; amino acids 1–323; see Fig. 5B). Thus, the alternatively spliced p120 N-terminal domain is both necessary and sufficient for kinesin interaction.

**Microtubule and Kinesin Binding Reduce Nuclear Accumulation of p120**—In addition to membrane delivery, microtubules can also regulate the transport of several proteins in and out the nucleus (22). Interestingly, overnight treatment of NIH3T3 cells with nocodazole causes a pronounced nuclear accumulation of p120 (data not shown). To test the possibility that microtubules regulate the nuclear accumulation of endogenous p120, we used cadherin-deficient MDA-MB-231 cells, which exhibit both nuclear and cytoplasmic p120 localization. Microtubule depolymerization with nocodazole caused a significant increase in nuclear p120 accumulation (Fig. 6A), whereas taxol (which stabilizes microtubules) caused nuclear exclusion. Thus, microtubules may prevent nuclear p120 import and/or promote nuclear export. p120 is known to have at least one nuclear localization signal and one nuclear exclusion signal (NES) (9, 20). The NES identified to date resides in the alternatively spliced exon B (9). However, treatment of p120-overexpressing NIH3T3 cells with the nuclear export inhibitor leptomycin B causes a pronounced nuclear accumulation of p120 isoform 1A, which lacks the B exon (Fig. 6B). Indeed, the existence of an additional p120 nuclear export signal has been suggested in another recent report (23).

To clarify whether kinesin binding affects the nuclear accumulation of p120, we localized p120 in cells following overexpression of KIF-HA or p120-(ΔArm). KIF-HA is a 103-amino acid fragment of KIF5C shown by yeast two-hybrid assay and co-immunoprecipitates (Fig. 4B-1) to bind p120. p120-(ΔArm) is a p120 fragment that lacks the Arm repeat domain and consists primarily of the N-terminal kinesin binding region (along with a short segment of the p120 C terminus). Thus, each fragment when overexpressed should selectively block the interaction between endogenous p120 and kinesin without significantly affecting other interactions. Interestingly, ectopic expression of either KIF-HA or p120-(ΔArm) caused the nuclear accumulation of co-expressed wild type p120-GFP (Fig. 6, B and C), suggesting that uncoupling the p120-kinesin interaction increases p120 levels in the nucleus. Similar results were also obtained after overexpression of the N1 p120 fragment (data not shown), which also binds kinesin (Fig. 5B).

We also evaluated the effects of overexpressing reconstituted functional kinesin by co-expressing KHC and KLC. Under these conditions, co-transfected p120 was strongly excluded from the nucleus (Fig. 6, B and C). Thus, overexpression of functional kinesin promotes nuclear exclusion of p120.

**Kinesin Binding Blocks Effects of p120 on Cell Contractility and Morphology—**p120 overexpression in NIH3T3 cells leads to an exaggerated branching phenotype associated with RhoA inhibition, reduced stress fibers, and loss of contractility (5, 7). To evaluate further the role of the p120-kinesin interaction, we tested whether kinesin binding affects the p120-mediated loss of stress fibers and the induction of the branching phenotype. Kinesin co-expression blocked both p120-induced branching (Fig. 7) and loss of stress fibers (data not shown). To test the specificity of this kinesin effect with respect to p120 binding, we co-expressed the N1 p120 fragment, which avidly binds kinesin and should uncouple the interaction of full-length p120–1A with kinesin. N1-p120, which has no branching activity on its own (20), reinstated p120-induced branching when co-expressed with kinesin (Fig. 7). In contrast, under the same conditions N3-p120, which associates poorly with kinesin (Fig. 5B), had no effect. These data strongly suggest that the interaction of p120 with kinesin blocks its ability to affect cell contractility and induce branching. In agreement with this conclusion, kinesin was unable to impair significantly the ability of p120 isoform 3A to induce branching, as would be predicted by the poor affinity of this isoform for kinesin.

**DISCUSSION**

The microtubule network is known to affect both cadherin function and cell motility. We show here that p120 catenin can associate with microtubules. Previous data suggested that the levels of cytoplasmic p120 might modulate the sessile versus motile behavior of cells (7, 8). The current studies provide an important first insight into the mechanism by which p120 targets its various sites of action.

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Results from three experiments were pooled to generate mean ± S.D. Student’s t test was used for statistical analysis. B, kinesin association promotes nuclear exclusion of p120. NIH3T3 cells were co-transfected with p120-GFP and control pcDNA3 vector, p120-GFP and Δ(Arm)-HA, or p120-GFP together with KHC and KLC. After 24 h, cells were fixed in paraformaldehyde to preserve GFP staining and immunostained for HA tag (red) by using the anti-HA mAb 12CA5. Representative images of p120 nuclear accumulation were captured for each group, both under high (×60) and low magnification (×20). Control experiments verified that Myc-KHC and HA-KLC were co-expressed in more than 95% of the cells. B, bar graph representation of the effect of kinesin binding on nuclear localization of p120. By using the same assay described previously, we quantified the nuclear exclusion or accumulation of p120 under each treatment. In one set of experiments, p120-overexpressing NIH3T3 cells were treated with the nuclear export inhibitor leptomycin B (LMB, 20 μM) or methanol (control) for 6 h, prior to fixation and immunofluorescence detection of p120. In the other group, p120 nuclear localization was detected in cells co-expressing p120-GFP together with control pcDNA3 vector, KIF-HA, Δ(Arm)-HA, or KHC + KLC. Co-transfected cells were detected by HA immunostaining. KIF-HA and Δ(Arm)-HA are expected to uncouple the p120-kinesin interaction, whereas KHC and KLC reconstitute functional kinesin. Cells exhibiting p120-GFP nuclear exclusion or accumulation (filled and open bars, respectively) under each condition were counted, and the results are depicted in a bar graph format. Methanol was used as control for leptomycin B (LMB) (asterisk), and pcDNA3 as control for the other conditions (plus sign). + represents p < 0.05; **+/+++ represents p < 0.01; and ++++ represents p < 0.001 (Student’s t test).
We have observed two different modes of p120-microtubule interaction, one that is likely direct and requires p120 armadillo repeats, and an indirect one mediated by kinesin association with the p120 N terminus. The co-precipitation of recombinant p120 with highly purified, polymerized tubulin suggests that at least some of the p120 co-localizing with microtubules is directly associated. In principle, non-specific interactions based on charge effects could be enhanced in vitro or by p120-overexpression in cells. On the other hand, most proteins do not decorate microtubules when overexpressed. Moreover, in cadherin-deficient cells endogenous p120 sometimes exhibited weak microtubule co-localization without the need for overexpression, probably because of the increased pool of cytoplasmic p120 under these conditions. In any case, the interaction was effectively competed by co-expression with E-cadherin, suggesting a mutually exclusive interaction. At this point, the physiologic relevance of the direct p120-tubulin interaction is not known. However, perturbing the microtubule network with nocodazole or taxol profoundly affected the balance of endogenous p120 in the cytoplasm or nucleus in cadherin-deficient cells (Fig. 6A).

Upon overexpression, 28% of MDCK cells exhibited filamentous p120 staining. However, we did not observe a direct correlation between the level of p120 expression and its localization to microtubules. Indeed, we found that p120-GFP rapidly localizes to dynamic microtubules, in cells selected for their low expression of p120-GFP. Interestingly, the association of p120-GFP with microtubules seems to be a regulated process, as p120 could be seen to rapidly associate and dissociate from microtubules (see Supplemental Material, Fig. 1 and movie 2). A first indication on the possible mechanism of action for this process comes from the observation that staurosporine, a general PKC inhibitor, induces p120 localization to microtubules. The mechanism by which staurosporine acts is still unclear, but PKC phosphorylates p120 (24), and staurosporine treatment has been shown previously to affect p120 function (25). Therefore, it is possible that p120 phosphorylation regulates its interaction with microtubules.

p120 also associated indirectly with microtubules via a novel interaction with kinesin heavy chains KIF5C and KIF5B. Whereas KIF5C is brain-specific, KIF5B is expressed ubiquitously (11). The yeast two-hybrid data are consistent with a direct interaction between these proteins. Furthermore, the observation that the interaction of p120 with kinesin persists in the absence of microtubules (after nocodazole treatment) suggests that the putative direct association of p120 with microtubules cannot account for the p120-kinesin interaction. Although co-immunoprecipitation of p120 and kinesin was not efficient, it was nonetheless reproducible enough to easily map the interaction region, and there are reasons to believe that the interaction may be better than that represented by the co-precipitation data. First, one would not necessarily expect significant amounts of p120 and kinesin to be associated under normal conditions. The interaction between p120 and kinesin is relatively transient given that only a small fraction of total p120 is trafficking on kinesin at any given point in time. The cytoplasmic pool of p120 is insignificant anyway compared with the p120 levels associated with cell junctions and cadherins. A technical issue associated with the co-precipitation reactions is that much of the microtubule-associated p120 and kinesin is probably removed prior to co-immunoprecipitation when the cell lysate is clarified by centrifugation. These issues suggest that the increased p120-kinesin interaction observed when p120 is overexpressed (Fig. 4B) reflects a physiologically relevant interaction.

We have observed both retrograde and anterograde movement of p120-GFP along microtubule tracks. Kinesin binding is likely to account for the plus end-directed anterograde movement, suggesting that p120 may associate with other microtubule motor proteins to move in the opposite direction. The observation that in Drosophila (26), as well as in fish and murine fibroblasts, p120 can localize at centrosomes is consistent with this conclusion. Another possibility is that the retrograde movement (toward the centrosome) observed relates to p120 degradation. Degrading proteins often accumulate in “aggresomes” which traffic along microtubules toward the cell interior (27). This possibility would still be unable to account for the plus-ended movement of p120-GFP, which is likely to reflect the trafficking of kinesin-bound p120.

Although p120 binding to cadherin and microtubules was mutually exclusive, kinesin and cadherin binding (12) map to different sites on p120, suggesting that p120 may interact simultaneously with cadherins and kinesins. Thus, it is possible that p120 mediates trafficking of cadherin complexes to cell-cell junctions. This is consistent with the observation that kinesin is implicated in N-cadherin trafficking to these sites (28). On the other hand, we have reported previously (13) that p120-uncoupled E-cadherin mutants localize properly to areas of cell-cell contact. In addition, expression of the kinesin-uncoupled p120 isoform 4 in p120-deficient SW48 cells results in E-cadherin stabilization and localization at cell-cell junctions (12). Thus, the p120-kinesin interaction is clearly not essential for cadherin delivery to these sites.

The association of p120 with microtubules also affected the nuclear and cytoplasmic distribution of p120. Nuclear levels of p120 were substantially increased by both microtubule depolymerization and by experiments designed to selectively uncouple the p120-kinesin interaction. In contrast, p120 nuclear exclusion was induced by co-expression of KHC and KLC, which reconstitute functional kinesin, and to a lesser extent by KHC alone (data not shown). Thus, kinesin is likely to play a crucial role in the nucleocytoplasmic shuttling of p120. Unlike β-catenin, little is known about the nuclear functions of p120. However, p120 is thought to affect gene transcription by binding Kaiso (29, 30) and inhibiting its transcriptional repressor function (31). The recent observation (32) that Kaiso represses gene transcription by being a core component of and targeting the N-CoR deacetylase complex to methylated DNA loci suggests a potentially important role for nuclear p120 in regulating this process.

It is probably not accidental that the nuclear localization of p120 is highly regulated by alternative splicing. Indeed, all 32 putative p120 isoforms are thought to bind cadherins, but most of them exhibit a differential ability to localize in the nucleus, due either to the introduction of NESs (exon B) (9) or to the elimination or modulation of nuclear localization signals (20) (e.g. isoform 4 and exon C). The fact that p120-1A binds kinesin better than p120-3A may account for the reported increased ability of p120-3A to accumulate in the nucleus (23). In pancreatic cancer, cytoplasmic mislocalization, up-regulation and nuclear accumulation of p120 correlate with increased tumor growth and reduced survival rates (10), suggesting that like β-catenin, nuclear p120 could be important in tumor progression.

Kinesins associates with RanBP2, an important regulator of the nuclear export complex (33). RanBP2 also interacts with CRM1p (exportin1), which binds nuclear proteins via their NES domains and promotes their nuclear export. We show that p120 is actively exported from the nucleus. One possibility is that kinesin binding promotes the nuclear exclusion of p120 by removing it from the vicinity of the nuclear pores via its microtubule plus-end directed motor function. Alternatively, the di-
rect association of p120 to microtubules and/or kinesin may block its nuclear entry.

We reported previously that epithelial cells expressing p120-uncoupled E-cadherin exhibit primarily cytosolic p120 and reduced RhoA signaling (5), which correlates well with their inability to form compacted colonies (13). Upon p120 overexpression in NIH3T3 cells, p120 floods the cytoplasm causing loss of stress fibers and an exaggerated branching phenotype that is probably due to inhibition of RhoA activity (5). By using the branching phenotype as an assay for this p120 function, we show here that co-expression of p120 with kinesin heavy chain potently blocked both branching and loss of stress fibers. The effect is due to kinesin-p120 interaction, because branching is restored by co-expression of a small p120 fragment (N1) that binds avidly to kinesin but not by a p120 fragment (N3) that binds kinesin with very low affinity. Thus, binding of kinesin to p120 blocks p120 effects on contractility and cell morphology. Interestingly, kinesin co-expression blocked branching effects that were induced by p120-1A but not p120-3A, a result that parallels the relatively high and low affinities of kinesin for p120-1A and -3A, respectively (Fig. 7). These data provide evidence for a functional interaction between kinesin and p120 isoform 1, which is the predominant p120 isoform expressed in mesenchymal cells and epithelial tumors.

The mechanism by which kinesin affects p120-induced branching is unclear. One possibility is that kinesin binds competitively a region of p120 important for inhibition of RhoA. Another possibility is that kinesin sequesters or transports p120 away from an active site important for the manifestation of the branching phenotype (e.g. kinesin expression strongly decreased nuclear levels of p120, see Fig. 6C). In any case, kinesin promotes p120 delivery to cell membranes and appears to regulate its ability to inhibit contractility. Therefore, kinesin binding may regulate the reported ability of cytoplasmic p120 to promote cell motility (6, 7). We postulate that upon high level overexpression, p120 overwhelms both cadherin and kinesin binding resulting in loss of contractility and induction of the branching phenotype.

p120 is postulated to play a key role in the cross-talk between cell-cell adhesion and cell motility (8). Such effects are likely to be mediated by several factors, including precise and regulated targeting of p120 to cell junctions, protruding cell edges, and the nucleus. Our data suggest strongly that the kinesin-p120 interaction plays a fundamental role in these decisions and is therefore relevant to the postulated roles for p120 in tumor progression and metastasis.

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