A p120 CATENIN ISOFORM SWITCH AFFECTS RHO ACTIVITY, INDUCES TUMOR CELL INVASION AND PREDICTS METASTATIC DISEASE

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Running Title: A p120 isoform switch affects Rho activity and promotes invasion

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p120 catenin is a cadherin associated protein that regulates Rho GTPases and promotes the invasiveness of E-cadherin deficient cancer cells. Multiple p120 isoforms are expressed in cells via alternative splicing, and all of them are essential for HGF signaling to Rac1. However, only full-length p120 (isoform 1) promotes invasiveness. This selective ability of p120 isoform 1 is mediated by reduced RhoA activity, both under basal conditions and following HGF treatment. All p120 isoforms can bind RhoA in vitro, via a central RhoA binding site. However, only the co-operative binding of RhoA to the central p120 domain and to the alternatively spliced p120 N-terminus, stabilizes RhoA binding and inhibits RhoA activity. Consistent with this, increased expression of p120 isoform 1, when compared to other p120 isoforms, is predictive of renal tumor micrometastasis and systemic progression, following nephrectomy. Furthermore, ectopic expression of the RhoA-binding, N-terminal domain of p120 is sufficient to block the ability of p120 isoform 1 to inhibit RhoA, and to promote invasiveness. The data indicate that the increased expression of p120 isoform 1 during tumor progression contributes to the invasive phenotype of cadherin-deficient carcinomas, and that the N-terminal domain of p120 is a valid therapeutic target.

During epithelial tumor progression, tumor cells acquire the ability to invade surrounding tissues and eventually metastasize. A number of pathways have been uncovered to date that promote local invasiveness and metastatic tumor spread, and recent evidence argues that most of these converge on the loss of E-cadherin expression or function (1,2). E-cadherin is the main epithelial cell-cell adhesion molecule, and its loss in most of these tumors coincides with an epithelial to mesenchymal transition (EMT), where cells shed their epithelial characteristics and acquire a more mesenchymal phenotype. EMT is associated with normal development and wound healing, but its aberrant regulation contributes to cancer progression and metastasis (3). External cues, such as growth factors, can promote EMT via signaling pathways that are not well characterized, but may involve the function of Snail family transcriptional repressors. The best-characterized function of these transcription factors (i.e. Snail, Slug and SIP1) is down-regulation of E-cadherin expression (4-7). Consistent with this, experiments in transgenic mice strongly suggest that loss of E-cadherin directly promotes the transition of a benign adenoma into a carcinoma (8). Furthermore, re-establishing E-cadherin function in cadherin-deficient cell lines can reverse the invasive phenotype, suggesting that E-cadherin acts as a suppressor of cell invasion (9).

The mechanism by which E-cadherin promotes suppression of invasiveness is still unclear. However, the adhesive function, which is mediated by its extracellular domain, is not thought to be involved in this effect (10). The intracellular domain of E-cadherin interacts directly with β-catenin and p120 catenin (p120), via separate, conserved interaction domains. Recent data argue that both β-catenin and p120 binding are important for the anti-invasive properties of E-cadherin (10,11).
p120 binding promotes the stabilization of cadherin complexes on the plasma membrane and thus strengthens cell-cell adhesion (12,13). In addition, p120 overexpression induces dramatic changes in cell morphology and increases cell motility (14). These effects are apparently mediated by p120’s ability to suppress RhoA activity (15,16) and induce the activities of the related Rho GTPases, Rac1 and Cdc42 (16,17). When overexpressed in normal cells, p120 rapidly saturates available cadherin-binding sites, and then accumulates in the cytoplasm. Endogenous p120 also promotes the migration and invasiveness of E-cadherin-deficient cells (11). E-cadherin expression blocks p120 effects on cell morphology and motility, suggesting that the recruitment of p120 to E-cadherin complexes reduces its effects towards Rho GTPases, thereby determining the balance between sessile and motile states (11,15,17). Based on these observations, p120 could play two different roles in human cancer (reviewed in (18)). Loss of p120 expression can result in loss of E-cadherin stability and function, promoting invasiveness, while loss of E-cadherin expression during EMT can mislocalize p120 and promote migration and invasiveness via the p120-mediated regulation of Rho GTPases.

Most cell types express multiple variants (termed isoforms therein) of p120, which are derived by alternative splicing of a single gene (19,20). N-terminal splicing events lead to the use of four different translation start site ATGs (Keirsebilck et al., 1998), resulting in the expression of p120 isoforms type 1, 2, 3 or 4, respectively. Epithelial cells normally express p120 isoforms 3 and 4, while mesenchymal cells express predominantly full-length p120 isoform 1 (19-21). During epithelial tumor progression, the expression of the mesenchymal-specific p120 isoform 1 (full-length p120) is often turned on (19-21), resulting in a switch in the overall p120 isoform expression pattern. Recent data argue that expression of Snail, which induces an epithelial to mesenchymal transition (EMT), switches p120 isoform expression to isoform 1 in a cadherin-independent manner (22). The physiological significance of the isoform switch has been unclear. However, it is expected that p120 isoforms will have functional differences, as they differ in their inclusion of several conserved motifs, including a coiled-coil domain (N-terminal of isoform 1) and a “regulatory” domain (lacking in p120 isoform 4). Both domains are likely to mediate protein-protein interactions, either directly or indirectly following p120 phosphorylation, which occurs predominantly, but not exclusively, at the “regulatory” domain (23,24). Consistent with this, overexpression of a p120 truncation mutant lacking the entire N-terminal domain abrogated both EGF-stimulated cell motility and HGF-mediated cell scattering (25), suggesting that the N-terminal domain of p120 is important for receptor tyrosine kinase-induced cell motility.

In this report, the function of different p120 isoforms on cell invasion and the regulation of Rho GTPases are tested for the first time. Using E-cadherin-deficient cancer cells, we found that the ability of p120 to promote invasiveness depends on the p120 isoforms expressed. Expression of p120 isoform 1 promotes invasiveness, isoform 3 has no effect, while isoform 4 blocks invasiveness. The selective ability of p120 isoform 1 to induce invasiveness correlates with its ability to suppress RhoA activity. All p120 isoforms can bind RhoA via a central binding domain, however, co-operative interaction with the alternatively spliced N-terminal domain is required for RhoA inhibition. Testing a classic example of human EMT, clear cell renal cell carcinoma (ccRCC), we found that the ratio of p120 isoforms expressed predicts tumor micrometastasis and systemic progression following nephrectomy. Furthermore, we show that expression of the RhoA-binding, N-terminal domain of p120 is sufficient to block the ability of p120 isoform 1 to inhibit RhoA and to promote cell migration and invasiveness, validating p120’s potential as a therapeutic target.

**EXPERIMENTAL PROCEDURES**

**Cell culture, infections and transfections**

Culture conditions have been described previously (11). MDA-MB-231 cells expressing human p120-specific shRNA following retroviral infection (pRS vector) have also been described previously (11). As indicated, vector control (pRS) and p120-depleted cells were infected a second time with LZRS-neo or zeo control viruses,
viruses expressing murine p120 isoform 1A, 3A, 4A, or N1 truncation mutant, and selected with 1mg/ml G418 or 350μg/ml zeocin. Amaxa electroporations were performed according to the company’s protocol. In brief, 10^6 cells were resuspended in 100 μl solution T (Amaxa Inc.) containing 2μg of plasmid DNA. Electroporation was performed using program A-23. Electroporated cells were plated in 60mm dishes and incubated for 24 hours in normal culture medium. Cells were then washed in PBS and incubated for another 12 hours in serum-free Dulbecco’s minimal essential medium prior to performing invasion assays.

Constructs

LZRS-neo, LZRS-mp120 isoform 1A-neo, and LZRS-mp120 isoform 3A-neo were described previously (26). pRS human p120 shRNA was also described previously (11). LZRS-MS-zeocin was provided by Dr. Al Reynolds (Vanderbilt University, Nashville, TN) and encodes for zeocin instead of neomycin resistance. pcDNA3-N1 was also described previously (21). To generate LZRS-N1-zeo the pcDNA3-N1 plasmid was initially digested with XhoI, the ends filled using Klenow and the N1 fragment released following an additional digestion with EcoRI. This fragment was then ligated into the EcoRI and AfeI sites of LZRS-MS-zeocin. LZRS-mp120 isoform 4A-neo was generated by subcloning an EcoRI-XhoI fragment of LZRS-murine p120 4A-GFP (26), into the respective sites of the LZRS-murine p120 1A-neo vector. Finally, pcDNA3-RhoA-V14-myc (CA-RhoA) has been described previously (15). All constructs were verified by sequencing.

Western blotting

Western blotting procedures were conducted as described previously (11). Primary antibodies were used as follows: 0.25 μg/ml anti-p120 mAbs pp120 (BD Biosciences), 5 μg/ml anti-Flag tag mAb (M2; Sigma-Aldrich), 1 μg/ml anti-myc tag (9E10; Sigma-Aldrich), 0.5 μg/ml anti-cadherin 11 mAb (Zymed), 0.6 μg/ml anti-RhoA mAb (Santa Cruz), 0.75 μg/ml anti-Rac1 mAb (BD Biosciences) and 0.6 μg/ml anti-actin goat polyclonal antibody (I-19; Santa Cruz). Secondary antibodies were peroxidase-conjugated donkey anti–mouse IgG (Jackson Immunologicals) and donkey anti-goat IgG (Santa Cruz) used at 1:10,000.

Invasion and migration assays

Cell invasion was measured in vitro using BioCoat Matrigel-coated invasion chambers (8 μm pore size; Becton Dickinson), as previously described (11). Briefly, cells were serum-starved overnight, harvested using Cell Stripper (Mediatech, Inc.), and resuspended in serum-free medium at a density of 5 × 10^5 cells/ml. 100 μl (5 × 10^4 cells) of cell suspension was added to the top chamber, while serum-free medium containing either 20 ng/ml HGF (Peprotech, Inc.) or 5% FBS was added to the lower chamber, as a chemoattractant. Cells were allowed to invade the Matrigel and migrate to the underside of the invasion chamber for 20 h at 37°C in 5% CO2. Cells on the top surface of the chamber were removed by gentle scrubbing with a cotton swab, and cells on the underside were stained with 0.2% crystal violet and counted. Control experiments established that no significant growth differences existed between all cell lines tested under the conditions of this assay. Data from several experiments are expressed as percentage of control and represent the mean ± SEM of at least three independent determinations performed in duplicate. One and two asterisks represent P < 0.05 and P <0.01 respectively (t test, or one way ANOVA followed by post-hoc comparisons using the Newman-Keuls test). The Y27632 Rho kinase inhibitor (Calbiochem) was used in some experiments at 0.1 μM and 0.01 μM.

Rho/Rac activity assays

The activity of RhoA and Rac1 was determined in MDA-231 cells using specific pull-down assays for the activated forms of these proteins as reported previously (11). Rhotekin RBD or PAK-1 PBD (Upstate Biotechnology) bound to glutathione agarose beads were used to precipitate GTP-bound RhoA and Rac1 from cell lysates, respectively. Active, GTP-bound RhoA or Rac1, as well as total RhoA/Rac1, were visualized by SDS-PAGE and Western blotting using either a RhoA-specific mAb (26C4; Santa Cruz Biotechnology, Inc.) or a Rac1-specific mAb (BD Biosciences).
Guanine-nucleotide exchange assay.

The \[^3H\] GDP/GTP exchange of RhoA was measured at 25°C as described (15). In brief, 2 mg of \[^3H\] GDP-bound RhoA was incubated with ~4mg GST, GST–p120-1A, GST–p120-4A, GST-p120-4A–ΔRho or 0.5 mg His–TrioC (a Rho-specific GEF) at ambient temperature in a GEF assay buffer (27). The exchange reactions were terminated at the indicated times by nitrocellulose filter binding, and the amount of \[^3H\]GDP remaining bound to RhoA was expressed as a percentage of the radionucleotide bound at time 0.

Expression and purification of recombinant proteins

E.coli (BL21-DE3) containing a His-RhoA plasmid were cultured overnight at 37°C in 5 ml 2X YT with 50 μg/ml ampicillin. The culture was then expanded 8-fold into fresh culture medium (40 ml final volume), and incubated for 90 minutes at 37°C. His-RhoA expression was induced by incubating the bacteria with 1mM IPTG for 3 hours at 37°C. Bacteria were harvested (6000xg for 10 minutes at 4°C) and lysed in 1.5 ml lysis buffer (PBS containing 0.5% NP40, 10mM imidazole and protease inhibitors). Following a brief sonication, lysates were cleared of insoluble debris (14,000 x g at 4°C for 5 minutes), and 80-100 μl of Nickel Affinity Gel (SIGMA-Aldrich) was added to the supernatants. After two hours rotation at 4°C, the gel was washed 3X in Wash Buffer (50 mM H2NaPO4, pH 8.0, 0.3 M NaCl, 10 mM imidazole) and 2X in GDP binding buffer (20 mM Tris pH 7.5, 100 mM NaCl, 10 mM MgCl2, 1 mM dithiothreitol (DTT), 0.1 % Triton X-100) and mixed with 30 μl of purified His-RhoA (above) in 750 μl of protein binding buffer. Following 25 min rotation at room temperature, the agarose beads were washed 5X in protein binding buffer, and then boiled in 50 μl 2x LSB to remove associated proteins.

Recombinant GST fusion proteins (GST-p120 isoforms and mutants) were purified from BL21(DE3) E.coli, as previously described (15). Briefly, following IPTG induction and generation of bacterial lysates (in PBS containing 0.5% NP40 and protease inhibitors), clarified supernatants were incubated for two hours under rotation at 4°C with 50 μl of Glutathione-Agarose (SIGMA, G4510). GST-fusion proteins on agarose beads were then washed three times in PBS containing 0.5% NP40, and two times in protein binding buffer (50 mM Tris-HCl pH 7.3, 150 mM NaCl, 3 mM MgCl2, 1mM EDTA, 1 mM dithiothreitol (DTT), 0.1 % Triton X-100).

In vitro binding assays

GST-fusion proteins on agarose beads (GST, GST-p120 1N, GST-p120 4A, GST-p120 1A–ΔRho, GST-p120 4A–ΔRho, and GST-N-terminal truncation mutants of p120) were washed 2X in protein binding buffer (50 mM Tris-HCl pH 7.3, 150 mM NaCl, 3 mM MgCl2, 1mM EDTA, 1 mM dithiothreitol (DTT), 0.1 % Triton X-100) and mixed with 30 μl of purified His-RhoA (above) in 750 μl of protein binding buffer. Following 25 min rotation at room temperature, the agarose beads were washed 5X in protein binding buffer, and then boiled in 50 μl 2x LSB to remove associated proteins.

Human RCC tumor processing and analysis

H&E-stained sections of matched normal and renal tumor tissues were initially analyzed by a pathologist to confirm diagnosis, staging, and overall integrity of the tissue samples. Samples eligible for inclusion were from consented individuals treated by nephrectomy for newly diagnosed, histologically confirmed, localized clear cell RCC at Mayo Clinic between January 1, 2000 and July 30, 2004. Forty de-identified cases of ccRCC [20 that progressed during a 3-6 year follow-up, and 20 that did not progress] and matched fresh frozen normal renal tissues were chosen for protein extraction, and processed following the approval of the Institutional Review Board. Ten 10-µm-thick slices were cut from each frozen tissue block. Protein was isolated by direct solubilization in SDS-PAGE sample buffer.

Analysis of p120 protein expression in human renal tissue

Protein from human tumor samples was quantified using nitric acid mediated nitration of tyrosine (28). Equal amounts of protein (~30 μg) from each sample was resolved on 7% SDS-PAGE gels, transferred to polyvinylidene difluoride (PVDF) membrane (Immoblin-P, Millipore, Billerica, MA), and subjected to immunoblot
analysis using the appropriate antibodies (mAb HECD-1 for E-cadherin, mAb 15D2 recognizing all p120 isoforms, and mAb C-28 for c-Met) and Enhanced Chemiluminescence (ECL) Plus detection (Amersham, Piscataway, NJ) as described previously (29). Images were obtained on X-omat AR film and antigens quantified by fluorescence detection using a Typhoon 9410 Variable Mode Imager. The fluorescent signal was analyzed using ImageQuant 5.2 software (Amersham).

Immunohistochemistry was done on paraffin-embedded sections of primary tumor and normal renal tissues. The tissue was deparaffinized by placing slides into three changes of xylene and rehydrated in a graded ethanol series. The rehydrated tissue samples were rinsed in water and subjected to antigen retrieval in citrate buffer (pH 6.0) as described by the manufacturer (DAKO, Carpinteria, CA). Slides were treated with 3% H2O2 for 5 minutes to reduce endogenous peroxidase activity and washed with PBS containing 0.5% (w/v) Tween 20. E-cadherin, p120 and c-Met were detected using specific antibodies at a 1:1000, 1:400, and 1:200, dilution, respectively, in PBS/Tween and visualized using the Envision Plus Dual Labeled Polymer Kit following the manufacturer’s instructions (DAKO). Images were captured using ImagePro software.

RESULTS

Differential effect of p120 isoforms on cell invasiveness

Previously, we reported that depletion of endogenous p120 in E-cadherin-deficient cells significantly reduced cell migration and invasiveness, and that re-expression of full-length p120 (isoform 1) rescues these effects (11). To test the hypothesis that p120 isoforms differentially affect cell migration, invasiveness and Rho GTPase signaling, we have infected p120-depleted MDA-MB-231 cells (11) with amphotropic retroviruses expressing different murine p120 isoforms (Figure 1A). Polyclonal cell lines expressing control neo (shRNA-neo), or murine p120 isoform 1A, 3A, or 4A were generated after 10 days incubation with 1mg/ml G418. After selection, more than 95% of cells expressed a given p120 isoform. Invasiveness in vitro towards a gradient of HGF (20ng/ml) was measured using Matrigel-coated transwell filters (BD Biosciences), as described in the methods section. HGF was used as a chemoattractant, as previous studies have shown that blocking HGF signaling prevents MDA-MB-231 metastasis in nude mice (30). As expected from previous data (11), ectopic expression of p120 isoform 1A promoted cell invasiveness, when compared to the shRNA-neo control (Figure 1B). Interestingly, expression of p120 isoform 3A had no significant effect, while expression of p120 isoform 4A blocked invasiveness (Figure 1B). Identical results were also obtained when these p120 isoforms were overexpressed in NIH3T3 fibroblasts, using FBS as the chemoattractant (Figure 1C). Therefore, the N-terminal domain of p120, including its regulatory domain, is required for p120’s ability to promote invasiveness.

The data are consistent with the hypothesis that p120 isoforms 3 and 4 play a role in maintaining the sessile phenotype of epithelial cells, while the predominant expression of p120 isoform 1 in mesenchymal cells and epithelial carcinomas promotes cell invasiveness.

Differential effect of p120 isoforms on Rho GTPase activities

To test the hypothesis that the differential effects of p120 isoforms on cell migration and invasion are due to differential regulation of Rho family GTPases, we measured the levels of GTP-bound, active RhoA and Rac1 by performing Rhotekin and PAK pull-down assays, respectively. Initial experiments focused on p120 isoforms 1 and 4, which exhibited the biggest differences in cell invasiveness. First, we studied Rac1 activity under basal conditions, with cells serum-starved overnight. As can be seen in Figure 2A, depletion of endogenous p120 by constitutive expression of the human p120-specific shRNA (shRNA-neo) decreased Rac1 activity. As shown previously (11), re-expression of murine p120 isoform 1 rescued Rac1 activation. Re-expression of p120 isoform 4 was also able to induce activation of Rac1, to levels comparable to those induced by p120 isoform 1 (Figure 2A).
To characterize further the effects of specific p120 isoforms on Rac1 activity we performed time-course experiments where we determined the state of Rac1 activation following HGF treatment. Serum-starved cells were treated for the indicated times (up to 40 min) with HGF. Cell lysates were then generated, split in two, and levels of GTP-bound (active) or total Rac1 were measured in the same samples (see Methods section). Following densitometric analysis of GTP-bound and total GTPase from the western blots, activation data was expressed either as 1) % total (GTP-bound vs. total Rac1 x 100), which represents the percentage of total GTPase in the cell that is at the active, GTP-bound state (Figure 2B); or 2) % control (GTP-bound at time x' vs. GTP-bound at time 0' x 100), which represents the percentage of active GTPase in the cell at any given time point following HGF treatment, as compared to time 0 (Figure 2C). As can be seen in Figure 2B, HGF treatment caused a time-dependent increase in Rac1 activity of control (pRS) MDA-MB-231 cells. However, in repeated experiments, p120-depleted cells failed to activate Rac1 in response to HGF, while re-expression of either p120 isoform 1 or isoform 4 was able to rescue the HGF-induced Rac1 activation. Similar conclusions were obtained when the results were expressed as % control (Figure 2C), indicating that p120 depletion prevents HGF-induced Rac1 activation and that both p120 isoforms can rescue this effect. Combined, the data argue that the N-terminal domain of p120 is not required for HGF-mediated activation of Rac1 and thus, cannot explain the differential ability of these p120 isoforms to promote cell invasion.

Unlike Rac1 activation, the N-terminal domain of p120 is essential for the inhibition of RhoA. Figure 2D shows that under basal, serum-starved conditions, ectopic expression of p120 isoform 1, but not isoform 4, inhibits RhoA activity. Time course experiments, examining RhoA activation following HGF treatment, were also performed. The percentage of total active RhoA was significantly higher (up to 10 fold) in p120-depleted cells (shRNA) than in either control cells (pRS), or cells expressing murine p120 isoform 1 (Figure 2E). Furthermore, in repeated experiments, the expression of p120 isoform 4 resulted in significantly higher levels of activated RhoA, when compared to the other cell lines (Figure 2E). Interestingly, treatment of control cells (pRS) with HGF induced a transient and moderate increase in GTP-bound RhoA at 5 min, which was then followed by a decrease in active RhoA levels at later time points (Figure 2F). p120 depletion (shRNA) prevented both the initial increase and the late decrease of HGF-induced RhoA activity. p120 isoform 1 expressing cells responded to HGF treatment with a time-dependent decrease in RhoA activity, lacking the initial activation phase. In contrast, cells expressing p120 isoform 4 exhibited RhoA activation at 5 min of HGF treatment, which was persistent and not followed by the late decrease in Rho activation (Figure 2F).

The data argue that p120 is an important modulator of HGF-induced RhoA activity. As control MDA-MB-231 cells express p120 isoforms 1, 3 and 4 (not shown), the data also suggest that different p120 isoforms mediate the early (4A) and late (1A) effects of HGF on RhoA activity. Therefore, it is possible that the differential effects of p120 isoforms on cell invasion are mediated by differential regulation of RhoA activity.

The RhoA-Rho kinase pathway mediates p120 isoform effects on cell invasiveness.

Our previous data suggest that p120 is downstream of the c-Met receptor tyrosine kinase in the regulation of Rho family GTPases. p120 isoform 1 promotes the activation of Rac1 in response to HGF and suppresses RhoA activity. As a result, we postulated that cells expressing p120 isoform 1 exhibit higher protrusive ability (due to Rac1 activation) and reduced substrate adhesion (due to RhoA inhibition), leading to increased cell migration and invasiveness (Figure 3A). Expression of p120 isoform 4 also promotes the activation of Rac1; however, instead of inhibiting it causes a marked induction of RhoA activity in response to HGF. As the activation of RhoA could lead to increased contractility and substrate adhesion, we hypothesized that increased RhoA activation in p120 isoform 4 expressing cells is responsible for the reduced rates of cell invasion (Figure 3B). To test directly these hypotheses we either induced, or inhibited, the
activity of the RhoA-Rho kinase (ROCK) pathway in our cells and tested cell invasiveness in vitro. As can be seen in Figure 3C, ectopic expression of constitutively active (V14) RhoA in MDA-MB-231 cells expressing murine p120 isoform 1 suppressed in vitro invasion towards a gradient of HGF. Furthermore, treatment of MDA-MB-231 cells expressing p120 isoform 4 with the Rho kinase inhibitor Y27632 resulted in an increase of cell motility (Figure 3D) at doses that are at or below the Ki (0.14µM) of this inhibitor for Rho kinase (31). At higher doses (≥1µM) Y27632 failed to induce cell invasion, either due to the complete inhibition of cell contractility, or due to off target effects of the inhibitor (data not shown). An induction of invasiveness was also obtained by expressing the weak dominant negative N19-RhoA in the p120-4A expressing cells (data not shown). Treatment of p120-depleted cells, or cells expressing p120 isoform 1A with Y27632 failed to significantly alter invasiveness (data not shown). Taken together, these data support the hypothesis that the differential effects of p120 isoforms on cell invasion are due to the differential regulation of RhoA and its downstream effectors.

The N-terminal domain of p120 is required for RhoA inhibition

Previous work has shown that amino acids 622 to 628 within p120’s second loop region (L2; see Figure 1A) are required for its ability to induce morphological changes and to inhibit RhoA in cells when overexpressed, or to act as a GDP-dissociation inhibitor in vitro (15). Furthermore, recent data have demonstrated that the N-terminal domain of p120 binds directly to RhoA, but is not sufficient for RhoA inhibition (32). To better understand the mechanism by which p120 binds and regulates RhoA activity, we performed a series of in vitro binding experiments, as well as GDP dissociation assays, as described in Experimental Procedures. p120 isoforms 1 and 4, with or without amino acids 622-628 (ΔRho deletion; Figure 1A), were expressed in bacteria as GST-fusion proteins. Purified p120 proteins were incubated together with bacterially expressed His-tagged RhoA for 30 min, and precipitated using glutathione-agarose beads. The presence of associated RhoA was determined in the p120 precipitates following SDS Page and Western Blotting. As can be seen in Figure 4A, the binding of RhoA was impaired, but not completely blocked, by deletion of p120’s N-terminal domain (isoform 4), or deletion of amino acids 622-628 (isoform 1-ΔRho) alone, suggesting that both p120 regions are capable of binding RhoA. Consistent with this, a p120 deletion mutant lacking the N-terminal domain and amino acids 622-628 (isoform 4-ΔRho) was unable to bind RhoA in vitro.

To understand further the effect of p120 isoforms on RhoA activity, the GDP/GTP exchange rate of RhoA was measured using purified proteins in vitro. Incubation of [³H] GDP-loaded RhoA with TrioC, a fragment of Trio that has Rho exchange activity, significantly increased the exchange rate of Rho-bound GDP for GTP, as compared to incubation with control GST (Figure 4B). Incubation with p120 isoform 1 significantly blocked [³H] GDP dissociation from RhoA, as previously reported (15). In contrast, incubation of RhoA with either recombinant p120 isoform 1-ΔRho, or p120 isoform 4, had no effect on the rate of RhoA [³H] GDP dissociation: the dissociation rate was identical to that of the GST control (Figure 4B).

Finally, Figure 4C shows that the N-terminal 232 amino acids of p120 can directly interact with RhoA, in vitro. The data argue that two different p120 domains are involved in RhoA binding and that the presence of both is required for p120’s ability to act as a GDI (Figure 4D). We show that all N-terminal p120 isoforms can potentially associate with RhoA. However, our data suggest that the presence of p120’s N-terminal domain is essential for p120-mediated RhoA inhibition (see model in Figure 4D). This is consistent with the inability of p120 isoform 4 to inhibit RhoA in cells and to promote invasion. Since multiple p120 isoforms are expressed in cells, our data also raise the possibility that the relative abundance of p120 isoform 1, compared to N-terminally-truncated isoforms, determines p120 effects towards RhoA, cell migration and invasiveness.

An N-terminal p120 fragment blocks invasiveness
Next, we postulated that ectopic expression of the N-terminal domain of p120 would compete with endogenous p120 isoform 1, thus increasing RhoA activity and blocking invasiveness. Indeed, expression of an N-terminal fragment of p120 (N1) in MDA-MB-231 cells depleted of endogenous p120 and re-expressing murine p120 isoform 1A, caused a significant increase in basal RhoA activity (Figure 5A), effectively reversing the murine p120-mediated inhibition of RhoA activity (Figure 2D). The p120 fragment corresponds to the full-length N-terminal domain of p120 (N1; amino acids 1-323). Unlike for RhoA activity, N1 expression failed to affect the activity of Rac1, or to change the levels of endogenous cadherin 11, the predominant cadherin expressed in MDA-MB-231 cells. Furthermore, expression of N1 failed to increase RhoA activity in cells depleted of endogenous p120 (data not shown).

Consistent with its effects on RhoA activity, ectopic expression of the N1 p120 fragment in NIH3T3 cells inhibited their invasiveness in vitro (Figure 5B). Similar results were also obtained when N1 was expressed in MDA-MB-231 cells (Figure 5B), or UMRC3 ccRCC cells (not shown). The data argue that N-terminal p120 fragments can block invasiveness by effectively reversing the ability of endogenous p120 isoform 1 to affect Rho signaling. The data also suggest that targeting the N-terminal domain of p120 may be of therapeutic value, at least for a subset of human tumors.

A p120 isoform switch correlates with ccRCC micrometastasis and recurrence

Predominant expression of p120 isoform 1, compared to other p120 isoforms, is often observed in tumors that lack E-cadherin expression, and is termed p120 isoform switch (19). E-cadherin loss during EMT is an important event in tumor progression. However, the switch in p120 isoform expression is thought to be independent of E-cadherin (22). To test whether the increased expression of p120 isoform 1 in tumors undergoing EMT indeed correlates with invasiveness, we studied micrometastasis and systemic progression in clear renal cell carcinoma (ccRCC). The only effective therapy for ccRCC is nephrectomy. Unfortunately, even patients exhibiting early stage tumors with no evidence of metastasis have a high risk of recurrence (~35%) after nephrectomy. Micrometastasis, or spread of a small number of tumor cells not detectable by current methods, is thought to account for these recurrences.

In preliminary work we verified previous reports that E-cadherin loss, both at the RNA and protein levels, is very common in ccRCC (33-36), and also verified the upregulation of mesenchymal markers including increased expression of N-cadherin (not shown). Figure 6A shows immunohistochemical staining of one such patient with ccRCC. As can be seen, the E-cadherin staining in areas adjacent to tumor (normal) is concentrated in proximal tubule epithelial cells, the presumed precursor cells of ccRCC. In contrast, E-cadherin staining is lost in the tumor. p120 is predominantly expressed at the interfaces between proximal epithelial cells in normal tissue, and is maintained in the tumor, but becomes largely delocalized to the cytoplasm. Finally, c-Met expression (the HGF receptor) is absent in normal renal tissue but becomes highly expressed in the tumor, suggesting the potential for increased HGF signaling. The data argue that at least in a subset of ccRCC tumors that exhibit reduced E-cadherin expression and increased expression of c-Met, conditions exist that favor the involvement of p120 isoform 1 in invasiveness.

To test directly the hypothesis that a p120 isoform switch correlates with increased invasiveness in human tumors, we obtained fresh-frozen tissue from 40 ccRCC patients who underwent nephrectomy at Mayo Clinic between 2000 and 2004. All patients exhibited unilateral ccRCC with no evidence of metastasis at the time of nephrectomy, while 20 of them exhibited systemic cancer progression during a three-year follow-up. The expression of p120 isoforms was examined in the lysates of these ccRCC tumor samples by Western Blotting with a p120 antibody that binds all p120 isoforms (mAb 15D2). p120 isoform 4 was hardly detectable in normal, uninvolved tissue from these patients, or in tumors (not shown). p120 isoforms predominantly expressed in normal renal tissue are isoforms 1 and 3, at a ratio of ~1:2 (see Figure 6B). Following densitometric analysis of the p120
isoform bands, we arbitrarily designated tumors with p120 isoform switch to be those that exhibited a ratio of isoform 1 to 3 that was greater than 1. Using this criterion, tumor T2 but not T1 (Figure 6B) exhibits a p120 isoform switch.

When isoform switch data for 36 patients was compared to ccRCC systemic progression (tumor recurrence) and therefore micrometastasis, a significant correlation was uncovered. Indeed, approximately 90% of tumors that had progressed exhibited p120 isoform switch, compared to only 25% of localized tumors that did not metastasize. Despite a relatively small number of samples, the correlation between p120 isoform switch and ccRCC metastasis was highly significant (p<0.001), suggesting that the increased expression of p120 isoform 1, compared to other N-terminally-truncated p120 isoforms, promotes ccRCC invasiveness. Interestingly, neither E-cadherin expression per se, nor expression of p120 isoform 1 alone, correlated significantly with ccRCC progression (data not shown).

DISCUSSION

Epithelial cells primarily express p120 isoform 3, which lacks N-terminal sequences including a coiled-coil domain (19,20). However, highly motile mesenchymal cells and epithelial carcinomas express predominantly full-length p120 isoform 1 (19-21). Information on the expression of p120 isoform 4, which lacks the entire p120 N-terminal including the coiled-coil and regulatory domains, is limited, but is thought to be predominantly expressed in epithelial cells and lost in their tumor counterparts (21). The differential expression of p120 isoforms in tumor vs. normal epithelial cells has led to speculation of a possible role in tumor progression, and/or invasiveness (19). Our data indicate that p120 isoform expression is relevant to cancer cell migration and invasion. Only the mesenchymally expressed p120 isoform 1, whose expression is also turned on during epithelial tumor progression, is capable of promoting cell motility, whereas p120 isoform 4 suppresses migration/invasion and promotes a sessile cellular phenotype. Combined, the data suggest a model in which E-cadherin loss and p120 isoform 1 expression cooperate to promote tumor cell invasiveness.

Our data indicate that the N-terminal region of p120 is essential for promoting cell migration and invasiveness, and is required for p120’s ability to suppress RhoA activity. Indeed, basal levels of RhoA activity are high in cells expressing p120 isoform 4 and low in cells expressing full-length p120 isoform 1. Furthermore, induction of RhoA activity in p120 isoform 1 expressing cells suppresses cell motility, while suppression of the RhoA/ROCK pathway in p120 isoform 4 expressing cells induces motility and invasiveness. Thus, the regulation of RhoA activity by p120 is necessary for and underlies the different effects of p120 isoforms on the invasiveness of E-cadherin deficient cells. Our data with purified recombinant RhoA and p120 proteins indicate that all p120 isoforms are capable of binding RhoA via the central Armadillo domain region of p120 (a.a. 622-628; ∆Rho region). However, the interaction of p120 isoform 1 with RhoA is further stabilized via additional interaction of RhoA with the N-terminal p120 domain. GDP-dissociation assays indicate that the presence of both p120’s N-terminal and ∆Rho domains is essential for blocking GDP dissociation and inhibiting RhoA activity.

Interestingly, all p120 isoforms tested were able to induce Rac1 activation. The ability of p120 to induce Rac1 plays an important role in promoting cell migration and invasiveness (11). Nonetheless, the differential effect of N-terminal p120 isoforms on cell invasion is Rho-specific. While the exact mechanisms by which p120 affects RhoA and Rac1 activities are still a matter of investigation, our data indicate that different p120 domains mediate these effects. Therefore, the mechanism by which p120 inhibits RhoA activity is distinct from the mechanism by which p120 activates Rac1.

p120 depletion, as well as expression of murine p120 isoforms in endogenous p120-depleted cells, had profound effects on the basal activities of RhoA and Rac1. However, as our in vitro invasion studies utilized primarily HGF as the chemoattractant, we also tested the activation of Rho GTPases in our cell lines as a function of HGF treatment. The ability of HGF to promote cell scattering and migration depends on the regulation of Rho GTPases (37-39). Previous studies have established that Rac1 activity is
required for HGF-induced migration, while RhoA activation blocks the HGF effect (38,39). Consistent with these observations, HGF treatment induces a short-term increase (5-10 min) followed by a long-term reduction of RhoA activity in control MDA-MB-231 cells (see Figure 2F), as well as long-term activation of Rac1 activity (Figure 2C). However, despite an overall increase in endogenous RhoA activity, p120-depleted cells fail to alter either RhoA or Rac1 activities in response to HGF. The data suggest that p120 is an important modulator of HGF-induced Rac1 and RhoA activities. The mechanism by which p120 mediates HGF-induced Rac1 activation is currently under investigation, however, the p120 N-terminal domain is not involved as re-expression of either p120 isoform 1 or 4 rescues Rac1 activation. In contrast, p120 isoforms 1 and 4 had dramatically different effects on RhoA activity. Expression of p120 isoform 1 promoted the long-term reduction of RhoA activity by HGF, but failed to induce the short-term activation phase (Figure 2F). In contrast, isoform 4 expressing cells were able to induce RhoA in response to HGF but lacked the ability to induce the long-term inhibition of RhoA. As most cells, including control MDA-MB-231, express a mix of p120 isoforms 1, 3 and 4, the data suggest that different p120 isoforms mediate the early and late effects of HGF on RhoA activity. We postulate that the ability of all p120 isoforms to bind RhoA, but only of p120 isoform 1 to inhibit RhoA activation, is relevant in these HGF effects. Our data suggest that the presence of p120, and the particular isoforms expressed, is essential for HGF signaling to Rho GTPases and downstream effects of HGF towards Rho GTPases and cell migration.

The previous data suggested that targeting p120 function may be of therapeutic value in human cancer. As p120 is essential for cadherin stability, we reasoned that overall inhibition of p120 function would be detrimental to normal epithelial cells. However, since the N-terminal domain of p120 can bind RhoA directly and is required for p120-mediated RhoA inhibition, we postulated that overexpressing this p120 fragment would block the ability of p120 isoform 1 to promote invasiveness by inhibiting RhoA activity. The ability of the p120 N-terminal fragment N1 to block p120-mediated inhibition of RhoA and cell invasiveness indicates that uncoupling the p120 isoform 1-RhoA interaction may be a valid means of therapeutic intervention in invasive/metastatic tumors lacking E-cadherin expression.

Next, we examined whether altered p120 isoform expression correlates with more invasive/metastatic cancer. Both E-cadherin loss and increased HGF signaling are thought to be involved in the pathophysiology of renal cancer, and especially clear cell renal cell carcinoma (ccRCC). A classic EMT appears to be promoted in ccRCC via the transcriptional regulation of E-cadherin and other genes, at least in part through the von Hippel-Lindau – hypoxia-inducible factor pathway (reviewed in (33). Overall five-year survival for patients with ccRCC is approximately 60% (40,41). Once ccRCC spreads beyond the confines of the kidney, five year survival rates drop from 60% to less than 10% (42). The mainstay of treatment for patients with localized ccRCC is surgical excision. However, it is estimated that approximately 35% of patients with no evidence of metastasis at time of surgery will subsequently develop distant metastasis (i.e. experience disease progression)(43-45). We previously showed that endogenous p120 promotes the invasiveness of E-cadherin-deficient UMRC3 renal cell carcinoma cells in culture (11). Here, we examined p120 expression in a set of approximately 40 well-annotated human ccRCC tissue samples and correlated our data to micrometastasis and systemic progression. Despite the small data set, the data strongly support the hypothesis that increased expression of p120 isoform 1 compared to other p120 isoforms promotes ccRCC micrometastasis, and is predictive of tumor progression/recurrence. Consistent with our previous observations, it is the ratio of p120 isoforms that correlates with ccRCC micrometastasis, rather than simply the expression level of p120 isoform 1 alone. Combined, the data argue that a switch in p120 isoform expression and subsequent misregulation of RhoA are critical events that promote invasion and micrometastasis, at least in a subset of human tumors that lose E-cadherin expression during tumor progression.

Finally, we have focused our studies on p120 isoforms 1 and 4 as they exhibit the strongest differences in the regulation of RhoA and cell migration/invasiveness. Clearly, more studies are...
needed to clarify the roles of other p120 isoforms, including isoforms 2 and 3. Expression of p120 isoform 3 failed to induce migration and invasion in a number of cell lines, and unlike N1, expression of the isoform 3 N-terminal domain (N3 p120 fragment) failed to suppress invasion (data not shown). The addition of 6 amino acids in the central ΔRho region of p120 by expression of the alternatively spliced exon C may also have dramatic effects on p120’s ability to bind RhoA and regulate its activity. Finally, RhoA binding and subsequent inhibition may be regulated by p120 phosphorylation events that are just starting to become appreciated (32).

In summary, our data support an important role for p120 as a regulator of cell motility and invasiveness in epithelial cells. Under normal conditions, epithelial cells express p120 isoforms 3 and 4, which associate with E-cadherin and promote cell adhesion. In cancer, p120 is either mislocalized to the cytoplasm/nucleus upon loss of E-cadherin expression, or is lost altogether (18), presumably leading to the subsequent loss of cadherin-catenin complexes. In the first case, E-cadherin loss is often accompanied by preferential expression of p120 isoform 1, which, according to our data, promotes cell invasiveness. E-cadherin loss is a common event in many metastatic tumors, suggesting that p120 isoform 1 acts as a tumor promoter by contributing to the invasive phenotype of these cadherin-deficient carcinomas.

Whether loss of p120 expression during cancer progression also contributes to invasiveness is currently unknown. It is possible however that p120 isoforms 3 and 4 act as tumor suppressors by stabilizing cadherin complexes and suppressing motility and invasiveness. External cues, such as growth factors, are thought to promote EMT, a process thought to be important in tumor invasiveness. At least one such growth factor, HGF, requires p120 isoform 1 for its motogenic effects. Our data suggest that the increased expression of p120 isoform 1 during tumor progression contributes to the invasive phenotype of E-cadherin-deficient renal cell carcinomas, and that the N-terminal domain of p120 is a valid therapeutic target.

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FIGURE LEGENDS

Figure 1. Differential effects of p120 isoforms on cell invasiveness. A. p120 isoforms tested in this study include the naturally-occurring isoforms 1A, 3A and 4A. Murine p120 isoform 1A contains a coiled-coil domain and a regulatory phosphorylation domain in its N-terminal, a central armadillo domain and the alternatively spliced exon A in the C-terminal. Murine p120 isoforms 3A and 4A lack the coiled-coil, or the entire N-terminal domain, respectively. The ΔRho p120 mutant is identical to p120 isoform 1A with an in-frame deletion of amino-acids 622-628. Finally, N1 is a flag-tagged N-terminal mutant of p120 lacking the central and C-terminal domains. B. MDA-MB-231 cells with knocked down expression of endogenous p120 (shRNA) were infected with retroviruses expressing neomycin resistance alone (neo), or together with murine p120 isoforms 1A, 3A, or 4A. After G418 selection, the invasiveness of p120 re-expressing cells was tested in vitro towards a gradient of HGF, as described in Experimental Procedures. After a 20 hour incubation, cells on the underside of a Matrigel-coated transwell membrane were counted under a 20X objective. Data are expressed as % control and represent the mean ± standard error (S.E.) of three independent determinations performed in duplicate. ** represent p<0.001 as compared to the neo control (Student’s t-test). Re-expression of murine p120 isoform 1A promotes cell invasiveness, while expression of p120 isoforms 3A or 4A has no effect, or suppresses invasiveness, respectively (n=6). Lower 2 panels: Lysates from all cell lines were subjected to SDS-PAGE and western blotted for expression of p120 (mAb pp120) and actin.

Figure 2. Differential effect of p120 isoforms on Rho GTPase activities. A. Levels of total and GTP-bound Rac1 were determined in serum-starved MDA-MB-231 cells infected with control pRS and LZRS-neo viruses (pRS + neo), p120-depleted cells (shRNA + neo), or cells re-expressing p120 isoform 1A (shRNA + p120 1A), or 4A (shRNA + p120 4A). p120-depleted cells exhibited lower Rac1 activity under these basal conditions. Expression of either murine p120 isoform 1A or 4A rescued Rac1 activation (n=3). B. Levels of total and GTP-bound Rac1 were determined in the same serum-starved cell lines as above, following incubation with 20ng/ml HGF for the indicated times. Following western blotting and densitometry, mean ± SEM activity data from at least 3 independent determinations was expressed as % of total (active vs. total x 100). p120-depleted cells exhibited no Rac1 activation at any time following HGF addition, while control cells and cells re-expressing p120 isoforms 1A or 4A exhibited HGF-induced Rac1 activation with a peak at 10 min (p<0.01; ANOVA). C. The above data was also expressed as % of control (active Rac1 at time x’ vs. active at time 0 x100). As can be seen in the graph, both p120 isoforms 1A and 4A rescue Rac1 activation. D. Similar to Rac1, levels of total and active RhoA were also measured in control serum-starved MDA-MB-231 cells (pRS + neo), p120-depleted cells (shRNA + neo), or cells re-expressing p120 isoform 1A (shRNA + p120 1A), or 4A (shRNA + p120 4A). p120-depleted cells exhibited higher RhoA activity under these basal conditions, as reported previously (11). Expression of p120 isoform 1A blocked Rho activation, while expression of the N-terminally truncated p120 isoform 4A was unable to block RhoA activation, despite its ability to induce the activation of Rac1 (n=6). E. Levels of total and GTP-bound RhoA were also determined in HGF-treated cells. When mean ± SEM RhoA activity data were expressed as % of total, both basal and HGF-induced RhoA activities were much higher (2-10 fold) in shRNA expressing cells than in control cells (pRS) or cells expressing p120 isoform 1A (p120-1A). Cells expressing p120 isoform 4A exhibited 10-20 fold higher levels of activated RhoA than control cells (n≥3). F. When the same data were expressed as % control a biphasic response to HGF was uncovered. p120-depleted cells lacked both an initial activation phase and a late decrease in RhoA activity. Cells expressing murine p120 1A were
unable to induce RhoA activation in response to HGF, but retained the long-term inhibition phase (p<0.05; ANOVA), while cells expressing p120 isoform 4A induced RhoA within 5 min (p<0.05; ANOVA) but failed to inhibit RhoA in the long-term (n=3). Representative raw western blot data for figures 2B and 2E are shown in Supplemental figure 1.

Figure 3. RhoA-ROCK signaling mediates the differential effects of p120 isoforms on cell invasion. A. Effect of p120 isoform 1 on HGF-mediated migration/invasiveness. HGF-mediated cell scattering, migration and invasiveness require the upregulation of Rac1 signaling (right) and downregulation of cell contractility, promoted by RhoA-ROCK signaling (left). p120 depletion causes the upregulation of basal RhoA activity and blocks HGF-mediated Rac1 activation. Our data suggest that p120 isoform 1 mediates both the activation of Rac1 in response to HGF, and maintains RhoA signaling at a low level, thus promoting cell motility. This model explains why p120 depletion suppresses migration and invasiveness. It also predicts that activation of RhoA signaling in cells expressing p120 isoform 1 would suppress cell invasion. B. Effect of p120 isoform 4 on HGF-mediated migration/invasiveness. Our data indicate that cells expressing p120 isoform 4 can activate Rac1 in response to HGF. However, these cells have very high basal RhoA activity, which is further increased by HGF treatment. This model suggests that the increased RhoA-ROCK signaling in p120 isoform 4 expressing cells is able to suppress overall cell invasiveness. It also predicts that inhibition of RhoA-ROCK signaling would promote motility and invasiveness in cells expressing p120 isoform 4. C. p120-depleted MDA-MB-231 cells stably expressing murine p120 isoform 1A were electroporated and transfected transiently with pcDNA3-RhoA-V14-myc (V14-RhoA), or pcDNA control. Control immunofluorescence experiments verified that more than 60% of cells expressed CA-RhoA. One day after transfection, cells were tested in vitro for invasion towards HGF, as described in Fig. 1. In agreement with our hypothesis (Fig. 3A), overexpression of CA-RhoA significantly suppressed the invasiveness of p120 isoform 1A expressing cells (n=6). D. The in vitro invasiveness of p120-depleted cells stably expressing murine p120 isoform 4A was tested under control conditions, or following treatment with the ROCK inhibitor Y27632 (0.01 – 0.1μM). In agreement with our hypothesis (Fig. 3B), HGF-induced invasiveness of p120 isoform 4 expressing cells was significantly increased in cells treated for 24 hours with Y27632.

Figure 4. N-terminal and central regions of p120 cooperate to promote RhoA binding and inhibit RhoA activity. A. Immunoblot analysis of GDP-RhoA association with various p120 isoforms and deletion mutants. His-tagged RhoA and GST-p120 proteins were bacterially expressed and purified. The extent of GDP-RhoA association with recombinant p120 isoforms 1 vs. 4, with or without the 622-628 (ΔRho) deletion, was determined in binding assays, in vitro. Both the central ΔRho p120 region and the N-terminal domain are required for efficient RhoA association. Deletion of both domains abrogates RhoA binding. Data shown is representative of at least 4 independent determinations. B. 2μg [3H]GDP bound RhoA (in the presence of GST; GST) were incubated alone, with ~4μg GST-p120 isoform 1A, GST-p120 isoform 4A, GST-p120 isoform 1-ΔRho (p120-ΔRho), or with 0.5μg of His-TrioC, a RhoA-specific GEF. Incubations were carried out at ambient temperature in a GEF assay buffer (15). The amount of RhoA-bound radionucleotide at time 0 was taken as 100%. As previously shown, p120 isoform 1 inhibited the intrinsic guanine nucleotide exchange activity of RhoA. p120 proteins lacking the N-terminal domain (p120-4A), or ΔRho region (p120-ΔRho) had no effect on RhoA exchange activity. Identical results were obtained in at least 3 independent determinations. C. The extent of GDP-RhoA association with a recombinant p120 N-terminal mutant containing amino acids 1-232 was determined in binding assays, in vitro. The N-terminal 232 amino acids of p120 associate with RhoA-GDP (upper panel). Lower panel shows the expression of different GST proteins. D. Model of p120-RhoA interactions. Our data suggest that naturally-occurring p120 isoforms, including those lacking the N-terminal domain (like isoform 4), can associate with RhoA via their central ΔRho region. The N-terminal p120 domain is required for stabilization of RhoA binding (increased affinity interaction) and for inhibiting the dissociation of GDP from RhoA. Therefore, interaction of p120 isoform 1 with RhoA results in local inhibition of RhoA activity, while p120 isoform 4 fails to inhibit RhoA, but may promote
the recruitment of RhoA at sites where it can be activated by upstream signaling events (i.e. HGF signaling).

**Figure 5. An N-terminal p120 fragment suppresses invasiveness.** A. Effect of p120 N-terminal fragment overexpression on RhoA and Rac1 activities. p120-depleted MDA-MB-231 cells re-expressing murine p120 isoform 1A were infected with retroviruses expressing zeocin resistance alone (zeo), or together with a flag-tagged N-terminal p120 truncation mutant containing amino acids 1-323 (N1). The relative levels of active RhoA and Rac1 were determined in stable polyclonal cell lines using pull-down assays and western blots as described earlier. Expression of N1 did not affect endogenous levels of cadherin 11, or Rac1 activation, but caused a significant increase in basal RhoA activity. B. NIH3T3 fibroblasts and MDA-MB-231 cells stably expressing zeocin resistance alone (zeo), or together with the N1 p120 fragment (N1) were subjected to invasion assays to determine the effect of N1 on cell invasiveness. Results are the mean ± S.E. of three independent determinations performed in duplicate. ** represent p<0.001 as compared to the zeo control (Student’s t-test). Lower 2 panels: Lysates from all cell lines were subjected to SDS-PAGE and western blotted for expression of N1 (using the anti-flag tag mAb M2) and actin.

**Figure 6. p120 isoforms and ccRCC.** A. Immunohistochemical analysis of a clear cell renal cell carcinoma (ccRCC) sample compared to patient-matched normal kidney. E-cadherin expression is lost in this ccRCC tumor sample, as it is in the majority of ccRCC cases. Expression of p120 is maintained, but largely mislocalized to the cytoplasm. Expression of the HGF receptor c-Met is absent in normal tissue, but becomes highly induced in the tumor. B. To determine whether endogenous p120 isoforms correlate with invasiveness in ccRCC we initially determined the relative p120 isoform expression in 36 tumor and patient-matched normal fresh-frozen kidney samples (see Experimental Procedures). The ratio of p120 isoform 1 (which induces invasion in vitro) to the shorter p120 isoform 3 (lower band; which does not induce invasion in vitro) was calculated in both normal (N) and ccRCC tumor (T) samples. p120 isoform 1 and 3 expression in MDA-MB-231 cells is also shown as an additional control (C). Normal kidney exhibited a p120 isoform 1A:3A ratio of ~0.4. p120 isoform switch is indicated by a higher relative expression of p120 isoform 1 in tumor samples (ratio larger than 1). According to this analysis, the second tumor (T2) exhibits a p120 isoform switch, while the first one does not. C. When p120 isoform switch data was compared with disease progression, a significant correlation was uncovered between p120 isoform switch and the incidence of metastatic ccRCC disease. 89.2% of ccRCC tumors that metastasized following nephrectomy within a 5 year follow-up exhibited a p120 isoform switch, versus only 25% of localized tumors.
Figure 1: Yanagisawa et al.

A

![Diagram of Armadillo domain and shRNA constructs](image)

B

![Bar graph showing invading cells](image)

C

![Bar graph showing invading cells](image)
Figure 2: Yanagisawa et al.

A

Active Rac
Total Rac

pRS + neo  shRNA + neo  shRNA + p120 1A  shRNA + p120 4A

B

Rac activity (% total)
HGF treatment (min)

C

Rac activity (% control)
HGF treatment (min)

D

Active RhoA
Total RhoA

pRS + neo  shRNA + neo  shRNA + p120 1A  shRNA + p120 4A

E

RhoA activity (% total)
HGF treatment (min)

F

RhoA activity (% control)
HGF treatment (min)
Figure 3: Yanagisawa et al.

A

B

C

D

HGF

\[ \text{shRNA} \]

\[ \text{p120-1A} \]

\[ \text{CA-RhoA} \rightarrow \text{RhoA} \rightarrow \text{Rac1} \]

\[ \text{ROCK} \]

\[ \text{Migration/Invasion} \]

\[ \text{shRNA} \]

\[ \text{p120-4A} \]

\[ \text{RhoA} \rightarrow \text{Rac1} \]

\[ \text{ROCK} \]

\[ \text{Migration/Invasion} \]

\[ \frac{1}{2} \]

\[ \text{Invasion (% control)} \]

\[ \frac{1}{2} \]

\[ \text{Invading cells (% control)} \]

\[ \text{shRNA + p120 1A} \]

\[ \text{shRNA + p120 4A} \]

\[ \text{Control} \]

\[ \text{0.01 \mu M} \]

\[ \text{0.1 \mu M} \]

\[ \text{Y27632} \]

\[ \text{WB: Myc-tag} \]

\[ \text{WB: actin} \]

\[ \text{pCDNA3} \]

\[ \text{V14-RhoA} \]
Figure 4: Yanagisawa et al.

A

Isoform 1  Isoform 1-ΔRho  Isoform 4  Isoform 4-ΔRho

GDP-RhoA

p120

B

Bound [3H]GDP remaining %

Time (min)

+GST

+ΔRho

+p120-4A

+p120-1A

C

GST  p120(1-232)  His-RhoA

GDP-RhoA

GST-p120(1-232a.a.)

GST

D

Isoform 1

Isoform 4

(aa. 622-628)

RhoA-GDP

Increased affinity

Low affinity interaction

Inhibition of GDP dissociation

No effect on GDP dissociation

Reduced RhoA activity
Figure 5: Yanagisawa et al.

A

- Active RhoA
- Total RhoA
- Active Rac1
- Total Rac1
- Cadherin 11
- N1 fragment
- Actin

Zeo | N1
---|---

B

**Invading cells (% control)**

A bar graph showing the comparison between Zeo and N1 conditions for different proteins.

**WB: M2**

- Zeo
- N1

**WB: actin**

- Zeo
- N1

NIH3T3

MDA-MB-231

**NIH3T3**

**MDA-MB-231**
Figure 6: Yanagisawa et al.

A

Normal  cRCC

E-cadherin

p120 (15D2)

c-Met

B

| N1 | T1 | C | N2 | T2 |
|----|----|---|----|----|
| 1A |    |   | 3A |    |   |

Ratio: 0.45 0.8 0.8 0.37 1.69

C

Isoform switch (% total)

Metastatic: 89.2
Local: 25.0

Odds Ratio = 24.2, p<0.001, n=36
95% Confidence Interval = 3.8-156.8
A p120 catenin isoform switch affects Rho activity, induces tumor cell invasion and predicts metastatic disease
Masahiro Yanagisawa, Deborah Huveldt, Pamela Kreinest, Christine M. Lohse, John C. Cheville, Alexander S. Parker, John A. Copland and Panos Z. Anastasiadis

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