Interaction of p190RhoGAP with C-terminal domain of p120-catenin modulates endothelial cytoskeleton and permeability

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Running title: p120-catenin–p190RhoGAP binding

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Background: p120-catenin protein interactions regulate vascular permeability

Results: We identified p190RhoGAP-binding domain of p120-catenin and evaluated its functional significance

Conclusion: Binding of p190RhoGAP occurs at (AA 820-843) domain of p120-catenin and promotes activation of Rac and downregulation of Rho signaling leading to increased endothelial barrier.

Significance: These data demonstrate functional significance of uncoupling the p120-catenin – p190RhoGAP interaction in the context of agonist-induced endothelial permeability

SUMMARY

p120-Catenin is a multidomain intracellular protein which mediates a number of cellular functions including stabilization of cell-cell transmembrane cadherin complexes as well as regulation of actin dynamics associated with barrier function, lamellipodia formation and cell migration via modulation of the activities of small GTPases. One mechanism involves p120 catenin interaction with Rho GTPase activating protein (p190RhoGAP) leading to p190RhoGAP recruitment to cell periphery and local inhibition of Rho activity. In this study, we have identified a stretch of 23 amino acids within the C terminal domain of p120 catenin as the minimal sequence responsible for the recruitment of p190RhoGAP (herein referred to as CRAD: Catenin-RhoGAP-Association Domain). Expression of the p120-catenin truncated mutant lacking the CRAD in endothelial cells attenuates effects of barrier protective oxidized phospholipid, OxPAPC. This effect was accompanied by inhibition of membrane translocation of p190RhoGAP, increased Rho signaling as well as suppressed activation of Rac1 and its cytoskeletal effectors.
PAK1 and cortactin. Expression of p120 catenin truncated mutant lacking CRAD also delayed the recovery process after thrombin-induced endothelial barrier disruption. Concomitantly, RhoA activation and downstream signaling were sustained for a longer period of time while Rac signaling was inhibited. These data demonstrate a critical role for p120-catenin (AA 820-843) domain in the p120-catenin - p190RhoGAP signaling complex assembly, membrane targeting and stimulation of p190RhoGAP activity towards inhibition of Rho pathway and reciprocal upregulation of Rac signaling critical for endothelial barrier regulation.

**INTRODUCTION**

p120-catenin is a multidomain intracellular protein containing N-terminal regulatory domain, a central domain with 10 Armadillo repeats and a less well functionally defined C-terminal tail (1-4). One of the major functional roles of p120-catenin in mammalian cells is stabilizing cell-cell transmembrane cadherin molecules at the cell membrane by modulating their trafficking and degradation either through direct binding to the cadherin cytoplasmic tail or indirectly through regulating the trafficking machinery (1,4-8). By virtue of its main localization in adherens junctions, p120-catenin plays an essential role in the maintenance of cell-cell adhesion and in the control of intercellular permeability of epithelial as well as endothelial cell monolayers in vitro and in vivo (9-12). In addition, a number of recent studies have shown that it plays an important role in vascular development, in inflammation and in tumor progression and metastasis (13-17).

One of the many important roles of p120-catenin consists of its regulatory activity on actin dynamics associated with barrier function, lamellipodia formation and cell migration through modulation of the activities of small GTPases RhoA, Rac, and Cdc42 (17-22). Although p120-catenin-mediated inhibition of RhoA activity was shown to occur via a direct binding of RhoA to a region within the N-terminal regulatory domain (23), other studies suggest that it may also do so indirectly through recruitment and binding of the Rho family exchange factor Vav2 (24) or through functional interaction with Rho GTPase activating protein (p190RhoGAP) (25). Consistent with the latter, we have recently shown that p190RhoGAP plays a key role in integrating the opposed activities of Rac and Rho and the actin remodeling events involved in endothelial barrier enhancement by oxidized 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphorylcholine (OxPAPC) (26). OxPAPC treatment of human pulmonary artery endothelial cells led to tyrosine phosphorylation and recruitment of p190RhoGAP to the plasma membrane in a Rac-dependent manner where it then formed a complex with adherens junction protein p120 catenin (26). In this study, we have sought to identify the minimal sequence responsible for the interaction between p120-catenin and p190RhoGAP using a series of deletion and C-terminal truncation mutants and to characterize the functional significance of p120-catenin – p190RhoGAP molecular uncoupling. Tagged constructs were exogenously expressed in human embryonic kidney cells and analyzed by pull-down assays followed by Western blotting. The effects of p120-catenin mutants on Rac- and Rho-dependent signaling, cytoskeletal remodeling and agonist-induced permeability changes were investigated upon transient transfection in primary culture of human pulmonary artery endothelial cells (HPAEC).

**EXPERIMENTAL PROCEDURES**

**Reagents and cell culture.** Unless specified otherwise, biochemical reagents were obtained from Sigma (St. Louis, MO). Reagents for immunofluorescence were purchased from Molecular Probes (Eugene, OR). Antibodies to diphospho-Ser19/Thr18 myosin light chain (MLC), phospho-Ser199–PAK1 and phospho-Ser423–PAK1 were from Cell Signaling Inc (Beverly, MA). Antibodies to phospho-Thr850 myosin-associated phosphatase (MYPT) and phospho-Tyr421–cortactin were from Millipore (Billerica, MA); antibodies to p120-catenin and p190RhoGAP were from BD Transduction Laboratories (San Diego, CA). Primary antibodies to GST tag were from Santa Cruz (Santa Cruz, CA), antibody to His tag from QED Bioscience (San Diego, CA), and fluorescently labeled (Alexa Fluor 488 and 555) secondary antibodies were from Invitrogen (Grand Island, NY).

Human Embryonic Kidney (HEK) 293 T cells were cultured in DMEM/high glucose (Invitrogen, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS, Mediatech) and
maintained in a humidified tissue culture incubator at 37°C and 5% CO₂. HEK 293 transfections were carried out using Lipofectamine 2000 (Invitrogen, Grand Island, NY) as recommended by the manufacturer. Cells were used 48 hours after transfection. Human pulmonary artery endothelial cells (HPAEC) were obtained from Lonza (Allendale, NJ), maintained in a complete culture medium according to the manufacturer’s recommendations and used for experiments at passages 5-7. Transient transfections of HPAEC were carried out using PolyJet reagent from Signagen Laboratories (Rockville, MD) as recommended by the manufacturer. Cells were used 24 hours after transfection.

**Constructs.** The original p120-catenin and p190RhoGAP constructs described previously (6,22,27,28) were subcloned into the Gateway entry vector (pENTR/D-TOPO) then transferred by recombination reactions using LR clonase to the destination vectors pDEST26 (N-terminal Histidine fusion) and pDEST27 (N-terminal GST fusion) (Invitrogen, Grand Island, NY). All constructs were verified by sequencing prior to use.

**PCR-based mutagenesis.** Stop codons were introduced at different positions within the C-terminal tail of p120-catenin (Table 1) using a PCR-based mutagenesis approach. Briefly, full length p120 catenin construct was amplified by PCR in the presence of a selected pair of primers containing the desired mutation (Table 1). The PCR reaction mixture was then treated with DpnI restriction enzyme to eliminate the original methylated template plasmid DNA before retransforming into E. coli bacteria. All mutations were confirmed by sequencing prior to use.

**GST/His pull down.** 48 hours after transfection, cells were washed in cold phosphate buffered saline (PBS) and lysed on ice with cold TBS-NP40 lysis buffer (20 mM Tris pH 7.4, 150 mM NaCl, 1% NP40) supplemented with protease and phosphatase inhibitor cocktails (Roche, Indianapolis, IN). Clarified lysates were then incubated with either Glutathione magnetic beads (Thermo Scientific, Rockford, IL) or His-Tag Dynabeads (Invitrogen, Grand Island, NY) overnight at 4°C, washed 3-4 times with TBS-NP40 lysis buffer and the complexes were analyzed by Western blotting using appropriate antibodies. The fluorescent signals were acquired and analyzed using Pharos FX Plus molecular imager (Bio-Rad, Hercules, CA) as recommended by the manufacturer.

**Rac and Rho activation assay.** Rac and Rho activation was assessed using the GTP-bound GTPase pulldown assays as described previously (29,30). Briefly, after the incubation with agonist, cell lysates were collected, and GTP-bound Rac was captured using pull-down assay with PAK-1 PBD agarose, while GTP-bound Rho was captured using pull-down assay with rhotoxin agarose. The levels of activated Rac1 and Rho as well as total Rac1 and Rho content were evaluated by Western blot analysis and quantified by scanning densitometry of the autoradiography films. The levels of activated GTPases were normalized to total Rac or Rho content in cell lysates.

**Measurements of transendothelial electrical resistance (TER).** TER measurements were performed in HPAEC monolayers after transfection using an electrical cell-substrate impedance sensing system (Applied Biophysics, Troy, NY) as described previously (29).

**Immunofluorescence and image analysis.** Endothelial monolayers plated on glass cover slips were transfected with corresponding plasmids using PolyJet reagent (Signagen Laboratories, Rockville, MD). 48 Hours after transfection, cells were subjected to immunofluorescence staining with GST antibody to detect transfected cells, VE-cadherin to visualize adherens junctions, and DAPI to visualize nuclei, as described previously (31,32)

**Western blot analysis of MYPT, MLC, cortactin and PAK1 phosphorylation.** Analysis of MYPT and MLC phosphorylation was used to monitor activation of Rho signaling, and levels of phosphorylated cortactin and PAK1 were assessed as readouts of Rac activation, as previously described (32,33).

**Statistical analysis.** Results are expressed as mean ± SD of three to six independent experiments. Experimental samples were
compared to controls by unpaired Student’s t-test. For multiple-group comparisons, a one-way variance analysis (ANOVA) and post hoc multiple comparisons tests were used. P<0.05 was considered statistically significant.

RESULTS

Interaction between p190RhoGAP and p120-catenin. Full length p120-catenin or deletion mutants containing an N-terminal (Nt) GST tag and lacking either N-terminus part, or reported Rho binding site, or clusters of Armadillo domains (Figure 1A) were co-transfected in HEK 293T cells together with a His-tagged p190RhoGAP construct. After 48 hours, clarified cell lysates were incubated either with His-Tag magnetic beads or Glutathione magnetic beads, and the resulting complexes were analyzed by western blotting as described in the Materials and Methods section. All p120-catenin constructs used in this analysis (asterisk) were detected in the His-p190RhoGAP pulldown complexes (Figure 1B). The additional few bands detected by Western blotting with p120-catenin antibody are likely to correspond to the different isoforms of endogenous p120-catenin associating with p190RhoGAP. These bands were also detected when GST alone was cotransfected with p190RhoGAP.

Similar results were obtained when GST pulldown was performed (Figure 2). p190RhoGAP was not detected when GST alone was expressed whereas in all the other GST-p120-catenin constructs, p190RhoGAP was detected in the precipitate (Figure 2, bottom panel). Similar interactions were observed when full length p120-catenin was co-transfected with p190RhoGAP mutants lacking either the GTP-binding domain (GBD) or the carboxy (C)-terminal Rho family GTPase activating protein (GAP) domain (data not shown). Taken together these data suggest that p190RhoGAP association with p120-catenin requires the 23 amino acid stretch immediately following the last armadillo domain of p120-catenin. We will refer to this domain as CRAD (Catenin-RhoGAP-Association Domain) and to the GST-p120 1A truncated mutants as p120(1-820) and p120(1-843).

p120(1-820) Catenin mutant attenuates agonist-induced EC barrier enhancement. The functional consequence of abrogated p120-catenin–p190RhoGAP complex formation was tested in the model of agonist-induced permeability regulation in human pulmonary endothelial cells (HPAEC). Cells were transfected either with full-length p120-catenin, with p120(1-820) mutant which lacks CRAD domain and the ability to recruit p190RhoGAP, or with the CRAD-containing truncated mutant p120(1-843). Endothelial cell response to barrier enhancing agonist OxPAPC was evaluated by measurement of the electrical resistance (TER) across the EC monolayer. Ectopic expression of full-length p120-catenin (p120 1A) showed trend to increased TER elevation in response to OxPAPC, although this difference did not reach statistically significant levels. In turn, expression of p120(1-820) mutant which lacks CRAD domain and the ability to recruit p190RhoGAP, or with the CRAD-containing truncated mutant p120(1-843). 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OxPAPC-induced TER changes in EC expressing p120 mutants is presented in Figure 4D. p120(1-820) Catenin mutant colocalizes with VE-cadherin at the adherens junctions and inhibits agonist-induced adherens junction enhancement. OxPAPC-induced barrier enhancement is associated with increased assembly of adherens junction (AJ) complexes and peripheral accumulation of VE-cadherin (26,33,34). The next experiments tested whether the lack of p190RhoGAP binding to p120 catenin would affect increased localization of p120 catenin and VE-Cadherin to the plasma membrane caused by OxPAPC, which reflects increased AJ formation. Similar to endogenous proteins, ectopically expressed full length p120-catenin (p1201A) and truncated p120(1-820) mutant co-localized with VE-cadherin at the adherens junctions of non-stimulated HPAEC (Figure 5A). However, OxPAPC-induced adherens junction enhancement detected by staining of p120-catenin mutants was suppressed in cells expressing p120(1-820) (Figure 5B).

p120(1-820) Catenin attenuates OxPAPC-induced p190RhoGAP membrane translocation and modulates Rho and Rac signaling pathways. The translocation of p190RhoGAP to the cell membrane is facilitated by p120-catenin (25,26). We examined whether this event is affected by expression of p120(1-820) mutant. Indeed, expression of p120(1-820) inhibited both the basal and OxPAPC-induced p190RhoGAP accumulation in the membrane fraction (Figure 6A). Since the observed decrease in the pool of membrane-associated p190RhoGAP could affect local Rho signaling, we assessed the effect of the presence or the absence of CRAD on the levels of MLC phosphorylation reflecting basal Rho activation. After transfection with full length p120 catenin, p120(1-820) or p120(1-843) constructs, HPAEC were analyzed by Western blotting using anti pp-MLC antibody. Unlike the CRAD containing-constructs (full length and p120(1-843)), expression of p120(1-820) lacking CRAD domain led to higher basal level of Rho activity and increased MLC phosphorylation mediated by Rho (Figure 6B,C). Increased basal Rho activation was also observed in p120(1-820) expressing HeLa cells (Figure 6B,C) suggesting a fundamental mechanism of Rho regulation by CRAD domain of p120-catenin.

In order to analyze the role of the CRAD sequence in OxPAPC-induced Rac1 activation we transfected either full length p120 1A, or p120(1-820), or p120(1-843) into HPAEC, and OxPAPC-induced Rac1 activation was assessed using the pull down assay described in Materials and Methods. In cells transfected with p120 1A, a transient increase in the amount of GTP-loaded Rac1 is observed at 5 minutes. In samples where the deletion mutant lacking CRAD was used, OxPAPC failed to induce this transient increase in activated Rac1, whereas OxPAPC-induced Rac1 activation in EC expressing the mutant containing CRAD (p120(1-843)) was preserved (Figure 6D). Consistent with this observation, expression of p120(1-820) catenin also suppressed activation and phosphorylation of Rac effectors PAK1 and cortactin upon OxPAPC treatment (Figure 6E).

p120(1-820) Catenin suppresses EC barrier recovery after thrombin. Stimulation of endothelial cells with thrombin causes rapid and reversible Rho-dependent increase in endothelial permeability (30). In the present study, CRAD-containing, full length p120 1A or the mutant p120(1-820) devoid of CRAD were expressed in HPAEC, and monolayer integrity was assessed by TER upon treatment with thrombin. Thrombin challenge of non-transfected HPAEC (data not shown) and cells expressing full length p120 catenin caused a prominent but reversible permeability increase as detected by TER measurements (Figure 7A). The initial phase of thrombin-mediated monolayer disruption was similar between the two constructs. However, expression of p120(1-820) markedly delayed the recovery of EC barrier after thrombin challenge (Figure 7A).

Expression of p120(1-820) catenin mutant prolongs thrombin-induced RhoA activation in HPAEC. Downregulation of Rho signaling is an essential prerequisite of endothelial barrier recovery after agonist stimulation. We analyzed the level of Rho activation during the recovery phase following thrombin-induced monolayer disruption of endothelial cells expressing either the full length p120 1A or p120(1-820) mutant lacking the p190RhoGAP-binding motif. Unlike p120 1A
expressing cells, cells expressing p120(1-820) exhibited delayed Rho activation which was observed at later time points (30 and 60 minutes after thrombin challenge), the times normally corresponding to development of the recovery phase, Rho downregulation and reciprocal activation of Rac signaling (Figure 7A, B). The recovery process can also be monitored by assessing the level of phosphorylation of downstream Rho targets, MYPT and MLC. A rapid and transient activation of RhoA-dependent signaling was evident in thrombin-stimulated HPAEC expressing full length p120 catenin where basal levels were reestablished less than 30 min after thrombin treatment (Figure 7C). In contrast, Rho signaling in HPAEC expressing p120(1-820) remained elevated even after 30 min of thrombin treatment (Figure 7C). In turn, Rac-dependent phosphorylation of cortactin at later times after thrombin challenge, which reflects reciprocal upregulation of Rac signaling leading to EC barrier restoration, was suppressed by expression of p120(1-820)-catenin.

DISCUSSION
This study identified for the first time the p120-catenin domain involved in the functional interaction with p190RhoGAP and evaluated functional significance of uncoupled p120-catenin – p190RhoGAP interaction in the context of agonist-induced endothelial permeability regulation. Targeting of p190RhoGAP to cell junctions by p120-catenin represents an important mechanism of local regulation of Rho GTPases, which control cell-cell interactions, monolayer integrity and vascular endothelial permeability. This study sought to identify the region within p120-catenin that is responsible for the formation of p120-catenin - p190RhoGAP functional complex.

Published studies identified p120-catenin Armadillo domains as a region interacting with the juxtamembrane domain (JMD) of cadherins (35,36). Based on three-dimensional structure analysis, Ishiyama et al., have shown that the bound JMD core peptide stretches along the N-terminal half of the p120-catenin ARM domain (ARM repeats 1-5) in the opposite orientation (4) suggesting that these regions are less likely to be involved in the interaction with other p120-catenin binding partners including p190RhoGAP. We still tested involvement of N-terminus and Armadillo domains in p120-catenin interaction with p190RhoGAP using N-terminus and Armadillo domains p120-catenin deletion mutants. However, taking these published data into consideration, we have focused on the C-terminal tail of p120-catenin. Using a combination of deletion mutants and subsequently a series of PCR-generated truncations of the C terminal tail of p120-catenin, we have identified a stretch of 23 amino acids (AA 821-843) following Armadillo domain-10, designated as CRAD domain, which mediates the formation of functional p120-catenin - p190RhoGAP complex. We further performed analysis of the CRAD domain homology with other binding proteins. Results of BLAST search for homologies were negative and did not yield any positive hits suggesting that this is a unique sequence rather than consensus sequence shared by other p190RhoGAP interactors. It is also important to note that, although the role of CRAD domain in p120-catenin – p190RhoGAP association was tested extensively in the forward and reverse pulldown assays of p190RhoGAP and p120-catenin truncation mutants co-expressed in mammalian cell system, we cannot exclude the presence of intermediate linker protein involved in these interactions.

Control of Rho signaling by p190RhoGAP is critical for dynamic EC barrier regulation. We have previously discovered that stimulation of pulmonary endothelium with OxPAPC promoted p120-catenin/p190RhoGAP association, tyrosine phosphorylation and recruitment of p190RhoGAP to adherens junctions leading to enhancement of endothelial barrier and reciprocal reduction of Rho activity and elevation of Rac signaling via Rac-Rho crosstalk mechanisms (26). Important role of p190RhoGAP activity in control of vascular endothelial barrier was demonstrated in experiments with molecular inhibition of p190RhoGAP, which exacerbated Rho-dependent endothelial permeability in vitro and lung vascular leak in the animal models of ventilator induced lung injury (26).

Uncoupling of p120-catenin - p190RhoGAP association achieved in the present study by expression of p120-catenin mutant lacking p190RhoGAP binding site had important functional implications. Expression of p120 1A(1-820) mutant lacking the CRAD domain suppressed
the OxPAPC-induced p190RhoGAP translocation to cell membrane compartment, caused increase in basal Rho signaling and reciprocal decrease in basal Rac activation in cells expressing p120 1A(1-820) mutant. As a result, expression of truncated mutant lacking the CRAD domain (p120 1A(1-820)) attenuated endothelial barrier enhancing response to OxPAPC. In contrast, expression of the full length construct or the truncation mutant containing the CRAD domain (p120 1A(1-843)) did not significantly alter the OxPAPC-induced endothelial barrier enhancement when compared to non-transfected cells. Under conditions of thrombin-induced, Rho mediated increase in EC permeability, expression of p120(1-820) led to a sustained activation of Rho and its downstream signaling after thrombin challenge and delayed activation of Rac signaling. As a result, EC monolayers expressing the truncated mutant lacking CRAD exhibited a lower ability to recover from the disruptive effect of thrombin. Taken together, these data suggest that p120-catenin coordinates the activities of Rho and Rac pathways in response to OxPAPC and that the C-terminus domain of p120-catenin containing AA 821-843 is important for this switch mechanism. Whether all observed effects can be attributable to the CRAD fragment itself or to other portions of the C-terminus domain of p120-catenin is yet to be clarified.

Previous reports described p120-catenin functional interactions with the small GTPases Rho, Rac and Cdc42 (18,24,25,37,38). Among these three GTPases, only direct interaction with RhoA was reported (22,24). Such an interaction, mediated through p120-catenin Armadillo domain and inhibited by cadherin binding, inhibited RhoA activity which led the authors to propose a mechanism whereby p120-catenin would play a role of a “recruiting agent” for RhoA to nascent cell contacts. Further evidence has shown that p120-catenin constitutes a hub regulating small GTPase activities as exemplified by its interaction with regulators of Rac and Rho GTPases as the guanine nucleotide exchange factor (GEF) Vav2 (24) and p190RhoGAP (25,26,39).

Interestingly, our current study shows additional p120-catenin associated inhibitory effect towards Rho via p190RhoGAP binding to p120-catenin AA-820-843 domain in the C-terminal tail located immediately downstream of the last Armadillo domain-10. Incidentally, this region was recently shown to form two alpha helices that fold over the hydrophobic surface of Armadillo domain R9 (4) Taken together these observations suggest the existence of a regulatory mechanism mediated by intramolecular folding of p120-catenin in the recruitment of p190RhoGAP and ultimately in the local inhibition of Rho activity. These conformation changes are apparently induced by cell stimulation with barrier-enhancing agonists. However, the exact events involved in such a mechanism are yet to be identified but do reveal a potential regulatory role of the C terminal tail of p120-catenin.

In conclusion, our results suggest a key role for p120-catenin C-terminal CRAD domain (AA 821-843) in p190RhoGAP subcellular targeting, which is essential for local regulation of agonist-induced Rho and Rac activities and dynamic control of endothelial barrier under normal conditions and during vascular barrier dysfunction associated with acute lung injury, pulmonary and brain edema, atherosclerosis and other pathological conditions.

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FIGURE LEGENDS

Figure 1. Binding of p190RhoGAP to GST-tagged p120 catenin deletion mutants. GST-tagged full length p120 catenin 1A (GST-p120 1A) and related deletion mutants (A) were cotransfected in HEK 293T cells together with His-tagged p190RhoGAP. Interacting complexes were pulled down with His-Tag Dynabeads, and p120 catenin domains bound to p190RhoGAP were detected by Western blot with anti-GST and anti-p120-catenin antibodies (B). Bottom panel shows the protein content of recombinant p120-catenin and its deletion mutants in total whole cell lysates (WCL). Asterisks indicate the bands corresponding to p120-1A-catenin constructs expressed.

Figure 2. Binding of p120 catenin truncation mutants to His-tagged p190RhoGAP. GST-tagged full length p120 catenin 1A (GST-p120 1A) and its deletion mutants were cotransfected in HEK 293T cells together with His-tagged p190RhoGAP. Interacting complexes were pulled down with glutathione magnetic beads, and their content was analyzed by Western blotting using antibodies to the GST tag, p120 catenin or p190RhoGAP as indicated. Bottom panel depicts the protein content of recombinant His-tagged p190RhoGAP in whole cell lysates (WCL).

Figure 3. Identification of the C-term polypeptide sequence in p120-catenin responsible for recruitment of p190RhoGAP. GST-tagged full length p120-catenin 1A (GST-p120 1A), related deletion mutants as well as truncated mutants of p120-catenin created by site specific mutagenesis (A) were cotransfected in HEK 293T cells together with His-tagged p190RhoGAP. Interacting complexes were pulled down with His-Tag Dynabeads (B), and their content was analyzed by Western blotting using antibodies to the His tag, GST tag, p120-catenin or p190RhoGAP as indicated. Asterisks in (B) indicate the bands corresponding to p120-catenin constructs expressed. Bottom panel depicts the protein content of recombinant GST-tagged p120-catenin and its deletion mutants in whole cell lysates (WCL).

Figure 4. Role of the p120-catenin CRAD domain responsible for p190RhoGAP recruitment in the OxPAPC-induced barrier enhancement. GST-tagged full length p120 catenin (p120 1A) and truncated mutants p120(1-843) and p120(1-820) catenin were expressed in HPAEC followed by OxPAPC treatment. Time-resolved TER measurements of barrier integrity across transiently transfected HPAEC monolayers are shown in A, B and C. D - Statistical analysis of TER changes at 30 min and 2 hrs of OxPAPC stimulation. *P<0.05 vs. p120 1A; n=5 independent experiments.

Figure 5. p120(1-820) catenin mutant colocalizes with VE-cadherin to the adherens junctions but fails to accumulate at the plasma membrane upon OxPAPC treatment. GST-tagged full length p120 catenin (p120 1A) and truncated mutant p120(1-820) catenin were transiently transfected in HPAEC. A - Double immunostaining with antibodies to GST tag to visualize ectopically expressed p120-catenin (green) and endogenous VE-cadherin (red) was carried out 48 hours after transfection as described in Materials and Methods. B - Effect of OxPAPC treatment (20 µg/ml, 30 min) on the membrane localization of p120 1A and p120(1-820) mutant. Expressed proteins were analyzed by immunofluorescence using anti-GST antibody. Cell nuclei were visualized by DAPI counterstaining.

Figure 6. p120(1-820) catenin attenuates OxPAPC-induced p190RhoGAP membrane translocation and activation of Rac signaling. GST-tagged full length p120 catenin and truncation mutants were expressed in HPAEC followed by OxPAPC treatment (15 µg/ml) for the indicated periods of time. A – Western blot analysis of p190RhoGAP in the membrane fractions from control and OxPAPC-treated HPAEC expressing full-length or p120(1-820)-catenin mutant (A, top panel). Total p190RhoGAP content in the cell lysates (middle panel) and expression levels of the recombinant p120 proteins detected by western blot with anti-GST antibody (bottom panel) were used as normalization controls. B – Rho-GTP pull down assay from unstimulated HPAEC and HeLa cells showing basal Rho activation (top panel) and total (bottom panel) Rho levels in cells expressing p120 1A, p120(1-820) or p120(1-843) mutants. C – Western blot analysis of basal phospho-MLC levels in HPAEC expressing p120 1A,
p120(1-820) or p120(1-843) mutants. Samples for each condition are presented in duplicates. Western blot detection of GST tag (middle panel) was used to monitor recombinant p120-catenin expression; detection of β-tubulin (bottom panel) was used as normalization control. D – Rac-GTP pull down assay showing active Rac (top panel) and total (bottom panel) Rac levels. E – Western blot analysis of phospho-PAK1 and phospho-cortactin levels in OxpAPC-treated HPAEC expressing full-length and p120(1-820)-catenin mutant using phospho-site specific PAK1 and cortactin antibodies. GST and β-tubulin staining were included to account for construct expression levels and sample loading respectively. Bar graphs represent quantitative densitometry analysis of western blot data. *P<0.05 vs. p120 1A; n=5 independent experiments.

Figure 7. p120(1-820) catenin delays thrombin-induced barrier restoration and prolongs thrombin-induced activation of Rho signaling. GST-tagged full length p120 catenin (p120 1A) and its truncation mutant p120(1-820) were expressed in HPAEC followed by thrombin treatment (0.2 U/ml). A – TER measurements showing thrombin-induced permeability and barrier restoration in HPAEC monolayers. Thrombin-induced barrier dysfunction was markedly prolonged by expression of p120(1-820) catenin. B – Rho-GTP pulldown assay on thrombin-treated HPAEC expressing full-length or p120(1-820) catenin mutant during the recovery phase. C – Western blot analysis of phospho-MYPT, phospho-MLC and phospho-cortactin levels in HPAEC expressing full-length and p120(1-820) catenin mutant upon thrombin stimulation for the indicated periods of time. GST and β-tubulin staining were included in (C) to account for construct expression levels and sample loading respectively.
Table 1

| Primer Sequence (5'-3') | Orientation | Mutation | Mutant |
|-------------------------|-------------|----------|--------|
| GCAGCAGCTCTTGCTGTAGACAATCTGGGGCTATAAGG | Forward | Stop Codon at aa821 | GST-p120 1A(1-820) |
| CTTATAGCCCCAGATGTCTACAGGACAAGAGCTGCTGC | Reverse | | |
| GGATGGAAGAAATCAGACTTCTAGGTGAATCTAAACAATGC | Forward | Stop Codon at aa844 | GST-p120 1A(1-843) |
| GCATTGTCAAAGATTGAAGAGTTTACATCACATCATCC | Reverse | | |
| CATATGATAGACACCTCTGACTTCATTGACCAGAAATC | Forward | Stop Codon at aa865 | GST-p120 1A(1-864) |
| GATTCGGTCAATGACAGGACTCATTGCATDCTCATATG | Reverse | | |
| CCAATGAGCAATATGGGGTTAATAACACAAATCATAGATAAC | Forward | Stop Codon at aa889 | GST-p120 1A(1-888) |
| GATTCTAATGATTATGTGTATCCCATATGCTCTG | Reverse | | |
| GAGAGGGAGAACCACACTGAACACTTGACCGATCTGGGG | Forward | Stop Codon at aa909 | GST-p120 1A(1-908) |
| CCCAGATCGGTCCAGTGTTGCTGCTCTCTCTCTC | Reverse | | |

**Table 1.**

List of forward and reverse primers used to generate stop codons at the indicated positions within the C-terminal tail of p120 catenin.
A

Coiled
Regulatory Domain
Armadillo Repeat Domain
Tail

p120 1A
  1  2  3  4  5  6  7  8  9  10

p120 4A

p120 1A ΔR3-11

p120 1A ΔArm1

p120 1A Δ622-628

B

His-p190RhoGAP
GST-p120 1A
GST-p120 4A
GST-p120 1A ΔR3-11
GST-p120 1A ΔArm1
GST-p120 1A Δ622-628

His

p190RhoGAP

GST

p120

His Pulldown

WCL: GST
Zebda et al., Figure 2

GST

p120

p190 RhoGAP

WCL: His
A

p120 1A Full Length (1-932)

p120 1A (1-908)

p120 1A (1-888)

p120 1A (1-864)

p120 1A (1-843)

p120 1A (1-820)

p120 1A ΔR3-11

p120 4A

B

His-p190RhoGAP

+ GST

GST-p120 1A\(\Delta R3-11\)

GST-p120 1A

GST-p120 1A (1-820)

GST-p120 1A (1-843)

GST-p120 1A (1-864)

GST-p120 1A (1-888)

GST-p120 1A (1-908)

His Pulldown

WCL: GST

MW, kDa
A

OxPAPC

p120 1A

Non-TF

Time (hrs)

Norm. Resistance

0 1 2 3

0.9 1.0 1.1 1.2 1.3

B

OxPAPC

p120 1A

p120 1A (1-843)

Time (hrs)

Norm. Resistance

0 1 2 3

0.9 1.0 1.1 1.2 1.3

C

OxPAPC

p120 1A

p120 1A (1-820)

Time (hrs)

Norm. Resistance

0 1 2 3

0.9 1.0 1.1 1.2 1.3
Figure 4D

% of OxPAPC response (non-TF)

- non-TF
- P1201A
- P1201A (1,843)
- P1201A (1,820)

- non-TF
- P1201A
- P1201A (1,843)
- P1201A (1,820)

30 min

2 hrs

* Denotes significant difference from non-TF treatment.
A

p120 1A

GST (green) / VEC (red)

p120 1A (1-820)

GST (green) / DAPI (blue)

B

p120 1A: Vehicle

p120 1A: OxPAPC

GST (green) / DAPI (blue)

p120 1A (1-820): Vehicle

p120 1A (1-820): OxPAPC

p120 1A (1-820)
A

|        | p120 1A | p120 1A (1-820) |
|--------|---------|-----------------|
| OxPAPC | 0       | 0               |
|        | 15      | 15              |
|        | 30      | 30 (min)        |

p190RhoGAP

Membrane fraction

p190RhoGAP

GST

Total Lysate

B

|        | p120 1A (1-820) | p120 1A (1-843) |
|--------|-----------------|-----------------|
| Rho-GTP| HPAEC           |
| Rho total| HeLa          |
| Rho-GTP|                 |
| Rho total|              |
| GST    |                 |

C

|        | p120 1A      | p120 1A (1-820) | p120 1A (1-843) |
|--------|--------------|-----------------|-----------------|
| pp-MLC |              |                 |
| MLC    |              |                 |
| GST    |              |                 |
| β-Tubulin |          |                 |

RDU

0  50  100  150  200

*
A

![Graph showing normalized resistance over time for different treatments.](Image)

- **Thrombin**
- **p120 1A**
- **p120 1A (1-820)**

B

| Thrombin recovery | p120 1A | p120 1A (1-820) |
|-------------------|---------|-----------------|
|                   | 0       | 0               |
|                   | 30      | 30              |
|                   | 60      | 60 (min)        |

- **Rho-GTP**
- **Rho total**
|            | Thrombin: | p120 1A | p120 1A (1-820) |
|------------|-----------|---------|-----------------|
| p-MYPT-Thr850 | 0 5 15 30 |        |                 |
| MYPT       |           |        |                 |
| RDU        |           | 0 100 200 | 0 100 200 300 |
| pp-MLC     |           |        |                 |
| MLC        |           |        |                 |
| RDU        |           | 0 100 200 | 0 100 200 300 |
| p-Cortactin-Tyr421 | 0 5 15 30 |        |                 |
| Cortactin  |           |        |                 |
| RDU        |           | 0 100 200 | 0 100 200 300 |
| GST        |           |        |                 |
| β-Tubulin  |           |        |                 |
Interaction of p190RhoGAP with C-terminal domain of p120-catenin modulates endothelial cytoskeleton and permeability

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