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Research Article

Pressure activates colon cancer cell adhesion *via* paxillin phosphorylation, Crk, Cas, and Rac1

C. Downey[a], D. H. Craig[a] and M. D. Basson[a, b, c, *]

[a] Department of Surgery, John D. Dingell VA Medical Center and Wayne State University, 4646 John R. Street, Detroit, MI 48201 (USA), Fax: +1-313-576-1002, e-mail: marc.basson@va.gov
[b] Department of Anesthesiology, John D. Dingell VA Medical Center and Wayne State University, Detroit, MI 48201 (USA)
[c] Department of Anatomy and Cell Biology, John D. Dingell VA Medical Center and Wayne State University, Detroit, MI 48201 (USA)

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Abstract. Physical forces can activate colon cancer cell adhesion, critical for metastasis. Paxillin is phosphorylated by FAK and required for pressure-stimulated adhesion. However, whether paxillin acts as an inert scaffolding protein or whether paxillin phosphorylation is required is unknown. Transfection with paxillin point-phosphorylation mutants demonstrated that phosphorylation at tyrosines 31 and 118 together is necessary for pressure-stimulated adhesion. We further evaluated potential paxillin partners. Reducing the adaptor protein Crk or the focal adhesion protein $p130^{Cas}$ blocked pressure-stimulated adhesion. Furthermore, Crk and $p130^{Cas}$ both displayed increased co-immunoprecipitation with paxillin in response to increased pressure, except in cells transfected with a Y31Y118 paxillin mutant. Inhibiting the small GTPase Rac1 also abolished pressure-stimulated adhesion, and reducing paxillin by siRNA blocked Rac1 phosphorylation by pressure. Thus, paxillin phosphorylation at tyrosines 31 and 118 together is necessary for pressure-induced adhesion. Paxillin, Crk and Cas form a trimeric complex that activates Rac1 and mediates this effect.

Keywords. Adhesion, cancer, Cas, Crk, pressure, Paxillin, Rac1.

Introduction

Metastasis of a tumor cell is the leading cause of mortality for cancer patients. Tumor cell adhesion at a distant site is a pivotal step in the metastatic process. Physical forces, including a pathophysiologically relevant increase in extracellular pressure (15 mmHg), can mediate tumor cell adhesion to matrix proteins, endothelial cells and *in vivo* surgical sites by initiating intracellular signals that modulate integrin binding affinity [1–4]. This phenomenon has been observed in cell lines derived from human and murine colon cancers, glottal cancers, and breast cancers, as well as primary cells isolated directly from surgically resected human colon cancers [2–6]. Pressure-induced cell signaling depends upon an intact cytoskeleton, β1 integrin heterodimers, and activation of FAK, Src, Akt and PI-3-kinase [2–4, 7, 8]. Tumor cells may be exposed to pressure increases of this magnitude during tumor growth against a constraining stroma, surgical procedures, postoperative bowel edema and

* Corresponding author.

lymphatic transit [9–13]. Further elucidation of the mechanism by which tumor cells regulate adhesion may offer new therapeutic approaches to target metastasis.

Paxillin is a 68-kDa focal adhesion associated protein that facilitates protein–protein interactions, focal adhesion assembly and functions in cell signaling downstream of integrins [14]. These events influence diverse cellular behaviors, including cell migration, proliferation, survival and apoptosis. In some cases, paxillin functions as a structural protein that links other proteins and facilitates formation of focal adhesions [15]. However, in other instances, kinases may phosphorylate paxillin directly and alter its binding affinity [16]. Paxillin localizes to focal adhesions where it has been shown to interact directly with the α5 and α9 subunit of β1–integrin [17–19], providing a platform for tyrosine kinases including FAK and Src that can become activated in response to adhesion or growth factor stimulation [15, 20–22]. Paxillin phosphorylation may also regulate the recruitment of downstream molecules such as Crk that can further lead to activation of $p130^{Cas}$ [14, 23]. In addition, negative regulators of these pathways, including Csk and PTP-PEST bind directly to paxillin [15].

Paxillin appears to be required for extracellular pressure to increase cancer cell adhesion, since paxillin reduction by siRNA blocks the effect and paxillin overexpression seems to reproduce it. Increased extracellular pressure also stimulates paxillin phosphorylation at tyrosines 31 and 118 [5, 24]. However, it has not been clear whether paxillin acts simply as an adaptor protein or whether paxillin phosphorylation is necessary for the effects of extracellular pressure on cancer cell adhesion. This is important because understanding how paxillin acts may ultimately facilitate the design of therapeutic interventions to reduce cancer cell adhesion and metastasis.

We therefore sought to test the hypothesis that paxillin phosphorylation is important to the regulation of cancer cell adhesion using paxillin phosphorylation single mutants that cannot be phosphorylated at tyrosine 31, 118 or 181, a Y31Y118 double mutant and a Y31Y118Y181 triple mutant. We transfected these constructs into human SW620 colon cancer cells and examined their effects on pressure-stimulated cancer cell adhesion. We then determined the effects of increased pressure and transfection with non-phosphorylatable paxillin mutants on the potential paxillin-associated molecules Crk, Cas and Rac1 to further delineate signaling events downstream of paxillin. Next, we examined the association of paxillin with α-actinin, a molecule known to be involved in pressure-stimulated cancer cell adhesion [25], by reducing α-actinin levels and evaluating paxillin phosphorylation. Finally, we explored these signaling events in primary human colon cancer cells to confirm that our observations are not limited to a single cell line.

Materials and methods

Cell culture. SW620 colon cancer cells were cultured as previously described [2]. Primary human colon cancer cells were obtained from surgically resected specimens that were minced and collagenase digested into single-cell suspensions [26]. Viability of the isolated colonocytes was determined to be over 90 % by trypan blue exclusion. The Wayne State University Human Investigation Committee approved the use of all human samples.

Pressure regulation. Pressure was regulated using an airtight Lucite box that was pre-warmed to 37°C as described previously [2]. The box contained an inlet and outlet valve for gas application and a manometer connection, respectively. Temperature was regulated to within ±2°C and pressure was maintained to within 1.5 mmHg.

Cell adhesion assay. Six-well plates were precoated with a bicarbonate-based buffer (pH 9.4) containing 12.5 μg/ml fibrillar collagen I (Sigma, St. Louis, MO) as previously described [27], and then washed three times with PBS prior to use. SW620 cells in suspension were added for 30 min under ambient or 15 mmHg increased pressure (10^5 cells/well). After 30 min, the nonadherent cells were washed away with PBS and the adherent cells were fixed with formalin and stained with hematoxylin. Each well was divided into 20 random fields and cells were counted in each field using an inverted microscope [2].

To determine adhesion of SW620 cells transfected with the paxillin plasmid phosphorylation mutants or a wt $p130^{Cas}$ rescue plasmid, the cells were plated in collagen I-precoated six-well plates 48 h post transfection as described above. After 30 min of ambient or 15 mmHg increased pressure, the nonadherent cells were washed away and the adherent cells were permeabilized and incubated overnight with antibody to myc or human influenza hemagglutinin (HA) depending upon the tag of the mutant. After 24 h, the myc or HA antibody was removed, the cells were washed with PBS, and Texas Red (GE Healthcare, Piscataway, NJ) was added for 1 h. The cells were then counted under a fluorescence microscope.

Transfection. Selection and characterization of the paxillin plasmid phosphorylation mutants has been previously described [28]. Myc tagged wild-type and Y31Y118 paxillin plasmid mutants were compliments of Dr. M. Cecilia Subauste [28, 29]. HA-tagged wild-type, as well as Y31, Y118, Y181 and Y31Y118Y181 paxillin plasmid mutants were generous gifts of Dr. Ravi Salgia. All mutants had a Y→F mutation on their respective tyrosine residues. Using Lipofectamine 2000 (Invitrogen, Carlsbad, CA), SW620 cells were transfected with 2 μg/ml plasmids expressing myc-tagged wild-type or Y31Y118 mutant, or HA-tagged wild-type, Y31, Y118, Y181, or Y31Y118Y181 mutants.

Additionally, total Crk, p130cas and FAK levels were reduced using small interfering (si) RNA *SMART*-pools® produced by Dharmacon (Lafayette, CO), as well as Oligofectamine and Plus reagent according to the manufacturer's protocol (Invitrogen). Crk, p130cas and FAK expression was reduced using siRNA targeting the 5'-ACACTATTTGGACACTA-CA-3' segment of Crk mRNA, the 5'-GGTCGA-CAGTGGTGTGTAT-3' segment of p130cas mRNA and the 5'-AAGCAUGUGGCCUGCUAUGGA-3' segment of FAK mRNA. α-Actinin expression was reduced using siRNA as described previously [25]. A Dharmacon si*CONTROL*® non-targeting siRNA 1 was used as a control. Scrambled siRNA sequences for paxillin, Crk and Cas were also used as controls for selected studies. The scrambled sequences used were: paxillin 5'-TGGTGTCTTCCGTTTAGGG-3', Crk 5'-ATATAAGCAGCGTGATTCC-3' and Cas 5'-CAGTGATGGTATGTGCGGT-3'. All experiments were performed 48 h after transfection.

Inhibitor studies. The Src family kinase inhibitor PP2 (EMD Chemicals, Gibbstown, NJ) was dissolved in dimethyl sulfoxide (DMSO) and diluted in cell culture medium immediately prior to use. SW620 cells were treated with 10 μM PP2 or an equivalent amount of DMSO (vehicle control) for 1 h prior to adhesion or signaling studies. NSC23766 (EMD), an inhibitor of Rac1 activation was dissolved in sterile distilled water and diluted in cell culture medium immediately prior to use. SW620 cells were treated with 50 μM NSC23766 or cell culture medium (vehicle control) for 1 h prior to adhesion or signaling studies.

Immunoprecipitation and Western blotting. Signaling studies were performed on suspended cells that were plated on bacteriological dishes precoated with 1% heat-inactivated BSA to prevent adhesion. The cells were exposed to ambient or 15 mmHg increased pressure for 30 min and collected by centrifugation and lysed with a lysis buffer containing 50 mM Tris pH 7.4, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 1% deoxycholic acid, 0.1% SDS, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 1 mM sodium vanadate, 50 mM NaF, 10 mM sodium pyrophosphate, 2 μg/mL aprotinin, and 2 μg/mL leupeptin. Total protein concentrations of the cell lysates were determined using a bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL). For co-immunoprecipitation studies, 400 μg protein for each sample was incubated with its appropriate antibody for 1 h, after which time agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA) were added overnight. To perform Western blotting techniques, equal amounts of protein were loaded onto an SDS-PAGE gel and transferred to a Hybond nitrocellulose membrane (GE Healthcare, Little Chalfont, Buckinghamshire, UK). Mouse monoclonal antibodies to total paxillin, p130cas, Crk, total Rac (BD Transduction, San Diego, CA), GAPDH (Biodesign International, Saco, MN), actin (Sigma Aldrich), HA (Covance, Berkley, CA) and myc (Cell Signaling, Beverly, MA), as well as rabbit polyclonal antibodies for phospho-paxillin (pY31, Biosource, Camarillo, CA; pY118, Cell Signaling) and phospho-Rac (Cell Signaling) were used for immunoprecipitation studies and/or protein detection along with the appropriate horse-radish peroxidase–conjugated secondary antibodies. Proteins were visualized by ECL-Plus techniques (GE Healthcare) and imaged and quantitated using a Kodak Phosphoimager (Perkin Elmer, Boston, MA). All blots were studied within the linear range of exposure.

Rac1 activation assay. Rac activity was assessed using a pull-down assay (Millipore, Billerica, MA). SW620 cells exposed to ambient or increased pressure on dishes precoated with 1% heat-inactivated BSA were collected by centrifugation and lysed using buffer provided by the manufacturer. Protein concentration was determined by the BCA method. Active Rac1 levels were assessed by glutathione S-transferase-conjugated p21 binding domain (GST-PBD) of p21-activated kinase 1 pull down assays. The cell lysates were incubated with PAK-PBD beads for 1 h at 4°C on a rotator and the beads were pelleted by centrifugation at 5000 *g* for 2 min at 4°C. The resulting pellet was then resuspended in Laemmli buffer, resolved electrophoretically, transferred to nitrocellulose, and immunoblotted with monoclonal anti-Rac1 antibody (Pierce). Of each lysate, 20 μg was used for Western blotting for total Rac1.

Statistical analysis. SigmaStat (SPSS Inc., Chicago, IL) was used for paired and unpaired *t*-tests that sought to achieve a 95% confidence interval. Addi-

tionally, for adhesion studies, results of adhesion under ambient or increased pressure for each experimental group were normalized against ambient pressure adhesion for control cells transfected with non-targeting siRNA to permit evaluation of both potential differences in basal adhesion and differences in the effects of pressure on the adhesion of each transfectant.

Results

Paxillin phosphorylation at tyrosine 31 and tyrosine 118 is necessary for pressure-stimulated colon cancer cell adhesion. We have previously reported that increased extracellular pressure promotes paxillin phosphorylation in head and neck cancer cells [5]. However, we had yet to identify whether this phosphorylation is important to pressure-stimulated cancer cell adhesion. Cells transfected with single plasmid phosphorylation mutants all displayed increased adhesion in response to increased pressure. However, cell adhesion was not increased in response to pressure in cells transfected with the triple mutant (Fig. 1A, $n = 5$; $p < 0.05$).

Crk is necessary for pressure-stimulated cancer cell adhesion, and Crk interacts with paxillin. In some other systems, paxillin phosphorylation at tyrosine 31 and tyrosine 118 creates a docking site for the adaptor protein Crk [21, 29–31]. We therefore sought to determine whether Crk is also involved in pressure-stimulated adhesion. Crk was reduced in SW620 cells approximately 74 % by siRNA treatment and the cells were exposed to ambient or increased pressure (Fig. 2A). Pressure failed to stimulate cell adhesion when Crk was reduced (Fig. 2B, $n = 4$; $p < 0.05$).
To evaluate the potential association between paxillin and Crk under pressure, we performed co-immunoprecipitation studies using SW620 cells exposed to ambient or increased pressure. Indeed, pressure increased the association of paxillin with Crk by 36±8 % compared to ambient pressure controls (Fig. 2C, $n = 6$; $p < 0.05$). We therefore, next evaluated the association of Crk with paxillin in SW620 cells transfected with the paxillin single tyrosine mutants and the tyrosine double mutant by similar co-immunoprecipitation analysis. Paxillin-Crk interaction was increased under pressure by 34±9 %, 27±4 % and 32±6 % in the Y31, Y118 and Y181 transfected cells, respectively (Fig. 2D, $n = 6$; $p < 0.05$). However, the association between paxillin and Crk was not significantly increased in the cells transfected with the Y31Y118 double mutant (Fig. 2E, $n = 5$; $p < 0.05$).

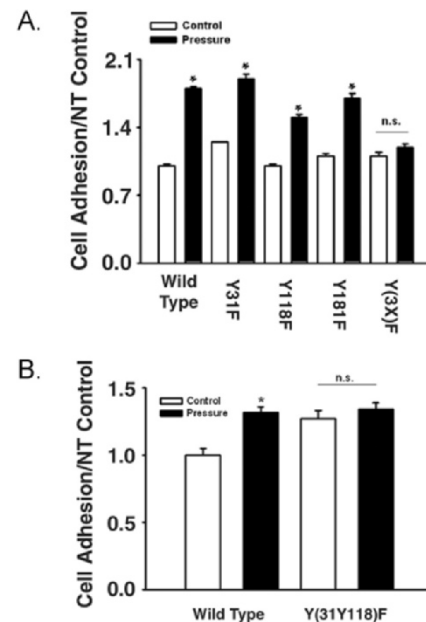

Figure 1. Effect of paxillin plasmid phosphorylation mutants on pressure-stimulated colon cancer cell adhesion. (*A*) Extracellular pressure increased by 15 mmHg (black bars) stimulated SW620 cell adhesion to type I collagen in cells transfected with an HA-tagged Y31, Y118 or Y181 paxillin phosphorylation mutant plasmid, to the same extent as that observed in cells transfected with the wild-type construct. However, cells transfected with a Y31Y118Y181 triple phosphorylation mutant plasmid did not respond to elevated pressure ($n = 5$; $p < 0.05$). (*B*) Adhesion to type I collagen was not increased inSW620 cells transfected with a Y31Y118 paxillin double phosphorylation mutant plasmid in response to elevated pressure (black bars) relative to ambient pressure controls (white bars) ($n = 4$; $p < 0.05$).

To further validate these results, we assessed the interaction of paxillin and Crk under ambient and increased pressure conditions in primary human colon cancer cells isolated from six surgically resected specimens. Primary colon cancer cells yielded results similar to SW620 cells: pressure increased paxillin and Crk interaction in these human cancer cells by 38±4 % (Fig. 2F, $p < 0.05$).

Cas is necessary for pressure-stimulated cancer cell adhesion, and Cas interacts with paxillin and Crk. Cas is a highly phosphorylated protein that may associate with Crk upon activation of Cas by Src [32–34]. We therefore investigated whether Cas is necessary for pressure-stimulated adhesion. We used siRNA to reduce Cas by approximately 62 % in SW620 cells and exposed the cells to ambient or increased pressure (Fig. 3A). Pressure-stimulated adhesion was abolished in cells treated with Cas specific siRNA (Fig. 3B, $n = 3$; $p < 0.05$). To determine whether Cas function in SW620 cells can be restored after siRNA treatment, we transfected the cells with human Cas-specific siRNA in the presence of a plasmid containing a rat

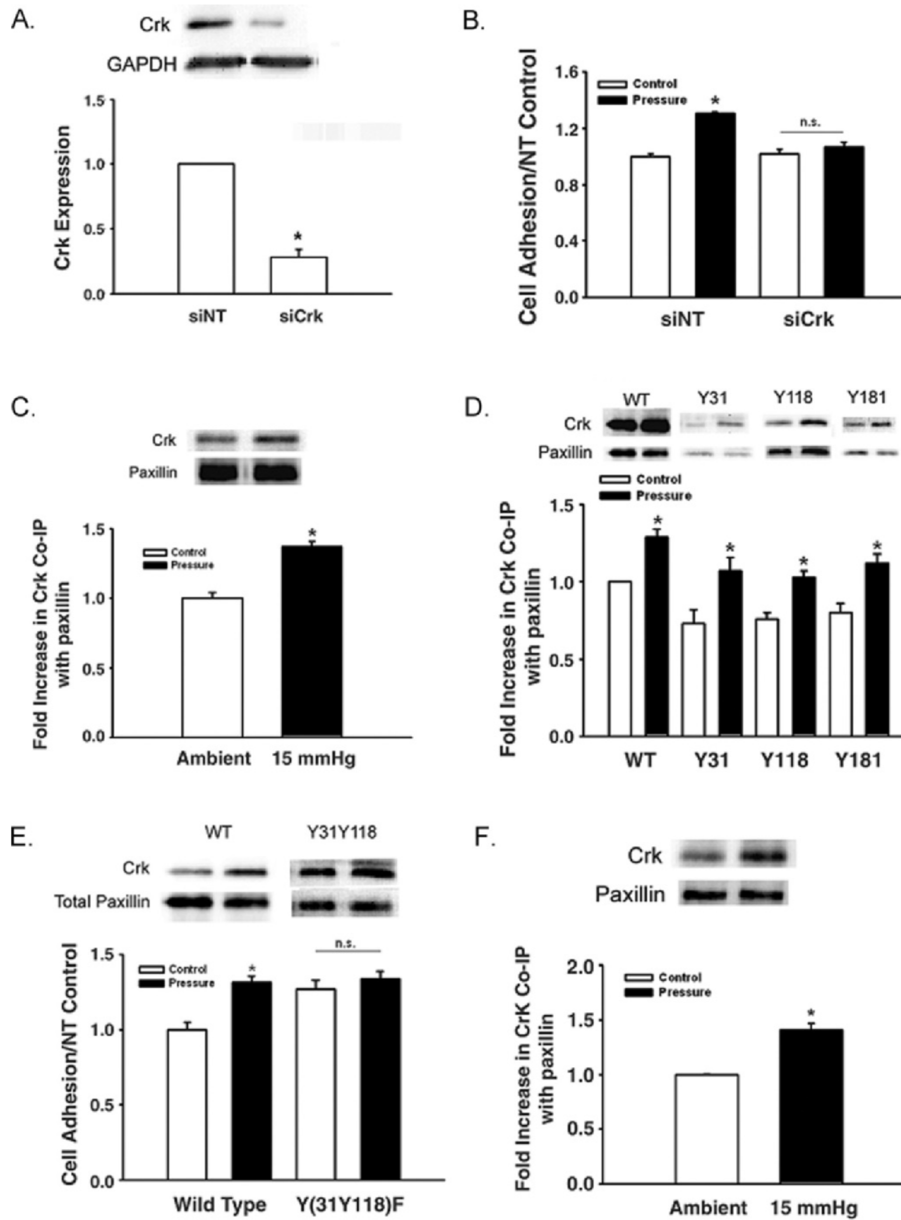

Figure 2. Crk is necessary for pressure-stimulated colon cancer cell adhesion and Crk interacts with paxillin. (*A*) Typical reduction of Crk in SW620 cells transfected with Crk-specific siRNA. GAPDH was used as a loading control and Crk protein expression was normalized against non-targeting siRNA controls ($n = 4$; $p < 0.05$). (*B*) Pressure elevated by 15 mmHg increased adhesion in cells transfected with a non-targeting siRNA control but not in cells transfected with Crk-specific siRNA ($n = 4$; $p < 0.05$). (*C*) Equal protein samples from SW620 cells that were exposed to ambient (white bar) or increased pressure (black bar) were immunoprecipitated for paxillin and probed for Crk by Western blot. Samples were normalized against ambient controls ($n = 6$; $p < 0.05$). Paxillin-Crk association was increased in pressure-treated cells. (*D*) Equal protein samples from SW620 cells transfected with an HA-tagged Y31, Y118 or Y181 paxillin phosphorylation mutant plasmid and exposed to ambient or increased pressure were utilized for co-immunoprecipitation studies. Crk co-immunoprecipitation with each mutant was significantly increased with elevated pressure (black bars) relative to ambient pressure controls (white bars) ($n = 6$; $p < 0.05$). (*E*) Co-immunoprecipitation studies on samples from SW620 cells transfected with a myc-tagged Y31Y118 paxillin double phosphorylation mutant plasmid and exposed to ambient or 15 mmHg increased pressure. demonstrate that Crk co-immunoprecipitation with myc tagged paxillin was not increased in response to elevated pressure ($n = 5$; $p < 0.05$). (*F*) Primary human colon cancer cells isolated from surgically resected specimens were exposed to ambient or 15 mmHg increased pressure. Protein samples were used for co-immunoprecipitation studies and analyzed by Western blot to evaluate the association of paxillin and Crk. Pressure increased paxillin-Crk association in primary colon cancer cells from six patients similarly to our observations in SW620 cells. ($p < 0.05$).

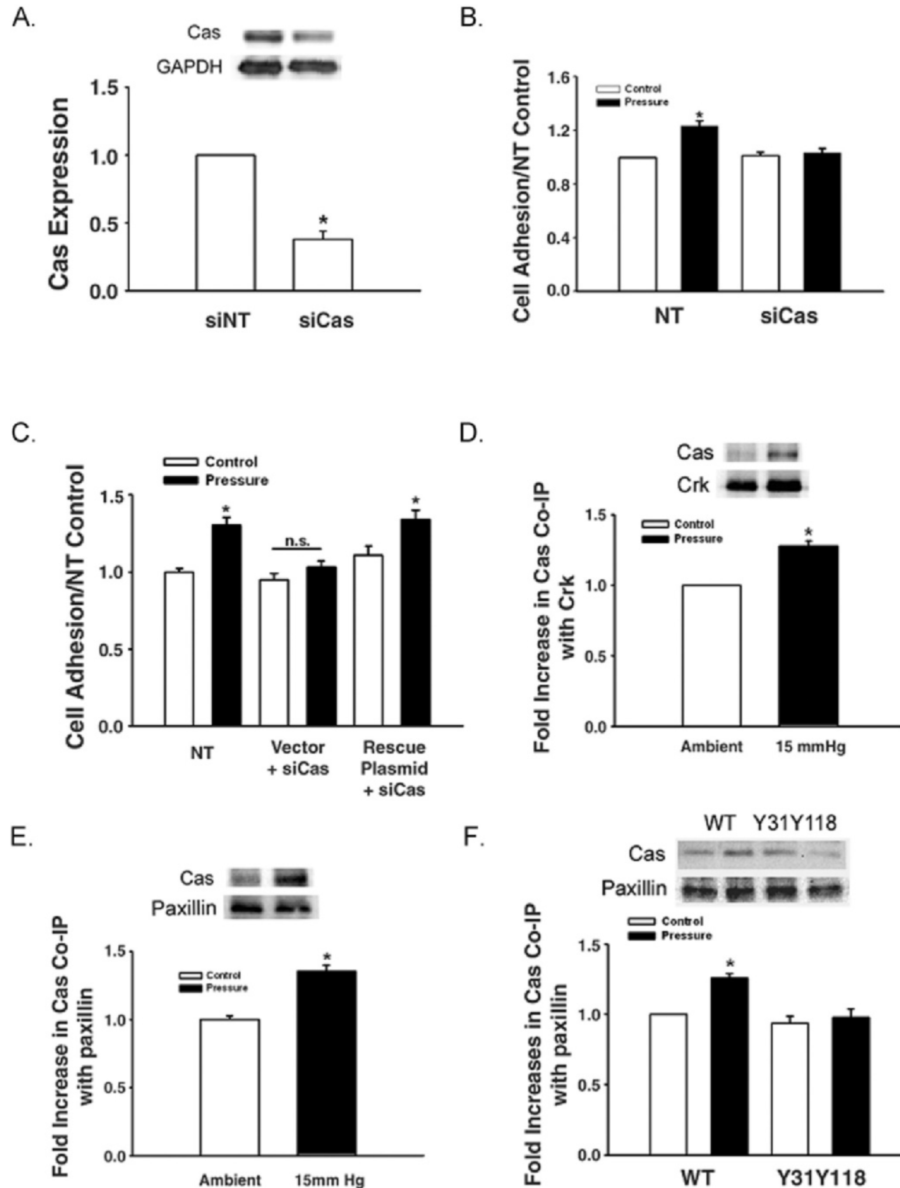

Figure 3. Cas is necessary for pressure-stimulated colon cancer cell adhesion and Cas interacts with Crk and paxillin. (*A*) Typical reduction of Cas in SW620 cells transfected with Cas siRNA. GAPDH was used as a loading control and Cas protein expression was normalized against non-targeting siRNA controls ($n=3$; $p<0.05$). (*B*) Pressure increase of 15 mmHg (black bars) stimulated adhesion of SW620 colon cancer cells transfected with a non-target siRNA control but had no effect in cells transfected with Cas-specific siRNA ($n=3$; $p<0.05$). (*C*) Co-transfection of SW620 cells with Cas specific siRNA with a plasmid rescue construct containing a rat Cas construct resulted in an increased adhesion in response to pressure similar to non-targeting controls. Pressure had no effect in cells co-transfected with Cas-specific siRNA and the empty vector control ($n=5$; $p<0.05$). (*D*) In co-immunoprecipitation experiments, lysates of SW620 cells that had been exposed to ambient or increased pressure were immunoprecipitated for Crk, probed for Cas by Western blot and normalized against total Crk. Pressure stimulated Crk-Cas association. ($n=6$; $p<0.05$). (*E*) Pressure stimulated paxillin-Cas association in SW620 cells were exposed to ambient or increased pressure. Paxillin was immunoprecipitated and Cas was probed for by Western blot. ($n=4$, $p<0.05$). (*F*) Pressure did not stimulate paxillin-Cas association in SW620 cells transfected with a Y31Y118 paxillin double phosphorylation mutant plasmid then exposed to ambient or increased pressure. Protein samples were immunoprecipitated for myc-tagged paxillin and Cas protein levels were evaluated by Western blot. ($n=5$; $p<0.05$).

Cas sequence or the empty vector. Cells transfected with both the siRNA and the rescue plasmid displayed increased pressure-stimulated adhesion. In contrast, cell co-transfected with Cas siRNA and the empty vector failed to respond to pressure (Fig. 3C, $n=5$; $p<0.05$).

Since Crk and Cas may interact [35], we compared Crk-Cas interaction under conditions of ambient and elevated pressure using co-immunoprecipitation. Elevated pressure increased the association of Crk and Cas by 38±7 % (Fig. 3D, $n=6$, $p<0.05$). We further evaluated whether Cas-paxillin association also

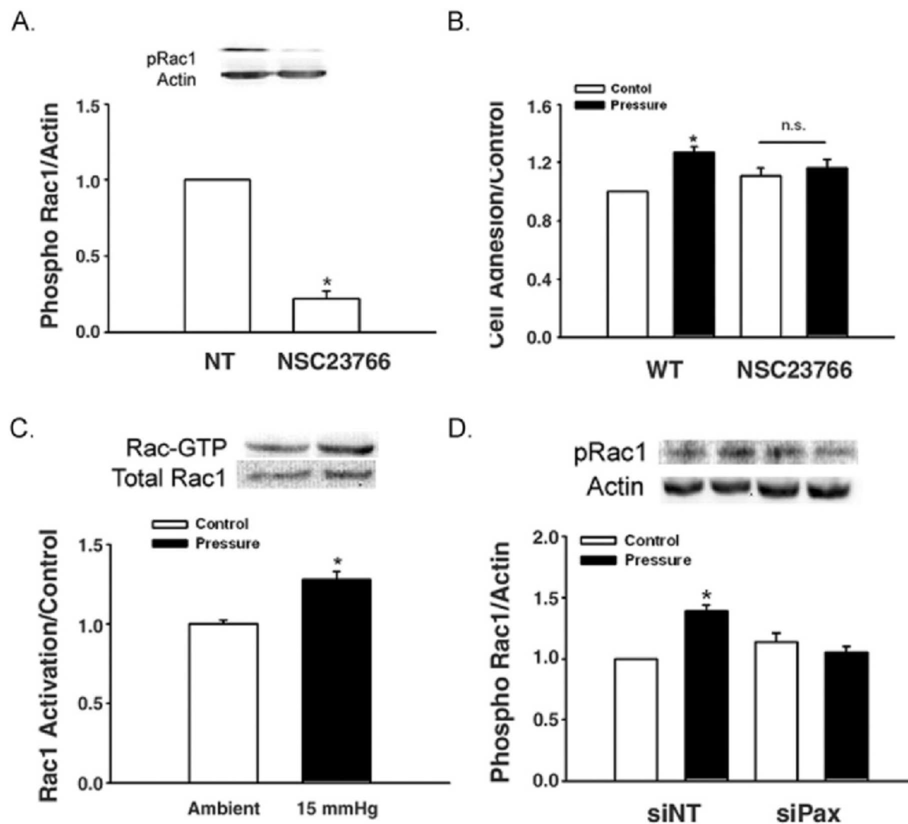

Figure 4. Rac1 is necessary for pressure-stimulated cancer cell adhesion and paxillin affects Rac1 phosphorylation. (*A*) Typical reduction in phosphorylated (active) Rac1 using NSC23766, an inhibitor of Rac1 activation. Phosphorylated Rac1 levels were normalized against actin ($n=5$; $p<0.05$). (*B*) Wild-type SW620 cells displayed increased adhesion in response to 15 mmHg increased pressure (black bars) relative to ambient controls. However, this effect was not seen in cells treated with NSC23766 ($n=5$; $p<0.05$). (*C*) A Rac1 pull-down assay was performed on SW620 cells that were exposed to ambient or increased pressure. Pressure increased Rac1activation by 28 % relative to ambient controls ($n=6$; $p<0.05$). (*D*) Rac1 phosphorylation in increased in SW620 cells transfected with a non-targeting siRNA (black bars) relative to ambient controls (white bars). Rac1 phosphorylation is not increased in response to elevated pressure in SW620 cells transfected with paxillin-specific siRNA ($n=7$; $p<0.05$).

changes in response to increased extracellular pressure. Cas did co-precipitate with paxillin under ambient pressure conditions, and the association increased 36±5 % under increased pressure conditions (Fig. 3E, $n=4$; $p<0.05$).

We then examined how the extent of pressure-stimulated paxillin phosphorylation affects its association with Cas. Paxillin-Cas association was increased by 30±9 %, 24±5 % and 32±6 % under increased pressure conditions in the cells transfected with the tyrosine 31, 118 and 181 plasmid mutants, respectively (data not shown; $n=6$; $p<0.05$). In contrast, the cells transfected with the double tyrosine mutant showed no increase in association between paxillin and Cas after exposure to increased pressure (Fig. 3F, $n=5$; $p<0.05$).

Rac1 is necessary for pressure-stimulated cancer cell adhesion and dependent on paxillin for phosphorylation. Activated Cas may phosphorylate and activate members of the small GTPase family, such as Rac1, that can in turn influence diverse cellular events including migration, motility and adhesion [36, 37]. We therefore treated SW620 cells with NSC23766, an inhibitor of the small GTPase Rac1 and reduced Rac1 activation by approximately 88 % (Fig. 4A). Pressure-stimulated adhesion was abolished in cells treated with this inhibitor (Fig. 4B, $n=5$; $p<0.05$). Rac1 activation was increased by 28±5 % in response to pressure by Rac1 pull-down assay (Fig. 4C, $n=6$; $p<0.05$). We therefore further evaluated Rac1 phosphorylation in response to increased extracellular pressure in SW620 cells transfected with either siRNA to reduce paxillin or a control non-targeted siRNA sequence. In the control cells, Rac1 phosphorylation was increased 38±4 % under elevated pressure conditions (Fig. 4D, $n=7$; $p<0.05$). However, the cells that were treated with siRNA to reduce paxillin showed no increase in phosphorylation in Rac1 in response to pressure.

FAK, Src and α-actinin affect paxillin phosphorylation. We have previously shown that pressure-stimulated cancer cell adhesion requires FAK, Src and α-actinin. Therefore, we sought to evaluate the effect of reducing these molecules on paxillin phosphorylation. We treated SW620 cells with siRNA to FAK and examined paxillin phosphorylation at Y31. The results indicated that paxillin Y31 phosphorylation is increased under elevated pressure conditions in cells transfected with a non-target siRNA control. In contrast, cells transfected with siFAK showed increased basal phosphorylation, but no additional

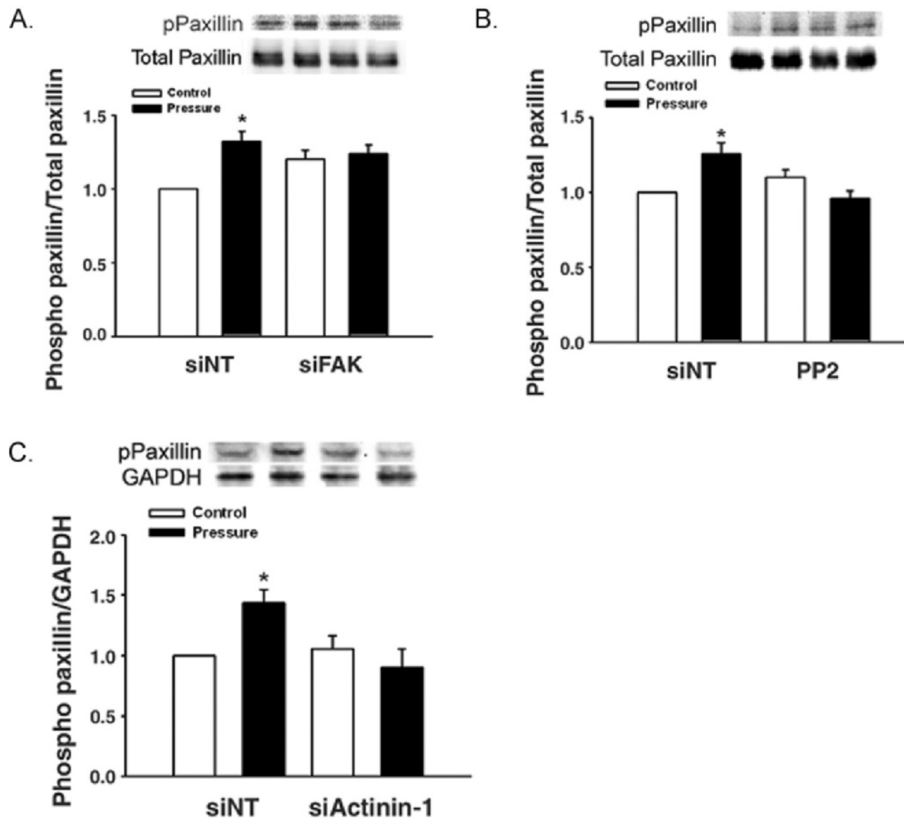

Figure 5. FAK, Src and α-actinin-1 influence paxillin phosphorylation. (*A*) Phosphorylation of paxillin at tyrosine 31 increased under pressure in SW620 cells transfected with a non-targeting control. This effect was abrogated in cells transfected with FAK specific siRNA ($n=4$; $p<0.05$). (*B*) Treatment of SW620 cells with PP2 blocked increased phosphorylation of paxillin at tyrosine 31 compared to non-targeting transfected control cells $n=5$; $p<.05$). (*C*) Paxillin phosphorylation in response to pressure was higher in SW620 cells transfected with a control non-targeting siRNA but not in cells transfected with α-actinin-1-specific siRNA ($n=6$; $p<0.05$).

increase in phosphorylation in response to elevated pressure (Fig. 5A, $n=4$; $p<0.05$).
Next, we treated SW620 cells with PP2, a potent and specific inhibitor of Src family kinases. Although paxillin Y31 phosphorylation was increased in response to pressure in DMSO-treated control cells, treatment with PP2 abolished the increase in paxillin Y31 phosphorylation in response to pressure (Fig. 5B, $n=5$; $p<0.05$). This result was consistent with similar observations in cells treated with Src-specific siRNA that were probed for paxillin phosphorylation at Y31 (data not shown).
Finally, we sought to determine if silencing α-actinin with siRNA would affect paxillin Y31 phosphorylation. Pressure increased Y31 phosphorylation in SW620 cells treated with a non-targeted siRNA control but not in cells treated with siRNA to reduce α-actinin. (Fig. 5C, $n=6$; $p<0.05$).

Scrambled siRNA does not affect pressure-stimulated colon cancer cell adhesion. Although commercially available non-targeting siRNA is a common control for siRNA studies [38], it remains possible that such non–targeted siRNA could potentially exert off-target effects that could conceivably bias the results of control studies performed with this agent. We therefore compared the effects of transfection with non-targeting siRNA with the effects of transfection with scrambled siRNA sequences for paxillin, Crk, and Cas on pressure-stimulated adhesion to demonstrate that the pressure-stimulated adhesion pathway is similar in each case. Cells transfected with scrambled paxillin, Crk and Cas siRNA all displayed increased adhesion in response to increased pressure that was not different from that seen in the non-targeted controls (Fig. 6, $n=5$; $p<0.05$). We have previously performed similar studies with scrambled controls for FAK [3] and with an siRNA targeted to a different isoform of α-actinin [39] with similar results.

Discussion

Metastasizing tumor cells are exposed to increased pressure during passage through the circulatory or lymphatic systems or during and after surgical procedures [9, 11–13]. The signals by which such pressures influence tumor cell adhesion may prove useful targets to inhibit metastasis. Although preliminary studies suggested that paxillin might be required for pressure to stimulate adhesion [5, 24], it was unclear whether paxillin was merely required for focal adhesion assembly and cytoskeletal linkage, or whether paxillin actually participated in a dynamic signal

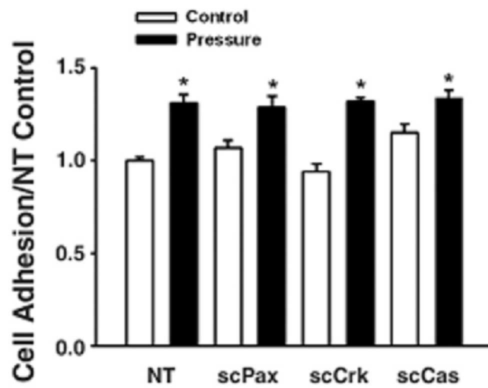

Figure 6. Scrambled siRNA does not inhibit pressure-stimulated colon cancer cell adhesion. Pressure elevation of 15 mmHg induced increases in adhesion in SW620 cells transfected with scrambled siRNA sequences for paxillin, Crk and Cas were similar to that in the non-targeting controls ($n = 5$; $p < 0.05$). Tyrosine 31 and tyrosine 118 on paxillin are phosphorylated by FAK in response to various cellular events, including adhesion [21]. Since FAK is involved in pressure-stimulated cancer cell adhesion, we next sought to block these two tyrosine phosphorylation sites specifically. We transfected cells with a myc-tagged plasmid mutant that is unable to be phosphorylated at both tyrosines 31 and 118 and similarly evaluated basal and pressure-stimulated adhesion. Transfection with this construct was sufficient to block pressure stimulated adhesion (Fig. 1B, $n = 4$; $p < 0.05$).

pathway stimulated by pressure. Our current data indicate that paxillin phosphorylation at both tyrosines 31 and 118 is necessary for pressure-induced adhesion. Phosphorylation at these two sites facilitates binding of Crk, p130Cas recruitment, and ultimately Rac1 activation. Each step seems required for pressure to activate cancer cell adhesion.

Paxillin is increased in some aggressive malignancies [40–42], participates in focal adhesion assembly and links the focal adhesion complex to integrins and the cytoskeleton [15, 43]. In some instances, paxillin acts only as a scaffolding protein offering docking sites for other proteins. For example, paxillin can bind directly to α_4 integrin subunits [16] or associate with FAK and Src [15]. Non-phosphorylated paxillin is essential for endothelial fibrillar adhesion formation [44]. In other situations, paxillin phosphorylation is necessary for downstream signaling and cellular functions. For instance, blocking paxillin phosphorylation at tyrosines 31 and 118 reduces rat bladder carcinoma cell motility on collagen [29].

In this study, we found that paxillin tyrosine phosphorylation was required for pressure to stimulate cancer cell adhesion. Pressure stimulated paxillin phosphorylation at tyrosines 31 and 118. Preventing phosphorylation at tyrosines 31 or 118 individually did not affect pressure-stimulated adhesion, but when both sites were blocked simultaneously, the pressure effect was abolished. In contrast, tyrosine 181 seemed irrelevant. These results are consistent with requirements for paxillin tyrosine 31 and 118 phosphorylation for adhesion, spreading, and migration in other systems [14, 45, 46]. Serine phosphorylation of paxillin may also influence epithelial adhesion [47, 48], and our studies of paxillin tyrosine phosphorylation do not preclude the possibility that pressure may also stimulate serine phosphorylation.

Crk is a multifunctional adaptor protein that binds diverse proteins, depending on the cell and stimulus studied. For instance, Crk interacts with phosphatidylinositol kinase, activating Akt [49], which also influences cancer cell adhesion in response to pressure [7]. Our results indicate that paxillin phosphorylation in human colon cancer cells in response to pressure facilitates paxillin-Crk binding, which is also required for the stimulation of adhesion. Further study may clarify whether pressure-induced signaling prior to adhesion also involves such known Crk-binding partners in adherent cells as DOCK180 or C3G [50, 51].

p130Cas is also an adaptor that promotes protein-protein interactions [34] mediating downstream events [32, 35, 52, 53]. Cas modulates cell motility, and integrin-mediated adhesion triggers Cas tyrosine phosphorylation, actin cytoskeleton reorganization and focal adhesion formation [32]. However, whether Cas influences the binding affinity of suspended cells is less clear. The substrate domain of p130Cas contains phosphorylation-dependent SH2 binding sites for Crk II [54, 55]. This may explain their increased association with pressure in our system and our results that siRNA for either p130Cas or Crk inhibited pressure-stimulated adhesion to the same extent. It also suggests that lowering protein expression of both with specific siRNA is not likely to cause greater inhibition of adhesion than that seen with individual siRNAs. As for Crk, paxillin-phosphorylation-dependent paxillin-Cas binding seems required for pressure to stimulate adhesion. We confirmed the specificity of our observations using re-expressed wild-type Cas to reverse the blockade of the pressure effect achieved by reducing human Cas. Cas associates with many proteins, but our results suggest that paxillin, Cas, and Crk form a trimeric complex in response to paxillin phosphorylation stimulated by pressure. This is consistent with reports in collagen-stimulated NBT-II cells [56] and FG-M carcinoma cells migrating on vitronectin [35], although Cas also binds Bmx in Cos-7 cells after adhesion [57].

Rac1 binds to Cas, although Rac1 also binds to and is activated by RhoG and aldosterone in other settings [58, 59]. We hypothesized that paxillin-Crk-Cas interaction might influence Rac1. Indeed, Rac1 was phosphorylated and activated in response to pressure in a paxillin-dependent manner. Whether paxillin

interacts directly with Rac1 or facilitates conformational changes that permit Cas to phosphorylate Rac1 awaits study. Others have reported that Rac1 is important in actin polymerization, lamellipodial extension, and cell migration, but not adhesion itself [60–63]. Indeed, blocking Rac1 did not inhibit basal adhesion in our studies. However, since Rac1 inhibition completely prevented the stimulation of adhesion by pressure, force-activated adhesion may be regulated by a very different pathway than basal adhesion. Reducing α-actinin also blocks pressure-stimulated adhesion despite minimal effect on basal adhesion [25].

If paxillin phosphorylation is critical for pressure-stimulated adhesion, what might trigger this phosphorylation in response to pressure? Modifications of microtubule dynamics can increase paxillin phosphorylation by an unclear mechanism [64]. FAK and Src influence adhesion in normal and tumor cells [20]. FAK is phosphorylated at tyrosine 397 in response to adhesion itself or pressure prior to adhesion [3, 65]. This phosphorylation facilitates Src binding and activation, leading to further FAK phosphorylation. α-Actinin enhances FAK-Src interaction, and reducing α-actinin blocks pressure-stimulated cancer cell adhesion [25]. The FAK-Src complex may control focal adhesion turnover and target paxillin and Cas for phosphorylation [66]. In our model, FAK reduction stimulated basal paxillin phosphorylation but prevented further phosphorylation in response to pressure. The increase in ambient pressure paxillin phosphorylation resembles that in FAK null fibroblasts [67]. This increase in basal paxillin phosphorylation in FAK null cells may reflect microtubular destabilization by FAK reduction, as occurs in neuronal cells and mouse fibroblasts [68, 69]. However, the failure of pressure to stimulate paxillin phosphorylation in FAK-reduced cells suggests FAK mediates the pressure effect. Src inhibition or reduction of α-actinin also prevented pressure stimulation of paxillin phosphorylation, although without altering basal paxillin phosphorylation, again suggesting that paxillin is probably downstream of FAK and Src and α-actinin in the pathway by which suspended cancer cells respond to pressure. Although previous studies suggested that pressure stimulates paxillin phosphorylation despite FAK-specific siRNA or PP2 [24], we reduced FAK more substantially (~70 %) and treated with PP2 for a longer time (4 h).

In conclusion, pressure-stimulated cancer cell adhesion requires FAK-, Src-, and α-actinin-dependent paxillin phosphorylation at tyrosines 31 and 118. This facilitates association with Crk and Cas and promotes Rac1 activation. This pressure-activated pathway seems clinically relevant, as increased extracellular pressure stimulates cancer cell adhesion to endothelial cells *in vitro* and surgical wounds *in vivo*, and influences tumor-free survival in mice [3, 4, 70]. Paxillin and its binding partners may offer innovative targets to modulate metastasis.

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