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Abstract

Phosphoinositides regulate several actin-binding proteins but their role at intercellular adhesions has not been defined. We found that phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$) was generated at sites of N-cadherin–mediated intercellular adhesion and was a critical regulator of intercellular adhesion strength. Immunostaining for $PI(4,5)P_2$ or transfection with GFP-PH-PLCδ showed that $PI(4,5)P_2$ was enriched at sites of N-cadherin adhesions and this enrichment required activated Rac1. Isoform-specific immunostaining for type I phosphatidylinositol 4-phosphate 5 kinase (PIP5KI) showed that PIP5KIγ was spatially associated with N-cadherin–Fc beads. Association of PIP5KIγ with N-cadherin adhesions was in part dependent on the activation of RhoA. Transfection with catalytically inactive PIP5KIγ blocked the enrichment of $PI(4,5)P_2$ around beads. Catalytically inactive PIP5KIγ or a cell-permeant peptide that mimics and competes for the $PI(4,5)P_2$-binding region of the actin-binding protein gelsolin inhibited incorporation of actin monomers in response to N-cadherin ligation and reduced intercellular adhesion strength by more than twofold. Gelsolin null fibroblasts transfected with a gelsolin severing mutant containing an intact $PI(4,5)P_2$ binding region, demonstrated intercellular adhesion strength similar to wild-type transfected controls. We conclude that PIP5KIγ-mediated generation of $PI(4,5)P_2$ at sites of N-cadherin contacts regulates intercellular adhesion strength, an effect due in part to $PI(4,5)P_2$-mediated regulation of gelsolin.

Comments

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Phosphatidylinositol-4,5 Bisphosphate Produced by PIP5KIγ Regulates Gelsolin, Actin Assembly, and Adhesion Strength of N-Cadherin Junctions[V]

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Phosphoinositides regulate several actin-binding proteins but their role at intercellular adhesions has not been defined. We found that phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$) was generated at sites of N-cadherin–mediated intercellular adhesion and was a critical regulator of intercellular adhesion strength. Immunostaining for $PI(4,5)P_2$ or transfection with GFP-PH-PLCδ showed that $PI(4,5)P_2$ was enriched at sites of N-cadherin adhesions and this enrichment required activated Rac1. Isoform-specific immunostaining for type I phosphatidylinositol 4-phosphate 5 kinase (PIP5KI) showed that PIP5KIγ was spatially associated with N-cadherin–Fc beads. Association of PIP5KIγ with N-cadherin adhesions was in part dependent on the activation of RhoA. Transfection with catalytically inactive PIP5KIγ blocked the enrichment of $PI(4,5)P_2$ around beads. Catalytically inactive PIP5KIγ or a cell-permeant peptide that mimics and competes for the $PI(4,5)P_2$-binding region of the actin-binding protein gelsolin inhibited incorporation of actin monomers in response to N-cadherin ligation and reduced intercellular adhesion strength by more than twofold. Gelsolin null fibroblasts transfected with a gelsolin severing mutant containing an intact $PI(4,5)P_2$ binding region, demonstrated intercellular adhesion strength similar to wild-type transfected controls. We conclude that PIP5KIγ-mediated generation of $PI(4,5)P_2$ at sites of N-cadherin contacts regulates intercellular adhesion strength, an effect due in part to $PI(4,5)P_2$-mediated regulation of gelsolin.

INTRODUCTION

The remodeling of cortical actin filaments associated with cadherins is critical for the formation, maturation, and maintenance of intercellular contacts. Several actin-binding proteins have been implicated in regulating the assembly of cadherin-associated actin filaments including VASP, vinculin, zyxin, formins, Arp 2/3, cortactin, and gelsolin (Hazan *et al.*, 1997; Vasioukhin *et al.*, 2000; Kovacs *et al.*, 2002b; Chan *et al.*, 2004; El Sayegh *et al.*, 2004; Kobielak *et al.*, 2004). These proteins can localize to adherens junctions and significantly impact cadherin function because of their role in regulating actin assembly. However the mechanisms by which these actin-binding proteins are regulated at intercellular contact sites remain undefined. Identification of these regulatory systems could enhance our understanding of the nature of intercellular contact formation and dynamics.

Although several regulatory systems including Rho GTPases, Fak/paxillin, Src and Fer nonreceptor tyrosine kinases, growth factor receptor signaling, and others (Behrens *et al.*, 1993; Zanetti *et al.*, 2002; Schaller, 2004; Erez *et al.*, 2005; Lilien and Balsamo, 2005) have been described at sites of cadherin-dependent contacts, much less attention has been devoted to plasma membrane components and the role of lipid signaling. Phosphoinositides are important regulators of several actin-binding proteins and have been strongly implicated in control of cell shape, motility, and phagocytosis (Czech, 2000; Insall and Weiner, 2001; Simonsen *et al.*, 2001). The products of PI-3 kinase are localized to sites of intercellular contact (Watton and Downward, 1999; Kovacs *et al.*, 2002a), but little is known about other phospholipids, despite detailed information on their role in regulating membrane-localized actin filament assembly (Yin and Janmey, 2003). Indeed, $PI(4,5)P_2$ may directly link the cortical actin cytoskeleton to the plasma membrane (Raucher *et al.*, 2000) and stimulate de novo actin nucleation at the membrane (Prehoda *et al.*, 2000; Rohatgi *et al.*, 2000). Conceivably, the synthesis and turnover of phospholipids such as $PI(4,5)P_2$ could have an important local role at intercellular junctions because of their ability to regulate actin-binding proteins and as a result, the assembly and organization of actin filaments.

Enrichment of $PI(4,5)P_2$ at sites of early intercellular contact can be mediated by sequestration and activation of enzymes that convert PI(4)P to $PI(4,5)P_2$ (Anderson *et al.*, 1999). Recently, a type I phosphatidylinositol 4-phosphate 5 kinase (PIP5KI), specifically PIP5KIγ, has been shown to physically associate with a binding region of the highly

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conserved cytoplasmic tail of E-cadherin (Ling *et al.*, 2007). This PIP5KI isoform, which also localizes to focal adhesions, was shown to be important for the endocytic recycling of E-cadherin (Ling *et al.*, 2002, 2007). Here we tested the hypothesis that PIP5KI mediates localized synthesis of $PI(4,5)P_2$ at sites of N-cadherin ligation, thereby regulating the function of the actin-binding protein gelsolin and the strengthening of N-cadherin–mediated intercellular junctions. Our results demonstrate that localized $PI(4,5)P_2$ synthesis is required for actin assembly at sites of intercellular adhesion by a mechanism that involves uncapping of gelsolin from actin barbed ends.

MATERIALS AND METHODS

Cell Culture and Intercellular Adhesion

Rat-2 cells (CRL 1764; ATCC, Rockville MD), NIH3T3 cells (CRL1658, ATCC), and wild-type or gelsolin-null (Gsn −/−) fibroblasts obtained from day 12 mouse embryos were grown in DMEM (Invitrogen, Grand Island, NY) supplemented with 0.017% penicillin G, 0.01% gentamicin sulfate, and 5% fetal bovine serum. Cells were maintained at 37°C in 5% CO_2. To model intercellular adhesion processes we used the donor-acceptor model (Ko *et al.*, 2000) or plates or beads coated with a recombinant N-cad-Fc (chicken N-cadherin ectodomain fused to the Fc fragment of mouse IgG2b) protein (El Sayegh *et al.*, 2004). These model systems generate large numbers of synchronized, N-cadherin—mediated intercellular adhesions that facilitate biochemical and quantitative study with high temporal resolution. In the donor-acceptor model system, fibroblasts in suspension are added to the dorsal surfaces of substratum-bound, confluent monolayers of homotypic acceptor cells. The attachment of donor cells occurs via N-cadherin–mediated adherens junctions that are not confounded by integrin ligation to the substratum underlying the acceptor cells.

Preparation of N-cad-Fc Beads and Dishes

The N-cad-Fc protein was expressed in HEK-293 cells and collected as described (Lambert *et al.*, 2000). N-cad-Fc purification and coating of beads or substrata was conducted as described (El Sayegh *et al.*, 2004). Briefly, purified N-cad-Fc protein (concentrations ~100 μg/ml) obtained with Immunopure Plus immobilized protein G columns (Pierce Biotechnology, Rockford, IL) was used to coat protein A–conjugated polystyrene beads (3-μm diameter, Spherotech, Libertyville, IL) or adsorbed onto nontissue culture plates by overnight incubation at 4°C (at ~1.25 $\mu g/cm^2$).

Immunofluorescence and Videomicroscopy

Rat-2 cells incubated with N-cad-Fc protein–coated beads were immunostained for $PI(4,5)P_2$ (antibody from Echelon Biosciences, Salt Lake City, UT) or PIP5KI α and γ using isoform-specific polyclonal antibodies (from R.A.A.). Samples were fixed with a 3.7% paraformaldehyde-5% sucrose solution, permeabilized with 0.2% Triton X-100, stained with either polyclonal antibody for 1 h at 37°C, and counterstained with a Cy3-conjugated goat anti-rabbit Fab-specific secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA; 1:500 at 37°C for 60 min). Samples were visualized for Cy3 using a Leica TCS SL confocal microscope (excitation = 543 nm; emission = 565/10 nm; Deerfield, IL). Consecutive optical sections were obtained in the *z*-axis at a nominal thickness of 1 μm from the cell-substrate interface. Fluorescence intensity was quantified along line scans traversing regions of N-cadherin ligation and control, nonligated plasma membrane in images of bead-bound cells using Leica software.

For tracking $PI(4,5)P_2$ during intercellular adhesion formation, Rat-2 cells were seeded onto 35-mm fibronectin-coated glass-bottom microwell dishes (MatTek, Ashland, MA) overnight before transfection with a GFP-tagged Pleckstrin homology domain of phospholipase Cδ1 (GFP-PH-PLCδ). On achieving the desired level of confluence, samples were washed and replenished with fresh growth media (α-MEM without HCO_3^- containing 25 mM HEPES buffer and serum). Samples were placed on a heated stage and imaged for fluorescein isothiocyanate (FITC) fluorescence (excitation = 488 nm; emission = 520/10 nm) using a confocal microscope set with a scan speed set at 400 Hz. Regions of imminent intercellular contact were imaged every 60 s for up to several hours.

Immunoprecipitation and Immunoblotting

After treatments, samples were washed, extracted, precleared, and immunoprecipitated for N-cadherin as described previously (El Sayegh *et al.*, 2004). Briefly, after extraction, precleared lysates containing equal amounts of protein were incubated with primary antibodies recognizing N-cadherin (pan-cadherin antibody: Sigma, St. Louis, MO), and then protein A/G-Sepharose (Pierce Biotechnology, Rockford, IL) at 4°C each for >1 h. Washed immunocomplexes were eluted from beads using 2% Laemmli sample buffer and boiled for 5 min. Samples were fractionated on 10% SDS-PAGE, transferred to nitrocellulose membranes, and blocked with 5% nonfat milk in 0.1% Tween 20 in Tris-buffered saline solution. Membranes were washed and immunoblotted for polyclonal anti PIP5KIγ (from R.A.A.) or anti-gelsolin antibodies (affinity-purified and described previously; Arora *et al.*, 2005; Azuma *et al.*, 1998) and N-cadherin (GC-4, Sigma). Bound antibodies were detected with peroxidase-conjugated goat anti-mouse antibody (Jackson ImmunoResearch Laboratories) and chemiluminescence (Amersham, Oakville, ON, Canada).

N-Cadherin–mediated Adhesion Strength

Donor-acceptor cell preparations or N-cadherin–coated beads were subjected to shear forces by performing a logarithmic series of jet washes to estimate the relative strength of intercellular adhesion (Chou *et al.*, 1996). To assess the role of $PI(4,5)P_2$ in adhesion strengthening, Rat-2 fibroblasts were transfected with full-length, catalytically inactive, hemagglutinin (HA)-tagged-PIP5KIγ. Cells were transfected with FuGENE 6 Transfection Reagent (Roche, Laval, QC, Canada) and were compared with untransfected cells or cells in samples transfected with full-length wild-type HA-PIP5KIγ. To assess the role of $PI(4,5)P_2$ in the localized regulation of actin-binding proteins, cells were incubated with 30 μM of rhodamine B–conjugated PBP-10 as previously described (Cunningham *et al.*, 2001). The cell-permeant 10-residue peptide (QRLFQVKGRR) was based on the sequence of the $PI(4,5)P_2$-binding region of gelsolin (residues 160–169). Control cells were incubated with an equivalent concentration of rhodamine B–conjugated QRL peptide (Cunningham *et al.*, 2001). N-cad-Fc–coated polystyrene beads or donor cells were incubated with attached cells for 15 min. After jet washing, attached donor cells or bead-associated samples were fixed as described above. Data of cell or bead attachment were acquired from three, randomly chosen, low-power fluorescence microscopy fields. At least 30 cells were quantified per field, and at least three fields were assessed per sample.

Magnetic Bead Pulloff Assay

Cell surface proteins were enriched at sites of N-cadherin ligation with recombinant N-cad-Fc–coated beads. Bead-associated adhesion complexes were isolated and immunoblotted as described previously (Plopper and Ingber, 1993). Briefly, after designated incubation times, cells and attached N-cadherin-coated magnetic beads (Spherotech) were collected by scraping into ice-cold extraction buffer (CSK-EB: 0.5% Triton X-100, 50 nM NaCl, 300 mM sucrose, 3 mM $MgCl_2$, 2 mM AEBSF, 1 mM EDTA, 30 μM bestatin, 14 μM E-64, 1 μM leupeptin, 0.3 μM aprotinin, and 10 mM PIPES, pH 6.8). Beads were pelleted using a side-pull magnetic isolation apparatus (Dynal, Lake Placid, NY), and supernatants were collected. Isolated beads were resuspended, sonicated, homogenized, and washed three times in extraction buffer before PAGE and Western blot analysis.

Cell Sorting and PIP5KIγ Recruitment

Constitutively active and dominant negative enhanced green fluorescent protein (EGFP)-Rac– or Rho-transfected NIH 3T3 cells were harvested with 0.01% trypsin supplemented with 2 mM $CaCl_2$. Transfected cells were sorted by flow cytometry from untransfected cells (ALTRA, Beckman-Coulter, Mississauga, ON, Canada) with excitation at 488 nm. Sorted cells were allowed to bind to N-cad-Fc–coated 35-mm plates for 30 min before lysis, immunoprecipitation, and immunoblotting as described above. Quantification of recruitment of PIP5KIγ to adherens junctions was determined by dividing the band densities of PIP5KIγ with N-cadherin for each respective construct.

Actin Assembly

NIH 3T3 cells transfected with wild-type or catalytically inactive HA-PIP5KIγ constructs or cells previously incubated with PBP-10 or QRL peptides (30 μM) were allowed to attach to N-cad-Fc–coated, nontissue culture plates. In permeabilized cells incubated with rhodamine actin monomers, increases of rhodamine fluorescence due to incorporation into nascent actin filaments were measured (Hartwig, 1992; Hartwig *et al.*, 1995; Azuma *et al.*, 1998). Cells were permeabilized for 20 s using 0.1 vol of OG buffer (PHEM buffer containing 2% octyl glucoside and 2 μM phalloidin). Permeabilization was stopped by diluting the detergent with buffer. Immediately thereafter, freshly sedimented rhodamine actin monomer (0.23 μM) in buffer containing 120 mM KCl, 2 mM $MgCl_2$, 3 mM EGTA, 10 mM PIPES, and 0.1 mM ATP was added to the samples for 10 s followed by fixation with 3.7% formaldehyde. The samples were observed with a Nikon TE 300 microscope (Melville, NY) and rhodamine fluorescence in single cells was quantified using the PCI Imaging program (SimplePCI, Minneapolis, MN). For background correction, detergent treatments were omitted, fluorescence was quantified, and background signal were subtracted from experimental samples. Transfected cells were identified by immunostaining as described above using anti-HA antibodies (Covance, Denver, PA) and counterstained with FITC-conjugated Fab-specific goat anti-mouse antibody (Jackson ImmunoResearch Laboratories).

Electron Microscopy of Cytoskeletons

NIH3T3 cells incubated with PBP-10 or QRL peptides (30 μM) were allowed to attach onto N-cad-Fc–coated glass coverslips for 3 min before detergent extraction, fixation, and processing as described elsewhere (Svitkina *et al.*, 1998; Chan *et al.*, 2004). Briefly cells were extracted for 5 min with 1% Triton X-100, 4% PEG in PEM buffer (100 mM PIPES, pH 6.9, 1 mM $MgCl_2$), 1 mM EGTA supplemented 10 μM phalloidin, washed three times in PEM buffer, and fixed in 2% glutaraldehyde (EM grade) in 0.1 M sodium cacodylate, pH 7.3, for 20 min at room temperature and overnight at 4°C. Samples were subsequently fixed in 0.1% aqueous tannic acid and uranyl acid solutions for 20 min, respectively, before dehydration and critical point drying. Samples were gold-coated using a Polaron sputter coater with a rotary planetary stage. Samples were visualized and digital images were acquired at a working length of 3 nm using the upper detector of a Hitachi S-570 scanning electron microscope (Pleasanton, CA).

Statistical Analysis

For continuous variables, means and SEMs were computed. Comparisons between two groups were made with the unpaired Student's *t* test and multiple comparisons by ANOVA. Statistical significance was set at $p < 0.05$.

RESULTS

N-Cadherin Specifically Associates with PIP5KIγ

We investigated the association of the PIP5KIs with N-cadherin and determined PIPKI isoform expression specificity using biochemical analyses and confocal microscopy. From donor:acceptor samples established for 30 min, N-cadherin immunoprecipitates were immunoblotted with PIP5KIα or γ isoform-specific antibodies. Only the γ isoform was detected in immunoprecipitated material, indicating specific association of N-cadherin with the PIP5KIγ isoform (Figure 1A). As these results indicate the proteins associated with total cellular N-cadherin, we could not rule out that the immunoprecipitated proteins may be derived from cytosolic pools of N-cadherin. Accordingly, we characterized the spatial association of PIP5KIγ with surface-expressed N-cadherin by immunostaining and confocal microscopy. Rat-2 fibroblasts were incubated with recombinant N-cad-Fc fusion protein–coated beads. Staining for PIP5KIγ but not PIP5KIα was found at regions of bead-to-cell contacts (Figure 1B, ii and v). Quantification of fluorescence intensity plots from optical sections indicated marked spatial association of N-cadherin with PIP5KIγ. These findings were supported by magnetic bead pull-off experiments which biochemically verified an isoform specific association between surface expressed N-cadherin and PIP5KIγ (data not shown). These results demonstrate that PIP5KIγ indeed associates with surface-expressed, ligand-engaged N-cadherins (Figure 1B, vii and viii).

PIP_2 Is Generated at Sites of N-Cadherin Adhesion

As one of the products of PIP5KIs is $PI(4,5)P_2$, we determined if this phospholipid is generated at sites of N-cadherin ligation. Rat-2 fibroblasts were transfected with GFP-PH-PLCδ as a marker for $PI(4,5)P_2$. Quantitative analysis of fixed samples reveals significantly greater fluorescence intensity attributable to $PI(4,5)P_2$ at sites of intercellular contact compared with noncontact sites as determined by line scans (representative line scan seen in Figure 2A). Similar results were obtained using anti-PIP_2–specific antibodies (data not shown).

To ascertain the temporal relationship between contact formation and remodeling, and $PI(4,5)P_2$ synthesis we conducted long-span (>120 min) videomicroscopic, real-time analyses of GFP-PH-PLCδ transfected Rat-2 cells at low- and high-power magnifications. Analysis of videos reveal that $PI(4,5)P_2$ is formed subsequent to physical contact between adjacent cells. We also note that $PI(4,5)P_2$ is not present at contact sites that are about to form. Thus the enrichment of this phospholipid at intercellular contacts appears to be in response to intercellular contact formation and is not directly involved with the initiation of contacts. N-cadherin contacts are very dynamic structures in which there is constant remodeling and where some contacts may undergo several rounds of formation and dissolution (Sayegh *et al.*, 2005). Long-span videomicroscopic analysis of GFP-PH-PLCδ transfected cells also revealed that there is continued $PI(4,5)P_2$ synthesis throughout contact maturation or remodeling (Figure 2B, see also Supplementary Videos Fig2biVideo.mov and Fig2biiVideo.mov). Mature, broad contacts are marked with sustained enrichments of fluorescence attributable to $PI(4,5)P_2$. Remodeling contacts (or zones of contact extension) display fluctuations of fluorescence intensity, which presumably correspond to the cycles of formation and dissolution of physical contact (Figure 2C and Supplementary Video Fig2cVideo.mov). This analysis further supports other results indicating that $PI(4,5)P_2$ is formed in response to contact formation. Regions of intercellular contact are complex structures containing numerous cell surface receptors potentially capable of eliciting the noted enrichment of $PI(4,5)P_2$. We next investigated the role of N-cadherin, a major mediator of intercellular contacts in fibroblasts, in stimulating the formation of $PI(4,5)P_2$. Cells were incubated with N-cad-Fc–coated beads and evaluated by confocal microscopy. Enhanced fluorescence attributable to $PI(4,5)P_2$ was found at sites of N-cadherin ligation compared with poly-L-lysine–coated controls (Figure 2D; also see Figures 3 and 5). These findings demonstrate that $PI(4,5)P_2$ is generated after physical contact at sites intercellular adhesion and that N-cadherin ligation is specifically required to elicit this response.

PIP_2 Is Generated at Sites of N-Cadherin in a PIP5KIγ-dependent Manner

PIP_2 enrichment at intercellular contacts may due to either localized synthesis, inhibition of phospholipid phosphatases, or recruitment of buffering proteins (McLaughlin *et al.*, 2002). Although cadherin-associated PIP5KIγ functions have been implicated in the regulation of endocytic recycling and surface trafficking of cadherins (Ling *et al.*, 2007), we considered that PIP5KIγ may be responsible for the localized production of $PI(4,5)P_2$ at intercellular contacts. We cotransfected Rat-2 cells with GFP-PH-PLCδ and with full-length, HA-tagged, wild-type-PIP5KIγ, followed by incubation with N-cad-Fc beads for 15 min. Cells were fixed and immunostained with an anti-HA antibody and Cy3-conjugated second antibody. In these preparations staining for both GFP-PH-PLCδ and wild-type-PIP5KIγ colocalized to sites of bead-cell binding (Figure 3A, ii and iii) and at regions of intercellular contacts (Figure 3A, insets). Fluorescence intensity line scans showed that peak fluorescence was restricted to zones of bead-cell contacts for both constructs (Figure 3I, v and viii), indicating that PIP5KIγ is involved in the production of $PI(4,5)P_2$ at sites of N-cadherin ligation. No enrichment of either GFP-PH-PLCδ or the PIP5KIγ construct was present at sites on cells where there was binding to poly-L-lysine–coated beads (Figure 3A).

In more definitive experiments to evaluate the role of PIP5KIγ in the production of $PI(4,5)P_2$, we cotransfected Rat-2 cells with full-length, catalytically inactive PIP5KIγ and GFP-PH-PLCδ constructs, and evaluated fluorescence intensity around N-cad-Fc beads. Confocal microscopy showed that cells expressing both constructs demonstrated very little bead localized GFP-PH-PLCδ fluorescence. This pattern was found at all optical section levels around bead-cell binding

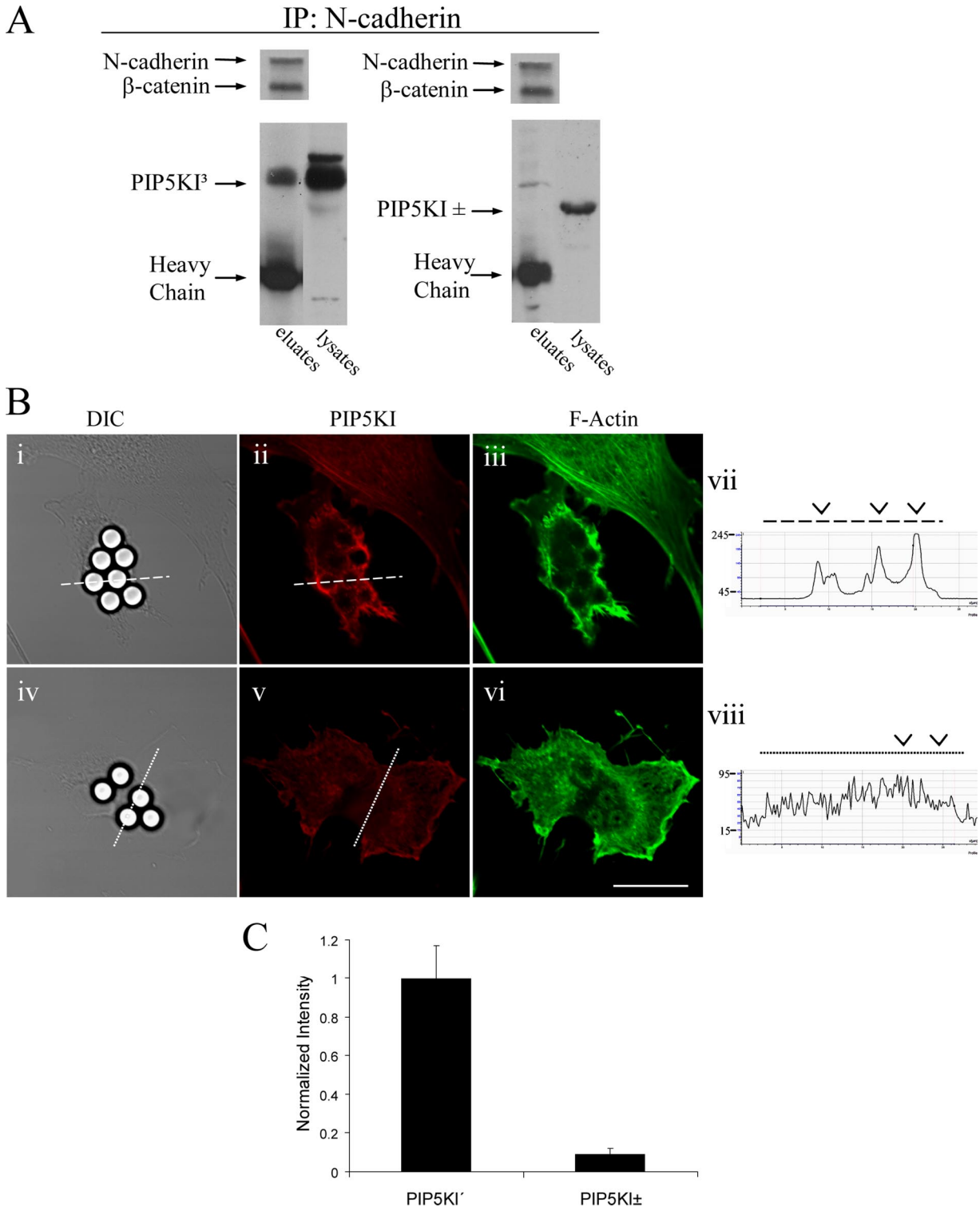

Figure 1. γ-Isoform of PIP5KI associates specifically with N-cadherin adhesions. (A) Cell lysates were prepared from Rat-2 donor-acceptor samples incubated for 30 min, immunoprecipitated for N-cadherin, and blotted with PIP5KIγ and PIP5KIα-specific antibodies. (B) Confocal microscope rendered 1-μm optical sections of bead-cell binding interface of N-cad-Fc–coated bead bound Rat-2 cells stained with PIP5KIγ (ii) and PIP5KIα antibodies (v) and Alexa fluor 488 phalloidin (iii and vi). Solid bar, 20 μm. Fluorescence intensity plots (vii and viii) for images in the red emission channel (ii and v), with regions corresponding to bead-binding sites indicated by the open arrows. Note different scales used for *y*-axis. (C) Maximal fluorescence intensity of bead-bound sites in 32 cells stained with PIP5KIγ and PIP5KIα isoform-specific antibodies. Data shown are mean values.

sites (Figure 3B, open arrow). In contrast, cells that did not express the catalytically inactive PIP5KIγ construct demonstrated significantly higher GFP-PH-PLCδ fluorescence intensity at bead-cell binding sites (Figure 3B, closed arrow).

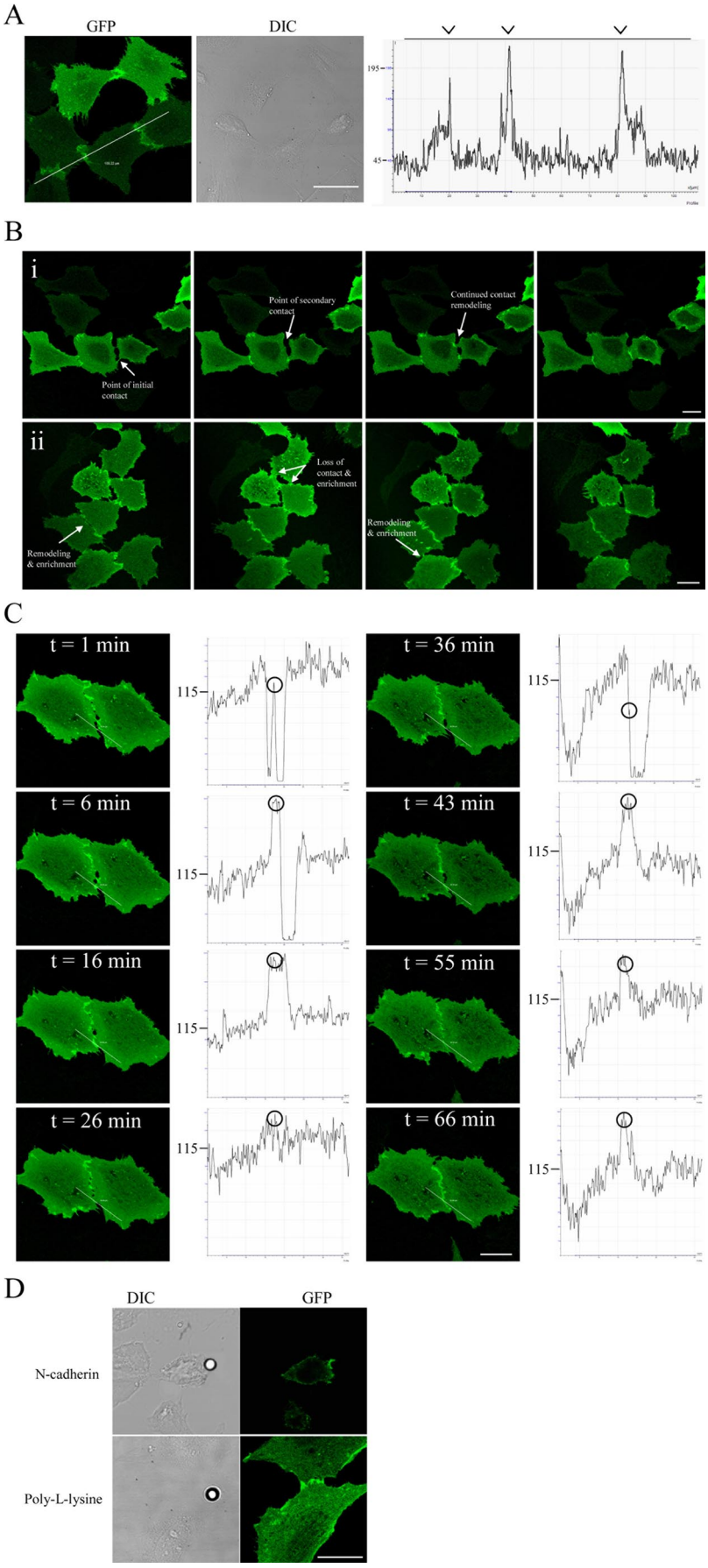

Figure 2. Phosphatidylinositol (4,5) bisphosphate (PIP_2) is generated at sites of N-cadherin adhesions. (A) Rat-2 cells transfected with GFP-PH-PLCγ1 and fixed. Fluorescence intensity plot indicates enrichment of construct at sites of intercellular adhesion. (B) (i and ii) Still images from long-span videos of Rat-2 cells transfected with GFP-PH-PLCγ1 during intercellular junction formation reveal enrichment of GFP-PH-PLCγ1 only after contact formation and at broad mature contacts. (ii) Loss of enrichment noted after loss of intercellular contact. Total video time (i) t = 170 min (10,195 s) and (ii) t = 307 min (18,427 s). (C) Still images and corresponding line scans from long-span video of Rat-2 cells transfected with GFP-PH-PLCγ1 during intercellular junction formation reveal fluctuations in fluorescence intensity corresponding to periods of intercellular contact at zone of contact extension. Circles on line scans highlight readings at tracked contact zone. Total video time t = 184 min (11,040 s) (D) Samples of Rat-2 cells transfected with GFP-PH-PLCγ1 were allowed to bind to N-cadherin-Fc–coated beads and fixed after 15 min of bead binding and counterstained for F-actin using rhodamine phalloidin. All bars, 20 μm.

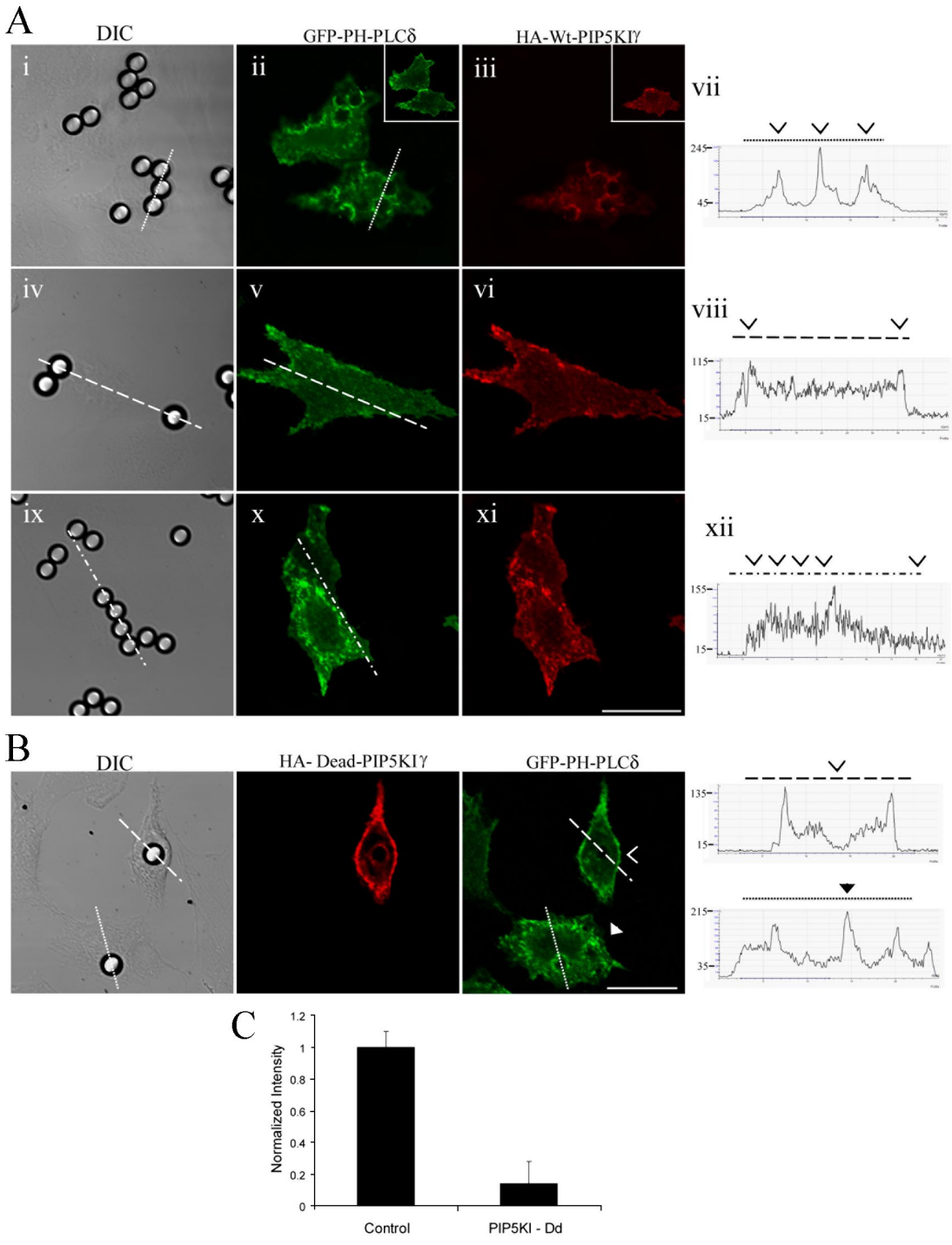

Figure 3. PIP$_2$ at sites of N-cadherin ligation is generated in a PIP5KIγ-dependent manner. (A) Rat-2 cells double-transfected with GFP-PH-PLCγ1 and wt-PIP5KIγ attached onto glass coverslips were incubated with N-cad-Fc–coated (i–iii), bare (iv–vi), or poly-L-lysine–coated (ix–xi) beads for 15 min. Insets demonstrate accumulation of constructs at native intercellular contacts. Open arrows on fluorescence intensity plots (vii, viii, and xii) of the green emission channel indicate regions of bead binding. Note different scales used for *y*-axis. (B) Rat-2 cells double-transfected with GFP-PH-PLCγ1 and kinase-dead PIP5KIγ-bound N-cad-Fc–coated beads for 15 min demonstrate loss of accumulated PIP$_2$ around beads bound by kinase dead transfected cells. Compare open versus closed arrows. Fluorescence intensity plots for images in the green emission channel with regions corresponding to bead-binding sites indicated by an open arrow and long-dash marker or closed arrow and dotted marker for each respective cell as displayed. Note different scales used for *y*-axis. (C) Normalized means of maximal fluorescence intensity of bead-bound sites in 24 cells, each either expressing both constructs or only GFP-PH-PLCγ. Bars, 20 μm.

No differences of plasma membrane-associated GFP-PH-PLCδ fluorescence were found in cells transfected with the kinase-dead PIP5KIγ. Collectively these results indicate that the increase of PI(4,5)P$_2$ concentration at zones of intercellular contact is attributable in part to the enzymatic activity of PIP5KIγ.

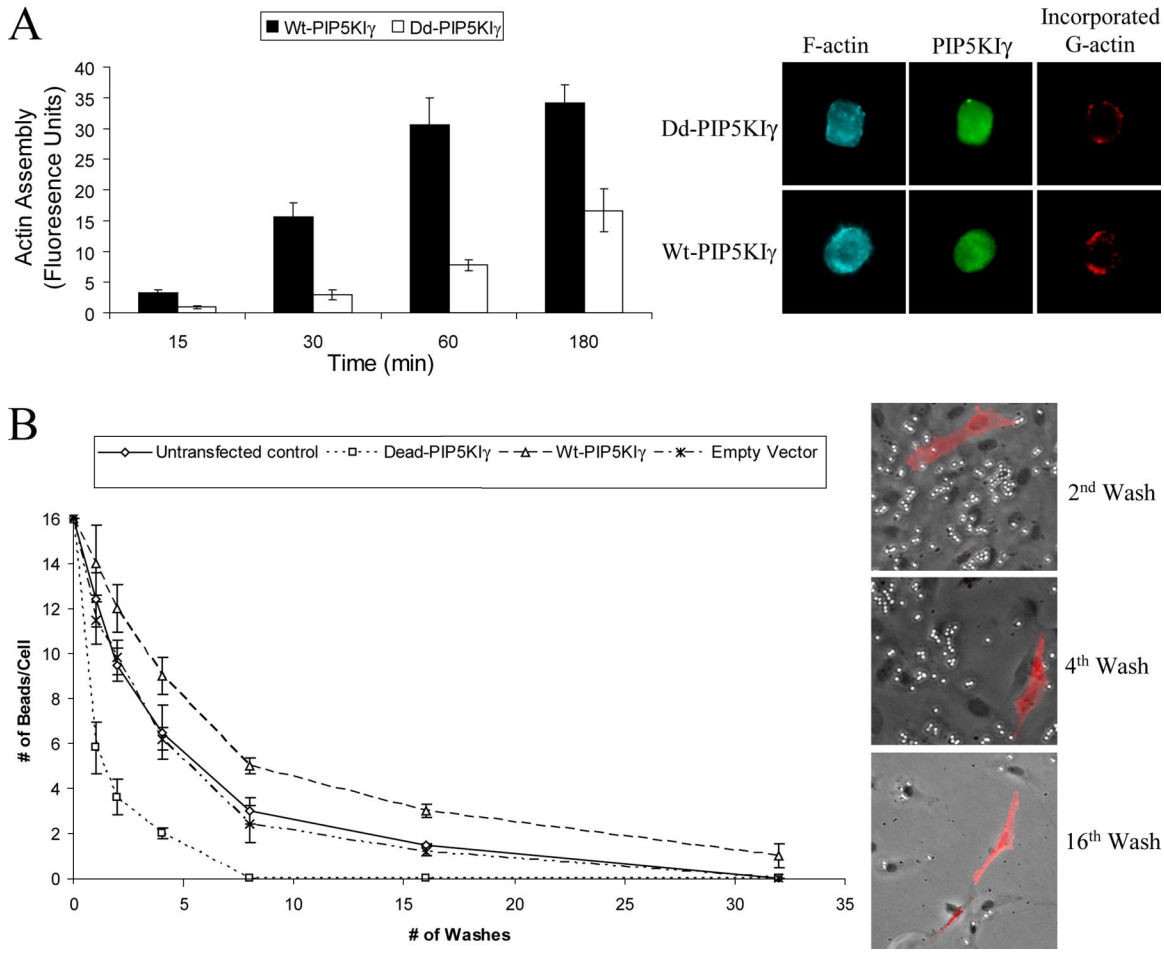

Figure 4. PIP5KIγ-mediated PIP_2 formation required for N-cadherin localized actin polymerization and adhesion strength. (A) NIH 3T3 cells transfected with either wild-type or dominant negative PIP5KIγ constructs and allowed to attach to N-cad-Fc–coated substrata for designated periods of time. Samples were processed with rhodamine-tagged G-actin and Alexa fluor 356 phalloidin, immunostained with anti-HA antibodies (green) to identify transfected cells, and quantification of actin assembly was completed. Representative micrographs are shown for 15-min samples. (B) Rat-2 cells transfected with either wild-type or dominant negative PIP5KIγ constructs or a GFP empty vector incubated with N-cad-Fc–coated beads were subjected to a shear wash-off assay. Overlay pictures presented of 2nd, 4th, and 16th washes are indicative of representative fields. Cells with red emission are transfected with PIP5KIγ dominant negative construct.

PI(4,5)P_2 Generation Regulates Localized Actin Assembly and N-Cadherin Adhesion Strength

PI(4,5)P_2 can interact with numerous actin-binding proteins to regulate actin polymerization at the plasma membrane (Yin and Janmey, 2003). As cadherin junctions are active sites of actin assembly (Lambert *et al.*, 2002), we evaluated the role of PIP5KIγ-generated PI(4,5)P_2 in localized actin assembly. NIH3T3 fibroblasts were transfected with either full-length wild-type or catalytically inactive PIP5KIγ constructs, allowed to attach and spread on to N-cad-Fc–coated substrata for 15–180 min and processed to determine rhodamine actin monomer incorporation into filaments. Appropriate transfection of cells was verified by fixation and immunofluorescence labeling of HA epitopes in the transfected constructs (Figure 4A, green). The greatest amount of incorporated actin occurred during the first 60 min with little activity after that. There were about fourfold reductions of rhodamine actin fluorescence between 15 and 60 min for cells transfected with PIP5KIγ catalytically inactive constructs compared with wild-type constructs. To evaluate the specificity of these results, transfected cells were allowed to attach and spread on control poly-L-lysine substrates. Although there was a general reduction in actin monomer incorporation in control samples, there were no significant differences noted in monomer incorporation rates between the wild-type or catalytically inactive transfected cells (data not shown).

We evaluated the effect of inhibiting locally-generated PI(4,5)P_2 formation on N-cadherin–mediated intercellular adhesion strength. N-cad-Fc–coated beads were incubated with an underlying, nonconfluent layer of Rat-2 cells transfected with either the full-length catalytically inactive PIP5KIγ or with wild-type PIP5KIγ for 15 min and then subjected to shear wash-off assays. Quantification of the number of beads bound per cell provides estimates of N-cadherin–binding strength in transfected and nontransfected cells (El Sayegh *et al.*, 2004). The expression of catalytically inactive PIP5KIγ constructs reduced by about threefold N-cadherin–mediated adhesion strength compared with untransfected controls or cells transfected with an empty vector (GFP) or the wild-type PIP5KIγ ($p < 0.05$; Figure 4B).

PIP_2 Generation and PIP5KIγ Recruitment to N-Cadherin Is Dependent on Rho GTPase Signaling

Cadherin adhesions exhibit activation of rho GTPases (Noren *et al.*, 2001; Charrasse *et al.*, 2002; Sayegh *et al.*, 2005) that in turn generate complex signaling cascades which may impact the localized synthesis of $PI(4,5)P_2$ by regulating PIP5KIγ recruitment and activation state (Chong *et al.*, 1994; Tolias *et al.*, 2000; Weernink *et al.*, 2004). We first evaluated the impact of locally activated rhoA and rac1 on the generation of $PI(4,5)P_2$ at sites of N-cadherin ligation. NIH3T3 cells were transfected with both GFP-PH-PLCδ and Myc-tagged, constitutively active or dominant negative rac1 or rhoA constructs. Cells were then allowed to bind N-cad-Fc–coated beads for 15 min, and the fluorescence intensity of GFP-PH-PLCδ was quantified at regions of bead-to-cell contacts. Compared with the other constructs, constitutively active rac increased GFP-PH-PLCδ fluorescence sevenfold, indicating enhanced formation of $PI(4,5)P_2$ formation ($p < 0.001$; Figure 5A). Constitutively active rhoA also significantly increased the fluorescence intensity of GFP-PH-PLCδ compared with control, untransfected cells; however, this increase was much lower in magnitude than that noted for rac1 (Figure 5A).

We next investigated if rac1 or rhoA activation impacts the recruitment of PIP5KIγ. We used flow cytometry to purify cells successfully transfected and plated the cells on N-cad-Fc–coated substrata. This approach allowed us to synchronize contact formation between different samples, each consisting of large numbers of cells transfected with various rho GTPase constructs. Specifically, NIH3T3 cells were transfected with GFP-tagged, constitutively active or dominant negative rac1 or rhoA constructs. Cells were sorted by flow cytometry into purified populations that expressed these constructs before attachment to N-cad-Fc–coated substrata for 30 min. Cell lysates were immunoprecipitated for N-cadherin and immunoblotted for PIP5KIγ. Densitometric analysis showed that constitutively active rhoA but not rac1 enhanced the association of PIP5KIγ with N-cadherin (Figure 5B).

Gelsolin is an important downstream effector of rac1-mediated actin assembly (Azuma *et al.*, 1998). As rac is activated transiently after N-cadherin ligation (Sayegh *et al.*, 2005) in a timeline that coincides with the association of gelsolin with N-cadherin adhesions (Chan *et al.*, 2004), we investigated if gelsolin recruitment to sites of N-cadherin ligation is also regulated by rho or rac activation. When blots of the experiment described above were stripped and reprobed with a polyclonal anti-gelsolin antibody, there was no detectable temporal association between gelsolin recruitment to N-cadherin and the activation state of rhoA or rac1. Taken together these results suggest that the rho GTPases have independent functions in regulating $PI(4,5)P_2$ production at intercellular contacts. RhoA is primarily responsible for recruitment of PIP5KIγ, whereas Rac1 may be required only for the activation of PIP5KIγ and enhanced $PI(4,5)P_2$ production.

PIP_2-binding Peptide Influences Adhesion Strength and Actin Polymerization and Architecture

In addition to coupling the function of actin-binding proteins with actin assembly at the plasma membrane, phosphoinositides may regulate other cellular functions including ion channel activation and molecular trafficking, all of which may impact cadherin-dependent adhesion processes (Downes *et al.*, 2005; Rohacs *et al.*, 2005). To investigate the effects of uncoupling the interactions between phosphoinositide-regulated actin-binding proteins and PIP_2 on N-cadherin adhesion strength, we used a previously characterized 10-residue peptide (PBP-10; Cunningham *et al.*, 2001) that is based on the sequence of the $PI(4,5)P_2$ binding site of gelsolin. This membrane-permeant peptide competes with gelsolin in binding $PI(4,5)P_2$, thereby displacing gelsolin (and potentially other actin-binding proteins with similar sequences) and altering function. Notably, not all actin-binding proteins share similar $PI(4,5)P_2$ sequences as gelsolin, and consequently the function of these other actin-binding proteins is not expected to be altered (for a review of different $PI(4,5)P_2$ binding domains see Sechi and Wehland, 2000). Donor-acceptor model cultures were established for 15 min from cells preloaded with either rhodamine-tagged PBP-10 or a control peptide (RhodamineB-QRL, a truncated PDP10 that is cell permeant but does not bind PIP_2), and subjected to shear wash-off assays. Samples incubated with PBP-10 exhibited nearly threefold reductions of adhesion strength at all time points compared with QRL peptide controls (Figure 6A). Localization of the peptides was distinctly different: PBP-10 accumulated at the periphery of contact zone extensions, whereas the QRL peptide was diffusively distributed throughout the cell (Figure 6A, inset). Similar results were obtained when N-cad-Fc–coated beads were used (data not shown).

We examined the impact of uncoupling phosphoinositide regulation of gelsolin and potentially other actin-binding proteins on actin polymerization at sites of N-cadherin ligation. NIH 3T3 cells were preincubated with PBP-10 or QRL peptides for 15 min and allowed to attach and spread on N-cad-Fc–coated substrata in media containing the peptides for designated time periods (15–180 min). Actin polymerization was reduced about threefold in samples incubated with PBP-10 peptide compared with QRL controls at all time points, but the largest reduction was during the first 60 min ($p < 0.001$; Figure 6B). We investigated the effect of these peptides on actin architecture with scanning electron microscopy of NIH 3T3 cells preincubated with either PBP-10 or QRL as described (Chan *et al.*, 2004). Compared with controls, cells incubated with PBP-10 exhibited much less well-developed actin architecture, with longer filaments and with reduced branching along the filament lengths (Figure 6C). These results demonstrate that $PI(4,5)P_2$ regulation of gelsolin and possibly the function of other associated actin-binding proteins impacts N-cadherin adhesion strength by influencing local actin polymerization and actin architecture.

PIP_2 Regulation of Gelsolin Modulates N-Cadherin Adhesion Strength

As gelsolin may regulate N-cadherin adhesion strength (Chan *et al.*, 2004) and in view of the data obtained with the PBP-10 peptides suggesting a PIP_2-dependent mechanism for uncapping gelsolin from actin filaments, we investigated the impact of PIP_2 binding to gelsolin and gelsolin severing deficiency on N-cadherin adhesion strength. We used a previously described, full-length gelsolin mutant (Arora *et al.*, 2005) that preserves $PI(4,5)P_2$ binding and $PI(4,5)P_2$-mediated barbed end actin filament uncapping function but exhibits dramatically reduced actin-severing activity. N-cad-Fc–coated beads were incubated with either gelsolin null fibroblasts or with wild-type fibroblasts or with gelsolin null cells transfected with GFP-tagged, full-length wild-type or mutant gelsolins. N-cad-

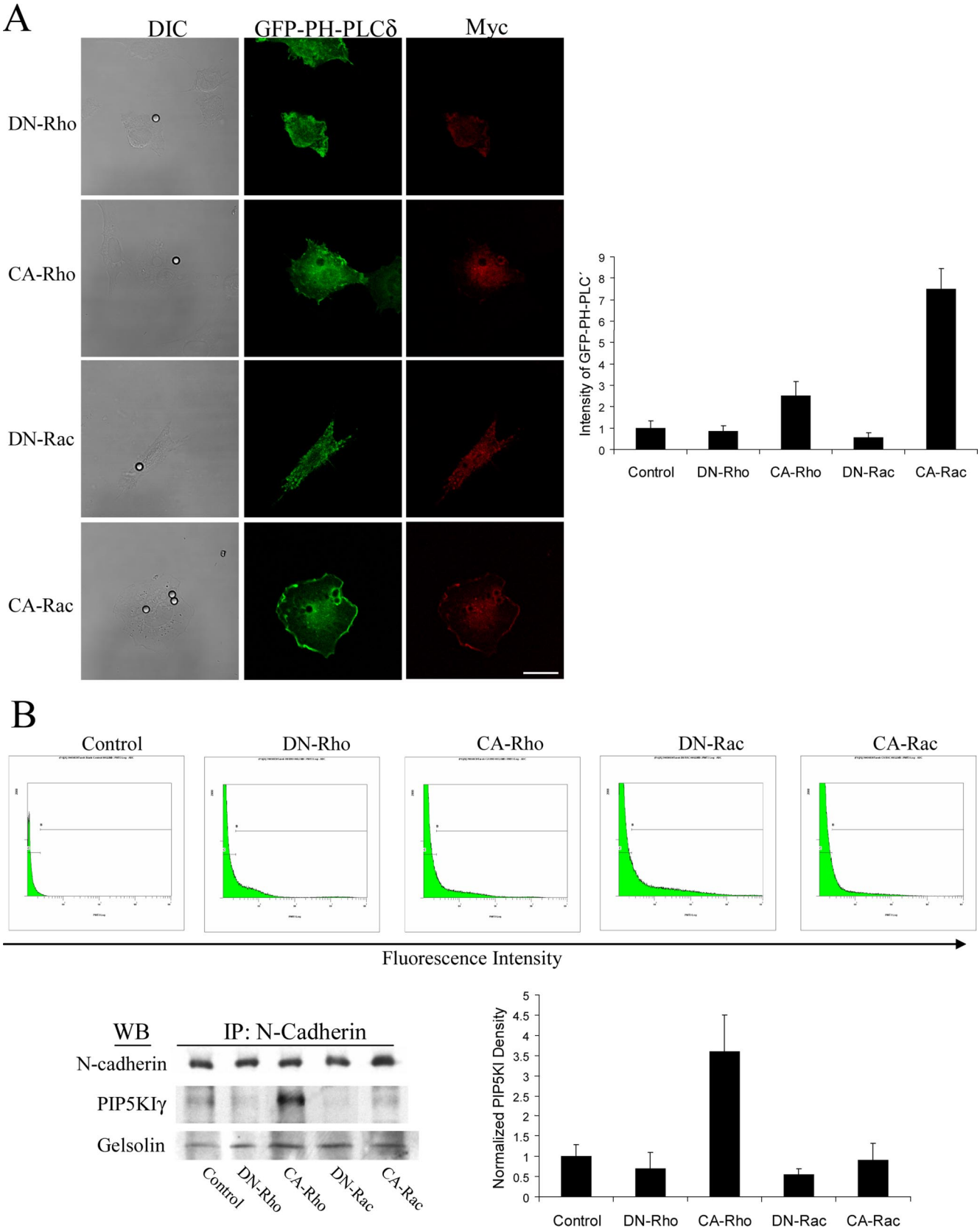

Figure 5. Recruitment and activation of PIP5KIγ is mediated by localized activation of Rho GTPases. (A) N-cad-Fc beads were allowed to bind for 15 min to Rat-2 cells transfected with either constitutively active or dominant negative myc-tagged RhoA or Rac1 constructs and GFP-PH-PLCγ1. Normalized means of maximal fluorescence intensity of bead-bound sites in 20 double-transfected cells are demonstrated. Bar, 20 μm. (B) N-cadherin immunoprecipitation of sorted NIH 3T3 cells transfected with GFP-tagged Rho and Rac constructs that have been

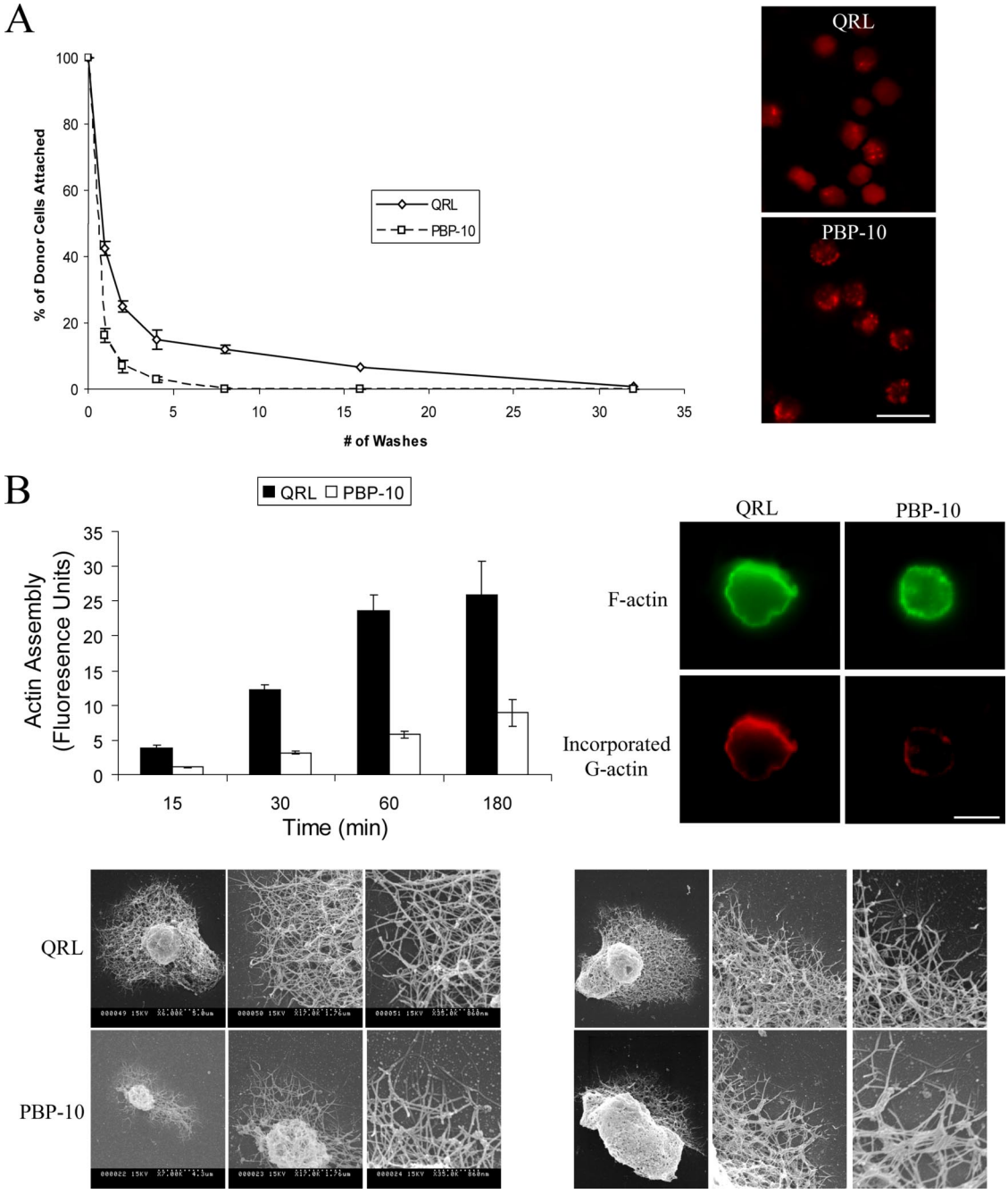

Figure 6. Competitive binding of PBP-10 (10-mer peptide derived from gelsolin PIP$_2$-binding site) influences adhesion strength and localized actin polymerization, and architecture. (A) Rat-2 cells were incubated with rhodamine-tagged PBP-10 or QRL control peptide before and during to intercellular adhesion contact for 15 min using the donor-acceptor model and subjected to a shear wash-off assay. Donor-focused confocal optical section of bound cells after one wash, revealing diffuse staining of QRL control peptide and intense peripheral PBP-10 localization. Bar, 10 μm. (B) Actin assembly of rhodamine-tagged PBP-10 or QRL treated NIH 3T3 fibroblasts spreading onto N-cad-Fc substrates for designated periods of time. Samples were processed with rhodamine-tagged G-actin, and Alexa fluor 488 phalloidin and de novo actin assembly was quantified. Representative micrographs shown for 15-min samples. Bar, 10 μm. Scanning electron microscopy of cells treated with either PBP-10 or QRL and allowed to attach for 5 min on N-cad-Fc–coated glass coverslips. Two different cells per treatment group at equivalent magnifications are shown.

Figure 5 (cont). allowed to attach and spread onto N-cad-Fc substrates for 30 min. Recruitment of gelsolin is independent of Rho GTPase activation. Blots were probed with antibodies specific for N-cadherin, PIP5KIγ, and gelsolin. Histogram presents means from densitometric quantification of three independent experiments.

herin adhesion to gelsolin null cells was reduced compared with wild-type cells; this reduction was rescued by transfection with full-length, wild-type gelsolin (Figure 7), similar to previous findings (Chan *et al.*, 2004). Transfection of gelsolin null cells with the gelsolin mutant that preserved PIP$_2$ binding but with inhibited severing also restored N-cadherin adhesion strength (Figure 7). Cells transfected with this mutant gelsolin

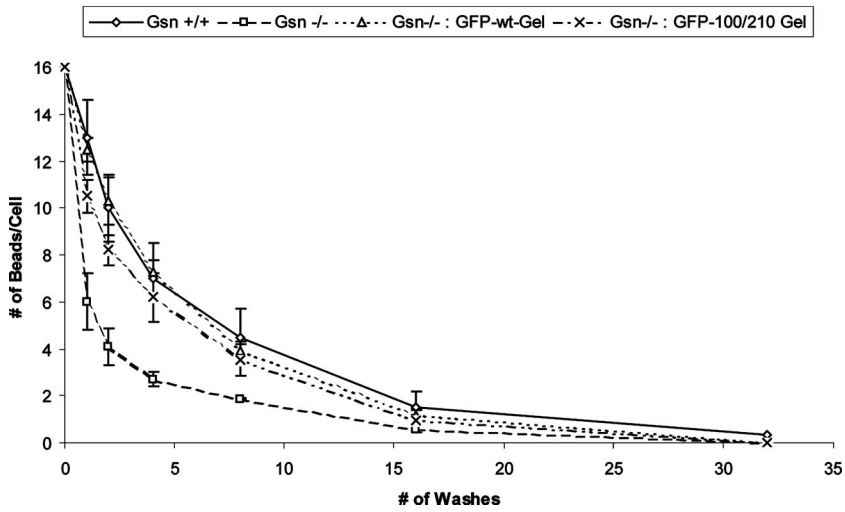

Figure 7. Gelsolin actin filament–capping function is critically important for N-cadherin–mediated adhesion strength. Gelsolin wild-type (Gsn+/+), gelsolin null (Gsn−/−) or gelsolin null cells transfected with GFP-tagged constructs for wild-type gelsolin (Gsn−/− GFP-wt-Gel) or severing mutant (Gsn−/− GFP-100/210 Gel) were incubated with N-cad-Fc beads for 30 min and subjected to a shear wash-off assay. Quantification of mean values of beads bound per cell are presented for every treatment condition.

had an adhesion strengthen profile that was slightly lower, but not statistically different ($p > 0.2$) than gelsolin wild-type or gelsolin null cells transfected with wild-type gelsolin. These results suggest that PIP_2-mediated uncapping of gelsolin from the barbed ends of actin filaments plays an important role in the function of N-cadherin adhesions.

DISCUSSION

Cadherin junctions are critically dependent on actin remodeling for adhesion zone extension and maturation (Adams and Nelson, 1998; Yap and Kovacs, 2003). We considered that this process requires precise regulation of the production of $PI(4,5)P_2$. Here we report that locally activated Rho GTPases mediate the recruitment and activation of PIP5KIγ at sites of N-cadherin ligation, resulting in the synthesis of $PI(4,5)P_2$, a potent regulator of gelsolin and other actin-binding proteins. The generation of $PI(4,5)P_2$ subsequent to N-cadherin ligation and its interaction with gelsolin was important for localized actin assembly at N-cadherin adhesions. Indeed, reconstitution of gelsolin null cells with gelsolin-severing mutants containing intact $PI(4,5)P_2$-binding sites restored N-cadherin adhesion strength. These data underscore the importance of $PI(4,5)P_2$ as a local regulator of gelsolin function and demonstrate the significance of uncapping actin filament barbed ends for strengthening intercellular adhesions.

Rho GTPases Regulate PIP5KIγ Recruitment and $PI(4,5)P_2$ Synthesis

Cadherin-mediated intercellular adhesions are important sites of rho GTPase signaling, and both rhoA and rac1 are activated subsequent to N-cadherin ligation (Noren *et al.*, 2001; Charrasse *et al.*, 2002; Sayegh *et al.*, 2005). Further, rhoA and rac1 can bind to all isoforms of PIP5KIs and can stimulate the synthesis of $PI(4,5)P_2$ (Chong *et al.*, 1994; Weernink *et al.*, 2004). We showed that activation of RhoA mediates, in part, the recruitment of PIP5KIγ to sites of N-cadherin–mediated adhesions, whereas activation of Rac1 facilitates localized $PI(4,5)P_2$ production. These results suggest independent functions of the rho GTPases at sites of N-cadherin ligation and are in agreement with recent findings that Rac1 and RhoA regulate PIP5KI by independent pathways (Weernink *et al.*, 2004). RhoA can bind constitutively to the PIP5KIs and thus may facilitate transport to distinct cellular locations, sites where RhoA activation enhances binding of effectors such as Rho kinase, which uncouples RhoA-PIP5KI interactions (Weernink *et al.*, 2004). Indeed, direct substrates of Rho kinase signaling have been detected at sites of E-cadherin adhesions (Shewan *et al.*, 2005), supporting the importance of RhoA activation at sites of cadherin adhesion and its role in localization of PIP5KIγ. We found that expression of a GDP-bound Rac construct reduced $PI(4,5)P_2$ production, whereas activated forms of Rac1, and to a lesser extent RhoA, resulted in significant increases of $PI(4,5)P_2$ concentrations, in agreement with earlier observations (Hartwig *et al.*, 1995; Chatah and Abrams, 2001; Weernink *et al.*, 2004). We recognize that the increase in $PI(4,5)P_2$ associated with RhoA activation may simply be a result of the increased concentrations of PIP5KIγ available locally for activation or due to a direct effect of RhoA on PIP5KIγ activation.

$PI(4,5)P_2$ Regulation of N-Cadherin Adhesions

Phosphoinositides, specifically $PI(3,4,5)P_3$ and associated PI-3 kinase activity, have been detected at cadherin adhesions where they may be important in cadherin signaling and in interactions with rac (Watton and Downward, 1999; Kovacs *et al.*, 2002a; Tran *et al.*, 2002). Although $PI(3,4,5)P_3$ has been implicated in some cellular processes that are dependent on actin polymerization (Wang *et al.*, 2002), $PI(4,5)P_2$may have a predominant role in the regulation of actin-binding proteins (Yin and Janmey, 2003). Analysis of cells by videomicroscopy demonstrated that $PI(4,5)P_2$ was generated at sites of N-cadherin ligation and that its synthesis never preceded contact formation. Rather $PI(4,5)P_2$ was only formed after intercellular contact and its enrichment at these zones was lost subsequent to contact dissolution. Although $PI(4,5)P_2$ has diverse functions in the cell and binds to several actin-binding proteins (Sechi and Wehland, 2000), our data demonstrating significantly decreased levels of de novo actin incorporation after incubation with a competitive peptide derived from the gelsolin $PI(4,5)P_2$ binding domain strongly suggests a role for $PI(4,5)P_2$ in regulating actin-binding proteins and actin filament assembly.

Gelsolin is a Ca^{2+}- and $PI(4,5)P_2$-regulated actin-severing and barbed end–capping protein (Kwiatkowski, 1999) that has been previously associated with strengthening of nascent N-cadherin adhesions (Chan *et al.*, 2004). $PI(4,5)P_2$ in-

hibits gelsolin severing and dissociates gelsolin from actin filaments, thereby facilitating barbed-end uncapping (Janmey and Stossel, 1987; Hartwig *et al.*, 1995). Based on the markedly reduced adhesion strength in the gelsolin null cells, it is evident that gelsolin is a particularly important, $PI(4,5)P_2$-regulated actin-binding protein in this process. Although gelsolin's severing activity may also be involved in facilitating actin assembly at nascent contacts, our data from the severing defective mutant gelsolin showed no major impact on adhesion strengthening. Instead, locally generated $PI(4,5)P_2$ may inhibit excessive severing and enhance barbed end filament uncapping to favor growth of actin networks necessary for adhesion zone extension and intercellular contact strengthening.

Although $PI(4,5)P_2$ appears to have an important role in regulating N-cadherin–associated pools of gelsolin, it is likely that the impact of $PI(4,5)P_2$ is not restricted to this actin-binding protein. Indeed, other cadherin-associated, actin-binding proteins such as vinculin, Arp 2/3, and cortactin may be directly or indirectly regulated by PIP5KIγ-mediated $PI(4,5)P_2$ synthesis (Gilmore and Burridge, 1996; He *et al.*, 1998; Rohatgi *et al.*, 1999). Notably, $PI(4,5)P_2$ has an important role in coupling actin assembly to plasma membrane extension (Raucher *et al.*, 2000) and may enhance the formation of intercellular projections and contact zone extension (Vasioukhin *et al.*, 2000). We conclude that at N-cadherin contacts, Rac1 activation stimulates production of $PI(4,5)P_2$ in a PIP5KIγ-dependent manner; the activation of RhoA appears necessary for the recruitment of PIP5KIγ. In response to locally generated $PI(4,5)P_2$, gelsolin and possibly other actin-binding proteins mediate actin assembly, a process critical for regulating N-cadherin adhesion strength.

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