Molecular Mechanism for Orienting Membrane and Actin Dynamics to Nascent Cell-Cell Contacts in Epithelial Cells

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Summary

The small GTPase Rac1 has been implicated in regulation of cell migration and cell-cell adhesion in epithelial cells. Little is known, however, about the spatial and temporal coordination of Rac1 activity required to balance these competing processes. We fractionated endogenous Rac1-containing protein complexes from membranes of MDCK cells and identified 3 major complexes comprising a Rac1-PAK (p21-activated kinase) complex, and 11S and 16S Rac1 complexes. Significantly, Rac1 shifts from the 11S to a 16S particle during initiation of cell-cell adhesion. This shift may reflect a diffusion trapping mechanism by which these Rac1 complexes are localized to cadherin-mediated cell-cell contacts through an interaction with annexin II.
Introduction

The actin cytoskeleton is highly regulated and organized. Though recent work has provided great insight into how actin dynamics are regulated in cells, mechanisms linking spatial organization with specific functions remains poorly understood. Migratory cells assemble actin-rich lamellae and filopodial at the leading edge, a distinct subdomain of the plasma membrane, ensuring directional movement. In fibroblasts, the small GTPases Rac1 and Cdc42 regulate formation of lamellae and filopodia, respectively (1). In epithelial cells, membrane dynamics observed at developing cell-cell contacts are reminiscent of those observed at the leading edge (2-4). Levels of active Cdc42 and Rac1 increase following initiation of cadherin-mediated adhesion (5-7). Rac1 accumulation within cell-cell contacts is juxtaposed with high levels of PI-3 kinase products and greatest lamellae activity, and occurs concomitantly with depletion of Rac1 from non-contacting membranes (2). However, molecular mechanisms that specify Rac1 localization and retention at cell-cell contacts are not understood.

To investigate how Rho family members become localized to cell-cell contacts, we isolated protein complexes containing endogenous Rac1 from membranes of MDCK cells undergoing cell-cell contact formation. We demonstrate that Rac1 shifts from an 11S to a 16S particle in response to cadherin-based adhesion. This shift requires PI-3 kinase activity and occurs concomitantly with recruitment and retention of Rac1 at nascent cell contacts. We also show that annexin II interacts with Rac1 complexes and that its localization to cell-cell contacts also requires PI-3 kinase, implicating annexin II as the determinant of specific Rac1 localization.

Annexins are peripheral membrane proteins that bind anionic phospholipids, such as PI-3 kinase products, in a Ca\(^{2+}\)-dependent manner (8). Annexin II has been
identified in preparations of lamellae (9), implicated in regulation of migration (10), and observed in actin comet tails of rocketing macropinocytic vesicles where it plays a critical, but unidentified role (11). Annexin II also binds CD44/H-CAM, resulting in lamellipodia formation in a Rac1-dependent manner (12).

Experimental Procedures

Cell Culture
MDCK cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM) + 10% FBS. To generate contact naïve cultures(13), MDCK cells were passaged at low density (2x10^6 cells/15cm dish) for two 24-hour periods. Cells were then replated at confluent density (10x10^6 cells/7.5cm dish) in Low Calcium Medium (LCM) + 10% dialyzed FBS and cultured for 3 hours. To initiate adhesion synchronously(13), DMEM + 10% FCS was perfused into the culture and the cells incubated for the appropriate time. For suspension culture, MDCK cells were suspended in the appropriate medium and incubated in culture dishes coated with 1% agar. MDCK cells which express Rac1 mutants under control of a tetracycline repressor(14, 15) were maintained in 20ng/ml doxycycline. To induce expression, cells were replated in the absence of doxycycline for 24-48 hours. LE cells (16) were cultured in DMEM + 5% FBS. To induce expression of E-cadherin, cells were cultured in the presence of dexamethasone for 16 hours.

Subcellular Fractionation
MDCK cells were washed with and scraped in ice-cold Ringer’s saline (+/- 1.8mM CaCl₂) and scraped. Cells were recovered by centrifugation at 1500rpm for 12 minutes
at 4°C and resuspended in Buffer A (100mM NaCl, 20mM HEPES pH7.4, 5mM Mg Cl₂, 
0.25M sucrose, 1mM PMSF, and 10μg/ml leupeptin, pepstatin A, and antipain). Cells 
were disrupted by brief sonication and the resulting homogenate pre-cleared by 
centrifugation at 3000rpm for 5 minutes at 4°C. Solubility properties of proteins were 
determined by supplementing homogenates to 1M KCl, 4M Urea, 0.5M Na₂CO₃, 0.5% 
Triton X-100, or 1% octylglucoside and incubating on ice for 30 minutes prior to 
ultracentrifugation.

**Centrifugation**

Homogenates were separated into particulate or soluble fractions by differential 
ultracentrifugation at 100,000g for 45 minutes at 4°C. Membrane-association was 
determined by diluting homogenates or the particulate material from the above to a 
final concentration of 30% iodixanol (OptiPrep). Samples were subjected to 
centrifugation at 67,500rpm for 65 minutes at 4°C in a vTi80 rotor. Gradients were 
fractionated from the top into 12 0.5ml fractions. Size-based separation of membrane 
proteins was accomplished by solubilizing the 100,000g particulate fraction in Buffer A 
supplemented with 1% octylglucoside on ice for 1 hour. Resulting extract was loaded 
onto 4ml 10-40% (w/v) sucrose gradients and subjected to centrifugation at 48,000rpm 
for 9 hours at 4°C in an SW60 rotor. Gradients were fractionated from the top into 20 
200μl fractions.

**Western Blot Analysis**

Proteins were separated by SDS polyacrylamide gel electrophoresis and processed for 
Western blot analysis on PVDF membranes. Antibodies used were: Rac1
Antibody-protein binding was detected using HRP-conjugated secondary antibodies and subsequent ECL.

**Immunofluorescence**

Cells were cultured on collagen-coated glass cover slips. Cells were washed and fixed in 4% paraformaldehyde on ice or methanol at –20°C. Cells were then prepared for fluorescence using the appropriate antibodies followed by FITC- and rhodamine-conjugated secondary antibodies. Actin was visualized with rhodamine-phalloidin. Slides were viewed using a Zeiss microscope. Antibodies used were: 3G8 or E2 (E-cadherin), 9E10 (myc), annexin I and II (Transduction), α-catenin, p120catenin (Transduction), fodrin (spectrin), adducin, paxillin (Transduction), FAK (Transduction), and actin (Santa Cruz).

**Results**

**Rac1 Shifts from an 11S to a 16S Complex During Cell-Cell Contact Formation**

Previous studies of Rac1 complexes have often relied upon affinity binding of solubilized proteins to Rac1 in vitro (17). We sought to define endogenous Rac1 complexes in membranes isolated from whole MDCK cells, and examine whether these Rac1 complexes are dynamically altered during cell-cell contact assembly. Membranes isolated from a mixed population of migratory and cell-cell adherent MDCK cells were extracted in octylglucoside-containing buffer to solubilize membrane-bound Rac1; note
that <5% of endogenous Rac1 is tightly membrane-bound, but can be quantitatively removed by extraction with detergents (18). Solubilized proteins were separated by size in sucrose density gradients. The Rac1 profile, as determined by Western blotting, reveals three separate peaks of Rac1 distribution (Figure 1A). First (fractions 3-5) is the Rac1-PAK effector complex reported previously (18), the second (fractions 8 and 9) sediments as an 11S particle, and the third (fractions 11 to 15) sediments as a 16S particle.

We next determined if the relative amounts of Rac1 in these complexes changed during establishment of cell-cell adhesion by isolating membranes from homogeneous populations of MDCK cells. In gradients of membrane extracts from “contact naïve” MDCK cells, Rac1 sedimented in three peaks: 34% of membrane-bound Rac1 occurs in a complex with PAK, 51% in the 11S complex, and 15% in the 16S complex, as determined by densitometric scans of Western blots (Figure 1B, C). Levels of the 11S complex are selectively increased in non-contacting, migratory MDCK cells.

Cell-cell adhesion was induced synchronously in confluent cultures of contact naïve MDCK cells by a calcium switch and allowed to proceed for 2.5 hours. Following sucrose density sedimentation of membrane extracts prepared from these cells, the Rac1-PAK complex accounts for 41% of membrane-bound Rac1, the 11S complex accounts for 5%, and the 16S complex accounts for 54% (Figure 1B, C). Thus, during formation of cell-cell contacts the 11S complex is replaced with the 16S complex. To examine this transition in more detail, the amount of Rac1 in the 11S and 16S complexes was determined at thirty minute intervals following initiation of cell-cell adhesion, revealing that the disappearance of the 11S complex and the appearance of the 16S complex occur concomitantly (Figure 1C). Cells were also fractionated into cytosol and membranes at thirty minute intervals following the initiation of cell-cell adhesion, and
the percentage of membrane-associated Rac1 determined (Figure 1D). Results show that there was a slight increase in the amount of Rac1 on membranes during initiation of cell-cell adhesion, but the overall levels to do not change significantly during the period in which the transition of Rac1 from the 11S to the 16S particle occurs.

**Cadherin-Based Adhesion Is Sufficient to Trigger Assembly of the 16S Rac1 Complex**

In order to distinguish a specific role for cadherin in the transition of Rac1 from the 11S complex to 16S complex, we analyzed Rac1 complex formation in LE fibroblasts. LE cells express E-cadherin under control of a dexamethasone (dex) inducible promoter and express ~100-fold higher levels of E-cadherin in the presence of dex than in its absence (16). LE cells do not assemble tight contacts or desmosomes, and cell-cell adhesion is, therefore, mediated only by ectopic E-cadherin (16). LE cells were cultured overnight in the presence or absence of 1nM dex. Membrane extracts were generated, separated in sucrose density gradients, and the Rac1 sedimentation profile determined by Western analysis (Figure 1E). Following dex-induced E-cadherin expression, Rac1-PAK and 16S Rac1 complexes are detected. Some 11S complex is detected, though at much lower levels than those in cells lacking cadherin expression. This experiment confirms the result in MDCK cells: initiation of cadherin-based cell-cell adhesion induces a transition of Rac1 from the 11S to the 16S complex without affecting assembly of the Rac1-PAK complex.
Rac1 Mutant Expression Alters Incorporation of Endogenous Rac1 into 11S and 16S Complexes

Rac1 mutants are thought to exert biological activity by remaining in a specific nucleotide binding state (19, 20). Dominant negative forms of Rac1 (T17N) preferentially bind GDP and sequester machinery required to activate endogenous Rac1. Constitutively active Rac1 mutants (G12V) have reduced GTPase activity and remain GTP bound for long periods, thereby generating a persistent signal through downstream effector proteins. We examined the effects of expression of Rac1 mutants on incorporation of endogenous Rac1 into protein complexes using Rac1 mutants whose expression is under the control of a tet-repressor in well-characterized MDCK cell lines (14, 15).

Cells expressing either dominant-negative (T17N) or constitutively active (G12V) Rac1 mutants were used to generate membrane extracts for fractionation by differential or isopycnic centrifugation. Rac1T17N and Rac1G12V are expressed at ~600% and 100% of the level of endogenous Rac1, respectively, in non-confluent cultures of cells (14). However, in confluent monolayers of MDCK cells, expression levels of these tet-regulated proteins is reduced to levels similar to, or slightly greater than, that of endogenous Rac1. Unlike endogenous Rac1, which is >95% cytosolic at steady state (17), T17N and G12V Rac1 mutants quantitatively partition into the particulate fraction (Figure 2A); note that Rac1 mutants are tagged with a myc epitope, resulting in their slower migration in SDS-PAGE compared to endogenous Rac1. As reported previously for Rac1T17N (17), Rac1G12V also floated with membranes upon isopycnic gradient centrifugation, consistent with its membrane localization (Figure 2B).

Since mutant forms of Rac1 are associated with membranes, we anticipated that they might compete with endogenous Rac1 for membrane binding sites. Membrane
extracts prepared from Rac1T17N expressing cells were separated by rate-zonal centrifugation, and profiles of endogenous Rac1 and ectopic Rac1T17N distribution were examined by Western blotting with a Rac1 antibody (Figure 2C). Endogenous Rac1 appeared as a single peak corresponding to the Rac1-PAK complex, but was absent from the 11S and 16S complexes. Rac1T17N sedimented as a single broad peak that had a distribution similar to that of endogenous Rac1 in the 11S complex of control cells. Expression and localization of Rac1T17N to membranes displaced endogenous Rac1 from the 11S and 16S complexes, but not from the Rac1-PAK complex, even though Rac1T17N is expressed at a level similar to that of endogenous Rac1. We note, however, that our previous study showed that the Rac1-PAK complex is assembled in the cytosol (18). Since all of the Rac1T17N is present on the membrane, we speculate that membrane bound Rac1T17N, despite being expressed at a relatively high level, is unable to compete with endogenous Rac1 for binding to PAK in the cytosol.

An identical analysis of fractionated membrane extracts from Rac1G12V expressing MDCK cells on sucrose density gradients revealed that endogenous Rac1 was detected in the Rac1:PAK protein complex, as well as in the 11S and 16S complexes (Figure 2D). Rac1G12V (slower migrating protein) co-sedimented with endogenous Rac1 in two peaks that correspond to the 11S and 16S complexes.

**Rac1G12V, but not Rac1T17N, is Rapidly Recruited to Cell-Cell Contacts**

To determine if Rac1 mutants both redistribute to cell-cell contacts, we synchronously induced cell-cell adhesion in MDCK cells expressing either Rac1G12V or Rac1T17N and processed them for immunofluorescence microscopy (Figure 2E). After 2 hours of adhesion Rac1G12V localized strongly to cell-cell contacts while Rac1T17N
localized diffusely to the entire membrane surface. Similarly, Ehrlich et al. (2) observe rapid expansion and strengthening of cell-cell contacts in cells expressing constitutively active Rac1 (Q61L, G12V), accompanied by the rapid accumulation of Rac1Q61L-GFP at cell-cell contacts. In contrast, slow expansion and strengthening of cell-cell contacts are observe in cells expressing dominant negative Rac1 (T17N), and little accumulation of Rac1T17N-GFP at cell-cell contacts occurred (2).

**PI-3 Kinase Inhibition Blocks the Cadherin-Induced Change in Rac1 Complexes**

PI 3-kinase inhibitors have been shown to block Rac1 activation in response to cell-cell adhesion (6), and PI-3 kinase products localize strongly to cell-cell contacts (2, 21). We therefore examined the effect of PI 3-kinase inhibition on Rac1 complexes using the specific kinase inhibitor LY294002 (LY; 22). Although LY blocks PI 3-kinase activity at cell-cell contacts within minutes (2), we allowed additional time for turnover of residual Rac1 membrane complexes during treatment. Extracts prepared from cultures of adherent MDCK cells incubated for two hours with LY (Figure 3A), during which time LY remains active (2), were separated in sucrose gradients. In control cells, Rac1 occurred in peaks corresponding to the Rac1-PAK and 16S complexes, as expected. In the presence of LY, Rac1 occurred in the Rac1-PAK complex, and a peak corresponding to the 11S complex, but no longer appeared in the 16S complex. Thus, LY causes an accumulation of Rac1 in the 11S complex and blocks transition of Rac1 into the 16S complex.

We next examined whether LY treatment affected cell-cell contact structure. Immunostaining with antibodies to cell-cell contact components (α-catenin, adducin, cadherin, p120catenin, and fodrin) revealed little or no detectable difference in cell-cell contact organization between control and LY-treated MDCK cells (Figure 3B). Though
unexpected, this result is corroborated by the observation of Ehrlich et al. (2) that LY treatment does not disrupt expansion or strengthening of cell-cell contacts, despite the immediate reduction in PI-3 kinase activity at cell-cell contacts. Rhodamine-phalloidin staining also revealed little differences in actin organization at cell-cell contacts between control and LY-treated MDCK cells (Figure 3C). More striking changes in actin arrangement occurred at non-contacting regions of the cell periphery. In LY-treated cells, the peripheral actin cytoskeleton appears less bundled and is arranged into protrusive, migratory structures that radiate from the cell body. In control cells, the peripheral actin cytoskeleton is bundled into a single thick cable and few radial protrusions are observed. Radially oriented fibers of LY-treated cells terminate in focal complexes, as determined by immunostaining with FAK and paxillin antibodies (Figure 3D). We conclude that PI-3 kinase activity is not required for cell-cell contact assembly, but rather to orient membrane activity and actin dynamics to the contact site.

PI-3 Kinase Activity is Required for Rapid Accumulation of Rac1 Sites of Cell-Cell Contact

Since PI-3 kinase inhibition prevents formation of the 16S Rac1 complex, but does not affect cell-cell contact structure, we propose that PI-3 kinase functions in cell-cell adhesion to recruit specific proteins to nascent cell-cell contacts, reducing their availability at other sites such as non-contacting membranes. We examined the effect of PI-3 kinase inhibitors (LY) on Rac1G12V recruitment to cell-cell contacts (Figure 3E). MDCK cells were synchronously induced to form cell-cell contacts in the presence or absence of LY. In control cells, levels of ectopic Rac1G12V at sites of cell-cell contact formation (barbed arrowheads) are far greater than those at non-contacting surfaces.
(arrowheads). In LY-treated cells, Rac1G12V levels at cell-cell contacts are similar to those at non-contacting membranes.

**Annexin II Interacts with Rac1 Complexes**

Preventing the shift of Rac1 from the 11S to 16S complex using either a dominant negative Rac1 mutant or inhibitors of PI-3 kinase results in decreased accumulation of endogenous Rac1 at cell-cell contacts and lack of orientation of actin-rich membrane protrusions to cell-cell contacts. We therefore sought to identify proteins that mediate Rac1 accumulation at cell-cell contact sites. Protein distributions on sucrose gradients were compared to that of Rac1. A candidate binding protein was determined to be either annexin I or II by mass spectrometry. After sucrose gradient sedimentation of membrane extracts from non-contacting MDCK cells, annexin I occurs as a single peak in fraction 9, whereas annexin II occurs in a broader peak in fractions 8-12 (Figure 4A, C). After sucrose gradient sedimentation of membrane extracts from adherent MDCK cells, annexin I occurs in two peaks: the first peak occurs in fractions 6-8 and the second occurs in fractions 9-12. Annexin II occurs as a broad peak in fractions 13-17 (Figure 4B, C).

As annexins have been implicated in Rac1-dependent processes and bind anionic phospholipids, we sought to examine whether annexins bind Rac1 complexes. Rac1 and Rac1G12V cosediment with annexin I but we could not detect co-immunoprecipitation of Rac1G12V with annexin I. Despite poor co-sedimentation with Rac1, we found that annexin II co-precipitated with Rac1G12V from MDCK cell membrane extracts (Figure 4D). We note, however, that Rac1 complexes occur in low abundance whereas annexin II is a highly abundant protein, and hence a small peak of a Rac1-annexin II complex
may be masked by broader annexin II peak(s) in sucrose gradients. The efficiency of the
annexin II immunoprecipitation is <20%, accounting for the low yield of Rac1G12V in
the precipitates. In addition, annexin II antibodies co-precipitate annexin I (Figure 4D).
Annexins form tetramers consisting of two annexin polypeptides and two p11 subunits
(23). It is therefore possible that annexin complexes could consist of two different
annexin subunits, such as annexins I and II.

In order to test whether annexin II required PI-3 kinase activity to localize to cell-
cell contacts, control and LY-treated MDCK cells were fixed and stained with annexin
antibodies (Figure 4F). In control cells, annexin I and II localize strongly to cell-cell
contacts (Figure 4E). While the localization of annexin I to cell-cell contacts is unaffected
by LY treatment, that of annexin II only occurs between cells that are in the centers of
large colonies. In cells at the edge of colonies annexin II is no longer localized to cell-cell
contacts. These data indicate that PI-3 kinase activity is not required to maintain the
localization of annexin II to stable, well-established cell-cell contacts, such as those
found in the interior of colonies, but is required to initially localize annexin II to nascent
cell-cell contacts, such as those found at the periphery of colonies of MDCK cells.

Discussion

In this report, we have identified protein complexes containing endogenous Rac1
associated tightly with membranes from MDCK cells. We demonstrate that Rac1 shifts
from an 11S particle to a 16S particle at the membrane of epithelial cells upon initiation
of cadherin-based cell-cell adhesion. This shift likely results from interactions between
the 11S Rac1 complex and additional proteins in response to cell-cell adhesion, giving
rise to the 16S complex. Such a precursor (11S)–product (16S) relationship is supported by the effect of dominant-negative Rac1 in displacing endogenous Rac1 from the 16S complex, and the accumulation of the 11S Rac1 complex when assembly of the 16S Rac1 complex is blocked with inhibitors of PI-3 kinase. The shift of Rac1 from 11S to 16S complexes may reflect one of several events following initiation of cell-cell adhesion. Rac1, inactive in the 11S complex, may become activated following cadherin engagement and then bind additional proteins. Importantly, PI-3 kinase inhibitors block 16S complex assembly and cause 11S complex accumulation, and have been proposed to block Rac1 activation in response to cell-cell adhesion (6). We attempted to affinity precipitate active Rac1 from these fractions using GST-GBD pull-down assays, but without success (MDHH, unpublished results). Although it could be inferred that Rac1 is inactive in these fractions, we note that this assay does not precipitate Rac1 from the Rac1-PAK complex, which remains stable in the sucrose gradient (18). Therefore, it remains a possibility that Rac1 is active in these sucrose gradient fractions, and is not available to bind exogenous GST-GBD because of a stable interaction with effectors molecules.

Another possibility is that Rac1 is active in both the 11S and 16S complexes. This idea is supported by our observation of Rac1GFP at tips of lamellae in both non-contacting and contacting MDCK cells (2), despite differences in the complement of Rac1 complexes in these cells. If Rac1 is indeed active in both complexes, additional protein-protein interactions could serve two purposes. First, protein-protein interactions could modulate Rac1 activity to generate distinct biological effects. That Rac1GFP is observed at the tips of lamellae both prior to and following the initiation of cell-cell adhesion (2) suggests that any altered output is subtle. Note also that the small increase in the amount of Rac1 on the membrane of cells that accompanies cell-cell
adhesion occurs over a much longer time period than the transition of Rac1 from the 11S to the 16S complex. This indicates that changes in membrane activity associated with Rac1 was a consequence of reorganization of Rac1 from one membrane site (non-contacting membranes) to another (contacting membranes), rather than recruitment of additional Rac1 to a specific membrane site. Second, protein-protein interactions could serve to act as a diffusion trap, localizing Rac1 to specific membrane sites. In support of such a model, Rac1G12V (in 11S and 16S complex) does rapidly accumulate at cell-cell contacts, while Rac1T17N (in an 11S complex) does not. A diffusion trap model with active Rac1 in 11S complexes would explain how LY-treated MDCK cells are still able to assemble normal cell-cell contacts. The randomly localized 11S Rac1 complexes provide sufficient activity to expand and strengthen nascent cell-cell contacts. In cells expressing Rac1T17N active Rac1 is displaced from the 11S and 16S complex, resulting in inhibited expansion and strengthening of cell-cell contacts (2).

In a diffusion trap model, PI-3 kinase activity may provide spatial information to mark sites of Rac1 accumulation. In support of this, we observe that PI-3 kinase inhibitors reduce accumulation of Rac1G12V at cell-cell contacts, indicating that PI-3 kinase activity is important to localize Rac1. The PI-3 kinase-dependent increase in GTP-bound Rac1 levels in response to cell-cell adhesion observed by Nakagawaa et al. (6) could be explained by increased stability of Rac1 at cell-cell contacts. Interestingly, Ehrlich et al. (2) noted increased perdurance of lamellae at contacting relative to non-contacting membranes, indicating that Rac1 may remain active for longer periods when localized at cell-cell contacts.

Identification of annexin II as a binding partner of Rac1 complexes gives novel insight into a mechanism of PI-3 kinase-mediated Rac1 localization. An interaction between annexins and anionic phospholipids, such as products of PI-3 kinase, has been
reported (8). We observe that annexin II localizes rapidly to cell-cell contacts, and that this requires PI-3 kinase activity. Although further work is needed to define the nature of the Rac1-annexin II complex, an interaction between annexin II and Rac1 complexes, as described here, could serve to recruit and retain diffusive Rac1 11S complexes at areas of high PI-3 kinase activity, such as those observed at nascent cell-cell contacts of MDCK cells (2) or at the leading edge of polarized migratory cells (23). Note that the C terminus of annexin II binds directly to actin as a monomer (24), perhaps providing a mechanism to reinforce the localization of such a complex at cell-cell contacts as actin reorganization occurs. We suggest that in the absence of cell-cell adhesion, regulatory machinery such as 11S Rac1 complexes is freely diffusive and actin remodeling is not directionally oriented. Cadherin engagement results in the local activation of PI-3 kinase and the generation of specific anionic phospholipids. Phospholipid binding proteins bind diffusive complexes and trap them within larger lipid microdomains, limiting their diffusion. Such a system would result in the majority of actin remodeling activity accumulating at sites of stimulus and removing from the rest of the plasma membrane. The observation of the phospholipid-binding protein annexin II as a component of Rac1 complexes implicates it as the link between lipid accumulations that mark plasma membrane sites and actin dynamics.
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Figure Legends

Figure 1. Cell-Cell Adhesion Initiates Changes In Rac1 Interactions
A. Sedimentation profile of endogenous Rac1 from membrane extracts derived from a mixed population of MDCK cells. B. Sedimentation profile of endogenous Rac1 from membrane extracts derived from homogeneous populations of "contact-naïve" or 2.5h contacting MDCK cells. C. Amount of membrane-associated Rac1 occurring in the 11 and 16S complexes during the initiation of cell-cell adhesion, as determined by densitometry. D. Amount of Rac1 associated with the membrane following the initiation of cell-cell adhesion. E. Sedimentation profiles of endogenous Rac1 from membrane extracts derived from LE fibroblasts, which express cadherin under a dexamethasone-inducible promoter.

Figure 2. Rac1 Mutants Alter Appearance of the Rac1 16S Complex
A. Separation of endogenous and mutant forms of Rac1 into soluble (S) and particulate (P) fractions by differential centrifugation. B. Sedimentation profile of ectopic Rac1G12V from whole MDCK cell homogenates. C. Sedimentation profile of endogenous and mutant Rac1 from membrane extracts taken from T17N Rac1-expressing MDCK cells. D. Sedimentation profile of endogenous and mutant Rac1 from membrane extracts taken from G12V Rac1-expressing MDCK cells. E. Immunolocalization of Rac1 mutants in MDCK cells following 2 hours of cell-cell contact formation. Scale bar= 40 microns.
Figure 3. Role of PI-3 kinase in Rac1 Complex Assembly and Localization.

A. Sedimentation profile of endogenous Rac1 from membrane extracts derived from contacting MDCK cells treated with the PI-3 kinase inhibitor, LY294002 (LY). B. Immunolocalization of cadherin, fodrin, adducin, and p120catenin in control and LY-treated MDCK cells. C. Rhodamine-phalloidin staining of control and LY-treated MDCK cells. D. Costaining of FAK or paxillin with rhodamine phalloidin in LY-treated MDCK cells. E. Localization of Rac1G12V to cell-cell contacts (barbed arrowheads) and non-contacting membranes (arrowheads) of MDCK cells following 2 hours of cell-cell contact formation in the presence or absence of LY294002. Scale bars= 40 microns.

Figure 4. Interaction of Annexin II with Rac1 Complexes.

A. Sedimentation profiles of proteins in membrane extracts from contact naïve MDCK cells. B. Sedimentation profiles of proteins in membrane extracts from 2.5h contacting MDCK cells. C. Quantification of the sedimentation profiles in A and B. D. Western blot analysis of annexin immunoprecipitates from Rac1G12V-expressing MDCK cells. E. Colocalization of annexin I or II and E-cadherin in 2h contacting MDCK cells. F. Immunolocalization of annexin I and II to cell-cell contacts in LY294002-treated and –untreated MDCK cells. The dotted line denotes the free cell periphery. Scale bars= 40 microns.
A. MDCK Cells

B. Contact
Naive
Contacting

C. % of membrane-bound Rac1

D. Time following adhesion (min)

E. Control
Dex

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

11S Complex
16S Complex

0 10 20 30 40 50 60

0 30 60 90 120 150 180 210

Rac1:PAK

Rac1:PAK

Rac1:PAK

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A. 

Control

LY

Rac1:PAK
11S
16S

B. 

α-catenin
adducin
cadherin
fodrin
p120

Control

LY

C. 

Control

LY

D. 

Actin/Paxillin
Actin/FAK

E. 

Control

LY294002

Rac1G12V

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A. Non-Contacting

Rac1
Annexin I
Annexin II

B. Contacting

Rac1
Annexin I
Annexin II

C. Rac1

% of Membrane-Bound Protein

D. Blot

Annexin I
Annexin II
Rac1G12V

E. Annexin I
Cadherin

F. Annexin I

Control
LY294002

Annexin II

Control
LY294002
Molecular mechanism for orienting membrane and actin dynamics to nascent cell-cell contacts in Epithelial cells
Marc D. H. Hansen, Jason S. Ehrlich and W. James Nelson

J. Biol. Chem. published online September 18, 2002

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