Integrins Regulate Rac Targeting by Internalization of Membrane Domains

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Translocation of the small GTP-binding protein Rac1 to the cell plasma membrane is essential for activating downstream effectors and requires integrin-mediated adhesion of cells to extracellular matrix. We report that active Rac1 binds preferentially to low-density, cholesterol-rich membranes, and specificity is determined at least in part by membrane lipids. Cell detachment triggered internalization of plasma membrane cholesterol and lipid raft markers. Preventing internalization maintained Rac1 membrane targeting and effector activation in nonadherent cells. Regulation of lipid rafts by integrin signals may regulate the location of membrane domains such as lipid rafts and thereby control domain-specific signaling events in anchor-dependent cells.

Integrin-mediated cell adhesion not only initiates signals directly but also modulates transmission of signals downstream of growth factor receptors (1). Among these signals are the Rho family of small GTP-binding proteins that regulate cell polarization and migration, membrane trafficking, cell cycle progression, gene expression, and oncogenic transformation (2). Integrins control the activation of Rho proteins and separately regulate the translocation of activated (GTP-bound) Rac1 and Cdc42 to the plasma membrane (3, 4). Consequently, GTP-Rac1 in nonadherent cells remains in the cytoplasm bound to Rho guanine nucleotide dissociation inhibitor (RhoGDI) and thus is uncoupled from downstream signaling. This regulatory mechanism may account for a variety of effects of integrins in anchorage-dependent cells.

The plasma membrane is thought to contain domains enriched in cholesterol, sphingolipids, and proteins including caveolins, flotillins, src-family kinases, and glycosphingolipid phospholipid (GPI)-linked proteins (5–7). The size and composition of these domains in vivo are still uncertain (8–10), and there is evidence for different types (11). Known collectively as lipid rafts, they represent cholesterol-rich regions of higher order and lower buoyant density than bulk plasma membrane. Sphingolipids, including gangliosides such as G_{Ms}, are proposed structural components of lipid rafts (12). These domains have been proposed to compartmentalize and organize signal transduction at the plasma membrane (5, 6, 13).

GTP-Rac1 binds more effectively to membranes from adherent than from suspended fibroblasts, indicating that integrins regulate Rac1 membrane binding sites at the cell surface (3). RhoA and Rac1 are also thought to be concentrated in lipid rafts and caveolae (14, 15). We therefore investigated the involvement of such membrane domains in Rac1 membrane targeting and elucidated how they are regulated by integrins. Membrane domains such as lipid rafts can be disrupted by depleting membrane cholesterol with methyl-β-cyclohexyldene

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Depletion of cholesterol from adherent 3T3 cells did not alter activation of endogenous Rac1 but prevented its translocation to the membrane (Fig. 1A and B, and fig. S1A), consistent with a recent study on A431 epidermoid carcinoma cells (16). A constitutively active mutant, V12Rac1, behaved similarly (Fig. 1C and D, and fig. S1B), further indicating that changes in Rac1 GTP loading were not involved. In vitro binding of V12Rac1 to isolated membranes was similarly inhibited by cholesterol depletion (Fig. 1E). Treatment of adherent cells with CD reversibly inhibited serum stimulation of the Rac1 effector p21-activated kinase (PAK) (Fig. 1F and fig. S1A), consistent with the requirement for membrane translocation in effector activation (4). Depletion of membrane cholesterol therefore mimics the effect of cell detachment on Rac1 targeting and effector activation (3, 4).

These and other findings (14–16) suggest a role for cholesterol-rich domains in the translocation of Rac1 to membranes. To further investigate Rac1 targeting, we used a recombinant, isoprenylated human Rac1-RhoGDI complex purified from yeast (17) (fig. S2A). Rac1 showed selective and GTP-dependent binding to membranes isolated from adherent 3T3 cells, whereas RhoGDI showed no membrane association (fig. S2B). Plasma membranes from adherent fibroblasts were then fractionated with a detergent-free density gradient centrifugation method (17) (fig. S2C). Rac1 showed greater GTP-dependent binding to the low-density, cholesterol- and caveolin-1–enriched fraction relative to whole plasma membranes and no detectable GTP-dependent binding to the high-density, cholesterol-depleted fraction (Fig. 2A).

When liposomes were prepared from purified lipids, an equimolar mixture of phosphatidylycholine (PC), cholesterol, and sphingomyelin (Sph) that is in a liquid-ordered state that may be similar to that of endogenous lipid rafts (5, 6, 18) supported greater GTP-dependent Rac1 binding than other lipid mixtures (Fig. 2, B and C). Phosphorus assays showed equal recovery of liposomes of all compositions (17). RhoGDI did not bind under any circumstance and remained in the supernatant (19). In contrast, the phospholipid-binding bee venom peptide melittin (17) bound better to liposomes with greater PC content (Fig. 2, B and C). The physical state of the lipids may therefore contribute to the binding of Rac1 to specific membrane domains.

Although lipid rafts are thought to be heterogeneous, ganglioside GM1 is a widely used marker for these domains (12, 20). Therefore, localization of the GM1–binding cholera toxin subunit B (CTxB) was compared with that of Rac1. Following treatment of cells with serum to activate Rac1, Rac1 and CTxB colocalized extensively, primarily at cell edges, whereas little colocalization was observed in unstimulated cells (Fig. 2D). Rac1 and GM1 showed negligible colocalization in nonadherent cells, and staining of adherent cells with wheat germ agglutinin to label the entire cell surface showed no concentration of stain at cell edges (19). Furthermore, Rac1 colocalized with GM1 that was clustered with CTxB-coated latex beads (5 μm). Rac1 was not observed around control beads coated with antibody to transferrin receptor (TIR) (Fig. 2E). Activation of Rac1 by CTxB beads was not detected (fig. S5B), confirming an effect on Rac1 targeting rather than on GTP loading. Thus, Rac1 preferentially associates in vivo with regions of the membrane enriched in GM1.

If low-density, cholesterol-rich domains provide the membrane binding sites for Rac1, then these domains may be the targets for integrin regulation of Rac1 recruitment to the membrane. We therefore assayed the effects of cell adhesion on GM1 localization. 3T3 cells, either attached to glass cover slips or incubated in suspension for various times, were chilled on ice and incubated with fluorescently labeled CTxB [fluorescein isothiocyanate (FITC)]–CTxB to label surface GM1. FITC–CTxB efficiently labeled the cell surface before or immediately after detachment, but surface staining rapidly decreased with time in suspension (Fig. 3A). CTxB staining decreased ~10-fold after detachment, whereas surface staining of CD44 increased, and α5 integrin was unchanged (Fig. 3B). Total GM1 levels were unchanged (19) but showed accumulation inside the cells (fig. S3).

To follow the fate of surface GM1, we first labeled adherent cells with FITC–CTxB and detached them from the substratum. CTxB localized sharply at the cell surface immediately after detachment but was subsequently...
internalized, and within 30 to 60 min accumulated in a central region of the cell (Fig. 3C). Adherent cells showed slower and less complete internalization during this time (fig. S4), consistent with published results (21). Replacing cells on fibronectin (FN) or anti-β1 integrin, but not on anti-CD44, reversed these effects (Fig. 3C). A second lipid raft marker, aerolysin, which labels GPI-anchored proteins, showed a similar shift in localization (Fig. 3D).

To determine whether movement of these lipid raft markers correlated with changes in cholesterol distribution, we stained cells with the fluorescent cholesterol-binding antibiotic filipin (22). As in other cell types (22), adherent 3T3 cells showed perinuclear and vesicular staining, and less intense but positive plasma membrane staining. Cells labeled immediately after detachment also showed both plasma membrane and internal staining (Fig. 3E). Further incubation in suspension decreased surface staining and increased accumulation of cholesterol in a central compartment, similar to the effects on G<sub>M1</sub> and GPI-linked proteins. Replacing cells on FN restored surface cholesterol (Fig. 3, E and F). Caveolin-1 also moved from the cell surface to an internal compartment upon loss of adhesion (19). Therefore, loss of integrin-mediated adhesion induced internalization of components thought to be in lipid rafts. When cold Triton-X detergent extracts of cells were separated on sucrose gradients, G<sub>M1</sub> shifted from the light to the heavy fractions (percent in the light fraction: 50 ± 6% in adherent versus 27 ± 3% in suspended cells), suggesting that membrane domain structure is altered when cells become nonadherent.

The effects of integrins on Rac1 membrane targeting can be exerted locally (4). Therefore, to determine whether integrins can affect membrane domains locally, we incubated cells with beads coated with antibodies to β1 integrin or to CD44 as a control. Binding was specific because beads coated with anti-β1 integrin and anti-CD44 bound 7.77 ± 2.01 and 9.07 ± 1.56 beads per cell, respectively, whereas nonspecific rat immunoglobulin G-coated beads bound 0.25 ± 0.13 beads per cell. Only beads coated with anti-β1 integrin triggered local accumulation of G<sub>M1</sub> and green fluorescent protein (GFP)–V12Rac1 (fig. S5A).

To determine whether integrin-regulated internalization of membrane domains mediates loss of Rac1 membrane targeting and downstream signaling, we blocked internalization of at least a portion of the G<sub>M1</sub>. Adherent cells were treated with CtxB beads before detachment and incubation in suspension for 2 hours. Beads remained bound to the cell surface and were not engulfed (19). Cells were then treated with 10% serum for 10 min to activate Rac1. Trapping G<sub>M1</sub>-containing.

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**Fig. 3.** Cell adhesion regulates lipid raft marker localization. (A) 3T3 cells that were adherent (Att) or suspended (Sus) for the indicated times were chilled and labeled with FITC-CtxB to visualize G<sub>M1</sub> on the cell surface. (B) Cells were surface labeled with FITC-CtxB or with antibodies to CD44 or to α5 integrin immediately or 2 hours after detachment, then analyzed by flow cytometry. Black line, nonspecific staining; red line, suspended for 2 hours; blue line, suspended for 30 s. (C) 3T3 cells were surface labeled with FITC-CtxB, rinsed, then suspended at 37°C for the indicated times. Some cells that had been suspended for 2 hours were replated for 1 hour on cover slips coated with FN, anti–β1 integrin, or anti–mouse CD44. (D) Adherent (Att) 3T3 cells or cells suspended for the indicated times were fixed, permeabilized, and stained with aerolysin to detect GPI-anchored proteins. (E) 3T3 cells were adherent, suspended for the indicated times, or suspended for 2 hours then replated on FN. Cells were fixed and stained with filipin to visualize cholesterol. (F) Pixel intensity for filipin was assessed starting at the cell edge and moving toward the cell center (17). Values are means ± SEM (n = 3). Arrows indicate cell surface (red) or intracellular (green) staining.

**Fig. 4.** Internalization of G<sub>M1</sub>-containing membrane domains regulates Rac1 signaling. 3T3 cells were incubated with beads coated with antibodies to CtxB or to TIR and then placed in suspension for 2 hours. Bound beads per cell were scored in an identical experiment: CtxB beads: 3.3 ± 0.2; TIR beads: 3.8 ± 0.4. After stimulation with 10% serum for 10 min, cells were fixed and stained for endogenous Rac1 (A) or assayed for PAK kinase activity (B). Arrows indicate the area of cell-bead contact. Bar, 5 μm (n = 3).
domains at the surface of suspended cells maintained Rac1 localization at the plasma membrane (Fig. 4A) and PKA activation (Fig. 4B). Control TRx-Beads bound to cells but had no effect. CtxB beads had no effect on Rac1 GTP loading (fig. S5B). Additionally, no integrin B1 staining was detected at the bead surface (fig. S5C). These experiments demonstrate that loss of G_{M1}-containing domains from the cell surface in suspended cells is required for the loss of Rac1 targeting and subsequent effector activation.

Rac1 association with the plasma membrane and activation of effectors require membrane binding sites that are controlled by integrins (3, 4). These binding sites are components of cholesterol-rich membrane domains. Integrin-mediated adhesion maintains membrane domains at the plasma membrane. When cells are detached, domains are cleared from the cell surface through internalization. Preventing internalization maintains Rac1 plasma membrane localization and Rac1 signaling in suspended cells. Although selectivity of Rac1 for membrane domains is unexpectedly determined to some extent by the state of the lipids themselves, it is unlikely that lipids alone completely account for this effect. This effect may also provide a means by which adhesion can influence many growth factor pathways that are dependent on integrins (1) to confer anchorage dependence of growth. Local regulation of membrane domains by integrins may explain their ability to locally regulate Rac1 targeting (4) and to recruit many signaling proteins thought to associate with domains (23). Regulation of Rac1 localization by integrins is likely to be important for cell migration and polarity in many systems where precise spatiotemporal control of guanosine triphosphatase function is crucial. Although fibroblasts provide a good model for integrin signaling in anchorage-dependent cells, Rac1 binding sites in specific membrane domains may diverge between epithelial, mesenchymal, and hematopoietic cells, where Rac1 function can also differ (24).

References and Notes
17. Materials and methods are available as supporting material on Science Online.
19. M. A. del Pozo, data not shown.
25. We thank P. Read and R. Nakamoto for the Rac1 and RhoGDI vectors; I. S. Trowbridge for H68 antibody; and P. Liu, M. Zhu, S. Revak, and E. M. Moreno for advice and technical assistance. This work was supported by a Lady Tata Memorial Trust International Award for Research in Leukemia, a Special Fellow Award from the Leukemia and Lymphoma Society of America, and MCYT (Ministerio de Ciencia y Tecnologia) grant SAF2002-02425 to M.A.d.P.; U.S. Public Health Service grant RO1 GM47214 to M.A.S.; and grants from NIH (HL 20948, GM52016) and the Perot Family Foundation to R.C.W.A. This is manuscript no. 15179-V8 from The Scripps Research Institute.

Supporting Online Material
www.sciencemag.org/cgi/content/full/303/5659/839/DC1
Materials and Methods
SOM Text
Figs. S1 to S5
References and Notes
14 October 2003; accepted 23 December 2003

Large Shifts in Pathogen Virulence Relate to Host Population Structure
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Theory on the evolution of virulence generally predicts selection for an optimal level of virulence determined by trade-offs with transmission and/or recovery. Here we consider the evolution of pathogen virulence in hosts who acquire long-lived immunity and live in a spatially structured population. We show theoretically that large shifts in virulence may occur in pathogen populations as a result of a bistability in evolutionary dynamics caused by the local contact or social population structure of the host. This model provides an explanation for the rapid emergence of the highly virulent strains of rabbit hemorrhagic disease virus.

Over the past 30 years, emerging diseases have caused unexpected and, in some localities, significant human mortality (1). This increase in the prevalence of novel diseases has generally been associated with anthropogenic changes of the environment, such as a change in farming practices or urbanization, as well as the zoonotic transfer of pathogens from wildlife to humans (2). However, there is increasing concern that some pathogens may emerge as a consequence of evolutionary changes in virulence. Here we show that rapid evolution of virulence can occur as a consequence of bistability in the evolutionary dynamics of pathogens associated with changes in host social structure. Evidence from molecular epidemiology studies leads us to suppose that this may have occurred in the emergence of the virulent pathogen rabbit hemorrhagic disease virus (RHDV).

General theory on the evolution of virulence (the death rate due to infection) is focused on the maximization of the epidemiological basic reproductive number of the pathogen R_{0} (3); single infections in completely mixed host populations should evolve in a manner that maximizes R_{0}. In turn, this suggests that the evolutionarily stable (ES) transmission rate will be the maximum possible, the recovery rate the lowest possible, and the ES virulence the minimum possible. Virulence can, therefore, be seen as a consequence of trade-offs with transmission and recovery, due to underlying mechanisms associated with factors such as pathogen replication rates (4). Specifically, a finite ES virulence will occur when fitness benefits to the parasite in terms of increased transmission or decreased recovery rates become increasingly costly in terms of increased virulence. Evolution toward an evolutionarily stable strategy (ESS) with higher virulence (determined by a trade-off relationship) would typically be gradual, and we would not expect the rapid emergence of a highly virulent strain.

One important assumption within these classical virulence models is that the host population is free-mixing, whereas in nature hosts typically live in spatially structured