Localized Stabilization of Microtubules by Integrin- and FAK-Facilitated Rho Signaling

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Microtubule (MT) stabilization is regulated by the small guanosine triphosphate (GTP)–binding protein Rho and its effector, mammalian homolog of Diaphanous (mDia), in migrating cells, but factors responsible for localized stabilization at the leading edge are unknown. We report that integrin-mediated activation of focal adhesion kinase (FAK) at the leading edge is required for MT stabilization by the Rho-mDia signaling pathway in mouse fibroblasts. MT stabilization also involved FAK-regulated localization of a lipid raft marker, ganglioside G_{M1}, to the leading edge. The integrin-FAK signaling pathway may facilitate Rho-mDia signaling through G_{M1}, or through a specialized membrane domain containing G_{M1}, to stabilize MTs in the leading edge of migrating cells.

Dynamic MTs are locally stabilized during many morphogenetic events, including cell migration, muscle development, neurite outgrowth, and epithelial polarization (*1*, *2*). Localized stabilization may result from capping of MT plus-ends to prevent subunit exchange (*3*). The tubulin in stabilized MTs becomes posttranslationally modified by detyrosination and other modifications (*1*, *2*). Stable MTs with detyrosinated α-tubulin (Glu tubulin, named for the newly exposed C-terminal glutamate residue) may contribute to polarization by serving as unique tracks for motors, membrane transport, and intermediate filaments (*4*, *5*). The dynamics of MTs allows them to search the cell for stabilization sites (*6*), but the factors localizing MT stabilization are unknown.

In fibroblasts migrating into a wound, stable MTs containing Glu tubulin (Glu MTs) are formed selectively near the leading edge (*7*, *8*). The serum factor lysophosphatidic acid (LPA) stimulates MT stabilization through Rho guanosine triphosphatase (GTPase) and its effector protein mDia in wounded monolayers of serum-starved fibroblasts (*7*, *8*). However, Rho and mDia are not restricted to the leading edge (*8*, *9*), so other factors must be involved in localizing MT stabilization.

Focal adhesions, sites of integrin binding to the extracellular matrix, form in the leading edge during cell migration. Although Glu MT ends are not localized in focal adhesions (*3*, *9*), focal adhesions are sites of signaling (*10*) that could direct the Rho-mDia signaling pathway to stabilize MTs in the leading edge. To test this hypothesis, we examined cells in which focal adhesions are lost upon detachment from the substratum. When 3T3 fibroblasts were detached, Glu tubulin became undetectable within 5 min (Fig. 1A). This resulted from selective loss of Glu MTs because suspended fibroblasts maintained an array of MTs, but no Glu MTs (Fig. 1B) (fig. S1). Taxol treatment, which stabilizes MTs, resulted in the accumulation of Glu MTs in suspended cells (Fig. 1, B and C). Similar results were observed for acetylated tubulin (fig. S2), another modification of stable MTs (*1*, *2*). Thus, suspended cells lack stable MTs that serve as substrates for tubulin-modifying enzymes.

Suspended cells replated on the integrin ligands fibronectin (FN) or vitronectin (*9*), but not on the integrin-independent substratum polylysine (PL), generated Glu tubulin and Glu MTs (Fig. 1, C and D). Similar results were observed for acetylated tubulin (fig. S2). These results correlated with integrin stimulation, indicated by activation (phosphorylation of Tyr^{397}) of FAK (Fig. 1C) and formation of focal adhesions (*9*). Suspended cells treated with FN-coated beads stimulated FAK phosphorylation and generated Glu tubulin (fig. S3), indicating that FN-stimulated formation of stable MTs occurs through integrin signaling rather than cell spreading.

We screened fibroblasts from knockout mice to analyze the pathway of integrin signaling that generates stable MTs. FAK knockout fibroblasts ($FAK^{-/-}$) (*11*) did not generate Glu MTs upon adhesion to FN, whereas paxillin (*12*), Cas (*13*), and Src-Yes-Fyn knockout fibroblasts ($SYF^{-/-}$) (*14*) did (Fig. 2, A and B) (table S1). Formation of Glu MTs on FN was restored in FAK knockout cell lines (DA2 and DP3) that reexpressed FAK (Fig. 2, A and B; also see below). $FAK^{-/-}$ fibroblasts treated with Taxol accumulated tubulin modifications, which indicates that these cells are defective in stabilizing but not modifying MTs (Fig. 2B). In addition, expression of dominant negative constructs of FAK [FAK-related non-kinase (FRNK) (*15*) or membrane-targeted CD2-FAK-Y397F (*9*, *16*)] in 3T3 fibroblasts blocked Glu MT formation on FN (Fig. 2, C and D).

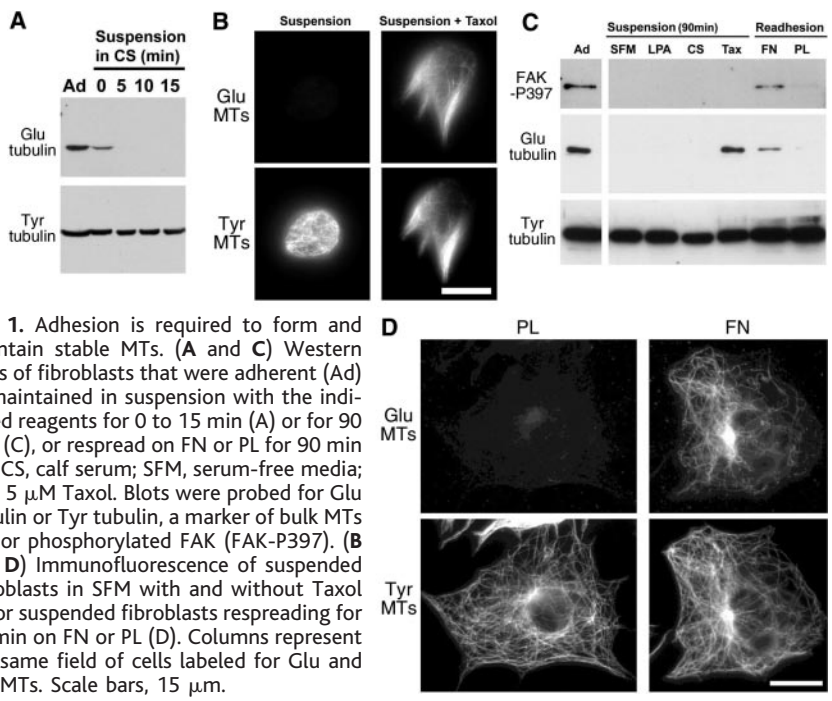

Fig. 1. Adhesion is required to form and maintain stable MTs. (**A** and **C**) Western blots of fibroblasts that were adherent (Ad) or maintained in suspension with the indicated reagents for 0 to 15 min (A) or for 90 min (C), or respread on FN or PL for 90 min (C). CS, calf serum; SFM, serum-free media; Tax, 5 μM Taxol. Blots were probed for Glu tubulin or Tyr tubulin, a marker of bulk MTs (*1*), or phosphorylated FAK (FAK-P397). (**B** and **D**) Immunofluorescence of suspended fibroblasts in SFM with and without Taxol (B) or suspended fibroblasts respreading for 90 min on FN or PL (D). Columns represent the same field of cells labeled for Glu and Tyr MTs. Scale bars, 15 μm.

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Glu tubulin and Glu MTs were undetectable in suspended cells treated with LPA or calf serum (CS) (Fig. 1, B and C), although both trigger formation of Glu MTs in adherent cells (*7*). Because Rho is activated by LPA and CS in suspended 3T3 cells (*17*) and in FAK$^{-/-}$ cells on FN (*18*), these results suggest that active Rho cannot stimulate MT stabilization without FAK activity. Consistent with this idea, constitutively active Rho (V14Rho) weakly activated MT stabilization in cells plated on PL (Fig. 2D) and in cells that also expressed FRNK (Fig. 2, C and D). Cells expressing a Rho inhibitor (C3 toxin) did not form Glu MTs on FN (Fig. 2D). Thus, although active Rho is not sufficient, it is required for integrin-stimulated Glu MT formation.

Active mDia could bypass the integrin-FAK signaling pathway requirement for MT stabilization, as shown by expression or microinjection of active forms of mDia. The levels of Glu MTs in 3T3 cells on PL expressing constitutively active ΔGBDmDia1 (*19*) or the mDia autoinhibitory domain (DAD), which activates endogenous mDia1 (*20*), were as high as those in cells on FN (Fig. 2D). Cells expressing DAD with FRNK or CD2-FAK-Y397F also formed stable MTs (Fig. 2, C and D). DAD did not stimulate formation of focal adhesions on PL; hence, focal adhesions per se are not required for Glu MTs (*9*). Thus, activated mDia1 functions downstream of FAK and RhoA.

Although FAK might phosphorylate RhoA or mDia1, we did not detect tyrosine phosphorylation of either (*9*). This finding suggested that FAK plays another role. Integrins in T cells regulate the distribution of lipid microdomains ("rafts") (*21*), so we examined raft markers in fibroblasts. Localization of the raft marker ganglioside G_{M1} with cholera toxin (*22*) showed that, relative to cells plated on PL, cell surface G_{M1} increased in DP3 and DA2 cells plated on FN, but not in FAK$^{-/-}$ cells plated on FN (Fig. 3A) (*9*). Cell surface G_{M1} localization was normal in SYF$^{-/-}$ and Cas$^{-/-}$ cells (*9*). Upon permeabilization, G_{M1} was detected in a perinuclear compartment in FAK$^{-/-}$ and DP3 cells (Fig. 3A). Other raft markers, such as CD44 (fig. S4) and caveolin 1 (*9*), and cholesterol (fig. S4), which is enriched in lipid rafts, were distributed similarly in FAK$^{-/-}$, DP3, and DA2 cells plated on FN or PL (fig. S4) (*9*). NIH3T3 fibroblasts overexpressing a marker for lipid rafts (green fluorescent protein linked to a glycosyl phosphatidylinositol lipid tail; GFP-GPI) showed similar GFP-GPI localization to the cell surface when plated on either FN or PL (*9*). Lipid rafts have been characterized biochemically by extraction of their components by cold Triton detergent into detergent-resistant membranes (DRMs) (*22*). Although DRMs isolated from both FAK$^{-/-}$ and DA2 cells contained almost all of the cell's caveolin 1, FAK$^{-/-}$ cells had no detectable G_{M1} in DRMs (Fig. 3, B and C). Transferrin receptor, a nonraft membrane protein, and Rho-GTP dissociation inhibitor, a cytoplasmic protein, were recovered in the soluble fraction (Fig. 3C) (*9*). Thus, G_{M1} distribution becomes altered upon adhesion, and this requires FAK.

Such a shift in G_{M1} distribution could be indicative of alterations in membrane domain composition, and this could affect Rho signaling because Rho GTPase must be membrane-associated to stimulate most of its responses (*23*). Consistent with this idea, expression of V14RhoA C190S [active RhoA with a mutant CAAX box to prevent prenylation and membrane association (*23*)] did not effectively induce stable MTs when compared with V14RhoA (Fig. 3D). To test this further, we treated 3T3 cells with methyl-β-cyclodextrin (MβCD) to extract cholesterol, which disrupts the integrity of membrane microdomains (*22*). Cells treated with 5 mM MβCD failed to form Glu MTs on FN, but spread well, formed focal adhesions, and activated FAK (Fig. 3, E and F). This inhibition was reversed by cholesterol addition (*9*).

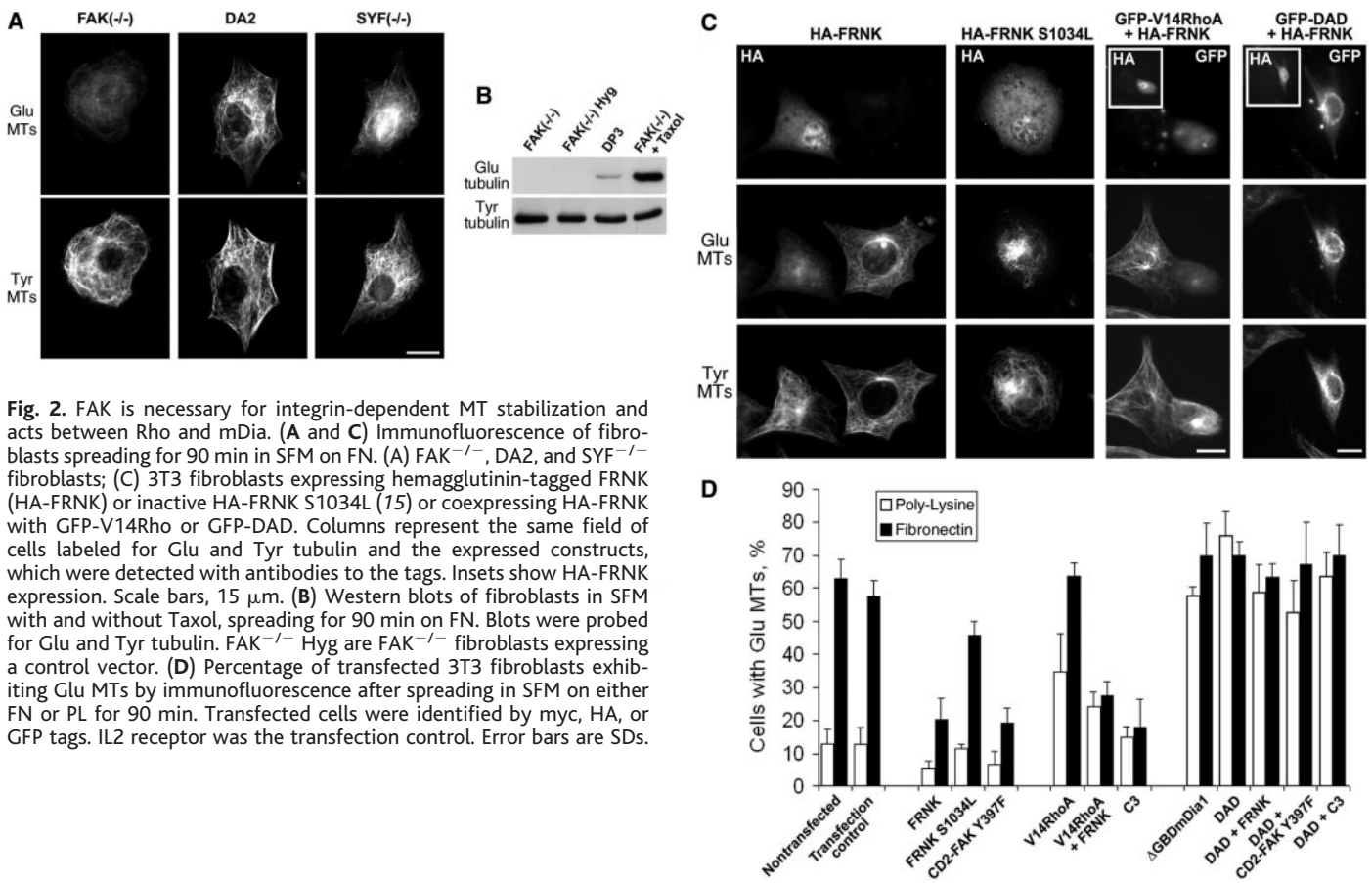

Fig. 2. FAK is necessary for integrin-dependent MT stabilization and acts between Rho and mDia. (**A** and **C**) Immunofluorescence of fibroblasts spreading for 90 min in SFM on FN. (A) FAK$^{-/-}$, DA2, and SYF$^{-/-}$ fibroblasts; (C) 3T3 fibroblasts expressing hemagglutinin-tagged FRNK (HA-FRNK) or inactive HA-FRNK S1034L (*15*) or coexpressing HA-FRNK with GFP-V14Rho or GFP-DAD. Columns represent the same field of cells labeled for Glu and Tyr tubulin and the expressed constructs, which were detected with antibodies to the tags. Insets show HA-FRNK expression. Scale bars, 15 μm. (**B**) Western blots of fibroblasts in SFM with and without Taxol, spreading for 90 min on FN. Blots were probed for Glu and Tyr tubulin. FAK$^{-/-}$ Hyg are FAK$^{-/-}$ fibroblasts expressing a control vector. (**D**) Percentage of transfected 3T3 fibroblasts exhibiting Glu MTs by immunofluorescence after spreading in SFM on either FN or PL for 90 min. Transfected cells were identified by myc, HA, or GFP tags. IL2 receptor was the transfection control. Error bars are SDs.

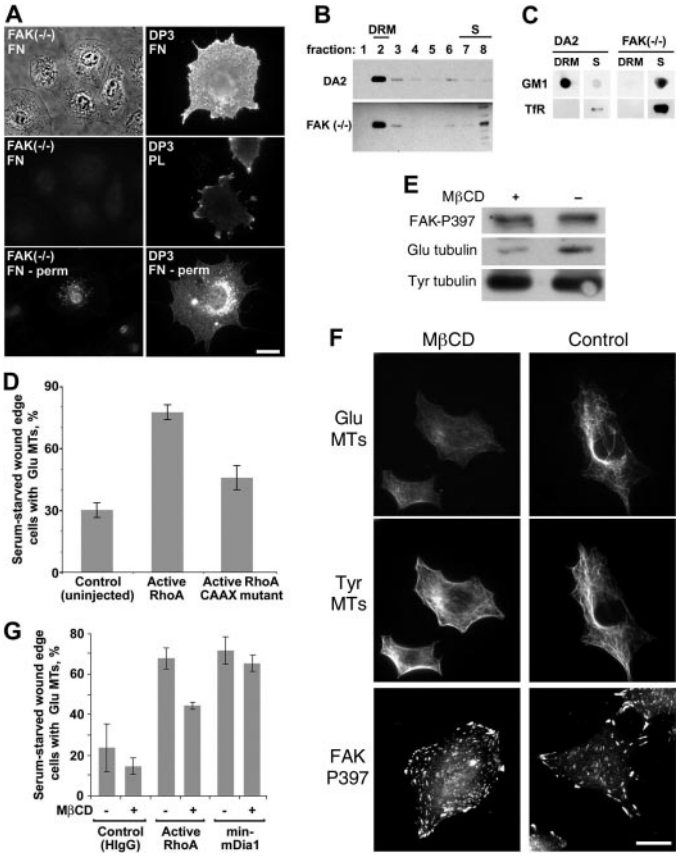

Fig. 3. Cell surface G_{M1} lipid domains are regulated by integrins and FAK and function between FAK and RhomDia in MT stabilization. (**A**) $FAK^{-/-}$ and DP3 cells plated on FN or PL for 30 min, fixed and stained, or permeabilized (Perm) and stained for G_{M1} with cholera toxin. $FAK^{-/-}$ cells had no detectable surface G_{M1} staining (phase image shows cells in the field). Scale bar, 15 μm. (**B** and **C**) Western blots and dot blots of fractions from a sucrose gradient (fraction 1 is top) of DRM and soluble fractions (S) from fibroblasts spreading on FN for 30 min. Samples were blotted for caveolin 1 (B) and for G_{M1} and transferrin receptor (TfR) (C). $FAK^{-/-}$ fibroblasts had higher TfR levels. (**D**) Percentage of serum-starved wound edge 3T3 fibroblasts exhibiting Glu MTs after expression of microinjected DNA encoding GFP-V14RhoA (active RhoA) or V14RhoA C190S (CAAX mutant). Error bars are SDs. (**E** and **F**) Western blots (E) and immunofluorescence (F) of 3T3 fibroblasts in SFM with and without 5 mM MβCD and plated on FN for 90 min. Scale bar, 15 μm. Blots in (E) were probed for FAK-P397, Glu, and Tyr tubulin. In (F), top two panels in each column represent the same field of cells; bottom panels represent different fields. (**G**) Percentage of serum-starved wound edge 3T3 fibroblasts exhibiting Glu MTs after treatment with 5 mM MβCD and microinjection of GST-L63RhoA protein (active RhoA) or His-tagged minimal active mDia1 protein (min-mDia) (*9*). Human IgG (HIgG) was a microinjection control. Error bars are SDs.

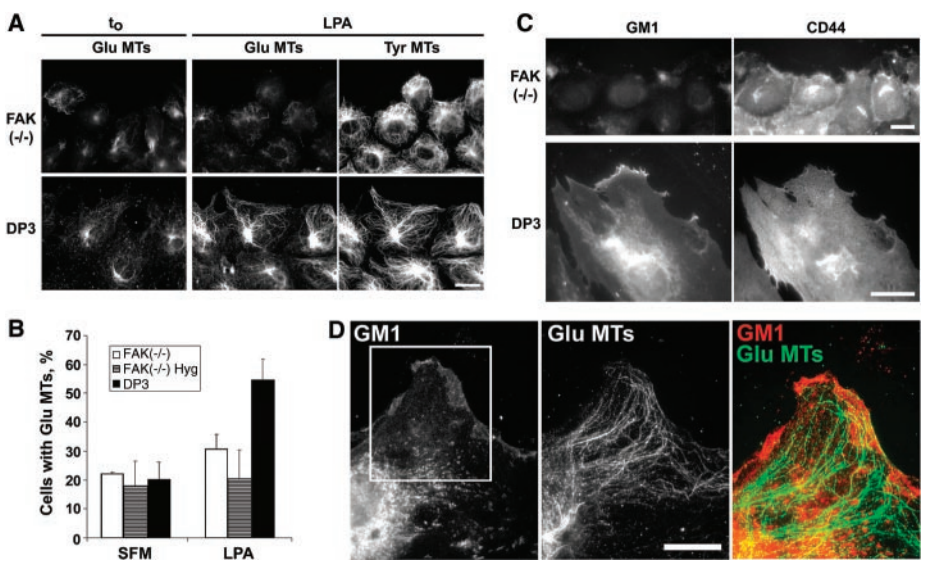

Fig. 4. Glu MTs and G_{M1} are localized near the leading edge in a FAK- and LPA-dependent manner. (**A**) Glu and Tyr MT immunofluorescence of wounded monolayers of serum-starved fibroblasts before (t_o) and after LPA treatment (60 min). (**B**) Percentage of cells in (A) exhibiting Glu MTs after LPA stimulation. (**C** and **D**) Cholera toxin staining of G_{M1} in wounded and LPA-treated (30 min) serum-starved fibroblasts. Cells were costained for CD44 (C) or Glu MTs (D). The rightmost panel in (D) is a color overlay of the boxed region; red and green overlap appears in yellow. Scale bars, 15 μm.

MβCD inhibited active RhoA-stimulated, but not active mDia-stimulated, Glu MT formation in serum-starved 3T3 fibroblasts (Fig. 3G). Thus, cholesterol-dependent membrane microdomains are required for RhoA- and FN-stimulated, but not for mDia-stimulated, formation of Glu MTs.

We examined the distribution of Glu MTs and G_{M1} in cells at the edge of a wounded monolayer. LPA stimulated Glu MT stabilization in the leading edge of DP3 cells, but not in $FAK^{-/-}$ cells (Fig. 4, A and B). In DP3, but not $FAK^{-/-}$, cells, G_{M1} was enriched at the leading edge (Fig. 4C). This localization was specific, as CD44 (Fig. 4C), caveolin 1 (*9*), and a lipophilic dye (fig. S5) were not enriched at this site. In double-stained DP3 cells, Glu MTs were oriented toward the G_{M1} staining in the leading edge (Fig. 4D). Thus, FAK is necessary for polarization of both G_{M1} and Glu MTs in the leading edge. In contrast, another MT reorganization—MT organizing center reorientation, which is regulated by Cdc42 (*24*)—occurred normally in DP3 and $FAK^{-/-}$ cells (*9*).

Our data show that the integrin-FAK signaling pathway enables Rho to activate its effectors. This "adhesion checkpoint" ensures that the output of a Rho-regulated pathway, such as MT stabilization, is coordinated both spatially and temporally with integrin engagement. Integrins have also been implicated in regulating the coupling of the related GTPase Rac to its effector Pak (*25*). FAK appears to regulate G_{M1} localization to the cell surface, perhaps through its effects on endocytic recycling, as suggested by the redistribution of G_{M1} during adhesion and the alteration in transferrin receptor levels in $FAK^{-/-}$ cells (Fig. 3, A and C). G_{M1} may be a component of a specialized membrane domain that harbors regulatory proteins that enable Rho to stimulate mDia, thus acting as an intermediate between the two signaling pathways.

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Supporting Online Material
www.sciencemag.org/cgi/content/full/303/5659/836/DC1
Materials and Methods
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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 anchorage-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 glycosylphosphatidylinositol (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_{M1}, 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-cyclodex-

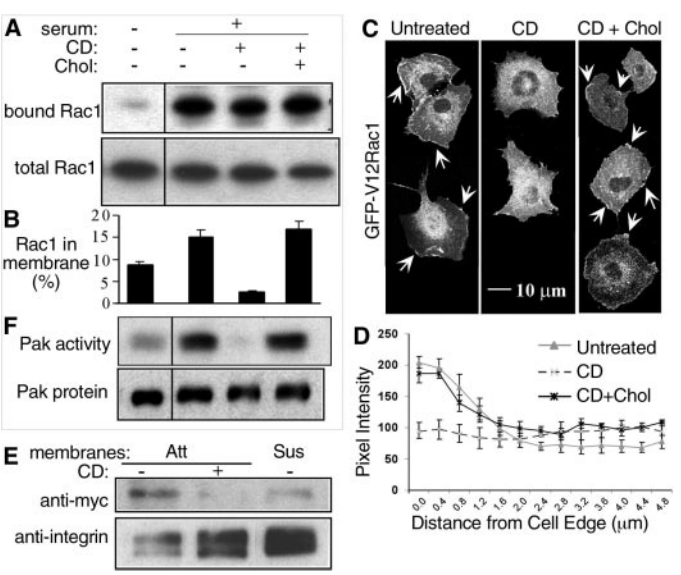

Fig. 1. Cholesterol depletion mimics loss of adhesion. (**A**) Adherent, serum-starved 3T3 cells were incubated with or without 10 mM methyl-β-cyclodextrin (CD) for 1 hour at 37°C. Some CD-treated cells were then incubated with cholesterol (16 μg/ml)–0.15 mM CD [+chol in (A), (B), (C), (D), and (F)] for 1 hour at 37°C to replenish cholesterol. Cells were stimulated with 10% serum for 10 min, and Rac1 activity was assayed ($n = 5$ experiments). (**B**) Cells treated as in (A) were separated into particulate and cytosolic fractions. Rac1 association with membranes was determined by Western blot (*17*). Values are means ± SEM ($n = 3$). (**C**) GFP-V12Rac1–expressing 3T3 cells treated as in (A). Arrows indicate V12Rac1 at the plasma membrane. (**D**) Pixel intensity for GFP-V12Rac1 was assessed starting at the cell edge (*18*). GFP-V12Rac1 is uniformly distributed across CD-treated cells but is concentrated at the edges in control and cholesterol-replenished (+chol) cells. Values are means ± SEM ($n = 3$). (**E**) Isolated plasma membranes from adherent (Att) 3T3 cells were treated with or without CD; plasma membranes from suspended (Sus) cells were analyzed as a control. Binding of cytosolic myc-tagged V12Rac1 was measured by Western blot. β1 integrin blot shows levels of membrane protein for normalization. (**F**) PAK protein was immunoprecipitated and kinase activity assayed in cells treated as in (A) ($n = 3$). PAK protein is indicated by Western blot.

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