Integrin-Matrix Clusters Form Podosome-like Adhesions in the Absence of Traction Forces

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SUMMARY

Matrix-activated integrins can form different adhesion structures. We report that nontransformed fibroblasts develop podosome-like adhesions when spread on fluid Arg-Gly-Asp peptide (RGD)-lipid surfaces, whereas they habitually form focal adhesions on rigid RGD-glass surfaces. Similar to classic macrophage podosomes, the podosome-like adhesions are protrusive and characterized by doughnut-shaped RGD rings that surround characteristic core components including F-actin, N-WASP, and Arp2/Arp3. Furthermore, there are 18 podosome markers in these adhesions, though they lack matrix metalloproteinases that characterize invadopodia and podosomes of Src-transformed cells. When nontransformed cells develop force on integrin-RGD clusters by pulling RGD lipids to pre-fabricated rigid barriers (metal lines spaced by 1–2 μm), these podosomes fail to form and instead form focal adhesions. The formation of podosomes on fluid surfaces is mediated by local activation of phosphoinositide 3-kinase (PI3K) and the production of phosphatidylinositol-(3,4,5)-triphosphate (PIP3) in a FAK/PYK2-dependent manner. Enrichment of PIP3 precedes N-WASP activation and the recruitment of RhoA-GAP ARAP3. We propose that adhesion structures can be modulated by traction force development and that production of PIP3 stimulates podosome formation and subsequent RhoA downregulation in the absence of traction force.

INTRODUCTION

Activation of integrin receptors by extracellular ligand binding mediates the formation of cell-matrix adhesions (Miranti and Brugge, 2002). The clustering of activated integrins and integrin-associated proteins locally promotes the activation of downstream signal transduction paths leading to events such as cell migration (Huttenlocher and Horwitz, 2011), differentiation (Engler et al., 2006), and cancer metastasis (Leventai et al., 2009). The recruitment of actin-binding proteins, such as talin and vinculin, provides structured linkages between integrins and the actin cytoskeleton (Vogel and Sheetz, 2006; Wehrle-Haller, 2012). While the initial clustering of integrin receptors upon binding mobile Arg-Gly-Asp (RGD) moieties is independent of traction forces (Yu et al., 2011, 2012a), contraction-mediated maturation of integrin clusters results in stable adhesion formation (Moore et al., 2010). More importantly, the physical characteristics of extracellular matrix (ECM) can initiate differential assembly of the actomyosin cytoskeletal network (Geiger et al., 2009). For example, fibroblasts on a rigid ECM substrate (100 kPa) are flat, polarized cells with actin stress fibers across the cell body. On softer but chemically identical ECM substrates (10 kPa), fibroblasts fail to polarize and exhibit fewer and less robust actin stress fibers (Prager-Khutorsky et al., 2011). Despite numerous studies, the interplay among actin assembly, force generation, and adhesion structure remains unclear.

Podosomes and focal adhesions are both integrin-mediated multimolecular assemblies for cell adhesion (Calle et al., 2006; Geiger et al., 2001; Machesky et al., 2008). Many adherent cells, such as epithelial cells or stromal fibroblasts cultured in vitro, maintain stable adhesions to the substratum via focal adhesions, adhesion structures interconnected by an actomyosin contractile network (Cai and Sheetz, 2009; Vogel and Sheetz, 2009). On the other hand, monocytic-lineage-derived cells such as macrophages utilize an alternative structure known as a podosome as their primary adhesion machinery (Cox et al., 2012; Murphy and Courtenidge, 2011). Podosomes characteristically contain WASP, cortactin Arp2/Arp3, and actin filaments in the center (podosome core, usually 1 μm in diameter and 2 μm in height), which is surrounded by a ring of integrin and integrin-associated proteins, such as talin, vinculin, and paxillin. Alternatively, transformation of fibroblasts by constitutively active Src kinase will also drive podosome or invadopodia formation with N-WASP substituting for leukocyte-restricted WASP. N-WASP/WASP and Arp2/Arp3 are regarded as markers of podosomes as they are not seen at focal adhesions, but otherwise the two adhesive structures share many molecular components, though
Figure 1. Podosome Formation on RGD Membrane

(A) REF52 fibroblast forms regular focal adhesion on RGD glass but developed podosomes on RGD membrane after 45 min of initial adhesion.

(B) Percentage of REF52 fibroblast cells forming regular adhesion and podosomes on RGD-coated glass or RGD membrane. A total 321 cells in four experiments were used.

(legend continued on next page)
the spatial organization of these components is very different (Gi-
mona et al., 2008). Although a wealth of experimental detail is
now available, the underlying mechanism of podosome assem-
by and whether it is force dependent comparable to the situation
with focal adhesions are largely unknown.

Mobile RGD ligands on nanopatterned supported lipid mem-
branes provide a simple means to study force-mediated signal
transduction events at the cell membrane and have been widely
used in various cell biological investigations, such as studies of
the immunological synapse (Mossman et al., 2005), ephrin-
mediated cancer metastasis (Salaita et al., 2010), and force-
modulated integrin adhesion (Yu et al., 2011, 2012a). Previously,
we have utilized RGD-tagged lipids in supported membranes
(RGD biotin bound to Cascade blue neuravidin bound to biotin
lipid) with or without nanopatterned lines to trigger integrin acti-
vation and to investigate force-dependent and independent functions
during early cell spreading (Yu et al., 2011). Continuous films
of RGD membranes generally exhibited long-range lateral
diffusivity (diffusion coefficient 2 \( \mu m^2/s \)) and were substrates
with infinitesimal elasticity (Evans and Yeung, 1994; Evans and
Hochmuth, 1978) (zero rigidity/shear moduli, equivalent film
viscosity 0.1 N-s/m²). When the long-range mobility of the
RGD membrane was locally restricted by fabricating metal lines
as nanoparticles within the bilayers (typically 100 nm line width
and 5 nm thick with 1–4 \( \mu m \) line pitch, passivated by BSA or
-casein) (Yu and Groves, 2010; Yu et al., 2010), mobile RGD-
integrin clusters assembled stable adhesions across the adja-
cent partitions with 1 and 2 \( \mu m \), but not with 4 \( \mu m \), pitch through
force generation and adhesion maturation. This system was ideal
for testing matrix-dependent mechanical regulation of
adhesion formation.

Various signal transduction pathways can regulate cell-matrix
adhesions. Anionic phospholipids, such as phosphatidylinositol
(4,5)-bisphosphate (PI(2)) and phosphatidylinositol (3,4,5)-
triphosphate (PI(3)) are dynamically regulated in plasma
membranes (McLaughlin et al., 2002; Xu et al., 2003). Local enrich-
ment of these negatively charged lipids can initiate
N-WASP/WASp-mediated actin polymerization at plasma mem-
branes (Papayannopoulos et al., 2005; Pollitt and Insall, 2009).
Class Ia phosphoinositide 3-kinase (PI3K) is composed of two
subunits, p85 (regulatory) and p110 (catalytic), that phosphor-
ylate PIP2 to generate PIP3 (Vanhaesebroeck et al., 2012), raising
local PIP3 concentrations. On the other hand, PTEN dephos-
phorylates PIP3 to PIP2, decreasing PIP3 concentrations. While
the biochemical interaction of p85beta and focal adhesion ki-
nase (FAK) has been reported previously (Chen et al., 1996),
N-WASP and Arp2/3 actin polymerization complexes are
absent at tension-loaded focal adhesions. Here, we report that
spatiotemporal recruitment of PI3K and local enrichment of
PIP3 at integrin-mediated adhesion sites on traction-force free
RGD membranes play an important role in differential signal
transduction leading to podosome formation.

RESULTS

Formation of Podosome-like Adhesion Follows Initial
Integrin Clustering of RGD Lipids

Although THP1 monocytic cells treated with transforming growth
factor \( \beta \) have been used as a model system to study podo-
somes on regular matrix-coated substrates (Monypenny et al.,
2011), fibroblasts generally do not form podosomes on matrix-
coated substrates unless transformed by Src (Oikawa et al.,
2008; Tarone et al., 1985). It was therefore surprising to see
that nontransformed fibroblasts (RPTP\( \alpha ^{++/} \) mouse embryonic
fibroblasts and REF52 rat fibroblasts) formed podosome-like adhesions
when plated on freely diffusive RGD lipids (Figure 1A)
without artificially elevated Src activity. About 70% of REF52 fi-
broblasts developed podosome-like adhesions after 45 min of
initial adhesion (Figure 1B; total of 321 cells in four experiments),
while the same cells seeded on immobilized RGD-coated glass
consistently formed classic focal adhesions (Figure 1A). In paral-
el, we also observed podosome formation in THP1 cells on
RGD-supported bilayers (Figure 1D; Movie S1).

To understand the development of podosome-like adhesions
in fibroblast cells, we examined the process of adhesion forma-
tion on RGD membranes. We found that cells assembled RGD-
integrin clusters during the early adhesion process, as we
observed previously (Yu et al., 2011, 2012a). Similar to the
case of focal complex and focal adhesion formation, the acti-
vated RGD-integrin receptors promptly recruited various integ-
rin-binding proteins such as talin and paxillin and nucleated
micrometer-sized clusters as nascent adhesion structures.
However, RGD-integrin clusters on continuous RGD-lipid bil-
ayers were not interconnected by actin stress fibers and devel-
oped into podosomes after 45 min of initial adhesion (Figure 1C;
Movie S2). The formation of podosome-like adhesions on RGD
membranes was characterized by actin filament assembly in
the podosome core at the center of individual RGD-integrin
clusters (Figure 1D). Integrin-associated proteins, such as talin,
paxillin, and vinculin, were consequently repartitioned into the
ring structure (podosome ring) surrounding the actin core (Fig-
ures 1D and 1F).

Podosome-like Adhesions Have the Same Components
as Classic Podosomes

To better identify the molecular organizations of podosome-like
adhesion on RGD membranes, we rigorously examined more
than 20 different molecular components (Table S1) that have

(C) Transition from initial RGD-integrin clusters to podosomes in REF52 fibroblast adhered on RGD membrane (Movie S2). Inset: podosomes (red arrowheads)
were identified by ring formations of both RGD and YFP paxillin. (D) THP1 monocytes and REF52 fibroblasts both formed podosomes on RGD membrane. The dense actin core is surrounded by adhesion proteins, such as paxillin, vinculin, and RGD-integrin clusters. The center of the podosome ring is depleted from RGD. (E) Interference reflection microscopy reveals tight contacts at the protrusive podosome core in REF52 fibroblast cells. Inset (top to bottom): RICM, CF594 phalloidin (F-actin staining), and YFP paxillin. (F) Development of podosomes (4 \( \times \) 4 \( \mu m \) each frame) in REF52 fibroblast cells. Integrin j3, talin, and RGD clusters are reorganized to form the podosome ring. Arp3 and WIP are enriched at the podosome core.

Error estimates are SEM. The scale bar represents 10 \( \mu m \).

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been identified previously (Linder and Kopp, 2005). We found that podosome-like adhesions shared identical components in classic podosomes in macrophages. Therefore, we refer to podosome-like adhesions on RGD membranes as "podosomes" in the rest of this article. Podosome cores were enriched in F-actin and other characteristic molecular markers, such as Arp2/Arp3, WIP, and N-WASP, that were absent in classic focal adhesions (Figures 1F and S2A). The metalloproteinase, MMP-14 (MT1-MMP), was not enriched at podosomes of nontransformed fibroblasts or THP1 macrophages (Figure S1B). However, Src-transformed fibroblasts exhibited a high level of MMP-14 at invadopodia or long-lasting podosomes (Figure S1B), as previously reported (Wu et al., 2005). In addition, Tks5, a key adaptor protein in invadopodia formation, was not enriched at podosomes in nontransformed fibroblast on RGD membranes (Figure S1C).

**Podosomes Are Dynamic Structures Formed by Arp2/Arp3-Mediated Actin Polymerization**

Intense F-actin polymerization within RGD-integrin clusters as visualized by LifeAct was a signature of podosome formation (Figures 1F and S2A). The metalloproteinase, MMP-14 (MT1-MMP), was not enriched at podosomes of nontransformed fibroblasts or THP1 macrophages (Figure S1B). However, Src-transformed fibroblasts exhibited a high level of MMP-14 at invadopodia or long-lasting podosomes (Figure S1B), as previously reported (Wu et al., 2005). In addition, Tks5, a key adaptor protein in invadopodia formation, was not enriched at podosomes in nontransformed fibroblast on RGD membranes (Figure S1C).

Force Generation in Nanopartitioned RGD Membranes Suppresses Podosome Formation

To test whether or not force on adhesions would affect podosome formation, we spread cells on nanoparticulated RGD bilayers where it was previously shown that cells would generate force on adhesions and stabilize them (Yu et al., 2011). The RGD bilayers were partitioned by nanofabricated metal lines (typically 100nm line width and 5nm in height) that provided passive resistance to adhesion movement. Previously, we demonstrated that the line pitch of partitioning barriers on an RGD membrane was inversely related to cell adhesion area. A smaller line pitch between barriers provided a higher density of barriers to RGD-integrin cluster movement and cells spread over larger areas. Cells formed focal adhesions with stress fibers, but they did not form podosomes when plated on nanoparticulated RGD membranes with a 1 μm line pitch (10% surface density). The immobilized RGD-integrin clusters were linear and were linked by actin fibers (hollow arrows in Figure 2A) after 60 min of initial adhesion. Furthermore, when cells were plated on nanoparticulated RGD-membranes with a 4 μm line pitch that did not support force generation, podosome formation was restored. At the beginning, cells nucleated RGD clusters adjacent to nanopatterned lines with a 4 μm pitch, but there were no actin stress fibers between RGD clusters and cells had a smaller spread area. After 60 min of adhesion, these RGD clusters were also converted to podosomes (white arrowheads in Figure 2A). Interestingly, when RGD membranes were partitioned by dot arrays (300 × 300 nm² metal areas with 1 μm pitch, also 9% of surface density) that provided no spatial confinement of RGD ligands, cells failed to develop force-stabilized RGD-integrin clusters and formed podosomes (Figure 2A). With increasing distance between the membrane partitions, fewer force-stabilized adhesion sites were nucleated, and podosome formation consequently increased (Figure 2C; total of 47 cells in three experiments). More surprisingly, when a single cell adhered to both a continuous and a partitioned RGD membrane, podosomes formed only on the continuous region and did not form at the partitioning lines (Figure 2B; Movie S4). Thus, we suggest that force generation by contraction to the lines produced a local signal that suppressed podosome formation within spatially restricted regions.
Recruitment of p85beta Precedes Podosome Formation and Local Enrichment of PIP3

To determine what factors might be involved in stimulating podosome formation in the absence of force, we looked at Src kinase activity and PIP3 formation. After inhibition of Src by PP2 (10–20 μM, 2 hr), podosomes still formed, but at only 40% of the frequency of control cells (Figure S6A; total of 104 cells in three experiments). In the case of PIP3 formation, we found the localized recruitment of class 1A PI3K regulatory subunit p85beta at prepodosomal RGD clusters preceded actin assembly and appeared to trigger podosome formation (Figures 3A and 3B, arrow). During the initial phase of podosome formation, EYFP-p85beta was initially recruited to a subset of preexisting integrin-RGD clusters (Figure S4A) and then it expanded to the podosome rings (Figure 3B; Movie S5). In parallel, we monitored the time-dependence of PIP3 production by measuring the level of Akt-PH binding and we measured a marked increase in Akt-PH binding during the transition from RGD clusters to podosomes (Figure 3C). In addition, N-WASP was recruited at podosome cores (Figure 3D).

To determine if local enrichment of PIP3 triggered podosome formation, we inhibited PI3K activity by Wortmannin (100 nM). Fibroblasts can still develop initial RGD clusters after PI3K inhibition, but podosome formation on RGD membranes was blocked (Figures 4C and S6C; total of 108 cells in four experiments). While p85beta was also found at regular focal adhesions (Figure S5A), there were no significant changes in local PIP3 levels at the adhesions (Figure S5B), and N-WASP was not recruited at focal adhesions (Figure S5C). Thus, we suggest the recruitment of class

Figure 2. RGD Membrane with Dense Partitioning Barriers Prevents Podosome Formation

(A) Nanopatterned lines (100 nm line width, with 1 to 4 μm line pitch) were prefabricated on glass substrate before RGD-membrane deposition. The cell formed regular adhesion and actin stress fibers (white hollow arrows) on line-partitioned RGD membrane with 1 μm pitch. However, podosome formation (white arrowheads) remained when the cell adheres on RGD membrane with a 4-μm-pitch line partition, as well as 1-μm-pitch dot arrays (each dot area: 300 x 300 nm²).

(B) When a single cell adhered to both a continuous and a partitioned RGD membrane, podosomes (white arrowheads) formed only on the continuous region and did not form between the partitioning lines (Movie S4).

(C) Percentage of REF52 fibroblast cells forming podosomes when adhering on various patterned RGD membranes. Denser line partitions in RGD membranes result in less podosome formation. A total of 47 cells in three experiments were used. Error estimates are SEM. The scale bar represents 10 μm.
Figure 3. Dynamical p85beta Recruitment and Increased PIP3 Level Turn RGD Cluster into Podosomes

(A) Class IA PI3K regulatory subunit p85beta was recruited at podosomes.

(B) Increased recruitment of p85beta at RGD clusters preceded F-actin polymerization. Spatial-temporal recruitment of F-actin and p85beta were analyzed by kymographs and intensity-time plot. p85beta was recruited at a subset of RGD clusters that subsequently turned into podosomes. As dot-like F-actin started to polymerize podosome core, p85beta reorganized from podosome core to podosome ring (Movie S5).

(C) Local enrichment of PIP3 during podosome formation. PIP3 levels were monitored by Akt-PH. The PIP3 level increased during the void formation within the RGD cluster (zone I) as a result of podosome formation on RGD membranes. The PIP3 level remains unaltered in stable RGD clusters (zone II).

(D) N-WASP was recruited at podosome cores. The scale bar represents 5 μm.
IA PI3K caused the rise in PIP3 levels that led to F-actin assembly in the transformation of prepodosomal RGD-integrin clusters to podosomes on traction-force-free RGD membranes.

In terms of the degradation of PIP3 and podosome disassembly, PTEN was found at podosomes, but it only appeared after p85beta. It was often found above the focal plane of RGD-integrin clusters and decreased as F-actin disassembled (Figure S4B). In addition, the level of PIP3 decreased with the disassembly of the F-actin core. This suggests that a sustained level of PIP3 is necessary for maintenance of the F-actin core.

**Tyrosine Autophosphorylation of Both FAK and PYK2 Regulates p85beta Recruitment**

As p85beta is known to bind to substrates with phosphotyrosines via SH2 domains (Songyang et al., 1993), we looked for possible tyrosine kinases that may have been involved. Classical PI3K activation often involved autophosphorylation of receptor tyrosine kinase (RTK), as well as focal adhesion kinase (FAK) (Chen et al., 1996). After testing a number of RTK inhibitors that did not block podosome formation, we tested the dual FAK and Pyk2 kinase inhibitor PF-562271 (Roberts et al., 2008) and found that it efficiently suppressed podosome formation on RGD membranes. We found that FAK was recruited to integrin-RGD clusters (Figure 4A) and Y397 of FAK was phosphorylated and colocalized with EYFP-p85beta at podosomes (Figure 4B). When FAK/Pyk2 autophosphorylation was inhibited by PF-562271 (10 μM, 4 hr pretreated), fibroblasts could still form initial RGD clusters, but p85beta recruitment was suppressed. Only 25% of fibroblasts were able to form podosomes on RGD membranes (Figures 4C and S6D; total of 139 cells in three experiments). Thus, we suggest autophosphorylation of FAK (Y397) and Pyk2 (Y407) recruited p85beta that triggered local enrichment of PIP3 at pre-podosomal RGD clusters.

**RhoA-GTP Levels Are Decreased upon Cell Adhesion to Mobile RGD Membranes**

Since artificially upregulated RhoA-GTP levels and cellular contractility abolished podosome formation (Schramp et al., 2008; van Helden et al., 2008), we decided to determine if reduced RhoA-GTP was also correlated with podosome formation. Inhibiting Rho-associated protein kinase (ROCK) activity (Y-27632, 10 μM) or downregulating myosin-II-mediated contractility (blebbistatin, 50 μM) did not affect podosome formation on RGD membranes (Figures 4C and S6D; total of 139 cells in three experiments, respectively). In contrast, artificially upregulating cellular contractility by the RhoA agonist lysophosphatidic acid (LPA, 40 μM) or expressing a constitutively active RhoA-Q63L mutant effectively inhibited podosome formation (Figure S6B; total of 62 and 87 cells examined in four experiments, respectively). Furthermore, we utilized a fluorescence resonance energy transfer (FRET)-based RhoA biosensor (Pertz et al., 2006) to measure RhoA activity when cells adhered to different substrates (Figure 5B). REF52 cells plated on the mobile RGD membrane had a significantly lower FRET efficiency (0.45 ± 0.01 SEM, n = 30; Figure 5C) than on RGD-coated glass (0.73 ± 0.02 SEM, n = 25; Figure 5C; p value < 0.0001, two-sample t test, two-tailed). Thus, low levels of RhoA-GTP seem to be important for podosome formation.

To further investigate RhoA regulation, we tested two RhoA GTPase-activating proteins (GAPs), DLC1 and ARAP3, which localized to podosomes. While DLC1 was recruited at both focal
Figure 5. Low RhoA-GTP Levels Accompany Podosome Formation

(A) ARAP3, a PIP3-bound RhoA GAP, was recruited at the podosome core. Recruitment of ARAP3 increased as the F-actin podosome core developed and then decreased before F-actin disassembled. Spatial-temporal recruitment of F-actin and ARAP3 were analyzed by kymographs and intensity time plot.

(B) Color-coded heatmap images of RhoA activity in vivo. RhoA activity measured by a FRET-based RhoA biosensor indicated lower RhoA-GTP levels when REF52 fibroblast cells adhered and formed podosomes on RGD membranes. Higher RhoA-GTP levels were measured as cells adhered and formed focal adhesions on RGD-coated glass.

(C) Comparison of RhoA activity via FRET efficiency between cells adhered on RGD membranes (0.45 ± 0.01 SEM, n = 30) and RGD glass (0.73 ± 0.02 SEM, n = 25); p value < 0.0001 (two-sample t test, two tailed). When PI3K was inhibited by Wortmannin, the FRET efficiency of the RhoA biosensor became high (0.85 ± 0.01 SEM, n = 29), even when cells adhered to the mobile RGD membrane.

(D) Differential effects of overexpressing two dominant-negative RhoA GAP mutants, DLC1-R677E and ARAP3-R982A. Podosome formation was suppressed to 25% in the case of ARAP3-R982A but unaltered in the case of DLC1-R677E. Error estimates are SEM. The scale bar represents 5 μm.
adhesion and podosome rings through tensin (Schramp et al., 2008), ARAP3 has been shown to bind to PIP3 at plasma membranes (Krugmann et al., 2002, 2004). Indeed, we found that ARAP3 was recruited at PIP3-enriched podosome cores after F-actin core formation (Figure 5A; Movie S9). However, ARAP3 was not recruited to focal adhesions (Figure S5D). We tested whether DLC1 or ARAP3 played important roles in podosome formation by transiently overexpressing dominant-negative RhoA GAP mutants DLC1-R677E and ARAP3-R982A, respectively. Podosome formation was suppressed to 25% in the case of ARAP3-R982A (Figure 5D), but not significantly disrupted in the case of DLC1-R677E (total of 149 and 114 cells in three experiments, respectively). When PI3K was inhibited, the FRET efficiency of the RhoA biosensor increased significantly (0.85 ± 0.01 SEM, n = 29), even when cells adhered to the mobile RGD membrane (Figure 5G), and podosome formation was suppressed. These observations indicate that the RhoA-GTP level is inversely correlated with podosome formation and that recruitment of ARAP3 and possibly other RhoA-GAPs by local enrichment of PIP3 provided a positive feedback to downregulate cellular RhoA level in podosome-forming cells.

**DISCUSSION**

In these studies, we have demonstrated for that plating cells onto RGD ligands linked to fluid lipid bilayers caused the formation of integrin-based podosome-like adhesions. Surprisingly, such a response was characteristic not only of cells that produce podosomes under normal culture conditions, such as the macrophage line used here, but also of fibroblasts, which do not produce podosomes when plated onto “normal” rigid substrates. Although large traction forces mediated by RhoA are important for focal adhesion maturation in fibroblasts, there is no evidence that they are major determinants in podosome formation. Notably, macrophages do not develop large traction forces on matrix-coated substrates (Féreol et al., 2009) or mature focal adhesions, but they do form podosomes. The implications are that the components needed to form podosomes are present in fibroblasts and immune cells, but the combination of cell contractility and matrix mechanics plays the critical role in determining which type of adhesion is formed.

The podosome-like adhesions that form in the absence of force in nontransformed fibroblasts are indistinguishable from podosomes in macrophages in terms of morphology, components (Table S1), and protrusive dynamics (Figure 1E). However, their physiological functions, such as chemotaxis and endocytosis/exocytosis, need to be further examined. Spatial depletion of mobile RGD ligands and destructive interference at podosomes by IRM indicated that there was active protrusion of the podosome core (Figures 1E and S3B). While ligands on supported membranes were freely diffusive, the observed spatial exclusion of RGD-neuraminidase (5.4 ± 5.8 nm footprint in the x-y dimension; Hendrickson et al., 1989) at the podosome core could not be explained by a simple partitioning effect from ligand with integrin receptor (8 ± 12 nm footprint in the x-y dimension; Nermut et al., 1988). Nevertheless, supported lipid membrane remains evenly distributed at the podosome core (Figure S3A). The vertical force required to physically penetrate a lipid membrane via biomimetic stealth probes (200 nm in diameter) has been reported as 58 nN (Armquist and Melosh, 2010).

We conclude that the protrusive force at podosomes was less than needed to penetrate the membrane but sufficient to block diffusion of RGD neuraminidase into the contact region.

Invadopodia or long-lived stable podosomes (more than 30 min) have been widely investigated in Src-transformed cells (induction of constitutively activated Src kinase) and invasive cancer cell lines (Huveneers et al., 2008; Okawa et al., 2008). However, podosome-like adhesions on RGD membranes and invadopodia in Src-transformed cells are different in both dynamics and molecular components (Table S1). Constitutively activated Src causes hyperactivation of various downstream targets, such as ARHGEF5 RhoA-GEF (Kuroiwa et al., 2011), phosphorylation of Tks5/Grb2 complexes (Okawa et al., 2008), and MMP-14 secretion (Poincloux et al., 2009; Wu et al., 2005; Yu et al., 2012b). Notably, Tks5 (Figure S1C) is not enriched at podosomes in nontransformed fibroblast on RGD membranes. In addition, we have examined the potential recruitment of MMP-14 and found that it was present at invadopodia in invasive cancer cells and Src-transformed fibroblasts. However, in nontransformed fibroblasts, most of the MMP-14 remained in endocytic vesicles and there was only a weak recruitment of MMP-14 around the podosomes (Figure S1B). This is all consistent with the hypothesis that podosomes formed in the absence of force are aided by but do not require Src activity.

Likewise, diaphanous-related formins are required for invadopodia formation in invasive MDA-MB-231 breast adenocarcinoma cells (Lizárraga et al., 2009). However, when we treated nontransformed fibroblasts and THP1-differentiated macrophages with the formin inhibitor SMIFH2, we still observed podosome formation at a similar density to control cells. Podosomes and invadopodia share many molecular components, but most likely not all. We suggest that podosomes on RGD membranes indeed differ from long-lived invadopodia in Src-transformed cells or invasive cancer cells in their lifespan, formin involvement, Tks5 recruitment, and MMP-14 secretion.

Our data suggest that conventional traction force development and myosin-II activities are dispensable in podosome formation. Mobile RGD membranes with nanoparticling systems provide a unique platform to examine the force-regulated adhesion structure transformation (Figure 2A). As cells adhere to RGD membranes, initial integrin activation results in RGD clustering without traction force. With dense line-partitioned RGD membranes, such as with 1 or 2 μm line pitch, forces can be generated on RGD-ligated integrins to form traction-force loaded adhesions (Figure 2A) after activation of initial spreading. Because the local contraction units are unable to span the 4 μm spacing (Ghassemi et al., 2012; Yu et al., 2011), the podosomes form as well on the larger line spacing as on continuous bilayers. In addition, myosin-II is not recruited during podosome formation on RGD membrane (Figure S4C). Thus, our data indicate that podosome formation requires minimum traction force development and that the local force generation between RGD-integrin adhesion clusters at dense line-partitioned RGD membranes inhibits podosome formation. When the same cell covers both the 2 μm pitch lines and a continuous membrane (Figure 2B), there is a remarkably local formation of focal...
adhesions at the lines while podosomes form over continuous membrane regions, indicating that the effect of contractility is local and may involve spatial contact signals.

Although we do not fully know how local contractions are translated into inhibition of podosome formation, we find that peak production of PIP3 is the key upstream event to trigger translated into inhibition of podosome formation, we find that local and may involve spatial contact signals.

membrane regions, indicating that the effect of contractility is local and may involve spatial contact signals.

Figure 6. Summary of Force-Mediated Adhesion Transformation Pathway

Early RGD-integrin activation triggered actomyosin contraction. When substratum provides traction force (1 μm line-pitch RGD membranes), cells form classic focal adhesions. When substratum provides no traction force, RGD-integrin clusters can turn into podosomes after 45 min of initial adhesion. Local enrichment of PIP3 by PI3K activation at the prepodosomal RGD cluster triggers N-WASP and Arp2/Arp3-mediated actin polymerization that initiated podosome formation. Recruitment of ARAP3 via local enrichment of PIP3 serves as a positive-feedback mechanism to downregulate RhoA-GTP in podosome-forming cells.

In conclusion, we suggest that the development of podosomes as adhesion structures implicates the absence of traction forces between integrin receptors and matrix ligands. Lack of could be off-target inhibition of other kinases by PF-562271. In addition, FAK can phosphorylate N-WASP and promote actin polymerization, and inhibition of FAK kinase activity suppresses N-WASP activity (Tang et al., 2013). However, N-WASP can still be phosphorylated by other kinases, such as Src family kinases (Dovas and Cox, 2010) or Abi kinase (Burton et al., 2005). While N-WASP could be activated by other kinases, N-WASP may fail to be recruited at RGD clusters without local enrichment of PIP3. We suggest that inhibition of FAK and Pyk2 autophosphorylation provides a mechanism to abolish local production of PIP3 by perturbing p85/PI3K association. As expected, inhibition of PI3K also suppresses podosome formation (Figure 4C).

Another protein that binds to PI3P is PTEN, and it dephosphorylates PI3P, thereby causing the loss of actin polymerizing proteins. It has previously been shown that PTEN is present in and regulates podosome/invadopodia formation (Hoshino et al., 2012; Poon et al., 2010). PTEN associates with podosomes after the actin core is formed and contributes to the disassembly of the podosome F-actin core. Thus, it seems that the cycle of podosome formation and disassembly is primarily dependent upon the local levels of PIP3 on plasma membranes. This can explain the regional differences in podosome formation we see in single cells (Figure 2B; Movie S4) through slower two-dimensional diffusion of PIP3 lipids rather than fast diffusive cytosolic signals.

A factor that could contribute indirectly to the formation of podosomes is RhoA activity. Using the FRET-based RhoA biosensor, lower RhoA-GTP levels are observed when cells develop podosomes, and pharmacologically activating RhoA-mediated contractility using LPA is seen to abolish podosome formation on RGD membranes, in agreement with previous reports (Schramp et al., 2008; van Helden et al., 2008). More than 70 Rho GAPs have been identified in eukaryotes (Tcherkezian and Lamine-Vane, 2007), and it remains unclear how RhoA activities are differentially regulated during adhesion formation. While DLC1 is linked to downregulation of RhoA in Src-transformed cells (Schramp et al., 2008), we find that the PI3P-binding protein ARAP3 is another RhoA-regulating factor recruited at podosome cores. ARAP3 contains both RhoA GAP and Arf6 GAP domains, and the RhoA GAP function of ARAP3 is activated by Rap-GTP (Krugmann et al., 2002, 2004). ARAP3’s Arf6 GAP function is in vivo, however, is still under investigation (Gambardella et al., 2011). Inhibition of PI3K upregulates RhoA-GTP and cellular contractility (Krugmann et al., 2004; Orlova et al., 2007), and our RhoA biosensor measurements also agree with previous findings (Figure 5C). Using overexpressed dominant-negative RhoA GAP mutants, we find ARAP3-R982A moderately suppresses podosome formation, while DLC1-R677E has no significant effect. However, ARAP3 is recruited largely after podosomes are formed. Recruitment of ARAP3 provides a positive-feedback mechanism to downregulate RhoA-GTP. Thus, our results indicate that manipulations of traction force development at integrin-matrix clusters can serve as a mechanical signal to modulate adhesion phenotype switching and RhoA activities.

In conclusion, we suggest that the development of podosomes as adhesion structures implicates the absence of traction forces between integrin receptors and matrix ligands. Lack of
traction forces at activated RGD-integrin clusters results in spatial-temporal recruitment of pSβeta and local enrichment of PIP3, which is not observed in force-loaded focal adhesions. This PIP3-dependent pathway of podosome formation does not require the induction of constitutively activated Src kinase and is further aided by the inactivation of RhoA by PIP3-mediated recruitment of ARP3. We suggest that local contractions may directly inhibit podosome formation while facilitating focal adhesion formation through a block of the PIP3-dependent pathway. The transformation between prepodosomal RGD-integrin clusters and podosomes is a remarkable example of mechanosensing through cell-adhesion processes. The reorganization of adhesion structures triggered by changing microenvironments has become an emerging theme of adaptive regulation in cellular signaling. Force and matrix ligand and integrin composition are all critical factors regulating adhesion phenotype and turnover.

EXPERIMENTAL PROCEDURES

Cell Culture and Fluorescent Fusion Proteins

Nontransformed RPTPα-/- mouse embryonic fibroblasts (Su et al., 1999), rat embryonic fibroblast (REF52), and THP-1 (human monocytic leukemia cells) were used in this study. Detailed information regarding cell culture, transfection protocol, the plasmids of fluorescent fusion proteins, and microscopy methods can be found in the Supplemental Experimental Procedures.

RGD-Supported Lipid Bilayer Membranes

1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and 1,2-dipalmityl-sn-glycero-3-phosphoethanolamine-N-(cap biotinyl) (16:0 biotinyl-Cap-PE) were purchased from Avanti Polar Lipids. The lipids (0.2 mol% of biotinyl-Cap-PE and 99.8 mol% of DOPC) were mixed with an equal volume of 1× PBS and then pipetted onto cleaned glass substrates for the self-assembly processes. A total of 0.1 µg/ml of Cascade blue neutraevin (Life Technologies) or DyLight 680 neutraevin (Thermo Fisher Scientific) was added onto supported lipid membranes, followed by 1 µg/ml of biotinylated RGD, cyclo(Arg-Gly-Asp-D-Phe-Lys-[Biotin-PEG-PEG]; Peptides International). Detailed information regarding lipid preparation and membrane functionalization can be found in the Supplemental Experimental Procedures.

Nanopatterned Glass Substrate

Nanoprint lithography was utilized to fabricate the physical barriers on glass substrates, and detailed preparation methods were previously described (Yu et al., 2011). In brief, a silicon-based imprint mold was fabricated by electron-beam lithography and anisotropic etching processes. First, Coverglasses (Warner Instruments) were cleaned by Piranha solution (sulfuric acid and hydrogen peroxide, mixed in 3:1 ratio) for 15 min, rinsed with deionized water, and then spin-coated with UV-curable imprint polymers. Patterns were then transferred from the mold to the glass by high-pressure stamping the imprint mold onto the polymer-coated coverglass and curing the polymer by UV exposure. After demolding, oxygen plasma etching was used to extend imprinted trenches vertically to the surface of the coverglass. A thin chromium metal layer was deposited onto the exposed glass surface by thermal evaporation. The chromium on imprinted polymers was removed by resist lift-off processing. Typically, metal lines were 100 nm in width and 5 nm in height with a gap distance ranging from 1 to 4 µm. The density of the metal lines remains constant and is about 10% per µm².

SUPPLEMENTAL INFORMATION

Supplemental information includes Supplemental Experimental Procedures, six figures, one table, and six movies and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2013.10.040.

AUTHOR CONTRIBUTIONS

C.-H.Y. and N.B.M.R. conducted and analyzed most of the experiments. A.K. and K.L.H. assisted in sample preparations. C.-H.Y., G.E.J., A.D.B., and M.P.S. supervised this study and prepared the manuscript.

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