Sequential signals toward podosome formation in NIH-src cells

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Introduction

Podosomes (also termed invadopodia in cancer cells) are actin-rich adhesion structures with matrix degradation activity that develop in various cell types. Despite their significant physiological importance, the molecular mechanism of podosome formation is largely unknown. In this study, we investigated the molecular mechanisms of podosome formation. The expression of various phosphoinositide-binding domains revealed that the podosomes in Src-transformed NIH3T3 (NIH-src) cells are enriched with PtdIns(3,4)P2, suggesting an important role of this phosphoinositide in podosome formation. Live-cell imaging analysis revealed that Src-expression stimulated podosome formation at focal adhesions of NIH3T3 cells after PtdIns(3,4)P2 accumulation. The adaptor protein Tks5/FISH, which is essential for podosome formation, was found to form a complex with Grb2 at adhesion sites in an Src-dependent manner. Further, it was found that N-WASP bound all SH3 domains of Tks5/FISH, which facilitated circular podosome formation. These results indicate that augmentation of the N-WASP–Arp2/3 signal was accomplished on the platform of Tks5/FISH-Grb2 complex at focal adhesions, which is stabilized by PtdIns(3,4)P2.

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Abbreviations used in this paper: FA, focal adhesion; FAK, focal adhesion kinase; PH, pleckstrin homology; PX, phox homology; SH3, Src homology 3; TIRF, total internal reflection fluorescence; WASP, Wiskott-Aldrich syndrome protein. The online version of this paper contains supplemental material.
and has been shown to play a pivotal role in podosome formation and ECM degradation (Seals et al., 2005). Although the importance of the PX domain and several binding proteins of the SH3 domains are suggested in all the research on Tks5/FISH, a comprehensive understanding regarding its role in podosome formation is yet to be achieved.

Substantial evidence has recently revealed that phosphoinositides bind to and regulate a number of cytoskeletal proteins (Takenawa and Itoh, 2001). Among them, PtdIns(3)-kinase products such as PtdIns(3,4,5)P3 and PtdIns(3,4)P2, which are present in negligible amounts under resting conditions, are formed at the plasma membrane in response to extracellular stimuli. Further, these locally produced PtdIns(3,4,5)P3 and PtdIns(3,4)P2 recruit cytosolic proteins to the plasma membrane. Consequently, proteins that specifically bind these phosphoinositides play important roles in extracellular signal-dependent cytoskeletal remodelling, membrane trafficking, cell proliferation, and apoptosis (Itoh and Takenawa, 2004; Takenawa and Itoh, 2006). The proteins within podosomes are presumably influenced by phosphoinositides. For example, PtdIns(3,4,5)P3 is able to modulate the functions of small GTPases of the Rho family, such as Rho, Rac, and Cdc42; these proteins are believed to be involved in podosome formation (Chellaiah, 2006). This modulation occurs via the action of guanine nucleotide exchange factors (GEFs) such as Tiam1, Vav1, and SWAP70 on these proteins; all these factors possess pleckstrin homology (PH) domains via which they interact with PtdIns(3,4,5)P3. However, it remains unclear how these lipids participate in podosome formation.

Here, we demonstrate that PtdIns(3,4)P2 and Grb2 at focal adhesions cooperatively recruit the adaptor protein Tks5/FISH before actin polymerization at podosomes. Further, N-WASP was found to bind Tks5/FISH depending on its SH3 domains, and it contributes to circular podosome formation. Based on these observations, we propose a novel model for podosome formation, particularly the initiation phase.

Results

PtdIns(3,4)P2 plays an important role in podosome formation in NIH-src cells

We first examined the involvement of PtdIns(3)-kinase in podosome formation because of its importance in cell migration and in establishing cell polarity. As with PP2 (an Src kinase inhibitor), treatment with LY294002 (a PtdIns(3)-kinase inhibitor) resulted in the disappearance of podosomes, indicating the involvement of PtdIns(3)-kinase activity in podosome formation (Fig. S1 A, available at http://www.jcb.org/cgi/content/full/jcb.200801042/DC1). This ability to suppress podosome formation correlated with the inhibition of invasion (Fig. S1, B and C), and it supports the role of podosomes in ECM invasion. Next, we used various phosphoinositide-binding domains to detect the localization of intracellular phosphoinositides (Furutani et al., 2006). The FYVE domain of Hrs binds specifically to PtdIns(3)P, and it was distributed at endosome-like dots in NIH-src cells (Fig. 1 A). The PH domains of Fapp1 and PLC5/1 bound specifically to PtdIns(4)P and PtdIns(4,5)P2, respectively, and they were localized at the Golgi apparatus and plasma membrane (Fig. 1 A). It is noteworthy that PtdIns(3,4)P2, which was specifically detected by the PH domain of Tapp1 (hereafter referred to as Tapp1PH), was strongly concentrated at the podosomes (Fig. 1 A). On the other hand, PtdIns(3,4,5)P3, which was detected by the PH domain of Grp1 (Grp1PH), was localized at the podosomes, although it was also present at other areas, including at ruffles and intracellular vesicles (Fig. 1 A). Expectedly, the localization of the PH domain of Akt, which recognizes both PtdIns(3,4)P2 and PtdIns(3,4,5)P3, was like a superimposed image of Tapp1PH and Grp1PH (Fig. 1 A). Tapp1PH, when weakly expressed, favored localization at podosomes; however, its overexpression (including weak and strong expression) tended to suppress podosome formation. Approximately 20% suppression was observed when Tapp1PH was expressed as compared with when its lipid-binding inactive form (Tapp1PH R212L) was expressed (Fig. 1 B). Compared with its mutants (AktPH R25A and AktPH K14A), AktPH also suppressed podosome formation by ~35% (Fig. 1 B). These results indicate that the plasma membrane of podosomes is enriched with PtdIns(3,4)P2 and that the interference of binding between this phosphoinositide and its targets inhibits podosome formation. However, because the inhibitory effect of Tapp1PH expression was not so efficient, there is a possibility that other mechanisms are also involved in the formation of podosomes.

Constitutive active Src induces PtdIns(3,4)P2 production at focal adhesions, which triggers podosome formation

Because Tks5/FISH—a recently identified adaptor molecule essential for podosome formation (Seals et al., 2005)—contains a PX domain in its N terminus (Fig. S2 A, available at http://www.jcb.org/cgi/content/full/jcb.200801042/DC1), we expected this domain to be one of the targets of phosphoinositides at podosomes. Actually, the PX domain of Tks5/FISH is reported to bind with a high affinity to PtdIns(3)P and PtdIns(3,4)P2 (Abram et al., 2003). In support of this, the wild type of the PX domain accumulated on both endosome-like dots and podosomes, whereas its lipid-binding inactive form (PX R42A/R93A) did not accumulate on them (Fig. S2, B–D). Further, the expression of the PX domain tended to suppress podosome formation to ~20%, which is significant when compared with the PX R42A/R93A mutant or the delta-PX mutant of Tks5/FISH (Fig. S2 E). This suggests that PtdIns(3,4)P2 plays an important role in podosome formation by interacting with Tks5/FISH via its PX domain. More importantly, the PX R42A/R93A mutant also significantly suppressed podosome formation as compared with the delta-PX mutant (Fig. S2 E); this ascribes the inhibitory effect by the PX domain to additional mechanisms involving lipid interaction, which would explain the relatively effective suppression by the PX domain as compared with that by Tapp1PH or AktPH.

To determine whether the accumulation of PtdIns(3,4)P2 at podosomes is a result or cause of podosome formation, we examined the localization of this phosphoinositide in NIH-src cells with reduced Tks5/FISH expression. Reduced Tks5/FISH
SrcY530F plasmid 3 h before starting the observation, and found that some cells began to bear podosomes in 1–2 h. At the transition state, polarization of 2×Tapp1PH was observed in the vicinity of newly formed vinculin-positive FAs (Fig. 2 C, arrowheads), resulting in rearrangement of vinculin (Fig. 2 C, arrows) and ring-shaped podosomes (Fig. 2 C). On the other hand, NIH-src cells also began to assemble podosomes at FA-related adhesions when spreading (Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb.200801042/DC1), supporting the notion that podosomes are initiated at FAs. These results indicate that the constitutive activation of Src alters the phosphoinositides at the proximal membrane of the FAs, and that the resulting FA-related adhesions are essential for the subsequent actin polymerization at podosomes.

Grb2 aids Tks5/FISH recruitment to focal adhesions in an Src-dependent manner

To examine the idea that Tks5/FISH recruitment to FAs is responsible for podosome formation, we used an immunoprecipitation assay with antibodies against FA molecules. As the adaptor
proteins Nck, Crk, and Grb2 have been reported to play important roles in adhesion-mediated cytoskeletal changes through interaction with adhesion molecules such as FAK or p130Cas, we examined their involvement in podosome formation and binding ability to Tks5/FISH. When expressed in NIH-src cells, the SH2 domains of Grb2 and Crk significantly suppressed podosome formation, whereas the SH2 domain of Nck did not (Fig. 3 A). Further, reduced expression of Grb2 and Crk, but not Nck1 (Fig. S4, A–C; available at http://www.jcb.org/cgi/content/full/jcb.200801042/DC1) resulted in the suppression of podosomes, indicating that Grb2 and Crk play important roles in podosome formation (Fig. 3 A). On the other hand, invasion activity of NIH-src cells with reduced Nck1 expression did not correlate with podosome formation activity; they showed significant suppression of invasion as did the cells with reduced expression of Grb2 and Crk (Fig. S4 D). These results suggest that Nck1 is
involved in matrix invasion via other mechanisms than podosome formation. Then, we performed an immunoprecipitation assay by using anti-Grb2 or anti-Crk antibody, and we found that only Grb2 precipitated Tks5/FISH in an Src-dependent manner (Fig. 3 B, N-WASP as a positive control; discussed in Fig. 6). This interaction was confirmed by immunoprecipitation with an anti-Tks5/FISH antibody; Grb2 coimmunoprecipitated in an Src-dependent manner (Fig. 3 C), whereas Crk was not (unpublished data; the involvement of Crk is presented in the Discussion section). This interaction is thought to be mediated by the poly-proline sequences of Tks5/FISH (sporadically found between the SH3 domains) with the N-terminal SH3 domain of Grb2 (Fig. 3 D). To visualize the Src-dependent translocation of Grb2 or Tks5/FISH, we observed endogenous Grb2 or Tks5/FISH together with vinculin in NIH-ctr and NIH-src cells. In NIH-ctr cells, neither Grb2 (Fig. 3 E, top panels) nor Tks5/FISH (Fig. 3 F, top panels) was concentrated at vinculin-positive adhesion sites. In contrast, both were translocated involved in matrix invasion via other mechanisms than podosome formation. Then, we performed an immunoprecipitation assay by using anti-Grb2 or anti-Crk antibody, and we found that only Grb2 precipitated Tks5/FISH in an Src-dependent manner (Fig. 3 B, N-WASP as a positive control; discussed in Fig. 6). This interaction was confirmed by immunoprecipitation with an anti-Tks5/FISH antibody; Grb2 coimmunoprecipitated in an Src-dependent manner (Fig. 3 C), whereas Crk was not (unpublished data; the involvement of Crk is presented in the Discussion section). This interaction is thought to be mediated by the poly-proline sequences of Tks5/FISH (sporadically found between the SH3 domains) with the N-terminal SH3 domain of Grb2 (Fig. 3 D). To visualize the Src-dependent translocation of Grb2 or Tks5/FISH, we observed endogenous Grb2 or Tks5/FISH together with vinculin in NIH-ctr and NIH-src cells. In NIH-ctr cells, neither Grb2 (Fig. 3 E, top panels) nor Tks5/FISH (Fig. 3 F, top panels) was concentrated at vinculin-positive adhesion sites. In contrast, both were translocated...
not only to podosomes but also to vinculin-positive FA-related adhesions in NIH-src cells (Fig. 3, E and F; bottom panels, arrowheads). Grb2 was still observed at PtdIns(3,4)P2-enriched FA-related adhesions of NIH-src cells with reduced Tks5/FISH expression, suggesting the translocation was independent of Tks5/FISH (Fig. S4 E). However, compared with Tks5/FISH, Grb2 showed weaker concentration at podosomes; this would explain the small amount of Tks5/FISH in anti-Grb2 immunoprecipitates (Fig. 3 B), while the substantial amount of Grb2 was detected in anti-Tks5/FISH immunoprecipitates (Fig. 3 C). Collectively, these results indicate that the constitutive activation of c-Src facilitates a complex formation of Grb2-Tks5/FISH in parallel with PtdIns(3,4)P2 generation at FA-related adhesions, which is a hallmark of podosomes-to-be. To localize correctly, Tks5/FISH must be recruited to podosomes not only through PtdIns(3,4)P2 but also through protein complex formation.

**Grb2 and Tks5/FISH translocate to focal adhesions in an Src-dependent and F-actin-independent manner**

To clearly elucidate the molecular events that occur before actin polymerization, the Src-dependent and F-actin–independent translocation of these molecules in connection with PtdIns(3,4)P2 generation was observed. NIH-src cells expressing these molecules were treated with PP2 and latrunculin B followed by only latrunculin B treatment. This treatment enabled the recovery of Src activity in 40 min (Fig. 4, A and B; phospho-Src [Y416] column) with F-actin kept depolymerized. As Src recovered its activity within 40 min after PP2 wash out, PtdIns(3,4)P2 detected by 2 × Tapp1PH or Grb2 was observed at phospho-Src (Y416)-positive FA-related adhesions (Fig. 4, A and B; bottom panels). Tks5/FISH, on the other hand, was found to have merged with these sites (Fig. 4, A and B; bottom panels, arrowheads). These results indicate that constitutive Src activation leads to PtdIns(3,4)P2 generation and Grb2 recruitment to FA-related adhesions, where Tks5/FISH is also recruited independent of actin polymerization. Thus, the puncta of Grb2 and Tks5/FISH observed in Fig. 4 B are considered podosome precursors that would develop into podosomes.

**Live-cell imaging analysis of podosome-precursor formation**

To confirm the molecular dynamics of podosome-precursor formation at the proximal part of the adhesion sites, the localization of PtdIns(3,4)P2, Grb2, and Tks5/FISH was detected in live cells by using total internal reflection fluorescence (TIRF) microscopy. First, we observed NIH-src cells expressing either RFP-2 × Tapp1PH or RFP-Grb2 together with GFP-Tks5/FISH. For the same purpose as the previous section, the cells expressing these molecules were treated with PP2 and latrunculin B followed by only latrunculin B treatment. RFP-2 × Tapp1PH began to distribute in a polarized way right after the PP2 wash out, followed by the gradual accumulation of GFP-Tks5/FISH (Fig. 5 A, arrowheads). The relative pixel intensity of the region of interest (ROI, RFP/GFP) tended to show a sharp increase due to early PtdIns(3,4)P2 accumulation, and it then converged to a constant value when Tks5/FISH was recruited to this region (Fig. 5 A, chart). Grb2 accumulation appeared to precede Tks5/FISH recruitment, and they were observed to move synchronously to form podosome-shaped accumulations (Fig. 5 B, arrowheads). Next, we examined whether the FA-related adhesions with PtdIns(3,4)P2, observed in Figs. 4 A and 5 A, would develop into a podosome. NIH-src cells expressing RFP-2 × Tapp1PH and YFP-actin were pretreated with PP2 and latrunculin B to block podosome formation, and then the drugs were completely washed out. As expected, FA-related adhesion with persistent PtdIns(3,4)P2 production resulted in circular podosome formation (Fig. 5 C). The relative pixel intensity of the ROI (RFP/YFP) tended to show a similarity with that of Fig. 5 A, indicating that Tks5/FISH recruitment correlates with actin polymerization. These results suggest that the Src-driven formation of the Tks5/FISH-Grb2 complex (podosome precursor) occurs on the site of action; i.e., PtdIns(3,4)P2-enriched FA-related adhesions before actin polymerization, and then circular podosomes develop on this site.

**Tks5/FISH enhances N-WASP-dependent actin polymerization**

Accumulated evidence has demonstrated that the N-WASP-Arp2/3 pathway is indispensable for actin polymerization in podosomes/invadopodia of many cell types (Linder et al., 1999, 2000; Mizutani et al., 2002; Yamaguchi et al., 2005). We examined whether Tks5/FISH mediates N-WASP–dependent actin polymerization by interacting with it. Immunoprecipitation of Tks5/FISH identified N-WASP as a binding partner, although the binding was constitutive (Fig. 3 C). To identify other molecules involved in podosome-precursor formation, we used a pull-down assay using each domain of Tks5/FISH (Fig. S2 A). To this end, GST-tagged PX or SH3 domains of Tks5/FISH were immobilized on glutathione-sepharose beads, incubated with NIH-src cell lysates, and then washed intensively. The proteins that bound to the PX domain or any of the SH3 domains of Tks5/FISH were resolved by SDS-PAGE and then subjected to mass spectrometry (Fig. 6 A, left panel). The following proteins were identified with no ambiguity: dynamin 2 (bound to SH3A and E; band a); N-WASP (bound to SH3A, B, C, D, and E; band b); WIP (bound to SH3C and E); tubulin (bound to SH3C); zyxin (bound to SH3C and E); nogo-B (bound to SH3E); and actin (bound to SH3E). The interaction of N-WASP and dynamin with the SH3 domains of Tks5/FISH was validated by performing Western blotting using anti-N-WASP and antidy-namin antibodies, respectively (Fig. 6 A, right). However, we could not detect dynamin in Tks5/FISH immunoprecipitates (unpublished data). Because all the SH3 domains of Tks5/FISH can bind to N-WASP, we expected Tks5/FISH to assemble a large amount of N-WASP at FA-related adhesions, leading to strong actin polymerization by the Arp2/3 complex. Actually, Grb2 precipitated more N-WASP from the cell lysate of NIH-src cells than from that of NIH-ctr cells (Fig. 3 B). Three truncated constructs containing different number of the SH3 domains were synthesized to test whether Tks5/FISH binding to N-WASP depends on these domains (Fig. 6 B). The cell lysates of the NIH3T3 cells expressing GFP-tagged-Tks5/FISH constructs were immunoprecipitated using anti-GFP antibody,
that were either fragmented or not sufficiently thick (Fig. 6 E, arrowheads). These aberrant podosomes resemble those observed in v-Src + /N-WASP/H11002 fibroblasts expressing a mutant N-WASP that cannot interact with barbed ends (Co et al., 2007). Thus, they are considered to originate from the attenuation of N-WASP recruitment to the sites of action.

Delta-VCA mutant of N-WASP forms a ring-shaped podosome-like structure

Because the verproline-homology (V) and collin-homology and acidic (CA) regions of the WASP family proteins contribute to rapid actin polymerization by binding G-actin and the Arp2/3 complex, respectively, mutants that lack the VCA region (delta-VCA) serve as dominant-negative forms of N-WASP function (Fig. S5, A and B; available at http://www.jcb.org/cgi/content/
and D), colocalized with Tks5/FISH (Fig. S5 E, arrowheads); however, it was devoid of F-actin and cortactin (Fig. S5 F, arrows). This structure is only formed when N-W ASP/H9004 expression is moderate in a cell, thus enabling its proper regulation. These data suggest that a podosome precursor comprising Tks5/FISH, Grb2, and N-W ASP is formed before actin polymerization itself (the remaining 95% localized broadly in the cytoplasm with suppressed podosome formation). This structure possessed phosphorylated Src (at Y416) and FAK at its center (Fig. S5, C and D), colocalized with Tks5/FISH (Fig. S5 E, arrowheads); however, it was devoid of F-actin and cortactin (Fig. S5 F, arrows). This structure is only formed when N-WASPΔVCA expression is moderate in a cell, thus enabling its proper regulation. These data suggest that a podosome precursor comprising Tks5/FISH, Grb2, and N-WASP is formed before actin polymerization.

Figure 5. Live-cell imaging of PtdIns(3,4)P2 generation, Grb2-Tks5/FISH recruitment, and actin polymerization at podosomes. NIH-src cells expressing RFP-2×Tapp1PH and GFP-Tks5/FISH (A), RFP-Grb2 and GFP-Tks5/FISH (B), or RFP-2×Tapp1PH and YFP-actin (C) were cultured in a fresh medium with (A and B) or without (C) latrunculin B (0.5 μg/ml) after the treatment with PP2 (10 μM) and latrunculin B (0.5 μg/ml) for 30 min. White boxed areas are enlarged to reveal the accumulation of each molecule. Arrowheads indicate Tks5/FISH or Grb2 being recruited to this region. The time that elapsed from the start of the observation is indicated above each column. Representative charts of the relative pixel intensity (RFP/GFP or RFP/YFP) from the enlarged area are also indicated.
and that Tks5/FISH may play a role in arranging N-WASP as a podosome launcher at FAs.

Thus, the molecular mechanisms involved in podosome formation can be summarized as follows: (1) constitutive activation of c-Src accompanied by PtdIns(3)-kinase activation leads to tyrosine phosphorylation of FAK and other FA machineries as well as the generation of PtdIns(3,4)P2 in the vicinity of FAs. (2) Grb2 is targeted to this FA-related adhesion sites (probably through interacting with the phosphorylated site of FAK [Y925]; Mitra et al., 2005) together with Tks5/FISH. Then, the localization of Tks5/FISH at the FA-related adhesion is stabilized via PX-lipid interaction. Simultaneously, dynamin is considered to be recruited to FA-related adhesions via Grb2, and it might help Tks5/FISH recruitment to this site. (3) N-WASP is effectively concentrated near FA-related adhesions by Tks5/FISH, and this leads to intent actin polymerization at these sites (Fig. 7).

Discussion

In this study, we have identified a stepwise mechanism for podosome formation, particularly its initiation. By using phosphoinositide-binding domains, PtdIns(3,4)P2 was shown to be the most concentrated phosphoinositide in podosomes. Further, although Tapp1PH inhibited PtdIns(3,4)P2 signaling, this inhibition was weaker than that of AktPH, which binds both PtdIns(3,4)P2 and PtdIns(3,4,5)P3 (Fig. 1B). Because the expression of these domains may not be sufficient for blocking phosphoinositides from interacting with downstream targets, it is important to investigate the pathways by which PtdIns(3,4)P2 and PtdIns(3,4,5)P3 are generated. An intriguing aspect would be to investigate the manner in which antecedent PtdIns(3,4)P2 production occurs at adhesion sites in an Src-dependent manner (Fig. 2, A–C; and Fig S2 I). PtdIns(4,5)P2 and type I phosphatidylinositol phosphate kinase isoform-H9253, an enzyme that produces PtdIns(4,5)P2, have been suggested to be responsible for the modulation of FAs by their binding to talin and other FA proteins (Di Paolo et al., 2002; Ling et al., 2002, 2006). Likewise, PtdIns(3)-kinase is also known to be activated via the Src-FAK pathway (Hanks S3E of Tks5/FISH. (C) The ability of Tks5/FISH to bind N-WASP depends on its SH3 domains. Cell lysates of NIH3T3 cells expressing GFP-tagged Tks5/FISH constructs were immunoprecipitated by using anti-GFP antibody, and the bound proteins were detected by using anti-N-WASP antibody. The blot of anti-GFP for lower molecular weight was separately shown. (D) The expression of the poly-proline sequence of N-WASP (rat N-WASP, residues 267–390) suppresses podosome formation. *, P < 0.01 by Student’s t test compared with mock-transfected cells. A minimum of 50 cells were counted in each round. Error bars represent standard deviations of three different experiments. (E) GFP-Tks5/FISH constructs rescue podosome formation in NIH-src cells having reduced Tks5/FISH expression. GFP-tagged Tks5/FISH constructs were transfected into NIH-src cells, followed by transfection with Tks5/FISH siRNA. The cells were stained with rhodamine-phalloidin (red) to visualize F-actin and with anti-Tks5/FISH antibody (blue). Arrowheads indicate aberrant podosomes. (F) Cells that exhibited a GFP signal and a weak (Full) or no (GFP, PX-A, PX-C) anti-Tks5/FISH signal were grouped under three categories based on their podosome morphology (no podosomes, aberrant podosomes, or circular podosomes). ***, P < 0.02; ***, P < 0.05 by Student’s t test compared with full length. A minimum of 50 cells were counted in each round. Error bars represent standard deviations of three different experiments. Bars, 20 μm.

Figure 6. Tks5/FISH–N-WASP binding promotes podosome formation in NIH-src cells. (A, left) NIH-src cell lysates were mixed with the GST-FX or GST-SH3 domains of Tks5/FISH immobilized on beads; the bound proteins were resolved by SDS-PAGE, stained with Coomassie Brilliant Blue (CBB), and then subjected to mass spectrometry. (A, right) NIH-src cell lysates were mixed with the GST-SH3 domains of Tks5/FISH immobilized on beads, and the bound proteins were analyzed by using anti-dynamin or anti-N-WASP antibody. (B) Schematic diagrams of the truncated Tks5/FISH constructs used in the assay. Anti-Tks5/FISH antibody (Santa Cruz Biotechnology, Inc.) is raised against amino acid residues expanding SH3D and
The nature of adhesion sites with PtdIns(3,4)P2 observed in NIH-src cells might be different from that of normal FAs. Live-cell imaging analysis of NIH3T3 cells transfected with active Src revealed that although PtdIns(3,4)P2 production was observed at the proximal of newly formed FAs, it was distinct from FAs themselves (Fig. 2 C). Additionally, vinculin-rings of podosomes were newly assembled at PtdIns(3,4)P2-enriched adhesion sites rather than that preexisting vinculin of FAs were modified into podosomes.

The PX domain of Tks5/FISH has been reported to bind both PtdIns(3)P and PtdIns(3,4)P2 (Abram et al., 2003), and this binding is indispensable for Tks5/FISH localization to podosomes and for Src-dependent podosome formation (Seals et al., 2003). Therefore, the most likely pathway for PtdIns(3,4)P2 production is via dephosphorylation of PtdIns(3,4,5)P3 by PtdIns(3,4,5)P3 5-phosphatases such as SHIP2, synaptojanin2, and/or SKIP. In fact, synaptojanin2 is inferred to be involved in podosome formation and invasion in NIH-src cells (Fig. S1, D–F) and is reported to play an essential role in invadopodium formation in glioma cells (Chuang et al., 2004). Further, because synaptojanin2 has been identified as a downstream effector for Rac (Malecz et al., 2000) and Rac activity is essential for podosome formation (unpublished data), the hypothesis that the p130Cas-Crk-Rac-synaptojanin2 pathway is involved in PtdIns(3,4)P2 production is plausible (the involvement of Crk is described in Fig. 3 A and Fig. S4 D).

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et al., 2005). Given the specific targeting of Tks5/FISH to PtdIns(3,4,5)P2-enriched FA-related adhesions, the adaptor protein Grb2 is the most likely to help targeting Tks5/FISH at the sites of action because their binding was Src dependent (Fig. 3, B and C). Grb2 is suggested to be involved in podosome formation, whereas another adaptor protein, Nck1, is not (Fig. 3 A). However, a contradictory result was reported: Nck1 but not Grb2 is involved in EGF-induced invadopodium formation in mammary carcinoma cells (Yamaguchi et al., 2005). It is intriguing to speculate that the difference between the integrin-based and growth factor–based triggering mechanisms may explain this discrepancy. In support of this speculation, integrin-mediated invadopodia are not expected to move laterally across the membrane because such movement might be limited by integrin adhesion to a substrate; however, the nascent invadopodia observed by Yamaguchi et al. (2005) are actively motile despite limitations provided to the membrane by a clearly identified moving actin-rich object. Moreover, although the suppressed expression of Nck1 seemed to have little effect on podosome formation itself (Fig. 3 A), significant effect on invasion was observed (Fig. S4 D). Our results suggest that Nck1 would participate in matrix degradation via other mechanisms than podosome formation, where future works should be addressed.

While investigating for proteins that bind to the adaptor molecule Tks5/FISH, we identified dynamin as a binding partner of Tks5/FISH. Dynamin localizes to podosomes in NIH-src cells, and RNAi in dynamin expression suppresses podosome formation (unpublished data), indicating that dynamin plays an essential role in podosome formation. Dynamin has been shown to interact with FAK (probably through Grb2), and it plays a role in microtubule-induced FA disassembly (Ezratty et al., 2005). Thus, it is possible that the FA-related adhesions in NIH-src cells are susceptible to disassembly by the dynamin-FAK system, and this leads to the frequent turnover of podosomes.

We observed that the delta-VCA mutant of N-WASP localizes to form a podosome-like structure and inhibits actin polymerization in NIH-src cells. By using antibodies against several endogenous proteins, Tks5/FISH was found to colocalize with rings of N-WASP&Delta;VCA, whereas cortactin was absent (Fig. S5, E and F). Recently, live-cell imaging analysis of Src-transformed breast carcinoma cells has revealed that actin polymerization in NIH-src cells is blocked by the V867G point mutation. To produce GST-tagged PX and SH3 domains of Tks5/FISH, the cDNA was cloned into pGEX6P-1 (GE Healthcare), respectively. To generate GFP and RFP fusion proteins, cDNA encoding full-length and truncated regions of Tks5/FISH or Grb2 was cloned into pEGFP C-1 (Clontech Laboratories, Inc.) and pTagRFP-C (Evrogen). Further, cDNA of the lipid-binding domains (Furutani et al., 2006) and the PX domains of Tks5/FISH were cloned into pCMV3B (Stratagene), pCMVHA, or pTagRFP-C. Site-specific mutagenesis was performed by PCR using mutated primers.

Materials and methods

Reagents and antibodies

The following antibodies were used: rabbit anti-Tks5/FISH, anti-Grb2, anti-synaptojanin2, and monoclonal mouse anti-my c (Santa Cruz Biotechno logy, Inc.); monoclonal mouse anti-FAK, monoclonal mouse anti-Grb2, and anti-dynamin (BD Biosciences); rabbit anti-Nck, monoclonal mouse anti-c-Src, and anti-cortactin (Millipore); rabbit anti-c-Src, anti-phospho-Src, anti-Crk, anti-myc, and monoclonal mouse anti-HA (Cell Signaling Technology); monoclonal mouse anti-vinculin and anti-py tubulin (Sigma-Aldrich); rabbit anti-GFP (MBL International); and rabbit anti-N-WASP. Latrunculin B and LY294002 were purchased from Sigma-Aldrich. Pp2 was purchased from BIOMOL International, LP.

Plasmid construction and mutagenesis

All Tks5/FISH constructs were generated by polymerase chain reaction (PCR) using mouse cDNA as the template, and their identity was confirmed by sequencing. The 2,600th nucleotide of the Tks5/FISH cDNA was observed to be adenine in three different cDNA libraries (BD Biosciences), although it is registered as thymine in a database (GenBank/EMBL/DDBJ) accession no. AJ007012). This mutation results in a V867G-point mutation. To produce GST-tagged PX and SH3 domains of Tks5/FISH, the cDNA was cloned into pGASTBAC and pGEX6P-1 (GE Healthcare), respectively. To generate GFP and RFP fusion proteins, cDNA encoding full-length and truncated regions of Tks5/FISH or Grb2 was cloned into pEGFP C-1 (Clontech Laboratories, Inc.) and pTagRFP-C (Evrogen). Further, cDNA of the lipid-binding domains (Furutani et al., 2006) and the PX domains of Tks5/FISH were cloned into pCMV3B (Stratagene), pCMVHA, or pTagRFP-C. Site-specific mutagenesis was performed by PCR using mutated primers.

Generation of stable NIH3T3 cell lines

NIH3T3 cells were transfected with pcDNA or pcDNA-SrcY530F, encoding a mutant form of human Src encompassing an activating Y530F substitution, with Lipofectamine 2000 (Invitrogen). Single colonies were isolated after 2–3 wk of G418 selection (1.0 mg/ml). Protein expression was assessed by immunoblotting of cell lysates with anti-phospho-Src antibody. The resultant cells were termed NIH-hct or NIH-src.

Cell culture and transfection

NIH3T3, NIH-hct, and NIH-src cells were cultured at 37°C in DME containing 10% calf serum (CS) and penicillin/streptomycin under 5% CO2. To obtain transient expression, the cells were plated at 40% confluence on a 6-well plate, and on the following day they were incubated with 1.6 μg DNA and 3.5 μl Lipofectamine 2000 (Invitrogen) in Opti-MEM (Invitrogen) for 6 h. After this, they were transferred to a complete medium and cultured for 18 h. The cells were then replated onto type I-C collagen (Nitta Gelatin)-coated coverslips and cultured for an additional 24 h before analysis. For siRNA transfection, the cells were plated at 20% confluence on a 6-well plate, and on the following day they were incubated with 160 pmol siRNA and 4.0 μl Lipofectamine 2000 (Invitrogen) or 6.0 μl RNAi MAX (Invitrogen) in Opti-MEM (Invitrogen) for 6 h. They were then cultured in a complete medium for 18 h and then fixed for 20 min. For total RNA transfection, the cells were then replated and analyzed as described above. To prevent PtdIns(3,5)-kinase activity, the cells were treated with 25 μM LY294002 for 40 min before fixation. To prevent Src-kinase activity, the cells were treated with 10 μM PP2 for 40 min before fixation.

Matrigel invasion assay

The NIH-src cells (2.0 × 104 cells) were plated on a BioCoat Matrigel Invasion Chamber (BD Biosciences) for 18 h with DMSO, PP2, or LY294002. For transfection analysis, two rounds of siRNA or DNA transfection was performed 48/72 h before plating on the chamber. After this, the chambers were fixed in 3.7% formaldehyde in PBS for over 30 min. The chambers were then washed in PBS, and the invaded cells were stained with crystal violet. After washing the cells with PBS more than three times, cells from three different sample points on the lower surface of the chamber were counted.
RNAi experiments
The following stealth siRNA [invitrogen] derived from mouse N-WASP, Tks5/FISH, Nck1, Grb2, Crk, or synaptotagmin2 cDNA, respectively, were used for the RNAi experiments: siRNA for N-WASP (5'-AUAGUCACA-CAUUUUCUUGGGAGA-3' or 5'-AAGGCGGCCACACUGCUCCUCUG-UCC-3'), Tks5/FISH (5'-UGGCUGGAAGAGGGAUAACUCC-3' or 5'-GAUAGGAGUGGGAUGAGGAUCG-UCC-3'), or synaptotagmin2 (5'-AUGGCGUCCGAAUAAGAACACC-3' or 5'-AUUAAACUUACUUACCCAG3'), and scrambled [invitrogen] for 30–60 min at 3–5 min intervals by using a TIRF microscopy system.

Immunofluorescence
The cells cultured on type 1-C collagen-coated coverslips were fixed in 3.7% formaldehyde in PBS or 2% paraformaldehyde in PBS. These were permeabilized with 0.1% Triton X-100 in PBS for 5 min and then incubated with primary antibodies for over 60 min. They were then washed with PBS and incubated with secondary antibodies and rhodamine-phalloidin (invitrogen) for 30 min. Finally, the cells were washed with PBS and mounted on glass slides.

Live-cell imaging of NIH-src cells
NIH-src cells transfected with the indicated constructs were grown on type 1-C collagen-coated glass-base dishes (IWAKI). After treatment with PP2 (10 μM) and latrunculin B (0.5 μg/ml), the medium was replaced with new medium with or without latrunculin B (0.5 μg/ml). The images were acquired in Fig. 2C at 37 °C. 40 × objective, NA 0.90 (Olympus).

Protein expression and purification
The His-GST-tagged PX domain of the Tks5/FISH proteins was expressed in SF9 cells and purified on glutathione-agarose beads (GE Healthcare) according to a standard method. The GST-tagged SH3 domains of Tks5/FISH, Grb2, and the SH2 domain of Grb2 were expressed in Escherichia coli and bound to glutathione-agarose beads for the pull-down assay.

Statistics
Statistically significant differences were determined using the Student’s t-test. Differences were considered significant if P < 0.05.

Online supplemental material
Fig. S1 shows the involvement of Src, PtdIns(3)kinase, and synaptotagmin2 in podosome formation and invasion activity of NIH-src cells. Fig. S2 shows the importance of the PX domain of Tks5/FISH in targeting PtdIns(3,4)P2 in podosome formation and invasion activity of NIH-src cells. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200801042/DC1.

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References
Abram, C.L., D.F. Seals, I. Pass, D. Salinsky, L. Maurer, T.M. Roth, and S.A. Courtneidge. 2003. The adaptor protein fish associates with members of the ADAMS family and localizes to podosomes of Src-transformed cells. J. Biol. Chem. 278:16844–16851.
Alvarez, R.H., H.M. Kantarjian, and J.E. Cortes. 2006. The role of Src in solid and hemotropic malignancies: development of new-generation Src inhibitors. Cancer. 107:1918–1929.
Artym, V.V., Y. Zhang, F. Seilier-Moiseiwitsch, K.M. Yamada, and S.C. Mueller. 2006. Dynamic interactions of cortactin and membrane type 1 matrix metalloproteinase at invadopodia: defining the stages of invadopodia formation and function. Cancer Res. 66:3034–3043.
Buccione, R., J.D. Orth, and M.A. McNiven. 2004. Foot and mouth: podosomes, invadopodia and circular dorsal ruffles. Nat. Rev. Mol. Cell Biol. 5:647–657.
Carman, C.V., P.T. Sage, T.E. Scuito, M.A. de la Fuente, R.S. Geha, H.D. Ochs, H.F. Dvorak, A.M. Dvorak, and T.A. Springer. 2007. Transcellular diapedesis is initiated by invasive podosomes. Immunity. 26:784–797.
Chellaiya, M.A. 2006. Regulation of podosomes by integrin alphaVbeta3 and Rho GTPase-facilitated phosphomositide signaling. Eur. J. Cell Biol. 85:311–317.
Chuang, Y.Y., N.L. Tran, N. Rusk, M. Nakada, M.E. Berens, and M. Symons. 2004. Role of synaptotagmin 2 in glioma cell migration and invasion. Cancer Res. 64:8271–8275.
Co, C., D.T. Wong, S. Gierke, V. Chang, and J. Tuantoon. 2007. Mechanism of actin network attachment to moving membranes: barbed end capture by N-WASP WH2 domains. Cell. 128:901–913.
David-Pfeuty, T., and S.J. Singer. 1980. Altered distributions of the cytoskeletal proteins vinculin and alpha-actinin in cultured fibroblasts transformed by Rous sarcoma virus. Proc. Natl. Acad. Sci. USA. 77:6687–6691.
Destang, O., F. Sautel, J.C. Geminaire, P. Juhre, and F. Bard. 2003. Podosomes display actin turnover and dynamic self-organization in osteoclasts expressing actin-green fluorescent protein. Mol. Biol. Cell. 14:407–416.
Di Paolo, G., L. Pellegrini, K. Letinic, G. Cestra, R. Zoncu, S. Voronov, S. Chang, J. Guo, M.R. Wenk, and F. De Camilli. 2002. Recruitment and regulation of phosphatidylinositol phosphate kinase type 1 gamma by the FERM domain of talin. Nature. 420:85–89.
Ezraty, E.J., M.A. Partridge, and G.G. Gundersen. 2005. Microtubule-induced focal adhesion disassembly is mediated by dynamin and focal adhesion kinase. Nat. Cell Biol. 7:581–590.
Furutani, M., K. Tsujita, T. Itoh, T. Ijim, and T. Takenawa. 2006. Application of phosphomositide-binding domains for the detection and quantification of specific phosphomositides. Anal. Biochem. 355:8–18.
