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Nck1 and Grb2 localization patterns can distinguish invadopodia from podosomes

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Abstract

Invadopodia are matrix-degrading ventral cell surface structures formed in invasive carcinoma cells. Podosomes are matrix-degrading structures formed in normal cell types including macrophages, endothelial cells, and smooth muscle cells that are believed to be related to invadopodia in function. Both invadopodia and podosomes are enriched in proteins that regulate actin polymerization including proteins involved in N-WASP/WASP-dependent Arp2/3-complex activation. However, it is unclear whether invadopodia and podosomes use distinct mediators for N-WASP/WASP-dependent Arp2/3-complex activation. We investigated the localization patterns of the upstream N-WASP/WASP activators Nck1 and Grb2 in invadopodia of metastatic mammary carcinoma cells, podosomes formed in macrophages, and degradative structures formed in Src-transformed fibroblasts and PMA-stimulated endothelial cells. We provide evidence that Nck1 specifically localizes to invadopodia, but not to podosomes formed in macrophages or degradative structures formed in Src-transformed fibroblasts and PMA-stimulated endothelial cells. In contrast, Grb2 specifically localizes to degradative structures formed in Src-transformed fibroblasts and PMA-stimulated endothelial cells, but not invadopodia or podosomes formed in macrophages. These findings suggest that distinct upstream activators are responsible for N-WASP/WASP activation in invadopodia and podosomes, and that all these ventral cell surface degradative structures have distinguishing molecular as well as structural characteristics. These patterns of Nck1 and Grb2 localization, identified in our study, can be used to sub classify ventral cell surface degradative structures.

Keywords

cancer invasion; invadopodia; podosomes; Src-transformed fibroblasts; Nck1; Grb2; N-WASP

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Introduction

Podosomes and invadopodia were both initially identified in Rous Sarcoma Virus-Transformed (described hereafter as Src-transformed) fibroblasts (Chen, 1989). Podosomes were first described as adhesive structures in close contact with the substratum containing actin and vinculin (Tarone et al, 1985). Similar to podosomes, invadopodia were also identified as ventral cell surface structures enriched with actin and vinculin, but the added feature of invadopodia was their ability to focally degrade the underlying extracellular matrix (ECM) using matrix metalloprotease (MMP) activity (Chen, 1989). Several studies have now demonstrated that podosomes formed in many cell types also have the ability to degrade the underlying ECM using MMP activity (Linder, 2007; Yamaguchi et al, 2006) suggesting that the ventral cell surface structures formed in Src-transformed fibroblasts, originally termed invadopodia and podosomes (Chen, 1989; Tarone et al, 1985), were essentially the same structures.

Since podosomes were identified in Src-transformed fibroblasts, researchers began to discover that many nontransformed normal cell types form podosomes in vitro including: myeloid-derived cells such as primary monocytes (Worthylake et al, 2001), macrophages, dendritic cells, osteoclasts and neutrophils (Szczer et al, 2006), smooth muscle cells, endothelial cells and lymphocytes (Carman et al, 2007; Redondo-Munoz et al, 2006) (for reviews see (Linder, 2007; Linder, 2009). In these cell types, podosomes could form in the absence of Src transformation suggesting that they play physiological, rather than pathological, roles in adhesion, protrusion and matrix remodeling during normal physiological processes. Currently, podosomes in non-transformed cells are structurally defined as ventral cell surface structures that contain an F-actin core accompanied by other actin regulatory proteins including Wiskott Aldrich Syndrome protein (WASp), neural (N)-WASp, cortactin, the Arp2/3 complex, surrounded by a ring of adhesion plaque proteins including vinculin and paxillin, that are linked to the ECM via integrins (Gimona et al, 2008; Linder et al, 1999).

Since invadopodia were identified in Src-transformed fibroblasts (Chen, 1989), it was discovered that several invasive cancer cell types form invadopodia including: metastatic mammary carcinoma cells, melanoma cells, and aggressive head and neck squamous carcinoma cells (Baldassarre et al, 2003; Clark et al, 2007; Yamaguchi et al, 2005). Invadopodia that form in invasive cancer cells share many similar structural and functional features to the invadopodia first identified in Src-transformed fibroblasts, although their morphological features are distinct. Invadopodia formed in invasive cancer cells are relatively small (~1–2 μm diameter) circular punctate structures (Yamaguchi et al, 2005), while the invadopodia formed in Src-transformed fibroblasts appear as large (~5 μm diameter) rosettes (Webb et al, 2007). Today, invadopodia are defined as F-actin enriched membrane protrusions with matrix degradation activity formed on the ventral cell surface of invasive tumor cells (Caldieri et al, 2009; Lizarraga et al, 2009; Oser & Condeelis, 2009; Weaver, 2008b; Yamaguchi & Condeelis, 2007). Invadopodia are believed to play an important role in tumor cell invasion by degrading the ECM via MMP activity (Poincloux et al, 2009; Yamaguchi & Condeelis, 2007).

Both invadopodia and podosomes are enriched in F-actin and proteins involved in regulating the actin cytoskeleton including: cortactin, N-WASp/WASp, cofilin, and the Arp2/3 complex (Albiges-Rizo et al, 2009; Linder, 2007; Oser et al, 2009). In addition, both invadopodium and podosome formation requires the coordination of many cell biological processes including integrin and growth factor receptor signaling (Buccione et al, 2009; Calle, 2006; Yamaguchi et al, 2005), membrane trafficking (Liu et al, 2009; Sakurai-Yageta et al, 2008), and MMP localization, activation, and secretion (Artym et al, 2006; Clark et al,

2007; Sakurai-Yageta et al, 2008). In fact, podosomes and invadopodia share so many features that the umbrella term “invadosome” has been used to group these ventral cell surface degradative structures (Linder, 2009).

Although invadopodia and podosomes share many proteins in common and functional characteristics, there are also several unique features of each organelle that suggest that they may have some distinct functions. For example, invadopodia do not contain an adhesion plaque ring (containing vinculin and paxillin) that surrounds the F-actin core, which is a defining feature of podosomes, indicating that invadopodia may not mediate adhesion to the ECM. In fact, the absence of vinculin in invadopodia may be a useful structural marker to distinguish invadopodia from podosomes (Chan et al, 2009; Gimona, 2008; Linder, 2009). Apart from some structural differences, invadopodia have longer lifetimes, a greater protrusive capacity, and increased matrix degradation activity compared to podosomes (Linder, 2007). These characteristics support the hypothesis that invadopodia are critical for tumor cell invasion during metastasis (Condeelis & Segall, 2003; Philippart et al, 2008). Podosomes are smaller in size, but each podosome-forming cell generates a much greater number of podosomes compared to invadopodia (Linder, 2007). Lastly, invadopodia form in invasive cancer cells, while podosomes form in normal, non-pathological cell types.

Ironically, although the terms invadopodia and podosomes were first coined in studies of Src-transformed fibroblasts (Chen, 1989; Tarone et al, 1985), the ventral cell surface degradative structures formed in Src-transformed fibroblasts (referred to hereafter as degradative structures formed in Src-transformed fibroblasts) have several features that suggest they are unique from what is currently described as invadopodia and podosomes. Unlike podosomes, degradative structures formed in Src-transformed fibroblasts do not contain an adhesion ring with vinculin and paxillin surrounding a punctate F-actin core, but rather appear as much larger rosette-like structures with matrix degradation activity containing adhesion proteins (e.g. vinculin and paxillin) that colocalize with the F-actin core (Buschman et al, 2009; Oikawa et al, 2008). In that respect, both podosomes and degradative structures formed in Src-transformed fibroblasts appear to be *bona fide* adhesion structures. Unlike both podosomes and degradative structures formed in Src-transformed fibroblasts, invadopodia are not enriched with vinculin (Chan et al, 2009).

Recent evidence suggests that invadopodia, podosomes, and ventral cell surface degradative structures formed in Src-transformed fibroblasts may contain a distinct set of proteins used to regulate the actin cytoskeleton (Oikawa et al, 2008; Yamaguchi et al, 2005). Although N-WASp/WASp localizes to and is necessary for the formation of invadopodia, degradative structures formed in Src-transformed fibroblasts, and podosomes formed in macrophages (Linder et al, 1999; Mizutani et al, 2002; Oikawa et al, 2008; Yamaguchi et al, 2005), distinct activators of N-WASp are responsible for invadopodium formation in metastatic mammary carcinoma cells compared to degradative structure formed in Src-transformed fibroblasts (Oikawa et al, 2008; Oser et al, 2009; Yamaguchi et al, 2005). Specifically, Nck1, an upstream activator of N-WASp, localizes to invadopodia and is important for invadopodium formation and matrix degradation activity of invadopodia in both metastatic mammary carcinoma cells (Yamaguchi et al, 2005) and melanoma cells (Stylli et al, 2009). In contrast, Grb2, another upstream activator of N-WASp, does not localize to nor is important for invadopodium formation in the same mammary carcinoma cell type (Yamaguchi et al, 2005). However, Grb2 localizes to degradative structures formed in Src-transformed fibroblasts early during their assembly and is critical for their formation, while Nck1 knockdown has no effect (Oikawa et al, 2008). These findings suggest that the specific localization patterns of Nck1 and Grb2 can be used to distinguish invadopodia from degradative structures formed in Src-transformed fibroblasts. It is not known whether Nck1 or Grb2 localizes to podosomes formed in non-transformed cell types, such as macrophages.

Based on these results, we hypothesized that Nck1 and Grb2 localization patterns could also distinguish invadopodia from podosomes formed in macrophages.

In this short communication, we investigated whether the endogenous localization patterns of Nck1 and Grb2 could be used to distinguish between the degradative structures formed in metastatic mammary carcinoma cells, macrophages, Src-transformed fibroblasts, and PMA-stimulated endothelial cells. We hypothesized that Nck1 and Grb2 localization patterns could be used as markers to distinguish among these structures.

Materials and methods

Antibodies

For immunofluorescence (IF), cortactin (ab-33333) and Nck1 (ab-14588) were from Abcam, and Grb2 (sc-255(C-23)) was from Santa Cruz. 4G10 anti-phosphotyrosine monoclonal antibody was from Millipore. Monoclonal antibody against vinculin (hVin1) was from Sigma. Secondary antibodies Alexa488 donkey anti-rabbit, and Alexa568 donkey anti-mouse, and Alexa647-phalloidin were from Invitrogen, and Cy5-conjugated goat anti-mouse was from Jackson Laboratories. For immunoblot analysis, Nck1 (ab-14588) was from Abcam, Grb2 (sc-255(C-23)) was from Santa Cruz, β -actin (AC-15) monoclonal antibody was from Sigma and GAPDH mouse antibody was from Biodesign. Horseradish peroxidase (HRP)-conjugated secondary antibodies against rabbit and mouse IgG were from Jackson Immuno research.

Cell Culture

For all experiments, MDA-MB-231 cells were cultured in D-MEM supplemented with 10% FBS and antibiotics, MTLn3 cells were cultured in α -MEM supplemented with 5% FBS and antibiotics. RAW/LR5 cells were grown in RPMI 1640 supplemented with 10% newborn calf serum and antibiotics. CD14⁺ human peripheral blood monocytes were provided by Dr Gabriel Bricard (Department of Infection and Immunology, Albert Einstein College of Medicine) and differentiated in hMDMs by culturing on bacterial plastic dishes in RPMI 1640 supplemented with 10% FCS, antibiotics and 10,000 U/ml of human recombinant CSF-1. Human umbilical vein endothelial cells (HUVECs) were obtained from ATCC and cultured in M199 supplemented with 15% FBS, 10 ng/ml recombinant FGF-1 (Peprotech), 5 μ g/ml heparin, and antibiotics. NIH3T3 cells were provided by Dr Louis Hodgson (Anatomy and Structural Biology, Albert Einstein College of Medicine) and maintained in D-MEM supplemented with 10% FBS and antibiotics.

Invadopodium Assay and Immunofluorescence

For MDA-MB-231 and MTLn3 IF experiments, 100,000 MDA-MB-231 or MTLn3 cells were plated on Alexa405-labeled thin gelatin matrix 4 hours prior to fixation. Macrophages were plated on glass coverslips for 16 hours prior to fixation as described below. For NIH3T3 experiments, 10,000 cells were plated on glass coverslips, overnight and transfected with 0.5 μ g v-src plasmid using Fugene HD (Roche) for 14 hours. PMA stimulation of HUVECs was performed as described by Tatin (Tatin et al, 2006) by incubating for 30 minutes with 50 ng/ml of PMA and fixing the cells as described below. For Nck1 IF experiments, the cells were fixed in 3.7% PFA for 20 minutes at RT, and IF was performed as described previously (Yamaguchi et al, 2005). For Grb2 IF experiments, the cells were fixed with 2% PFA for 10 minutes at RT and IF was performed as described previously (Oikawa et al, 2008). The Alexa405 (Invitrogen-A30000) was conjugated to gelatin (Sigma-G2500) as described previously (Artym et al, 2009) and thin matrix Alexa405-gelatin matrix was prepared as previously described (Artym et al, 2006).

Immunoblot Analysis

For protein immunoblot analysis, whole cell lysates were prepared by washing 2X with cold PBS before direct extraction in SDS-PAGE sample buffer. The samples were resolved by 10 % SDS-PAGE, transferred onto PVDF membranes (Immobilon-P; Millipore), blocked in 5% milk-TBST and incubated with the indicated antibodies. Signals were detected using the Super Signal West Pico chemiluminescent substrate (Pierce) on a Kodak Image Station 440.

Results

Endogenous Nck1 localizes specifically to invadopodia

Nck1, but not Grb2, localizes to invadopodia and plays an important role in invadopodium formation and matrix degradation in the MTLn3 metastatic mammary carcinoma cell line (Yamaguchi et al, 2005). In contrast, in Src-transformed fibroblasts, Grb2 localizes to degradative structures early during their formation and Grb2, but not Nck1, is required for their formation (Oikawa et al, 2008). Based on these studies, we investigated the localization patterns of endogenous Nck1 and Grb2 in several cell types including: podosomes formed in macrophages, invadopodia formed in metastatic mammary carcinoma cells, and degradative structures formed in Src-transformed fibroblasts and PMA-stimulated endothelial cells. We performed immunofluorescence (IF) and stained for endogenous Nck1 and Grb2 in two metastatic breast cancer cell lines (the human MDA-MB-231 and the rat MTLn3 cell lines), the mouse monocyte/macrophage cell line RAW/LR5 (Dovas et al, 2009), human monocyte-derived macrophages (hMDM), mouse Src-transformed fibroblasts (Oikawa et al, 2008), and human PMA-stimulated HUVEC cells (Tatin et al, 2006). Thus, ventral invasive structures found in both human and rodent cell types were examined. Immunoblot analysis confirmed protein expression of both Nck1 and Grb2 in all cell types used in this study (Figure S1). As has been reported for invadopodia in metastatic mammary carcinoma cells and melanoma cells using fluorescently tagged Nck1 (Stylli et al, 2009; Yamaguchi et al, 2005), we found that endogenous Nck1 localized to invadopodia in MDA-MB-231 cells (Figure 1A) and MTLn3 cells (data not shown). GFP-Nck1 also localized to invadopodia in both mammary carcinoma cell types (data not shown). In contrast, endogenous Nck1 did not localize to podosomes formed in either hMDMs (Figure 1B), podosome-forming RAW/LR5 cell line (data not shown), or degradative structures formed in Src-transformed fibroblasts and PMA-stimulated HUVEC cells (Figure 2A–C). PMA-stimulated HUVEC cells primarily formed rosette-like structures and rarely formed individual dot-like structures. Consistent with a previous study (Osiak et al, 2005), Nck1 failed to localize to either morphological structure in HUVEC cells (Figure 2B & C). Together, these results show that endogenous Nck1 specifically localizes to invadopodia and that the presence of endogenous Nck1 in a ventral cell surface degradative structure uniquely identifies invadopodia.

Endogenous Grb2 does not localize to invadopodia and podosomes, but localizes to degradative structures formed in Src-transformed fibroblasts and PMA-stimulated HUVEC cells

As described above, Grb2 localizes to the degradative structures formed in Src-transformed fibroblasts, but GFP-Grb2 does not localize to invadopodia (Oikawa et al, 2008; Yamaguchi et al, 2005). Based on these studies, we investigated the localization of endogenous Grb2 using IF (Oikawa et al, 2008) in all cell types described above. Consistent with previous results using GFP-Grb2 in MTLn3 cells (Yamaguchi et al, 2005), endogenous Grb2 did not localize to invadopodia in either MDA-MB-231 (Figure 3A) or in MTLn3 cells (data not shown). Interestingly, endogenous Grb2 did not localize to podosomes in both hMDMs (Figure 3B) and RAW/LR5 cells (data not shown). GFP-Grb2 also failed to localize to invadopodia and podosomes in all of these cell types (data not shown). Consistent with previously published data, Grb2 localized to degradative structures formed in Src-

transformed fibroblasts (Figure 4A) (Oikawa et al, 2008). Interestingly, Grb2 also localized to both rosette-like (Figure 4B) and individual dot-like (Figure 4C) degradative structures formed in PMA-stimulated HUVEC cells suggesting that these structures may be more similar to degradative structures formed in Src-transformed fibroblasts than to invadopodia formed in metastatic mammary carcinoma cells or podosomes formed in macrophages. Taken together, these results show that Grb2 specifically localizes to degradative structures formed in Src-transformed fibroblasts and PMA-stimulated HUVEC cells, and suggest that these structures have a unique molecular composition and actin cytoskeletal regulation compared to invadopodia formed in metastatic mammary carcinoma cells and podosomes formed in macrophages.

Discussion

Src transformation of fibroblasts causes a redistribution of cytosolic proteins to form a degradative compartment previously described as both a podosome and an invadopodium (Mizutani et al, 2002; Seals et al, 2005; Webb et al, 2007). Although invadopodia formed in invasive cancer cells, podosomes formed in macrophages, and degradative structures formed in Src-transformed fibroblasts share a common function, there is recent evidence to suggest that each structure contains a unique molecular protein composition (Chan et al, 2009; Gimona et al, 2008; Oikawa et al, 2008; Yamaguchi et al, 2005). Whether degradative structures formed in Src-transformed fibroblasts should be categorized as either podosomes or invadopodia has been an ongoing debate in the field of adhesion and tissue invasion, and here we provide evidence that although all three structures have many similarities, each structure has an important molecular difference that can be identified using IF. Here and previously (Yamaguchi et al, 2005), we show that the Nck1 and Grb2 protein localization patterns are unique among each of the ventral cell surface degradative structures. Our data demonstrate that the presence of endogenous Nck1 in a ventral cell surface degradative structure uniquely identifies an invadopodium, while our work and previous work (Oikawa et al, 2008) indicates that Grb2 is specifically localized to degradative structures formed in Src-transformed fibroblast and PMA-stimulated HUVEC cells, while neither are present in podosomes formed in macrophages.

Distinct upstream N-WASp/WASp activators in invadopodia, podosomes, and degradative structures formed in Src-transformed fibroblasts

We show that the enrichment of endogenous Nck1 in a ventral cell surface degradative structure uniquely identifies an invadopodium (Figure 5 (model)). It was recently demonstrated that Nck1 is critical for initiation of actin polymerization in the invadopodia of metastatic mammary carcinoma cell and melanoma cells (Oser et al, 2009; Stylli et al, 2009) through the activation of N-WASp (Oser et al, 2009). In addition to the requirement of Nck1 for N-WASp activation, Cdc42 is critical for invadopodium formation in several metastatic cancer cell types (Ayala et al, 2009; Sakurai-Yageta et al, 2008; Yamaguchi et al, 2005). Furthermore, Fgd1, a Cdc42-specific GEF, localizes to and is important for invadopodium formation and degradation activity in melanoma cells (Ayala et al, 2009). Together with our results, this suggests that invadopodia in metastatic cancer cells require both Nck1- and Cdc42-dependent N-WASp activation (Figure 5 (model)). Future studies will investigate the interplay between Nck1- and Cdc42-dependent N-WASp activation in invadopodia.

Our study also provides evidence that Grb2 does not localize to invadopodia formed in metastatic mammary carcinoma cells nor in podosomes formed in macrophages, which is consistent with previous findings in mammary carcinoma cells (Yamaguchi et al, 2005). Another study demonstrated Grb2 localizes early and is critical for assembly of degradative structures formed in Src-transformed fibroblasts (Oikawa et al, 2008). Interestingly, we show that Grb2, but not Nck1, localizes to both rosettes and individual dot-like structures

formed in PMA-stimulated HUVEC cells-the same localization pattern observed in the degradative compartment formed in Src-transformed fibroblasts. Together, these results show that the enrichment of Grb2 identifies a degradative compartment formed in Src-transformed fibroblasts and HUVEC cells (Figure 5 (model)). Furthermore, it suggests that these structures may specifically utilize Grb2 to activate N-WASp for initiation of actin polymerization (Figure 5 (model)). In addition to Grb2, Cdc42 is also involved in the assembly of degradative structures formed in both Src-transformed fibroblasts (Gelman & Gao, 2006) and endothelial cells (Billottet et al, 2008; Moreau et al, 2006).

We show that podosomes formed in macrophages are not enriched with either endogenous Nck1 or Grb2 suggesting that podosomes may require a distinct upstream regulation of WASp. Consistent with this, many studies have shown that Cdc42 is required for podosome formation in macrophages (Dovas et al, 2009; Linder et al, 1999) and dendritic cells (Burns et al, 2001) (Figure 5 (model)). The convergence of tyrosine kinases on WASp could also act synergistically with Cdc42 in the establishment of mature, functional podosomes (Figure 5 (model)). Indeed, Cdc42 appears to be required for efficient WASp tyrosine phosphorylation in vitro and in cells (Cammer et al, 2009; Park & Cox, 2009), while the tyrosine phosphorylation of WASp appears to affect correct targeting of active WASp to podosomes, and podosome formation and dynamics in macrophages (Blundell et al, 2009; Dovas et al, 2009). While the contribution of Cdc42 to WASp activation and phosphorylation has been well established, the targeting mechanisms of WASp to sites of activity have been linked to its binding to SH3 domain containing proteins, such as Nck (Zeng et al, 2003). While we were unable to detect Nck enrichment in podosomes, the possibility of Nck acting upstream in the targeting of WASp for the formation of nascent podosomes remains a possibility. Additionally, distinct SH3 domain containing proteins may target WASp to podosomes, e.g. Crk adaptors. Future studies will investigate more precise mechanisms used by invadopodia, podosomes, and degradative structures formed in Src-transformed fibroblasts to regulate the temporal and spatial activation of distinct N-WASp/WASp activators.

Mechanisms that regulate endogenous Nck1 localization to invadopodia, but not podosomes

Here we show that endogenous Nck1 enrichment is specific to invadopodia formed in invasive cancer cells. Previously, we have demonstrated that cortactin tyrosine phosphorylation is critical for Nck1 recruitment to invadopodia (Oser et al, 2009) and presumably involves a previously described direct binding interaction between phosphorylated cortactin and Nck1 (Tehrani et al, 2007). In melanoma cells, it was also recently demonstrated that Tks5 phosphorylation by Src is important for Nck1 recruitment to invadopodia (Stylli et al, 2009). Thus, it appears that Nck1 is recruited to invadopodia by phosphorylated cortactin and phosphorylated Tks5 (Oser et al, 2009; Stylli et al, 2009). We show here that endogenous Nck1 is not enriched in podosomes formed in macrophages. This may be explained by the observations that cortactin is highly overexpressed in many human cancers including head and neck squamous carcinomas, breast, and ovarian cancers (Weaver, 2008a). In contrast, some macrophage cell lines express HS1, a hematopoietic homologue of cortactin, but do not express high levels of cortactin (Tehrani et al, 2006). Thus, the targeting of endogenous Nck1 to invadopodia, but not podosomes, may be a result of cortactin overexpression in highly aggressive cancer cells that form invadopodia (Ammer & Weed, 2008; Weaver, 2008a).

Src transformation alters the stability of ventral cell surface degradative structures

Src transformation of fibroblasts causes a redistribution of cytosolic proteins to form a ventral cell surface degradative compartment previously described as both an invadopodium

and a podosome (Buschman et al, 2009; Mizutani et al, 2002; Seals et al, 2005). Interestingly, Src transformation can also induce invadopodium formation in metastatic mammary carcinoma cells (Artym et al, 2006; Cortesio et al, 2008; Oser et al, 2009), but the invadopodia formed in response to Src are short-lived structures that disassemble before acquiring the ability to degrade the ECM (Oser et al, 2009). Consistent with this is the observation that podosomes formed in Src^{-/-} osteoclasts are long-lived structures with slower rates of actin turnover (Destaing et al, 2008). Interestingly, one notable difference between invadopodia and podosomes is the average lifetime or persistence of each structure (Linder, 2007). While invadopodia are relatively stable with an average lifetimes of >30 minutes (Yamaguchi et al, 2005), podosomes have much shorter lifetimes (average lifetime <10 minutes) (Linder, 2007). Future studies will investigate whether different levels of Src activity are responsible for the differences in lifetimes among these degradative structures.

Transformation and cross-talk between focal adhesions and ventral cell surface degradative structures

Grb2 localizes early and is critical for the formation of degradative structures in Src-transformed fibroblasts (Oikawa et al, 2008). The same study also demonstrated these structures arise from focal adhesions after Src-transformation induces Grb2-Tks5 complex formation and increased PI(3,4)P₂ at the plasma membrane. Tks5 then binds to PI(3,4)P₂-enriched membrane domains to function as a scaffold for N-WASp recruitment and activation (Oikawa et al, 2008). Grb2 localizes to focal adhesions before complete transformation into the degradative structure (Oikawa et al, 2008). Thus, the specific localization of Grb2 in these structures could be a result of their focal adhesion-like properties. This may also explain why other focal adhesion proteins, such as vinculin, are present in degradative structures in Src-transformed fibroblasts, but are excluded from invadopodia (Chan et al, 2009; Oikawa et al, 2008). Interestingly, we show here that Grb2 is excluded from podosomes formed in macrophages suggesting that, unlike the degradative structures formed in Src-transformed fibroblasts (Oikawa et al, 2008), podosome biogenesis in macrophages may not require the formation of a Grb2-Tks5 complex.

Focal adhesions can regulate invadopodium formation in metastatic mammary carcinoma cells through the regulation of a FAK-Src pathway (Chan et al, 2009). However, in these cells the two structures remain spatially distinct (Chan et al, 2009). Thus, it is reasonable to hypothesize that in Src-transformed fibroblasts, increased Src activity leads to the fusion of focal adhesions and invadopodia (Oikawa et al, 2008), and the FAK-Src pathway is no longer able to regulate the formation of the invadopodium as a spatially distinct structure from a focal adhesion. This may lead to the fusion of focal adhesions and invadopodia as observed in Src-transformed fibroblasts (Oikawa et al, 2008). These results may explain why Grb2 is enriched in degradative structures in Src-transformed fibroblasts, but not enriched in invadopodia nor podosomes formed in the absence of Src. It will be interesting to determine whether controlled regulation of the FAK-Src pathway is critical for the spatial segregation of focal adhesions and invadopodia, and whether different localization patterns between invadopodia and degradative structures formed in Src-transformed fibroblasts are a result of focal adhesion-like properties due to increased Src activity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Endogenous Nck1 localizes to invadopodia formed in metastatic mammary carcinoma cells, but does not localize to podosomes formed in macrophages

(A) Representative images of MDA-MB-231 cells fixed, and stained for cortactin and Nck1 showing that Nck1 colocalizes with cortactin in matrix degrading invadopodia (insets). Bar, 10 μ m. (B) Representative images of human monocyte-derived macrophages fixed and stained for F-actin, vinculin, and Nck1 showing that Nck1 does not colocalize to podosomes (insets). Bar, 10 μ m.

Figure 2. Endogenous Nck1 does not localize to ventral cell surface rosettes formed in Src-transformed fibroblasts or PMA-stimulated HUVEC cells

(A) Representative images of Src-transformed fibroblasts fixed and stained for F-actin and Nck1 showing that Nck1 does not colocalize with F-actin in ventral cell surface rosettes (insets). Bar, 10 μ m. (B) Representative images of PMA-stimulated HUVEC cells fixed and stained for F-actin, vinculin, and Nck1 showing that Nck1 does not colocalize to ventral cell surface rosettes (insets). Bar, 10 μ m. (C) Representative images of PMA-stimulated HUVEC cells fixed and stained for F-actin, vinculin, and Nck1 showing that Nck1 does not colocalize to individual dot-like ventral surface structures (insets). Bar, 10 μ m.

Figure 3. Endogenous Grb2 does not localize to invadopodia formed in metastatic mammary carcinoma cells nor podosomes formed in macrophages

(A) Representative images of MDA-MB-231 cells fixed, and stained for cortactin and Grb2 showing that Grb2 does not colocalize with cortactin in matrix degrading invadopodia (insets). Bar, 10 μ m. (B) Representative images of human monocyte-derived macrophages fixed and stained for F-actin, vinculin, and Grb2 showing that Grb2 does not colocalize to podosomes (insets). Bar, 10 μ m.

Figure 4. Endogenous Grb2 localizes to ventral cell surface rosettes formed in Src-transformed fibroblasts and PMA-stimulated HUVEC cells

(A) Representative images of Src-transformed fibroblasts fixed, and stained for F-actin and Grb2 showing that Grb2 colocalizes with F-actin in ventral cell surface rosettes (insets). Bar, 10 μ m. (B) Representative images of PMA-stimulated HUVEC cells fixed and stained for F-actin, vinculin, and Grb2 showing that Grb2 colocalizes to ventral cell surface rosettes (insets). Bar, 10 μ m. (C) Representative images of PMA-stimulated HUVEC cells fixed and stained for F-actin, vinculin, and Grb2 showing that Grb2 colocalizes to individual dot-like ventral surface structures (insets). Bar, 10 μ m.

Figure 5. Distinct upstream activators are involved in N-WASP/WASp regulation in invadopodia, podosomes, and ventral cell surface degradative structures formed in Src-transformed fibroblasts

(Left) Cartoon of a metastatic cancer cell (top left) showing that Nck1 (Oser et al, 2009; Stylli et al, 2009; Yamaguchi et al, 2005) and Cdc42 (Ayala et al, 2009; Sakurai-Yageta et al, 2008; Yamaguchi et al, 2005) are involved in regulating N-WASP activation (bottom left) in invadopodia. Nck1 is shown in blue to highlight a unique marker of invadopodia. (Middle) Cartoon of a macrophage (top middle) showing that Cdc42 (Billottet et al, 2008; Burns et al, 2001; Dovas et al, 2009; Linder et al, 1999), and tyrosine phosphorylation by non-receptor tyrosine kinases (Blundell et al, 2009; Dovas et al, 2009) regulate WASp activation (bottom middle) in podosomes. (Right) Cartoon of an Src-transformed fibroblast or a HUVEC cell (top right) showing that Grb2 (Oikawa et al, 2008), and Cdc42 (Billottet et al, 2008; Gelman & Gao, 2006; Moreau et al, 2006), are involved in regulating N-WASP activation (bottom right) in degradative structures formed in Src-transformed fibroblasts and PMA-stimulated HUVEC cells. Grb2 is shown in red to highlight a unique marker of degradative structures formed in Src-transformed fibroblasts and PMA-stimulated HUVEC cells. PM=plasma membrane.