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Spatiotemporal regulation of Src and its substrates at invadosomes

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Abstract

In the past decade, substantial progress has been made in understanding how Src family kinases regulate the formation and function of invadosomes. Invadosomes are organized actin-rich structures that contain an F-actin core surrounded by an adhesive ring and mediate invasive migration. Src kinases orchestrate, either directly or indirectly, each phase of the invadosome life cycle including invadosome assembly, maturation and matrix degradation and disassembly. Complex arrays of Src effector proteins are involved at different stages of invadosome maturation and their spatiotemporal activity must be tightly regulated to achieve effective invasive migration. In this review, we highlight some recent progress and the challenges of understanding how Src is regulated temporally and spatially to orchestrate the dynamics of invadosomes and mediate cell invasion.

Keywords

Src; invasion; invadosome; invadopodia; podosome; rosette; Rho; Cdc42; FAK; Cortactin; mAbp1; ROS; MT1-MMP

Introduction

Invadosomes are highly dynamic, actin-rich, protrusive structures that promote adhesion to and degradation of the extracellular matrix (ECM), facilitating invasive cell migration. The collective term invadosomes includes podosomes that form in macrophages, dendritic cells, osteoclasts and endothelial cells, and invadopodia that are associated with cancer cells (Saltel et al., 2011). Invadosomes are generally composed of an actin-rich core with actin-nucleating components including cortactin, N-WASP and Arp2/3, surrounded by a ring of adhesion and adaptor proteins such as vinculin, paxillin, and integrins. These protrusive structures promote localized secretion of degradative enzymes including matrix metalloproteinases (MMPs) and can exist independently as dot-like structures or they can be arranged into complex metastructures such as clusters and rosettes (Figure 1). In osteoclasts, podosomes can mature further into a sealing belt that forms a cavity to mediate bone

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degradation and resorption (Jurdic et al., 2006). The dynamic formation, disassembly and degradation activity of both podosomes and invadopodia have been implicated in invasive cell migration (Linder et al., 2011; Pan et al., 2011; Badowski et al., 2008; Calle et al., 2006; Varon et al., 2006; Seals et al., 2005).

Cell migration and invasion are necessary for a variety of physiological functions including leukocyte trafficking, development, and wound repair. Defective podosome formation can be seen in inherited disorders including Wiskott Aldrich syndrome (Linder et al., 1999; Nusblat et al., 2011), PAPA syndrome (Cortesio et al., 2010), and potentially Frank-Ter-Haar syndrome (Iqbal et al., 2010; Buschman et al., 2009), while defects in osteoclast podosomes are associated with osteopetrosis (Gil-Henn et al., 2007). Moreover, cancer invasion and metastasis have been associated with the formation of dynamic, actin rich invadopodia with the capacity for matrix degradation both in vitro and in vivo (Eckert et al., 2011; Gertler and Condeelis, 2010; Philippar et al., 2008; Packard et al., 2009). Although podosomes and invadopodia are important during invasive migration, it has been suggested that podosome rosettes of smooth muscle cells, vascular endothelial cells, aortic endothelial cells, or fibroblasts may also function in ECM remodeling (Daubon et al., 2011; Rottiers et al., 2009), mechanosensing (Collin et al., 2008) and adhesion to the ECM (Boateng et al., 2012; Kocher et al., 2009; Collin et al., 2006).

Podosomes and invadopodia are highly dynamic and require tight regulation to control their rapid formation and turnover. In contrast to other adhesion structures like focal adhesions, podosomes and invadopodia are primary sites of rapid actin polymerization and are not associated with stabilized actin filament bundles (Destaing et al., 2003; Ochoa et al., 2000). Invadosome cores contain signaling molecules such as Rho GTPases (Bravo-Cordero et al., 2011) and Src family kinases (Gavazzi et al., 1989), as well as actin regulatory proteins including cortactin (Bowden et al., 1999), WASP (Linder et al., 1999), and the actin nucleating Arp 2/3 complex (Yamaguchi et al., 2005). Other components generally concentrate in the surrounding ring structure including integrins and integrin-associated proteins like vinculin, talin and paxillin (Gavazzi et al., 1989; Marchisio et al., 1988; Linder and Aepfelbacher, 2003). As highly dynamic structures, invadosomes can assemble and disassemble within minutes, but in some cases can stabilize and exist for hours.

The invadosome lifetime is divided into stages including assembly, maturation and disassembly (reviewed by Murphy and Courtneidge, 2011). During invadosome precursor formation, signaling proteins such as transmembrane growth factor receptors (Rottiers et al., 2009; Varon et al., 2006) and/or cytoplasmic kinases, Src and protein kinase C (PKC) (Tatin et al., 2006; Gatesman et al., 2004), organize with structural and adaptor proteins including Tks5, Nck1, and cortactin (Gatesman et al., 2004; Stylii et al., 2009; Oser et al., 2009; Crimaldi et al., 2009; Oser et al., 2010) to recruit the Arp2/3 complex and mediate actin polymerization (Yamaguchi et al, 2005). Under some conditions, actin may be organized into metastructures such as clusters and rosettes. Next, the maturation stage includes protrusion mediated by actin bundling or cross-linking proteins (Li et al., 2010; Guiet et al., 2012) and microtubules (Schoumacher et al., 2010), stabilization of actin filaments through cortactin (Oser et al., 2009) and secretion or localization of proteases for ECM degradation (Clark et al., 2007; Chen and Wang, 1999; Nakahara, 2007). Finally, during disassembly, the actin core is dismantled and invadosome components disassociate (Badowski et al., 2008; Cortesio et al., 2008). Understanding the signaling mechanisms and functional components of invadosome formation and turnover has been a key focus for invadosome research and has implications to developing drug targets that control cell invasion.

A major candidate therapeutic target is the non-receptor tyrosine kinase, Src (Wadhawan et al., 2011). Src kinase, often referred to as “the oldest oncogene”, has received considerable

attention due to its role in cell transformation and cancer cell invasion. v-Src was initially discovered as the transforming agent of the rous sarcoma virus (David-Pfeuty and Singer, 1980; Tarone et al., 1985), and its cellular counterpart, c-Src, have been the focus of intensive investigation in cancer research. Src is over-expressed or constitutively active in many cancers including breast (Biscardi et al., 1998; Ottenhoff-Kalff et al., 1992), prostate (Nam et al., 2005), and colon cancer (Cartwright et al., 1989; Talamonti et al., 1993), and plays an integral role in regulating each stage of the formation and turnover of invadosomes by targeting distinct substrates. The Src family kinases (SFKs) are composed of nine members: Src, Yes, Fyn, Fgr, Yrk, Hck, Lck, Lyn and Blk (Martin, 2001), with Src, Fyn, and Yes being ubiquitously expressed in non-hematopoietic cells. Src is a non-receptor tyrosine kinase and its mechanism of activation has been well studied over the past several decades (Martin, 2001; Sicheri et al., 1997; Xu et al., 1997; Yeatman, 2004). At the amino terminus, Src has an SH3 and SH2 domain that mediate protein-protein interactions, followed by a linker region and a kinase domain at the C-terminus. During its inactive state, Src is phosphorylated at Y527, which maintains inhibitory intramolecular interactions. When active, the SH2 and SH3 domains are released to initiate intermolecular interactions, and the kinase domain autophosphorylates tyrosine 416 in the activation loop of the catalytic domain for full activity (Kmiecik et al., 1988).

Src can be regulated by kinases and phosphatases, or protein-protein interactions with its SH2 and SH3 domains. Negative regulators of Src kinase activity include the non-receptor C-terminal Src kinase, Csk (Ia et al., 2010; Okada and Nakagawa, 1989) and the Csk homologous kinase, Chk (Zrihan-Licht et al., 1997). Csk and Chk phosphorylate Src at Y527 to induce Src folding and autoinhibition. Conversely, phosphatases that activate Src by dephosphorylation of the C-terminal phosphotyrosine, including PTPα (Zheng et al., 1992), SHP-1 (Somani et al., 1997), SHP-2 (Hakak et al., 2000), PTP1B (Bjorge et al., 2000; Cortesio et al., 2008), and PTP-PEST(Chellaiah and Schaller, 2009), release the autoinhibitory configuration of Src, thereby leading to its activation. Both PTP1B and PTP-PEST regulate Src activity at invadopodia and podosomes, respectively (Cortesio et al., 2008; Chellaiah and Schaller, 2009). PTP1B regulates Src phosphorylation at the C-terminal tyrosine during invadopodia formation and proteolysis and activation of PTP1B by calpain-2 can amplify Src activity during invadopodia assembly (Cortesio et al., 2008). PTP-PEST localizes to osteoclast podosomes (Chellaiah et al., 2001) and is important for the control of rosette formation in Src-transformed fibroblasts (Diaz et al., 2009); however, how PTP-PEST regulates Src activity at podosomes is not clear.

In this review, we focus on the challenges of understanding how Src participates in different steps during the invadosome lifecycle, and further, how Src activity can be involved in opposing functions, including roles in assembly and disassembly of invadosomes. Despite substantial progress in understanding the role of Src kinases at invadosomes, fundamental questions remain unanswered. What is the switch that determines the change between Src-induced invadosome formation and disassembly? How does Src target multiple different substrates to mediate invadosome formation, maturation and disassembly? How is Src activity regulated temporally and spatially to control invadosome dynamics and invasion? Here, we focus on several recent examples from the literature that highlight the challenges of understanding the role of Src at invadosomes. Specifically, we discuss mechanisms that regulate the spatiotemporal control of Src activity at invadosomes and how Src phosphorylation of different substrates can orchestrate distinct stages of the invadosome lifecycle. For additional reviews on Src or invadosome regulation we refer the readers to other sources (Destaing et al., 2011; Frame, 2004; Murphy and Courtneidge, 2011).

Spatiotemporal regulation of Src localization and activity at invadosomes

The temporal and spatial localization and activation of Src at podosomes and invadopodia remains poorly understood. However, there has been substantial progress in understanding Src localization to endosomes and in identifying mechanisms that control Src targeting to other related adhesion structures such as focal adhesions. Src localizes to the perinuclear region where it is generally inactive (Rohrschneider, 1979; Welham and Wyke, 1988; Sandilands et al., 2004) and mutation or dephosphorylation of tyrosine 527 can induce the translocation of Src to focal adhesions independent of its catalytic activity (Kaplan et al., 1994; Timpson et al., 2001). Different members of the Rho GTPase family direct the localization of c-Src to distinct intracellular structures. For example, RhoA directs Src targeting to focal adhesions, Rac1 mediates Src localization at lamellipodia, and Cdc42 induces Src translocation to filopodia in Swiss 3T3 fibroblasts (Timpson et al., 2001). By contrast, the mechanisms that direct Src localization to podosomes or invadopodia remain largely unknown.

Trafficking of Src-associated endosomes to focal adhesions at the cell periphery is dependent on a functional actin cytoskeleton, as well as Rho GTPase signaling (Fincham et al., 1996; Timpson et al., 2001; Sandilands et al., 2004). It is likely that Src trafficking to invadosomes is also dependent on the actin cytoskeleton, however the mechanism of endosome delivery to invadosomes may differ, since, in contrast to focal adhesions, stress fiber formation is often disrupted during podosome or invadopodia formation (Berdeaux et al., 2004; Badowski et al., 2008; Albiges-Rizo et al., 2009). Therefore, it is possible that the dynamic actin “cloud” or “comet tail” polymerization associated with RhoB-containing endosomes may provide a mechanism for the transport of Src to invadosomes (Sandilands et al., 2004). In addition to trafficking of Src from the perinuclear region or cytoplasm to the cell periphery during invadosome formation, Src activity must also be tightly regulated at invadosomes. At the onset of invadosome formation, exogenous factors, including the growth factors EGF, TGF-β and PDGF, lead to Src activation (Kypta et al., 1990; Stover et al., 1995) and induce the formation of podosomes or invadopodia to facilitate invasive migration (Eckert et al., 2011; Mader et al., 2011;Luttrell et al., 1994; Marcotte et al., 2009; Varon et al., 2006). Integrin-mediated adhesion can also activate Src at invadosomes (Destaing et al., 2011).

The importance of having tight regulation of the temporal and spatial activation of Src at invadosomes is highlighted by its complex role during different stages of the invadosome lifecycle including formation, degradation and disassembly. For example, v-Src induces the formation of invadopodia precursors but their lifetimes decrease (Oser et al., 2009), indicating the essential role for Src activity in invadopodia formation as well as their disassembly. Accordingly, v-Src expression enhanced overall matrix degradation, but the amount of degradation at individual invadopodia precursors decreased (Oser et al., 2009). Moreover, expression of constitutively active Src in $SYF^{-/-}$ fibroblast cells (c-Src, Yes, and Fyn null cells) is sufficient for invadopodia formation, but not for their maturation and degradation activity (Kelley et al., 2010). Expression of wild type c-Src was sufficient to rescue the defect in invadosome maturation in these cells, supporting the idea that the temporal and spatial activation of endogenous c-Src is critical for distinct stages of the invadosome lifecycle. The challenge is to understand how Src activity is regulated during invadosome formation, maturation and disassembly. Surprisingly few studies have directly addressed how endogenous Src activity is regulated at invadosomes. In large part, progress has been limited due to the lack of tools to reliably detect changes in endogenous Src activity temporally and spatially, but with the development of new biosensors it is anticipated that there will be rapid progress in this area (Gulyani et al., 2011; Welman et al., 2010).

Redox regulation of Src family kinases at invadosomes

In addition to phosphorylation and SH2/SH3 domain interactions, Src family kinases can be regulated by oxidation of specific cysteine residues by reactive oxygen species (ROS), such as hydrogen peroxide (Giannoni et al., 2005; Kemble and Sun, 2009; Yoo et al., 2011). Moreover, recent studies suggest that ROS are generated at invadopodia and NADPH oxidases and ROS are necessary for invadopodia formation (Diaz et al., 2009; Gianni et al., 2010). It is intriguing to speculate that ROS mediate invadosome assembly through the localized activation of Src kinases. Interestingly, the invadosome component Tks5 associates directly with components of the NADPH oxidase complex, including p22phox and promotes its activity (Diaz et al., 2009), providing a potential positive feedback mechanism through ROS, Src and Tks5 that could amplify signaling to mediate invadopodia assembly.

In addition to Src, the serine kinase PKC can be activated by ROS (Gopalakrishna and Anderson, 1989; Gopalakrishna and Anderson, 1991; Gopalakrishna et al., 1995; Wu et al., 2006) and substantial evidence supports the idea that PKC and Src function coordinately to regulate invadosomes (Burger et al., 2011; Li et al., 2010; Wang et al., 2010; Tatin et al., 2006; Gatesman et al., 2004). For example, podosome formation is induced in THP-1 macrophage cells and endothelial cells by PKC-activating PMA treatment, and inhibition of PKC or Src ablates podosome formation (Burger et al., 2011; Wang et al., 2010; Tatin et al., 2006). Further, the interactions between PKC and Src at podosomes may be orchestrated through the adaptor protein, AFAP110, which contains binding domains for both PKC and Src (Guappone et al., 1998; Qian et al., 2002). In support of this idea, AFAP110-deficient cancer cells have impaired Src activation and podosome formation induced by PKC-activating phorbol myristate acetate treatment. By contrast, ectopic expression of an active AFAP-110 enables Src activation and podosome formation (Gatesman et al., 2004). ROS can activate PKC by oxidation of several cysteine residues in the regulatory domain, causing the release of the closed auto-inhibitory conformation of PKC, while oxidation of cysteine residues in the catalytic domain may have an inhibitory effect on enzyme activity (reviewed by Gopalakrishna and Jaken, 2000). The mechanism of PKC function at invadosomes remains unclear, however phosphorylation of fascin at S39 (a PKC site), is required for its actin bundling activity (Yamakita et al., 1996) as well as invadopodia formation and degradation activity in melanoma cells (Li et al., 2010). These reports suggest that redox signaling may play a key role in regulating PKC as well as Src at invadosomes.

In addition to Src or PKC, redox signaling also regulates MMPs (Rajagopalan et al., 1996; Brenneisen et al., 1997; Yoon et al., 2002; Grote et al., 2003), and Rho GTPases (Lander et al., 1995; Savitsky and Finkel, 2002; Heo and Campbell, 2005; Heo et al., 2006) that regulate invadopodia function; however, these mechanisms are less well characterized. ROS can also amplify phospho-tyrosine signals (Denu and Tanner, 1998; Terada, 2006) at invadosomes by inhibiting phosphatases including PTP1B (Chen et al., 2008; Lee et al., 1998), SHP-2 (Meng et al., 2002), and PTP-PEST (Wu et al., 2005; Diaz et al., 2009). While the majority of evidence points to a positive feedback loop between Src and ROS, oxidation of some substrates can reduce Src activity. For example, PTP1B inhibition by ROS (Bogeski et al., 2006) could provide negative feedback to Src and invadopodia formation. A key challenge is to understand how Src and ROS cooperate to regulate invadosomes and how this feedback loop is regulated during different stages of the invadosome life cycle. Phosphatases that regulate Src activity, such as PTP1B and PTP-PEST, are well-positioned to modulate this feedback loop due to their regulation by redox signaling, and thus represent intriguing targets for future studies.

Focal adhesion kinase (FAK): a key spatiotemporal Src regulator

Src activity is necessary for the formation of invadopodia, podosomes and organized rosette structures (Ammer et al., 2009; Pichot et al., 2009; Destaing et al., 2008; Varon et al., 2006), but not for the formation of focal adhesions (McLean et al., 2000; Volberg et al., 2001; Hamadi et al., 2005). On the other hand, Src activity has been implicated in the disassembly and turnover of both focal adhesions and invadosomes (Fincham and Frame, 1998; McLean et al., 2000; Frame et al., 2002; Hamadi et al., 2005). Several lines of evidence suggest that FAK plays a key role in regulating Src activity at both focal adhesions and invadosomes. Integrin-mediated adhesion to the ECM induces the activation of FAK and Src signaling at focal adhesions (Guan et al., 1991; Kornberg et al., 1992; Arias-Salgado et al., 2003). Adhesion induces FAK autophosphorylation on tyrosine 397, which recruits Src to focal adhesions (Schaller et al., 1994). Subsequent Src-mediated phosphorylation of FAK at Y576/577 renders FAK fully active and the FAK-Src complex phosphorylates multiple scaffolding and signaling proteins at focal adhesions that are involved in adhesion dynamics and motility (Huttenlocher and Horwitz, 2011; Mitra and Schlaepfer, 2006; Playford and Schaller, 2004).

However, how FAK and Src are regulated temporally and spatially to modulate invadopodia or podosomes remains unclear. The SH2 and SH3 domains are important for the localization of Src at adhesions (Destaing et al., 2008), suggesting that specific binding partners, like FAK, may regulate Src localization and activity at invadosomes. Interestingly, in some breast cancer cells, FAK plays an inhibitory role in invadopodia formation by binding and sequestering Src activity at focal adhesions (Figure 2A,B; Chan et al., 2009). Depletion of FAK releases Src from focal adhesions and switches phospho-tyrosine containing proteins from focal adhesions to invadopodia leading to the formation of more invadopodia (Chan et al., 2009). In accordance with an inhibitory role for FAK in invadopodia, α3β1 integrin/laminin-332 sequesters FAK-Src activity at focal adhesions, and FAK inhibition or laminin-332 knockdown enhances invadopodia formation (Liu et al., 2010). These studies suggest that FAK-Src activity is sequestered at focal adhesions and loss of FAK results in redistribution of Src and phospho-tyrosine containing proteins to invadopodia resulting in invadopodia formation. In colon cancer cells and Src-transformed fibroblasts, like breast cancer cells, FAK is not required for invadopodia or dot podosome formation, respectively (Vitale et al., 2008; Pan et al., 2011). It is important to note that although invadopodia produced in FAK-deficient cells are functional, FAK depletion impairs invasive migration of breast cancer cells due to the requirement of FAK for cell migration (Chan et al., 2009). This is in agreement with many studies that have supported a key role for FAK in invasive migration (Hauck et al., 2002; Alexander et al., 2008). However, FAK localization and function at invadopodia seems to be dependent on the mechanical properties of the matrix, since FAK is activated at invadopodia and enhances invadopodia degradation activity on rigid matrices (Figure 2B; Alexander et al., 2008).

Although endogenous FAK is not necessarily required for invadosome formation, recent evidence suggests that FAK plays an important role in the formation of organized podosome rosettes (Pan et al., 2011). FAK-Src signaling induces the assembly of podosome rosettes through FAK-mediated docking of p130Cas and suppression of Rho signaling (Figure 2A,B; Pan et al., 2011). Moreover, FAK-Src signaling at focal adhesions can serve as a platform for rosette formation in Src-transformed fibroblasts (Oikawa et al., 2008). Specifically, Src activation at focal adhesions induces the switch to podosome rosettes through PtdIns(3,4)P_2-mediated recruitment of Tks5 and Grb2 (Oikawa et al., 2008). The focal adhesion-PtdIns(3,4)P_2-Tks5-Grb2 complex can function as an early precursor before the actin polymerization machinery is recruited to mediate the switch from focal adhesions to podosome rosettes (Figure 2A,B; Oikawa et al., 2008). Collectively, FAK is a key adaptor protein that spatiotemporally regulates Src activity at adhesions to regulate invadosome

dynamics; however the effects of FAK on invadosomes are likely context-dependent and can be influenced by the mechanical properties or composition of the matrix.

Src targets multiple substrates to regulate different stages of invadosome dynamics

Src is necessary and sufficient for invadosome formation: ectopic expression of activated Src induces invadopodia and podosomes while Src inhibition impairs their formation. Src promotes invadosome formation by phosphorylating many different substrates including the adaptor protein Tks5 and the actin regulatory proteins cortactin and N-WASP (for a list of Src substrates, see Table 1; Park et al., 2005; Stylli et al., 2009; Tehrani et al., 2007). Src also regulates the degradation activity of invadopodia by affecting MT1-MMP endocytosis through CIP4 (Hu et al., 2011) and endophilin A2 (Wu et al., 2005b), and disassembly through the targeting of specific substrates such as paxillin (Badowski et al., 2008) or indirectly through calpain-mediated substrate proteolysis (Calle et al., 2006; Cortesio et al., 2008). Currently, we do not fully understand how Src targets so many different substrates to orchestrate invadosome formation and turnover during invasive motility. We speculate that Src achieves its specificity at least in part through the temporal and spatial localization of its substrates, in addition to its localized activation. Here we focus on a few specific Src targets that are involved in distinct stages of invadosome formation and turnover.

Src regulates Rho GTPase signaling during invadosome formation

Rho GTPases are required for podosome and invadopodia formation, and Rho GTPase activity is tightly regulated, in part by Src-mediated phosphorylation of both GTPase activating proteins (GAPs) and guanine nucleotide exchange factors (GEFs). For example, Src phosphorylates Fgd1, a GEF for Cdc42 (Miyamoto et al., 2003) and active Cdc42 works in concert with N-WASP to facilitate actin polymerization during invadopodia formation (Ayala et al., 2009). In addition, Src can activate both Cdc42 and RhoA by activating the Rho GTPase GEF, Arhgef5, to mediate podosome and invadopodia formation (Kuroiwa et al., 2011).

Several lines of evidence suggest that RhoA activity is important for podosome rosette formation, however, RhoA activity must be tightly regulated (Berdeaux et al., 2004; Pan et al., 2011; Schramp et al., 2008; Chellaiah et al., 2000). Src activates RhoA at rosettes by phosphorylating RhoGDI, to limit its inhibitory effects on RhoA (DerMardirossian et al., 2006). By contrast, mechanisms that limit RhoA activity during rosette formation include Src-mediated activation of FAK (Pan et al., 2011) and ERK5 (Schramp et al., 2008), and possibly through p190RhoGAP (Bravo-Cordero et al., 2011; Fincham et al., 1999). The targeting of both GAPs and GEFs by Src provides an additional challenge for understanding the spatiotemporal control of downstream effectors during podosome and invadopodia formation and turnover. Recent work provides an example of strict spatiotemporal organization of active RhoC at invadopodia. Within the invadopodia core, p190RhoGAP can inhibit RhoC activity which results in cofilin-mediated generation of barbed ends and actin polymerization (Bravo-Cordero et al., 2011). Conversely, surrounding the core, p190RhoGEF can activate RhoC, and cofilin activity is sequestered (Bravo-Cordero et al., 2011). It is an intriguing idea that Src may regulate the spatiotemporal activation of RhoC at invadopodia through the opposing functions of temporally and spatially restricted GAPs and GEFs.

Src targets actin regulatory proteins with different roles at invadosomes

Cortactin and mammalian actin-binding protein-1 (mAbp1) are actin regulatory proteins with high structural homology, and both have been implicated in regulating sites of dynamic

F-actin such as lamellipodia and invadosomes. Cortactin is a multi-functional Src substrate that is involved in both the assembly and maturation of invadosomes and is important for invasive cell migration (Bowden et al., 1999; Clark et al., 2007; Oser et al., 2009; Desmarais et al., 2009). Src phosphorylates cortactin at three sites, Y421, Y466 and Y482 (Huang et al., 1998), and cortactin phosphorylation is important for barbed end formation at invadopodia precursors (Figure 3A), but not for precursor formation itself (Oser et al., 2009). For example, phosphorylated cortactin releases its inhibitory grip on cofilin to allow for cofilin-mediated actin severing and barbed end formation at invadopodia (Figure 3B; Oser et al., 2009). In addition to its effects on the actin-severing activity of cofilin, phosphorylated cortactin facilitates actin polymerization through its interactions with N-WASp and Arp2/3 (Figure 3B; Oser et al., 2009; Tehrani et al., 2007; Martinez-Quiles et al., 2004), therefore, cofilin and the Nck1-cortactin-N-WASp-Arp2/3 complex, work synergistically to create barbed ends and mediate actin polymerization at invadopodia (DesMarais et al., 2009; Ichetovkin et al., 2002).

mAbp1, in contrast to cortactin, impairs invadopodia formation and invasive cell migration (Boateng et al., 2012). mAbp1 is phosphorylated by Src at two sites, Y337 and Y347 (Larbolette, 1999; Lock et al., 1998), and phosphorylation of these sites is important for mAbp1-mediated inhibition of podosome dot formation and invasion (Boateng et al., 2012). Since these two actin-binding Src substrates appear to have opposing roles during invadopodia/podosome dot formation, then the spatiotemporal phosphorylation of these substrates will help to elucidate how they work together to regulate invadosome dynamics. Live imaging experiments suggest that cortactin is present at invadopodia during early assembly, stabilization, and early degradation, but not during late degradation activity (Artym et al., 2006), while mAbp1 appears to arrive at mature podosome dots and facilitates the formation of podosome rosettes (Boateng et al., 2012). These results suggest that Src may target different actin-binding proteins during distinct stages of invadosome formation and maturation to regulate actin dynamics and invadosome function.

Src regulates degradation activity and MT1-MMP function

MT1-MMP is a transmembrane matrix metalloproteinase that is critical for the degradative functions of invadopodia (Poincloux et al., 2009; Williams and Coppolino, 2011) and its trafficking to invadopodia is a growing area of interest. MT1-MMP is a target of Src-mediated phosphorylation and Src appears to modulate the function of MT1-MMP by regulating its endocytosis rather than directly controlling its protease activity (Nyalendo et al., 2007; Wu et al., 2005b). Src can directly phosphorylate MT1-MMP on Y573 at the cytoplasmic tail, downstream of EGF signaling (Figure 3B). This phosphorylated tyrosine is important for tumor cell proliferation and 3D invasion of collagen matrices (Nyalendo et al., 2008) and inhibition of MTI-MMP tyrosine phosphorylation at Y573 in mice impairs tumor progression (Nyalendo et al., 2010). Interestingly, this Src-phosphorylated tyrosine is part of a critical sequence (LLY573) that is required for clathrin-mediated uptake of MT1-MMP, which has been implicated in cell invasion (Uekita et al., 2001). Whether Src-phosphorylation of MT1-MMP specifically promotes or inhibits clathrin-mediated endocytosis remains to be determined. On the other hand, Src phosphorylates endophilin A2 to inhibit MT1-MMP endocytosis and enhance matrix degradation at podosome rosettes (Wu et al., 2005b). Further, Src phosphorylates the Cdc42 interacting protein (CIP4) to inhibit CIP4-mediated endocytosis of MTI-MMP and enhance degradation activity at invadopodia (Hu et al., 2011). It is likely that Src-effects on MT1-MMP trafficking and activity are complex, but this regulation provides critical control of the late stages of invadosome function and matrix degradation.

Src activity during invadosome disassembly

Src activity is necessary not only for assembly of invadosomes, but also for their disassembly (Badowski et al., 2008; Destaing et al., 2008). Osteoclasts from $Src^{-/-}$ mice form fewer podosomes that are longer-lived and have impaired podosome turnover compared to osteoclasts from control mice (Destaing et al., 2008). Conversely, overexpression of v-Src enhances the turnover of invadopodia in MTLn3 cells leading to shorter-lived invadopodia (Oser et al., 2009). Targeting of specific substrates can mediate invadosome turnover. For example, Src-mediated paxillin phosphorylation on Y31 and Y118 is necessary for the disassembly and turnover of invadosomes (Badowski et al., 2008). Specifically, phosphorylated paxillin can activate calpain through Erk signaling to mediate disassembly (Figure 3C; Badowski et al., 2008; Glading et al., 2001). Calpain-2 is a cysteine protease that cleaves multiple adaptor proteins, such as talin, and paxillin, that are components of invadosomes (Calle et al., 2006). Calpain-2 also cleaves cortactin (Cortesio et al., 2008), WASP (Macpherson et al., 2012) and Pyk2 (Calle et al., 2006) to mediate invadosome disassembly. Accordingly, expression of a calpain-resistant cortactin decreases the rate of invadopodia disassembly (Cortesio et al., 2008). Finally, calpain-2 can positively regulate Src activity through the proteolysis and activation of PTP1B (Cortesio et al., 2008), a phosphatase that activates Src, thereby potentially creating a feedback loop between Src and calpain-2 through PTP1B. Collectively, these studies suggest that Src and calpain-2 coordinately regulate invadosome turnover. The remaining challenge is to determine the spatiotemporal mechanisms by which Src mediates the switch to invadosome disassembly.

Concluding Remarks

There has been substantial progress in understanding how Src regulates invadosomes and invasive migration. However, fundamental questions about how Src functions to mediate the different stages of invadosome formation, maturation and turnover remain unanswered. Challenges include understanding how Src is spatiotemporally regulated during invasive migration. The recent development of biosensors that detect Src activity have suggested that membrane tension can induce localized Src activity (Wang et al., 2005) and the future application of Src biosensors to understand how Src is regulated temporally and spatially will advance the field (Gulyani et al., 2011; Welman et al., 2010). Moreover, tools to read out activation of different Src substrates—such as the phosphorylation of cortactin versus mAbp1 at different stages of invadosome formation and turnover would provide further understanding about how the targeting of different substrates can have such diverse effects. These types of advances will allow us to identify strategies to target Src activity or specific Src substrates to aid in the development of new targeted therapies to modulate invasive cell migration.

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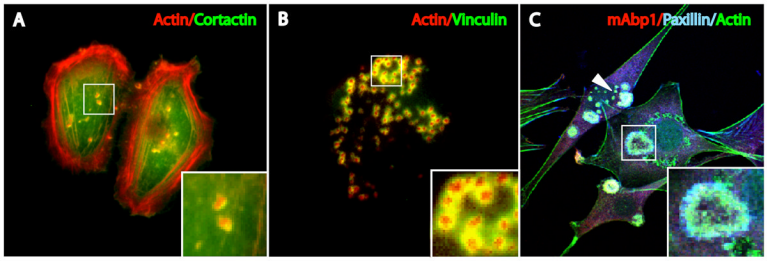

Figure 1. Podosomes and invadopodia from different cell types

(A) Actin and cortactin co-localize at invadopodia in human MDA-231-MD breast cancer cells. (B) Vinculin forms a ring around the actin cores of podosomes in primary human macrophages. (C) NIH 3T3 cells transformed with constitutively active c-Src527F form both dot (arrowhead) and rosette podosomes (inset). Magnified views of podosomes or invadopodia are shown within insets.

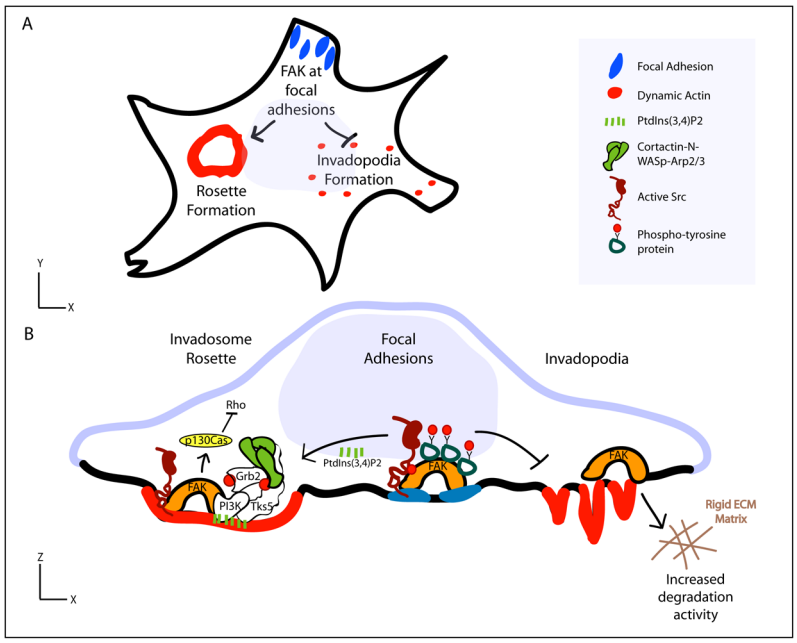

Figure 2. FAK regulation of Src activity

(A) Schematic of FAK regulation of invadosome formation in cells that form both focal adhesions and invadosomes. FAK sequesters Src and phosphotyrosine proteins at focal adhesions to limit invadopodia formation. (B) At focal adhesions, FAK enhances the formation of rosettes. Phosphoinositides and FAK recruit Tks5 and Grb2, which interact with cortactin, N-WASp, and Arp2/3 to mediate dynamic actin polymerization. FAK interacts with p130Cas to inhibit Rho signaling during rosette formation. Although FAK at focal adhesions can sequester phosphotyrosine proteins and impair invadopodia formation, overexpression of FAK can enhance the degradation activity of invadopodia on rigid ECM substrates and promote invasive migration.

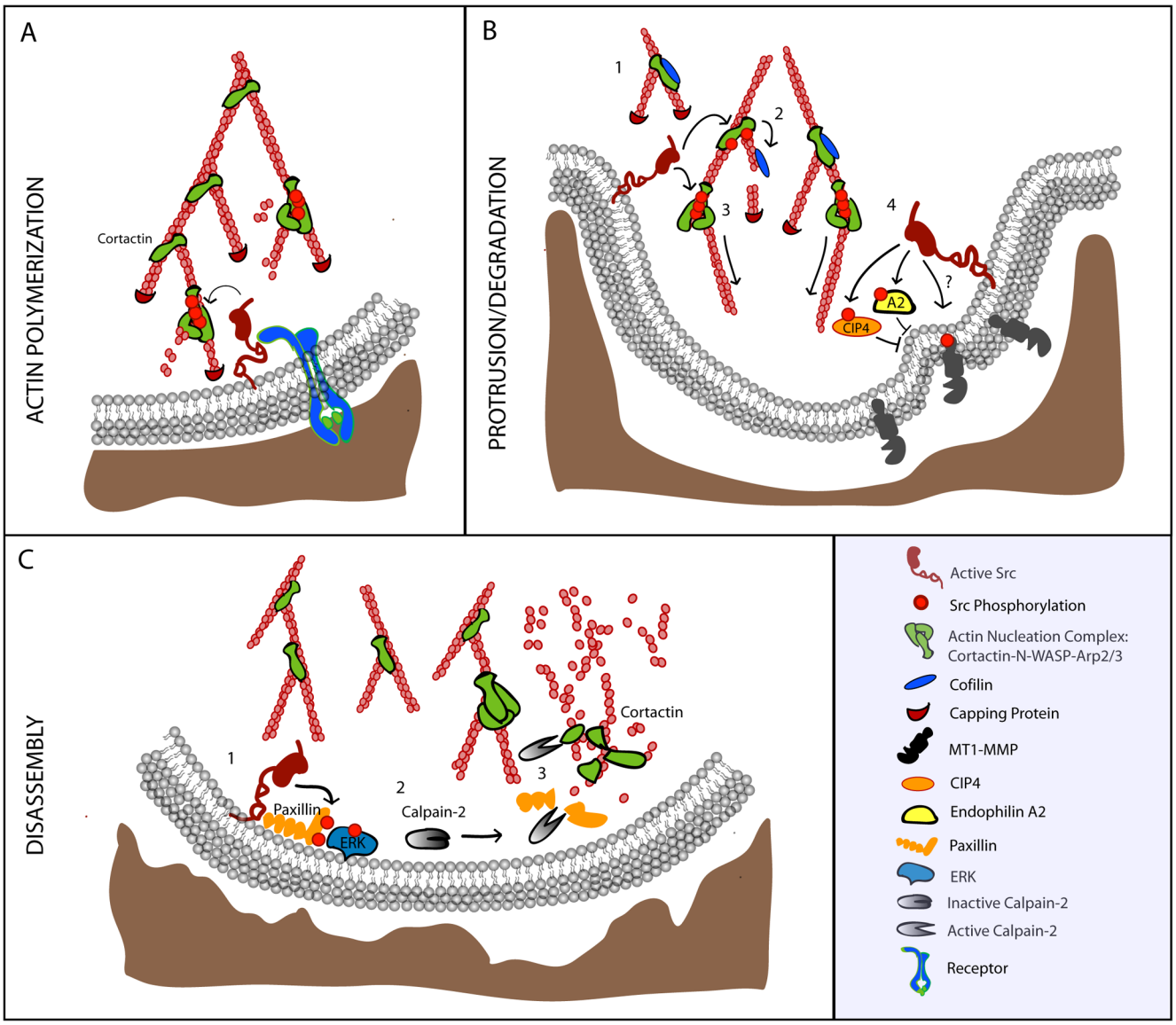

Figure 3. Src regulation of invadosomes

(A) Src becomes activated downstream of receptor and/or integrin-mediated signaling and phosphorylates multiple substrates to induce actin polymerization. Cortactin is phosphorylated at three tyrosine residues and interacts with WASP and Arp2/3 to nucleate new actin branches. (B) 1: New actin branches are stabilized by dephosphorylated cortactin which binds cofilin in an inactive state. 2: During later stages, cortactin is phosphorylated and cofilin is released. Cofilin cleaves actin filaments resulting in the formation of new barbed ends. 3: Cortactin interacts with the N-WASP/Arp2/3 complex to nucleate de novo actin branches. 4: Src can regulate MT1-MMP matrix degradation activity by phosphorylating the cytoplasmic tail at Y573 which may regulate its trafficking. Alternatively, Src can phosphorylate endophilin A2 or the Cdc42 interacting protein (CIP4) to inhibit endocytosis of MT1-MMP thereby increasing surface expression. (C) During disassembly, Src phosphorylates paxillin which activates Erk signaling. Erk leads to the activation of the protease Calpain-2 which cleaves multiple invadosome proteins including paxillin and cortactin to mediate invadosome disassembly.

Table I

Src substrates at invadosomes

Protein	Phosphosite(s)	Invadosome Function	Notes	References
AFAP-110	Y93, Y94, Y451, Y453	Podosome formation	Amplifies Src activity	Guappone et al., 1998; Gatesman et al., 2004
ASAP-1	Y782	Podosome and invadopodia formation	pY782 is required for podosome formation	Bharti et al., 2007
Caveolin	Y14	ND	pY14 involved in podosome rosette-like formation	Colonna and Podesta, 2005
CIP4	Y471	Degradation	p471 inhibits endocytosis of MT1-MMP at invadopodia	Hu et al., 2011
Cortactin	Y421, Y466	Actin polymerization	pY421 and pY466 interact with Nck1 to facilitate actin polymerization	Oser et al., 2010; Tehrani et al., 2007; Head et al., 2003
	Y482	Degradation	Y421D enhances degradation; 3YF inhibits degradation	Ayala et al., 2008
Dynamin	Y231, Y597	ND	Function of Y231 or Y597 unknown at invadosomes	Ahn et al., 1999; Ochoa et al., 2000
	ND	Degradation	Dominant-negative mutant blocks degradation	Baldassarre et al., 2003
Endophilin A2	Y315	Degradation	pY315 inhibits endocytosis of MT1-MMP at podosomes	Wu et al., 2005b
Ezrin	Y477	ND	pY477 is crucial for 3D invasion	Heiska et al., 2011
	ND	Podosome formation	Localizes with cortactin to form rosettes	Kocher et al., 2009
FAK	Y576, Y577	Podosome formation	Necessary for rosette formation	Pan et al., 2011
	Y925	ND	pY925 interacts with Grb2	Schlaepfer et al., 1994
	Y861	ND	pY861 interacts with p130Cas	Lim et al., 2004
	ND	Inhibits invadopodia	Sequesters pY proteins at focal adhesions	Chan et al., 2009
HS1	ND	Podosome organization	Important for podosome array organization, function of tyrosine sites unknown.	Dehring et al., 2011
mAbp1	Y337, Y347	Actin polymerization	pY337 and pY347 Inhibit dot podosome formation	Boateng et al., 2012
		Actin organization	pY347 necessary for rosette formation	Boateng et al., 2012
MT1-MMP	Y573	Degradation	Phosphorylation may have a role in regulating MT1-MMP endocytosis	Nyalendo et al., 2007; Uekita et al., 2001
Nox-A1	Y110	Invadopodia formation	pY110 interacts with Tks4 at invadopodia	Gianni et al., 2010
N-WASP	Y253	Actin polymerization	Interacts with Arp2/3	Park et al., 2005
p130Cas	Y165, Y249, Y410	ND	Src phosphorylation sites reported at focal adhesions, and p130Cas localizes to some invadopodia	Meenderink et al., 2010; Chan et al., 2009
	ND	Podosome formation	FAK-p130Cas complex mediates rosette formation	Wang and McNiven, 2012
p190RhoGAP	ND	Formation/Degradation	Antibodies against p190 inhibit matrix degradation	Nakahara et al., 1998

Protein	Phosphosite(s)	Invadosome Function	Notes	References
Paxillin	Y31, Y118	Disassembly	pY31 and pY118 important for disassembly of inner ring of podosome rosettes	Badowsky et al., 2008; Vindis et al., 2004
Pyk2	Y579, Y580, Y881	Podosome formation	Phosphorylation function unknown at invadosomes. Y881 associates with Grb2	Lakkakorpi et al., 2003; Duong et al., 1998; Schlaepfer et al., 1999
	Y1105	ND	pY1105 associates with p120RasGAP, mediates disassembly of stress fibers	Roof et al., 1998; Fincham et al., 1999
RhoGDI	Y156	ND	pY156 induces release of RhoA, Cdc42 and Rac1 from RhoGDI inhibition	DerMardirossian et al., 2006
Tensin	Y1173, Y1206, Y1256	ND	Src phosphorylation sites identified at focal adhesions. Tensin localizes to podosomes in osteoclasts	Hiura et al., 1995; Qian et al., 2009
	Y25, Y373	Podosome formation	3YF has defect in podosome rosette formation	Buschman et al., 2008
	Y508	Maturation	pY508 interacts with NoxA1 to generate ROS at invadopodia	Gianni et al., 2010
Tks4	Y508	Degradation	3YF cannot degrade matrix at podosomes	Buschman et al., 2008
	Y557, Y619	Podosome and Invadopodia formation	pY557 and pY619 interact with N-WASp to promote actin polymerization	Stylli et al., 2009
Tks5	Y557	Degradation	pY557 interacts with Nck1/2 to promote ECM degradation at invadopodia	