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Significance of Kinase Activity in the Dynamic Invadosome

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Abstract

Invadosomes are actin rich protrusive structures that facilitate invasive migration in multiple cell types. Comprised of invadopodia and podosomes, these highly dynamic structures adhere to and degrade the extracellular matrix, and are also thought to play a role in mechanosensing. Many extracellular signals have been implicated in invadosome stimulation, activating complex signalling cascades to drive the formation, activity and turnover of invadosomes. While the structural components of invadosomes have been well studied, the regulation of invadosome dynamics is still poorly understood. Protein kinases are essential to this regulation, affecting all stages of invadosome dynamics and allowing tight spatiotemporal control of their activity. Invadosome organisation and function have been linked to pathophysiological states such as cancer invasion and metastasis; therapeutic targeting of invadosome regulatory components is thus warranted. In this review, we discuss the involvement of kinase signalling in every stage of the invadosome life cycle and evaluate its significance.

Keywords

Invadopodia; Podosomes; Kinases; Src; Tyrosine phosphorylation; Actin

Introduction

Cell migration is an essential physiological process in development, wound healing and immune system-mediated protection from pathogens. Many different cell types must migrate through tissues and basement membranes as part of their normal function or in response to environmental cues. Methods of cell migration vary depending on the cell type and extracellular environment; cells can move collectively or as single entities, using amoeboid or mesenchymal modes of migration (Friedl and Wolf, 2010). Cancer cells make use of various migration methods to pathologically invade tissues and disseminate from primary tumours, forming new tumours around the body in a process called metastasis (Bravo-Cordero et al., 2012).

In order to facilitate migration, tissue cells can adhere to the extracellular matrix (ECM), produce force via the modulation of the actomyosin cytoskeleton, and degrade the surrounding matrix and basement membranes blocking the path of migration (Parsons et al., 2010). A key feature of migrating cells is cell-matrix adhesions, of which there are different classes with varying functions. This review will focus on two closely related cell-matrix adhesions: podosomes and invadopodia, collectively termed invadosomes (Murphy and Courtneidge, 2011). These are small multi-molecular complexes formed at the plasma membrane, linking the actin cytoskeleton to the extracellular membrane via integrin receptors. While some types of adhesion such as focal adhesions predominantly function to anchor the cell to the ECM, invadosomes are utilised for cell invasion.

Invadosomes consist of a filamentous actin (f-actin) core which contains proteins regulating actin nucleation, elongation and branching, and a ring structure of integrins and integrin-associated proteins (Murphy and Courtneidge, 2011; Schachtner et al., 2013b). Integrins provide a transmembrane link between the actin cytoskeleton and the ECM; in invadosomes, proteins within the ring structure such as paxillin, talin and vinculin link integrins to the f-actin core. As well as cell-matrix adhesion, invadosomes are sites of matrix degradation. At invadosomes, cells secrete various matrix metalloproteases (MMPs) to degrade ECM proteins and basement membranes and consequently facilitate cell migration through tissues (Saltel et al., 2011). Invadosomes also exhibit rigidity sensing capabilities (Alexander et al., 2008; Collin et al., 2008; Labernadie et al., 2014). Atomic force microscopy revealed that podosomes exert traction on the substrate as well as being protrusive (Labernadie et al., 2014) and thus are sensitive to matrix rigidity, suggesting that they play a role in both outside-in and inside-out mechanosensing (Collin et al., 2008).

Invadosomes are formed by many different cell types in response to different signalling events. Monocyte derived cells including macrophages, immature dendritic cells, osteoclasts and megakaryocytes constitutively form podosomes in order to carry out their inherent immune or cell specific functions (Dovas and Cox, 2011; Schachtner et al., 2013b). Macrophages and dendritic cells produce podosomes in order to adhere to the ECM and migrate through tissues towards sites of infection or tissue damage; osteoclast podosomes enable bone remodelling via the formation of podosome superstructures called sealing zones, which act to confine the area of bone resorption; megakaryocyte podosomes penetrate basement membranes to allow proplatelet arm extension into blood vessels for platelet release (Schachtner et al., 2013a). In all these cell types, podosome assembly occurs upon adhesion to ECM proteins and engagement of integrin receptors. Matrix stiffness therefore plays an important role in podosome assembly, and can also affect podosome lifetime and invasiveness (Collin et al., 2008). Clinically, the importance of podosomes is highlighted

in Wiskott-Aldrich syndrome, a well-described immunodeficiency disorder (Kirchhausen, 1998; Thrasher and Burns, 2010). Additionally, osteopetrosis is characterised by increased bone mass due to impaired osteoclast-mediated bone resorption (Tolar et al., 2004).

Invadopodia can be described as a cancer cell's mimic of a podosome. They are formed as adhesive and invasive structures to enable migrating cancer cells to penetrate tissues during metastasis and contain many of the same molecular components as podosomes (Revach and Geiger, 2014). Despite their similarities however, invadopodia are generally observed to be longer lived, sometimes lasting several hours (Sharma et al., 2013) compared to the highly dynamic podosome which typically lasts less than ten minutes (Calle et al., 2004; Evans et al., 2003; Yamaguchi et al., 2005). Also, invadopodia protrude further from the cell body (Enderling et al., 2008) and are more degradative than podosomes (Artym et al., 2011); this is in keeping with the notion that cancer cells adapt normal cellular processes for their own pathological function (Murphy and Courtneidge, 2011). The clinical relevance of invadopodia in cancer progression has been studied in animal models (Eckert et al., 2011; Lohmer et al., 2014). The recent development of multiphoton microscopy in intravital imaging has transformed our ability to visualise cancer cell movement *in vivo* and confirmed the importance of invadopodia in tumour cell intravasation during metastasis (Condeelis and Segall, 2003; Gligorijevic et al., 2012).

The exact mechanisms that regulate invadosome dynamics are not fully understood, however many proteins are known to be involved and regulation is thought to be cell-type specific (Murphy and Courtneidge, 2011). Invadosome formation is initiated in response to signalling events promoting a migratory and invasive phenotype. This includes a variety of stimuli, including matrix adhesion, growth factors and cytokines (Destaing et al., 2011; Hoshino et al., 2013). At the plasma membrane, integrins and receptor kinases relay extracellular signals to downstream signalling molecules such as kinases, GTPases and adaptor proteins. These signalling pathways initiate invadosome formation via the nucleation of branched actin filaments by Wiskott-Aldrich Syndrome Protein (WASP) family members and the actin-related protein 2/3 (Arp2/3) complex; this will form the invadosome actin core (Beaty and Condeelis, 2014; Calle et al., 2008; Hurst et al., 2004; Kaverina et al., 2003; Machesky and Insall, 1998; Thrasher and Burns, 2010).

The invadosome is subsequently stabilised by elongation and cross-linking of actin filaments, and recruitment of various molecules to the region surrounding the core to make up the invadosome ring. The ring structure has been proposed to form a link between integrin receptors contacting the ECM and the intracellular actin core (Murphy and Courtneidge, 2011; Schachtner et al., 2013b). Mature, invadosomes can then recruit and secrete MMPs (Marchesin et al., 2015). Less is known about invadosome disassembly; in dendritic cells, myosin IIA has been suggested to induce contractions of the actin cytoskeleton, which disrupt the structural integrity of podosomes and lead to their dissolution (van Helden et al., 2008). Calpain, a cysteine protease, has also been linked to podosome disassembly, through its cleaving of podosome-related proteins such as talin and WASP (Calle et al., 2006; Macpherson et al., 2012).

While the invadosome life cycle can be described in various stages including formation, proteolytic activity and dissolution (Artym et al., 2006; Branch et al., 2012; Hoshino et al., 2013), the identification of regulatory factors acting at a precise stage has proved experimentally challenging due to the dynamic nature of these structures. This is further complicated by the observations that

podosome f-actin cores can undergo oscillations in stiffness in response to localised actomyosin contractility (Labernadie et al., 2010) and can also undergo fission and fusion events (Evans et al., 2003). Following perturbation of a regulatory factor, the presence or absence of invadosomes can be used to determine whether this factor is necessary for the initiation and formation of the invadosome. The number of adhesions per cell allows further conclusions to be drawn: an increased number of invadosomes per cell would suggest either a promotion of invadosome formation or a defect in invadosome dissolution (Hoshino et al., 2013). Live cell imaging is crucial in determining between these stages, allowing invadosome lifetime measurements, as well as comparisons between the timing of regulatory factor localisation and recruitment of ring proteins or MMPs (Artym et al., 2006; Branch et al., 2012). Additionally, studies have assessed the size of invadosomes or tertiary structures, as well as their ability to degrade matrices such as gelatin (Beaty and Condeelis, 2014; Schachtner et al., 2013b).

Using a combination of such analytical techniques, the key proteins that make up the structure of the invadosome have been well described (Beaty and Condeelis, 2014; Schachtner et al., 2013b), however less is known about the regulatory proteins involved. Kinases are a large group of protein enzymes which function to add phosphate groups to their substrates, resulting in extremely diverse cellular actions. Kinases often act as signal transducers within intracellular signalling cascades in order to affect cell physiology or phenotype; in relation to invadosomes, many different kinases have been implicated in the dynamics of these structures (Hoshino et al., 2013; Saykali and El-Sibai, 2014; Winograd-Katz et al., 2011). Each stage of the invadosome is tightly regulated by signalling factors influencing where and when invadosomes are formed. Kinases are integral components of these signalling pathways, and while certain phosphorylation events have been identified as important, there is much still to be explored. In this review, we aim to describe known kinase involvement in invadosome stimulation, formation, stabilisation, activity and dissolution (Figure 1), and highlight where current knowledge is lacking.

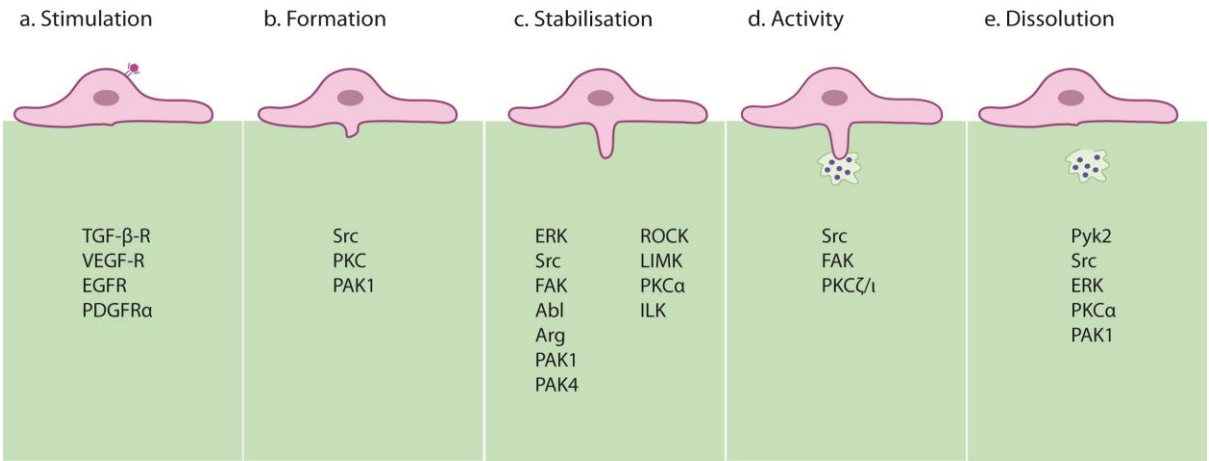

Figure 1. Kinases involved in each stage of the invadosome life cycle. Cell surface receptors such as EGFR become activated by extracellular stimuli and induce signaling cascades leading to the phosphorylation of major regulators of invadosome formation. Kinase activity controls the regulation of effector proteins that in turn dictate the elongation and stability of the actin core, the delivery and secretion of MMPs as well as the turnover of protrusions. Classifying the kinases involved according to the different stages of the invadosome life cycle reveals kinases such as PAK1 being active in more than one stage. This suggests that kinase activity is under tight spatiotemporal regulation resulting in differential roles and functions. Moreover, Src appears to be a major regulator across the invadosome life cycle.

Kinases in invadosome stimulation

The stimuli inducing invadosome formation are varied; they include growth factors, cytokines, ECM proteins, micro RNAs, calcium and reactive oxygen species (ROS) (Murphy and Courtneidge, 2011). The extracellular signals inducing invadosome formation are well studied (Hoshino et al., 2013), as well as the downstream involvement of Rho GTPases (Spuul et al., 2014) and the molecules responsible for the formation of branched f-actin, such as WASP/N-WASP and the Arp2/3 complex (Saykali and El-Sibai, 2014; Schachtner et al., 2013b); however, little is known about the factors regulating these processes. Kinases play a critical role as signal transducers to regulate invadosome initiation; many kinases and phosphorylation events have been shown to be involved, yet it is still unclear how these signals are integrated.

Signalling via enzyme-linked cell surface receptors such as vascular endothelial growth factor receptors (VEGFRs), epidermal growth factor receptor (EGFR) and transforming growth factor-β receptors (TGF-β-Rs) can induce invadosome formation in multiple cell types (Hoshino et al., 2013). Activated receptors act as serine/threonine or tyrosine kinases in order to intracellularly transduce a signal. For example, endothelial cells (ECs) form podosomes in response to growth factor and cytokine signalling, in order to degrade the ECM and basement membranes to allow vascular remodeling and angiogenesis. VEGF signals via the receptor tyrosine kinase VEGFR-2 to stimulate angiogenesis (Meyer et al., 2003); this requires VEGF-induced EC podosome formation. VEGF treatment of ECs results in the activation of the non-receptor tyrosine kinase c-Src, which phosphorylates G protein-coupled receptor kinase-interacting protein 1 (GIT1). C-Src, GIT1 and phospholipase C-γ (PLCγ) are required for podosome formation downstream of VEGF (Wang et al., 2009). VEGF-stimulation also enhances the formation of podosome rosettes by upregulating α6β1 integrin; these rosettes are suggested to be precursors of new blood vessel branches (Seano et al., 2014). TGF-β signalling can also induce podosome formation in ECs. TGF-β-stimulated aortic ECs form podosomes following the Src-mediated activation of Fgd1, which promotes its interaction with Cdc42 and the subsequent recruitment of the actin polymerisation factors Arp2/3 and N-WASP (Daubon et al., 2011; Rottiers et al., 2009; Varon et al., 2006). Furthermore, monocyte-derived cells form podosomes following growth factor signalling. In monocytic THP-1 cells, TGF-β treatment induces podosome formation and a macrophage-like phenotype (Bombara and Ignotz, 1992; Vijayakumar et al., 2015), although the downstream signalling involved is as yet undiscovered.

Similarly, receptor kinase signalling induces invadopodia formation in many different cancer cell types (Gould and Courtneidge, 2014). Activation of EGFR induces invadopodia formation in breast cancer cells, and increases matrix degradation (Busco et al., 2010; Yamaguchi et al., 2005; Zhou et al., 2014). EGFR signalling has been suggested to mediate branched f-actin formation at sites of invadopodia initiation, through the regulation of cortactin via Src and Arg kinases (Mader et al., 2011). EGFR signalling also promotes invadopodia formation in head and neck squamous cell carcinoma, pancreatic cancer and lung cancer cells (Diaz et al., 2013), and signalling is enhanced in hypoxic environments (Md Hashim et al., 2013). While EGFR involvement in metastasis is well studied, other growth factor receptors have also been shown to promote invadopodia formation; for example, TGF-β treatment of breast cancer cells enhances the formation of invadopodia, in a mechanism dependent on phosphoinositide 3-kinase (PI3K) and Src activity (Mandal et al., 2008). TGF-β treatment also induces invadopodia formation in normal breast epithelial cells and promotes epithelial-to-mesenchymal transition (EMT), enhancing the invasive potential of the cell (Pignatelli et

al., 2012). Additionally, activation of the platelet-derived growth factor receptor α (PDGFRα) induces EMT in normal mammary epithelial cells through activating Src and promoting invadopodia formation (Eckert et al., 2011). Activation of the receptor tyrosine kinase Met by its ligand hepatocyte growth factor (HGF) also induces invadopodia formation in fibroblasts, and enhances invadopodia formation in breast cancer cells (Rajadurai et al., 2012).

Alongside cell surface receptor activation, plasma membrane lipids and their modification by PI3Ks have been shown to be important for invadosome formation. Inhibition of PI3K disrupts podosome formation in bone marrow-derived macrophages (Wheeler et al., 2006) and invadopodia of breast cancer cells (Yamaguchi et al., 2010). Expression of active PI3K increases invadosome formation in various cancer cell types (Hoshino et al., 2012; Yamaguchi et al., 2011). PI3K is known to be activated downstream of Src-focal adhesion kinase (FAK) signalling (Hanks et al., 2003), which is upregulated in response to invadosome-stimulating signals. PI3K-mediated phosphorylation of phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$) to form phosphatidylinositol 3,4,5-trisphosphate (PIP_3) results in the downstream activation of 3-phosphoinositide-dependent protein kinase 1 (PDK1) and Akt signalling (Cantley, 2002), a pathway implicated in podosome formation; however the downstream targets of this signalling pathway remain unclear (Hoshino et al., 2013; Yamaguchi et al., 2011). PIP_3 can be dephosphorylated by the phosphatases SHIP2 and SYNJ2 to form phosphatidylinositol 3,4-bisphosphate ($PI(3,4)P_2$), which has been localised to invadosomes of Src-transformed fibroblasts (Oikawa et al., 2008). $PI(3,4)P_2$ is suggested to act as an anchor for the invadosome (Beaty and Condeelis, 2014), via its interaction with tyrosine kinase-substrate 5 (Tks5) (Oikawa et al., 2008; Seals et al., 2005).

In addition to receptor kinase and membrane lipid signalling, invadosome formation can be induced by other extracellular signaling factors, such as ROS, miRNAs and calcium, or by adhesion to ECM components and activation of integrin receptors (Hoshino et al., 2013). While these signalling factors do not directly involve kinases, they are often regulated by kinase activity, and act via kinases to promote invadosome initiation. For example, loss of β1A integrin in Src-transformed fibroblasts abrogates invadosome formation, and it is suggested that direct phosphorylation of β1A integrin by protein kinase C (PKC) promotes downstream invadosome formation (Destaing et al., 2010). Also, signalling via the chemokine receptor CXCR4 activates the kinases Abl and Arg, which phosphorylate and interact with N-WASP, Abi and WAVE2 to affect actin dynamics and promote invadopodia formation (Smith-Pearson et al., 2010).

Kinases in invadosome formation

Src: a major regulator of invadosome formation

While the extracellular factors promoting invadosome formation are varied and complex, these signalling pathways converge on a set of crucial kinases involved in initiating the invadosome actin core. Src is considered the most important kinase in invadosome regulation, being both necessary and sufficient for podosome formation (Berdeaux et al., 2004; Sanjay et al., 2001), and functioning in multiple stages of the invadosome cycle (Figure 1), including formation, stabilisation, MMP secretion and dissolution (Boateng and Huttenlocher, 2012). Additionally, expression of constitutively active Src in fibroblasts induces invadosome formation (Berdeaux et al., 2004; Tarone et al., 1985). The Src family kinases consist of 9 non-receptor tyrosine kinases, of which Src, Yes and Fyn are ubiquitously

expressed (Martin, 2001). The mechanism of Src activation has been well studied; in its inactive state, Src is phosphorylated at Y527, and activation requires a conformational change to release the kinase domain and enable autophosphorylation at Y416 (Kmiecik et al., 1988). Src activation is itself highly regulated; the kinases Csk and Chk phosphorylate Src at Y527 to maintain its inactive conformation (Ia et al., 2010; Zrihan-Licht et al., 1997), and the phosphatases PTPα, PTP1B, PTP-PEST, SHP-1 and SHP-2 remove this inhibition (Boateng and Huttenlocher, 2012; Chellaiah and Schaller, 2009; Cortesio et al., 2008).

In invadosome formation, Src can be activated following integrin-mediated adhesion (Destaing et al., 2011), TGF-β, EGF and PDGF signalling (Eckert et al., 2011; Kypta et al., 1990; Luttrell et al., 1994; Mader et al., 2011; Stover et al., 1995). Src is also activated by ROS-mediated oxidation (Giannoni et al., 2005; Kemble and Sun, 2009; Yoo et al., 2011); ROS are produced at invadosomes, and promote invadosome formation (Diaz et al., 2009; Gianni et al., 2010). PKC can also be activated by ROS (Gopalakrishna and Anderson, 1991; Wu et al., 2006), and has been shown to activate Src via actin filament-associated protein-110 (AFAP-110) to stimulate podosome formation (Gatesman et al., 2004; Tatin et al., 2006).

How Src is able to function in both invadosome formation and turnover remains an important question. Indeed, a siRNA screen of factors affecting actin cytoskeleton regulation in MEF-SrcY527F cells identified 35 phosphatases, MAPKs and tyrosine kinases functioning downstream of Src to influence invadosome dynamics (Winograd-Katz et al., 2011). To add to this complexity, Src can promote the formation of both focal adhesions and invadosomes, despite these structures being thought to be somewhat antagonistic (Boateng and Huttenlocher, 2012). It has been suggested that the various roles of Src are balanced by its spatiotemporal control; for example, FAK may act to sequester Src at focal adhesions (Chan et al., 2009). FAK is activated downstream of integrin-mediated adhesion to the ECM, and in turn recruits Src to focal adhesions, where Src phosphorylates FAK (Calalb et al., 1995; Webb et al., 2004). This Src-FAK complex then acts to regulate multiple focal adhesion components to affect adhesion dynamics. FAK has been shown to inhibit invadopodia in some breast cancer cells by sequestering Src at focal adhesions (Chan et al., 2009; Liu et al., 2010). Down-regulation of FAK results in increased invadosome formation; this is suggested to be due to the relocalisation of Src activity (Boateng and Huttenlocher, 2012). However, the role of FAK-Src interaction in invadosomes is unclear, since FAK activity is reported to be increased at invadopodia and FAK promotes invadopodia-mediated matrix degradation (Alexander et al., 2008).

Src acts to promote invadosome formation by phosphorylation of many varied substrates, with diverse effects (Boateng and Huttenlocher, 2012). The adaptor proteins Tks4 and Tks5 are important Src substrates (Courtneidge et al., 2005; Lock et al., 1998). Tks5 is necessary for invadosome formation in Src-transformed fibroblasts (Seals et al., 2005), and its expression is upregulated in THP-1 cells following PMA-mediated stimulation of podosome formation (Burger et al., 2011). In invadosomes, Tks5 is thought to act as a scaffold protein downstream of Src, interacting with Nck, N-WASP and Grb2 to regulate f-actin dynamics (Oikawa et al., 2008; Stylli et al., 2009). Tks4, a close relative of Tks5, is also phosphorylated by Src and localises to podosome rosettes in Src-transformed fibroblasts (Buschman et al., 2009). It is suggested that both Tks4 and Tks5 are necessary for complete formation and degradative activity of invadosomes (Buschman et al., 2009). Both Tks4 and Tks5 interact with another Src substrate Nox-A1, to promote invadopodia formation (Diaz et al., 2009) as well as the localised production of ROS at invadosomes (Gianni et al., 2010).

Rho GTPases make up a key class of proteins regulated by Src activity at invadosomes (Spuul et al., 2014). Src has been shown to regulate various GEFs, GAPs and GDIs to affect Rho GTPase function at invadosomes; for example, Src phosphorylates RhoGDI to suppress its activity, thus upregulating RhoA activity (DerMardirossian et al., 2006). Src also activates Arhgef5, which in turn activates RhoA and Cdc42 to promote invadosome formation (Kuroiwa et al., 2011). Cdc42 activity is also enhanced at invadosomes by Src-mediated phosphorylation and activation of Fgd1 (Daubon et al., 2011; Miyamoto et al., 2003). Rho GTPases act via effector proteins to regulate cytoskeletal dynamics, however little is known about the role of Rho GTPase effectors in invadosome formation. One important family of Rho GTPase effectors is the P21-activated kinase (PAK) proteins, which have well-established roles in actin cytoskeleton dynamics and cell migration (Dart and Wells, 2013; King et al., 2014). PAKs have known functions in focal adhesions downstream of Rho GTPases (Dart et al., 2015; Delorme-Walker et al., 2011; Wells et al., 2010), and have been implicated in invadosome formation (Gringel et al., 2006; Webb et al., 2005). The interaction of PAK1 with PIX is required for the PIX-GIT complex to translocate to focal complexes and induce cytoskeletal remodeling leading to podosome formation in vSMCs (Webb et al., 2005).

Cell type-specific kinases

To further complicate matters, cell type-specific kinases can play complementary roles in invadosome formation. Pyk2, a FAK family adhesion kinase expressed in the central nervous system and haematopoietic cells, may have similar functions to FAK in some contexts, but is suggested to have a distinct role in haematopoietic cell invadosomes (Duong and Rodan, 2000). In macrophages, Pyk2 colocalises with the podosome ring proteins vinculin, talin and paxillin. Macrophage adhesion to fibronectin and fibrinogen induces autophosphorylation at Y402 and activation of Pyk2; this is mediated by integrin signalling (Duong and Rodan, 2000). Pyk2 has been shown to directly interact with integrin αVβ3 and paxillin in osteoclast podosomes (Pfaff and Jurdic, 2001), and it is suggested that integrin activation recruits Pyk2 to the podosome ring, where Pyk2 phosphorylates paxillin, perhaps stabilizing the ring structure. Furthermore, Pyk2 association with Src is important for podosome formation; Pyk2 kinase activity is thought to be irrelevant, although autophosphorylation at Y402 is required for interaction with Src (Lakkakorpi et al., 2003). Notch2 signalling has also been implicated in the regulation of osteoclast podosome formation, by promoting Pyk2 autophosphorylation and therefore Pyk2-Src interaction (Jin et al., 2016). Additionally, in Pyk2 null mice, podosomes fail to form a belt, implicating the disruption of podosome spatial organisation in the compromised bone resorption observed (Gil-Henn et al., 2007).

The Src-family kinase Hck is another cell type specific kinase involved in invadosome formation (Dovas and Cox, 2011). Hck is expressed in leukocytes and has been shown to phosphorylate WASP, stimulating actin polymerisation in filopodia (Cory et al., 2002). Expression of constitutively-active Hck results in the formation of invadosomes in fibroblasts and macrophages (Cougoule et al., 2005; Poincloux et al., 2006), and is suggested to be important in the formation of podosome tertiary structures. Since both Hck and Src are present in leukocyte podosomes, and Hck levels are increased in $Src^{-/-}$ osteoclasts (Guiet et al., 2008), it is likely that the functions of Hck and Src at podosomes are overlapping.

Kinases in invadosome stabilisation

Invadosome stabilisation refers here to the elongation and stability of the protrusion via actin polymerisation and the cross-linking of actin filaments (Beaty and Condeelis, 2014; Murphy and Courtneidge, 2011). Since much research has focused on the simple parameters of presence or absence of invadosomes, current knowledge of factors influencing their growth and turnover is limited. Despite this, many kinases have been shown to be involved in invadosome dynamics and function, acting to transduce regulatory signals and spatiotemporally control factors influencing invadosomes.

The first step towards the maturation of invadopodia precursors is actin polymerisation, with Src-mediated phosphorylation of WASP/N-WASP (Park et al., 2005) and cortactin (Tehrani et al., 2006) being essential at this stage. Src kinase activity has been shown to be crucial for podosome formation, structure and organisation at the peripheral belt of osteoclasts (Destaing et al., 2008; Luxenburg et al., 2006). Cortactin becomes phosphorylated by extracellular signal-regulated kinase (ERK) on serine residues and by Src on tyrosine residues (Campbell et al., 1999; Desmarais et al., 2009; Head et al., 2003; Martinez-Quiles et al., 2004; Tehrani et al., 2007). ERK phosphorylated cortactin has been shown to bind and thus activate N-WASP while Src phosphorylated cortactin was reported unable to bind N-WASP (Martinez-Quiles et al., 2004). Indeed, cortactin phosphorylation by ERK activates the Nck-N-WASP-Arp2/3 pathway thereby mediating actin nucleation (Oser et al., 2009). At the same time, cortactin phosphorylation releases cofilin from its inactive state thereby initiating cofilin's actin severing activity (Oser et al., 2009). Dephosphorylation of cortactin (which prevents Nck binding) is required for the consolidation of stable long-lived protrusions (Garcia et al., 2016; Oser et al., 2009).

The Abl tyrosine kinases (Abl and Arg) also act downstream of Src to phosphorylate cortactin, promoting adhesion-dependent cell edge protrusion and invadopodia elongation (Lapetina et al., 2009; Smith-Pearson et al., 2010). Arg becomes phosphorylated by Src downstream of EGFR activation and in turn phosphorylates cortactin (Mader et al., 2011). Meanwhile, activation of β1 integrin in immature invadopodia and its subsequent association with Arg drive the maturation of the protrusions to degradation competent invadopodia (Beaty et al., 2013). However, neither Src-mediated Arg activation nor β1 integrin-Arg interaction were enough for complete Arg activation (Beaty and Condeelis, 2014; Beaty et al., 2013), suggesting that multiple signals must converge in order for invadosome stabilisation to occur.

The PAKs make up another major kinase family that is active in invadosomes. Studies suggest that PAKs are involved in the regulation of cytoskeletal dynamics but whether they are important for invadosome dynamics has not been determined (Duan et al., 2012; Webb et al., 2005). PAK4 has been localised to podosomes in primary human macrophages (Gringel et al., 2006; Wells and Jones, 2010), and is suggested to coordinate with αPIX to regulate actin core dynamics, influencing the size of podosomes (Gringel et al., 2006). This suggests a role for PAKs in the maturation and stability of the protrusion rather than its formation. This is further reinforced by the fact that PAK3 phosphorylates cortactin on S113 of the actin binding region (Webb et al., 2006). Indeed, transfection of the autoinhibitory domain of PAK1 (PAK-AID) as well as a non-phosphorylatable cortactin mutant, cortactin^{S113A}, decreased ECM degradation in A375MM cells (Ayala et al., 2008).

Stability of the invadosome actin core requires a synergy of events to occur, with cortactin dephosphorylation and subsequent cofilin inactivation being crucial (Oser et al., 2009). Cofilin has

been shown to be phosphorylated and thus inactivated by LIM motif-containing protein kinase (LIMK) (Yang et al., 1998). The spatial distribution of p190RhoGEF and p190RhoGAP defines the activation state of RhoC in and around invadopodia; RhoC is only active around the invadopodia and can therefore regulate cofilin inactivation via the ROCK-LIMK pathway. As a result, cofilin activity is spatially restricted within the invadopodium core thereby contributing to the directional elongation of the protrusion (Bravo-Cordero et al., 2011).

Likewise, cross-linking of actin filaments by actin-bundling proteins contributes to invadosome stabilisation; this class of proteins can be regulated by kinase activity. Fascin is a cross linking protein providing structural support in filopodia (Vignjevic et al., 2006), and localizing to invadosomes (Li et al., 2010). Fascin has been shown to become stabilised in invadopodia, and a phosphomimic mutant of fascin reduces invadosome-mediated matrix degradation (Li et al., 2010). Phosphorylation of fascin at S39 by PKCα blocks the N-terminal actin binding site thus rendering it inactive (Adams, 2004).

Invadosomes are further stabilised by the recruitment of adhesion proteins and formation of a ring structure surrounding the actin core. Focal adhesion components vinculin and talin were first shown to localise in podosomes of malignant B lymphocytes (Marchisio et al., 1988). Vinculin interacts with talin and other focal adhesion components around the actin core thereby forming a ring that provides support and structural stability in podosomes (Schachtner et al., 2013b). More recently, it was shown that the accumulation of vinculin and talin at the podosome ring is induced by Integrin linked kinase (ILK) thus implicating ILK in podosome stability (Griera et al., 2014). Knockdown of ILK has been shown to reduce the number of invadopodia as well as the delivery of MT1-MMP in head and neck squamous cell carcinoma cells (Branch et al., 2012). However, it is not known whether the kinase activity of ILK is responsible for the process as ILK has mainly been described as an adaptor protein linking integrins with the actin cytoskeleton (Bottcher et al., 2009).

Invadosomes can be organised into tertiary structures such as rosettes or belts. In some cell types this organisation is required for the full function of the mature invadosomes, such as for bone resorption at osteoclast sealing zones (Jurdic et al., 2006). Kinases may act to regulate the formation of these tertiary structures. For example, Src-mediated phosphorylation of mammalian actin-binding protein 1 (mAbp1) is necessary for the formation of invadosome rosettes in Src-transformed fibroblasts (Boateng et al., 2012). A role for FAK, known to also bind Arp2/3 (Serrels et al., 2007; Swaminathan et al., 2016), has also been found in the formation of podosome rosettes; FAK-Src signalling results in the phosphorylation of p130Cas and the suppression of Rho-ROCK signalling, resulting in rosette formation (Pan et al., 2011).

Kinases in invadosome activity

The final stage of invadosome maturity is marked by the delivery of MT1-MMP to the protrusion and the secretion of MMPs (Murphy and Courtneidge, 2011). In addition, MT1-MMP islets have recently been shown to co-localise with podosomes, persist longer than the podosome lifetime and enable podosome re-emergence in primary macrophages (El Azzouzi et al., 2016). This suggests that MT1-MMP accumulation not only regulates the activity of mature invadosomes but also influences their reformation.

In invadosome activity, cortactin once again appears to play an important role, as it promotes the secretion of MMP2 and MMP9 as well as the presentation of MT1-MMP (also known as MMP14) on the cell surface (Clark et al., 2007). Interestingly, the recruitment of cortactin to MT1-MMP endosomes is controlled by LIMK-mediated tyrosine phosphorylation of MT1-MMP. LIMK is a novel MT1-MMP binding partner and regulator of effective matrix degradation (Lagoutte et al., 2016). Additionally, Tks5 and Tks4 appear to have some overlapping function in the formation of podosomes; however, only Tks4 was shown to be crucial for the completion of podosome maturation and recruitment of MT1-MMP, ultimately regulating ECM degradation (Buschman et al., 2009). Phosphorylation of Tks4 was deemed necessary for this activity with Src being the probable responsible kinase (Buschman et al., 2009). This, together with the fact that cortactin is also a known Src substrate (Tehrani et al., 2007), implicates Src in the process of MMP delivery and invadosome-mediated ECM degradation. However, a more systematic investigation of the role of Src in this process needs to be performed (Buschman et al., 2009).

MT1-MMP recycling and cell membrane presentation is crucial for mature invadosome activity (Poincloux et al., 2009). Interestingly, Src phosphorylates MT1-MMP on Y573 which was found to be important in invasion assays *in vitro* as well as tumour growth *in vivo* (Nyalendo et al., 2008; Nyalendo et al., 2007). Additionally, phosphorylation of endophilin A2 by Src when Src is found in a scaffolding complex with FAK inhibited the re-uptake of MT1-MMP thereby prolonging its presentation on the cell surface and enhancing the invasive capacity of Src transformed cells (Wu et al., 2005). Furthermore, MT1-MMP trafficking and exocytosis is under the regulation of Atypical PKCζ/ι which phosphorylates cortactin on late endosomes containing MT1-MMP in triple negative breast cancer cells (Rosse et al., 2014). It thus appears that invadosome-mediated ECM degradation is heavily regulated by kinase activity.

Kinases in invadosome dissolution

Invadosome dissolution is arguably the least well studied stage of invadosome dynamics. Nonetheless, effective invadopodia and podosome turnover contribute to tumour and immune cell migration and invasion respectively. Therefore, the regulation of invadosome dissolution should not be overlooked (Murphy and Courtneidge, 2011; Schachtner et al., 2013b). Our knowledge regarding the regulation of invadosome disassembly is mainly derived from work on podosomes. In dendritic cells calpain appears to be crucial for podosome turnover by cleaving Pyk2 among other podosome regulatory proteins such as WASP and talin (Calle et al., 2006). Phosphorylation of WASP allows the cleaving activity of calpain that leads to the disassembly of podosomes in dendritic cells (Macpherson et al., 2012). A number of kinases are known to phosphorylate WASP including Src (Blundell et al., 2009). Paxillin, another known Src target, has been shown to be involved in the disassembly of adhesion complexes (Webb et al., 2004). Phosphorylation on paxillin residues 31 and 118 leads to ERK phosphorylation which in turn activates Calpain, thereby promoting podosome disassembly and turnover (Badowski et al., 2008). Phosphorylation of cortactin by Src also regulates podosome turnover as high levels of phospho-cortactin have been associated with a longer f-actin lifespan. This suggests that dephosphorylation of cortactin is crucial for effective podosome dynamics (Luxenburg et al., 2006). The involvement of Src and Pyk2 in podosome disassembly is also highlighted in osteoclasts (Shyu et al., 2007). It has recently been shown that osteoprotegerin induces podosome disassembly in osteoclasts by dephosphorylating Pyk2 at Y402, thus disrupting the association between Pyk2 and Src (Zhao et al., 2016).

PKC activity is implicated in both stabilisation and turnover of invadosomes. The cross-linking activity of the adaptor protein AFAP-110 is dependent on phosphorylation by PKCα (Qian et al., 2002); AFAP-110 acts to cross-link actin filaments, as well as couple signalling proteins to the actin cytoskeleton (Baisden et al., 2001). More recently, it was suggested that phosphorylation of AFAP-110 on Ser 277 by PKCα is important for podosome disassembly in vascular smooth muscle cells, as the transfection of mutant AFAP-110^{S277A} prolonged the podosome life-span (Dorfleutner et al., 2008). Additionally, PAK1 has been shown to phosphorylate cortactin downstream of Rac1, leading to the detachment of cortactin from the actin cytoskeleton and thereby inducing invadopodia dissolution in breast cancer cells (Moshfegh et al., 2014). Together, these studies suggest that kinase-mediated destabilisation of actin filaments in the invadosome core is a significant contributing factor towards invadosome disassembly.

Conclusion

This review has highlighted the involvement and importance of kinase signaling in every stage of the invadosome life cycle. Given that podosome deregulation and invadopodia function are both associated with pathophysiological states, studies investigating the potential clinical benefit of inhibiting the invadosome kinases are warranted.

It is evident that Src is not only implicated in every stage of the invadopodia life cycle but also in many other processes of tumourigenesis, such as proliferation, survival, angiogenesis and motility. It is thus not surprising that several Src inhibitors have been developed. Pre-clinical studies suggested that inhibition of Src could suppress metastasis. However, clinical trials of dasatinib, bosutinib and saracatinib (Aleshin and Finn, 2010) did not significantly improve patient outcome when inhibitors were administered as single agents (Aleshin and Finn, 2010; Lieu and Kopetz, 2010).

The complexity of Src activity in the invadosome may in part account for the lack of clinical benefit of Src inhibition. Indeed, we have observed that studies often implicate kinase activity through looking at specific phosphorylation events on a substrate of interest, without necessarily identifying the responsible kinase. Whilst this approach has significantly expanded our knowledge of invadosome components and signaling pathways regulating invadosome dynamics the increasing evidence of the clinical relevance of invadosome targeting highlights a need to better address kinase specificity. Thus a more focused and systematic investigation of the invadosome kinome is warranted.

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