**β1A Integrin Is a Master Regulator of Invadosome Organization and Function**

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Invadosomes are adhesion structures involved in tissue invasion that are characterized by an intense actin polymerization–depolymerization associated with β1 and β3 integrins and coupled to extracellular matrix (ECM) degradation activity. We induced the formation of invadosomes by expressing the constitutive active form of Src, SrcYF, in different cell types. Use of ECM surfaces micropatterned at the subcellular scale clearly showed that in mesenchymal cells, integrin signaling controls invadosome activity. Using β1A−/− or β3−/− cells, it seemed that β1A but not β3 integrins are essential for initiation of invadosome formation. Protein kinase C activity was shown to regulate autoassembly of invadosomes into a ring-like metastructure (rosette), probably by phosphorylation of Ser785 on the β1A tail. Moreover, our study clearly showed that β1A links actin dynamics and ECM degradation in invadosomes. Finally, a new strategy based on fusion of the photosensitizer KillerRed to the β1A cytoplasmic domain allowed specific and immediate loss of function of β1A, resulting in disorganization and disassembly of invadosomes and formation of focal adhesions.

INTRODUCTION

Invadosome is a general term for structures implicated in tissue invasion processes that share similarities in organization, composition, dynamics, or function with podosomes, present in nontransformed cells (such as macrophages, osteoclasts, dendritic cells, endothelial cells, and smooth muscle cells), or invadopodia, present in cancer cells. This class of structures can be simply defined as an adhesion structure centered on an actin column, where intense actin activity takes place, and is linked with localized extracellular matrix (ECM) degradation activity. Invadosomes can form isolated dot-like structures centered on a rapidly polymerizing actin column associated with actin regulators (such as cortactin, Wiskott-Aldrich syndrome protein, Rho GTPases, and fascin), adhesion molecules (such as paxillin, talin, and integrins), regulators of membrane dynamics (such as Tsk5, IQ motif containing GTPase activating protein 1, and vesicle-associated membrane protein 7), metalloproteases, and regulatory kinases (such as focal adhesion kinase [FAK]/Pyk2, p21-activated kinase, and Src) (Linder and Aepfelbacher, 2003; Weaver, 2006; Ori et al., 2008; Vignjevic and Montagnac, 2008; Albiges-Rizo et al., 2009; Poincloux et al., 2009). Moreover, like podosomes, invadosomes have the capacity to self-assemble into round metastructures known as rosettes or rings that can expand in diameter and fuse with each other. This treadmilling behavior is based on the coordinated assembly of new, individual actin columns, connected to each other by a “cloud” of F-actin at the outer rim, with disassembly of the original structure occurring at the inner rim of the rosette (Destaing et al., 2003, 2005; Jurdic et al., 2006; Badowski et al., 2008).

Invadosomes were originally described in cells expressing the oncogene v-Src and have been reported in many subsequent studies (Marchisio et al., 1984; Tarone et al., 1985; Mueller et al., 1992; Linder and Aepfelbacher, 2003; Bowden et al., 2006). The essential role of the nonreceptor tyrosine kinase c-Src in podosomes has been shown in Src−/− osteoclasts, where reduced bone resorption is associated with poorly functioning podosomes (Yoneda et al., 1993; Hall et al., 1994). The action of c-Src in podosomes is complex and essential for initiation of podosome assembly, intensity of the actin flux, and architecture and disassembly of the structure (Luxenburg et al., 2006; Ayala et al., 2006; Destaing et al., 2008). These adhesion structures also can be induced by activation of protein kinase Cs (PKCs) by phorbol ester treatment, suggesting a synergistic activity of this pathway with the tyrosine kinase Src (Hai et al., 2002; Tatin et al., 2006).

Thus, it seems that both invadosome formation and self-assembly involve an inside-out signaling process. The question of which specific stimuli from the external environment...
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MATERIALS AND METHODS

**Antibodies and Reagents**

Antibodies for immunoblotting and immunofluorescence were obtained from the following commercial sources: rabbit anti-phospho-Tyr 418 Src and anti-phospho-p85γ FAK (Innogenetics, Carlsbad, CA); mouse anti-Src (BD1; Millipore, Billerica, MA), mouse anti-actin (Sigma-Aldrich, St. Louis, MO), mouse anti-paxillin (BD Biosciences, Franklin Lakes, NJ), mouse anti-human beta 1 integrin (clone 4B7R; Becton Dickinson, Le Pont de Claix, France), rat anti-mouse beta 1 integrin (MBL2; Millipore), and rat anti-mouse beta 3 integrin (clone LucA5; Emret Analytics, Würzburg, Germany).

Alexa 546-phallolidin, as well as Alexa 488, gelatin-Oregon green, and Alexa 488-, 546-, and 633-conjugated secondary antibodies were from Invitrogen.

**Plasmids**

pEGFP-actin vector was obtained from Clontech (Palo Alto, CA); pBabe green fluorescent protein (GFP)-paxillin was provided by Dr. M. Hiraishi (Department of Molecular Biology, Osaka Bioscience Institute, Suita, Osaka, Japan); pBabe red fluorescent protein (RFP)- cortactin was engineered from the initial constructs; pBabe+xl-cortactin was from Dr. P. Juric (ENS Lyon, France), and pcDNA3-mRFP was from Addgen (Cambridge, MA).

Plasmids pB8-Neo-human beta 1-GFP, vaso dilator-stimulated phosphoprotein-RFP, and pBabe-Src Y527F were the generous gifts of Dr. M. Humphries (University of Manchester, Manchester, United Kingdom), Prof. Geltier (The David H. Koch Institute for Integrative Cancer Research, Massachusetts Institute of Technology, Cambridge, MA), and Dr. H. Gil-Ham (Yale University, New Haven, CT), respectively.

For the pTRFP or KillerRed-tagged human beta 1 construct, the pTRFP or KillerRed coding sequences were polymerase chain reaction (PCR) amplified from the original pTagRFP-N or pKillerRed-N vectors (Evrogen, Moscow, Russia) and used to replace GFP of pB8-Neo-human beta 1-GFP to generate an in-frame C-terminal fusion. pCLMGF-human beta 1 wild-type (WT), pCLMGF-human beta 1 D759A, pCLMGF-human beta 1 S785A, and pCLMGF-human beta 1 S785E constructs were made using the QuickChange XL site-directed mutagenesis kit (Stratagene, La Jolla, CA) using the following primer pairs: D799A: forward, 5'-gtttaatgataattacttccaagggaggtgg-3' and reverse, 5'-ggggagaatccttctGGGcattaaatcagttgg-3' and reverse, 5'-ccaaagatctgcgtggcatgagttg-3' and reverse, 5'-ccaaagatctgcgtggcatgagttg-3'.

**Cell Culture and Infection**

MEF beta 1+/+ and beta 1−/− cells were the generous gift of Dr. Richard Hynes (The David H. Koch Institute for Integrative Cancer Research). Mouse embryonic fibroblasts (MEFs) isolated from beta 1+/+ and beta 1−/− mice by embryonic day 12 and postnatal day 1, as described in Ferguson et al. (2009), were the generous gift of Prof. Reinhard Flassler (Max Planck Institute of Biochemistry, Martinsried, Germany).

A population of primary mouse osteoblast-enriched cells was isolated from newborn mouse calvaria by using a mixture of 0.3 mg/ml collagenase I (Sigma-Aldrich) and 0.25% trypsin (Invitrogen), as described previously (Bouvard et al., 1986; Bouvard et al., 2007). Cells were grown in e-minimal essential medium containing 10% fetal calf serum (ICS). Primary osteosarcoma cell line 2 were immortalized by transduction with mouse parvovirus expressing the large simian virus 40 T antigen (Fassler et al., 1995); cloned; and tested for their ability to express alkaline phosphatase upon differentiation (Mansukhani et al., 2000), as described previously (Bouvard et al., 2007). At least five clones from cloned mice were isolated. Rescue of beta 1 integrin expression in cells was performed via retroviral infection using the pCLMGF-beta 1 vectors, as described previously (Bouvard et al., 2007; Millon et al., 2008). Cells were grown in DMEM containing glucose and supplemented with 10% FCS and 1% penicillin/streptomycin. CRE adenovirus was purchased from the University of Iowa Gene Transfer Vector Core (Iowa City, IA). Minimal beta 1 depletion was achieved within 4 d. Cells were generally used for experiments between 6 and 9 d after Cre recombinase delivery. In most experiments, CDNA were delivered via retroviral transduction after packaging in Phoenix-Eco or Phoenix-Ampho cells (American Type Culture Collection, Manassas, VA). Supernatant containing viral particles from transduced cells was harvested; filtered; and after addition of 8 μg/ml polybrene (Sigma-Aldrich), it was used to infect fibroblasts. For rescue experiments, cells were infected with exogenous beta 1 constructs, before addition of the CRE recombinase, to remove endogenous integrin. Before use, the cells were either serum starved for 14 h to have SrcYF activity as the main signaling pathway to the cells or treated for 1 h with 50 μM sodium butyrate (PMA). Either treatment allowed the development of many invadosomes and rosettes.

**Micropatternning and Functionalization**

Patterned protein glass coverslips were performed according to Guillou et al. (2008), with slight modifications. Glass coverslips (22 × 22 mm) were washed

(those participating in outside-in signaling) are able to induce invadosome formation remains unanswered. This led us to explore the functions of the integrin receptor families in the regulation of invadosome activity because these receptors integrate the composition, concentration, and compliance of the ECM. Integrins are heterodimers that oscillate between conformations having low and high affinity for the ECM. This switch corresponds to integrin activation and is favored by the disruption of the salt bridge between the α and β subunits induced by talin interaction with the β- cytosolic domain of the integrin molecules (Shattil et al., 2010). Moreover, this class of proteins has a strong link with ECM degradation, interacting directly with the membrane-associated metalloprotease MT1-MMP (also known as MMP14; Galvez et al., 2002). Although numerous studies have localized different integrins in podosomes and invadosomes, the specific functions of integrins in invadosomes have been poorly described. Indeed, αvβ3 is found in osteoclast podosomes and in invadosomes and invadopodia in many cancer cells (Zambonin-Zallone et al., 1989): αβ1 in 804G carcinoma cells (Spinardi et al., 2004), αβ1 in monocytic podosomes and β2 integrins in macrophage podosomes (Duong and Rodan, 2000), and β1 integrins in osteoclast podosomes (Helfrich et al., 1996). Due to the role of podosomes in bone resorption by osteoclasts, a major role was assigned to αvβ3 integrin in this process based on the correlation between the strong increase in the expression of αvβ3 and the formation of podosomes during osteoclast differentiation (Mimura et al., 1994; Pfaff and Juricd, 2001; Destaing et al., 2003; Juricd et al., 2006). Moreover, perturbation of αvβ3 integrin by disintegrin modulates osteoclast migration and podosome formation (Nakamura et al., 1999; Blair et al., 2009). However, β3−/− mice show only a slight increase in bone mass in comparison with Src−/− osteoclasts, which are only associated with a disorganization of podosomes belts (McHugh et al., 2000; Faccio et al., 2003). In macrophages, Rho B inactivation results in decreased surfation of invadosomes. Finally, a new method to induce quick loss of function of β1A by photoactivation revealed that this integrin also plays a key role in invadosome metastructure stabilization. Thus, it seems that β1A is a key modulator of invadosome function in invasion processes.
in a solution of sulfuric acid and hydrogen peroxide (7.3, vol/vol) for 30 min, dried, and then dipped for 1 h in a solution of octadecyltrimethoxysilane and aminopropyltrimethoxysilane (3.1, mol/mol; Sigma-Aldrich) in toluene. Positively charged resin (Shipley, S1805, Richmond, VA) was spin coated and cured according to the manufacturer’s protocol to form a uniform, UV-sensitive film 0.5 μm in thickness. The coated coverslips were then insolated with UV light using a Karl Süss aligner (MJBI, SÜSS MicroTec, Saint-Justiere, France) at 436 nm and 15 mJ/cm² through a chromium mask. The irradiated pattern was revealed with microcospot developer concentrate in deionized water (1:1, vol/vol; Shipley, MD CD-26, Rhom & Haas Electronic Materials). The patterned coverslips were incubated for 1 h at 37°C in a solution of gelatin-rhodamine isothiocyanate (RITC) and 10 μg/ml vitronectin in phosphate-buffered saline (PBS). Substrates were rinsed in PBS and then in absolute ethanol in an ultrasonic water bath to dissolve the photoresist resin. Finally, either antiadhesive triblock copolymer Pluronic F127 (Sigma-Aldrich) at a concentration of 4% in water for 1 h at 37°C, or a solution of FN7–10-FITC (fibronectin type III domains 7–10 conjugated to fluorescein isothiocyanate [FITC]) at 5–15 μg/ml in PBS was adsorbed to the complementary pattern revealed after resin dissolution by ethanol for 1 h at 37°C. After a last rinse in PBS, cells (155 cells/mm²) were seeded and incubated overnight, before fixation and staining.

Degradation Assays

Coverslips were coated with 1 mg/ml gelatin-Oregon green, fixed with 4% (wt/vol) paraformaldehyde/0.5% glutaraldehyde for 30 min at 4°C, washed with 30 mg/ml sodium borohydride in PBS, stabilized with 70% ethanol, and rinsed once with PBS. Cells were seeded on the coverslips in culture medium for 2 h before being imaged for 14 h with an Axiovert 200M microscope (Carl Zeiss Microimaging, Gottingen, Germany) equipped with a MicroMax 5-MHz, 10× (numerical aperture [NA] 0.25) LDplann objective.

Microscopy and Photoactivation

For immunofluorescence analysis, cells were fixed with 4% paraformaldehyde in PBS, pH 7.4, and imaged with an Axiovert 200M microscope equipped with CoolSNAP HQ, 65× (NA 1.4) Plan Apochromat and 100× (NA 1.4) Plan Apochromat objectives and a filter set to specifically detect Alexa 488/GFP or Alexa 546/FRFP/KillerRed. At least 700 podosome structures were analyzed for each condition; representative data from to three to five independent experiments are presented. For live imaging, cells were seeded at subconfluent densities in 35-mm serum-coated glass-bottomed dishes (1.5 mm in thickness; MatTek, Ashland, MA) and allowed to grow for 12–48 h before imaging. DMEM was replaced by a CO₂-independent medium (Invitrogen), placed on a heated 37°C stage (Carl Zeiss Microimaging), and imaged with the same Axiovert 200M microscope setup described above. Total internal reflection fluorescence (TIRF) microscopy was carried out with the same setup as described above and equipped with the TIRF 1 slider (Carl Zeiss Microimaging).

For photoactivation experiments, an Axiovert 200M microscope equipped with a 63× (NA 1.4) Plan Apochromat objective and triple-filter set 25HE (excitation, TBF 405 + 495 + 575 [HE]; beam splitter, TFF 435 + 510 + 600 [HE]; emission, TBF 460 + 530 + 625 [HE]) (Carl Zeiss Microimaging) was used for illumination. Cells were imaged as described for live imaging, followed by a pause to allow illumination of the whole field of observation for 40–50 s (average, 45 s) with a 100-W HBO mercury lamp at 100% power. For fluorescence recovery after photobleaching experiments, an LSM510 ConfoCor microscope equipped with a 40× (NA 1.2) Plan apochromat objective (Carl Zeiss Microimaging) was used. The fluorescence recovery after bleaching time (3.2 s) was observed primarily in actin spots that persisted throughout the entire recovery time. Imaging Series 7.0 software (Molecular Devices, Sunnyvale, CA) was used to mount .avi movies from image stacks. Images extracted from stacks were processed with Photoshop CS2 (Adobe Systems, San Jose, CA) and ImageJ (http://rsb.info.nih.gov/ij/). Significance of the differences between standard deviations was analyzed in Excel (Microsoft, Redmond, WA) with an F-test.

RESULTS

The ECM Controls the Assembly of Invadosomes

To determine the role of ECM signaling on invadosome activity, we plated MEFs transformed with constitutively active SrcYF on a layer of ECM micropatterned at the subcellular scale. Cells were seeded on alternate stripes of adhesive (gelatin-RITC mixed with vitronectin; Figure 1Aa, red areas) and nonadhesive areas (Pluronic F127; Figure 1Aa, black areas), 5 and 10 μm in width, respectively. The invadosome rosettes were present on the adhesive stripes 95% of the time and continued to expand along the axis of these stripes (Figure 1Aa). These rosette formations suggested that the initiation of invadosome assembly is promoted by ECM signaling. Because integrins are the major actors in matrix sensing, we wanted to precisely define the roles played by specific subclasses of this receptor family. Therefore, we plated MEF-SrcYF onto patterned surfaces composed of alternating 5-μm stripes of RITC-vitronectin, which preferentially binds β3 integrins, and unlabeled fibronectin, which preferentially binds β1 integrins. Rosettes were more abundant on vitronectin, although the cells spread on both adhesive surfaces. In contrast, individual podosomes were seen on both surfaces (Figure 1B). However, on glass coverslips homogenously coated with fibronectin or vitronectin, MEF-SrcYF generated similar individual invadosomes and rosettes (data not shown). This result was reproduced with double patterned coverslips with either laminin and vitronectin, or collagen and vitronectin (Supplemental Figure S1A, B and C). These data indicated that invadosomes have a preference for vitronectin-coated areas, suggesting an essential role of β3 integrin in invadosome activity (Figure 1B).

To determine the importance of β1 and β3 integrins in invadosome activity, we analyzed the locations of these molecules and compared them with those of β1 integrins after spreading of the cells on endogenous matrix (48-h culture on glass coverslips). Because F-actin was found to be a reliable marker of nascent and mature invadosomes (Badowski et al., 2008), double staining with either β1 and paxillin, or β3 antibodies and phallolidin, was carried out. Confocal scanning microscopy showed colocalization of β3 and F-actin, whereas β1 integrins were mostly excluded from the rosettes and accumulated at the outer rim, suggesting a possible function in newly formed invadosomes (Figure 1C). The early assembled invadosomes contain mostly the actin core, including cortactin. Detection of the active conformation of β1A was carried out after external addition in the medium of 9FG7 antibody directly conjugated to FITC and visualized by TIRF microscopy on living cells expressing cortactin-monomeric(m)RFP. This analysis revealed that β3 integrins did not colocalize with cortactin; rather, they were in proximity to the rim of the invadosome rosette (Figure 1D). Although β1 integrins accumulate around rosettes, our results did not support a structural role for them, in contrast to β3 integrins, which mediate adhesion and are a component of the mature invadosome.

β1 Integrins Are Key Regulators of Invadosome Formation

To investigate the involvement of β3 integrins in invadosome assembly, we induced these structures in β3+/+ and β3−/− MEFs by expressing the constitutively active form of Src. Surprisingly, MEF-SrcYF β3−/− cells displayed invadosome rosettes, although these structures were narrower and exhibited less intense actin staining than those observed in MEF-SrcYF β3+/+ cells (Figure 2A). In line with these findings, MEF-YF src established invadosomes rosettes on the β1-specific substrates laminin111 and collagen (data not shown). Conversely, β1 depletion in MEF-SrcYF β1loxP/loxP cells, generated by adenoviral delivery of Cre recombinase, resulted in invadosome formation in <5% of cells (Figure 2B, top; and C). The invadosomes observed in this small percentage of cells were probably due to a lack of Cre expression in some uninfected cells, as shown by quantita-
tive PCR (Figure 2D). B1 depletion in MEF-SrcYF B1loxP/loxP was characterized by an increase in cell spreading and reorganization of F-actin into more abundant stress fibers (Figure 2B, bottom). To confirm this unexpected and dramatic effect of removal of B1 integrins and generalize this finding to other cell types, we applied the same strategy of inducing invadosomes through SrcYF expression in selected preosteoblastic pOBL B1loxP/loxP cells purified after Cre treatment. As for MEFs, the expression of SrcYF induced the formation of individual invadosomes and rosettes in B1/3 integrin preosteoblasts but not in their B1−/− counterparts. This loss of B1 was also accompanied by an increase in stress fibers within the cell bodies (Figure 3, A and D). These modifications suggested some impairment of Src activity. However, Western analysis of cell lysates did not reveal significant changes in either Y416 phosphorylation, which characterizes Src activation, or in total Src expression (Figure 3B). Thus, our data suggested that B1 integrins have an essential role in the initiation of invadosome assembly.

**Activation of B1 Integrins Stimulates Invadosome Autoassembly into Rosettes**

Most of the B1 integrins around the rosettes were stained with the 9EG7 monoclonal antibody, indicating that these receptors were bound to their ECM ligands and therefore probably in their high-affinity state (Figure 1D). To test the role of integrin activation in invadopodia dynamics, we carried out a structure–function study and re-expressed exogenous human B1A chains bearing the point mutation D759A, which is known to modulate B1A activation state, in B1−/− pOBL-SrcYF cells (Figure 3C). This mutation promotes the integrin high-affinity state by breaking the saline bridge to the nearby arginine residue on the a chain (Shattil et al., 2010). This mutant showed a dramatic increase in rosette number compared with wild-type cells (Figure 3D). In conclusion, in addition to the involvement of B1A in the initiation of assembly of individual podosomes, the activation of B1A integrins seems to potentiate invadosome autoassembly.

**PKC Directly Targets B1A Integrin to Control Invadosome Autoassembly**

Previous studies in the literature show that in addition to Src signaling, the activation of conventional PKCs promotes invadosome formation in a variety of cell types (Hai et al., 2002; Tatin et al., 2006). Indeed, under standard cell culture conditions, PMA treatment stimulated invadosome autoassembly into rosettes in a manner similar to conditions of serum starvation (Figure 4A). Based on the treadmilling process in invadosome turnover (Badowski et al., 2008), the increase in invadosome autoassembly could be due to an increase in either rosette stability or dynamics. To address this question, live-cell imaging was carried out on pOBL-

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**Figure 1.** Extracellular matrix sensing by B1 and B3 integrins controls invadosome formation and localization. (A) Most SrcYF-expressing cells formed invadosome rosettes (visualized by F-actin staining, in green) only on the adhesive surface (gelatin-tetramethylrhodamine B isothiocyanate [TRITC], in red, mixed with vitronectin) and not on antiadhesive areas (Pluronic F127, black areas). (B) Invadosome rosettes stained by phalloidin-TRITC show higher affinity for vitronectin-FITC (light gray bands), sensed by members of the B3 integrin family, than for fibronectin (black bands), sensed by members of both the B1 and B3 integrin families. (C) The B1 and B3 integrins show distinct patterns of localization, observed in MEF-SrcYF cells. B3 (in green in merge) highly colocalized with F-actin, whereas B1 staining is limited to the rosette periphery. (D) 9EG7 antibody B1 staining is specific of the activated form of the integrin. Antibody against activated form of B1 directly conjugated to FITC (9EG7-FITC) was added externally and localized around the invadosome visualized by cortactin-mRFP (in red in merge) expressed in live MEF-SrcYF cells. Bars, 5 μm (A and B), 4 μm (C), and 2 μm (D).
SrcYF cells stably expressing GFP-actin, and cells were grown in serum-supplemented DMEM in the presence or absence of PMA. In the absence of PMA, the few rosettes that could be detected were quite stable over 30 min. Conversely, activation of PKCs largely decreased invadosome life span to 10 min, resulting in a dramatic increase in rosette expansion and turnover (Figure 4B). In contrast, inhibition of PKCs by the general inhibitor bisindolylmaleimide I (BIM) resulted in rapid dissociation of rosettes, whereas the formation of individual invadosomes was still observed (Figure 4B). In the cytosolic domain of the $\beta$1 integrin subunit, many serine and threonine residues have been shown to be potential PKC phosphorylation sites modulating integrin function (Stroeken et al., 2000; Mulrooney et al., 2001; Figure 4C). Among those residues, Ser785 was shown previously to be strongly phosphorylated upon Src overexpression (Sakai et al., 2001). To investigate the importance of the PKC-dependent signaling pathway on $\beta$1A in the invadosome autoassembly process, we expressed the $\beta$1 integrin mutants S785A, or S785D or S785E (nonphosphorylatable or phosphomimetic forms, respectively), in a null genetic background and modulated PKC activity with an activator (PMA) or inhibitor (BIM). Consistent with the view that Ser785 is a direct target of PKC, only the phosphomimetic mutants S785D and S785E were able to stimulate invadosome autoassembly into rosettes in the absence of PMA (Figure 4D). The same phosphomimetic mutants were partly resistant to BIM inhibition compared with wild type and the nonphosphorylatable S785A mutant (Figure 4E). In addition, the S785A mutant poorly rescued rosette assembly after PMA treatment (Supplemental Figure S2). Together, these data support the view that Ser785 on the $\beta$1 subunit is one of the primary targets of PKC in the regulatory pathway of invadosome rosette autoassembly in cells.

$\beta$1 Integrin Downstream Signaling Links Invadosome Formation and ECM Degradation Properties

Because invadosomes are major sites of ECM degradation, we investigated the involvement of $\beta$1 integrins in this crucial invadosome function by quantifying gelatin-Oregon...
green surface degradation, normalized per cell, over time. Even in the absence of invadosomes, pOBL-SrcYF δ1 cells showed very low, but significant, ECM degradation activity 4 h after plating on gelatin (Figure 5, A and C). Quantification of the average fluorescence intensity of the digested area is an efficient way to determine the extent or the depth of digestion. It was clearly shown that surfaces degraded by pOBL-SrcYF δ1 cells were poorly digested (Figure 5B) compared with rescued cells. The same results were obtained for MEF-SrcYF cells (data not shown). As shown previously, this structure–function study allowed exploration of the function of δ1A in the ECM degradative activity of invadosomes. Re-expression of wild-type human δ1A integrin rescued the degradation phenotype of pOBL-SrcYF δ1 cells in terms of extent and depth of the ECM digestion (Figure 5, A and B). In contrast, the rescue of pOBL-SrcYF δ1 cells with human δ1A D759A, a mutant that preactivates the integrin, led to the formation of numerous invadosomes, but which poorly digested ECM. Because wild-type integrin cycles between low- and high-affinity states, this loss of function when the high-affinity state is promoted suggests the importance of the cycle of δ1A integrin activation–inactivation in this process. Thus, modulation of the δ1A integrin activation state allowed, for the first time, the uncoupling of invadosome formation and ECM degradation activity. Moreover, regulation of invadosomes by PKC through phosphorylation of Ser785 was shown to be an essential step in activating invadosome ECM degradation properties. Indeed, the nonphosphorylatable mutant S785A of δ1A strongly reduced the average surface area digested per cell, without affecting the depth of the degradation (Figure 5B). Conversely, the phosphomimetic δ1A mutant S785D dramatically increased invadosome ECM degradation activity. Thus, not only the affinity state of δ1 but also signaling pathways downstream of δ1A, δ5, and δ6 phosphorylation (probably by PKC) control the coupling of invadosome assembly and ECM degradation activity.

**δ1 Integrins Stabilizes Both Rosettes and Individual Invadosomes**

Having established that δ1A integrins are important in initiating formation of individual invadosomes in the autoassembly of rosettes, and in invadosome degradative function, we wanted to determine whether signaling from δ1A integrins located at the rosette periphery was required to simply initiate or to maintain rosette structure and dynamics. To address this question, we developed an improved photoinactivation strategy based on the use of the photosensitizer KillerRed to achieve inducible loss of the δ1A integrin chain. In brief, we fused KillerRed to δ1A in place of GFP and expressed it in MEF-SrcYF δ1loxP/loxP cells. The endogenous δ1 gene was deleted in almost 100% of the cells 7 d post-Cre expression, as monitored by quantitative (q)PCR analysis of mouse δ1 mRNA (data not shown). In this genetic null background, the δ1-KillerRed was functional because it allowed the assembly of rosettes and was correctly localized at their periphery. GFP-actin dynamics was followed after 45-s irradiation with red light in the KillerRed excitation spectrum (585–615 nm). KillerRed locally produces reactive oxygen species (ROS), resulting in the rapid inactivation of the
PKC regulates invadosome autoassembly by phosphorylating Ser785 of β1A integrins. (A) PKC activation by a 60-min treatment of pOBL-SrcYF cells with 2 μM PMA induces a massive increase in invadosome rosette autoassembly, visualized by phalloidin staining. (B) Extracted images from time series (in minutes) from representative observations of pOBL-SrcYF cells expressing GFP-actin and treated with either dimethyl sulfoxide (DMSO; control), the PKC activator PMA (2 μM), and the PKC inhibitor BIM (5 mM). PKC activity regulates the dynamics and maintenance of the invadosome autoassembly state. (C) Amino-acyl sequence of the cytoplasmic domain of β1A integrin and the location of the main PKC targets. (D) Quantification of invadosomes per Airy unit (AU) shows that a mutation mimicking a constitutive phosphorylated form of Ser785 (Asp or Glu) dramatically increases the formation of rosettes in pOBL-SrcYF β1−/− cells. The nonphosphorylatable mutant of β1 at this site (S785A) has no effect on rosette formation. (E) The number of rosettes/AU was quantified in pOBL-SrcYF cells treated with 2 μM PMA or simultaneously with PMA and 5 mM BIM for 60 min. The inhibitory effect of BIM was determined by calculating the percent inhibition of invadosome rosette formation. Mutants mimicking a constitutively phosphorylated Ser785 (S785D and S785E) show only 50% inhibition after BIM treatment, indicating that this residue on β1 is a major target of PKC in the regulation of invadosome autoassembly. Bars, 20 μm (A) and 5 μm (B).
Figure 5. β1 activity and signaling control invadosome ECM degradation activity. (A) Quantification of the degraded surface of gelatin-Oregon green per cell reveals that β1 has an essential function in this invadosome function. Surprisingly, expression of the activated mutant of β1 (D759A) induces numerous invadosome rosettes associated with poor degradation activity. Moreover, constitutive activation of the signaling pathway downstream of phosphorylation of Ser785 strongly stimulates ECM degradation. (B) β1 also controls the quality of the degradation, as revealed by quantification of the average intensity of the digested areas. The few areas degraded in the pOBL-SrcYF β1−/− cells are poorly digested because they are close to the maximal fluorescence intensity of a nondigested surface (25%) in comparison with pOBL-SrcYF β1+/− cells (values closer to 0 indicate a totally black, completely digested area). Between 600 and 1050 cells were counted per condition. (C) Representative images extracted from the time series of pOBL-SrcYF β1−/− cells expressing or not expressing either human β1 WT, D759A, S785A, or S785D spread on a layer of degradable gelatin-Oregon green. Bar, 10 μm.

protein to which it is fused. Indeed, photoinactivation of β1-KillerRed resulted in a 95% decrease in red fluorescence, which was not recovered over the time of the observation (a maximum of 40 min after irradiation). This loss of β1A function led to the massive disorganization of the rosettes in <20 min (Figure 6, A and D).

ROS production in the cells could potentially have multiple indirect effects. To clearly show the specificity of this strategy, we first confirmed that the 45-s excitation with red light that resulted in KillerRed inactivation had no effect on invadosome structure, organization, and dynamics in cells expressing KillerRed alone in the cytosol (data not shown). Thus, nonlocalized ROS production after KillerRed irradiation had no effect on invadosomes or focal adhesions. To evaluate the potential toxicity of the 45-s red light irradiation and adsorption, KillerRed was replaced in the β1A fusion protein by TagRFP, another photostable fluorescent molecule with an excitation/emission spectra similar to that of KillerRed (Supplemental Movie 2). Indeed, 45-s red light excitation of β1A-TagRFP had no effect on invadosomes, which remained stable for more than 40 min after irradiation (Figure 6, B and D). Finally, the nonspecific effects of ROS production on proteins and membranes in the vicinity of β1 after KillerRed excitation were investigated by simultaneous expression of β1A-GFP and β1A-KillerRed in a β1−/− background, because the two proteins were probably located in proximity to each other (Supplemental Movie 3). In this environment, photoinactivation of β1-KillerRed had no effect on β1A-GFP, which still allowed the formation of characteristic invadosome rings, as seen in nontreated invadosome visualized by β1A-GFP and cortactin-TagRFP (Figure 6C, bottom; and Supplemental Movie 8). These data clearly show that ROS produced by photoinactivation of KillerRed act only on the KillerRed-tagged protein and not on surrounding macromolecules (Figure 6, C and D).

This photoinactivation strategy allowed us to monitor the specific effects of loss of β1A on pre-existing rosettes on a time scale of minutes. It is noteworthy that despite its specific localization around invadosome rings and its role in their initiation, inducible β1A depletion did not lead to progressive dissociation of the actin cytoskeleton from the inside to the outside of the rosettes, as would be expected because new actin structures are formed at the outer rim of the ring, whereas older parts of the structure are disassembled at its inner rim (Figure 6A). Soon after photoinactivation, the polymerization of GFP-actin was not blocked but rather formed unstable waves inside the rosette before the disorganization of these structures (Supplemental Movies 1 and 7). Instability in the actin polymerization domains within the rosette was followed by the complete collapse of the structure and increased cell spreading associated with the rapid formation of stress fibers (Supplemental Movie 1). Occasionally, GFP-actin signals reminiscent of the localization of invadosomes remained (Figure 6A).

Photoinactivation of β1A Integrins Reveals the Integrin Functions in Invadosome Organization

To understand the mechanism underlying the GFP-actin polymerization defect seen after loss of β1A function, the dynamics of the activity of the actin regulator cortactin fused to GFP was monitored (Supplemental Movie 4). The β1 photoinactivation was rapidly followed by the dissociation of cortactin from invadosomes, explaining the perturbation of actin dynamics (Figure 7A). Similarly, the dynamics of adhesion molecules such as paxillin and β3-GFP integrins was analyzed (Supplemental Movies 5 and 6). In particular, the induction of β1 loss of function led to paxillin disorganization in invadosomes, although a fraction of this GFP-tagged protein remained localized at the previous site in the rosette (Figure 7B). This was also the case for β3-GFP, but with this specific marker, it was clearer that invadosome disorganization was associated with massive new formation or growth of focal adhesions (Figure 7C, red arrows). This rapid reorganization of the cellular adhesive structures was consistent with our observations of the formation of multiple stress fibers in response to β1 photoinactivation (Supplemental Movie 1). In conclusion, despite its peripheral localization in the rosette, β1A integrin is a master regulator of invadosome assembly, organization, and stability.
DISCUSSION

Based on their high content and variety of integrins, invadosomes are integrin-dependent adhesive structures associated with intense actin dynamics and responsible for local ECM degradation. The role of a specific integrin type in invadosome regulation is unclear, due to transdominant activities among the integrin classes and the lack of specificity of the tools used such as specific inhibitors or antibodies. Herein, we used the power of genetics to understand the role of two major integrin classes (those pairing with \(\alpha_1\) and \(\alpha_3\) chains) in the formation and dynamics of invadosomes. This strategy is based on the induction of invadosomes by SrcYF expression in a \(\alpha_1\) or \(\alpha_3\) null genetic background. Thus, we are coupling the historical experiment by Tarone et al. (1985) with the re-expression of wild-type or mutant \(\beta\) chains, allowing a straightforward reverse genetic analysis of \(\beta_1\) or \(\beta_3\) integrins functions in invadosomes.

Although the rosette assembly associated with the colocalization of \(\beta_3\) and F-actin on patterned surfaces has clearly shown preferential invadosome localization on vitronectin, MEF SrcYF \(\beta_3^{-/-}\) cells form invadosome rosettes, indicating that other integrin types can compensate the loss of \(\beta_3\). This finding confirms previous data showing that \(\beta_3\) osteoclasts are able to form podosomes (Faccio et al., 2003).

On the contrary, the specific role of the \(\beta_1\) chain in initiating invadosome assembly is highlighted by the fact that the loss of \(\beta_1\) resulted in the disappearance of both individual and self-assembled invadopodia in primary cells and cell lines. These data do not fit with an earlier study reporting the presence of invadosome rosettes in the \(\beta_1\) null cell lines G\(\beta_{11}\) and GD25 transformed with SrcYF. In that case, however, the re-expression of the \(\beta_1A\) double mutant (Y783F, Y795F) that poorly responds to Src transformation (Sakai et al., 2001) clearly showed a trans dominant-negative effect on invadosome formation, pointing to a major role of this integrin chain in invadosome formation (Huveneers et al., 2008). Because these studies were using immortalized cells lines from knockout embryonic cells, one may assume that other

Figure 6. Photoinactivation revealed role of \(\beta_1\) in maintaining invadosome self-assembly. (A) Representative images extracted from time series of MEF-SrcYF \(\beta_1^{-/-}\) cells expressing human \(\beta_1\)-KillerRed and GFP-actin. Exogenous human \(\beta_1\)-KillerRed is functional in rescuing invadosome formation and its proper localization at the periphery. Exposure of KillerRed to light for 45 s is followed by fluctuations in intensity and disorganization of GFP-actin in the invadosome rosette. (B) Representative images extracted from time series of MEF-SrcYF \(\beta_1^{-/-}\) cells expressing human \(\beta_1\)-pTagRFP, which has the same excitation and emission spectrum but is much more photostable than KillerRed, and GFP-actin. Light irradiation without ROS production is not sufficient to dissociate invadosomes. (C) Representative images extracted from time series of MEF-SrcYF \(\beta_1^{-/-}\) cells expressing human \(\beta_1\)-pTagRFP, which has the same excitation and emission spectrum but is much more photostable than KillerRed, and GFP-actin. Light irradiation without ROS production is not sufficient to dissociate invadosomes. (D) Photoinactivation of \(\beta_1\)-KillerRed leads to rapid and specific disorganization of invadosomes. Histograms, show the distribution of the percentage of cells where invadosomes are disorganized at various times after light irradiation. The x-axis shows time in minutes. Twelve to 39cells per condition were monitored. Bars, 3 \(\mu\)m (A–C).
compensatory mechanisms may have been at work. Indeed, in our hands, isolated clones of pOBL SrcYF β1/−/− cells that normally do not form invadosomes, can form few invadosome rosettes after numerous passages in culture, suggesting that these immortalized cells lines start to set up compensatory mechanisms that rescue the formation of these adhesion structures (data not shown). At a first glance, and despite its localization around the rosette, the importance of β1 integrin is surprising because this integrin class appeared in wild-type cells to be excluded from invadosomes, which contain mostly β3 integrin chains. However, immunostaining revealed an increased concentration of β1 at the outer rim of the rosettes. Because no colocalization with cortactin, a marker of nascent and mature invadopodia and invadosomes (Artym et al., 2006; Badowski et al., 2008) was observed, it is likely that β1 integrins do not belong to the F-actin architecture composing invadosomes but rather form a signaling platform to initiate invadosome assembly. To test this hypothesis, we developed a new approach to inactivate integrins at the minute time scale. The specific photoinactivation of β1-KillerRed showed that this integrin loss induced a rapid collapse of the whole structure, indicating that the β1 chain is required not only to initiate invadosome assembly but also to control the stability of the structure during the entire life span of the rosette. This β1 functional ablation allowed us also to observe the rosette collapse from the outside to the inside. Thus, it seems that β1 integrins can signal at distance to control the disassembly processes occurring at the inner rim of invadosomes (Badowski et al., 2008). Moreover, some residual F-actin, not associated with GFP-cortactin, forms an imprint after F-actin disassembly after photoinactivation corresponding to the initial rosette. This strongly suggests the presence of two distinct actin network in the rosette: the actin cores with high cortactin content that depend on β1 signaling, and a more stable actin cloud around individual podosomes that is reminiscent of the radial actin arrays observed in macrophage or in the sealing zone of osteoclasts (Evans et al., 2003; Luxenburg et al., 2007).

Figure 7. β1 photoinactivation leads to the loss of cortactin, disorganization of adhesion molecules within the invadosome metastructures, and induction of large, β3-rich focal adhesions. (A) Representative images extracted from time series of MEF-SrcYF β1/−/− cells expressing human β1-KillerRed and GFP-cortactin. Photoinactivation of β1-KillerRed is followed by a slow decrease in GFP-cortactin fluorescence and its disappearance. (B) Representative images extracted from time series of MEF-SrcYF β1/−/− cells expressing both human β1-KillerRed and GFP-paxillin. β1 photoinactivation leads to GFP-paxillin disorganization and decrease in intensity, but in contrast to what is observed with GFP-cortactin, GFP-paxillin remains associated with the invadosome. (C) Representative images extracted from time series of MEF-SrcYF β1/−/− cells expressing human β1-KillerRed and β3-GFP. β1 photoinactivation leads to slow dissociation of invadosomes and the massive formation of β3-rich focal adhesions (red arrows). Bars, 3 μm (A–C).
Finally, photoinactivation of B1A-KillerRed is followed by the formation of focal adhesions, providing another example of the well described competition between focal adhesions and invadosomes.

To explore the B1A-dependent control mechanisms of invadosomes, we focus our attention on PKC that in addition to Src is a major inducer of invadosomes or podosomes (Hai et al., 2002; Tatin et al., 2006). Indeed, under standard cell culture conditions, PKC activity induced by PMA treatment greatly stimulated invadosome and rosette formation. The precise role of PKC activity in invadosomes is unclear. PKCα and PKCδ were shown to allow β1 integrin activation (Brenner et al., 2008). This latter finding could be indirect because another PKC, PKCβ, was shown to regulate the integrin activator Rap1 (Letchka et al., 2008). The other possibility is that PKC directly phosphorylates integrins. Indeed, Ser785 and threonine 788 and 789 on the cytoplasmic tail of this integrin are potential targets of PKC. Because it was shown previously that Ser785 phosphorylation alters cell spreading (Mulrooney et al., 2001) and that this particular residue was overphosphorylated upon Src overexpression (Sakai et al., 2001), phosphomimetic and the nonphosphorylatable B1A mutants S785E and S785D or S785A, respectively, were expressed in β1A−/− SrcYF MEF and preosteoblasts. Phosphomimetic mutant expression strongly stimulated invadosome self-assembly in the absence of PMA and the mutant became partially resistant to the general PKC inhibitor BIM. These results strongly suggest that the direct phosphorylation of β1A on Ser785 by PKC is an important event in the regulation of invadosome assembly. However, the partial inhibition of phosphomimetic β1A mutants by BIM treatment also suggests that in addition to the direct phosphorylation of the β1A chain, PKC stimulation by PMA acts at other different levels of invadosome assembly. This view is also confirmed by the fact that BIM treatment does not fully mimic the collapse of the rosette after B1A-KillerRed photoinactivation but rather leads to the dissociation of the rosette into individual invadosomes. The characterization of the ECM degradation by the multiple cell lines that we generated allowed us to determine the involvement of β1 integrins in this process and in invasion. This integrin is essential for proteolytic function of invadosomes. We observed a dramatic decrease in ECM degradation in pOBL β1A−/− SrcYF cells. This result was expected because despite the transformation of the cells by SrcYF, the loss of the integrin chain was accompanied by an almost complete loss of invadosomes. More surprisingly, the level of ECM degradation did not correlate with the increase in rosette assembly. Indeed, the expression of the preactivated β1A mutant D759A in a β1 null genetic background resulted in a two- to threefold increase in the rosette number, which was associated with a significant decrease in the ECM degradation. To our knowledge, this is the first report of the uncoupling of invadosome formation and matrix degradation. This could be due to the property of β1A integrin to interact with the plasma membrane-associated metalloprotease MT1-MMP (also called MMP14), which localized to invadosomes and is essential for the ECM degradation (Galvez et al., 2002; Steffen et al., 2008). Interference with MT1–MMP trafficking at the plasma membrane revealed that MT1–MMP activation and cell invasiveness are tightly coupled (Uekita et al., 2001; Steffen et al., 2008). Several lines of evidence have now established that in addition to being endosomal passengers, an important function of integrins is to direct the trafficking of other receptors and cargos (for review, see Caswell et al., 2009). One can hypothesize that the affinity state of B1A integrin could modulate MT1–MMP trafficking at the plasma membrane and therefore impair its activity at the cell surface. This hypothesis is supported by the fact that PKCα increases integrin trafficking (Ng et al., 1999) and that expression of β1A S785D or S785E, which mimic PKC activation, results in increased ECM degradation.

In conclusion, our results support a central role of β1 integrin-dependent outside-in signaling pathways in the regulation of the multiple cell compartments involved in invadosome functions.

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REFERENCES


β1 Integrin Control of Invadosomes


