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Paxillin Phosphorylation Controls Invadopodia/Podosomes Spatiotemporal Organization

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In Rous sarcoma virus (RSV)-transformed baby hamster kidney (BHK) cells, invadopodia can self-organize into rings and belts, similarly to podosome distribution during osteoclast differentiation. The composition of individual invadopodia is spatiotemporally regulated and depends on invadopodia localization along the ring section: the actin core assembly precedes the recruitment of surrounding integrins and integrin-linked proteins, whereas the loss of the actin core was a prerequisite to invadopodia disassembly. We have shown that invadopodia ring expansion is controlled by paxillin phosphorylations on tyrosine 31 and 118, which allows invadopodia disassembly. In BHK-RSV cells, ectopic expression of the paxillin mutant Y31F-Y118F induces a delay in invadopodia disassembly and impairs their self-organization. A similar mechanism is unraveled in osteoclasts by using paxillin knockdown. Lack of paxillin phosphorylation, calpain or extracellular signal-regulated kinase inhibition, resulted in similar phenotype, suggesting that these proteins belong to the same regulatory pathways. Indeed, we have shown that paxillin phosphorylation promotes Erk activation that in turn activates calpain. Finally, we observed that invadopodia/podosomes ring expansion is required for efficient extracellular matrix degradation both in BHK-RSV cells and primary osteoclasts, and for transmigration through a cell monolayer.

INTRODUCTION

Podosomes are dot-like actin-rich structures involved in cell/extracellular matrix (ECM) adhesion found in some specific cell types such as cells belonging to the monocytic lineage including osteoclasts, macrophages, and dendritic cells. They have also been described in aortic endothelial cells (Moreau et al., 2003), smooth muscular cells (Gimona et al., 2003), and Src-transformed cells (Tarone et al., 1985; Marchisio et al., 1987; Gavazzi et al., 1989; Ochoa et al., 2000; Abram et al., 2003). In osteoclasts, rosettes also called podosome rings were shown to expand and fuse in a microtubule-dependent manner, leading to the formation of podosome belts localized at the cell periphery (Destaing et al., 2003, 2005; Jurdic et al., 2006). Due to the oncogenic activity of v-Src, Src-transformed cells are closer to cancer rather than normal cells; thus, their adhesive structures are more related to invadopodia (Linder, 2007). Indeed, invadopodia are protrusions emanating from the ventral surface of invasive cancer cells (Mueller and Chen, 1991; Yamaguchi et al., 2005; Gimona and Buccione, 2006) exhibiting a very high proteolytic activity toward the extracellular matrix (Mueller and Chen, 1991; Mueller et al., 1992), one aspect that was reported in many articles to be the hallmark these structures (Baldassarre et al., 2003; Buccione et al., 2004; McNiven et al., 2004; Linder and Kopp, 2005; Ayala et al., 2006; Gimona and Buccione, 2006; Linder, 2007). Invadopodia of Src-transformed cells were shown to self-organize into rosettes similarly to what was observed for podosomes in osteoclasts (Destaing et al., 2003) and endothelial cells (Moreau et al., 2003).

Podosomes and invadopodia share many components and structural features (Linder and Aepfelbacher, 2003; Buccione et al., 2004; Gimona and Buccione, 2006). They have two distinct parts: the core is an actin-rich column (Marchisio et al., 1984; Pfaff and Jurdic, 2001; Baldassarre et al., 2006) containing proteins involved in actin nucleation such as Wiskott–Aldrich syndrome protein (WASP) (Linder et al., 1999; Mizutani et al., 2002) and cortactin (Bowden et al., 1999; Linder et al., 2000; Pfaff and Jurdic, 2001; Artym et al., 2006; Bowden et al., 2006; Luxenburg et al., 2006; Tehrani et al., 2006; Webb et al., 2007). The second part is a multimolecular complex surrounding the core and composed of integrin receptors and integrin-associated proteins also found in focal adhesions such as vinculin and talin (Linder and Aepfelbacher, 2003) or paxillin (Bowden et al., 1999; Pfaff and Jurdic, 2001). Paxillin is an adaptor protein initially found at cell extracellular matrix contacts in focal adhesions (Turner et al., 1990). It is highly phosphorylated on tyrosyl residues (Turner et al., 1990; Schaller, 2001), and it has been shown to regulate focal adhesion dynamics and cell migration (Nakamura et al., 2000; Petit et al., 2000; Brown and Turner, 2004; Vindis et al., 2004; Webb et al., 2004). The tyrosines 31 (Y31) and 118 (Y118) are preferential sites for
phosphorylation inducing focal adhesion turnover and cell migration possibly through the binding of Crk (Birge et al., 1993; Salgia et al., 1995; Schaller and Parsons, 1995; Nakamura et al., 2000; Petit et al., 2000; Zaidel-Bar et al., 2007).

Podosome/invadopodia dynamics and functions were reported to be regulated by the Rho family GTPases (Chellaiah et al., 2000; Moreau et al., 2003; Destaing et al., 2005; Yamaguchi et al., 2005; Gimona and Buccone, 2006), dynamin (Lee and De Camilli, 2002), and by Src-induced tyrosine phosphorylations (Marchisio et al., 1984; Tarone et al., 1985; Mueller et al., 1992; Linder and Aepfelbacher, 2003; Bowden et al., 2006). More specifically, c-Src was identified as a key regulator of osteoclast-mediated bone resorption (Yoneda et al., 1993; Hall et al., 1994).

Here, we described that Rous sarcoma virus (RSV)-transformed baby hamster kidney (BHK) cells have the ability to form successively invadopodia clusters, rings, and belt-like structures under the control of tyrosine phosphorylations. During these processes, invadopodia underwent a spatiotemporal organization that influenced their contact with the ECM. Paxillin phosphorylation on tyrosines 31 and 118 specifically stimulates invadopodia disassembly at the inner rim of the ring. The role of paxillin in controlling invadopodia ring dynamics was extended to the organization of osteoclast podosomes into rings and belts. The lack of paxillin phosphorylation, calpain or extracellular signal-regulated kinase (Erk) inhibition, resulted in similar phenotype, suggesting that these proteins belong to the same regulatory pathways. Indeed, paxillin phosphorylation promotes Erk activation that in turn was reported to activate calpain.

Finally, we have shown that invadopodia/podosomes ring expansion is required for efficient extracellular matrix degradation both in BHK-RSV cells and primary osteoclasts, and for transmigration through a cell monolayer.

**MATERIALS AND METHODS**

**Antibodies and Reagents**

The following antibodies were used: monoclonal anti-paxillin (clone 349; BD Biosciences, Le Pont de Claix, France), polyclonal anti-paxillin (Biosource Europe, Nivelles, Belgium), monoclonal anti-cortactin (clone 4F11; Upstate Biotechnology, Charlottesville, VA), anti-β, integrin EDL1 (generously given by Dr. B. Niewandt, Wuerzburg, Germany), monoclonal anti-phospho-tyrosine (clone 4G10; Upstate Biotechnology), monoclonal anti-vinculin (Millipore/Chemicon, St Quentin-Yvelines, France; AbCys, Paris, France) monoclonal anti-P-Erk (T202, Y204; Santa Cruz Biotechnology, Heidelberg, Germany), and polyclonal anti-Erk 1/2 (New England Biolabs, Ipswich, MA; Ozyme, St.-Quentin-en-Yvelines, France). Horseradish peroxidase (HRP)-conjugated goat anti-mouse or goat anti-rabbit immunoglobulin (IgG) were from Bio-Rad (Marnes-la-Coquette, France) and Jackson ImmunoResearch Laboratories (Soham, United Kingdom), respectively. Secondary antibodies Alexa 488- or Alexa 546-conjugated goat anti-rabbit or goat anti-mouse Igs were purchased from Invitrogen (Cergy Pontoise, France). Fluorescein isothiocyanate- (FITC) and tetramethylrhodamine B isothiocyanate (TRITC)-conjugated phalloidin were from Sigma (Isle d’Abeau, France). Sodium orthovanadate was from LC Laboratories (Woburn, MA), and it was prepared as a 100 mM stock solution in water. The calpain inhibitor was from Calbiochem (VWR International, Strasbourg, France), and it was prepared as a 24 mM stock solution in dimethyl sulfoxide (DMSO) and used at the final concentration 50 μM. The mitogen-activated protein kinase kinase (MEK)/Erk inhibitor U0126 was from Promega (Madison, WI), and it was prepared as a 10 mM stock solution in DMSO and used at the final concentration 100 μM.

**cDNA Constructs**

Human wild-type (WT) and Y31F/Y118F (YF) paxillin cDNAs were subcloned from plasmid vectors generously provided by Dr. M. Hiraishi (Department of Molecular Biology, Osaka Bioscience Institute, Suita, Osaka), into pcDNA3.1 (BD Biosciences) by using EcoRI and BspEI restriction enzymes, and T4 DNA ligase. Vectors encoding pEGFP-cortactin and pDsRed-N1-cortactin were from Dr. P. Jurdic (Ecole Normale Superieure, Lyon, France) (Destaing et al., 2003).

**Cell Culture and Transfection**

Baby hamster kidney cells transformed by Rous sarcoma virus (BHK-RSV cells) were maintained in DMEM containing 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin in a humidified air, 5% CO₂ atmosphere. For transient transfections, BHK-RSV cells were plated in 24-well plates 24 h before Exgen 500-mediated DNA transfection (Qiagen, Germany). For tranfection, 24 h after transfection by using a transfection/EDTA solution and plated on Lab-Tek chambers (Nalgene; Nalge Nunc International, Rochester, NY) in a 37°C incubator under a 5% CO₂ atmosphere. Cells were imaged 24 h later at room temperature, in DMEM medium supplemented with 10% fetal calf serum (FCS) and 10 mM HEPES. For conventional immunofluorescence, cells were processed 48 h after transfection.

**Knockdown of Paxillin in Primary Osteoclasts**

Mice differentiated osteoclasts from spleen were transfected twice with duplex of oligonucleotides targeting paxillin mRNA, in presence of Oligofectamine reagent (Invitrogen). Two RNA duplexes were used: sense 5'-GAG-CCC-ÚCA-CCU-ACC-GUC-AU-TTT-3' and antisense 5'-AU-GAC-CCU-GUG-UGG-GCU-UTT-3' alternatively, sense 5'-GUG-UGG-AGC-CCU-UGU-U-3' and antisense 5'-C-CAA-AAG-GCU-CCA-CAC-CTT-3'. The two sequences targeted at paxillin mRNA did not overlap. For transfection, 50 μM of small interfering RNA (siRNA) at 20 μM was added to 250 μl of Opti-MEM (Invitrogen) and 50 μl of Oligoectamine to 250 μl of Opti-MEM. Both solutions were mixed and incubated 20 min at room temperature, and then they were added to the cells. After transfection, the cells were fixed with 3% phosphate-buffered saline (PBS)-paraffinumaldehyde (PFA), permeabilized in 0.1% PBS-Triton, and incubated with phalloidin-FITC for 1 h at 37°C for actin staining. Efficiency of siRNA-mediated paxillin silencing was checked by Western blotting and immunofluorescence analysis.

**Immunofluorescence Microscopy**

Cells grown on glass coverslips were fixed with 2.5% paraformaldehyde and 5% sucrose in PBS for 10 min at room temperature (RT), and then they were permeabilized in 0.1% Triton X-100 PBS for 5 min. Coverslips were washed twice with PBS, blocked in 1% bovine serum albumin (BSA) in PBS and incubated for 1 h at RT with primary antibodies. Cells were rinsed in PBS, and secondary antibodies and TRITC-phalloidin were added for 1 h at RT. Coverslips were permanently mounted in Mowiol from Calbiochem (VWR International, Strasbourg, France) containing 4′-6-diamidino-2-phenylindole. Fixed cells were examined using a confocal laser-scanning microscope (LSM 510; Carl Zeiss, Le Pecq, France), equipped with a 40× Plan-Neo Fluor oil immersion objective. For Scan Z analyses, successive planes in three-dimensional (3D) image stacks were taken every 0.1 μm. Images of interference reflection microscopy were obtained using the same objective equipped with the Wollaston prism and a 633-nm HeNe laser. The polarized light reflection was detected in channel 1 of a confocal microscope (LSM 510) equipped with polarization analyzer.

**Videomicroscopy**

Living BHK-RSV cells were imaged at room temperature by using the inverted confocal laser-scanning microscope with a 40×/numerical aperture (NA) 1.0 water immersion objective. Cells were imaged at 24 h after transfection. Mouse differentiated osteoclasts from spleen were transfected twice with oligonucleotides targeting paxillin mRNA, in presence of Oligofectamine reagent (Invitrogen). Two RNA duplexes were used: sense 5′-GAG-CCC-ÚCA-CCU-ACC-GUC-AU-TTT-3′ and antisense 5′-AU-GAC-CCU-GUG-UGG-GCU-UTT-3′ alternatively, sense 5′-GUG-UGG-AGC-CCU-UGU-U-3′ and antisense 5′-C-CAA-AAG-GCU-CCA-CAC-CTT-3′. The two sequences targeted at paxillin mRNA did not overlap. For transfection, 50 μM of small interfering RNA (siRNA) at 20 μM was added to 250 μl of Opti-MEM (Invitrogen) and 50 μl of Oligoectamine to 250 μl of Opti-MEM. Both solutions were mixed and incubated 20 min at room temperature, and then they were added to the cells. After transfection, the cells were fixed with 3% phosphate-buffered saline (PBS)-paraffinumaldehyde (PFA), permeabilized in 0.1% PBS-Triton, and incubated with phalloidin-FITC for 1 h at 37°C for actin staining. Efficiency of siRNA-mediated paxillin silencing was checked by Western blotting and immunofluorescence analysis.

**Molecular Biology of the Cell**
minated to SDS-PAGE electrophoresis and Western blotting.

coated beads (overnight at 4°C). After centrifugation, the pellets were sub-
was successively incubated with IgG 2A-coated beads and paxillin antibody-
paxillin monoclonal antibody (mAb) for 2 h at 4°C. Then, 1 mg of total lysate
protein A-Sepharose beads (GE Healthcare) were then incubated with either

West Grove, PA, respectively) were detected by enhanced chemiluminescence
ies. HRP-conjugated goat anti-mouse or goat anti-rabbit secondary antibodies
transferred to nitrocellulose membranes, and probed with primary antibod-

Roche Diagnostics). After a first incubation with 3% PBS-BSA for 1 h at 4°C,
protein A-Sepharose beads (GE Healthcare Europe, Saclay, France). Equal loading was assessed by pro-
tein staining on nitrocellulose membrane with Ponceau S (Sigma).

Coimmunoprecipitation
Whole cell lysates were prepared in 50 mM Tris-HCl, pH 7.3, 75 mM NaCl, 50
mM NaF, 40 mM sodium pyrophosphate, 1 mM Na3VO4, 1 mM EDTA, 1 mM
PMFSF, and 0.02% (vol/vol) protease inhibitor cocktail (complete EDTA-free;
Roche Diagnostics, Mannheim, Germany). Then, 20 μg of proteins from each
cell lysate was submitted to SDS-polyacrylamide gel electrophoresis (PAGE),
transferred to nitrocellulose membranes, and probed with primary antibod-
ies. HRP-conjugated goat anti-mouse or goat anti-rabbit secondary antibodies
(from Bio-Rad, Hercules, CA, and Jackson ImmunoResearch Laboratories,
West Grove, PA, respectively) were detected by enhanced chemiluminescence

GE Healthcare Europe, Saclay, France). Equal loading was assessed by pro-
tein staining on nitrocellulose membrane with Ponceau S (Sigma).

Extracellular Matrix Degradation Assays
The extracellular matrix was carried out by coating coverslips with TRITC-
labeled gelatin diluted at 10 μg/ml in DMEM, for 24 h at 4°C. Then, the
gelatin-TRITC–coated coverslips were fixed in 3% paraformaldehyde, 5%
sucrose in PBS for 10 min at room temperature. After two washes with sterile
PBS, the coverslips were coated with human vitronectin (BioSource Interna-
tional, Camarillo, CA) at 5 μg/ml in DMEM for 24 h at 4°C. Then, the
coverslips were fixed and washed twice with sterile PBS. Finally, BHK-RSV
cells, or K-RSV cells transfected with paxillin-GFP were plated onto the
covered coverslips in DMEM containing 10% FCS. After 24 h of culture, cells
were fixed and observed with an inverted confocal laser-scanning microscope
(LSM 510) equipped with a 40× numerical aperture (NA) 1.2 Plan Neo Fluor
oil immersion objective Degradation of the extracellular matrix was evaluated
by visualizing dark holes in the red matrix. Quantification of the degradation
ability was established by calculating the relative degradation index: DR
was calculated using Volocity 2.6.3 Software
extracted from stacks and treated with Adobe Photoshop (Adobe Systems,
imaging at 37°C every 3 min with both TIRF and epifluorescence. Images were
extracted from stacks and treated with Adobe Photoshop (Adobe Systems,
Mountain View, CA) for artificial colors attribution. Then, quantification of
signals intensity along the ring was made using Volocity 2.6.3 Software

Invadopodia in BHK-RSV Cells Reorganize Sequentially
into Clusters, Rings, and Belt-like Structures
In BHK-RSV cells plated on glass coverslips, invadopodia
core staining by an anti-cortactin mAb revealed that indi-
vidual invadopodia could self-assemble into various pat-
terns. These structures evolved over time: 24 h after plating,
invadopodia were mostly organized into clusters and small
rings sometimes incompletely formed or displaying irregular
shapes with diameters <10 μm (Figure 1, A and C).

Within 48 h, >50% of invadopodia rings had increased in
diameter (>10 μm). At this stage, invadopodia were found
in large circular-shaped rings and belt-like structures (Figure
1A). These observations suggested that invadopodia clusters
and small rings self-organize during the early stages
of cell attachment and maturate into larger rings and belt-
like structures later, a process that has been described in
differentiated osteoclasts (Destaing et al., 2003).

Dynamic of Invadopodia Is Regulated by Tyrosine
Phosphorylations
Invadopodia are very active sites of phosphorylation on
tyrosyl residues (Marchisio et al., 1984; Tarone et al., 1985;
Gavazzi et al., 1989; Mueller et al., 1992; Bowden et al., 2006).
To address the role of this posttranslational modification, we

Figure 1. Dynamics of invadopodia in src-transformed BHK cells. (A) BHK-RSV cells were plated on glass coverslips for 24 or 48 h at
37°C, and then they were immunostained with an anti-cortactin
mAb. Bar, 20 μm. (B) BHK-RSV cells plated for 24 h (control; 0 min) were treated either with the orthovanadate vehicle (H2O) (control;
30 min) or with 5 mM orthovanadate for 30 min (+ Na3VO4; 30 min)
or 90 min (+ Na3VO4; 90 min) before immunostaining for cortactin
or actin. Bar, 20 μm. (C) Size distribution of invadopodia rings in
BHK-RSV cells plated for 24 h without orthovanadate (blue line)
and 24-h–plated cells and treated with 5 mM orthovanadate during
30 min (yellow line) or 90 min (red line), respectively (n = 60 cells
for each condition).
tase inhibitor Na$_3$VO$_4$ supplemented to the medium at the final concentration of 5 mM, after 24 h of preculture under standard conditions. Whereas untreated cells mainly displayed small rings whose diameter did not significantly increase within a subsequent 30-min period, orthovanadate addition induced, within 30 min, a dramatic increase in the size of invadopodia rings that was amplified together with the enhanced formation of belt-like structures at longer incubation times (Figure 1B). Quantification of these data is shown in Figure 1C. These results strongly suggest that the expansion of invadopodia rings is regulated by tyrosine phosphorylation events leading to the fast maturation of invadopodia rings into belt-like structures.

Tyrosine Phosphorylation Accelerates Invadopodia Turnover Resulting in Faster Ring Enlargement

To visualize the process of invadopodia ring enlargement, we performed time-lapse videomicroscopy analyses of BHK-RSV cells transfected with an invadopodia core protein, cortactin fused to GFP. In control cells, invadopodia rings displayed no diameter increase during the time course of the experiment (Figure 2 and Supplemental Movie 1), whereas during the same period in orthovanadate-treated cells invadopodia rings expanded and fused, leading to the formation of peripheral belt-like structures (Figure 2 and Supplemental Movie 2). Image deconvolution resolved the rings into individual invadopodia (Figure 2). The use of false colors and image merging at 0 min (red), 80 min (blue), and 130 min (green) allowed following each invadopodia along the time course of the experiment. According to the color pattern, five regions could be defined from the center to the periphery of the ring that reflected both the spatiotemporal organization of invadopodia within the ring and the relative immobility of the individual invadopodia.

Figure 2. Orthovanadate-induced invadopodia ring expansion results from the coordinated processes of invadopodia assembly at the periphery and disassembly at the ring center. BHK-RSV cells plated for 24 h were transiently transfected with cDNA encoding cortactin-GFP. The expressed fusion protein was followed by time-lapse videomicroscopy for 130 min at room temperature, in the absence (control) or in the presence of 5 mM orthovanadate (+Na$_3$VO$_4$) by using a confocal microscope. Bar, 10 μm. Image deconvolution and zoom, using MetaMorph software revealed the individual invadopodia within the invadopodia rings. Merge of the images in false colors from 0 min (red), 80 min (blue), and 130 min (green) allowed following each invadopodia along the time course of the experiment. According to the color pattern, five regions could be defined from the center to the periphery of the ring that reflected both the spatiotemporal organization of invadopodia within the ring and the relative immobility of the individual invadopodia.
moted by orthovanadate treatment favored both invadopodia assembly at the outer rim and dissociation at the inner rim of the ring, respectively. Consequently, these coordinated processes should result in the shortening of invadopodia lifetime. This was confirmed by direct measurement in orthovanadate-treated cells compared with untreated cells (59 ± 19 vs. 122 ± 13 min, respectively; n = 34; p = 0.014).

Phospho-paxillin Accumulates at Invadopodia Ring Center

Surprisingly, immunostaining of phospho-tyrosines in invadopodia ring of untreated BHK-RSV extended toward the ring center where actin was not detected by phalloidin-TRITC, and it was not present at the outer rim of the ring (Figure 3A, a–d). Because invadopodia were dissociated at the inner rim of the ring, this observation suggested that dismantling of invadopodia at the inner rim of the ring started with actin core disassembly at sites where a strong accumulation of tyrosyl-phosphorylated proteins occurred. Paxillin is a major target for tyrosine kinases, and it was reported to promote focal adhesion disassembly upon phosphorylation on tyrosines 31 and 118 (Brown and Turner, 2004; Zaidel-Bar et al., 2007). Thereby, we hypothesized that

Paxillin, through its phosphorylation, could be responsible of invadopodia disassembly at the inner rim of the ring. Indeed, immunostaining of control BHK-RSV cells with a monoclonal anti-paxillin antibody (Figure 3A, e–h) or phospho-paxillin–specific antibodies (Figure 3A, i–p) revealed a similar localization than phospho-tyrosine staining, indicating that endogenous paxillin accumulated in a phosphorylated state on both tyrosine 118 (PY118; j) and on tyrosine 31 (PY31; n) (i and m are actin staining). Changing the secondary antibody labeling did not modify the respective localization of paxillin (q) at the center of the ring and cortactin (r) at the periphery (merge images, s; zoom, t). Bar, 6 μm. Bottom, triple merged images. Zoom on four invadopodia (P1–P4) localized from the periphery (P1) to the ring center (P4) is presented: V, P, C, and M are vinculin, PY118 paxillin, cortactin-DsRed, and merge staining, respectively. Each invadopodia showed a specific composition. (C) BHK-RSV cells were transiently transfected with cortactin-DsRed, plated for 24 h, and treated subsequently with 5 mM Na₃VO₄ for 30 min before staining with a monoclonal anti-β3 integrin antibody. Z scanning along the white arrow (A → B) reveals an increase in the length of invadopodia core along a cross section of the ring and β3 integrins recruitment in invadopodia located at the inner rim of the ring. Bar, 10 μm. (D) BHK-RSV cells plated for 24 h were stained with anti-β3 integrin antibody and phospho-specific polyclonal PY31 or PY118 paxillin antibodies. Bar, 10 μm.
Vinculin, phospho-paxillin, and talin (data not shown) accumulated at invadopodia located at the inner rim of the ring whereas the core protein cortactin was only present in invadopodia of the outer rim together with other core proteins such as actin, α-actinin, and dynamin (data not shown). These differential stainings clearly show a spatiotemporal change in invadopodia structure and composition: newly assembled invadopodia contained exclusively core proteins such as cortactin (Figure 3B, P1). Mature invadopodia were constituted by core proteins (cortactin), with surrounding proteins such as phospho-paxillin and vinculin (Figure 3B, P2). Finally, older invadopodia at the inner rim were devoid of any core protein (Figure 3B, P3), and eventually only vinculin was detected in the remaining structures (Figure 3B, P4).

The spatiotemporal organization of paxillin and cortactin was confirmed by time-lapse experiments performed on living BHK-RSV cells transiently transfected with both GFP-paxillin and cortactin-DsRed and then treated with 5 mM orthovanadate (Supplemental Figure S1 and Supplemental Movie 3). We observed that GFP-paxillin always maintained an internal localization compared with cortactin-DsRed and that it accumulated at sites where invadopodia cores progressively disappeared.

In podosome/invadopodia, paxillin is recruited indirectly by integrins (Gimona and Buccione, 2006). Immunofluorescence microscopy with anti-β1 and anti-β3 integrin antibodies in orthovanadate-treated BHK-RSV cells showed that β1 integrins were excluded from invadopodia (data not shown), whereas β3 integrin accumulated at invadopodia located at the inner rim of the ring (Figure 3C) where it was colocalized with phospho-paxillin (Figure 3D) suggesting that paxillin recruitment at the inner rim of the ring depends on integrins.

Differential ECM/Cell Contacts at Invadopodia Rings

Z Scan analysis showed that at the outer rim of the ring, invadopodia were mostly constituted of a cortactin core...
devoid of any β3 integrins (Figure 3C). At a more central localization, invadopodia cores became longer and recruited β3 integrins. Finally, at the inner rim of the rings, the cortactin core was no longer present, but the integrins remained. The increase in the core height during invadopodia maturation and the late recruitment of β3 integrins suggests that invadopodia may not be in contact with the ECM during the early stages of core assembly at the ring periphery. This hypothesis was addressed by TIRF microscopy that allowed visualization of invadopodia components in proximity to the extracellular matrix. In this experiment, BHK-RSV cells were transiently transfected with both cortactin-DsRed and GFP-paxillin and treated with 5 mM orthovanadate. We compared images of cortactin-DsRed cores in contact with the matrix visualized by TIRF, with images of the whole Cortactin-DsRed cores obtained using classical epifluorescence (a similar analysis was carried out with GFP-paxillin). False colors were used to compare the overlaid images (Figure 4). Cortactin stainings visualized by epifluorescence (green) and TIRF (red), respectively, did not fully match: epifluorescence signal extended beyond the periphery of the TIRF signal. This difference (colored gray) was interpreted as the amounts of nascent invadopodia at the ring periphery that had not yet reached the extracellular matrix. Conversely, no significant difference between GFP-paxillin epifluorescence, and TIRF signal was noticed indicating that all invadopodia containing paxillin were in contact with the support.

Lack of Paxillin Tyrosine Phosphorylation Impairs Invadopodia Disassembly at the Inner Rim of the Ring

Transient transfections of BHK-RSV cells with cDNAs encoding wild-type or mutant Y31F/Y118F GFP-paxillin named WT GFP-paxillin or YF GFP-paxillin, respectively, were carried out to address the role played by paxillin phosphorylation in invadopodia dynamics. Overexpression of paxillin mutants had a marked dominant-negative effect on the overall phosphorylation of paxillin within the cell, either in the presence (Figure 7C and Supplemental Figure S2) or absence (data not shown) of vanadate. The transfected cells were cultivated for 24 h on coverslips, and subsequently they were stained for actin with phalloidin-TRITC. Untreated cells expressing WT GFP-paxillin displayed normal invadopodia rings, whereas YF GFP-paxillin–expressing cells preferentially formed invadopodia clusters (Supplemental Figure S3). Statistical analyses (n = 101 cells; p =

**Figure 6.** YF GFP-paxillin overexpression, or calpain inhibitor treatment, impairs invadopodia core disassembly. (A) BHK-RSV cells were double transfected with cortactin-DsRed and WT GFP-paxillin or YF GFP-paxillin and treated with 5 mM orthovanadate for 30 min. Then, cells were fixed with 3% paraformaldehyde. With WT GFP-paxillin cells, WT GFP-paxillin accumulated at the center of the rosette at sites where cortactin disassembled as shown on fluorescence intensity scans along the ring axis (bottom left). In YF GFP-paxillin cells, YF GFP-paxillin still accumulated at the center of the rosette, but cortactin did not dissociate at the rosette center, resulting in a much thicker ring and colocalization with YF GFP-paxillin (shown by fluorescence intensity scans along the ring axis; bottom right). (B) BHK-RSV cells treated with 50 μM ALLM calpain inhibitor and 5 mM orthovanadate for 30 min had thicker rings similar in structure and composition to those observed in YF GFP-paxillin cells. Bar, 5 μm.
0.021) revealed that YF GFP-paxillin-transfected cells displayed fewer invadopodia rings than WT GFP-paxillin-transfected cells (Figure 5A). When ring expansion was accelerated by a 30-min treatment with 5 mM orthovanadate, the cells expressing WT GFP-paxillin exhibited invadopodia rings that were similar to those of untransfected cells. Under these experimental conditions, however, overexpression of YF GFP-paxillin induced the formation of much thicker rings with a reduced lumen (Figure 5B and Supplemental Figure S3). Statistical measurements of the ratio internal diameter/external diameter confirmed this observation (Figure 5C) (n = 54; p value between control and WT, 0.5; p value between WT and YF, <0.001). This result suggested that in presence of YF GFP-paxillin, new invadopodia cores formation at the ring periphery was not modified because the ring external diameter still increased, but invadopodia disassembly was somehow impaired at the ring center.

**Blockade of Invadopodia Disassembly by Calpain Inhibition**

WT GFP-paxillin accumulation at the ring center was always associated with the disappearance of cortactin-DsRed–rich cores (Figure 6A, left), whereas YF GFP-paxillin mutant clearly colocalized with cortactin-DsRed at the inner rim of the ring, confirming the defect in invadopodia disassembly (Figure 6A, right), suggesting that phosphorylation on paxillin is a prerequisite for invadopodia dissociation. Calpain is another protein that was reported to regulate podosomes disassembly (Calle et al., 2006), possibly through cortactin proteolysis (Huang et al., 1997). Therefore, we investigated whether in BHK-RSV cells, calpain inhibition could mimic the YF GFP-paxillin phenotype. We applied the calpain inhibitor ALLM at the final concentration 50 μM associated with 5 mM orthovanadate treatment for 30 min (to promote fast ring expansion). Actin staining clearly indicated that calpain inhibition was sufficient to inhibit invadopodia cores disassembly at the ring center and generated thick rings similar to what was observed under transfection of YF GFP-paxillin (Figure 6B, right). Moreover, under these experimental conditions, Western blotting revealed a marked decrease in cortactin degradation (data not shown). Together, these findings suggested that calpain is positioned downstream of paxillin phosphorylation in the invadopodia disassembly regulatory pathway.

**Paxillin Phosphorylation on Tyrosine 31 and 118 Controls Erk Activation in Invadopodia Rings**

The mitogen-activated protein kinases Erk1 and 2 are involved in the FAK/Src/paxillin signaling pathway driving focal adhesions disassembly (Webb et al., 2004). Moreover, it has been reported that Erk can activate calpain (Glading et al., 2001). Therefore, we hypothesized that paxillin could activate calpain through Erk signaling, resulting in invadopodia disruption. Indeed, coimmunoprecipitation experiments carried out with lysates from BHK-RSV cells revealed that paxillin and Erk interacted within a same molecular complex (Figure 7B). Moreover, immunofluorescence analysis showed that phospho-Erk (P-Erk) was present not only in the nucleus but also was enriched with phospho-paxillin into invadopodia rings (Figure 7A). Western blotting with P-Erk and Erk antibodies revealed that without orthovanadate, the levels of P-Erk were similar in WT GFP-paxillin and YF GFP-paxillin cells. Conversely, stimulating tyrosine phosphorylation by orthovanadate markedly increased the phosphorylation level of Erk in WT GFP-paxillin cells, but it had no significant effect on P-Erk levels in YF GFP-paxillin cells (Figure 7C). Indeed, overexpression of YF GFP-paxillin had a dominant-negative effect on endogenous paxillin phosphorylation that is reduced by at least 45% (Figure 7C and Supplemental Figure S5). Together, these results suggested that Erk phosphorylation and thereby its activation is regulated by paxillin phosphorylation on tyrosine 31 and 118 in invadopodia rings.

**Erk Activation Is Required for Invadopodia Disassembly in Invadopodia Rings**

To address to possible role of Erk phosphorylation in invadopodia core disassembly, we treated BHK-RSV cells with U0126 (a specific noncompetitive inhibitor of Erk kinase activity; Favata et al., 1998) at the final concentration of 100 μM. Then, we measured the thickness of the rings after 30-min treatment with orthovanadate. Invadopodia core immunostaining with anti-cortactin revealed that U0126 inhibited invadopodia rosette thickening similarly to what was observed upon expression of YF GFP-paxillin, or calpain inhibition, suggesting that Erk inhibition prevented invadopodia cores disassembly at the inner rim of the ring (Figure 8, A and B). The similarities in the phenotypes suggested that Erk activation was acting upstream of calpain. This hypothesis was confirmed by intracellular calpain activity measurements indicating that calpain activity was
mostly wiped out by blocking Erk with U0126 (Figure 8C) and by Western blotting showing that Erk inhibition by U0126 induced a reduction in cortactin degradation (Figure 8D). Together, our results strongly suggested that Erk activation is responsible of calpain-driven disassembly of invadopodia with the rings.

**Generalization of Paxillin Role Invadopodia and Podosome Dynamics**

The role of paxillin in podosome/invadopodia dynamics in a more physiological context was tested in primary mature osteoclasts in culture that exhibited podosomes organized into a classical peripheral belt. Similarly to invadopodia ring of BHK-RSV cells, paxillin accumulated at the inner rim of the podosome belt (Figure 9A). The same distribution was observed using phospho-paxillin–specific antibodies (data not shown). This suggested that paxillin could be also involved in osteoclast podosomes dynamics. We knocked down paxillin using siRNA in differentiated mouse primary osteoclasts. The efficiency of the knockdown was checked by Western blotting (Figure 9B). After paxillin knockdown, podosomes were no longer self-organized into a belt, but rather they remained as a cluster of podosomes (Figure 9C). We concluded that paxillin was also required for organization of podosome rings in this physiological model.

**Lack of Invadopodia Ring Organization Impairs Extracellular Matrix Degradation**

We investigated the possible effects of Y31F/Y118F paxillin mutations on invadopodia functions, such as extracellular matrix degradation. We observed that 24-h plated BHK-RSV cells could efficiently degrade an extracellular matrix constituted by one layer of gelatin-TRITC covered by one layer of vitronectin (a ligand of α5β3 integrins) at invadopodia ring sites. Fully degraded matrix showed up as a dark trail left behind the invadopodia actin rings, probably due to the migration of the cell that displaced the ring underneath degradation took place (Figure 10A, control). It is noteworthy that differentiated primary mouse osteoclasts exhibiting podosomes could similarly degrade this organic matrix.

**Figure 8.** Inhibition of Erk phosphorylation by U0126 prevents invadopodia cores disassembly. (A) BHK-RSV cells were treated with 100 μM Erk inhibitor U0126. They formed thick invadopodia rings under orthovanadate treatment (5 mM; 30 min). Bar, 2 μm. (B) Statistical measures of invadopodia rings thickness in cells treated or not with U0126 after orthovanadate treatment. (C) BHK-RSV cells were treated with either DMSO or calpain inhibitor ALLM at 50 μM (1 h), orthovanadate at 5 mM (30 min), UO126 at 100 μM (1 h), UO126 at 100 μM (1 h), and then orthovanadate at 5 mM (30 min). After each treatment, cells were supplemented with CMAC t-Boc-Leu-Met (Invitrogen) at 50 μM (10 min) and fixed with 3% PBS-PFA. Cells were mounted and analyzed by fluorescence microscopy. Bar, 10 μm. (D) Western blotting showing that Erk inhibitor U0126 increased cortactin level either with or without orthovanadate treatment. Equal protein loading was checked by proteins staining on the nitrocellulose membrane with Red Ponceau S (data not shown).
without any need of mineralization (Figure 10B). Expression of WT GFP-paxillin did not impair matrix degradation (Figure 10A, GFP WT-paxillin). By contrast, expression of YF GFP-paxillin dramatically reduced extracellular matrix degradation. Although each invadopodia corresponded to a
small area of degradation, these degradation spots remained disperse, probably due to the lack of invadopodia self-organization into rings, resulting in an overall lower efficiency in matrix proteolysis (Figure 10A, YF GFP-paxillin). The impaired matrix degradation was quantified by the relative degradation index described in Materials and Methods. Statistical measurements of the relative degradation index indicated that the expression of YF GFP-paxillin reduced by 2.4-fold the ability of cells to degrade the matrix compared with WT GFP-paxillin–transfected cells (n = 48 cells; p < 0.001) (Figure 10C).

**Paxillin Y31F/Y118F Overexpression Alters Transmigration of BHK-RSV Cells through a Cell Monolayer**

Matrix degradation and cell transmigration are often associated with invasiveness of cancer cells (Yamaguchi et al., 2005). To check the possible role of invadopodia dynamics in transmigration, BHK-RSV cells were transfected with WT GFP-paxillin and plated on a HeLa cells monolayer. After 24 h, time-lapse videomicroscopy in 4D (3 space dimensions + time) was carried out and allowed us to visualize cells crossing the layer along the time from different side, top, and bottom views (Figure 11A and Supplementary Movies 4, A–C). The progressive sliding of the BHK-RSV cell under the cell layer (Figure 11A, side view) was associated with the expansion of an invadopodia ring located at the ventral membrane of the cell (Figure 11A, bottom view), suggesting that invadopodia ring expansion could play a role in this process. Immunofluorescence experiments with BHK-RSV cells fixed after 24-h plating on the cell monolayer and stained for actin with phalloidin-TRITC were carried out. Observation of the cells by confocal microscopy along the z-axis allowed us to have a better insight of invadopodia ring formation during transmigration. At the early stages, BHK-RSV cells on the top side of the cell layer sent a membrane protrusion through the cell layer along the time from different side, top, and bottom views (Figure 11A and Supplementary Movies 4, A–C). The progressive sliding of the BHK-RSV cell under the cell layer (Figure 11A, side view) was associated with the expansion of an invadopodia ring located at the ventral membrane of the cell (Figure 11A, bottom view), suggesting that invadopodia ring expansion could play a role in this process. Immunofluorescence experiments with BHK-RSV cells fixed after 24-h plating on the cell monolayer and stained for actin with phalloidin-TRITC were carried out. Observation of the cells by confocal microscopy along the z-axis allowed us to have a better insight of invadopodia ring formation during transmigration. At the early stages, BHK-RSV cells on the top side of the cell layer sent a membrane protrusion through the cell layer that reached the extracellular matrix. At this contact the membrane protrusion exhibited a small invadopodia ring (Figure 11B, stage 1). Subsequently, the cells displayed large invadopodia rings sliding under the neighboring cells of the layer (Figure 11B, stage 2). Later on, large well-formed invadopodia belt allowed the full spreading of the BHK-RSV cells beneath the cell layer (Figure 11B, stage 3), and finally, BHK-RSV cells were fully located on the bottom face of the cell monolayer (Figure 11B, stage 4).

According to these experiments, invadopodia dynamics seemed to be tightly related to transmigration. Because paxillin tyrosine phosphorylation could efficiently alters this process we investigated the role of paxillin phosphorylation in cell transmigration. We expressed YF GFP-paxillin into BHK-RSV cells. As expected, these cells presented markedly restricted spreading areas under the cell layer, compared with WT GFP-paxillin (Figure 11C; n = 58; p < 0.001) strongly suggesting that paxillin tyrosine phosphorylation through its involvement in invadopodia ring expansion favors the spreading of the transformed cells onto basal membrane that in turn triggered efficient transmigration through a cell layer.

**DISCUSSION**

Here, we describe that RSV-transformed BHK cells exhibit invadopodia that self-organize into large invadopodia rings and peripheral invadopodia belts, which are similar to podosomes found in macrophages and osteoclasts. These large structures were observed 48 h after plating the cells on glass; thus, they have much slower dynamics that podosomes in osteoclasts. However, orthovanadate, a general tyrosine phosphatase inhibitor, dramatically accelerates the process of invadopodia ring expansion into large rings and invadopodia belts. It is noteworthy that the time of preculture in serum supplemented media is likely sufficient to allow vitronectin and fibronectin coating on the glass slide. Previous studies carried out with RAW 264.7 cells differentiated into osteoclasts indicated that podosome ring expansion results from the assembly of new podosomes at the ring periphery together with podosome disassembly at inner rim of the ring (Destaing et al., 2003). These two processes are coordinated and maintain a relative constant ring width during expansion. Taking advantage of the acceleration of invadopodia ring expansion induced by orthovanadate in src-transformed BHK cells and using time-lapse videomicroscopy coupled with 2D deconvolution and object tracking, we demonstrated that invadopodia followed identical behavior in BHK-RSV cells. Because orthovanadate-treated invadopodia rings maintained a constant width during their expansion, tyrosine phosphorylations probably promote both new invadopodia formation at the ring periphery and invadopodia disassembly at the ring center: two mechanisms that account for the acceleration of the ring expansion. This view is consistent with the well-described action of Src-induced tyrosine phosphorylations that promote invadopodia assembly (Mueller et al., 1992; Bowden et al., 2006). Additionally, orthovanadate was shown to induce podosomes formation in monocytes and fibroblasts (Marchisio et al., 1988; Cory et al., 2002) confirming the importance of tyrosine phosphorylation in podosomes assembly.

Actin nucleating factors such as cortactin and WASP also seemed to play a major role in podosome assembly depending on their phosphorylation on tyrosine by Src (Linder and Aepfelbacher, 2003; Artym et al., 2006; Tehrani et al., 2006). Thus, it can be hypothesized that Src may promote a strong nucleation of actin polymerization at the locus of a new invadopodia formation. Additionally, optical flow analysis suggests that the initial organization into clusters and small rings may result from spontaneous self-organization of actin, whereas life span and distance between invadopodia/podosome depend on the substrate flexibility (Collin et al., 2006). By contrast, little is known on podosome/invadopodia disassembly. Interestingly, tyrosine phosphorylation has been described to promote focal adhesion turnover (Webb et al., 2004; Westhoff et al., 2004). More specifically, focal adhesion disassembly seems to be triggered by the phosphorylation of paxillin on tyrosine 31 and 118 (Brown and Turner, 2004; Zaidel-Bar et al., 2007). Because paxillin is also a major invadopodia/podosome component, it was tempting to make a parallel and to hypothesize that tyrosine-phosphorylation on paxillin is also involved in invadopodia core disassembly resulting in fine, in the complete dismantling of invadopodia. Consistent with this idea, we have shown that tyrosine 31/118 phosphorylated paxillin and β1 integrins accumulated preferentially at the inner rim of the ring where invadopodia had a tight contact with the matrix and progressively loosed their actin rich core. Moreover, overexpression of a phosphorylation-deficient paxillin-GFP Y31F/Y118F mutant clearly impaired invadopodia actin core disassembly. On orthovanadate treatment that favored fast ring expansion, this mutation resulted in the thickening of invadopodia rings with small lumens due to the inability of inner invadopodia to dismantle.

TIRF and Scan Z confocal microscopy indicated that at the early stages of invadopodia assembly, the actin core was increasing in height toward the matrix. At this stage, the newly formed invadopodia were not in contact with the extracellular matrix. Once this actin core-driven membrane
protrusion had reached the extracellular matrix, β3 integrin and integrin-associated proteins such as vinculin and paxillin were recruited and surrounded the actin core, giving rise to the classical invadopodia/podosome structure. Finally, paxillin phosphorylation seemed to be a prerequisite for core disassembly and eventually the complete disappearance of the structure. This spatiotemporal evolution is consistent with published data showing that invadopodia display different stages depending on the time contact with the extracellular matrix (Artym et al., 2006). These authors reported that during the last stage, invadopodia have lost their actin core, and in meantime massively recruited MT1-MMP promoting ECM degradation. Interestingly, we showed that podosomes in osteoclast cells could also induce the efficient degradation of the ECM, in vitro, although it has been commonly considered that these structures have a poor proteolytic activity.

Transfected the cells with YF GFP-paxillin, by using the calpain inhibitor ALLM or U0126, a specific Erk inhibitor, resulted in similar alteration of invadopodia rings in the presence of orthovanadate, strongly suggesting that all these signaling molecules belong to the same signaling pathway. Within this pathway, paxillin phosphorylation is likely acting upstream because the YN GFP mutant impairs Erk activation. Crk is a good potential candidate for signaling downstream of paxillin in invadopodia because it was shown to control migration and invasion of transformed cells (Rodriguez and Guan, 2005). The adaptor protein Crk-associated substrate (Cas), which links Crk, was also described as essential for invasion and metastasis of src-transformed cells by activating the small GTPase Rac1 (Brabek et al., 2005). Thereby, tyrosine phosphorylation of paxillin could induce Crk/Cas recruitment and promote Erk activation through Rac1 activation. In turn, we have shown that Erk was required to activate calpain in good agreement with previous work (Glading et al., 2001).

Finally, our work shows that efficient matrix degradation by BHK-RSV cells requires invadopodia ring assembly. This probably results from local concentration of matrix proteases within the ring. Indeed, with YF GFP-paxillin mutant, when invadopodia mostly self-organize into clusters, the dot-like matrix degradation suggests that matrix protease delivery at invadopodia still occurs, but the invadopodia disorganization seemed to prevent massive matrix degradation. Invadopodia ring expansion seems to be also involved in transmigration of BHK-RSV cells through a cell layer. The invadopodia ring is known to be a site of strong anchorage of the cell on the matrix while invadopodia touch the extracellular matrix beneath the cell monolayer. Scan Z confocal microscopy suggests that ring expansion may allow spreading that trigger the mechanical force that pulls the cell body through the monolayer while providing a localized site of matrix degradation. Such behavior may be a general mechanism for invasion of metastatic cells. Because these processes are upstream controlled by paxillin phosphorylation, this protein seems to be a potential target for the design of anti-metastatic drugs.

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