Multiple regulatory inputs converge on cortactin to control invadopodia biogenesis and extracellular matrix degradation

Inmaculada Ayala[1], Massimiliano Baldassarre[1,*], Giada Giacchetti[1], Giusi Caldieri[1], Stefano Tetè[2], Alberto Luini[3] and Roberto Buccione[1,§]

[1]Tumour Cell Invasion Laboratory, Department of Cell Biology and Oncology, Consorzio Mario Negri Sud, S. Maria Imbaro (Chieti), Italy
[2]Department of Oral Sciences, University "G. D'Annunzio", 66013, Chieti, Italy
[3]Membrane Traffic Laboratory, Department of Cell Biology and Oncology, Consorzio Mario Negri Sud, S. Maria Imbaro (Chieti), Italy
*Present address: Department of Pharmacology, Yale University School of Medicine, New Haven, CT, USA
§Author for correspondence (e-mail: buccione@negrisud.it)

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Summary

Invadopodia are proteolytically active protrusions formed by invasive tumoral cells when grown on an extracellular matrix (ECM) substratum. Although many molecular components have been defined, less is known of the formation and regulation of invadopodia. The multidomain protein cortactin, which is involved in the regulation of actin polymerisation, is one such component, but how cortactin is modulated to control the formation of invadopodia has not been elucidated. Here, a new invadopodia synchronization protocol is used to show that the cortactin N-terminal acidic and SH3 domains, involved in Arp2/3 complex and N-WASP binding and activation, respectively, are both required for invadopodia biogenesis. In addition, through a combination of RNA interference and a wide array of cortactin phosphorylation mutants, we were able to show that three convergent regulatory inputs based on the regulation of cortactin phosphorylation by Src-family kinases, Erk1/Erk2 and PAK are necessary for invadopodia formation and extracellular matrix degradation. These findings suggest that cortactin is a scaffold protein bringing together the different components necessary for the formation of the invadopodia, and that a fine balance between different phosphorylation events induces subtle changes in structure to calibrate cortactin function.

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Key words: Invadopodia, Invasion, Protein kinases, Cortactin

Introduction

Invasive tumoral or transformed cells grown on a flat extracellular matrix (ECM) substratum such as gelatin, fibronectin and others, extend proteolytically active protrusions into the matrix from their ventral surfaces; these protrusions have been termed invadopodia (Chen, 1989; Mueller and Chen, 1991). Invadopodial protrusions are actin-rich structures enriched in integrins, tyrosine kinase signaling machinery, soluble and membrane proteases including matrix metalloproteases (MMPs), and actin-associated proteins (Baldassarre et al., 2003; Bowden et al., 1999; Chen, 1996; Kelly et al., 1994; Monsky et al., 1994; Mueller et al., 1992; Nakahara et al., 1997). These features define them as powerhouses for the focal degradation of the ECM, made possible by a tight integration between the signaling, membrane trafficking and cytoskeletal machineries (reviewed by Buccione et al., 2004). In recent years, many molecular players have been defined. Invadopodia formation was shown to rely on the ubiquitous member of the Wiskott-Aldrich syndrome protein family (N-WASP) (Mizutani et al., 2002), an activator of the actin filament nucleator Arp2/3 complex (Olazabal and Machesky, 2001). Another study presented a morpho-functional approach to the understanding of invadopodia, reporting that Dynamin 2, an atypical GTPase implicated in the control of actin-driven cytoskeletal remodeling events and membrane transport, is required for focalized matrix degradation at invadopodia (Baldassarre et al., 2003). In addition, by a correlative confocal light-electron microscopy approach, invadopodia were found to be organized into a previously unrecognized structure consisting of a large invagination of the ventral plasma membrane surface in close spatial relationship with the Golgi complex (Baldassarre et al., 2003). ADP-ribosylation factor 6, a GTPase known to be involved in the regulation of actin assembly at the plasma membrane (Schafer et al., 2000), was also implicated in invadopodia formation and cell invasion (Hashimoto et al., 2004; Tague et al., 2004). Later, a combination of RNA interference and dominant-negative mutant expression studies confirmed the importance of N-WASP and the Arp2/3 complex in invadopodia formation, and suggested a role for some of their upstream regulators Nck1, Cdc42 and WIP (Yamaguchi et al., 2005). Cortactin (Olazabal and Machesky, 2001; Weed and Parsons, 2001) was also suggested to be localized and to function at invadopodia (Baldassarre et al., 2003; Bowden et al., 1999); this has been confirmed with RNA interference (Artym et al., 2002; Clark et al., 2007; Webb et al., 2007). More recently, analysis of the fine dynamics of invadopodia has shown that these are stable, persistent structures and that actin turnover is rapid and displays a striking comet-like organization resembling microbial pathogen-associated actin tails (Baldassarre et al., 2006).

Cortactin is an actin-binding protein and is involved in the coordination of cell migration, cytoskeleton remodeling and

intracellular protein transport (Cao et al., 2005; McNiven et al., 2000; Patel et al., 1998; Weed and Parsons, 2001). Human cortactin is encoded by *CTTN* (formerly *EMS1*) on chromosome 11q13, which is frequently amplified in breast, head and neck squamous carcinoma and bladder cancers (Bringuier et al., 1996; Schuuring, 1995). One consequence of the gene amplification is an increase in cortactin protein levels (Hui et al., 1998). Cortactin overexpression in vitro leads to increased motility and invasion (Patel et al., 1998); in vivo, it enhances the formation of bone metastases from a breast cancer cell line in a mouse model (Li et al., 2001). Consistent with its F-actin-binding ability, cortactin primarily localizes in peripheral cell structures such as lamellipodia, membrane ruffles (McNiven et al., 2000; Wu and Parsons, 1993) and invadopodia (Baldassarre et al., 2003; Bowden et al., 1999). Cortactin is a multidomain protein with an N-terminal acidic domain containing a conserved DDW motif that binds and weakly activates the Arp2/3 complex (Uruno et al., 2001). This is followed by a variable number of 37 amino acid repeats (depending on the splice variant) (van Rossum et al., 2003) constituting the actin-binding domain (ABD) of which only the fourth repeat is required for F-actin binding activity and has been suggested to stabilize the newly created branches between filaments (Weaver et al., 2001; Weaver et al., 2003). A proline-rich domain (PRD) and a C-terminal SH3 domain follow the ABD. The latter can bind and directly activate N-WASP (Martinez-Quiles et al., 2004). The SH3 domain of cortactin can also bind dynamin 2, which is required for invadopodia formation (Baldassarre et al., 2003) and this influences Arp2/3-dependent actin nucleation through cortactin (Schafer et al., 2002; Krueger et al., 2003).

Cortactin was originally identified as a major substrate of the *Src* oncogene (Wu et al., 1991) and was later confirmed to be phosphorylated on tyrosine in response to stimuli such as FGF, EGF and integrins, that induce remodeling of the cortical actin cytoskeleton (Vuori and Ruoslahti, 1995; Zhan et al., 1993). In particular, phosphorylation on Tyr421, Tyr466 and Tyr482 in the PRD is required for cell movement (Huang et al., 1998) and metastatic dissemination (Li et al., 2001), possibly by inhibition of F-actin stabilization (Huang et al., 1997). Cortactin can also be phosphorylated in vitro on Ser405 and Ser418 in the PRD by the extracellular-signal-regulated serine/threonine kinases ERK1/2 (Campbell et al., 1999). Although the precise mechanistic consequence of these regulatory events is unclear, the ability of cortactin to activate N-WASP was found to be positively regulated by ERK phosphorylation and negatively regulated by Src phosphorylation (Martinez-Quiles et al., 2004), thus suggesting a potential on-off switch mechanism.

More recently, the p21-activated serine/threonine kinase (PAK) has been shown to phosphorylate cortactin on serine residue 113 in vitro. As a consequence, cortactin binding to F-actin was reduced suggesting a role for PAK-dependent phosphorylation of cortactin in the regulation of branched actin filament dynamics (Webb et al., 2006). The PAK family comprises conventional PAK1, PAK2 and PAK3 isoenzymes, each containing an autoinhibitory domain, and a group of newly discovered members PAK4, PAK5 and PAK6 (reviewed by Kumar et al., 2006; Zhao and Manser, 2005). In general, the substrates of conventional PAKs are involved in the regulation of the cytoskeleton and adhesion. The *PAK1* gene maps to the 11q13 amplicon, the same region that contains the gene that encodes cortactin (Bekri et al., 1997). Hence, similarly to cortactin, *PAK1* overexpression has been documented in a number of cancers and T-cell lymphoma (Balasenthil et al., 2004; Carter et al., 2004; Mao et al., 2003; Schraml et al., 2003). Of note, cortactin was also shown to be constitutively associated with PAK1/2 in resting platelets. After thrombin stimulation, PAK and cortactin dissociate (Vidal et al., 2002), therefore possibly allowing phosphorylated cortactin to trigger the remodeling of the actin cytoskeleton.

In conclusion, the molecular complement of invadopodia and some structural aspects have been thoroughly investigated. Many studies have established key components and have centered on the actin-remodeling machinery governing invadopodia formation. Much less is known concerning invadopodia formation, dynamics and regulation. Cortactin is a complex, differentially phosphorylated protein that might act by integrating signaling cascades, cytoskeletal remodeling and membrane-trafficking events that operate at invadopodia and which are finalized towards the focal degradation of ECM components.

Here, we directly investigated the role of the various cortactin protein domains in the regulation of invadopodia formation and ECM degradation, and analyzed the functional consequences of phosphorylation events modifying cortactin. We applied a quantitative approach based on RNA interference, expression of mutant proteins and selective small molecule and protein inhibitors. This was made possible through an experimental protocol based on a reversible block, followed by a synchronized wave of spontaneous invadopodia formation.

Results

Invadopodia synchronization

The cell model used throughout this study was the invasive metastatic human melanoma cell line A375MM (Baldassarre et al., 2003; Kozlowski et al., 1984). Tumor cells placed on the appropriate ECM substrate spontaneously form proteolytically active invadopodia. Their activity can be monitored directly by observing and measuring the extent of degradation of the fluorophore-conjugated ECM substrate of choice (Baldassarre et al., 2003; Bowden et al., 2001). Hence, in some cases, and depending on the cell type, invadopodia formation and degradation of the ECM can be observed as early as 1 hour after plating the cells, even before spreading is complete. This poses specific technical and interpretive problems when applying experimental protocols that require long pretreatment times such as the transient expression of heterologous proteins (at least 24 hours) and even more so in RNA interference experiments (up to 60 hours or more). Ideally, one would like to block invadopodia formation until satisfactory levels of transfected protein expression or downregulation of targeted proteins have been achieved, thus uncoupling cell attachment and spreading processes from invadopodia formation.

We devised a protocol to solve this issue by applying a reversible block of invadopodia formation during which cell adhesion and spreading can occur, and treatments can be carried out, followed by release from the block and establishment of a synchronized wave of spontaneous invadopodia formation. To this end, we made use of the synthetic reversible broad-spectrum metalloprotease inhibitor batimastat (or BB94) that functions competitively and reversibly by fitting tightly in the active site of the enzyme (Brown, 1995; Brown and Giavazzi, 1995; Rasmussen and McCann, 1997). A375MM cells were then plated directly on crosslinked fluorescent gelatin in the presence of 5 μM BB94 and allowed to adhere and spread overnight. Cells could be kept for up to 96 hours in the presence of BB94 to allow for transfection of siRNA duplexes and

eventually re-expression of exogenous proteins. During this period, we observed no detectable effect on cell morphology, growth or behavior in general, but invadopodia did not form and therefore no degradation of the ECM occurred. Upon BB94 washout, the cells resumed the ability to form invadopodia and hence degrade the matrix (Fig. 1B). When BB94 was added after invadopodia establishment, progression of ECM degradation was halted but invadopodia were not disassembled (not shown).

Cortactin is required for invadopodia formation and ECM degradation

We first verified whether cortactin was required for the formation of invadopodial protrusions and ECM degradation in A375MM by siRNA-mediated knockdown of endogenous cortactin with a mix of four different duplexes specific for human cortactin. We observed a significant reduction (about 95%) of cortactin levels in the siRNA-treated cells as assessed by both western blotting and immunofluorescence (Fig. 2A-C). When BB94 was washed out, no invadopodia were formed and consequently gelatin was not degraded (Fig. 2C) in accord with previously described experiments (Artym et al., 2006). Cortactin-depleted cells did not appear to have an obviously altered general morphology although the actin cytoskeleton acquired a slightly diffuse pattern when compared with mock-treated cells.

To exclude off-target effects, we transfected a pool of four nontargeting siRNA duplexes. To further verify the specificity of the siRNA treatment we retransfected rat wild-type cortactin (cortactin^{WT}) in the knocked-down cells. This restored the ability of the depleted cells to form invadopodia and degrade the ECM (Fig. 2D). Thus, cortactin is an essential component of the ECM degrading machinery.

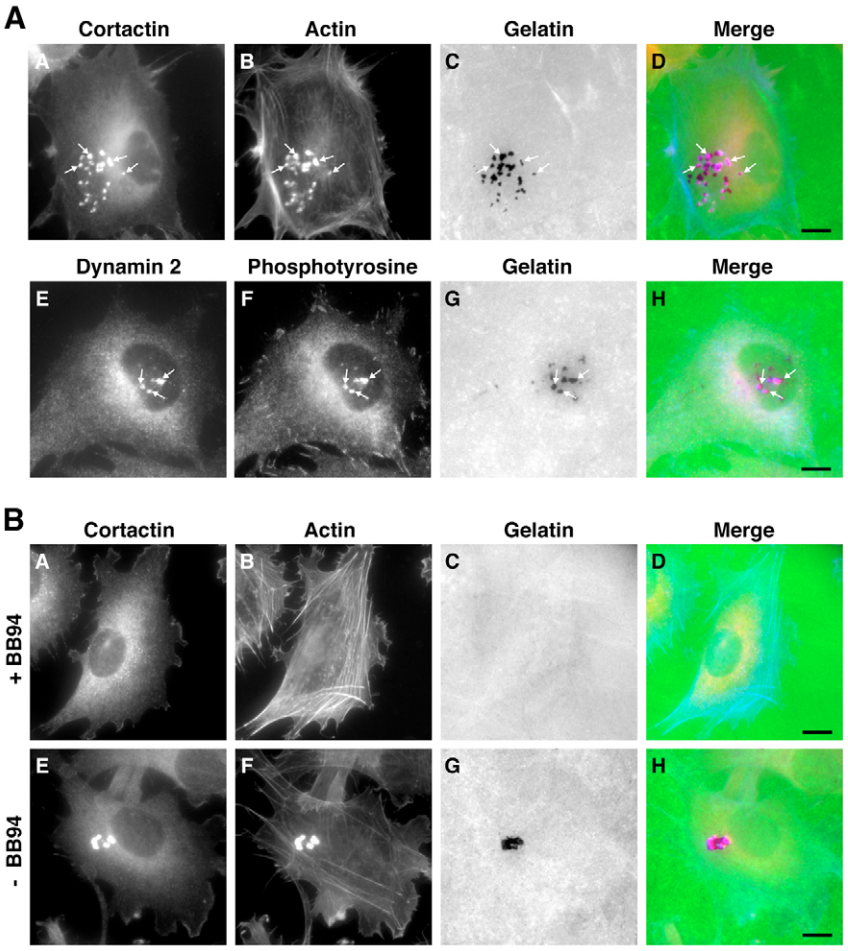

Fig. 1. Main features of invadopodia and effect of BB-94 treatment. (A) Triple staining of A375MM cells grown on crosslinked FITC-conjugated matrix for 16 hours and then fixed and stained with anti-cortactin (A) and Alexa Fluor 633-phalloidin (B), or anti-dynamin 2 (E) and anti-phosphotyrosine (F). All these proteins colocalized precisely to ECM degradation patches (C and G, arrows). (B) Anti-cortactin (A,E) and Alexa Fluor 633-phalloidin (B,F) labeling of cells grown for 16 hours on fluorescent matrix (C,G) in the continuous presence (+BB94) (A-D) and 3 hours after washout (–BB94) of the inhibitor (E-H). Scale bars: 10 μm.

Cortactin acidic and SH3 domains are required for ECM degradation at invadopodia

Cortactin is a multidomain protein with many binding partners. To better understand which of the many functional domains of cortactin are relevant for invadopodia function, cortactin^{WT} and various mutated forms were transiently expressed in A375MM cells. The mutant cortactin constructs used in this study are as outlined in Fig. 3. The N-terminal acidic Arp2/3-binding domain (NTA) has been shown to be important for in vitro actin polymerization. Hence, to evaluate the relevance of this domain, we used an N-terminal acidic domain deletion (cortactin$^{\Delta NTA}$) and a point mutant in amino acid position 22 (cortactin^{W22A}), that has been specifically shown to perturb the interaction with Arp2/3 (Uruno et al., 2001; Weaver et al., 2002; Weed et al., 2000). A deletion mutant (cortactinΔSH3) was used to examine the relevance of the C-terminal SH3 domain (Cao et al., 2005; Krueger et al., 2003; McNiven et al., 2000). Whereas overexpression of cortactin^{WT} did not significantly affect invadopodia formation and ECM degradation compared with mock-transfected cells, transient expression of cortactin$^{\Delta NTA}$, cortactin^{W22A} and cortactin$^{\Delta SH3}$ substantially inhibited both, compared with cortactin^{WT}-expressing cells (Fig. 4A).

To exclude nonspecific effects of the cortactin mutants, they were transfected after cortactin knockdown (Fig. 4B). None of the mutants, unlike cortactin^{WT}, was able to rescue the invasive phenotype (indicated by formation of invadopodia and matrix degradation; not shown).

Tyrosine phosphorylation of cortactin is required for invadopodia formation and ECM degradation

Previous work has shown that tyrosine phosphorylation of proteins by Src or Src-family tyrosine kinases (SFK) is required for ECM degradation at invadopodia (Hauck et al., 2002; Mueller et al., 1992) suggesting a main role for tyrosine phosphorylation in the formation of functional invadopodia. Also, immunoprecipitation of phosphotyrosine-containing proteins from invadopodia-enriched membrane fractions revealed that phosphorylated cortactin was highly enriched in this fraction, suggesting this actin-binding protein is a potential target for SFK at invadopodia (Bowden et al., 2006).

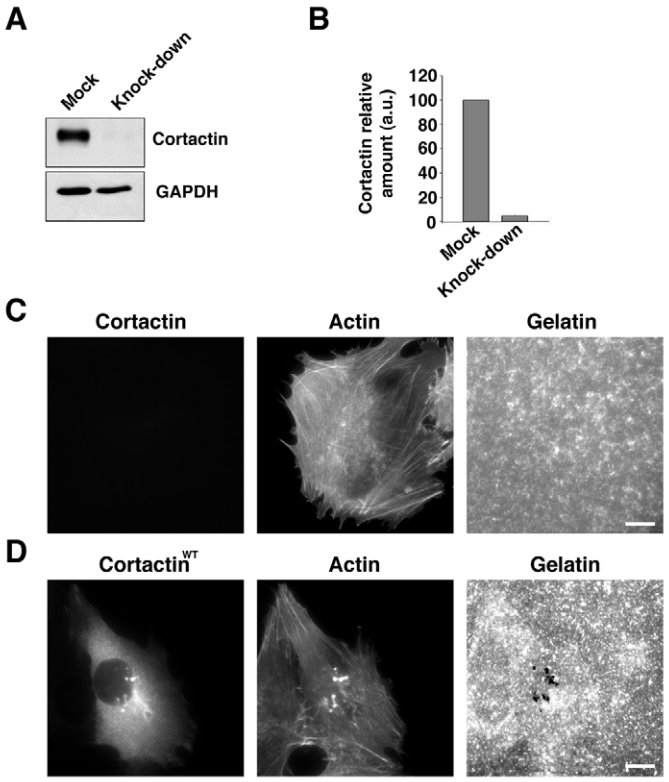

Fig. 2. Depletion of cortactin by RNA interference. (A) Lysates of A375MM cells 72 hours after mock or cortactin-specific siRNA transfection were subjected to SDS-PAGE, transferred to nitrocellulose and probed with anti-cortactin and anti-GAPDH as a loading control. (B) The relative amount of cortactin in mock-transfected and knockdown cells was measured. A 95% reduction in the amount of cortactin was observed. (C) Immunofluorescence labeling with anti-cortactin and phalloidin of cortactin siRNA-treated A375MM cells. Depletion of cortactin blocks the formation of invadopodia and hence, matrix degradation. (D) Transfection of cortactin^{WT} 48 hours after knockdown. Normal invadopodia form and degrade the extracellular matrix. Scale bars: 10 μm.

To verify that SFK activity is required for invadopodia formation in A375MM cells, we plated A375MM cells directly on fluorescent gelatin-coated coverslips in the presence of BB94. After adhesion and spreading, the cells were pretreated with the specific SFK inhibitor SU6656 for 30 minutes as well as with BB94. The protease inhibitor was washed out and the cells incubated with SU6656 for further 3 hours and finally fixed and stained. After this treatment, A375MM cells completely failed to form invadopodia and hence degrade the ECM (supplementary material Fig. S1A,B).

We then evaluated the relevance of specific cortactin tyrosine residues known to be phosphorylated by tyrosine kinases, in invadopodia function. This was achieved by transfecting A375MM cells with a mutant in which the primary SFK-phosphorylated residue was changed to aspartic acid to mimic phosphorylated cortactin (cortactin^{Y421D}) and a nonphosphorylatable mutant in which three relevant tyrosines were substituted by phenylalanine (cortactin^{Y421,466,482F}; Fig. 3). The latter mutant has been shown to act in a dominant-negative fashion in the motility of endothelial cells (Huang et al., 1998; Li et al., 2000) and metastatic dissemination of breast carcinoma cells (Li et al., 2001). We observed that cortactin^{Y421D} was present at invadopodia and increased the ability of the transfected cells to degrade the ECM

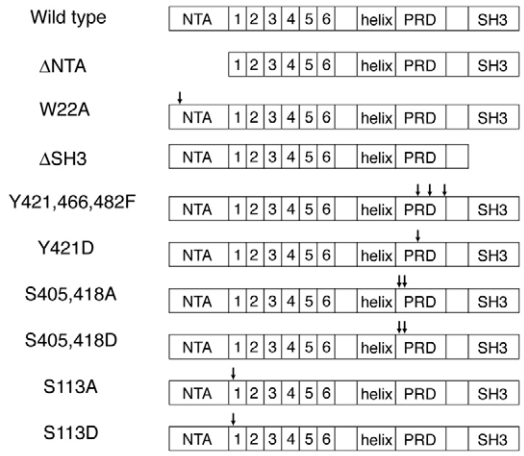

Fig. 3. Schematic diagram of the cortactin constructs used in this study. Cortactin features a N-terminal acidic domain (NTA) which specifically binds the Arp2/3 complex and is followed by a variable number of 37 amino acids repeats, the fourth of which binds F-actin. After an α-helix of undefined function, there is a proline-rich domain (PRD). Finally, the C-terminal is a Src-homology 3 domain (SH3) able to bind the proline-rich domain of several binding partners. The arrows indicate the positions of the mutated sites.

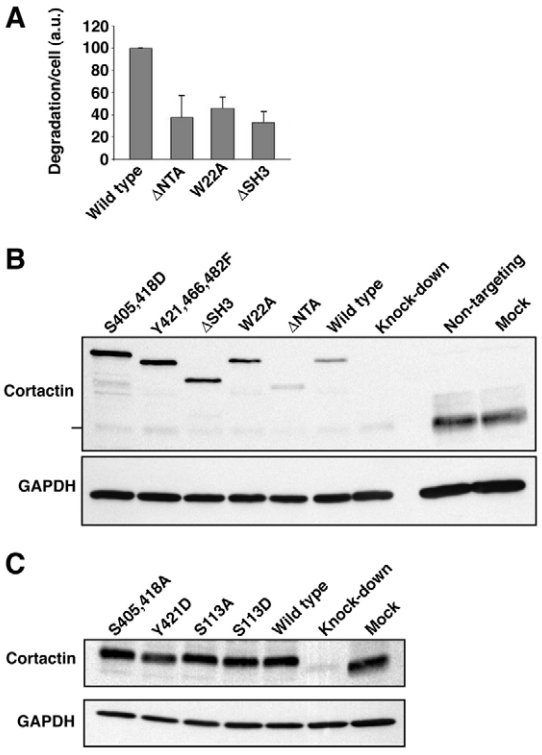

Fig. 4. Analysis of the cortactin functional domains required for ECM degradation at invadopodia. (A) A375MM cells transfected with mutant forms of cortactin, display a significantly reduced ability to degrade the ECM compared with control cells transfected with cortactin^{WT}. Data represent the mean ± s.d. of three independent experiments. Statistical significances were evaluated by Student's *t*-test: wild type vs ΔNTA, W22A and ΔSH3, $P<0.006$. (B) Transient re-expression of DsRed-tagged mutant and deleted forms of cortactin following knockdown as determined by immunoblotting. A typical experiment is shown. Line indicates endogenous cortactin. (C) Transient re-expression of FLAG-tagged mutant forms of cortactin following knockdown as determined by immunoblotting. A typical experiment is shown.

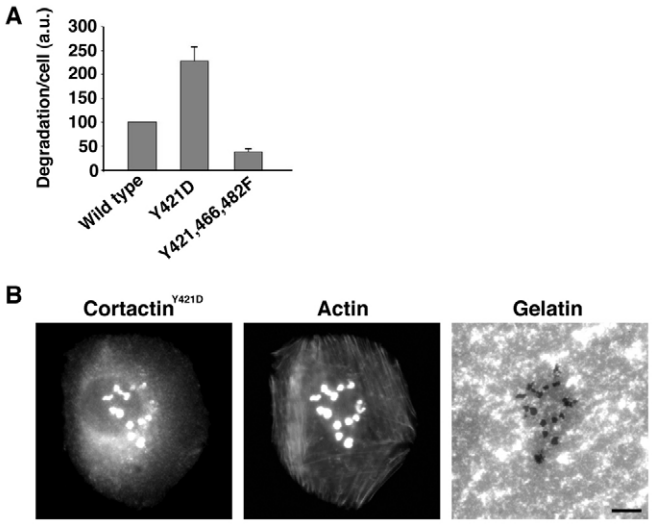

Fig. 5. Tyrosine phosphorylation of cortactin is required for ECM degradation at invadopodia. (A) A375MM cells transfected with cortactin^{Y421,466,482F} display a significantly reduced ability to degrade the ECM compared with control cells transfected with cortactin^{WT}. By contrast, cells transfected with the pseudophosphorylated cortactin^{Y421D} increased their capacity to degrade the matrix. Data represent the mean ± s.d. of three independent experiments. Statistical significance was evaluated by Student's *t*-test: wild type vs Y421D and Y421,466,482F, $P<0.002$. (B) Representative images of A375MM cells transfected with cortactin^{Y421D} 48 hours after cortactin depletion. Typically, cortactin^{Y421D} localizes to invadopodia. Scale bar: 10 μm.

more than twofold. By contrast, cortactin^{Y421,466,482F} caused more than 60% inhibition of degradation when compared with the control cortactin^{WT}-expressing cells (Fig. 5A).

A recent study has shown that the same three cortactin tyrosine residues can be phosphorylated by the Abl/Arg tyrosine kinase and that this modification is required for PDGF-induced dorsal wave formation (Boyle et al., 2007). To establish whether Abl/Arg kinase activity plays a role in invadopodia formation/function, we plated A375MM cells directly on fluorescent-gelatin-coated coverslips in the presence of BB94. After adhesion and spreading, the cells were pretreated with the specific Abl/Arg kinase inhibitor STI-571 for 30 minutes in the continued presence of BB94. The protease inhibitor was washed out and the cells incubated with STI-571 for further 3 hours and finally fixed and stained. After this treatment, A375MM cells formed normal invadopodia, in terms of both number and function (supplementary material Fig. S1E). This is taken to indicate that Abl/Arg kinase activity, and hence Abl/Arg-dependent phosphorylation of cortactin are not relevant for invadopodia biogenesis. To exclude nonspecific effects of the mutant cortactin forms, we verified the consequence of cortactin^{Y421,466,482F} and cortactin^{Y421D} expression after cortactin downregulation. To this end, cells were transfected with cortactin^{WT}, cortactin^{Y421,466,482F} and cortactin^{Y421D} after cortactin knockdown (Fig. 4B,C). The expression of cortactin^{Y421,466,482F}, as opposed to cortactin^{WT} did not restore the potential of A375MM cells to form invadopodia and degrade the ECM (data not shown), whereas the transfection of cortactin^{Y421D} in cortactin-depleted cells induced the formation of invadopodia (Fig. 5B) and degraded significantly more compared with cortactin^{WT}-expressing cells (not shown). Hence, tyrosine phosphorylation of cortactin possibly by SFK, is required for, and stimulates, invadopodia formation and consequently matrix degradation.

Serine phosphorylation of cortactin enhances ECM degradation

Cortactin is phosphorylated in vitro on Ser405 and Ser418 by ERK1/2 (Campbell et al., 1999), which is thought to enable cortactin-N-WASP binding in vitro (Martinez-Quiles et al., 2004). It is not known whether ERK1/2 are involved in invadopodia formation. We thus took advantage of U0126 (Favata et al., 1998), an inhibitor active on the mitogen-activated protein/ERK kinase (MEK), the prime activator of ERK1/2 (reviewed by Kolch, 2005). We found that U0126 treatment reduced the levels of phosphorylated ERK1/2 by 80% and reduced ECM degradation by 50% when compared with untreated cells (supplementary material Fig. S1D,E), suggesting that ERK1/2 activity supports invadopodia formation.

To study the relevance of serine phosphorylation of cortactin in invadopodia function, we used a mutant in which the two serines known to be phosphorylated by ERK1/2 were substituted by aspartic acid or alanine to generate pseudophosphorylated cortactin^{S405,418D} and nonphosphorylatable cortactin^{S405,418A}, respectively (Fig. 3). Overexpression of cortactin^{S405,418D} induced a 2.5-fold increase in the ability of the transfected cells to degrade the matrix compared with control cells. Conversely, the transfection of cortactin^{S405,418A} reduced degradation of the ECM by 70% (Fig. 6A).

Next, we investigated whether the phosphorylation-mimicking cortactin^{S405,418D} could overcome the inhibitory effect of U0126. To this end, we plated A375MM cells expressing cortactin^{S405,418D} directly on fluorescent-gelatin-coated coverslips in the presence of BB94. After adhesion and spreading, the cells were pretreated with 10 μM U0126 for 15 minutes in the presence of BB94. Next, BB94 was washed out and the cells incubated with UO126 for further 3 hours and finally fixed and stained. We found that expression of pseudophosphorylated cortactin^{S405,418D} alone was not sufficient to overcome the inhibitory effect of UO126 (data not shown), most probably because many other potential targets of ERK1/2 are involved in the regulation of the events upstream of cortactin.

Finally, to exclude nonspecific effects of the mutant cortactin forms, we investigated the effects of the expression of both mutants after siRNA-mediated endogenous cortactin knockdown (Fig. 4B,C). The re-expression of cortactin^{S405,418D} on a cortactin-depleted background induced formation of invadopodia (Fig. 6B) and led to an obvious increase of ECM degradation compared with levels observed using cortactin^{WT} (not shown). Cortactin^{S405,418A} transfection did not rescue the invasive phenotype (not shown). These results suggest that cortactin phosphorylation on residues Ser405 and Ser418 (presumably by ERK1/2) is a positive stimulus towards invadopodia formation and ECM degradation.

Cortactin can also be phosphorylated in vitro by the p21-activated serine/threonine kinase PAK (Vidal et al., 2002; Webb et al., 2006). Whether PAK itself plays a role in invadopodia biogenesis has not been investigated so far. To address this, we transfected A375MM cells with the autoinhibitory domain of PAK1 (PAK-AID), known to inhibit the endogenous kinase (Zhao et al., 1998). We found that PAK-AID expression induced a substantial decrease (50-70%) in ECM degradation (Fig. 6C), suggesting that PAK activity supports invadopodia formation.

The main PAK phosphorylation site on cortactin has been shown to be the Ser113 in the first actin-binding repeat (Webb et al., 2006). This event is thought to negatively regulate cortactin binding to F-actin in vitro (Webb et al., 2006). To test whether phosphorylation of cortactin on Ser113 has a regulatory role in invadopodia

biogenesis, A375MM cells were transfected with cortactin^{WT} as a control, and two cortactin phosphorylation mutants: a nonphosphorylatable S113A substitution and the phosphorylation-mimicking S113D (Fig. 3). Expression of cortactin^{S113A} reduced invadopodia formation and ECM degradation by 50% in contrast to cortactin^{S113D}, which instead produced a 1.6-fold increase in ECM degradation at invadopodia (Fig. 6D). Of note, cortactin^{S113D} localized to invadopodia (Fig. 6E). When coexpressed with PAK-AID, however, cortactin^{S113D} failed to revert the inhibitory effect (not shown), probably because many other potential targets of PAK are involved in the regulation of the cytoskeleton and adhesion (Zhao and Manser, 2005) and could thus act upstream of cortactin.

To gain confirmation, we transfected cells with cortactin^{S113A}, cortactin^{S113D} and cortactin^{WT} after siRNA-mediated endogenous cortactin knockdown (Fig. 4C). Expression of cortactin^{S113A} on a cortactin-depleted background failed to recover the normal phenotype compared with cortactin^{WT} (not shown). The phosphorylation-mimicking mutant cortactin^{S113D}, instead, induced formation of invadopodia (Fig. 7C) and increased levels of ECM degradation compared with cortactin^{WT}. These results indicate that cortactin phosphorylation on Ser113, presumably by PAK, positively modulates invadopodia formation/function.

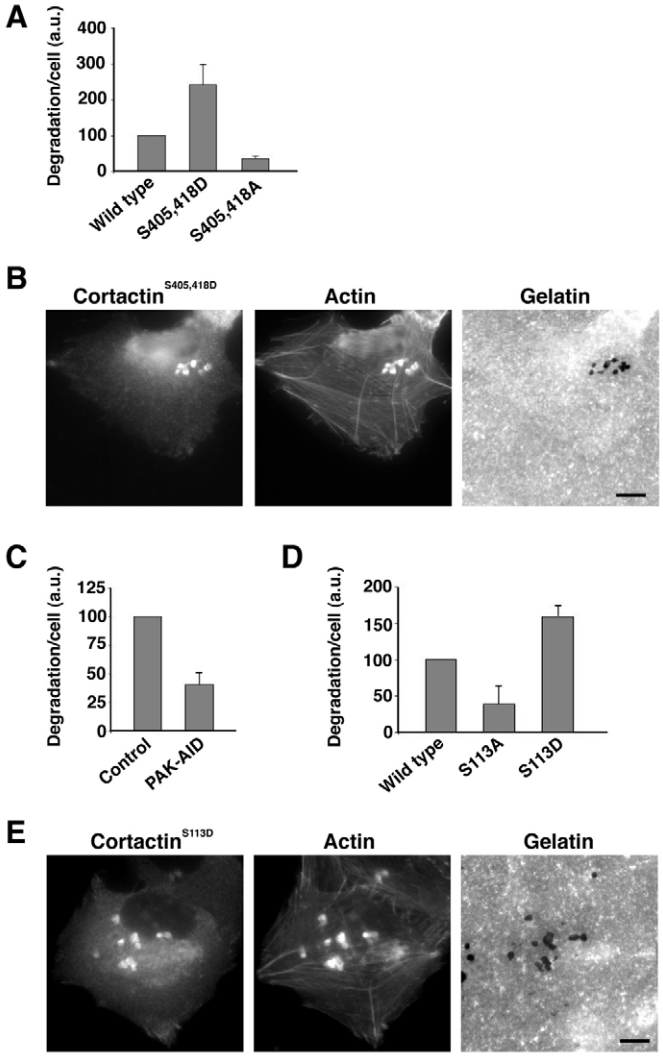

Fig. 6. Serine phosphorylation of cortactin is required for ECM degradation at invadopodia. (A) A375MM cells transfected with cortactin^{S405,418D} display a remarkably increased ability to degrade the ECM compared with control cells transfected with cortactin^{WT}. By contrast, transfection of cortactin^{S405,418A} markedly reduced ECM degradation. Data represent the mean ± s.d. of three independent experiments. Statistical significance evaluated by Student's *t*-test: wild type vs S405,418D, $P<0.01$; wild type vs S405,418A, $P<0.005$. (B) Representative image of A375MM cells transfected with cortactin^{S405,418D} 48 hours after cortactin depletion. Typically cortactin^{S405,418D} localizes to invadopodia. (C) A375MM cells transfected with the autoinhibitory domain of PAK (AID) display a reduced ability to degrade the matrix compared with mock-transfected cells. Data represent the mean ± s.d. of three independent experiments. Statistical significance was evaluated by Student's *t*-test: control vs autoinhibitory domain of PAK $P<0.0001$. (D) A375MM cells were transfected with cortactin^{WT}, nonphosphorylatable cortactin^{S113A} and pseudophosphorylated cortactin^{S113D}. The areas of degradation were then quantified. Data represent the mean ± s.d. of three independent experiments. Statistical significance was evaluated by Student's *t*-test: wild type vs S113A and S113D, $P<0.004$. (E) Representative immunofluorescence image of A375MM cells transfected with cortactin^{S113D} 48 hours after cortactin knockdown labeled with anti-FLAG antibodies. Cortactin^{S113D} clearly localizes to ECM degradation patches at invadopodia. Scale bars: 10 μm.

Discussion

We present a molecular dissection of the functional domains of cortactin relevant for invadopodia formation and function. In addition, through the expression of cortactin forms mutated in the residues previously found to be phosphorylated in vitro, we suggest the involvement of different kinases in the control of the ECM degradation machinery through the regulation of cortactin phosphorylation.

The overexpression of mutant or deleted forms of cortactin that are unable to bind the Arp2/3 complex, induces a substantial decrease in the ability of the cells to form invadopodia and hence degrade the ECM. In addition, the SH3 domain of cortactin, known to bind a number of relevant proteins such as N-WASP and dynamin 2, is also essential for invadopodia formation and ECM degradation. This is at variance with a recent report suggesting that the cortactin N-terminus is not required for invadopodia formation (Webb et al., 2007). A possible explanation is that the study was based on constitutively active Src-expressing NIH 3T3 cells (which normally do not form invadopodia). Our findings are based on a tumoral cell model that spontaneously forms invadopodia and altogether support the idea that cortactin is an active scaffold protein bringing together different components, each domain being necessary for the formation of invadopodia, the initial step of the invasive process.

Invadopodia are hotspots of protein tyrosine phosphorylation, mostly due to SFK (Baldassarre et al., 2003; Mueller et al., 1992). Furthermore, a clear colocalization between Src, cortactin and phosphotyrosine has been observed in active invadopodia, and tyrosine-phosphorylated cortactin was found to be associated to invadopodia-enriched membrane fractions (Bowden et al., 2006). This suggests a potential role for SFK-dependent tyrosine phosphorylation of cortactin at invadopodia. Here we demonstrate that phosphorylation of one or more of the cortactin tyrosine residues Tyr421, Tyr466 and Tyr482 is necessary for invadopodia formation and function. This provides a functional framework to interpret the finding that overexpression of the same tyrosine phosphorylation-deficient cortactin mutant used in this study inhibited bone metastasis formation in an in vivo mouse model (Li et al., 2001). The same cortactin tyrosine residues, however, have recently been shown to be targets of Abl/Arg kinase and their phosphorylation by the same kinase is relevant for PDGF-induced dorsal wave formation (Boyle et al., 2007). Our finding that the

Abl/Arg-specific inhibitor STI-571 does not affect invadopodia formation or function, would exclude this kinase from the regulatory circuit governing invadopodia formation.

A less studied aspect is the functional consequence of cortactin phosphorylation on serine. We observed that cortactin^{S405,418A} acted as a dominant negative when transiently transfected and was unable to rescue the defective phenotype in cortactin-knockdown cells. Strikingly, the phosphoserine-mimicking mutant cortactin^{S405,418D} induced a more than twofold increase in invadopodia formation and ECM degradation. The same pseudophosphorylated cortactin^{S405,418D} failed, however, to attenuate the inhibitory effect of the ERK1/2 inhibitor U0126. This finding is not surprising because ERK1/2 has multiple targets and regulates numerous functions in the cell (reviewed by Kolch, 2005), which could act upstream or in concert with cortactin in invadopodia formation and function. Since cortactin residues Ser405 and Ser418 are known targets of ERK1/2 (Campbell et al., 1999), our findings altogether suggest that ERK1/2 might be acting on cortactin to promote invadopodia formation.

Cortactin has also been shown in vitro to be a substrate of the p21-activated kinase (PAK) family of serine/threonine kinases (Vidal et al., 2002), known effectors of Cdc42 and Rac1 (reviewed by Bokoch, 2003). The main residue targeted by PAK is Ser113 (Webb et al., 2006). Our results show that pseudophosphorylated cortactin^{S113D} substantially enhances invadopodia formation and ECM degradation, whereas the corresponding nonphosphorylatable cortactin^{S113A} mutant acts in a dominant negative fashion. Co-expression of PAK-AID with pseudophosphorylated cortactin^{S113D}, however, was not sufficient to bypass the block induced by the PAK autoinhibitory domain. As in the case of ERK1/2, various PAK isoforms are required for the regulation of signaling pathways at multiple levels and hence possibly upstream of cortactin. Our experiments do suggest that PAK activity is involved in invadopodia formation and that cortactin might be one of its targets.

How can we reconcile our findings with the existing knowledge and current framework for cortactin function? Following N-WASP activation of the Arp2/3 complex, F-actin nucleation of barbed ends might be involved in the formation of invadopodia by pushing forward the plasma membrane (Baldassarre et al., 2006; Pollard, 2003; Weaver et al., 2003). Cortactin is thought to stabilize the branching point of dendritic actin networks by its interaction with the Arp2/3 complex via its N-terminal acidic domain and the mother filament by means of its actin-binding region (Olazabal and Machesky, 2001; Weaver et al., 2001). Cortactin may therefore act as a molecular scaffold essential to trigger invadopodia formation by tethering the Arp2/3 complex and its activator N-WASP, via its NTA and SH3 domains. Indeed, unlike other Arp2/3 complex activators, cortactin remains associated with the mother filament after binding and activation of Arp2/3 (Egile et al., 2005).

Phosphorylation of cortactin by tyrosine (possibly SFK) or serine/threonine (possibly PAK and ERK1/2) kinases, could directly or indirectly modulate the interaction and/or the binding of cortactin with its interacting partners. For instance, phosphorylation of cortactin on Ser113 (presumably by PAK) would stabilize branched actin networks but might also modulate its affinity for Arp2/3 because Ser113 is located adjacent to the N-terminal acidic domain (Webb et al., 2006). Hence, in our experiments cortactin^{S113D} could promote actin branching needed for invadopodia biogenesis. Based on in vitro experiments, it has been suggested, that tyrosine phosphorylation of cortactin stabilizes the intramolecular association of the SH3 domain with the PRD, whereas phosphorylation on Ser405 and Ser418 sets the SH3 domain free to bind and activate N-WASP (Martinez-Quiles et al., 2004). We observe however that the nonphosphorylatable cortactin^{Y421,466,482F} acted in a dominant negative fashion, suggesting again that the regulation of cortactin is based on a dynamic interplay between the different regulatory inputs. Cortactin has also been shown to interact with a number of other proteins through its PRD and SH3 domains during the formation and/or disassembly of actin networks at invadopodia. Among these is dynamin 2 (Krueger et al., 2003; Schafer, 2002), which is known to play a central role in invadopodia formation (Baldassarre et al., 2003). In this process, dynamin 2 could also affect the activity of the Arp2/3 complex indirectly through cortactin (Schafer et al., 2002; Krueger et al., 2003). Of note, recent findings have shown that Src-dependent phosphorylation of cortactin enhances its affinity for dynamin-2 more than threefold (Zhu et al., 2007), suggesting a possible molecular basis for the stimulatory effect of cortactin phosphorylation by SFK in invadopodia formation. An interesting twist might be provided by a recent report suggesting that cortactin functions in regulating matrix metalloprotease secretion at invadopodia (Clark et al., 2007). This would again bring dynamin 2 into the picture considering that cortactin has been reported to function in a complex with dynamin 2 in regulating anterograde membrane transport from the Golgi complex (Cao et al., 2005).

It is unclear whether cortactin phosphorylation by different kinases and on different residues takes place in a sequential and/or interdependent fashion. We performed a number of experiments in which the cortactin phosphorylation mutants described here were combined in double transfections in A375 cells. Specifically, nonphosphorylatable cortactin^{Y421,466,482F} was cotransfected with pseudophosphorylated cortactin^{S113D}, nonphosphorylatable cortactin^{S113A} with pseudophosphorylated cortactin^{S405,418D} and finally nonphosphorylatable cortactin^{S405,418A} together with pseudophosphorylated cortactin^{S113D}. We proceeded to assess ECM degradation as described above when comparable levels of cotransfection were achieved (as determined by western blotting; not shown). In all cases, the pseudophosphorylated cortactin mutants mimicking activity of one kinase class were able to overcome the effect of the nonphosphorylatable ones mimicking lack of activity of another class, leading to an increase in ECM degradation compared with cortactin^{WT} (supplementary material Fig. S2). These results support the hypothesis of independent but convergent signaling pathways regulating phosphorylation of cortactin at invadopodia. These pathways might not be necessarily be all activated depending on the engagement of different integrins or other membrane receptors by ECM components.

In summary, we show that at least three convergent routes may function to regulate cortactin activity by phosphorylation, but do not imply that these must act at the same time or in a sequential manner. Rather, a fine regulatory balance might exist through which cortactin controls invadopodia biogenesis, possibly acting as a trait d'union between signal generation at the plasma membrane triggered by integrin engagement and transduction to the appropriate cell machinery via tyrosine and serine/threonine kinases. In our interpretive model, the extent and quality of cortactin phosphorylation would be modulated by adhesion to matrix components via integrins and other membrane receptors. This would induce subtle changes in cortactin structure to calibrate its function.

More facets of the complex cortactin regulatory pattern by phosphorylation will perhaps emerge. For instance, protein kinase D has recently been found to colocalize with F-actin, cortactin and Arp3 at the leading edge of migrating cells and to phosphorylate cortactin in vitro (Eiseler et al., 2007). Also, mass spectrometry mapping has recently uncovered 17 new phosphorylated residues in cortactin (12 serine, 4 threonine and 1 tyrosine residues), and the same study also confirmed some of the known phosphorylation sites (S405,418 and Y421,466,482) thus validating the approach (Martin et al., 2006). These modifications might be functionally relevant as they map to well-conserved residues in functional domains. All the above would suggest that the complex regulatory pattern of cortactin and in particular its significance for invadopodia biology, is just beginning to be understood.

Materials and Methods

Constructs, antibodies and other reagents

Wild-type (WT) and mutant ΔNTA, W22A, ΔSH3 and Y421,466,482F rat cortactin constructs in pDsRedN1 (Clontech Laboratories, Mountain View, CA, USA) were a generous gift from M. McNiven (Mayo Clinic, Rochester, MN). Cortactin S405,418D in pGEX-6P2 (Martinez-Quiles et al., 2004), kindly provided by R. S. Geha (Div. Immunology, Children's Hospital, Boston, MA) was subcloned in pDsRedN1 by PCR using the following primers: Forward 5′-CGGAATTC-ATGTGGAAAGCCTCTGCAGGCC-3′ and Reverse 5′-CGGTGGATCCCGC-TGCCGCAGCTCCAC-3′. The previously described (Webb et al., 2006) FLAG-tagged mouse WT, S113A, S113D, S405,418A and Y421D cortactin in pcDNA 3.1 (–) constructs were a generous gift from A. S. Mak (Queen's University, Kingston, ON, Canada). The autoinhibitory domain (AID) of PAK1 (aa 83-149) in pXJ-FLAG (Zhao et al., 1998) was kindly provided by E. Manser (GSK-IMCB, Institute of Molecular and Cell Biology, Singapore). Polyclonal anti-dynamin 2 antibodies were provided by M. McNiven (Dyn2) (Henley and McNiven, 1996). The following commercial antibodies were also used: monoclonal anti-cortactin (Clone 4F11; Upstate Biotechnology, Lake Placid, NY), monoclonal anti-phosphotyrosine (Clone 4G10; Santa Cruz Biotechnology, CA), monoclonal anti-FLAG (M2; Sigma-Aldrich, St Louis, MO), polyclonal anti-glyceraldehyde phosphate dehydrogenase (GAPDH) (Biogenesis, Sandown, NH), monoclonal anti-phosphoERK1/2 (clone 12D4; Upstate Biotechnology), polyclonal anti-ERK1/2 (K-23; Santa Cruz Biotechnology), monoclonal anti-cSrc (GD11; Upstate Biotechnology) and polyclonal anti-phosphoSrc (Y418; Biosource International, Nivelles, Belgium). Alexa Fluor 633-, 546- and 488-conjugated secondary antibodies or phalloidin were from Molecular Probes Europe BV (Leiden, The Netherlands). Aliquots of 1 mM BB94 (British Biotech, UK) were prepared in 70% ethanol and kept at –20°C until use at 5 μM.

Cell culture

Human melanoma A375MM cells were cultured in DMEM/F-12 (1:1) (Invitrogen, Carlsbad, CA) containing 10% FCS. Cells were grown at 37°C in a humidified atmosphere containing 5% CO_2 as previously described (Baldassarre et al., 2003).

Immunofluorescence analysis of invadopodia

At the light microscopy level, invadopodia are identified by the colocalization of tyrosine-phosphorylated proteins, actin, cortactin and dynamin 2 at dark, nonfluorescent areas of degradation (Baldassarre et al., 2003; Bowden et al., 2006; Chen, 1996; Mueller et al., 1992) as shown in Fig. 1A. To this end, cells were fixed in 4% paraformaldehyde for 15 minutes, permeabilized for 30 minutes in phosphate-buffered saline (PBS) containing 0.02% saponin, 0.2% BSA and 50 mM NH_4Cl, incubated with the primary antibodies of interest or fluorophore-conjugated phalloidin (Molecular Probes) for 1 hour and then, where necessary, incubated with fluorophore-conjugated secondary antibodies for 45 minutes. Finally, coverslips were mounted in Mowiol (Calbiochem, La Jolla, CA). Experiments were observed using an Olympus videomicroscope equipped with T.I.L.L. Photonics software (T.I.L.L. Photonics GmbH, Gräfelfing, Germany).

ECM degradation assay

Fluorophore-conjugated gelatin was prepared with Fluorescein or Rhodamine B isothiocyanate (Sigma-Aldrich) and porcine gelatine (Sigma-Aldrich) according to the published procedure (Baldassarre et al., 2003; Bowden et al., 2001). Gelatin-coated coverslips were prepared and the assay carried out as described previously (Baldassarre et al., 2003; Bowden et al., 2001). Briefly, thin layers of Fluorescein- or Rhodamine B-conjugated gelatin (or unconjugated gelatin where specified) were placed on coverslips, crosslinked with 0.5% glutaraldehyde for 15 minutes at 0°C, washed three times with PBS and incubated for 3 minutes at room temperature with 5 mg/ml $NaBH_4$. Finally, after a wash and 10-minute incubation in 70% ethanol, coverslips were washed with complete medium for 1 hour at 37°C before cell plating. To quantify areas of degradation, for each experiment we considered 35 random fields containing at least one transfected cell; the area of each degradation patch was measured using the public domain ImageJ v.1.32 software. The total area was then normalized for the number of cells and the effect of each treatment was expressed as a percentage of control. When gelatin is referred to (whether fluorophore-conjugated or not), it is to be intended crosslinked as described above.

Invadopodia synchronization

A375MM cells were plated directly on fluorophore-conjugated or unconjugated gelatin in the presence of 5 μM BB94 to block invadopodia formation and incubated up to 96 hours, depending on the experiment. BB94 was then washed out to allow synchronous invadopodia formation.

Transfection

Cells were plated at 50% confluence in six-well plates and allowed to adhere and spread. The next day, monolayers were washed twice with DMEM/F-12 without FCS and incubated in 0.5 ml DMEM/F-12 without FCS with 2-4 μg DNA and TransFast reagent (Promega, Madison, WI) according to the manufacturer's instructions. Experiments were performed 24 hours after transfection.

Cortactin RNA interference and phenotype recovery

A375MM cells were plated directly on fluorophore-conjugated gelatin in the presence of BB94 to block invadopodia formation and were allowed to adhere overnight. Next, cells were transfected with the siGenome Smart pool reagent for human cortactin (code M010508-00-0005 Dharmacon, Lafayette, CA) using Oligofectamine (Invitrogen) according to the manufacturer's instructions. Mock and nontargeting siRNA (code D-001206, Dharmacon) transfections were performed as controls. After 72 hours, BB94 was washed out and the cells incubated for further 3 hours to allow the formation of invadopodia and degradation of the ECM. To assay for endogenous cortactin content after RNA interference, cells were lysed with RIPA buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) containing 2 μg/ml aprotinin, 0.5 μg/ml leupeptin, 2 μM pepstatin, 0.5 mM O-phenantroline, 1 mM PMSF and 1 μM benzamidine for 20 minutes on ice. Proteins from cell lysates were separated by SDS-PAGE, electroblotted to nitrocellulose and detected by enhanced chemiluminescence (ECL; GE Healthcare Life Sciences, Milano, Italy) with anti-cortactin and anti-GAPDH antibodies. Densitometric analysis of protein bands was performed with Image J 1.32. In some experiments, to evaluate the effect of cortactin mutant expression after cortactin depletion, cells were re-transfected 48 hours later with the various wild-type or mutant cortactin constructs. After a 24-hour incubation to allow for expression, BB94 was washed out and cells quantified for invadopodia formation and ECM degradation after a further 3-hour period.

Analysis of protein kinase inhibitor effects

A375MM cells were plated directly on fluorophore-conjugated gelatin in the presence of 5 μM BB94 and incubated for 16 hours. The following day, cells were preincubated with 10 μM U0126 (mitogen-activated protein/ERK kinase inhibitor; Promega), 5 μM SU6656 (Src-family kinase inhibitor; Sigma-Aldrich), 20 μM STI571 (Abl/Arg kinase inhibitor; kindly provided by Novartis Pharma AG) or vehicle for 15 minutes (UO126) or 30 minutes (SU6656 and STI571). BB94 was washed out and cells were treated with 10 μM U0126, 5 μM SU6656, 20 μM STI571 or vehicle for further 3 hours. Finally, cells were processed for invadopodia visualization and quantification of ECM degradation. To verify the effectiveness of inhibitor treatments, cells were lysed with 50 mM Tris-HCl pH 7.4, 100 mM NaCl, 50 mM NaF, 40 mM β-glycerophosphate, 200 μM sodium orthovanadate, 1% Triton X-100 with 2 μg/ml aprotinin, 0.5 μg/ml leupeptin, 2 μM pepstatin, 0.5 mM O-phenantroline, 1 mM PMSF and 1 μM benzamidine for 20 minutes on ice. Proteins from cell lysates were subjected to SDS-PAGE, transferred to nitrocellulose and probed with anti-phosphoERK1/2, anti-ERK1/2, anti-c-Src and anti-phosphoSrc antibodies.

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