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Jessica Lynn Crowley  
*University of Massachusetts Medical School*

*Et al.*

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Supervillin Reorganizes the Actin Cytoskeleton and Increases Invadopodial Efficiency

Jessica L. Crowley, Tara C. Smith, Zhiyou Fang, Norio Takizawa, and Elizabeth J. Luna

Department of Cell Biology and Cell Dynamics Program, University of Massachusetts Medical School, Worcester, MA 01605

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Tumor cells use actin-rich protrusions called invadopodia to degrade extracellular matrix (ECM) and invade tissues; related structures, termed podosomes, are sites of dynamic ECM interaction. We show here that supervillin (SV), a peripheral membrane protein that binds F-actin and myosin II, reorganizes the actin cytoskeleton and potentiates invadopodial function. Overexpressed SV induces redistribution of lamellipodial cortactin and lamellipodin/Raph1/Prel1 away from the cell periphery to internal sites and concomitantly increases the numbers of F-actin punctae. Most punctae are highly dynamic and colocalize with the podosome/invadopodial proteins, cortactin, Tks5, and cdc42. Cortactin binds SV sequences in vitro and contributes to the formation of enhanced green fluorescent protein (EGFP)-SV induced punctae. SV localizes to the cores of Src-generated podosomes in COS-7 cells and with invadopodia in MDA-MB-231 cells. EGFP-SV overexpression increases average numbers of ECM holes per cell; RNA interference-mediated knockdown of SV decreases these numbers. Although SV knockdown alone has no effect, simultaneous down-regulation of SV and the closely related protein gelsolin reduces invasion through ECM. Together, our results show that SV is a component of podosomes and invadopodia and that SV plays a role in invadopodial function, perhaps as a mediator of cortactin localization, activation state, and/or dynamics of metalloproteinases at the ventral cell surface.

INTRODUCTION

During metastasis, tumor cells invade the surrounding extracellular matrix (ECM), migrate into the bloodstream, and traverse endothelial barriers at secondary sites in distant organs (Condeelis et al., 2005). ECM degradation, in vitro, involves specialized membrane-associated F-actin structures known as podosomes and invadopodia (Weaver, 2006; Linder, 2007; Gimona et al., 2008; Machesky et al., 2008). Podosomes are endogenously found in cell types that cross tissue boundaries or are involved in tissue remodeling, e.g., leukocytes and osteoclasts, respectively (Gimona et al., 2008). Although podosomes contain many focal adhesion proteins and may mediate dynamic cell–ECM attachments, they are distinguishable from focal adhesions, which are ECM- and integrin-associated complexes that promote cell migration in fibroblasts (Zaidel-Bar et al., 2004; Evans and Matsudaira, 2006; Lo, 2006; Romer et al., 2006; Spinardi and Marchisio, 2006; Block et al., 2008; Broussard et al., 2008). Podosomes turn over much faster than do focal adhesions and contain cores of actin filaments that are surrounded by rings of vinculin and other focal adhesion proteins (Gavazzi et al., 1989; Ochoa et al., 2000; Destaing et al., 2003; Evans and Matsudaira, 2006). Electron micrographs of podosomes in Rous sarcoma virus-transformed fibroblasts show that the dense microfilamentous material in the core surrounds a central invaginating membrane tube (Nitsch et al., 1989; Ochoa et al., 2000). Invadopodia mediate ECM degradation, are larger and longer-lived than podosomes, and are characterized by surface protrusions and large membrane invaginations (Chen and Wang, 1999; Baldassarre et al., 2003; Yamaguchi and Condeelis, 2007; Styli et al., 2008).

Recent studies indicate many functional and compositional similarities between podosomes and invadopodia. Both these structures have been associated with secretion of matrix metalloproteinases (MMPs) and endocytosis of degraded matrix components (Ochoa et al., 2000; Baldassarre et al., 2003; Clark and Weaver, 2008). Also, both structures contain several proteins in common— including cortactin, Tks5/Fish, and Cdc42—in addition to many proteins also found at focal adhesions (Seals et al., 2005; Linder, 2007; Yamaguchi and Condeelis, 2007; Block et al., 2008). Current nomenclature is to use the term “invadopodia” for invasive structures in tumor cells and to use “podosome” for the invasive/adhesive structures in transformed fibroblasts and in cells—such as leukocytes, osteoclasts, and endothelial and smooth muscle cells— with endogenous or inducible podosomes (Gimona et al., 2008). However, vascular smooth muscle cells invade ECM, and tumor cells contain podosome-like, dynamic “preinvadopodial complexes” with short lifetimes that mature into longer lived invadopodia (Yamaguchi et al., 2005; Furmaniak-Kazmierczak et al., 2007). In addition, invadopodium formation involves sequential stages of cortactin recruitment to membranes, membrane type (MT) 1–MMP accumulation, matrix degradation, and

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Address correspondence to: Elizabeth J. Luna (elizabeth.luna@ umassmed.edu).

Abbreviations used: ECM, extracellular matrix; ERK, extracellular signal-regulated kinase; MMP, matrix metalloproteinase; pTyr, phosphorylasine; SmAV, smooth muscle isoform of supervillin; SV, supervillin.

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Supervillin and Invadopodia

cortactin dissociation (Artym et al., 2006). Thus, the distinction between “podosomes” and “invadopodia” may be as much temporal as compositional, i.e., invasion structures may arise from more dynamic podosome-like precursors in both normal tissues and tumors (Linder and Aepfelbacher, 2003; Gimona et al., 2008). Here, we use the current nomenclature for podosomes and invadopodia and refer to underfined structures as “actin punctae.”

Supervillin (SV) is one of the largest members of the villin/gelsolin family of actin-organizing proteins, which have both overlapping and distinct functions (Silacci et al., 2004; Archer et al., 2005; Khurana and George, 2008). Villin is associated with precancerous morphological changes in epithelia, and gelsolin is required for the formation and degradative activity of osteoclast podosomes; gelsolin also promotes cancer cell invasion and motility (MacLennan et al., 1999; Chellaiah et al., 2000; De Corte et al., 2002; Rieder et al., 2005; Van den Abbeele et al., 2007). Nonmuscle SV copurifies from neutrophil plasma membranes as a component of a lipid raft-like complex that also includes heterotrimeric G proteins, Src family kinases, and MT6-MMP (Nebel et al., 2002), all of which are involved in tumorigenesis and matrix degradation (Mareel and Leroy, 2003; Ingleby, 2008; Sohail et al., 2008). A smooth muscle isoform of SV, called SmAV, localizes to phorbol ester-induced podosomes in A7r5 cells with F-actin, nonmuscle myosin IIB, and extracellular signal-regulated kinases (ERKs) 1/2 (Gangopadhyay et al., 2004). SmAV is required for normal ERK1/2 activation downstream of phenylephrine signaling in ferret aorta (Gangopadhyay et al., 2004), but the functionality of SV isoforms in the formation of podosome-like structures has not been explored.

Based on its biochemical characteristics, SV is well positioned for a role in mediating alterations in the actin cytoskeleton at membranes. SV cofractionates with membranes and binds very tightly to the membrane bilayer, resisting extraction with buffered solutions at pH <12 (Pestonjamasp et al., 1997; Nebel et al., 2002; Oh et al., 2003). One site of membrane attachment is associated with SV amino acids 342–571 (SV342-571), which interact with the LIM domains of the focal adhesion proteins, thyroid receptor-interacting protein (TRIP) 6/zyxin-related protein 1, and lipoma-preferred partner (Takizawa et al., 2006). SV also binds avidly to F-actin at three sites through which it can either bundle or cross-link actin filaments; to nonmuscle myosins IIA and IIB; to the long isoform of myosin light chain kinase; and to calponin (Chen et al., 2003; Takizawa et al., 2007), a negative regulator of podosome formation in smooth muscle cells (Gimona et al., 2003).

SV potentiates a process that negatively regulates focal adhesion function. Overexpressed SV decreases cell adhesion to fibronectin, whereas RNA interference (RNAi)-mediated SV down-regulation increases cell adhesion (Takizawa et al., 2006). Sequences within the SV N terminus (SV1-171) disrupt stress fibers by inducing apparent hypercontractility of myosin II (Takizawa et al., 2006, 2007). Another SV sequence (SV342-571) counteracts stress fiber and large focal adhesion formation or stability through the interaction with TRIP6 (Takizawa et al., 2006). However, phalloidin staining of cytoplasmic F-actin is observed even after disruption of stress fibers in cells expressing elevated levels of SV or SV342-571 (Wulfkuhle et al., 1999; Takizawa et al., 2006). The nature of these phalloidin-stained structures has never been explored.

The mechanism by which SV and TRIP6 regulate adhesion is also imperfectly understood. Most investigations have focused on the roles of these proteins in “inside-out” signal-

MATERIALS AND METHODS

Antibodies, Reagents, and Constructs

Mouse monoclonal anti-vinculin (hVIN1) and anti-gelsolin (clone GS-2C4) antibodies were from Sigma-Aldrich (St. Louis, MO), as was affinity-isolated rabbit anti-hemagglutinin (HA) tag. Mouse monoclonal anti-cortactin (4F11) and anti-actin (clone C4) antibodies were from Millipore (Billerica, MA). Mouse monoclonal anti-phosphothreonine (pYyr-100) and anti-myc (71D10) antibodies were from Cell Signaling Technology (Danvers, MA). Mouse monoclonal anti-phospho-focal adhesion kinase (FAK) (pY397) antibody was from BD Biosciences (Franklin Lakes, NJ). AlexaFluor 488- and 568-conjugated secondary antibodies against rabbit and mouse immunoglobulin Gs (IgGs), and fluorescein- and Texas Red-conjugated phallolidin were from Invitrogen (Carlsbad, CA) and used at 1:2000 dilution. Affinity-purified rabbit polyclonal antibodies against FAK (Y762) and lamellipodin (3917) have been described previously (Krause et al., 2004; Nebel et al., 2002; Oh et al., 2003). EGFP-C1 and EGFP-SV vectors were described previously (Wulfkuhle et al., 1999). An untagged version of bovine SV was made by cloning the full-length SV cDNA into the KpnI and XbaI sites of pTracer-CMV (Invitrogen) (H340). His-tagged cortactin (ASH3, amino acids 1–506) was generated from pdsRed-cortactin (gifted by Dr. Alan S. Mak, Queen’s University, Kingston, ON, Canada) (Webb et al., 2006a) after digestion with BglII and cloning into pT310a vector (Invitrogen). Y527F-Src was from the Harold Varmus laboratory (National Institutes of Health, Bethesda, MD). Myc-tagged Tks5 (myc-Tks5) (Lock et al., 1998) and HA-tagged Cdc42 (Shinjo et al., 1990) (HA-Cdc42), both in the pE8-BOS expression vector (Mizushima and Nagata, 1990), were kind gifts from Drs. Sara Courtneidge (Burnham Institute for Medical Research, La Jolla, CA) and Dr. Kozo Kaibuchi (Nagoya University, Nagoya, Japan), respectively. EGFP-MT1-MMP was from Dr. Maria C. Montoya (Spanish National Cancer Research Center [CNIO], Madrid, Spain) (Galvez et al., 2002; Bravo-Cordero et al., 2007).

Immunoblotting

Whole cell lysates were prepared in 1% SDS, phosphate-buffered saline (PBS), 1 mM phenylmethylsulfonyl fluoride (PMSF), and a protease inhibitor cocktail containing: 1 μg/ml aprotinin, 1 μM antipain, 2 μM AMLM (calpain inhibitor II), 1 mM benzamidine, 10 μM E64, 1 μM leupeptin, and 1 mM pepstatin A. For each sample, 40 or 50 μg of protein, as determined by bicinchoninic acid protein assay (Pierce Chemical, Rockford, IL), were run SDS-polyacrylamide gels (Laemmli, 1970), transferred to nitrocellulose membranes (BioRad), and probed with primary antibodies against supervillin (H340), lamellipodin (3917), and calponin (H9262, 1:1000 dilution) was used as a loading control. Horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA), diluted 1:2000, were detected by the ECL Western Blotting substrate kit (Pierce Chemical, Rockford, IL). All blots were scanned using ImageJ (Rasband, National Institutes of Health, Bethesda, MD; http://rsb.info.nih.gov/ij/). EGFP-SV expression levels were determined by calculating band intensities with endogenous SV and correcting for transfection efficiency.

Cell Culture and Transfection

COS7-2 cells, a highly transfectable clone of COS7 (Kowalczyk et al., 1997), were a generous gift from Dr. Kathleen J. Green (Northwestern University, IL). COS7 cells were grown in high glucose DMEM (11995; Invitrogen) supplemented with 10% fetal calf serum (FCS) for ≥20 passages. MDA-MB-231 breast carcinoma cells were obtained from the American Type Culture Collection (Manassas, VA) and from the Tissue Culture Shared Resource of the Lombardi Cancer Center (c/o Dr. Susette Mueller, Georgetown University, Washington, DC). GC14-resistant MDA-MB-231 cells that stably overexpress wild-type c-Src (MDA-MB-231/WT Src cells) were a kind gift from Dr. Toshiyuki Yoneda (University of Texas Health Science Center, San Antonio, TX) and Dr. S. Mueller (Myoui et al., 2003; Bowden et al., 2006). MDA-MB-231 cells without and with WT c-Src overexpression were grown in DMEM supplemented with 10% FCS and 2 mM l-glutamine (29303; Invitrogen). Cultured cells for immunoblots were generously supplied by Drs. G. Sluder (human primary foreskin fibroblasts and hERT1–RPE1; Clontech, Mountain View, CA), L. Languino (LNCaP; LNCaP, C4-2, and DU-145), and A. Mercurio (MCF10A series, all from University of Massachusetts Medical School).

Transfection methods have been described previously (Takizawa et al., 2006). Briefly, for plasmids, COS7 cells were transfected using Effectene
Transfection Reagent (QiAGEN, Valencia, CA) for 24 h with the Y527F-Src, HA-Cdc42, and myc-Tks5 plasmids or for 24 or 48 h with the EGF-P-SV plasmid. For small interfering RNAs (siRNAs), COS7 cells were transfected with Lipofectamine 2000 (Invitrogen). MDA-MB-231/WT Src cells were transfected using the transfection system (Amaxa Biosystems, Gaithersburg, MD), according to manufacturer’s instructions. Briefly, for each Nucleofection, 1 × 10⁶ adherent cells were trypsinized, rinsed with Dulbecco’s PBS, modified (14:190), (Invitrogen), resuspended with 100 μl of solution V and with either 40 nM Stealth siRNA or 2 μg of plasmid, and treated using program X13. Cells treated with siRNA were incubated for 4 d before experiments, whereas cells treated with plasmid were incubated for 2 d.

The double-stranded (ds)RNAs targeting COS7 cell SV have been described previously (Takizawa et al., 2007). Two dsRNAs targeting human SV were used as Stealth duplexes (Invitrogen): human (h)SV1, 5′-GAGGAAAUUGGUGUGGAACAAGA-3′ (coding nucleotides 1680-1704); and hSV2, 5′-GCGAU- GCCUGCCGAGCAGAACA-3′ (coding nucleotides 226-250). The Stealth siRNA or 2 μg of plasmid, and treated using program X13. Cells used as a control dsRNA. A dsRNA targeting human geoslin was also made as a Stealth Duplex with the sequence 5′-GACTTCTATGGAGCCGAGAC-G-3′ (coding nucleotides 1460-1484). Two dsRNAs targeting primate cortactin were made as Stealth duplexes: CTI 5′-UCUUGUUCACCAAUUC-CUC-3′ and CT2 5′-GAGGAAAAAUUGGUGUGGAACAAGA-3′.

Recombinant Protein Purification and in Vitro Pull-Down Assay

Glutathione transferase (GST)-SV fusion fragment proteins were expressed after isopropyl β-D-thiogalactoside (IPTG) induction in BL21(DE3)EsR cells and purified with glutathione-Sepharose as described previously (Chen et al., 2003). His-cortactin(A3SH) was expressed after induction by IPTG and purified by using nickel-nitrirotiacetic acid Agarose (QiAGEN). Eluted proteins were dialyzed in dialysis buffer (50 mM Tris, pH 8.0, 300 mM NaCl, 1 mM dithiothreitol [DTT], and 10% glycerol). Dialyzed proteins were frozen quickly in liquid nitrogen and stored at −80°C. Purified GST-SV fragment proteins were mixed with His-cortactin(A3SH) in binding buffer (50 mM Tris, pH 8.0, 300 mM NaCl, 1% Triton X-100, 1 mM DTT, 10% glycerol), 1 mM PMSF, and protease inhibitor cocktail) and incubated with glutathione-Sepharose beads for 1 h at 4°C with shaking. The mixtures were then washed five times with binding buffer followed by centrifugation at 3000 rpm for 2 min. Beads were resuspended in 2× SDS sample buffer and heated to 95°C for 5 min. Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. Immunoblotting was performed using anti-cortactin antibodies.

Live Cell Imaging

COS7 cells transfected with EGFP-SV were cultured on 22-mm square coverslips and sealed into chambers with DMEM, 10% FCS, and 10 mM HEPES, without phenol red. After temperature equilibration in a Plexiglas environmental chamber heated to 37°C, images focused on the ventral cell surface were acquired for EGFP, by using an HCX PL Apo 63×/1.2 numerical aperture (NA) oil immersion objective lens on a DMIRE 2 inverted microscope (Leica Microsystems, Allendale, NJ), a Retiga Exi cooled charge-coupled device (CCD) camera (QImaging Corp., Burnaby BC, Canada), and OpenLab 3.5.2 software (Improvision, Waltham, MA). Images were processed for Z-series, images were acquired with a 100× APO objective lens (NA 1.35) by using step sizes of 0.2 μm on a DMIRE 2 inverted microscope–Newman–Keuls multiple comparisons post test. As noted in figure legends, some pairs of groups also were compared using unpaired t tests.

RESULTS

SV Promotes Restructuring of the Actin Cytoskeleton

SV Overexpression Increases F-Actin Punctae. Increased levels of SV decrease the numbers of COS7 cells containing stress fibers (Takizawa et al., 2006). Compared with overexpressed EGFP alone (Figure 1A, a–c), overexpression of either EGFP-tagged SV (EGFP-SV) (Figure 1A, d–f) or untagged SV (Figure 1A, g–i) increases the numbers of F-actin punctae (Figure 1A, e and h vs. b, insets). These punctae, which are ~0.5 μm in diameter, can be either round or irregularly shaped. They are prominent at the ventral surfaces of cells expressing EGFP-SV but are also found near nuclei, especially in EGFP-expressing control cells (Supplemental Figure S1). In wide-field fluorescence (Figure 1A), the mean numbers of ventral F-actin punctae in cells expressing EGFP-SV (Figure 1B) or untagged SV (Figure 1C) increase approximately sevenfold, compared with mock-transfected cells or cells expressing only EGFP, indicating...
specificity for SV sequences. The increase in ventral F-actin punctae in EGFP-SV–transfected cells was not due solely to protein expression levels because even with very high expression of EGFP, the number of these punctae does not increase to levels seen with EGFP-SV. This increase in ventral F-actin punctae is apparently a gain of function because RNAi-mediated reduction of endogenous SV to 5% of normal levels does not significantly reduce the low background numbers of F-actin punctae in COS7 cells (data not shown).

Most EGFP-SV Punctae Are Dynamic, but These Structures Are Probably Heterogeneous in Nature. In live cell imaging, ~94% of the EGFP-SV punctae in the cell periphery were highly dynamic and short lived (Figure 2 and Supplemental Video 1). Although occasional movements of ~1.0 μm/s were seen, the instantaneous velocities of these punctae averaged 0.36 ± 0.05 μm/s (mean ± SEM; n = 19) over their lifetimes of 2.9 ± 0.3 min (mean ± SEM, n = 93) (Figure 2, A and B). These dynamic short-lived punctae moved both toward and away from the cell periphery (Figure 2A, arrowheads; and Supplemental Video 1). The ~6% of the EGFP-SV punctae that were longer lived exhibited lifetimes of at least 30.5 ± 1.6 min (mean ± SEM; n = 6) (Figure 2B) and were largely restricted in localization (Figure 2C), with signal intensities that could vary over time (Figure 2A, double arrows). Other longer lived punctae tumbled in place, as though they might be associated with larger, vesicular structures and exhibited sporadic, rapid movements (Figure 2A, arrow; and C). The mean instantaneous velocity of EGFP-SV punctae is most consistent with directional movements propelled by kinesins (~0.5 μm/s), but the variable directionalities and the range of observed velocities suggest the possibility of multiple propulsion mechanisms, including actin comet tails (~0.17 μm/s), myosin Vb (0.22 μm/s), and dynein (~0.9 μm/s) (Porter et al., 1987; Woehlke et al., 1997; Benesch et al., 2002; Toba and Toyoshima, 2004; Paluch et al., 2006; Watanabe et al., 2006).

EGFP-SV Punctae Contain Podosome Markers, but Not Focal Adhesion Proteins. The lifetimes and dynamic behavior of most EGFP-SV punctae are reminiscent of podosomes or invadopodial precursors (Yamaguchi et al., 2005; Evans and Matsudaira, 2006). Although the local-
ization of some punctae above the ventral cell surface (Supplemental Figure 1) argues against a 1:1 identity with podosomes, most ventral EGFP-SV punctae do colocalize with podosome/invadopodial proteins (Figure 3). For example, ~91% of the EGFP-SV punctae colocalize with endogenous cortactin (Figure 3, A–C, boxes), a protein required for the formation and function of invadopodia (Bowden et al., 1999; Artym et al., 2006; Clark et al., 2007; Ayala et al., 2008; Clark and Weaver, 2008). Also, ~75 and ~50%, respectively, of the EGFP-SV punctae associate with coexpressed myc-Tks5/FISH (Figure 3, D–F) and HA-Cdc42 (Figure 3, G–I), other proteins important for invadopodial function (Nakahara et al., 2003; Courtneidge et al., 2005; Yamaguchi et al., 2005). Approximately 27% of the EGFP-SV punctae contain endogenous phosphoryrosine (Figure 3, J–L), a marker for invadopodia, tyrosine kinase-associated intracellular vesicles, and focal adhesions (Zamir et al., 1999; Bowden et al., 2006; Sandilands and Frame, 2008). By contrast, only ~3 or ~5%, respectively, of the EGFP-SV punctae colocalize with vinculin or pFAK (Figure 3, M–R), focal adhesion proteins that are present as rings in podosomes but absent from most invadopodia (Bowden et al., 2006; Machesky et al., 2008). Most of the overlap between EGFP-SV and vinculin or pFAK is at focal adhesions, as reported previously (Figure 3, M–R, arrows) (Wulfkuhle et al., 1999; Takizawa et al., 2006).

SV Causes a Redistribution of Cortactin. Because EGFP-SV reorganizes F-actin and overlaps extensively at punctae with cortactin, a protein that organizes actin at lamellipodial ruffles and is required for podosomes and invadopodia (Baday and Downward, 2007; Ammer and Weed, 2008; Weaver, 2008), we investigated cortactin localization in cells expressing EGFP-SV (Figure 4). In mock-transfected cells and in cells expressing EGFP alone for 48 h, ~70% (50–109 cells/experiment; n = 3) of the cells exhibit areas of contiguous, ~20-μm-long cortactin staining at their peripheries (Figure 4A, arrowheads; and B), as described originally (Wu and Parsons, 1993). By contrast, only 33.1 ± 5.7% (~50 cells/experiment; n = 6; p < 0.0001) of cells overexpressing EGFP-SV contained strong contiguous peripheral cortactin staining by 48 h after transfection (Figure 4A, d–f, arrows; and B); slight decreases were observed after only 24 h (data not shown). Cortactin was apparently redistributed to the cell interior, as opposed to being differentially stabilized or cleaved by calpain (Perrin et al., 2006), because FACS analyses showed no changes in total amounts of cellular cortactin with increasing expression of either EGFP or EGFP-SV (Figure 4C).

Other Lamellipodial Markers Also Are Displaced. To determine whether EGFP-SV promotes the selective redistribution of cortactin, we also monitored two other proteins characteristically concentrated at lamellipodia, lamellipodin/RAPH1/PREL1 (Figure 5, A–C) (Krause et al., 2004) and filamin...
A/ABP280 (Figure 5D-F) (Stendahl et al., 1980). EGFP-SV induces a significant loss of lamellipodin from the cell periphery (Figure 5, A and B). By contrast, filamin remains at the cell periphery in COS7 cells expressing EGFP-SV, but the extent and intensity of the peripheral filamin signal seems reduced (Figure 5, D and E). Unlike the nearly perfect overlap seen with cortactin antibody (Figure 3, A–C), most EGFP-SV punctae lack lamellipodin and filamin (Figure 5, C and F, arrowheads), with only ~38 and ~35%, respectively, of the EGFP-SV punctae overlapping with signal from antibodies against these two proteins (Figure 5, C and F, arrows).

**Cortactin Binds SV In Vitro.** Due to the redistribution of cortactin upon EGFP-SV overexpression (Figure 4) and the

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**Figure 3.** EGFP-SV punctae colocalize with cortactin and exogenous Tks5 and Cdc42 but not with phospho-FAK or vinculin. COS7 cells expressing EGFP-SV (A, D, G, J, M, and P) were stained with antibodies against endogenous cortactin (B), phosphotyrosine (pTyr; K), vinculin (N), or phosphorylated FAK (pFAK; Q) or with antibodies against tags on exogenously expressed Tks5 (E) or Cdc42 (H). EGFP-SV is green in the merged images (C, F, I, L, O, and R); other proteins are shown in magenta; overlap is white. Insets, fourfold enlargements of areas within boxes. Arrows denote focal adhesions. Bar, 10 μm.
striking amount of colocalization of the two proteins (Figure 3, A–C), compared with the relatively weak overlap with lamellipodin and filamin (Figure 5, C and F), we investigated whether cortactin binds SV directly. GST or GST-tagged SV N-terminal fragments were mixed with His-cortactin (SH3), and interacting proteins were co-sedimented with glutathione-Sepharose beads (Figure 6A). Even with significant His-cortactin (SH3) input (Figure 6A, a and b, lanes 1), there was no detectable binding with GST or beads only (lanes 2 and 3). His-cortactin (SH3) was pulled down with SV amino acids SV1-340 (lanes 4), SV570-830 (lanes 7), and SV340-830 (lanes 8). The lack of binding with SV171-340 (lanes 5) or SV340-570 (lanes 6) indicates likely cortactin binding sequences within SV1-171 and SV570-830.

Cortactin Increases the Numbers of EGFP-SV F-Actin Punctae. To see whether cortactin is necessary for the formation of EGFP-SV–induced F-actin punctae, we reduced cortactin levels by 74% of control-treated cells (Figure 6B, CON, lane 1) by using two different cortactin-specific siRNAs (CT1 and CT2; lanes 2 and 3). Cortactin knockdown cells were transfected with plasmids encoding EGFP or EGFP-SV and scored for the number of F-actin punctae (Figure 6C). As in the absence of siRNA treatment (Figure 1), there is a significant difference between EGFP- and EGFP-SV–transfected cells treated with the control siRNA (**p < 0.001) (Figure 6C). Although cortactin knockdown has no significant effect on the numbers of F-actin punctae in EGFP-transfected cells, the reduction in cortactin levels inhibits ~50% of the increase in numbers induced by EGFP-SV (*p < 0.05). Therefore, the podosome and invadopodial protein cortactin contributes to the formation of EGFP-SV punctae.

Role of SV at Podosomes and Invadopodia

Endogenous SV Localizes to Src-induced Podosomes and Areas of Matrix Degradation in COS7 cells. To further examine the relationship between SV and podosomes/invadopodia, we localized endogenous SV in COS7 cells containing podosomes induced by constitutively active c-Src-Y527F (Figure 7). Each Src-induced podosome consists of a core of F-actin, cortactin, and other proteins surrounded by a ring of focal adhesion proteins (Linder and Aepfelbacher, 2003). Endogenous SV colocalizes extensively with F-actin and cortactin at the podosome cores (Figure 7, A and B) and less

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Figure 4. Expression of EGFP-SV leads to redistribution of cortactin. (A) COS7 cells expressing EGFP alone (a; green in c) or EGFP-SV (d; green in f) for 48 h were immunostained for endogenous cortactin (b and e; red in c and f). Cells were scored as containing (arrowheads) or lacking (arrows) peripheral cortactin. Bar, 50 μm. (B) Percentages of cells with continuous peripheral cortactin staining 48 h after mock transfection (Mock) or expression of EGFP alone (EGFP) or EGFP-SV (EGFP-SV). Means ± SD; ***p < 0.0001. (C) Two-color FACS analyses showing no change in the levels of total cellular cortactin caused by expression of EGFP or EGFP-SV in COS7 cells. Control, secondary antibody alone.
well with α-actinin (Figure 7C), which associates with both the podosome core and ring (Linder and Aeppelbacher, 2003). Little overlap of SV with the focal adhesion proteins, phosphorylated FAK and vinculin, is observed in podosome rings (Figure 7, D and E). The overall SV staining pattern at podosome cores is generally more punctate than those of the other proteins (Figure 7, left, x-y inset), although foci of SV and cortactin show frequent overlaps (Figure 7B). Immunolocalization of SV shows a preferential exposure of the SV epitope at the apical ends of the podosomes, near the interface with the cytoplasm (Figure 7), regardless of whether SV is imaged with green (Figure 7) or red (Supplemental Figure S2A and S2B) fluorophores. However, colocalization of EGFP-SV and antibody-stained SV indicates that the N-terminal epitope recognized by the antibody is preferentially shielded near the base of the podosome cores (Supplemental Figure S2C). Thus, as has been reported for the transmembrane receptor CD44 (Chabadel et al., 2007), the peripheral membrane protein SV marks podosome cores. EGFP-SV also localizes to areas of matrix degradation in COS7 cells on fluorescently labeled gelatin although these cells do not efficiently degrade matrix (Supplemental Figure S3).

Many Tumor Cell Lines Contain Relatively High Levels of SV. To identify an invasive cell line in which we could examine SV function in matrix degradation and invasion, we screened a number of tumor cell lines for the presence of endogenous SV. Abundant levels of SV message have been reported in HeLa cervical adenocarcinoma, SW480 colorectal adenocarcinoma, and A549 lung carcinoma cells (Pope et al., 1998). In agreement with previous results (Pestonjamasp et al., 1997), we find that HeLa cells contain higher levels of endogenous SV than do untransformed cell lines (Supplemental Figure S4A, lanes 1–3). Several human prostate (LNCaP and C4-2, DU-145) (Stone et al., 1978; Thalmann et al., 1994) and breast (MCF10A1, MCF10AT1k.cl2, MCL10CA1h, MCF10ACA1a.cl1, and MDA-MB-231) (Tang et al., 2003) epithelial cell lines contain abundant endogenous SV (Supplemental Figure S4). The highest SV levels were seen in MDA-MB-231 metastatic breast carcinoma cells, which inherently form invadopodia on ECM substrates (Chen et al., 1994).

Endogenous SV Localizes to Invadopodia in MDA-MB-231 Cells. In MDA-MB-231 cells on gelatin, endogenous SV colocalizes with the large ventral accumulations of cortactin and F-actin characteristic of invadopodia (Figure 8A; Artym et al., 2006). In addition, mRFP-SV is found in proximity with 45% of the vesicles or invadopodia that contain EGFP-MT1-MMP (Figure 8B), the membrane-bound MMP responsible for most matrix degradation in MDA-MB-231 cells (Artym et al., 2006). Endogenous SV also is concentrated at and within regions of matrix degradation, as identified by black areas in thin films of TRITC-labeled gelatin (Figure 8C). In XZ and YZ views, SV is seen both within and above the holes in the gelatin matrix created by invadopodia (Figure 8C, a–d). By contrast, little overlap is seen between EGFP-SV and the pY-100 phosphotyrosine antibody (Bowden et al., 2006; Supplemental Figure S5). Together, these data support an association between SV and invadopodia.

SV Overexpression Increases the Numbers of Matrix Degradation Sites Formed Per Cell. We tested whether SV affects ECM degradation in highly invasive MDA-MB-231 cells that

Figure 5. EGFP-SV redistributes lamellipodin/RAPH1/PREL1, but not filamin A, within 24 h. (A and D) COS7 cells expressing EGFP alone (a) or EGFP-SV (c) for 24 h were immuno- stained for endogenous lamellipodin (b and d in A) or filamin A (b and d in D). Cells were scored as containing (arrowheads) or lacking (arrows) peripherally localized protein. Bars, 50 μm. Percentages of cells with peripheral (B) lamellipodin or (E) filamin staining 24 h after mock transfection (Mock) or expression of EGFP alone (EGFP) or EGFP-SV (EGFP-SV). Means ± SD; *p < 0.05; n = 2 (B); n = 3 (E). Overlap (arrows) or lack of overlap (arrowheads) of EGFP-SV punctae with endogenous lamellipodin (C) and filamin (F). Bars, 20 μm.
stably overexpress WT Src (MDA-MB-231/WT Src) (Myoui et al., 2003; Bowden et al., 2006). Transient overexpression of EGFP-SV to levels that were 4.9 ± 1.5 (mean ± SD; n = 3) times greater than endogenous SV levels doubled the average number of holes per cell in the fluorescent gelatin matrix (Figure 9B vs. A and C; and D). However, the percentage of cells that made holes did not differ significantly from controls (Figure 9E). Similar results are seen with MDA-MB-231 cells not transfected with c-Src (Supplemental Figure S6). This SV-promoted increase in the numbers of holes per cell corresponds to a 4.2-fold increase in the mean area of degraded matrix per cell (Supplemental Figure S7). These results are consistent with a SV-mediated increase in the numbers, efficiency, or dynamics of sites of ECM degradation.

**SV Underexpression Decreases Matrix Degradation, Especially in the Cell Center.** Conversely, reduction of endogenous SV levels reduces the apparent numbers of ECM-degrading sites (Figure 10). Each of two siRNAs reduces SV levels in MDA-MB-231/WT Src cells to 30% of endogenous levels without adversely affecting the expression level of the related protein, gelsolin (Figure 10A, lanes 1–2 vs. lane 3). Although these reduced levels of SV do not affect the percentages of cells associated with degraded matrix (Figure 10B), the mean numbers of holes per cell (Figure 10C) and the percentages of cells with holes underneath the central region of the cell are decreased, compared with cells treated with a control siRNA (Figure 10D; and E, e vs. b). By contrast, matrix degradation in these assays is unaffected by down-regulation of gelsolin (Figure 10A, lane 4; and B–D), an SV family member involved in podosome assembly in osteoclasts and matrix invasion of MDA-MB-231 cells (Chellalaiah et al., 2000; Van den Abbeele et al., 2007). Double knockdowns of SV and gelsolin (Figure 10A, lane 5) result in
the same number of total holes per cell as observed for controls (Figure 10C) and are not significantly more effective than knockdown of SV alone in decreasing the presence of central holes (Figure 10D). However, the locations of the holes in the matrix for the SV/gelsolin double knockdown tended to be shifted away from the cell centers toward the cell edges, as was also observed for knockdown of SV alone (Figure 10Eh). Thus, SV apparently affects both the numbers and locations of the holes in the fluorescent matrix.

SV Knockdown Does Not Decrease Ventral F-Actin Punctae. Although SV can increase F-actin bundling in vitro and in vivo (Wulfkuhle et al., 1999; Chen et al., 2003), SV probably is not required for the formation of ventral F-actin structures (Figure 10E). MDA-MB-231/WT Src cells with reduced levels of SV and significantly reduced sites of matrix degradation (Figure 10E, e, h vs. b) still contain apparently normal levels of F-actin structures within the ventral focal plane (Figure 10E, d, g vs. a). In addition, COS7 cells expressing constitutively active c-Src-Y527F form similar numbers of podosomes, regardless of whether their SV levels are normal or have been reduced to 20 ± 5% of endogenous levels (Supplemental Figure S8). The normal-looking appearance of the ventral F-actin structures in SV knockdown cells, coupled with the reduced numbers of associated ECM holes in MDA-MB-231/WT Src cells, suggest a defect in invadopodial function.

SV Knockdown Reduces Invasion, but Only in the Context of Reduced Levels of Gelsolin. As a direct assay for the roles of SV and gelsolin in invasion, we measured the effects of single and double SV and gelsolin knockdowns on the ability of MDA-MB-231/WT Src cells to invade Matrigel-coated transwell filters (Figure 11). Invasion indices are normalized to eliminate effects of changes in cell motility although no statistically significant differences are apparent in the rates of cell transmigration across uncoated filters at this long time point (data not shown). Knockdown of SV alone shows an upward trend in invasion index (Figure 11), and reduction of gelsolin alone shows a downward trend that compares favorably with previously published results (Van den Abbeele et al., 2007). However, assay-to-assay variences preclude statistical significance for single knockdowns of either SV or gelsolin. By contrast, simultaneously reducing SV and gelsolin significantly decreases the ability of MDA-MB-231 cells to invade Matrigel-coated filters.

DISCUSSION

We show here that SV reorganizes the cortical actin cytoskeleton, stimulating the formation or redistribution of dynamic basal punctae containing F-actin, cortactin, and other podosome/invadopodia proteins while decreasing the extents of cortactin and lamellipodin at the cell periphery. SV and cortactin bind directly in vitro and cooperate to redistribute F-actin in vivo. We also show that SV is a core component of Src-induced podosomes and invadopodia and that SV contributes to ECM degradation and Matrigel invasion by Src-expressing MDA-MB-231 breast carcinomas. These results are consistent with the report that the gene encoding SV, SVIL, is among the predisposing factors for nonBRCA1/2 (BRCAx) breast cancer (Hedenfalk et al., 2003).

One possible mechanism of SV action is alteration of signaling pathways upstream of cytoskeletal remodeling. The earliest change that we see in lamellipodial architecture in cells expressing EGFP-SV is the loss of peripheral lamellipodin (Figure 5). Lamellipodin regulates actin polymeriza-

Figure 8. Supervillin localizes at and within MDA-MB-231 cell invadopodia. (A) MDA-MB-231 cells were plated on unlabeled gelatin for 3 h, fixed, and stained with antibodies (SV and cortactin) and/or phalloidin (F-actin) as noted, and then they were imaged by wide-field microscopy. Merges show supervillin (red), cortactin (green), and F-actin (blue). Bars, 10 μm (top row) and 1 μm (bottom row). (B) MDA-MB-231/WT Src cells were transfected with mRFP-SV and EGFP-MT1-MMP. Cells were plated on unlabeled gelatin-coated coverslips for 6 h, fixed, and stained with phalloidin (F-actin) and imaged by wide-field microscopy. Merges show mRFP-SV (red), EGFP-MT1-MMP (green), and F-actin (blue). Arrows indicate colocalization; arrowheads denote proximate localization. (C) MDA-MB-231 cells were plated on TRITC-labeled gelatin for 6 h, fixed, and stained with anti-SV antibodies, and then they were imaged by confocal microscopy as a Z-series with 0.2-μm steps. Merges show supervillin (green) and TRITC-gelatin (red). An XY view of such a cell is shown (top row). Areas inside the boxes were enlarged fourfold (second row). Eightfold enlargements of XZ and YZ views corresponding to a, b, c, and d are shown below XY views. Bars, 10 μm (top row), 5 μm (second row), and 1 μm (bottom row).
tion mediated by Ena/VASP family proteins downstream of Ras activation (Krause et al., 2004; Jenzora et al., 2005). The lack of extensive overlap between lamellipodin and SV in punctae argues against a direct interaction between these two proteins. However, an indirect negative effect of SV on lamellipodial organization at the cell periphery could lead to the loss of broad arcs of peripheral F-actin, cortactin, and lamellipodin that we observe (Figures 4 and 5).

A second possibility is that SV-induced punctae may represent podosome/invadopodia “building blocks” associated with vesicles and/or molecular motors at various stages of assembly or intracellular transit. SV may help recruit cortactin and F-actin to intracellular membranes. Because SV, cortactin, and filamin all bind F-actin, the lack of filamin recruitment to punctae argues for a selective association with cortactin. A membrane-based mechanism for SV is likely based on its intracellular distribution and fractionation behavior (Pestonjamasp et al., 1997; Wulffkuhle et al., 1999; Nebl et al., 2002; Oh et al., 2003; Takizawa et al., 2006). The dot-like appearances of many EGFP-SV punctae (Figures 1 and 3) and XY views of endogenous SV at podosomes (Figure 7) are reminiscent of vesicles, as are the circular profiles that are occasionally apparent for larger structures associated with EGFP-SV (Figure 2 and Supplemental Video 1). EGFP-SV/F-actin/cortactin punctae also resemble cortactin structures described as occurring before actin recruitment in phorbol ester-induced podosomes (Webb et al., 2006a) or originating at lamellipodia and moving in association with endosomes (Kaksonen et al., 2000; Boguslavsky et al., 2007; Liadó et al., 2008). Although we did not detect consistent directional trafficking of SV punctae from the cell periphery to the basolateral cell surface, subtle effects on vesicle trafficking or

Figure 9. Overexpression of EGFP-SV increases the number of matrix holes formed per cell. MDA-MB-231/WT Src cells were transfected with either EGFP alone (A) or EGFP-SV (B) or they were mock transfected (C). Cells were plated on TRITC-labeled gelatin for 6 h before visualization by green (a) and red (b) fluorescence and phase contrast (d); merged green and red images also are shown (c). Bar, 20 μm. (D) The numbers of holes in the red gelatin matrix (representative of invadopodia) per cell were counted. EGFP-SV cells had a significant (***p < 0.001; n = 8) increase in the number of associated holes per cell. (E) The percentage of cells with some matrix degradation was not significantly different among the three conditions.
on cortactin recruitment to transport vesicles could lead to the observed shift in cortactin and F-actin distribution over time.

A selective effect of SV on basolateral targeting of cortactin and MMPs would be consistent both with the observed effects of SV knockdown on sites of matrix degradation under the cell center (Figure 10) and with the role of cortactin in MMP trafficking and matrix degradation (Bowden et al., 1999; Artym et al., 2006; Clark et al., 2007; Clark and Weaver, 2008). Cortactin recruitment to the ventral cell surface occurs in stages, mediated by at least three convergent regulatory signals (Artym et al., 2006; Webb et al., 2006a; Ayala et al., 2008). Activating mechanisms include cortactin phosphorylation by ERK1/2, Src family kinases, and PAK (Webb et al., 2006b; Tehrani et al., 2007; Ayala et al., 2008). In this context, we note that an isoform of SV is involved in stimulus-mediated activation of ERK1/2 in smooth muscle (Gangopadhyay et al., 2004) and that SV binds directly to and regulates myosin II (Takizawa et al., 2007), which directly affects exocytosis in a number of cell types (Togo and Steinhardt, 2004). The sequential regulated recruitment of MMPs and other proteins to invadopodia suggests that subsequent matrix degradation is a dynamic multistep process (Artym et al., 2006; Clark et al., 2007; Block et al., 2008). Our SV knockdown results (Figure 10) are consistent with SV-mediated increases in cortactin activation, enhanced cortactin- or myosin II-mediated MMP secretion, and/or elevation of the rate of invadopodia turnover.

The decreased matrix degradation generated by knockdowns of either SV, cortactin, or Tks5 and the colocalization of all three proteins at SV-induced punctae suggests functional cooperation (Seals et al., 2005; Artym et al., 2006; Clark et al., 2007; Webb et al., 2007; Ayala et al., 2008; Clark and Weaver, 2008). Participation in a common pathway is fur-
Figure 11. Invasion indices are decreased in cells depleted of both SV and gelsolin. MDA-MB-231/WT Src cells were treated with either control siRNA (Control) or with siRNAs targeting either SV (SV1 and SV2), gelsolin (Gel), or both proteins (SV1/Gel) and assayed for invasion through Matrigel-coated filters. Cells depleted in both SV and gelsolin were significantly less invasive than controls, based on unpaired t tests (*p < 0.05; n = 3).

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