Diaphanous-Related Formins Are Required for Invadopodia Formation and Invasion of Breast Tumor Cells

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Abstract
Proteolytic degradation of the extracellular matrix by metastatic tumor cells is initiated by the formation of invadopodia, i.e., actin-driven filopodia-like membrane protrusions endowed with matrix-degradative activity. A signaling cascade involving neural Wiskott-Aldrich syndrome protein and the Arp2/3 actin nucleating complex is involved in actin assembly at invadopodia. Yet, the mechanism of invadopodia formation is poorly understood. Based on their role as actin nucleators in cytoskeletal rearrangements, including filopodia formation, we examined the function of Diaphanous-related formins (DRF) in invadopodia formation and invasion by breast tumor cells. Using small interfering RNA silencing of protein expression in highly invasive MDA-MB-231 breast adenocarcinoma cells, we show that three members of the DRF family (DRF1–DRF3) are required for invadopodia formation and two-dimensional matrix proteolysis. We also report that invasion of a three-dimensional Matrigel matrix involves filopodia-like protrusions enriched for invadopodial proteins, including membrane type 1 matrix metalloproteinase, which depend on DRFs for their formation. These data identify DRFs as critical components of the invasive apparatus of tumor cells in two-dimensional and three-dimensional matrices and suggest that different types of actin nucleators cooperate during the formation of invadopodia. [Cancer Res 2009;69(7):2792–800]

Introduction
Tumor cell invasion across tissue boundaries and metastasis are dependent on the capacity of invasive cancer cells to breach the basement membrane (BM) and migrate through the three-dimensional interstitial collagen network (1, 2). One major route of invasion requires tumor cells to proteolytically cleave extracellular matrix (ECM) components via a mechanism involving matrix-degrading proteases (3). In particular, extracellular proteases belonging to the matrix metalloproteinase (MMP) family, including transmembrane membrane type 1 MMP (MT1-MMP), play a crucial role in cancer dissemination by degrading and remodeling ECM components (4–8).

Intriguingly, when invasive cancer cells are grown on a two-dimensional ECM substrate layered on glass, matrix proteolytic activity is restricted to invadopodia, which correspond to actin-rich finger-like structures protruding into the matrix and enriched in MT1-MMP (9–13). Neural Wiskott-Aldrich syndrome protein (N-WASP) and the Arp2/3 complex, both components of the actin polymerization machinery, are required for invadopodia formation and thus have been proposed to assemble actin filaments at invadopodia (14, 15). The cytoskeletal protein cortactin is also enriched at invadopodia and is critical for the formation and activity of these structures possibly through stabilization of the actin network and, as recently suggested, by controlling delivery/recruitment of MMPs at invadopodia (10, 11, 16–18). Furthermore, members of the Rho family of small GTPases are required for invadopodia formation (12, 15, 19). In particular, Cdc42 was shown to control the formation of invadopodia in human melanoma and rat mammary adenocarcinoma tumor cell lines through activation of the N-WASP/Arp2/3 complex cascade (14, 15), whereas we recently implicated Cdc42 and RhoA in the mechanism of invadopodia formation and MT1-MMP delivery in breast adenocarcinoma MDA-MB-231 cells (12). However, the complete machinery of invadopodia formation in cancer cells remains poorly understood. Based on the filopodia-like morphology of invadopodia (16, 20), it was postulated that formins might elongate actin filaments in invadopodia (14, 21).

Formins are filamentous actin (F-actin) nucleators that polymerize linear filaments through conserved formin homology domains (22). Among the formin family, Diaphanous-related formins (DRF) produce linear actin filaments that are the hallmarks of stress fibers and filapodia (23–27). In addition, DRFs are downstream effectors of active Rho GTPases, RhoA, and Cdc42 (22). Roles for DRF1 during formation of membrane protrusions by tumor cells and invasion have been recently reported (28, 29). However, the function of DRF proteins in invadopodia formation and in the acquisition of invasive phenotypes by cancer cells has not been thoroughly explored.

In this study, we assessed the contribution of DRF1, DRF2, and DRF3 to the invasion capacity of human MDA-MB-231 cells, a highly invasive cell line of basal-like breast tumor phenotype, the most aggressive form of breast cancers (30). Our data show a pivotal role of DRF proteins during invadopodia formation in two-dimensional and three-dimensional matrices for BM degradation and invasion by breast cancer cells.

Materials and Methods

Antibodies. Mouse monoclonal antibody for DRF1 was obtained from BD Biosciences. Goat polyclonal anti-DRF2 was purchased from Santa Cruz Biotechnology. Mouse MT1-MMP monoclonal antibody was a gift from...
Dr. M.C. Rio (Institut de Génétique et de Biologie Moléculaire et Cellulaire), Monoclonal mouse anti-β-actin antibody (clone AC15) was from Sigma-Aldrich. Monoclonal anti-RhoA was provided by Dr. J. Bertoglio (Institut National de la Sante et de la Recherche Médicale (INSM)). Monoclonal anti-cortactin (Clone 4F11) and anti-phosphotyrosine (pY; Clone 4G10) antibodies were obtained from Millipore. AlexaFluor-phalloidin, rabbit polyclonal anti-pY antibody, and antimouse IgG Alexa488 antibody were obtained from Invitrogen. Horseradish peroxidase-conjugated and fluorescently conjugated secondary antibodies were from Jackson ImmunoResearch Laboratories.

**Constructs.** EGFP-mDia2 and eSRC-Y527F expression constructs were kind gifts of Drs. T. Svitkina (University of Pennsylvania) and M. Arpin (Institut Curie), respectively. MT1-MMP with internal pHluorin was generated by PCR resulting in the insertion of pHluorin (super eclipptic variant, a gift from Dr. T. Galli) between amino acids 534 and 535, NH2 terminally to the transmembrane region. The sequence of Lifeact (31), kindly provided by Dr. R. Wedlich-Soldner (MPI Biochem), was fused to the aminoterminus of mCherry by PCR (mCh-Lifeact) with a GTP/VATP spacer and subcloned in pIRESpuro3 (Clontech).

**Cell culture, transfections, and stable cell lines.** Human breast adenocarcinoma cells MDA-MB-231 (American Type Culture Collection) were maintained in L-15 culture medium (Sigma-Aldrich) with 2 mmol/L glutamine and 15% FCS at 37°C in 1% CO2. For small interfering RNA (siRNA) treatment, MDA-MB-231 cells were treated with 10 to 100 nmol/L of specific siRNA (see Supplementary Table S1) with Oligofectamine (Invitrogen). Cells were analyzed after 24 h of treatment. MDA-MB-231 cells were transfected with expression constructs using Lipofectamine (Invitrogen). Cells were analyzed after 24 h of transfection. Stable lines of MDA-MB-231 cells expressing mCh-Lifeact alone or together with pHluorin-MT1-MMP were selected with 1 μg/mL puromycin or 1 μg/mL puromycin, together with 0.5 mg/mL G418, respectively.

**Reverse transcription–PCR.** Total RNA was obtained using the RNeasy Mini kit from QIAGEN (Hilden). cDNA synthesis was carried out using SuperScript III Reverse Transcriptase enzyme (Invitrogen). PCR reactions were performed using Platinum Taq DNA Polymerase (Invitrogen). Primers are listed in Supplementary Table S2.

**Fluorescent-gelatin degradation assay and quantification of invadopodia.** MDA-MB-231 cells were incubated for 5 h on FITC-conjugated or AlexaFluor 594-conjugated gelatin (Invitrogen) to quantify gelatin degradation and were stained for F-actin and cortactin to identify invadopodia-positive cells as described (12, 13). Statistical analysis was carried out using SigmaStat 3.5.

**Indirect immunofluorescence analysis.** MDA-MB-231 cells were cultured on gelatin-coated coverslips (Figs. 1 and 2) or on top of Matrigel (10 mg/mL, BD Biosciences; Fig 4 and Supplementary Figs. S4 and S5). Cells were preextracted with 0.3% Triton-X100 in 4% PFA and processed for immunofluorescence analysis as described (12, 13). Cells were imaged with a DM6000 B/M microscope (Leica Microsystems; Figs. 1 and 2), or with a Leica DMRA2 microscope with 100× PL APO HCX, 1.4 NA objective equipped with a piezoelectric driver (0.2-μm increment, Physik Instrumente; Fig. 4 and Supplementary Figs. S4 and S5). Microscopes were equipped with a CoolSnapHQ camera (Roper Scientific) and steered by Metamorph 6.0.7 (Molecular Devices Corporation).

**Live cell imaging.** MDA-MB-231 cells expressing mCh-Lifeact were plated on FITC-gelatin coated glass-base dishes (Iwaki) and kept in a humidified atmosphere at 37°C and 1% CO2. For Supplementary Videos S1 and S6, images were recorded using the 100× objective of a Leica DMIRE2 microscope equipped with a Cascade II camera (Roper Scientific). For Supplementary Video S2 and S4, images of mCh-Lifeact and FITC-gelatin were recorded with the 60× objective of an automated Nikon TE2000-E microscope equipped with a CoolSnapHQ camera. To allow representative sampling of mock and siRNA-treated cell populations, six fields per condition were recorded simultaneously.

**Scanning and transmission electron microscopy.** The upper chamber of a Transwell cell culture insert (BD Biosciences) was filled with 100 μL of Matrigel, and cells were added in serum-free L15 medium. The lower chamber contained L15 medium with 15% FCS. For scanning electron microscopy, cells were prefixed in 2.5% glutaraldehyde/0.1 mol/L sodium cacodylate (pH 7.4). After postfixation in 1% osmium tetroxide (in 0.2 mol/L cacodylate buffer), cells were dehydrated in a series of increasing ethanol concentrations and critical point dried using carbon dioxide. After coating with gold, cells were examined with a JEOL JSM-6700F scanning electron microscope. For transmission electron microscopy, 5 h after contact with Matrigel, cells were fixed overnight in 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 mol/L cacodylate buffer, postfixed with 2% OsO4 dehydrated in ethanol, and embedded in Epon. Ultrathin sections were prepared with a Reichert Ultracut-E Microtome (Leica), counterstained with 2% uranyl acetate in 70% methanol, and viewed with a Philips CM120 Transmission Electron Microscope (FEI Company) equipped with a Tecnoview camera (Olympus).

**Results**

**DRFs are required for invadopodia formation and matrix degradation.** Western blotting and semiquantitative reverse transcription–PCR analysis showed that MDA-MB-231 cells express all three members of the DRF family (Fig. 1A). To characterize the function of DRF proteins in matrix degradation and invadopodia activity in MDA-MB-231 cells, we silenced each DRF protein individually by using two independent siRNAs (Fig. 1A).

Mock and siRNA-treated cells were plated on a thin layer of fluorescently labeled gelatin for 5 hours in an assay monitoring matrix degradation (10, 12). We observed that ~20% of mock-treated cells degraded the fluorescent matrix (Fig. 1B; data not shown) and silencing of MT1-MMP reduced matrix proteolysis of MDA-MB-231 cells to ~10% of control (Fig. 1D), confirming recent reports (10, 12, 17). Next, we analyzed matrix proteolysis by DRF-depleted cells. Silencing of DRF1 to DRF3 with two independent siRNAs resulted in 70% to 80% reduction of degradation compared with mock-treated cells without significant perturbation of cell and actin cytoskeleton morphology or spreading on gelatin (Fig. 1B and D).

As described (9, 10, 16), proteolysis of the matrix by MDA-MB-231 cells was mainly focal and coincided with the presence of F-actin/cortactin-positive invadopodia at the ventral cell surface (Fig. 1C). Consistent with the proportion of cells able to degrade the matrix, invadopodia were observed in ~20% of mock-treated MDA-MB-231 cells plated on gelatin (6.0 ± 0.7 invadopodia per cell, n = 95 cells; Fig. 1D). Interestingly, this proportion dropped to 8% to 11% in cells depleted for each individual DRF (Fig. 1D). When cells were incubated on gelatin for a longer time (15 h), inhibition of matrix degradation and invadopodia formation was still observed in DRF-depleted cells compared with controls, indicating that loss of DRF function does not delay but rather inhibits invadopodia formation (not shown). No additional effect of simultaneously knocking down two DRF proteins was observed, suggesting that the availability of each DRF is limiting and that they are functionally linked (not shown). Of note, triple knockdown was inefficient for individual protein suppression (not shown). Altogether, these data show that DRF proteins are required for invadopodia formation and subsequent matrix degradation in MDA-MB-231 breast cancer cells.

To specify at which step of invadopodia formation DRF proteins are implicated, we performed live cell imaging of mock-depleted, DRF3-depleted, and MT1-MMP-depleted MDA-MB-231 cells stably expressing mCh-Lifeact (F-actin-binding peptide of Saccharomyces cerevisiae Abp140p fused to mCherry; ref. 31). MDA-MB-231 cells plated on gelatin displayed lamellipodial and membrane ruffling activities and exhibited random motility irrespective of siRNA.
treatment. In mock-treated degrading cells, invadopodia appeared as bright, static, and long-lived F-actin puncta, some being stable for at least 2 hours (Supplementary Fig. S1A; Supplementary Videos S1 and S2). Newly formed invadopodia appeared either as single isolated puncta (Supplementary Fig. S1A and B; Supplementary Videos S1 and S2) or formed collectively as a group of puncta originating from a wave of actin assembly (Supplementary Fig. S1A; Supplementary Video S1). Highly dynamic small actin dots could also be observed at the rear of extending lamellipodia that did not coincide nor precede matrix degradation and were not related to invadopodia (Supplementary Fig. S1B; Supplementary Video S2). These small actin dots were also present in cells depleted for DRF3 (Supplementary Fig. S1C; Supplementary Video S3). In contrast, long-lived degradative invadopodia were rarely observed in DRF3-ablated cells (Supplementary Video S3). Finally, F-actin recruitment or aggregation was virtually absent in MT1-MMP depleted cells (Supplementary Video S4). Altogether, these observations suggest that DRF3, as well as other DRF-family members, are important for the early stage of invadopodia formation.

**DRF localization at invadopodia.** Endogenous DRF1 was distributed throughout the cytoplasm of MDA-MB-231 cells plated on gelatin as observed in many different cell types (29, 32), with some dotty accumulations at the cell edge but no obvious localization at invadopodia (not shown).
lost in cells silenced for DRF1 (not shown). GFP-mDia2, the mouse orthologue of human DRF3, was similarly diffuse (not shown). As invadopodia are generally present at the center of cells where cytoplasmic signal was strongest, we analyzed DRF localization in MDA-MB-231 cells transiently expressing an active form of c-Src (Y527F) that triggers appearance of large peripheral invadopodia (10, 12). In these cells, endogenous DRF1 was detected into small dots. Some DRF1 dots were closely apposed to and surrounded F-actin–positive and pY-positive invadopodia (Fig. 2A and B). In addition, GFP-mDia2 also colocalized with F-actin and pY at invadopodia under these conditions (Fig. 2C and D). This association of DRF1 and mDia2/DRF3 to invadopodia argues for a direct role for DRFs in the formation of these structures.

**DRFs are required for invasion through a three-dimensional reconstituted BM.** As MT1-MMP–mediated proteolysis is critical for breaching of the BM by carcinoma cells (3, 5), we evaluated the role of DRFs during remodeling and invasion of Matrigel, a reconstituted matrix mimicking BM. For this purpose, MDA-MB-231 cells were plated on top of a thick layer of Matrigel (~3.5 mm) and were observed by scanning electron microscopy (SEM) after 2 to 14 hours on the matrix. MDA-MB-231 cells rapidly adhered to Matrigel and adopted a rounded morphology (Fig. 3A, 2 hours). Cells progressively invaded the matrix and were completely embedded in Matrigel after 12 to 14 hours (Fig. 3A and C). Of note, gel retraction during sample preparation created domes of Matrigel around invading cells (Fig. 3A). Plasma membrane folds, filopodia-like protrusions, and some membrane blebs were observed at the free surface of invading cells (see Figs. 3A and 5A). In contrast to mock-treated cells that invaded into the BM, cells silenced for MT1-MMP

![Figure 2.](image-url)
remained spread and formed chains on the surface of Matrigel (Fig. 3B and C).

SEM micrographs (Fig. 3C) allowed a precise quantification of cells completely buried within Matrigel after 14 hours and, hence, of the invasion capacity of the different cell populations (Fig. 3D). Strikingly, the invasion capacity of MT1-MMP–depleted and DRF1/ DRF3–depleted cells in Matrigel dropped to 18% to 37% compared with mock-treated cells (Fig. 3D). Invasion capacity of DRF-depleted or MT1-MMP–depleted cells was also significantly reduced compared with mock when assessed with commercial Matrigel invasion chambers (47–66% of control value; Supplementary Fig. S2). Interestingly, silencing of RhoA, a common regulator of the three DRF proteins also known to be necessary for gelatin degradation (12), phenocopied the effect of MT1-MMP and DRF depletion (Fig. 3B–D). Together, these data indicate that, similar to the effect observed on two-dimensional gelatin (Fig. 1), RhoA and each one of the DRF family members are required for invasion through Matrigel.

Invadopodia-like membrane protrusions are present during invasion of three-dimensional BM. Whether invadopodia are present in tumor cells invading three-dimensional ECM and share characteristics with those defined on a two-dimensional rigid matrix are critical issues still awaiting more detailed analysis (7, 16, 20). As an attempt to characterize mechanisms underlying three-dimensional matrix invasion and, in particular, requirement of DRFs, MDA-MB-231 cells either mock-treated or treated with siRNAs specific for DRF proteins were plated on Matrigel for 6 hours and the distribution of key invadopodial markers, i.e., F-actin, cortactin, and pY, was analyzed by indirect immunofluorescence and three-dimensional microscopy.

MDA-MB-231 cells labeled for F-actin and cortactin displayed the rounded morphology typical of invading cells in Matrigel (Supplementary Video S5). Focusing at the invasive surface of cells in contact with the matrix revealed a higher number of F-actin-rich protrusions compared with invadopodia in cells grown on two-dimensional gelatin (55.4 ± 7.0 protrusions per cell, n = 35 cells; see Fig. 4A; Supplementary Video S5). These structures were positive for cortactin and pY, with visible enrichment of both markers at the basis of the protrusions (Fig. 4B). In addition, examination of SEM micrographs of partially invading MDA-MB-231 cells revealed the presence of thin filopodia-like protrusions at the free dorsal side of cells partially embedded in Matrigel.

Figure 3. Depletion of RhoA and DRF impairs cell invasion through Matrigel. A–C, scanning electron micrographs of MDA-MB-231 cells invading a thick layer of Matrigel. A, mock MDA-MB-231 cells were plated on top of Matrigel and fixed at the indicated time points. Arrowheads point to the limit between cells and the matrix. B, MDA-MB-231 cells treated with the indicated siRNA were fixed after 4 h on Matrigel. C, low-magnification micrographs of siRNA-treated cells fixed 14 h after plating. Mock-treated cells display a rounded morphology and are partially or completely embedded within the matrix (arrow), whereas cells depleted for MT1-MMP, RhoA, or DRF proteins are spread and often form chains at the surface of Matrigel (arrowheads). Scale bar, 5 μm (A and B) and 100 μm (C). D, quantification of cell invasion through Matrigel from low-magnification SEM micrographs. Columns, mean invasion setting mock to 100%; bars, SE. *, all siRNA-treated cell populations are significantly different compared with mock-treated cells (see Supplementary Table S5).
(Fig. 5A and Supplementary Fig. S3). Some of these structures breaching through Matrigel likely represent proteolytically active structures. Consistent with this assumption, a fusion of MT1-MMP with pHluorin, a pH-sensitive GFP-emitting fluorescence only in the external milieu, colocalized with F-actin–positive protrusions labeled with mCh-Lifeact at the surface of MDA-MB-231 cells embedded in Matrigel (Supplementary Video S6).

The invasive interface of MDA-MB-231 cells with Matrigel was analyzed by transmission electron microscopy (TEM) on cross-sections perpendicular to the matrix. At an early time point (5 hours), cells that partially entered the matrix presented numerous short protrusions heterogeneous both in length and diameter (Fig. 5B). Furthermore, protrusions visible at the cell–matrix interface extending into Matrigel (Fig. 5B) were often surrounded by an electroluscent zone devoid of Matrigel, probably as a result of matrix degradation (Fig. 5B). At higher magnification, electron-dense regions of the cytoplasm devoid of organelles were visible at the cell cortex in contact with Matrigel (Fig. 5B). This cortical material consisting of fibrous structures extending within the protrusions (see Fig. 5B) is likely to correspond to cortical actin filament bundles. Altogether, these findings indicate that invadopodia-like structures are present at the invasive surface of MDA-MB-231 cells, invading a three-dimensional reconstituted BM and likely represent the sites of matrix proteolysis.

**Figure 4.** MDA-MB-231 cells display invadopodia-like protrusions in Matrigel that require DRF proteins for their formation. MDA-MB-231 cells either mock-treated (A and B) or treated with siRNA specific for DRF3 (C and D) were added on top of Matrigel, fixed after 6 h, and labeled for F-actin and cortactin or pY as indicated. Pictures are three-dimensional reconstructions from several planes corresponding to the ventral half of cells in contact with the matrix. Enlargements of boxed regions in A and C are shown in B (inset 1) and D (inset 3), respectively. For F-actin/pY double-labeling, only enlarged insets are shown (B, inset 2 and D, inset 4), corresponding to full-size pictures in Supplementary Fig. S4A (box 2) and B (box 4). In overlaid images, F-actin is pseudocolored in green and cortactin or pY is in red. Arrowheads in insets point to F-actin–rich invadopodia-like protrusions at the surface of mock-treated cells with their base enriched for cortactin (B, inset 1) or pY (B, inset 2). Arrows in D point to cytoplasmic aggregates of cortactin (inset 3) or pY (inset 4), with weak or no F-actin enrichment in cells silenced for DRF3. Scale bar, 10 μm (A and C) and 2 μm (B and D).
Three-dimensional invadopodia require DRFs. Cells depleted for each one of the DRF proteins remained elongated on Matrigel with some sparsely distributed F-actin and cortactin–positive or pY-positive protrusions at their surface (Fig. 4C and D and Supplementary Figs. S4B and S5B–D; Supplementary Video S7), corroborating results of SEM (Fig. 3). Numerous bright cortactin aggregates with little or no F-actin were observed in DRF-depleted cells. These seemed to be cytoplasmic on three-dimensional reconstruction of Z-stack of images (Supplementary Video S7; Fig. 4D, inset 3 and Supplementary Fig. S4B, inset 4). Similar observations were made in MDA-MB-231 cells silenced for RhoA or MT1-MMP (Supplementary Figs. S4C and S5A). In addition, the flat morphology of DRF3-depleted cells was also clearly visible on TEM analysis of cross-sections (Fig. 5C). More strikingly, although these cells developed some microvilli-like extensions on their free dorsal surface, they were almost devoid of membrane protrusions at the interface with Matrigel (Fig. 5C). DRF3 knocked-down cells remained closely apposed to the matrix, this being indicative of the absence of Matrigel proteolysis and remodeling, and a dense cortex was hardly visible underneath the plasma membrane (Fig. 5C). Therefore, as shown previously for two-dimensional invadopodia, three-dimensional invadopodia did not form in the absence of DRF1 to DRF3, further establishing the critical role of DRFs for invadopodia formation.
Discussion

We investigated the contributions of three members of the DRF family (DRF1–DRF3) to the mechanism of invadopodia formation and invasion of human breast cancer cells. Each of the three DRF proteins is required for the formation and activity of invadopodia when MDA-MB-231 cells are plated on a two-dimensional matrix. Similarly, DRF1 to DRF3 are required for invasion of breast tumor cells through a thick three-dimensional layer of Matrigel with a composition resembling BM matrix. Remodeling of the three-dimensional matrix occurs at the level of submicrometric finger-like actin-based protrusions possessing a composition similar to invadopodia, i.e., enriched in F-actin, cortactin, pY, and MT1-MMP, and depending on DRFs for their formation. Hence, our work adds DRFs to the list of proteins required for invasion by tumor cells and brings new insight into the mechanism of invasion in a three-dimensional matrix environment.

Invadopodia are viewed as dynamic filopodia-like extensions of the plasma membrane, wherein signaling components and cellular machineries involved in actin-driven membrane protrusion and exocytosis cooperate for delivering and concentrating active MMPs at sites of matrix degradation (10–13, 16, 20, 33, 34). The present data clearly implicate DRF proteins in the regulation of actin assembly during invadopodia formation as silencing of each DRF led to (a) a drastic reduction in cells with F-actin/cortactin–rich invadopodia in two-dimensional and three-dimensional matrices and (b) decreased matrix degradation and invasion capacity. Of note, no additional effect of knocking down simultaneously two DRF proteins was observed. One possibility is that DRF proteins may be functionally linked in the mechanism of invadopodia formation, which is supported by the observation that DRFs can form heterodimers (35, 36). Alternatively, each formin may be individually required for the induction of actin nucleation at invadopodia or for other individual roles. In this respect, DRF proteins are implicated in various cellular functions, including regulation of endosome motility, which could contribute to the delivery of MT1-MMP at invadopodia (13, 17). We did not find colocalization of GFP-mDia2 with endocytic markers at invadopodia. However, some association of mDia2 with transferrin-positive early endosomes was visible (not shown). Therefore, a role for DRF in membrane trafficking events related to invadopodia function cannot be excluded (37, 38). In addition, the known function of DRF proteins in the regulation of microtubule stability may be potentially relevant for the mechanism of invadopodia formation (37–39).

A signaling cascade based on N-WASP/Arp2/3 complex activation downstream of the Rho-GTP-binding protein Cdc42 is required for actin assembly during invadopodia formation in metastatic rat mammary adenocarcinoma cells (15). We confirmed that the Arp2/3 complex is required for invadopodia formation in two-dimensional and three-dimensional matrices in MDA-MB-231 cells.7 The Arp2/3 complex nucleates actin filaments and forms branched dendritic filament arrays, whereas formins produce unbranched actin filaments (22). Therefore, two types of actin-nucleating machineries cooperate during invadopodia formation in invasive cells. Using a FRET biosensor, N-WASP activation was visualized at the base of invadopodia, suggesting that Arp2/3–mediated actin nucleation is constrained to the base of invadopodia (14). In addition, the F-actin binding protein cortactin, which is recruited early at invadopodia concomitantly with F-actin assembly, is also essential for invadopodia formation and may act by stabilizing newly formed branches within the dendritic filament network (10, 16–18). Noticeably, cortactin and pY are enriched at the base of invadopodia that protrude from the invasive surface of MDA-MB-231 cells in a thick layer of Matrigel (see Fig. 4). Cells silenced for the Arp2/3 complex (15), cortactin (10, 17, 18), or DRFs are similarly impaired for invadopodia formation, indicating that both pathways of actin nucleation are required for invadopodia formation and cannot compensate for each other. Convergent extension of filopodia from an Arp2/3 complex–induced lamellipodial actin meshwork has been proposed as a mechanism for filopodia emergence (40), although this model is debated (41). In addition, transition from Arp2/3 to formin-mediated actin assembly may occur during actin dynamics associated with integrin-based adhesion sites (42). Invadopodia, which are enriched in adhesion proteins, includingintegrins and focal adhesion components, also correspond to cell-matrix adhesion sites (16, 43). Therefore, it is plausible that DRFs take over from N-WASP/Arp2/3/cortactin dendritic array at the base of invadopodia and elongate actin filaments into an invadopodial protrusion. DRF3/mDia2 and DRF1/mDia1 localize to filopodial tips and are involved in actin filament elongation during filopodia and membrane protrusion formation in mammalian cells including invasive tumor cells (23, 25, 27, 29, 44). In accordance, we observed endogenous DRF1 and mDia2-GFP at invadopodia in MDA-MB-231 cells. Although more work will be necessary to understand the cooperation between Arp2/3 complex and DRFs and unravel the ultrastructural architecture of invadopodial organization, the present study clearly identifies DRFs as important components involved in breast cancer cell invasion.

Rho GTP-binding proteins act as regulators of actin organization and membrane trafficking events in cells under physiologic conditions and have also been shown to contribute to various aspects of tumorigenesis including invasion of carcinoma cells (45). Several groups, including ours, found that Rho proteins control the formation of invadopodia in tumor-derived cell lines of diverse origins (15, 19). In particular, we recently reported that silencing of RhoA or Cdc42 abolishes invadopodia formation and matrix degradation by MDA-MB-231 cells cultured on a two-dimensional matrix (12). DRF proteins are downstream effectors of Rho GTPases (22). Our finding that RhoA and Cdc42 are required for invadopodia formation in both two-dimensional and three-dimensional matrices (ref. 12, and this study), suggests that beside the aforementioned regulation of Arp2/3 complex by Cdc42 (15), Cdc42/RhoA GTPases may regulate the function of DRFs in actin polymerization at invadopodia in invasive MDA-MB-231 cells. Along the same line, the formation of cellular protrusions associated with migration of rat mammary carcinoma cells involves a RhoA/DRF1 pathway acting in a coordinated network together with WASP family proteins and Arp2/3 complex (29). Interestingly, invasion of MDA-MB-435 human cancer cells in Matrigel is dependent on a RhoA/DRF1 pathway, but not DRF2 (DRF3 was not tested; ref. 28). The reason for this discrepancy with our findings is unclear and may involve the different origin of these two cell lines (30). It is worth noticing that, at a mechanistic level, MDA-MB-435 cells in Matrigel use a bleb-associated mode of invasion and lose the ability to form membrane blebbing when silenced for DRF1 (28). In contrast, invasion of MDA-MB-231 cells involves filopodia-like membrane protrusions that are enriched for MT1-MMP (see Supplementary Video S6) and require RhoA and DRFs for their formation.
In conclusion, this study highlights structural composition and mechanistic similarities between classic invadopodia, degradative structures of invasive cells cultured on two-dimensional matrix, and filopodial-like protrusions forming at sites of matrix degradation at the surface of breast tumor cells invading through three-dimensional Matrigel. It also reveals a new role for DRF proteins as essential components of the invasive machinery of metastatic cells through the regulation of actin assembly underlying the mechanism of invadopodia formation. Along this line, it is quite remarkable that analysis of gene expression array data (46) revealed a significant 2-fold to 2.5-fold overexpression of DRF2-encoding and DRF3-encoding transcripts in highly invasive basa-like breast tumors compared with normal human breast tissues (see Supplementary Materials). Understanding whether and how the multiple activities of DRF proteins contribute to the acquisition of specific invadopodial functions and to the invasive phenotype of cancer cells will be a challenge for future studies.

References


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