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Desmosome dynamics in migrating epithelial cells requires the actin cytoskeleton

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Abstract

Re-modeling of epithelial tissues requires that the cells in the tissue rearrange their adhesive contacts in order to allow cells to migrate relative to neighboring cells. Desmosomes are prominent adhesive structures found in a variety of epithelial tissues that are believed to inhibit cell migration and invasion. Mechanisms regulating desmosome assembly and stability in migrating cells are largely unknown. In this study we established a cell culture model to examine the fate of desmosomal components during scratch wound migration. Desmosomes are rapidly assembled between epithelial cells at the lateral edges of migrating cells and structures are transported in a retrograde fashion while the structures become larger and mature. Desmosome assembly and dynamics in this system are dependent on the actin cytoskeleton prior to being associated with the keratin intermediate filament cytoskeleton. These studies extend our understanding of desmosome assembly and provide a system to examine desmosome assembly and dynamics during epithelial cell migration.

Keywords

desmosome; desmocollin; plakophilin-3; actin; live cell microscopy; motility

Introduction

Cells in an epithelial tissue are anchored to neighboring cells by a number of distinct adhering type cell junctions. Generally, adhering junctions are comprised of a transmembrane component and a variety of cytoplasmic adapter proteins that in turn link cytoskeletal structures to sites of cell-cell contact (reviewed in [1]). Adhesive junction assembly and stability are tightly controlled processes and there must be mechanisms to allow cells to move with respect to their neighbors during normal physiologic processes (i.e. during normal development and wound healing). During tumorigenesis, cancer cells display altered adhesive characteristics and acquire the ability to detach from neighboring cells and migrate away from their normal niche. Alterations in cytoskeletal architecture and junction assembly often accompany increased motility and invasiveness of tumor cells and are believed to be a driving force in this process [2].

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Desmosomes are cadherin based cell-cell junctions present in a variety of epithelial tissues [3, 4]. These junctions are particularly prominent in stratified squamous epithelial tissues such as the skin and oral mucosa [5]. The desmosomal cadherins (desmogleins and desmocollins) are the transmembrane components of the desmosome and interact extracellularly to mediate cell-cell adhesion (reviewed in [4]). The cytoplasmic domain of the desmosomal cadherins interacts with the desmosomal plaque proteins that in turn recruit the keratin intermediate filament cytoskeleton to sites of cell-cell contact. Desmosomal plaque proteins include the armadillo repeat containing proteins plakoglobin and plakophilins as well as plakin family members such as desmoplakin. Together, the plaque proteins cluster the desmosomal cadherins in the plane of the plasma membrane and link the keratin intermediate filament cytoskeleton to the transmembrane cadherins [6–8]. Individual desmosomal components display remarkable stability in cultured cells and desmosomal junctions are maintained throughout the cell cycle [9]. Assembly of desmosomes is believed to provide many epithelial tissues the ability to withstand a variety of mechanical stresses. Additionally, a variety of carcinomas display a reduced number of desmosomes and decreased desmosome assembly in cultured cells compared to normal cells and decreased desmosome assembly correlates with increased motility [10–14].

While desmosomes provide robust linkage of intermediate filament cytoskeletal elements throughout an epithelial tissue, desmosome assembly and disassembly must be tightly regulated under conditions of controlled epithelial cell migration. For example, during normal wound healing keratinocytes must migrate in a controlled fashion to re-establish the epidermis. Conversely, a hallmark of tumor progression is the alteration of cell adhesion and acquisition of a migratory phenotype. In this study we sought to examine the fate and dynamics of desmosomal components in migrating epithelial cells. Scratch wounds of confluent oral squamous cell carcinoma cells were used as a model for migration and the subcellular localization of newly formed desmosomes as well as the fate of existing desmosomal components was observed in real time. This model system provided the opportunity to observe assembly of desmosomes in the migrating monolayer of cells and to establish the role of the actin cytoskeleton in desmosome dynamics in migrating cells.

Materials and Methods

Cells

UM-SCC-1 cells were a gift from Dr. Thomas Carey (University of Michigan, Ann Arbor, MI.) and were maintained in MEM medium containing 10% fetal bovine serum (Hyclone Laboratories, Logan, UT.). A431 cells were obtained from ATCC and HaCat keratinocytes were a gift from Dr. Pamela Jensen. Both cell lines were maintained in DMEM medium containing 10% fetal bovine serum (Hyclone Laboratories). Cells retrovirally expressing desmocollin 2/GFP and plakophilin-3/GFP were maintained in medium containing 400 µg/mL hygromycin or 1 µg/mL puromycin (Sigma Chemical Co., St. Louis, MO.) respectively. Nocodazole, bisindolylmaleimide I, cytochalasin D and blebbistatin were purchased from Sigma Chemical Co. (St. Louis, MO.)

Antibodies and Immunoblotting

Anti human desmocollin 2 (7G6), human desmoglein 2 (6D8), human desmoglein 3 (5H10), human plakoglobin (11E4) and human desmoplakin (20B6) have been previously described [15–17]. Anti plakophilin-3 (11F2) was generated as previously described [17, 18] using recombinantly expressed maltose binding protein fused to plakophilin-3 amino acids 1–307 as an antigen. Anti Green Fluorescent Protein (GFP) was purchased (Clontech, Mountain View, CA.). Immunoprecipitations, preparation of cell lysates and immunoblotting was performed as previously described [15, 16, 19].

Molecular Constructs

A cDNA encoding human desmocollin 2a was a gift from Dr. Kathleen Green (Northwestern University, Chicago, IL.). A cDNA encoding human plakophilin-3 (Genbank ID: BC000081) was purchased from Open Biosystems (Huntsville, AL.). Each cDNA was used as a target for PCR to remove the natural stop codon and introduce an appropriate restriction site to facilitate subcloning of the Dsc 2 and plakophilin-3 cDNAs upstream of GFP in pEGFP-N1 (Clontech, Mountainview, CA.). A fragment containing the entire open reading frame of Dsc2/GFP was subcloned into the retroviral expression vector LZRS-ms-hygro (for Dsc2/GFP) and LZRS-ms-neo (for pkp-3/GFP) [20]. Keratin 18/RFP was purchased (Evrogen, Moscow, Russia) and subcloned into LZRS-ms-puro using NheI and Not I. Retroviral particles were generated and stable cell lines were selected as previously described [21].

Microscopy

Live cell imaging was performed using a Marianas microscopy system controlled by SlideBook 5.0 image acquisition software (Intelligent Imaging Innovations Inc, Denver, CO.). For live cell imaging of migrating cells, cells expressing GFP fusion proteins were cultured on glass bottom 35 mm dishes (FluoroDish, World Precision Instruments Inc., Sarasota, FL.) and were allowed to reach 75–80% confluence. Cell cultures were scratched using a 200 μ L pipet tip and cultures were allowed to recover and begin migrating for 1.5–2 hours before imaging. Migrating cells were imaged using a Plan-NEOFLUAR 40 $\times$ 1.3 n.a. objective at 3 minute intervals. At each image acquisition, a z-stack was collected consisting of approximately 18 images with a 0.33 μ m step size. Individual images were deconvolved using the nearest neighbor algorithm in SlideBook 5.0 and projection images were generated by collapsing the deconvolved z-stack images at each time point. Projection images were used to assemble the time lapse movies.

Results

We sought to examine the dynamics of desmosomal junctions in migrating epithelial cells by live cell microscopy. To achieve this, we constructed a cDNA encoding desmocollin 2a/GFP (Dsc2/GFP) fusion protein in which GFP was fused to the carboxyl terminus of full-length human desmocollin 2a (Fig. 1A). The Dsc2/GFP fusion protein was retrovirally expressed in UM-SCC-1 cells and a stable population of Dsc2/GFP cells was isolated. UM-SCC-1 cells are an oral squamous cell carcinoma cell line that assembles endogenous desmosomes [22]. These cells express desmoglein 2 and 3, desmocollin 2 as well as all three isoforms of plakophilin ([14, 16] and data not shown). In addition to assembling endogenous desmosomes, UM-SCC-1 cells are relatively flat and are ideal for live cell imaging studies. In monolayer culture, the Dsc2/GFP fusion protein localized efficiently to sites of cell-cell contact in a punctate localization pattern characteristic of other desmosomal components (Fig. 1B–G). As expected, Dsc2/GFP co-localized with other desmosomal components (desmoglein 2 and desmoplakin) endogenously expressed in UM-SCC-1 cells.

Immunoblot analysis of cell lysates generated from UM-SCC-1 cells expressing Dsc2/GFP revealed a single band at approximately 150kDa using an antibody specific for GFP, the expected molecular weight of the Dsc2/GFP fusion protein. Additionally, this band was recognized by antibodies specific for desmocollin 2 (Fig. 1H). Expression of Dsc2/GFP resulted in a small reduction in expression of endogenous desmocollin2 (Fig. 1H). Triton X-100 soluble and insoluble fractions were prepared and these fractions were examined for the presence of Dsc2/GFP (Fig 1I). Similar to endogenous desmocollin 2, the Dsc2/GFP fusion protein was incorporated into the Triton X-100 insoluble fraction and approximately one-third of the total protein was present in a Triton X-100 soluble pool [8].

Immunoprecipitations were performed from lysates prepared in buffer containing TritonX-100 to verify that Dsc2/GFP associated with the desmosomal plaque protein, plakoglobin [15, 23, 24]. Anti plakoglobin antibodies were used to co-immunoprecipitate endogenous desmocollin 2 as well as retrovirally expressed Dsc2/GFP (Fig. 1J). Taken together these data suggest that Dsc2/GFP is efficiently expressed, associates with the desmosomal plaque protein plakoglobin and behaves in a manner similar to endogenous desmocollin 2 in UM-SCC-1 cells making this fusion protein a useful marker for desmosome assembly in living cells.

Desmocollin 2/GFP dynamics in migrating cells

In order to observe desmosome dynamics in migrating cells we chose to examine junction assembly in a scratch wound assay model. Cells were plated and allowed to reach 75–80% confluence before a pipette tip was used to make a scratch of the monolayer. The detached cells were washed away and the remaining cells were given fresh medium and allowed to recover for at least 1.5 hours before observation.

UM-SCC-1 cells were observed migrating into the denuded area as a monolayer in which cells maintained contact with their neighbors as they migrated to fill the wound. Dsc2/GFP localization and dynamics were observed in cells migrating to fill the wound (movies 1 and 2). The fusion protein was observed to accumulate in punctate structures between cells near the leading edge of the migrating monolayer. These punctate structures increased in size and fluorescence intensity as they migrated away from the leading edge of the migrating cells (Figure 2 and movies 1 and 2). In contrast, Dsc2/GFP present at the rear of the migrating cells, as well as in non-migrating cells, was present in what appear to be stable, non-motile desmosomes. This pool of desmosomes appeared quite stable and did not change significantly over the course of our observations. This stable appearance of Dsc2/GFP in desmosomes has been previously observed in epithelial cells [9] however the dynamic organization of desmosomes in migrating cells has not been reported. Additionally, we generated A431 and HaCat cells expressing Dsc2/GFP and compared the dynamics of Dsc2/GFP in these cell lines (movies 3 and 4). The assembly of punctate structures between cells near the leading edge of migrating cells was largely identical to that seen in UM-SCC-1 cells and demonstrates that the dynamics observed in UM-SCC-1 cells is not specific to this cell line but represents desmosome assembly in other migrating squamous epithelial cells.

The Dsc2/GFP structures appear to flow away from the leading edge of the migrating cells until they reach a position roughly in line with the nucleus at which point the rate of flow slows and the Dsc2/GFP structures appear to become less mobile. We termed this dynamic motion “retrograde streaming”. We calculated the migration of the Dsc2/GFP during streaming in UM-SCC-1 cells to be $0.373 \mu\text{m}/\text{min}$ ($\pm 0.074 \mu\text{m}/\text{min}$). At the rear of the cell there is little evidence of Dsc2/GFP structures being internalized or removed from the cell periphery and it is unclear how the cell balances the flow of Dsc2/GFP away from the leading edge with the accumulation of desmosomal components present at the rear of the migrating cells. Clearly there is a balance of desmosome assembly at the leading edge and desmosome turnover elsewhere in the migrating cells. Interestingly we rarely observed Dsc2/GFP endocytosis or movement of the fusion protein away from the plasma membrane suggesting that the degradative process may be more subtle than the assembly process occurring at cell borders in the migrating cells.

Co-localization of desmosomal components in migrating cells

The retrograde streaming Dsc2/GFP structures also contain other known desmosomal components such as desmoplakin and desmoglein 3 (Figure 3). In addition, expression of plakophilin-3/GFP fusion protein in SCC-1 cells displayed similar dynamics in migrating

cells to that seen for Dsc2/GFP (movie 5). In addition to cell border localization we also observed additional Pkp-3/GFP present in the cytoplasm of UM-SCC-1 cells. This observation is consistent with previously published immunofluorescence staining with anti plakophilin-3 antibodies ([22, 25, 26] and data not shown). Rather than plakophilin-3-containing particles being added from pre-formed cytoplasmic particles to established junctions at cell-cell borders, it appears the plakophilin-3/GFP monomers are assembled into junctions at the leading edge in migrating cells. Previously it has been demonstrated that particles containing desmoplakin and plakophilin-2 assemble in the cytosol of A431 cells and are actively transported to the cell periphery [27, 28]. Interestingly, we did not observe plakophilin-3/GFP particles migrating to the cell periphery as was previously described for plakophilin-2.

To examine the attachment of the keratin intermediate filament cytoskeleton to the streaming desmosomes, we co-expressed keratin 18/RFP in cells expressing Dsc2/GFP (Figure 4). In monolayer culture keratin 18/RFP was observed to label the endogenous keratin network and demonstrate keratin attachment at desmosomes at cell-cell borders (Figure 4B–F). Interestingly, very little keratin 18/RFP is observed to be co-localized with the streaming desmosomal components in migrating cells. This data suggests that desmosomal structures can assemble into punctate structures at the lateral edges of migrating cells but linkage to the keratin cytoskeleton likely occurs some distance from the leading edge in migrating cells. In contrast, the actin cytoskeleton was assembled at the leading edge of migrating cells (Figure 5) and was present at sites of cell-cell contact co-localized with the Dsc2/GFP streaming structures. Thus actin filaments are a good candidate for mediating the retrograde streaming of Dsc2/GFP puncta observed in migrating cells.

Retrograde streaming of the desmosomal components is actin dependent

We tested the requirement of the actin cytoskeleton in desmosome retrograde streaming by treating wounded cell cultures with agents to disrupt the actin cytoskeleton. Treatment with cytochalasin D resulted in rapid disruption of the actin cytoskeletal network and entirely prevented Dsc2/GFP retrograde streaming (movie 6). Prolonged incubation (greater than one hour) resulted in partial dissolution of cell adhesion in SCC-1 cells and is likely due to the global disruption of cytoskeletal systems (data not shown). To address more subtle involvement of the actin cytoskeleton, we treated migrating cells with the non-muscle myosin inhibitor blebbistatin [29, 30]. Disruption of actin-generated force by blebbistatin resulted in decreased Dsc2/GFP retrograde streaming without disruption of established punctate localization of Dsc2/GFP (movie 7) and Pkp-3/GFP (data not shown). Although these structures did not increase in size, no disassembly of the structures was detected over the course of our observations. Conversely, disruption of microtubules by nocodazole treatment resulted in subtle morphological changes in migrating cells. Cells became somewhat rounded at the leading edge, but the migrating edge was largely unaffected. Interestingly, Dsc2/GFP assembly of puncta and retrograde streaming of desmosomal structures was still observed in nocodazole-treated cells, although the velocity of retrograde streaming was reduced ($0.171 \mu\text{m}/\text{min} \pm 0.016 \mu\text{m}/\text{min}$) (movie 8). Together these data suggest that the actin cytoskeleton plays an important role in assembly and retrograde streaming of desmosomal components while microtubules play a lesser role in Dsc2/GFP dynamics.

Previous studies have suggested that protein kinase C isoforms are involved in desmosome assembly at cell borders [28] and were shown to promote desmosome assembly in colon carcinoma cells [31]. Therefore we examined the effect of PKC activation and inhibition on Dsc2/GFP retrograde streaming in migrating cells. Activation of PKC by treatment with TPA did not affect the retrograde streaming velocity of Dsc2/GFP (data not shown). However, incubation of migrating cells with the protein kinase C inhibitor

bisindolylmaleimide I (1 μM) resulted in a pronounced decrease in desmosome assembly between cells at leading edge and in retrograde streaming (0.005 $\mu\text{m}/\text{min}$ $\pm$ 0.001 $\mu\text{m}/\text{min}$). Stability of the punctate structures was not affected since Dsc2/GFP was still localized to punctate structures following bisindolylmaleimide I treatment (movie 9), suggesting that PKC activity is required for continued desmosome assembly at the leading edge of migrating cells. Immunofluorescence microscopy revealed that PKC inhibition led to disruption of the actin cytoskeleton in migrating cells (Figure 6). These data raise the possibility that PKC inhibition results in decreased retrograde streaming of desmosome puncta may be due to effects on the cytoskeleton rather than on recruitment of desmosomal components to the plasma membrane. Together these data suggest that desmosomal complexes assembled at the leading edge of migrating cells are dependent on the actin cytoskeleton as an early event. Subsequent linkage to the keratin cytoskeleton and decreased motility in the membrane occurs some distance away from the leading edge of migrating cells.

Discussion

In this study we describe a system to examine the assembly and the fate of desmosome components in migrating epithelial cells using live cell microscopy. GFP fusion constructs were prepared using desmocollin 2a and plakophilin-3 as fusion partners. Each fusion protein behaved similarly with respect to junction assembly and trafficking in migrating cells. Previous studies examined the assembly of the desmosomes in monolayer cultures and demonstrated that desmosomes are remarkably stable structures [9, 32, 33]. Interestingly, Windoffer et al. also observed rapid exchange of desmocollin protein within stable junctions by FRAP analysis suggesting that desmosomes are more dynamic structures than previously appreciated.

During the course of our study, we immediately recognized that assembly of desmosome structures primarily occurs at the lateral edges and near the leading edge of migrating cells at sites of cell-cell contact. Dsc2/GFP structures are observed to accumulate between cells and over time these structures migrate away from the leading edge. The accumulation of Dsc2/GFP into desmosomes appears to arise from fusion protein already at the cell surface rather than from a cytoplasmic store of preformed vesicles. No vesicular trafficking of Dsc2/GFP was observed in migrating cells or in stationary cells in monolayer culture. The same was true for plakophilin-3/GFP. Although plakophilin-3/GFP was observed in the cytoplasm, we did not observe plakophilin-3 particles or vesicles trafficking specifically to site of new junction assembly in migrating cells. This is in contrast to the findings of Bass et al. in which desmoplakin/plakophilin-2/PKC α particles were observed trafficking to the plasma membrane [28]. A potential explanation for the differences in our imaging is that in addition to our exogenous GFP fusion proteins, SCC-1 cells express endogenous plakophilins-2 and -3. It is possible that different plakophilins contribute to desmosome assembly using different pools of components. Alternatively, A431 cells may assemble plakophilin-2 particles while plakophilin-3 in SCC-1 cells is mainly recruited from a monomer pool in the cytosol. Characterization of plakophilin cytoplasmic oligomeric complexes is currently under investigation in our laboratory. Alternatively, plakophilin/DP/PKC α particles may be smaller and are simply not visible in our cells. However we have also performed the migration assays with A431 cells and we were unable to image cytoplasmic particles using our microscopy system.

A striking observation made during these studies was the dynamic nature of Dsc2/GFP at the leading edge of migrating cells. Dsc2/GFP labeled structures rapidly assembled near the leading edge and become larger in size as they “matured” and were pulled away from the leading edge in migrating cells. Initially, the migration of the desmosome structures away

from the leading edge is more rapid than the rate of cell migration into the wounded area. At some distance away from the leading edge, the Dsc2/GFP structures slowed their migration and eventually accumulated with other individual structures near the middle of the cell. The initial decrease in migration appears to coincide with the attachment of keratin intermediate filament cytoskeleton (Figure 4). This is in contrast to the rapidly migrating structures near the leading edge where no keratin filaments were observed. At the leading edge of migrating cells the actin cytoskeleton is actively involved in driving cell migration into the wounded area and as such is a site of extensive cytoskeletal remodeling. Disruption of the actin cytoskeleton using cytochalasin D or blebbistatin completely prevented the assembly of new Dsc2/GFP structures as well as the migration of previously formed structures at the leading edge. These data clearly demonstrate the important role of the actin cytoskeleton in the assembly and maturation of the desmosome in this system.

Based on the evidence presented here we propose a model of desmosome assembly in which transmembrane desmosomal components are clustered at sites of cell-cell contact nearest the leading edge of migrating cells. Clustering of the transmembrane components is likely to occur by recruiting monomers present at the cell surface. Assembled puncta subsequently associate with the actin cytoskeleton, possibly through interactions involving plakophilin and desmoplakin, and undergo maturation to increase in size by adding more Dsc2/GFP. Through interactions with the actin cytoskeleton, desmosome structures are transported along the boundary with neighboring cells during the maturation process. At a distance away from the leading edge the clustered desmosomal components become associated with the keratin intermediate filament cytoskeleton and migration of the desmosome is slowed. To balance the assembly of new structures the cell must dispose of desmosomes through a degradative process. However, disruption and disposal of desmosome complexes was not observed in our experiments and may be a subtle event. Evidence from Windoffer et al. suggests that a non-junctional desmocollin is rapidly exchanging with the desmosomal desmocollin and decreased exchange over time may therefore lead to decreases in desmosome complexes.

Conclusions

Our observations allow us to propose a mechanism by which migrating cells assemble desmosomal structures and coordinate epithelial cell migration during events such as wound healing or tumor cell migration. Importantly, our model suggests that the actin cytoskeleton is a significant component of desmosome assembly and maturation. However the nature of the association with the actin cytoskeleton is unknown and will require additional investigation. These studies provide the basis for future studies examining the dynamics of desmosome assembly in migrating epithelial cells

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Desmocollin 2/GFP labels endogenous desmosomes in SCC-1 cells

A. Schematic diagram depicting desmocollin 2a fused to green fluorescent protein at the carboxyl terminus (Dsc2a/GFP). SCC-1 cells expressing Dsc2a/GFP immunostained with anti desmoglein 2 (panels B–D) and desmoplakin (panels E–G). Anti GFP and anti desmocollin were used to immunoblot cell lysates prepared from SCC-1 cells expressing Dsc2a/GFP (I). Triton x-100 soluble and insoluble fractions were prepared from SCC-1 cells and SCC-1 cells expressing Dsc2a/GFP and immunoblotted using anti desmocollin 2 (J). Plakoglobin was immunoprecipitated from SCC-1 and SCC-1 cells expressing Dsc2/GFP and co-immunoprecipitated desmocollin was detected by immunoblotting with anti desmocollin 2 (H).

Figure 2. Dsc2a/GFP dynamics in migrating SCC-1 cells

SCC-1 cells expressing Dsc2a/GFP are observed migrating to fill a scratch wound on a glass bottom dish. Dsc2a/GFP fusion protein can be observed assembling punctate structures between migrating cells at the leading edge (white arrow). Dsc2a/GFP positive structures increased in size as they migrate away from the migrating edge (yellow arrow). The rate of migration is greatest near the leading edge and decreases as structures reach the rear of the cell. Select panels were selected from movie 1.

Figure 3. Desmosomal components co-localize with Dsc2/GFP in retrograde streaming structures in migrating cells

Migrating SCC-1 cells expressing Dsc2a/GFP were immunostained using anti desmoplakin (panels A–C) and anti desmoglein 3 (panels D–F). Note the extensive co-localization of these endogenous desmosomal components with retrograde streaming Dsc2a/GFP.

Figure 4. Keratin does not co-localize with Dsc2a/GFP in migrating cells
SCC-1 cells expressing Dsc2a/GFP and keratin 18/RFP were observed migrating into a scratch (panels A–C) or in monolayer culture (panels D–F). Keratin 18/RFP did not co-localize with Dsc2a/GFP in migrating cells but was extensively co-localized with desmosomes in non-migrating cells.

Figure 5. Actin co-localizes with retrograde streaming Dsc2a/GFP in migrating cells
SCC-1 cells expressing Dsc2a/GFP (A) were fixed and the actin cytoskeleton was labeled using rhodamine phalloidin (B). Actin was observed to accumulate between migrating cells and co-localized with Dsc2a/GFP (C).

Figure 6.

Bisindolylmaleimide I (BIM) treatment results in disrupted actin polymerization in migrating cells. Migrating UM-SCC-1 cells expressing Dsc2/GFP were stained using rhodamine phalloidin (panels A–C) and the same migrating cells are shown after one hour of Bisindolylmaleimide I (BIM) treatment (panels D–F). Note the disruption of the actin array at the leading edge of migrating cells.