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D Brennan  
*Thomas Jefferson University*

S Peltonen  
*University of Turku and Turku University Hospital, Turku, Finland*

A Dowling  
*Thomas Jefferson University*

W Medhat  
*Thomas Jefferson University*

K J Green  
*Northwestern University, Feinberg School of Medicine*

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ORIGINAL ARTICLE

A role for caveolin-1 in desmoglein binding and desmosome dynamics

D Brennan¹, S Peltonen², A Dowling¹, W Medhat¹, KJ Green³, JK Wahl III⁴, F Del Galdo⁵ and MG Mahoney¹

¹Department of Dermatology and Cutaneous Biology, Thomas Jefferson University, Philadelphia, PA, USA; ²Department of Dermatology, University of Turku and Turku University Hospital, Turku, Finland; ³Departments of Pathology and Dermatology, Northwestern University, Feinberg School of Medicine, Chicago, IL, USA; ⁴Department of Oral Biology, University of Nebraska Medical Center, Lincoln, NE, USA and ⁵Scleroderma Research Centre, Leeds Institute of Molecular Medicine, LMBU, University of Leeds, Leeds, UK

Desmoglein-2 (Dsg2) is a desmosomal cadherin that is aberrantly expressed in human skin carcinomas. In addition to its well-known role in mediating intercellular desmosomal adhesion, Dsg2 regulates mitogenic signaling that may promote cancer development and progression. However, the mechanisms by which Dsg2 activates these signaling pathways and the relative contribution of its signaling and adhesion functions in tumor progression are poorly understood. In this study we show that Dsg2 associates with caveolin-1 (Cav-1), the major protein of specialized membrane microdomains called caveolae, which functions in both membrane protein turnover and intracellular signaling. Sequence analysis revealed that Dsg2 contains a putative Cav-1-binding motif. A permeable competing peptide resembling the Cav-1 scaffolding domain bound to Dsg2, disrupted normal Dsg2 staining and interfered with the integrity of epithelial sheets in vitro. Additionally, we observed that Dsg2 is proteolytically processed; resulting in a 95-kDa ectodomain shed product and a 65-kDa membrane-spanning fragment, the latter of which localizes to lipid rafts along with full-length Dsg2. Disruption of lipid rafts shifted Dsg2 to the non-raft fractions, leading to the accumulation of these proteins. Interestingly, Dsg2 proteolytic products are elevated in vivo in skin tumors from transgenic mice overexpressing Dsg2. Collectively, these data are consistent with the possibility that accumulation of truncated Dsg2 protein interferes with desmosome assembly and/or maintenance to disrupt cell–cell adhesion. Furthermore, the association of Dsg2 with Cav-1 may provide a mechanism for regulating mitogenic signaling and modulating the cell-surface presentation of an important adhesion molecule, both of which could contribute to malignant transformation and tumor progression.

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Introduction

Desmogleins are the major transmembrane proteins of the cell–cell adhesion structures known as desmosomes. There are four distinct desmoglein genes (Dsg1–4) in humans, which are expressed in a tissue-type and differentiation-specific manner (Garrod et al., 2002; Cheng and Koch, 2004; Mahoney et al., 2006). Studies involving Dsg2-null mice revealed that Dsg2 contributes to embryonic stem cell proliferation, particularly in the inner cell mass of the developing blastocyst (Eshkind et al., 2002). Dsg2 is aberrantly expressed in select epithelial malignancies, including squamous cell carcinomas (Harada et al., 1996; Kurzen et al., 2003; Biedermann et al., 2005). Similarly, genetic profiling of prostate cancer cell lines showed increased expression of Dsg2 in a metastatic cell line, as compared with its non-metastatic syngeneic precursor cell (Trojan et al., 2005). Dsg2 expression is also upregulated in squamous cell carcinoma cell lines in comparison with cultured keratinocytes (Schäffer et al., 1994; Harada et al., 1996; Denning et al., 1998). We recently showed that Dsg2 is highly expressed in malignant skin carcinomas, including squamous cell carcinomas, basal cell carcinomas, sweat and sebaceous gland carcinomas, and adenocarcinomas (Brennan and Mahoney, 2009). Collectively, these results support a role for Dsg2 in epithelial cell growth, survival and malignant transformation. However, the mechanisms by which Dsg2 activates these signaling pathways and promotes tumor formation are unknown.

Caveolins are a family of hairpin-like, palmitoylated, integral membrane proteins that oligomerize and bind to cholesterol and sphingolipids to form specialized areas of the membrane distinct from the clathrin-coated pits. The caveolins form flask-shaped invaginations of 50–100 nm in diameter called caveolae (Severs, 1988). There are three caveolin isoforms: Cav-1 (α and β), Cav-2 and Cav-3. Whereas Cav-1 and Cav-2 are ubiquitously expressed, Cav-3 expression is predominantly restricted to muscle cells (Scherer et al., 1995; Tang et al., 1996). Caveolins and caveolae have been implicated as regulators of key cellular functions, including cholesterol transport and homeostasis (Fielding and Fielding, 1995; Smart et al., 1996), endocytosis and endocytic vesicle trafficking (Schnitzer and Oh, 1996), and cell
adhesion and apoptosis (Lisanti et al., 1994; Okamoto, 1998; Okamoto et al., 1998; Shaul and Anderson, 1998; Kurzchalia and Parton, 1999). Specific cell signals can be also transmitted through a spatially controlled organization of cell receptors into the caveolae. Indeed, the epidermal growth factor receptor has been shown to stimulate the phosphorylation of Cav-1, thus enhancing the epidermal growth factor receptor has been shown to be also transmitted through a spatially controlled Kurzchalia and Parton, 1999). Specific cell signals can modulate Wnt/β-catenin/Lef-1 signaling by regulating the intracellular localization of β-catenin (Galbiati et al., 2000). Consistent with these findings, mounting evidence suggests that diseases associated with deregulated signaling pathways often result from aberrant expression or localization of Cav-1. In cancer, the role for Cav-1 is complex, as it serves both as a modulator of tumor suppression as well as oncogenesis. Mutations in the Cav-1 gene have been linked to human breast cancer, suggesting that loss of Cav-1 function has a significant role in tumor initiation (Chen et al., 2004). Mice devoid of Cav-1 develop mammary epithelial cell hyperplasia (Capozza et al., 2003) and are susceptible toward mammary tumorigenesis (Park et al., 2002). In the skin, Cav-1-null mice are also more susceptible to epidermal hyperplasia and skin tumor formation in response to carcinogens (Capozza et al., 2003).

In this report, we provide strong evidence that Dsg2 interacts directly with Cav-1, and that these interactions may impact Dsg2 recycling, desmosome dynamics and cell adhesion; and furthermore, provide a mechanism by which Dsg2 mediates cell signaling.

Results

Colocalization of Cav-1 and Dsg2

We recently showed that overexpression of Dsg2 in the epidermis results in hyper-proliferation and the formation of pre-cancerous papillomas; additionally, Dsg2 transgenic mice are more susceptible to chemical-induced skin carcinogenesis (Brennan et al., 2007). Furthermore, Dsg2 overexpression in the skin of these mice results in the activation of several signaling pathways directly relevant to epithelial cell proliferation and survival, notably the phosphatidylinositol-3-kinase/akt, MEK/mitogen-activated protein kinase and nuclear factor-kB pathways. We thus searched for Dsg2-binding partners that could interact with the intracellular domain, and potentially lead to the activation of downstream signaling events. We generated glutathione-S-transferase (GST) fusion recombinant proteins of the intracellular domains (cytoplasmic tails) of Dsg1 and Dsg2. The GST fusion proteins were affinity-purified by using glutathione–sepharose beads, and bound proteins were eluted with glutathione elution buffer according to the manufacturer’s protocol (Figure 1a). To confirm the identity of the fusion proteins, we performed western blotting analysis by using the antibodies H-145 and DG3.10 (Figure 1b). We note here that there are two commercially available H-145 antibodies: one recognizes Dsg2 whereas the other, Dsg3. Throughout this report, we used the Dsg2 H-145. The antibody DG3.10 recognizes both Dsg1 and Dsg2. Immunoblot analysis showed that H-145 recognized only the GST-Dsg2.tail, whereas DG3.10 detected both GST-Dsg1.tail and the GST-Dsg2.tail.

The GST fusion proteins were used to affinity-purify proteins from A431 cell lysates. By western blot analysis, we first demonstrated that the desmosomal protein γ-catenin (plakoglobin), but not the adherens junction protein β-catenin, was able to bind to Dsg1 and Dsg2 (Figure 1c). It was demonstrated previously that Dsg2 localizes to lipid rafts (Delva et al., 2008) and here we identified Cav-1 as one of several novel Dsg2-binding proteins. Western blotting of cellular proteins eluted from the columns demonstrated that Cav-1 (22 kDa) interacted with Dsg2 and Dsg1, but not GST alone (Figure 1d).

Next, we performed immunoprecipitation assays to confirm the Dsg2–Cav-1 interaction. A431 cells were extracted into detergent-free and Triton X-100 (Tx)-containing protein fractions, incubated with antibody 10D2 (Keim et al., 2008) and the Dsg2 immunocomplexes were pulled down with Protein-A/G. The precipitated products were immunoblotted with H-145, to confirm the pull-down of the 160-kDa Dsg2 in the Tx fraction (Figure 1e). Cav-1 was detected in both detergent-free and Tx-containing fractions. However, the detergent-free fraction showed higher levels of Cav-1, suggesting perhaps that, whereas Cav-1 associated with both desmosome-bound and desmosome-free Dsg2, it was more likely to associate with Dsg2 outside of the desmosomal structure, that is, in lipid rafts. In summary, these results demonstrate that full-length Dsg2 binds to Cav-1.

To confirm Dsg2–Cav-1 colocalization at the cell level, we performed double-labeled immunofluorescence (IF) and laser-scanning confocal microscopy for Dsg2 (green) and Cav-1 (red) in A431 cells (Figure 2). We did not expect to see extensive colocalization of Dsg2 and Cav-1 as desmogleins are predominantly found in desmosomes and Cav-1 in lipid rafts. Indeed, we observed the hallmark punctate cell–cell border pattern for Dsg2, whereas Cav-1 had a diffuse cytoplasmic and cell-surface staining pattern. However, consistent with our immunoprecipitation results above, we did observe some colocalization of Dsg2 with Cav-1 at the membrane.

Next, we wanted to determine whether Cav-1 and desmogleins are expressed in similar cell compartments in normal human epidermis. We performed colocalization experiments for Cav-1 and Dsg1/Dsg2 (Supplementary Figure S1). We observed pronounced Cav-1 staining at the cell–cell borders, as well as in the...
Immediate suprabasal layers, which is in accordance with the immunoprecipitation for Dsg2 (antibody 10D2). The precipitated proteins were extracted from A431 cells and subjected to immunoblotting for Dsg2 (Ab H-145), Cav-1 and β-catenin. (c) Immunoprecipitation assay further confirms that Dsg2 binds to Cav-1. Tx-soluble (S) and -insoluble (I) proteins were extracted from A431 cells, and subjected to immunoprecipitation for Dsg2 (antibody 10D2). The precipitated products were immunoblotted for Dsg2 (Ab H-145), Cav-1 and mouse IgG (equal antibody loading). The panels to the right are overexposed. I, Tx-insoluble; S, Tx-soluble; Tx, Triton X-100.

cyttoplasm of keratinocytes in the basal and the immediate suprabasal layers, which is in accordance with the literature (Gassmann and Werner, 2000). In the same epidermal layers, we observed strong staining of Dsg1 and Dsg2 using antibody DG3.10. Merged image showed some colocalization, particularly at the cell–cell borders (arrows demarcate colocalization). These results demonstrate that, in human skin, Cav-1 is expressed in the basal and most immediate suprabasal epidermal layers where desmogleins could be found. Thus, consistent with our immunoprecipitation results above, we observed colocalization of desmogleins with Cav-1 primarily at the plasma membrane. In the hair follicle, where both Dsg2 and Cav-1 expression levels were high, we observed strong staining of both Dsg2 and Cav-1 in cells of the outer root sheath (not shown).

Dsg2 interacts with Cav-1 through the Dsg2 consensus binding motif and the Cav-1 scaffolding domain

Cav-1’s association with many protein-binding partners is mediated by a conserved 20-amino-acid domain called the caveolin-scaffolding domain, located between Asp82 and Arg101 (DGWIKASFTTFVKYWFYR) of Cav-1. This conserved domain binds to several consensus-binding motifs (φXφXXXφ, φXXXφXXφ and φXφXXXφXXφ; where φ are aromatic amino acids phenylalanine F, tyrosine Y and tryptophan W) present on signaling molecules (Lisanti et al., 1994; Okamoto et al., 1998). In some cases, hydrophobic amino acids are found in the place of aromatic amino acids, and the binding motifs may also be reversed in orientation (φXXXXφXφ, φXXXXφXXφ or φXXφXXXφXφ). Examination of the Dsg2 amino-acid sequence revealed a potential Cav-1-binding motif (776FTDKAASY783) in the cytoplasmic tail domain (Mahoney et al., 2002; Whittock, 2003) (Figure 3a). To demonstrate that Dsg2 can associate with the Cav-1 scaffolding domain, we generated a fusion peptide consisting of the Drosophila antennapedia (AP, RQPKIWFPNRRKPWWK) homeodomain, and a putative competing peptide resembling the scaffolding domain of Cav-1. The AP and AP-Cav-1 peptides are cell-permeable and are biotinylated at the N-terminus (Figure 3b). The bioactive Cav-1 fragment, AP–Cav-1, was previously used to restore Cav-1 bioavailability and abrogate transforming growth factor-β activation of cultured human dermal fibroblasts (Del Galdo et al., 2008). For our study, we reasoned that the peptide would disrupt the binding of Dsg2 with Cav-1. Thus, we treated A431 cells with either AP or AP-Cav-1 (5 μM) in serum-free medium for 1 h, and lysed in a 1% Tx lysis buffer. Cell lysates were incubated with the anti-Dsg2 antibody 10D2 and precipitated with Protein-A/G–agarose. The precipitated proteins were resolved by sodium dodecyl sulfate (SDS)–PAGE and immunoblotted using strep-avidin–horseradish peroxidase (HRP) (Figure 3c). The results showed that anti-Dsg2 pulled down biotinylated AP–Cav-1, but not biotinylated AP alone. These results demonstrate that Dsg2 associates with Cav-1, most likely through the Cav-1 scaffolding domain. To further assess whether Cav-1 has a role in maintaining desmosome dynamics, we treated A431

**Figure 1** Cav-1 is a binding partner of desmogleins. (a) Coomassie staining of purified GST and GST fusion proteins with intracellular domains of Dsg1 (GST–Dsg1) or Dsg2 (GST–Dsg2). (b) IB of GST, GST–Dsg1 and GST–Dsg2 using antibodies H-145 and DG3.10. H-145 recognized Dsg2 only, whereas DG3.10 recognized both Dsg1 and Dsg2. (c) These fusion proteins were used in a GST pull-down assay with A431 cell lysates. Whereas A431 cells expressed both γ-catenin and β-catenin, Dsg1 and Dsg2 were able to pull down γ-catenin but not β-catenin. (d) GST pull-down assay with GST, GST–Dsg1 and GST–Dsg2, and A431 cell lysates, followed by western blotting for Cav-1. Cav-1 was detected in the pull-down with Dsg1 and Dsg2 but not GST. (e) Immunoprecipitation assay further confirms that Dsg2 binds to Cav-1. Tx-soluble (S) and -insoluble (I) proteins were extracted from A431 cells, and subjected to immunoprecipitation for Dsg2 (antibody 10D2). The precipitated products were immunoblotted for Dsg2 (Ab H-145), Cav-1 and mouse IgG (equal antibody loading). The panels to the right are overexposed. I, Tx-insoluble; S, Tx-soluble; Tx, Triton X-100.
cells with the peptides AP and AP–Cav-1 for 24 h. Cells were then fixed and immunostained for Dsg2 using 6D8 or DG3.10 (Figure 4). Dsg2 was detected at the cell–cell border in cells treated with dimethyl sulfoxide or AP peptide. However, treatment with AP–Cav-1 peptides dramatically altered the localization of Dsg2 (Figure 4). We observed diffuse cell-surface staining with loss of the hallmark punctate cell–cell border staining. We note here that the haziness of the stainings of the AP–Cav-1-treated cells is consistent throughout our experiments with the use of this AP–Cav-1 peptide, and is not due to errors in photography. In summary, our results thus far demonstrate that Cav-1 binds to Dsg2, most likely through the Cav-1-binding domain. Disrupting Cav-1 binding with competing peptides results in profound changes in the cell-surface localization of Dsg2. We note here that although it is well established that caveolins localize to caveolae, structures that are defined by electron microscopy, as we did not use electron microscopy in this study, we will refer to these structures as lipid rafts or membrane microdomains.

**Colocalization of Dsg2 to membrane microdomains**
To further confirm that Dsg2 binds to Cav-1, and to determine whether Dsg2 colocalizes with Cav-1 in lipid-enriched rafts, we performed sucrose density-gradient fractionation. Lipid rafts are discrete specialized plasma membrane microdomains (Simons and Ikonen, 1997; Brown and London, 1998). Owing to their high cholesterol and sphingolipid content, they can be isolated based on their detergent insolubility and/or low buoyant density (detergent-free fractionation). We used 5–35% discontinuous sucrose-gradient ultracentrifugation to isolate caveolin-rich membrane microdomains from A431 cells as described previously (Song and Dohlman, 1996; Galbiati et al., 2000; Zheng et al., 2003). Proteins show a light buoyant density because they are encased in the ‘lipid shells’ of cholesterol and sphingolipids (Wang et al., 2003). We observed both Dsg2 and Cav-1 localized to the lighter gradient fractions of 4–5 (Figure 5a). In addition to Cav-1 and Cav-2, we detected other lipid raft proteins, including flotillins (Flo1 and Flo2). Additionally, we also detected the desmoglein-associated protein, plakoglobin (γ-catenin), in the lipid raft fractions. Proteins of the adherens junction did not co-fractionate under these conditions, and were primarily in the more dense fractions. Both E-cadherin and β-catenin were detected in fractions 6–12. Low levels of β-catenin were also detected in fractions 4 and 5. Interestingly, Dsg2 appeared evenly distributed throughout all sucrose fractions. We posit that Dsg2
may associate with other caveolae-free membrane microdomains such as low-density, Triton-resistant and glycosphingolipid-enriched membrane domains (Fra et al., 1994). However, as mentioned above, without using electron microscopy we cannot conclusively ascertain whether Dsg2 resides in caveolae or simply in microdomains similar to caveolae.

Methyl-β-cyclodextran (MβCD) and filipin are two widely accepted treatments for manipulating cholesterol-containing domains. Disruption of lipid rafts with MβCD results in the loss of compartmentalization and caveolae formation, and shifts Cav-1 out of lipid rafts and into denser gradient fractions (Furuchi and Anderson, 1998). Here, we observed a shift of both Cav-1 and Dsg2 into the higher density fractions 11 and 12 with MβCD treatment (Figure 5a, right panels). A shift in density fractionation was also observed with Cav-2, Flot1 and Flot2, and γ-catenin. On the other hand, MβCD did not alter the distribution of β-catenin, E-cadherin and actin; they all still localized mainly to the non-lipid raft fractions. Similar results, although to a lesser extent, were observed when cells were treated with filipin (data not shown), which also binds to cholesterol and alters membrane permeability (Laughlin et al., 2004).

Interestingly, in untreated control cells, we observed a band of weaker intensity at approximately 65 kDa that was recognized by using the Dsg2 antibody in fraction #4 (Figure 5a, arrowhead). Treatment with MβCD resulted in an accumulation of this 65-kDa fragment (Figure 5a, arrowhead), which was shifted to the high-density fractions along with the full-length 160 kDa Dsg2 (arrow). To further characterize the 65-kDa Dsg2 fragment, proteins from sucrose-gradient fractions #4 and #12 of cells both untreated and treated with MβCD were resolved over SDS–PAGE and immunoblotted with the Dsg2 antibodies 10D2 and DG3.10 (Figure 5b). Antibody 10D2, which recognizes the extracellular domain-1 (EC1) of Dsg2 (Keim et al., 2008; Brennan and Mahoney, 2009), detected only the 160-kDa full-length Dsg2 band in both fractions #4 and #12. Antibody DG3.10, which recognizes epitopes within the intracellular domain of Dsg2, recognized both the full-length protein and the truncated 65-kDa fragment (arrowhead). The antigenic epitopes of these antibodies have been characterized previously in detail (Brennan and Mahoney, 2009). Thus, our data demonstrate that the 160-kDa has been full-length and the truncated 65-kDa intracellular fragment both localized to lipid rafts. Disruption of lipid rafts led to the retention of the 65-kDa intracellular domain of Dsg2 in the non-lipid raft membrane fractions.

Next, we wanted to assess whether treatment with MβCD would alter the subcellular localization of Dsg2. Cells were fixed and stained for Dsg2 (antibody DG3.10) and Cav-1 (Figure 6). In control untreated cells, we observed diffuse cell-surface staining for Cav-1 (Figure 6a, red) and punctate cell–cell border staining for Dsg2 (Figure 6b, green). In response to MβCD treatment, we observed a detachment of keratinocytes and enhanced localization of Cav-1 to the cytoplasm (Figure 6e). Interestingly, treatment with MβCD also resulted in an increase in cytoplasmic staining for Dsg2. MβCD had little effect on the cell–cell border localization of Dsg2; however, the cell–cell contacts were no longer contiguous. Interestingly, at the points of cell–cell contacts, we observed an increase in colocalization of Dsg2 and Cav-1. We surmise that the treatment with MβCD disrupted caveolae formation, thereby releasing Cav-1 from these specialized membrane rafts. Cav-1 may then freely associate with other desmogleins or junctional proteins.

**Disruption of Cav-1 association altered Dsg2 localization and keratinocyte cell adhesion**

Next, we wanted to determine whether disrupting the Dsg2–Cav-1 interaction would alter Dsg2 localization within lipid rafts by examining the effect of AP–Cav-1 on Dsg2 distribution in light versus heavy membrane fractions following sucrose-gradient fractionation. A431 cells were treated with AP and AP–Cav-1 peptides...
Total cellular proteins were separated over a discontinuous sucrose gradient. Fractionated proteins were resolved over SDS–PAGE and immunobotted for Dsg2 and actin (Supplementary Figure S2). Disrupting the interaction between Dsg2 and Cav-1 with the Cav-1 competing antennapedia peptides partially shifted Dsg2 out of the low-density membrane lipid raft fractions. Furthermore, the Cav-1 competing peptides also induced a loss of keratinocyte adhesion. After treating A431 cells with AP or AP–Cav-1 peptides, cells were dislodged from the petri dish using dispase. Cell sheets were subjected to dispase-based keratinocyte dissociation assay, showing more fragmentation after treatment with AP–Cav-1 peptides, as compared with control untreated or when treated with AP alone.

(5 μM) for 2 h. Total cellular proteins were separated over a discontinuous sucrose gradient. Fractionated proteins were resolved over SDS–PAGE and immunobotted for Dsg2 and actin (Supplementary Figure S2). Disrupting the interaction between Dsg2 and Cav-1 with the Cav-1 competing antennapedia peptides partially shifted Dsg2 out of the low-density membrane lipid raft fractions. Furthermore, the Cav-1 competing peptides also induced a loss of keratinocyte adhesion. After treating A431 cells with AP or AP–Cav-1 peptides, cells were dislodged from the petri dish using dispase. Cell sheets were subjected to dispase-based keratinocyte dissociation assay, showing more fragmentation after treatment with AP–Cav-1 peptides, as compared with control untreated or when treated with AP alone.

Shown are representative results from three separate experiments (Supplementary Figure S3).

Proteolytic processing of Dsg2 during malignant transformation
It was demonstrated previously that shedding of the extracellular domain of Dsg2 protects epithelial cells from apoptosis (Nava et al., 2007). To determine whether the 65-kDa Dsg2 fragment observed here resulted from ectodomain shedding, we collected conditioned media from various epithelium-derived cell lines and performed immunoblot analysis to detect the shed fragment of Dsg2. Of the cell lines tested, the JAR choriocarcinoma cell line showed the highest level of

Figure 4 The Cav-1 consensus binding peptide perturbs membrane localization of Dsg2. Cells were treated with dimethyl sulfoxide, AP peptide or the AP–Cav-1 peptide for 24 h. They were then washed, fixed and immunostained for Dsg2 (antibodies 6D8 and DG3.10). Insets: Enlarged images. In response to the AP–Cav-1 peptide, but not dimethyl sulfoxide or AP peptide alone, the staining for Dsg2 appeared more diffuse and less punctate at the cell–cell border. Nuclei counterstained with DAPI (blue).
Dsg2 expression (Figure 7a). Cells were then grown to confluence and the conditioned medium was collected, concentrated and immunoblotted for Dsg2. The results showed a shed ectodomain product of approximately 95 kDa (barbed arrow), detected by 10D2, but not DG3.10 (Figure 7b, lanes M for medium). Tx-soluble and -insoluble fractions were prepared from JAR cells and immunoblot analysis revealed a 65-kDa fragment recognized by DG3.10, but not 10D2 (Figure 7b, arrowhead).

As A431 cells showed significantly less Dsg2 cleavage as compared with JAR cells, we treated A431 cells with the cytotoxic quinoline alkaloid camptothecin (10 μM) for 6 h to induce the apoptosis and processing of many proteins. Supernatant was collected, concentrated and proteins were immunoblotted with a series of antibodies against the extracellular (10D2, 10G11, Rb5, 6D8 and Ab10) and cytoplasmic (H-145 and DG3.10) domains of Dsg2 (Figure 7c). Treatment with camptothecin enhanced the ectodomain shedding of Dsg2, resulting in an accumulation of the 95-kDa fragment, which was recognized by antibodies to the extracellular domain but not antibodies to the intracellular domain (Figure 7c, + MβCD).

Figure 5 Localization of Dsg2 and Cav-1 to membrane lipid rafts. (a) A431 cells were treated with MβCD (10 mM) for 1 h and extracted in a Tris–NaCl–EDTA buffer containing Tx. Proteins were subjected to a discontinuous (5–35%) sucrose-gradient separation, resolved over SDS-PAGE and immunoblotted for Cav-1, Cav-2, Flo1, Flo2, β-Cat, γ-Cat, actin, E-Cad and Dsg2. IB revealed that Cav-1 localized predominantly to low-density fractions 4 and 5 (top left panel), corresponding to lipid rafts. Dsg2 was distributed through all fractions from 4 to 12. Treatment with MβCD (10 mM) for 1 h disrupted lipid rafts and shifted both Cav-1 and Dsg2 to the more dense fractions. In addition to the 160-kDa Dsg2 full-length protein, we observed a 65-kDa band in the lipid raft fraction 4 (vertical arrow). Accumulation of this fragment was enhanced and shifted to the denser fractions in the presence of MβCD (arrowhead). We note that β-Cat, γ-Cat, E-Cad and actin fractioned to the lower, denser fractions, and remained relatively unchanged in the presence of MβCD. (b) Proteins from fractions 4 (lipid raft fraction) and 12 (high-molecular-weight, non-raft fraction) above were resolved over SDS-PAGE and immunoblotted for Dsg2 using two different antibodies, 10D2 and DG3.10. Treatment with MβCD increased the level of the 65-kDa Dsg2 fragment as detected by DG3.10, but not 10D2.

Figure 6 Blocking of caveolae formation disrupts cell-cell adhesion. A431 cells were treated with DMSO (a–d) or MβCD (e–h) for 1 h, washed, fixed and subjected to IF staining for Dsg2 (green) and Cav-1 (red). Results show that blocking the formation of caveolae with MβCD resulted in a partial loss of cell-cell adhesion and enhanced the cytoplasmic staining of both Cav-1 and Dsg2. Nuclear staining with DAPI in blue.
immunoblotted with the antibodies Ab10 and H-145. Cav-1 antibodies, and the precipitated product was followed by western blotting analysis. Camptothecin binds to Cav-1, we performed immunoprecipitation in the medium of control untreated cells.

antibodies, 6D8 and Ab10, detected some shed Dsg2 in the barbed arrow. We note that the more sensitive Dsg2 antibodies, 6D8 and Ab10, detected some shed Dsg2 in the barbed arrow. We note that the more sensitive Dsg2 antibodies, 6D8 and Ab10, detected some shed Dsg2 in the barbed arrow. We note that the more sensitive Dsg2 antibodies, 6D8 and Ab10, detected some shed Dsg2 in the barbed arrow. We note that the more sensitive Dsg2 antibodies, 6D8 and Ab10, detected some shed Dsg2 in the barbed arrow. We note that the more sensitive Dsg2 antibodies, 6D8 and Ab10, detected some shed Dsg2 in the barbed arrow. We note that the more sensitive Dsg2 antibodies, 6D8 and Ab10, detected some shed Dsg2 in the barbed arrow. We note that the more sensitive Dsg2 antibodies, 6D8 and Ab10, detected some shed Dsg2 in the barbed arrow. We note that the more sensitive Dsg2 antibodies, 6D8 and Ab10, detected some shed Dsg2 in the barbed arrow. We note that the more sensitive Dsg2 antibodies, 6D8 and Ab10, detected some shed Dsg2 in the barbed arrow. We note that the more sensitive Dsg2 antibodies, 6D8 and Ab10, detected some shed Dsg2 in the barbed arrow. We note that the more sensitive Dsg2 antibodies, 6D8 and Ab10, detected some shed Dsg2 in the barbed arrow. We note that the more sensitive Dsg2 antibodies, 6D8 and Ab10, detected some shed Dsg2 in the barbed arrow. We note that the more sensitive Dsg2 antibodies, 6D8 and Ab10, detected some shed Dsg2 in the barbed arrow.

To determine whether the 65-kDa Dsg2 fragment binds to Cav-1, we performed immunoprecipitation by western blotting analysis. Camptothecin-treated A431 cell lysate was immunoprecipitated with Cav-1 antibodies, and the precipitated product was immunoassayed with the antibodies Ab10 and H-145 (Figure 7d). We note here that antibody Ab10 was raised against extracellular domain-5 (EC5) of Dsg2, the region adjacent to the transmembrane domain (Brennan and Mahoney, 2009). Ab10 recognized both the 95-kDa extracellular shed product as well as the 65-kDa membrane-spanning fragment (not shown). Immunoblots performed with Ab10 and H-145 detected a band migrating at approximately 65 kDa after precipitation for Cav-1. These findings further support our results above that the 65-kDa Dsg2 fragment associates with Cav-1. Our data thus far demonstrate that both the full-length 160-kDa and the truncated 65-kDa intracellular fragment localize to lipid rafts, and interact with Cav-1. Furthermore, although Cav-1 can bind to the full-length protein, it binds predominantly to the 65-kDa fragment. Disruption of lipid rafts may lead to the re-localization of this truncated 65-kDa product.

Finally, we wanted to determine whether Dsg2 undergoes proteolytic processing during skin cancer development, in vivo. For these experiments, we used a transgenic mouse line that was recently established in our laboratory, where Flag-tagged Dsg2 is expressed under the control of the involucrin (Inv) promoter (Brennan et al., 2007). Inv-Dsg2 transgenic and wild-type (WT) control littermates were subjected to DMBA (7,12-dimethyl-benz[a]anthracene)/TPA (12-O-tetradecanoylphorbol-13-acetate)-induced skin tumor development, as described previously, for 8 weeks (Brennan et al., 2007). Skin tumor tissues were extracted in Laemmli sample buffer, and proteins were resolved by SDS–PAGE and immunoassayed using a polyclonal anti-Flag antibody. We detected the 160-kDa Dsg2–Flag protein in the skin and tumors from transgenic, but not in WT mice (Figure 8a, arrow). Interestingly, although the level of full-length Dsg2 in the tumors of transgenic mice was comparable to unaffected skin in these mice, the level of proteolytic processing of Dsg2 protein was significantly enhanced in the tumor tissues. Among the many unique bands ranging from the 160-kDa full-length Dsg2 protein to a small 40-kDa fragment detected by the Flag antibody, we observed a major band of approximately 65 kDa (Figure 8a, arrowhead).

Several older Inv-Dsg2 transgenic mice developed spontaneous skin tumors (not shown). Thus far, none of the WT littermates have developed tumors, as expected of the tumor-resistant C57Bl6 background. To further confirm the proteolytic processing of Dsg2 in tumor tissues, we extracted proteins from spontaneously derived tumors and tumors derived after DMBA/TPA treatment. Again, proteins were resolved over SDS–PAGE for western blotting, but this time, using a monoclonal anti-Flag antibody (Figure 8b). Immunoblots showed that, in addition to the 160-kDa full-length protein (arrow), we observed a prominent 65-kDa band (arrowhead). Thus, we propose that the 65-kDa band observed here maybe the membrane-spanning intracellular Dsg2 product resulting from ectodomain shedding, and that generation of this product may have a role in tumor progression. Although we cannot rule out the in vitro proteolytic processing of Dsg2 during tissue...
During malignant transformation, cell–cell contacts are often reorganized, and desmosome assembly and stability are altered. However, there is conflicting evidence as to what roles desmosomal adhesion and/or desmosomal components have during cancer development and progression. We recently showed a correlation between Dsg2 expression and skin tumor progression, where we observed aberrant localization of Dsg2 in the cytoplasm and nuclei (Brennan and Mahoney, 2009). In the present study, we demonstrate colocalization of Dsg2 with Cav-1, which may have implications in the role of Dsg2 in carcinogenesis. We show that Dsg2 contains the necessary consensus-binding motifs to interact directly with the Cav-1 scaffolding domain. We believe that defining the interaction between Dsg2 and Cav-1 is important, as it may have an impact on cell adhesion (through regulation of turnover/dynamics), and possibly signaling, both of which could contribute to tumor progression.

We propose the following model of desmosome homeostasis (Figure 9) whereby desmosomes actively undergo assembly and disassembly. Junctional proteins such as desmogleins are subjected to dynamic turnover through a caveolae/lipid raft-dependent pathway. We show here that Dsg2 is proteolytically processed, resulting in a 95-kDa ectodomain shed product and a 65-kDa membrane-spanning fragment. The full-length and the truncated intracellular Dsg2 fragment associate with Cav-1 and are mobilized into membrane lipid rafts, where they are most likely fated for internalization and degradation. Altering the Dsg2–Cav-1 interaction, either by disrupting caveolae/lipid raft formation or with Cav-1-specific inhibitor peptides, leads to the retention of the 65-kDa fragment (Figure 5). We speculate that accumulation of this truncated Dsg2 fragment may disturb desmosome assembly and disrupt cell–cell adhesion.

The loss of cell–cell adhesion observed in our study is reminiscent of that reported using transgenic mice expressing NH2-terminally truncated Dsg3, which resulted in an accumulation of Dsg3DN, and disrupted desmosome assembly (Allen et al., 1993). If proteolytic processing and endocytic turnover of desmogleins are important for maintenance of desmosome dynamics, then results from studies using chimeric proteins, such as E-cadherin with Dsg3 (Andl and Stanley, 2001) or connexin with Dsg1 (Troyanovsky et al., 1993), may
also reflect the loss of appropriate ectodomain shedding and desmoglein recycling.

Dsg2 was recently identified as a proteolytic target of ADAM17 (Bech-Serra et al., 2006; Santiago-Josefat et al., 2007; Klessner et al., 2009), a member of the sheddase family. ADAMs are a class of enzymes involved in the ectodomain shedding of transmembrane proteins involved in receptor activation (Kenny and Bissell, 2007). ADAM17 cleaves Dsg2 in the region adjacent to the transmembrane domain, which would result in a shed ectodomain of approximately 95 kDa and an intracellular product of approximately 65 kDa. We believe the 65-kDa Dsg2 fragment is membrane-spanning, as we show that (1) antibodies recognizing extracellular epitopes detected this 95-kDa band, whereas antibodies raised against intracellular epitopes recognized the 65-kDa fragment, and (2) our polyclonal antibody Ab10, raised against the extracellular membrane-anchoring (EC5) domain, detected both the shed ectodomain and the membrane-bound fragment. Thus, if the cleavage site is within the EC5 domain, then Ab10 may contain antibodies recognizing the shed ectodomain, as well as antibodies to the membrane-spanning cytoplasmic domain.

Further supporting our hypothesis on the importance of Dsg2 proteolytic processing, in epithelial cancers epidermal growth factor receptor is often deregulated, and inhibition of epidermal growth factor receptor function promotes Dsg2 assembly into desmosomes (Lorch et al., 2009). Furthermore, this inhibition occurs at least in part through attenuation of ADAM-dependent cleavage of Dsg2, the latter of which contributes to its endocytic turnover (Klessner et al., 2009). At the onset of apoptosis in intestinal epithelial cells, Dsg2 is cleaved by cysteine proteases, and down-regulation of Dsg2 by small interfering RNA protects cells from apoptosis (Nava et al., 2007). Dsg2 has also been identified as a proteolytic target of caspase-3 (Cirillo et al., 2008), one of many caspases that comprise a family of proteins known to have critical roles in maintaining the cellular homeostasis between growth/survival and apoptosis (Rupinder et al., 2007; Denault and Salvesen, 2008). As mentioned above, ADAM17 is involved in the proteolytic processing of Dsg2. ADAM17 has been implicated in the development of cancer, and is being investigated as a possible target for anticancer therapies (Arribas et al., 2006). We are currently investigating the biological activity of the shed extracellular domain of Dsg2.

We reported previously that Dsg2 modulates cell growth and survival signaling pathways by demonstrating that ectopic expression of Dsg2 enhances epidermal proliferation and also increases susceptibility to two-step chemical-induced skin carcinogenesis (Brennan et al., 2007). In this report, we demonstrate enhanced expression and proteolytic processing of Dsg2 during skin tumor progression, which may contribute to the malignant phenotype, possibly through a caveolin-mediated pathway. This is a significant finding, as Cav-1 expression has been linked to many epithelium-derived cancers, and it has been shown that loss of Cav-1 function has a significant role in tumor initiation. It is proposed that Cav-1 binds to and inhibits kinases involved in mitogenic signaling pathways (Lajoie and Nabi, 2010). In many cancers, caveolins are down-regulated and the loss of caveolae may release these signaling molecules to activate mitogenic pathways. Interestingly, Cav-1 knockout mice display normal skin morphology, suggesting that, in addition to caveolin ablation, these mice may require an appropriate ‘oncogenic’ stimulus. We propose that during skin tumor development, the up-regulation of an oncogenic stimulus such as Dsg2 and the concomitant down-regulation of caveolins may provide the necessary stimuli for cell proliferation, signaling activation and malignant transformation. Furthermore, down-regulation of caveolins may result in an accumulation of the truncated Dsg2 fragment, which could potentially disrupt cell–cell adhesion, a process crucial for tumor cell migration and egression.

**Materials and methods**

**Antibodies**

Antibodies from our laboratories were Ab10 (1:10000 (immunoblotting (IB)), 6D8 (1:10 IF and 1:100 (IB)), 10D2 (1:10 (IF) and 1:100 (IB)), 6F9 β-catenin (1:1000 (IB)) and 11E4 γ-catenin (1:500 (IB)). The commercially purchased primary antibodies were Cav-1 (1:200 (IF) and 1:1000 (IB)) and H-145 (1:1000 (IB)) (from Santa Cruz Biotechnology (Santa Cruz, CA, USA)); E-cadherin (1:2500 (IB)), Cav-2 (1:1000 (IB)), flotillin-1 (1:500 (IB)) and flotillin-2 (1:5000 (IB)) (from BD Transduction Labs (Franklin Lakes, NJ, USA)); Flag M2 (1:1000 (IF) and 1:1000 (IB)) and Flag pAb (1:1000 (IB)) (from Sigma (St Louis, MO, USA)); DG3.10 (1:200 (IF) and 1:1000 (IB)) (from RDI Corp (Henderson, NV, USA)); β-actin (1:100 000 (IB)) (from Calbiochem (San Diego, CA, USA)); and GST (1:2500 (IB)) (from GE Healthcare (Piscataway, NJ, USA)). The secondary antibodies included were Alexa Fluor-594- and Alexa Fluor-488-conjugated (1:400; Molecular probes, Eugene, OR, USA) and HRP-conjugated (1:5000; Jackson labs, Bar Harbor, ME, USA).

**GST fusion proteins**

cDNA encoding the intracellular domains of Dsg1 (1921–3845) and Dsg2 (1911–3516) were generated by PCR and inserted in-frame cloning into the vector PGEX-4T-1 (GE Healthcare) at BamHI and SalI restriction sites for Dsg1 and at BamHI and NotI restriction sites for Dsg2. GST fusion proteins were produced in BL21 Escherichia coli cells after induction with isopropyl-β-D-thiogalactoside (1 mm) and purified as described previously in detail (Brennan and Mahoney, 2009).

**Cell culture, drug treatment and protein extraction**

A431, HaCaT and JAR cells (ATCC, Manassas, VA, USA) were cultured in Dulbecco’s modified Eagle’s medium supplemented with penicillin/streptomycin and 10% fetal bovine serum. To detect shedding of the Dsg2 ectodomain, medium was collected after 2 days in Dulbecco’s modified Eagle’s medium without fetal bovine serum and concentrated by 10-fold using Amicon Ultra concentrators (Millipore Corp., Billerica, MA, USA). To enhance shedding, A431 cells were treated with camptothecin (10 μM) in serum-free medium for 2 days in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum and 1000 units/ml penicillin and 1000 μg/ml streptomycin. A431 cells were treated with camptothecin (10 μM) in serum-free medium for
5–6 h. Cells were lysed in Laemmli buffer. For Tx-soluble proteins, cells were incubated on ice for 20 min in a 1% Tx-containing buffer (50 mM Tris–HCl (pH 7.5), 150 mM NaCl and 5 mM EDTA), complete with protease inhibitors (Roche Diagnostics, Indianapolis, IN, USA) and phosphatase inhibitors (Sigma). The insoluble pellet was resuspended in Laemmli buffer for Tx-insoluble proteins.

To disrupt lipid microdomains, cells were treated with MJCD (10 μm) or filipin (5 μg/ml) (Sigma). MJCD and filipin have been used extensively as the standard methods to disrupt raft-like microdomains. We are aware that there are limitations to the use of MJCD because it may affect both caveolin levels and caveolae structures, and may target both caveolar and non-caveolar rafts. However, the key point is that Dsg2 can be localized to lipid rafts and can interact with Cav1.

Dispase-based keratinocyte dissociation assay
A431 cells were grown in six-well culture dishes to confluence in Dulbecco’s modified Eagle’s medium +10% fetal calf serum and then treated with 5 μM AP or AP-Cav-1 peptides in serum-free medium for 2 h. Cells were washed with Hank’s Balanced Salt Solution and incubated with dispase-I (BD Biosciences, San Diego, CA, USA) for 20–30 min. The lifted cell sheets were subjected to dispase-based dissociation assay by pipetting five times by using a 1-ml pipette. Cell fragments were fixed in 10% formalin solution and stained with crystal violet.

IB, immunohistochemistry and immunoprecipitation
IB was performed with 2–25 μg of protein in each lane resolved over 5 or 12% SDS-PAGE (Bio-Rad Labs, Hercules, CA, USA) as described previously in detail (Brennan et al., 2007). Signals were detected by chemiluminescence (ECL; GE Healthcare). For western blotting of biotinylated AP and AP-Cav-1 peptides, immunoprecipitated proteins were resolved over 20% SDS-PAGE Tricine gel (Bio-Rad Labs) and electrotransferred for 30 min at a constant 80 V to a polyvinylidene difluoride membrane. Non-specific sites were blocked in Superblock (Thermo Scientific, Waltham, MA, USA); membranes were then incubated with strept-avidin–HRP (1:1000; Thermo Scientific) and immunoreactive bands visualized by chemiluminescence.

For IF, OCT-fixed human skin sections (5 μm) were prepared as described previously (Brennan et al., 2007). Briefly, tissue sections were fixed in 100% methanol and permeabilized in 1% Tx in phosphate-buffered saline. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) (Sigma) prior to mounting for viewing. Similar steps were performed for immunostaining of cultured cells. Fluorescent images were acquired by using a Hamamatsu monochromatic digital camera (Phase 3 Imaging Systems; Glen Mills, PA, USA; C4742-95), and analyzed by using the Image Pro 6.1 imaging software (Media Cybernetics, Bethesda, MD, USA). Confocal images were obtained by using a Zeiss LSM 510 META confocal scanning microscope system and software (Bioimaging Facility, Thomas Jefferson University, Philadelphia, PA, USA).

For immunoprecipitation, cells were solubilized in a buffer containing 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 1% Tx, 1 mM phenylmethylsulfonyl fluoride and protease and phosphatase inhibitors. Samples were pre-cleared with mouse or rabbit IgG agarose (25 μl; Sigma). Lysates were then incubated in antibodies (0.3 ml 6D8 or 5 μg/ml anti-Cav-1) and protein-A/G–agarose (30 μl; Pierce Biotechnology). Immune complexes were washed with 1% Tx-phosphate-buffered saline and suspended in Laemmli buffer for IB.

Isolation of caveolin-rich membrane fractions
Cells were washed twice with ice-cold PBS and scraped into TNE buffer (25 mM Tris–HCl (pH 7.5), 150 mM NaCl and 5 mM EDTA) containing 1% Tx, phenylmethylsulfonyl fluoride, protease and phosphatase inhibitors (Galliati et al., 2000). Cells were disrupted by using a loose-fitting Dounce Homogenizer (20 strokes), and sucrose concentration was brought to 45% by mixing 2 ml of cell lysates with equal volume of 90% sucrose. The mixture was placed at the bottom of an ultracentrifuge tube, and a discontinuous sucrose gradient was formed above the cell mixture by over-lying 4 ml each of 35% and 5% sucrose. The samples were centrifuged at 40 000 r.p.m. for 16–20 h in an SW41 rotor (Beckman Instruments, Fullerton, CA, USA). Twelve 1-ml fractions were collected from the top.

Skin tumor induction and tissue extraction
We recently established transgenic mice expressing Dsg2 in the differentiating layers of the epidermis under the control of the Inv promoter (Inv-Dsg2) (Brennan et al., 2007). Briefly mouse Dsg2-Flag cDNA was subcloned into the pH3700-pL2 parental vector epitope at the NotI restriction site downstream from the Inv promoter. The genotyping and characterization of the transgenic mice have been described previously in detail (Brennan et al., 2007). Adult WT and Inv-Dsg2 transgenic mice (6–8 weeks old) were treated once with DMBA (400 nmol in 200 μl of acetone) followed by TPA (17 nmol in 200 μl of acetone) twice weekly, as described previously (Brennan et al., 2007). Mouse back skin and tumors were processed in radioimmunoprecipitation assay buffer (50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS and protease inhibitors).

Conflict of interest
The authors declare no conflict of interest.

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