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Plakoglobin Rescues Adhesive Defects Induced by Ectodomain Truncation of the Desmosomal Cadherin Desmoglein 1

Implications for Exfoliative Toxin-Mediated Skin Blistering

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Desmoglein 1 (Dsg1) is a desmosomal cadherin that is essential to epidermal integrity. In the blistering diseases bullous impetigo and staphylococcal scalded-skin syndrome, pathogenesis depends on cleavage of Dsg1 by a bacterial protease, exfoliative toxin A, which removes residues 1 to 381 of the Dsg1 ectodomain. However, the cellular responses to Dsg1 cleavage that precipitate keratinocyte separation to induce blister formation are unknown. Here, we show that ectodomain-deleted Dsg1 (Δ381-Dsg1) mimics the toxin-cleaved cadherin, disrupts desmosomes, and reduces the mechanical integrity of keratinocyte sheets. In addition, we demonstrate that truncated Dsg1 remains associated with its catenin partner, plakoglobin, and causes a reduction in the levels of endogenous desmosomal cadherins in a dose-dependent manner, leading us to hypothesize that plakoglobin sequestration by truncated Dsg1 destabilizes other cadherins. Accordingly, a triple-point mutant of the ectodomain-deleted cadherin, which is uncoupled from plakoglobin, does not impair adhesion, indicating that this interaction is essential to the pathogenic potential of truncated Dsg1. Moreover, we demonstrate that increasing plakoglobin levels rescues cadherin expression, desmosome organization, and functional adhesion in cells expressing Δ381-Dsg1 or treated with exfoliative toxin A. Finally, we report that histone deacetylase inhibition up-regulates desmosomal cadherins and prevents the loss of adhesion induced by Dsg1 truncation. These findings further our understanding of the mechanism of exfo-

liative toxin-induced pathology and suggest novel strategies to suppress blistering in bulbous impetigo and staphylococcal scalded-skin syndrome. (Am J Pathol 2010, 177:2921–2937; DOI: 10.2353/ajpath.2010.100397)

Desmosomes are specialized intercellular adhesive organelles crucial for maintaining the integrity of tissues that endure mechanical stress, especially the epidermis.¹ The adhesive interface of desmosomes is built from specialized cadherin family members, including four desmogleins (Dsg1-4) and three desmocollins (Dsc1-3), assembled at junctions between epidermal keratinocytes.² Desmogleins and desmocollins cooperate to mediate adhesion with cadherins on neighboring cells via ectodo-

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main interactions, whereas their intracellular domains provide a platform for assembly of the desmosomal plaque components. The armadillo family members, plakoglobin (PG) and plakophilins, bind to cadherin tails and stably recruit desmoplakin (DP), which provides a direct link to intermediate filaments. Expression of desmosomal cadherins varies among the layers of the epidermis, and their roles in various aspects of cutaneous development and integrity have been explored in laboratory epidermal models and linked to human skin diseases.^{3–5}

Desmoglein 1 (Dsg1), in particular, is targeted in both inherited and acquired dermatological diseases.^{6,7} Although genetic haploinsufficiency of Dsg1 leads to skin thickening in striate palmoplantar keratoderma,^{8,9} targeting of this cadherin by autoantibodies in pemphigus foliaceus (PF) leads to epidermal blistering.¹⁰ In PF, anti-Dsg1 antibodies recognizing its ectodomain compromise the adhesive function of Dsg1 and lead to blistering only within the upper epidermal layers. Because Dsg1 is expressed throughout all suprabasal layers of the epidermis, the desmoglein compensation hypothesis has been invoked to explain the localization of pemphigus lesions, suggesting that blistering in PF occurs only within the layers where Dsg1 is the primary desmoglein and other desmogleins (Dsg2–4) are not expressed at high enough levels to compensate for the loss of its adhesive function.^{11,12}

Further confirming its requirement for epidermal integrity, Dsg1 is also targeted in an acquired blistering disease in which pathology can be localized or widespread, termed bullous impetigo (BI) or staphylococcal scalded-skin syndrome (SSSS), respectively.⁶ The histological appearance of blistering in BI and SSSS is identical to that observed in PF, and a connection between the two diseases was solidified by the identification of Dsg1 as a target of exfoliative toxin A (ETA), a protease secreted by *Staphylococcus aureus* and the pathological agent in BI and SSSS.¹³ ETA cleaves Dsg1, but not other desmosomal cadherins, hydrolyzing a single peptide bond within its ectodomain.¹⁴ The aim of current therapy for BI and SSSS is to eliminate the source of ETA by treating the underlying infection, but no treatment exists to directly halt what can become fatal blistering.^{15–17} The ectodomain of DSG1 contains five extracellular cadherin-typical domains (EC1–5),^{18,19} and the site of the direct adhesive interaction for Dsg1 is thought to lie within the most N-terminal portion of EC1, which is commonly the region targeted in pemphigus.^{20–22} This N-terminal adhesive region of Dsg1 is removed by ETA, which cleaves at amino acid 381 near the C-terminal end of EC3,¹⁴ thus producing a membrane-tethered, adhesion-deficient cadherin.

Beyond its ectodomain, the transmembrane, intracellular anchor, and intracellular catenin-binding segment (ICS) domains of Dsg1 are highly conserved.²³ Although they have not been crystallized, these domains are thought to exhibit a structure similar to that of classic cadherins and likewise serve as docking sites for armadillo proteins, including PG and plakophilins. Early interaction studies determined that an 87-amino acid sequence at the C terminus of the ICS domain of Dsg3 mediated its interaction with PG.²⁴ Furthermore, abolishing the Dsg-PG interaction impaired incorporation of the

cadherin into junctions, suggesting that PG may be essential to the trafficking of desmogleins. Moreover, for desmogleins and desmocollins to form functional adhesive complexes in nonadherent fibroblasts, coexpression of PG was required.^{25,26} Biochemical analysis predicted that several hydrophobic residues within the ICS domain would be essential to PG binding.^{27,28} Our own experiments have confirmed the failure of PG to interact with a mutant of Dsg1 harboring three alanine mutations at these sites.²⁹

PG can associate with both the ICS domain of desmosomal cadherins^{30–32} and the N terminus of desmoplakin,³³ thus serving as a critical linker in the chain of adhesive protein-protein interactions. PG is bound to desmogleins and desmocollins very soon after their synthesis and is known to traffic with the cadherins.³⁴ During the formation of intercellular junctions, PG plays a major role in the assembly of desmosomes. PG-null keratinocytes showed delayed incorporation of desmosomal components, including cadherins, into intercellular junctions, whereas adherens junctions were unaffected.³⁵ In fact, desmoglein levels were significantly reduced in PG-null cells, suggesting that PG might contribute to the production or stability of these adhesive molecules. How PG regulates the stability of desmosomal cadherins has not been fully explained. Together, these findings suggest that PG plays an essential role in the normal regulation of desmosomal cadherins. Of interest, PG has also been shown to be involved in pemphigus vulgaris, an autoimmune blistering disease that targets Dsg3. In response to autoantibodies, Dsg3 is internalized, but PG remains associated with the targeted cadherin and is required for keratin filament retraction during junction disassembly.^{36,37}

Here, we investigated the mechanism of blistering in BI and SSSS by expressing an N-terminal truncation of Dsg1 to mimic the end product of ETA cleavage (Δ 381-Dsg1) and assessed its effects on intercellular adhesion. Our findings indicate that truncated Dsg1 remains bound to its catenin partner, PG, and that this association is essential for disrupting desmosomes, reducing expression of other desmosomal cadherins, and compromising the adhesive strength of keratinocytes. Moreover, we show that increasing PG is sufficient to rescue cadherin expression and desmosome organization and strengthened intercellular adhesion in cells expressing truncated Dsg1 or treated with ETA. Finally, we found that an inhibitor of histone deacetylases (HDACs) previously linked to PG expression^{38–40} was sufficient to promote intercellular adhesion despite Δ 381-Dsg1 expression or ETA treatment. Taken together, these findings advance our understanding of the mechanism of exfoliative toxin-induced pathology and may provide therapeutic insight into the treatment of blistering diseases targeting the desmosome.

Materials and Methods

Generation of cDNA Constructs

The full-length, human Dsg1 cDNA in pBluescript KS (p536) was modified at its C terminus by PCR to add a

Flag epitope, followed by a stop sequence, and a BamHI site (p783). The Flag-tagged full-length Dsg1 cDNA was then excised by BamHI digestion and ligated into pTRE (Clontech, Mountain View, CA) (p812) for further manipulation. A Dsg1 construct lacking the region cleaved by exfoliative toxin (Δ 381-Dsg1; amino acids [AA] 382–end) was generated by PCR amplification of the entire sequence of p812 except amino acids 1 to 381 after the pro-domain. The linear product was recircularized by blunt ligation to link the pro-domain of Dsg1 directly to AA 382, forming a Dsg1 construct lacking AA 1 to 381. A Δ 381-Dsg1 construct deficient in PG binding (Δ 381-Dsg1AAA) was generated by site-directed mutagenesis (Stratagene, La Jolla, CA) to incorporate three alanine substitutions (Leu751Ala, Phe755Ala, and Leu758Ala) within the predicted binding region for PG.^{28,29} The Dsg1 cytoplasmic domain construct (Δ 569-Dsg1; AA 570–end) was generated, and a 5' BamHI site was inserted by PCR amplification of a fragment of p812.

For retroviral plasmids, the Dsg1 constructs described were digested with BamHI and then were ligated into the BamHI site of pLZRS-Linker (p989). Inserts were fully sequenced and orientation was confirmed by restriction enzyme digestion. pLZRS-Linker and pLZRS-BMN-enhanced green fluorescent protein (EGFP), used for production of EGFP retrovirus, were obtained from Mitchell Denning, Loyola University Medical Center, Maywood, IL.^{41,42}

An N-terminally EGFP-tagged PG was generated from a plasmid encoding the full-length human PG cDNA (p328), which was digested and ligated into pEGFP-C1 plasmid (Clontech) in-frame downstream of the EGFP sequence to form pEGFP-PG (p865). A retroviral construct encoding EGFP-PG was generated as follows. The retroviral vector, pLZRS-Linker, was digested with HindIII and then was treated with Klenow large fragment (New England Biolabs, Ipswich, MA) and dNTPs to fill in the nucleotide overhangs, followed by a second digestion with EcoRI. The insert was prepared by digesting pEGFP-PG with NheI and then treating with 0.5- μ l Klenow large fragment and 25 mmol/L dNTPs to fill in the nucleotide overhangs, followed by a second digestion with EcoRI. Insert and vector DNA bands were purified by electrophoresis in a 1% agarose gel. DNA bands were cut out of the gel, extracted using a DNA gel purification kit (Qiagen, Valencia, CA), and then joined by ligase treatment at 16°C overnight. The EGFP-PG insert was fully sequenced.

Antibodies and Reagents

The following previously described mouse monoclonal antibodies were used in this study: 4B2 (anti-Dsg1 cytodomain⁴³); 27B2 (anti-Dsg1 cytodomain, Zymed Laboratories, South San Francisco, CA); U100 and U114 (anti-Dsc1a/b and Dsc3a/b, respectively, RDI, Concord, MA); 115F (anti-desmoplakin, gift from David Garrod, University of Manchester, Manchester, UK); 11E4 (anti-plakoglobin, gift from James Wahl III, University of Nebraska, Lincoln, NE); HECD1 (anti-E-cadherin, Takara, Kyoto, Japan), 12G10 (anti- β -tubulin, Developmental Studies Hybridoma Bank,

University of Iowa, Ames, IA); and M2 (anti-Flag, Sigma-Aldrich, St. Louis, MO). Polyclonal antibodies used were NW6 (rabbit anti-desmoplakin⁴⁴), 1407 (chicken anti-plakoglobin, Aves Labs, Tigard, OR), C2206 (rabbit anti- β -catenin, Sigma-Aldrich); rabbit anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Abcam Inc., Cambridge, MA), and rabbit anti-Flag (Cell Signaling Technology, Danvers, MA). Secondary antibodies for Western blotting included goat anti-mouse, anti-rabbit, and anti-chicken peroxidase (Rockland Immunochemicals, Gilbertsville, PA, KPL, Inc., Gaithersburg, MD). Secondary antibodies for immunofluorescence included goat anti-mouse, anti-rabbit, anti-chicken, and anti-human linked to fluorophores of 350, 488, or 568 nm (Alexa Fluor, Invitrogen, Carlsbad, CA).

Polybrene (hexadimethrine bromide, Sigma-Aldrich) was used for retroviral transduction. 4',6-Diamidino-2-phenylindole (Sigma-Aldrich) was used for nuclear staining. Dimethyl sulfoxide (DMSO) (Fisher Scientific, Pittsburgh, PA) and trichostatin A (1 μ mol/L, Sigma-Aldrich) were used for treatment of keratinocyte cultures. Epidermal growth factor (Chemicon International, Temecula, CA) was used for organotypic culture growth.

Production of Exfoliative Toxin A

Noninfectious *S. aureus* lines RN4220 carrying constructs encoding His-tagged wild-type or protease-dead (S195A) ETA¹⁹ were provided by John Stanley, University of Pennsylvania (Philadelphia, PA). Bacterial cultures were grown in Luria broth overnight at 37°C. Supernatants were collected after centrifugation of cultures at 4°C for 20 minutes at 5000 rpm. ETA was harvested from bacterial supernatants using nickel-nitrilotriacetic acid-agarose beads (Qiagen) and then were washed and eluted with imidazole according to the manufacturer's instructions. The eluate was analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and Coomassie staining to confirm protein purity. ETA concentration was estimated using the BCA protein quantification kit according to the manufacturer's instructions (Pierce Chemical, Rockford, IL) and was used at 1 to 2 μ g/ml.

Primary Keratinocyte Cultures

Normal human epidermal keratinocytes (NHEKs) were isolated from neonatal human foreskin as described previously.⁴⁵ In brief, foreskin samples were incubated overnight at 4°C in 2.4 U/ml Dispase II (Roche Applied Science, Indianapolis, IN). The epidermal sheet was then separated from the underlying dermis using forceps. The epidermis was incubated in 0.05% trypsin for 15 minutes at 37°C. Then 1 ml of fetal bovine serum was added, and epidermal fragments were scraped against the cell culture dish using forceps. Keratinocytes were harvested after pouring the tissue debris through a 50- μ m strainer and centrifuging at 200 $\times$ *g* for 5 minutes. The cells were resuspended and propagated in Medium 154 supplemented with Human Keratinocyte Growth Supplement, 1000 $\times$ gentamicin/amphotericin B solution (Cascade

Biologics, Portland, OR), and 0.07 mmol/L CaCl_2 . For differentiation of submerged cultures, cells were grown to confluence and switched into Medium 154 containing 1.2 mmol/L CaCl_2 or E medium⁴⁶ containing 1.8 mmol/L CaCl_2 for 1 to 6 days.

Retroviral Production and Transduction of Keratinocytes

Retroviral supernatants were produced from Phoenix NIH-293 cells (kindly provided by Gary Nolan, Stanford University, Palo Alto, CA) grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 1× gentamicin/amphotericin B solution (Cascade Biologics). Phoenix cells were grown to 50% confluence and were transfected with pLZRS constructs as follows: 8 μg of DNA and 20 μl of Lipofectamine 2000 transfection reagent (Invitrogen) were diluted in separate volumes of 400 μl of Opti-MEM and were mixed and incubated at room temperature for 20 minutes. The transfection cocktail was then added to Phoenix cells, which were incubated for 8 to 12 hours before their medium was changed. After 24 hours of growth, transfected Phoenix cells were selected using 1 to 2 $\mu\text{g}/\text{ml}$ puromycin. Selected cells were grown to 80% confluence and were split at 1:5 to 1:10 in selection medium for propagation. For harvesting viral supernatants, selection medium was replaced with normal growth medium lacking puromycin. Cells were incubated at 32°C overnight. Retroviral supernatants were harvested and used directly for fresh transductions after addition of 4 $\mu\text{mol}/\text{L}$ Polybrene. For long-term storage, viral supernatants were concentrated by centrifugation at 5000 × *g* using 15 kDa molecular mass cutoff columns (Millipore Corporation, Billerica, MA). Concentrated supernatants were snap-frozen in liquid nitrogen and stored at −80°C. After thawing, virus was diluted in Medium 154 plus 4 $\mu\text{mol}/\text{L}$ Polybrene and applied as described above for transduction.

Dispase-Based Dissociation Assay

Mechanical integrity of cell monolayers was assessed as described previously.⁴⁷ Keratinocytes were grown to confluence in Medium 154 containing 0.07 mmol/L CaCl_2 and switched to medium containing 1.2 mmol/L CaCl_2 for 1 to 3 days. At that time, keratinocyte sheets were lifted from the culture dishes by treating with 2.4 U/ml Dispase II for 30 minutes at 37°C. Intact sheets were transferred to 15-ml conical tubes containing 5 ml of PBS. Tubes were placed in a rack and inverted together 10 to 30 times. Cellular fragments were transferred to 35-mm tissue culture plates and counted and imaged using a dissecting microscope. The average numbers of fragments among triplicate monolayers for each experimental condition were compared using Student's *t*-test. *P* > 0.05 were considered nonsignificant (NS). Data shown are representative of at least three independent experiments.

Organotypic Epidermal Cultures

For organotypic cultures, NHEKs were expanded and grown at an air-medium interface according to published protocols.^{46,48} In brief, J2-3T3 fibroblasts were grown in Dulbecco's modified Eagle's medium containing 10% heat-inactivated bovine serum and 1× gentamicin-amphotericin B solution (Cascade Biologics). To make "dermal" collagen plugs, fibroblasts were trypsinized and counted and 1 million cells for each organotypic culture were harvested by centrifugation. To make plugs of 1.5 ml each, fibroblasts were resuspended in 1/10 final volume of reconstitution buffer^{46,49} and mixed with 1/10 final volume of 10× Dulbecco's modified Eagle's medium. High concentration rat tail type I collagen (BD Biosciences, San Jose, CA) was added to a final concentration of 4 mg/ml and diluted to the final volume with water. The fibroblast-collagen slurry was mixed by inversion, and 1.5 ml was added to the upper chamber of each 0.6- μm transwell insert fitted into a deep-well plate (BD Biosciences). The plugs were allowed to solidify for 30 minutes at 37°C and submerged in J2-3T3 medium overnight.

Before seeding on the collagen plugs, keratinocytes were trypsinized and counted. One million cells per culture were harvested by centrifugation and resuspended in 2 ml of E medium supplemented with 5 ng/ml epidermal growth factor and seeded into the upper chamber of the transwell inserts atop the collagen plug. Then 13 ml of E medium (with 5 ng/ml epidermal growth factor) was added to the lower chamber to keep the keratinocytes submerged, and 48 hours later, the medium was removed from both chambers. The lower chamber was filled with 10 ml of E medium without epidermal growth factor supplementation to reach the bottom of the collagen plug, leaving the NHEKs exposed to air.

Organotypic cultures were maintained at an air-medium interface by feeding with E medium every other day for 3 to 12 days at which time they were lysed for protein analysis or fixed in 10% neutral buffered formalin and embedded in paraffin for standard histological analysis.

Protein Expression and Immunoprecipitation Analysis

Protein was extracted from monolayers or raft cultures using urea-SDS buffer (USB) for whole-cell lysates. For immunoprecipitations and protein solubility assessment, radioimmunoprecipitation assay (RIPA) buffer containing 1% protease inhibitor cocktail (Sigma-Aldrich) and 1% phosphatase inhibitor cocktails II and IV (EMD Biosciences, San Diego, CA) was used for lysis. Radioimmunoprecipitation assay buffer-insoluble fractions were solubilized in USB. Before immunoblotting, USB lysates were treated with 5% β -mercaptoethanol; radioimmunoprecipitation assay buffer lysates were denatured in 3× Laemmli buffer plus 5% β -mercaptoethanol at 95°C for 5 minutes before gel electrophoresis.

For immunoblotting, protein lysates were resolved by SDS-PAGE on a 10% polyacrylamide gel containing 1%

SDS by applying a current of 100 V in running buffer (25 mmol/L Tris, 250 mmol/L glycine, and 1% SDS). Proteins were transferred to nitrocellulose membranes at 20 V overnight in transfer buffer (20% methanol, 25 mmol/L Tris, and 250 mmol/L glycine). Protein transfer was assessed by staining with Ponceau S (Sigma-Aldrich). Membranes were blocked in 5% milk in PBS for 60 minutes at room temperature to prevent nonspecific antibody binding. Primary and secondary antibodies were diluted in 5% milk in PBS, added to blots, and incubated overnight at 4°C or for 1 hour at room temperature. Protein bands were visualized using enhanced chemiluminescence and exposure to X-ray film.

For immunoprecipitation studies, Flag-tagged proteins were precipitated with M2-agarose (Sigma-Aldrich). Immunoprecipitated complexes were denatured in 3× Laemmli buffer with 5% β -mercaptoethanol at 95°C for 5 minutes, resolved by SDS-PAGE, and immunoblotted as described above.

Histology, Immunohistochemistry, and Immunocytochemistry

Raft cultures were sectioned and processed for immunohistochemical or H&E staining using conventional methods. Antigen retrieval was performed by heating to 95°C in 0.01 mol/L citrate buffer containing 0.05% Tween 20. Sections were blocked in 10% normal goat serum (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) in PBS for 60 minutes at 37°C, incubated in primary antibody in 0.5% BSA in PBS overnight at 4°C, incubated in fluorophore-linked secondary antibody in 0.5% BSA in PBS for 60 minutes at 37°C, and mounted in polyvinyl alcohol. For immunocytochemistry of submerged cultures, cells were seeded on glass coverslips in 0.07 mmol/L CaCl_2 Medium 154, grown to confluence, and switched into 1.2 mmol/L CaCl_2 Medium 154 for 1 to 24 hours, at which time they were fixed in 3.7% formal saline for 10 minutes at room temperature, and permeabilized in acetone at -20°C for 2 minutes. Immunofluorescence was performed by dilution of primary antibody in 1% normal goat serum/2% bovine serum albumin in PBS. Primary antibody was applied to coverslips and incubated at 37°C for 60 minutes, followed by washing by submerging 30 times in 3 volumes of PBS. Secondary antibody was diluted in a similar fashion, applied, incubated at 37°C for 30 minutes, and then washed. 4',6-Diamidino-2-phenylindole was similarly diluted, applied, and incubated at room temperature for 2 minutes and then washed.

Images were obtained with a 40× PL Fluotar, NA 1.0 or 63× PL APO, NA 1.32 objective on a Leica DMR microscope using a charge-coupled device camera (Orca 100, model C4742-95, Hamamatsu, Bridgewater, NJ) and MetaMorph 6.1 software (MDS Analytical Technologies, Union City, CA) for fluorescence or a Leica DFC320 digital camera and Photoshop software (Adobe Systems, Mountain View, CA) for color images.

Fluorescence Intensity Measurement

Measurement of immunofluorescence at intercellular borders was performed as previously described⁵⁰ using MetaMorph 6.1 software to quantify the average fluorescence intensity within the area representing the intercellular border, circumscribed manually. At least 20 independent borders were quantified for each experiment and averaged. Data shown are representative of at least three independent experiments.

Results

Toxin-Cleaved Dsg1 Is Retained in Organotypic Epidermal Cultures in Areas of Desmosomal Disruption

To study exfoliative toxin-induced epidermal blistering in a physiological model, we used organotypic human epidermis consisting of primary keratinocytes grown at an air-medium interface.^{46,49} These cultures stratify to form all histological layers of epidermis and exhibit suprabasal expression of Dsg1 with concentration of this desmosomal cadherin in the uppermost granular layers as in human tissue.²⁹ For induction of blistering, raft cultures were matured for 8 days and then were treated for 24 hours with 2 $\mu\text{g}/\text{ml}$ recombinant wild-type ETA (ETA wt) or a protease-dead toxin (ETA mut).¹⁴ Of importance, treatment with ETA results in efficient cleavage of Dsg1 (Figure 1B) to generate a membrane-tethered cadherin (Figure 1C), inducing separation (acantholysis) of granular layer keratinocytes and subcorneal blister formation (Figure 1A).

To examine the fate of desmosomal components within the blistered tissue, immunohistochemical staining was used to highlight the localization of the toxin-targeted cadherin, Dsg1, its companion suprabasal desmosomal cadherin, desmocollin 1 (Dsc1), its catenin binding partner, PG, and an obligate component of the desmosomal plaque, DP (Figure 1D). In control cultures, desmosome components were localized at intercellular borders of keratinocytes, with increasing expression throughout the suprabasal layers. However, in the uppermost layers of cultures treated with ETA, localization of all desmosomal components examined was grossly perturbed, with a striking loss of staining (DP) or relocalization away from intercellular borders (Dsc1 and PG) (Figure 1D, Zoom). Toxin-cleaved Dsg1, stained with a cytoplasmic domain antibody, remained at intercellular borders in lower layers, where other desmosomal cadherins (eg, Dsg2/3) are more highly expressed and might stabilize adhesion-deficient Dsg1 in junctional complexes.¹² In contrast, the cleaved cadherin exhibited a drastic intracellular relocalization in the uppermost layers, where Dsg1 levels are highest and expression of another major desmoglein, Dsg3, is attenuated.¹¹ Despite its altered localization, Dsg1 staining and levels on immunoblot were still quite prominent in ETA-treated cultures, suggesting persistence of toxin-cleaved Dsg1 within the layers where blistering occurs, which could exert a negative influence on

Figure 1. Toxin-cleaved Dsg1 is retained in organotypic epidermal cultures in areas of desmosomal disruption within subcorneal blisters. NHEKs were grown as organotypic cultures for 9 days and then treated with ETA mut or ETA wt at 2 μ g/ml for 24 hours to cleave the ectodomain of Dsg1. **A:** H&E staining of sections from organotypic cultures shows that treatment with ETA wt results in subcorneal blister formation with separation of cells in the upper granular layers (Zoom) as is seen in ETA-induced pathology *in vivo*. **B:** Western blot of lysates from organotypic cultures shows that ETA efficiently cleaves full-length Dsg1 (FL) to generate a toxin-cleaved fragment (CL). Expression levels of other junctional proteins (DP and E-cadherin [E-cad]) are relatively constant, although Dsc1 and PG levels may decrease somewhat upon Dsg1 cleavage. GAPDH serves as a loading control. **C:** Diagram of Dsg1 FL and Dsg1CL, the cleaved product of ETA, which removes residues 1 to 381 of Dsg1 to produce a membrane-tethered, adhesion-deficient cadherin. ECTO, ectodomain; TM, transmembrane; CYTO, cytodomain; **D:** Immunohistochemical staining of organotypic cultures with an antibody recognizing its cytodomain indicated that Dsg1 may be internalized in the upper layers in response to ETA cleavage, whereas it remains at intercellular borders in control cultures (Zoom). Dsc1 and PG also seemed to re-localize to the cytoplasm on ETA treatment, whereas DP staining was reduced altogether in the uppermost granular layers.

desmosomal adhesion to cause separation of keratinocytes within the subcorneal layers.

N-Terminal Truncation of Dsg1 Mimics Toxin-Cleaved Cadherin, Disrupts Desmosomes, and Reduces Keratinocyte Adhesion in a Dose-Dependent Manner

To model ETA-induced pathology in a quantifiable and manipulable system, we used submerged keratinocyte cultures grown as confluent monolayers and differentiated in 1.2 mmol/L calcium to induce endogenous Dsg1 expression and then compared intercellular adhesive strength via a mechanical dissociation assay in control and ETA-treated cultures. Treatment of keratinocyte monolayers with ETA at 1 μ g/ml efficiently cleaved endogenous Dsg1 (Figure 2C) and significantly increased their fragmentation (Figure 2, A and B). To test whether an ectodomain-truncated Dsg1 mimicking the retained

toxin-generated cadherin product was sufficient to induce pathology, we engineered a Flag-tagged Dsg1 construct having its pre- and pro-domains linked directly to glycine 382 (Δ 381-Dsg1) (Figure 2D). This manipulation spliced out amino acids 1 to 381 of the mature ectodomain, eliminating the precise residues removed by the bacterial protease to reflect the disease state. Of importance, we verified that Δ 381-Dsg1 was the same size as wild-type Dsg1 after ETA cleavage as assessed by SDS-PAGE, exhibited comparable solubility and stability in keratinocytes, and localized to intercellular junctions similar to the toxin-cleaved cadherin (Supplemental Figure S1, A and B, see <http://ajp.amjpathol.org> and data not shown). In parallel, we used another truncation of Dsg1 lacking all extracellular and transmembrane residues (Δ 569-Dsg1) (Figure 2D), which exhibits a diffuse cytoplasmic, nonjunctional distribution (Figure 3A). Although nucleofection or adenoviral expression often leads to inefficient delivery or supraphysiological expression of ectopic proteins, retroviral delivery of truncated

Figure 2. $\Delta 381$ -Dsg1 mimics toxin-cleaved cadherin and compromises mechanical integrity of keratinocyte sheets. **A:** NHEK monolayers were cultured in 1.2 mmol/L calcium for 3 days to induce Dsg1 expression and then were treated with ETA wt or ETA mut at 1 μ g/ml. After Dispase treatment, detached monolayers were subjected to mechanical strain, and resulting fragments of the cell sheet were imaged. **B:** Triplicate monolayers were fragmented and counted to show a reduction in integrity on ETA wt treatment. A Student's *t*-test *P* value is shown. **C:** Western blot of monolayer lysates shows that Dsg1 was cleaved by ETA wt, but this did not affect levels of PG or GAPDH. **D:** Dsg1 constructs used included full-length wild-type Dsg1 (WT), Dsg1 lacking extracellular (EC) residues removed by ETA ($\Delta 381$ -Dsg1), or Dsg1 containing the cytoplasmic domain (CYTO), but lacking all extracellular (ECTO) and transmembrane (TM) residues ($\Delta 569$ -Dsg1). All constructs were Flag-tagged (FL) at their C terminus. NHEKs were transduced with Dsg1 constructs and allowed to differentiate in 1.2 mmol/L calcium for 48 hours before lysis or fragmentation. **E:** Triplicate monolayers were fragmented and counted to show a reduction in integrity caused by $\Delta 381$ -Dsg1 expression. Student's *t*-test *P* values are shown. **F:** Western blot shows the relative sizes and expression levels of Dsg1WT, $\Delta 381$ -Dsg1, and $\Delta 569$ -Dsg1. Expression of these constructs did not alter the levels of PG or GAPDH. **G:** Images of fragmented monolayers indicate that $\Delta 381$ -Dsg1 alone decreased adhesive strength.

Dsg1 to primary human keratinocytes resulted in efficient transduction with transgene expression comparable to endogenous cadherin levels (Figure 2F).

Expression of $\Delta 381$ -Dsg1 dramatically increased fragmentation of monolayers (Figure 2, E and G), indicating that ectopic introduction of the end product of toxin cleavage is sufficient to reduce functional adhesion and implying a dominant-negative effect. A similar dominant-negative effect on intercellular adhesion was seen when wild-type Dsg1 was ectopically expressed and treated with ETA to convert the functional cadherin into a truncated, adhesion-deficient protein (Supplemental Figure S1, C and D, see <http://ajp.amjpathol.org>), verifying that the ectodomain-truncated mutant ($\Delta 381$ -Dsg1) induces effects similar to those with the toxin-cleaved cadherin. On the other hand, the nonmembrane-tethered Dsg1 truncation mutant ($\Delta 569$ -Dsg1) did not increase monolayer fragmentation, despite its expression at a level similar to that of $\Delta 381$ -Dsg1 (Figure 2F), suggesting that localization of the truncated cadherin to junctional complexes via ectodomain and/or transmembrane residues retained in $\Delta 381$ -Dsg1 may be essential to its ability to disrupt adhesion. Interestingly, simply tethering the cytoplasmic domain of Dsg1 to the cell surface via linkage to the extracellular and transmembrane domains of the interleukin-2 receptor (IL2R:Dsg1)⁵¹ did not disrupt adhesion (data not shown). However, this chimeric Dsg1 construct did not colocalize well with other desmosomal

markers, indicating that specific targeting to desmosomes may be necessary for ectodomain-truncated Dsg1 to negatively affect desmosomal organization.

To examine the cellular consequences of $\Delta 381$ -Dsg1 expression, immunocytochemistry was performed on transduced keratinocytes grown as monolayers in 1.2 mmol/L calcium for 24 hours before fixation (Figure 3A). Similar to full-length wild-type Dsg1 (Dsg1WT), $\Delta 381$ -Dsg1 localized to intercellular borders, whereas $\Delta 569$ -Dsg1 exhibited a cytoplasmic distribution. Costaining for PG revealed recruitment of the catenin to intercellular borders in cells expressing either Dsg1WT or $\Delta 381$ -Dsg1 without a notable change in total PG levels (Figure 3B). These findings suggest that, similar to Dsg1WT, $\Delta 381$ -Dsg1 can bind and recruit PG to the cell surface but would presumably sequester this junctional component in a nonadhesive cadherin-catenin complex. Staining for other desmosome components present in submerged keratinocyte cultures and representing the membranous (Dsc3) and plaque (DP) compartments revealed a profound disruption of their localization in cells expressing $\Delta 381$ -Dsg1 (Flag-positive) versus internal control cells not expressing the transgene (Figure 3A). Quantification of Dsc3 and DP intensity at intercellular borders (Figure 3C and data not shown) between adjacent Flag-positive (Infected) or Flag-negative (Control) cells revealed a significant difference only for $\Delta 381$ -Dsg1 but not for Dsg1WT or $\Delta 569$ -Dsg1 despite similar transgene expres-

Figure 3. Δ381-Dsg1 impairs desmosomal organization, reducing desmosomal cadherin expression and intercellular adhesion in a dose-dependent manner. **A:** Immunofluorescence staining of NHEKs transduced with Dsg1WT, Δ381-Dsg1, or Δ569-Dsg1 showed that Δ381-Dsg1 was concentrated at intercellular borders and could recruit PG to these areas similar to Dsg1WT, whereas Δ569-Dsg1 was localized in the cytoplasm. Costaining for Flag and other desmosomal markers revealed that cells expressing Δ381-Dsg1 showed a marked loss of both Dsc3 and DP at intercellular borders, although E-cadherin (E-cad) was not affected. **B:** Western blot of NHEK lysates showed that expression of all three Dsg1 constructs was similar. Δ381-Dsg1 expression slightly decreased Dsc3 levels but did not grossly perturb expression of other junctional proteins (PG, DP, and E-cad) or GAPDH. **C:** Fluorescence intensity of Dsc3 staining (from **A**) at intercellular borders was significantly reduced in Flag-positive (Infected) cells expressing Δ381-Dsg1 versus Flag-negative (Control) cells. DP exhibited a similar trend (data not shown). Student's *t*-test *P* values are shown (not significant [NS]; >0.05). **D:** NHEKs were transduced with increasing amounts of Δ381-Dsg1, seeded as monolayers, and allowed to differentiate in 1.2 mmol/L calcium for 48 hours before lysis or mechanical dissociation. Western blot of monolayer lysates confirmed increasing expression of Δ381-Dsg1 and showed down-regulation of endogenous Dsg1 FL and Dsc3 at the highest level of Δ381-Dsg1 (×4). Other junctional protein (PG, DP, and E-cad) and GAPDH levels were relatively constant. **E:** Increasing amounts of Δ381-Dsg1 expression resulted in a near-linear increase in the fragmentation of keratinocyte monolayers, indicating a dose-dependent negative effect on intercellular adhesion. Student's *t*-test *P* values are shown. RV, retrovirus.

sion (Figure 3B). The junctional defect caused by Δ381-Dsg1 seemed limited to desmosomes because staining for the adherens junction component, E-cadherin, was not perturbed (Figure 3A).

To test the dose-dependence of the effects of Δ381-Dsg1 on intercellular adhesion, keratinocytes were infected with increasing amounts of Δ381-Dsg1 and differentiated as monolayers. The mechanical dissociation assay revealed a linear increase in monolayer fragmentation with increasing Δ381-Dsg1 expression (Figure 3E). Notably, at high levels of Δ381-Dsg1 (4×), there was a marked decrease in endogenous full-length Dsg1 (FL) and Dsc3 (Figure 3D), suggesting that these desmosomal cadherins are not simply absent from intercellular borders but are in fact reduced in expression. This observation is consistent with a previous report demonstrating dose-dependent loss of other desmosomal cadherins on ectopic expression of a chimeric Dsg1 construct lacking its normal ectodomain in simple epithelial cells.⁵² DP,

PG, and E-cadherin levels remained relatively constant (Figure 3D), suggesting that Δ381-Dsg1 specifically compromises the expression of desmosomal cadherins. These data indicate that Δ381-Dsg1 exerts a negative effect on desmosome organization at the cellular level and impairs the integrity of keratinocyte sheets. In addition, the dose dependence of its negative impact on adhesion is consistent with the hypothesis that larger amounts of Δ381-Dsg1 could sequester more of a stabilizing factor for other desmosomal cadherins.

Plakoglobin Binding Is Essential for Δ381-Dsg1 to Disrupt Desmosomes and Reduce Intercellular Adhesion

Previous studies have suggested that ectodomain-truncated cadherins might act in a dominant-negative fashion by sequestering stabilizing proteins, such as

Figure 4. Plakoglobin binding is essential for ectodomain-truncated Dsg1 to disrupt desmosomes. **A:** NHEKs were transduced with Dsg1 constructs and cultured in 1.2 mmol/L calcium for 24 hours before lysis and precipitation of Flag-tagged proteins. Full-length Dsg1 was able to associate with PG, as expected. Treatment with ETA at 1 μ g/ml cleaved Dsg1 but did not alter its interaction with PG. Likewise, Δ 381-Dsg1 was able to coprecipitate PG. Input of ectopic Dsg1 (Flag), PG, and GAPDH is shown. **B:** Immunofluorescence shows that although Δ 381-Dsg1 with a wild-type cytoplasmic domain (WT) enriched PG at intercellular borders, a triple-point mutant of the truncated cadherin (Δ 381-Dsg1AAA), engineered to be uncoupled from PG, did not. Moreover, Δ 381-Dsg1AAA did not significantly affect the localization of Dsc3 or DP, whereas cells expressing Δ 381-Dsg1WT showed a reduction in both Dsc3 and DP at intercellular borders. E-cadherin (E-cad) staining was not affected by any of the conditions. **C:** A triple mutation to alanine within the ICS domain of Δ 381-Dsg1 (AAA) designed to prohibit binding of PG was effective at uncoupling the truncated cadherin from its catenin partner. Accordingly, Δ 381-Dsg1AAA failed to precipitate PG compared with Δ 381-Dsg1 with a wild-type ICS (WT). Input of ectopic Dsg1 (Flag) and GAPDH is shown. **D:** NHEKs were transduced with GFP, Δ 381-Dsg1WT, or Δ 381-Dsg1AAA and allowed to differentiate in 1.2 mmol/L calcium for 24 hours. Western blot (WB) of monolayer lysates showed that Δ 381-Dsg1WT and Δ 381-Dsg1AAA were expressed at similar levels, but only Δ 381-Dsg1WT reduced the expression of endogenous Dsg1 (WT) and Dsc3. Other junctional protein (PG, DP, and E-cad) and GAPDH levels were relatively constant. **E:** Fluorescence intensity of Dsc3 staining (from **B**) at intercellular borders was significantly reduced in Flag-positive (Infected) cells expressing Δ 381-Dsg1WT versus Flag-negative (Control) cells, but the difference was not significant for Δ 381-Dsg1AAA. DP exhibited a similar trend (data not shown). Student's *t*-test *P* values are shown (not significant [NS], >0.05). IP, immunoprecipitation. RV, retrovirus.

catenins.^{30,53,54} PG, in particular, which is found in association with desmoglein shortly after its synthesis,³⁴ has been shown to regulate the levels of desmosomal cadherins in PG-null keratinocytes.³⁵ Thus, we hypothesized that ETA-cleaved Dsg1, which has an intact cytoplasmic domain, remained associated with its catenin partner, PG, and that this sequestration by an adhesion-deficient cadherin could contribute to its ability to destabilize other desmosomal cadherins and compromise adhesion. Accordingly, both full-length and toxin-cleaved Dsg1 coprecipitated PG from keratinocytes (Figure 4A); Δ 381-Dsg1 also associated with PG, consistent with its ability to enrich PG at intercellular borders as seen by immunocytochemistry (Figure 3A). To determine whether PG binding was essential to the negative effects of Δ 381-Dsg1 on desmosomal adhesion, a triple-point mutant of Δ 381-

Dsg1 (Δ 381-Dsg1AAA) was generated by substituting three alanines for hydrophobic residues within the PG-binding region of the intracellular catenin-binding segment.²⁸ While Δ 381-Dsg1 having a wild-type cytoplasmic domain (Δ 381-Dsg1WT) was able to coprecipitate its catenin partner from keratinocytes, Δ 381-Dsg1AAA was severely limited in its ability to complex with PG (Figure 4C).

Although Δ 381-Dsg1WT impaired the expression of endogenous Dsg1 and Dsc3, Δ 381-Dsg1AAA did not significantly alter the levels of other cadherins, suggesting that the ability to bind PG is essential to the destabilizing effect of the truncated Dsg1 (Figure 4D). Examination of desmosomal components by immunocytochemistry revealed that Δ 381-Dsg1WT again seemed to recruit PG to intercellular borders, but Δ 381-Dsg1AAA did not alter PG

Figure 5. Δ381-Dsg1AAA does not impair intercellular adhesion. NHEKs were transduced with GFP, Δ381-Dsg1WT, Δ381-Dsg1AAA, or Δ569-Dsg1 and seeded as confluent monolayers and cultured in 1.2 mmol/L calcium for 48 hours before lysis or mechanical dissociation. **A:** Fragmented monolayers demonstrate that Δ381-Dsg1WT alone reduced the integrity of keratinocyte sheets. **B:** Triplicate monolayers were fragmented and counted to show a reduction in integrity caused by Δ381-Dsg1WT but not Δ381-Dsg1AAA or Δ569-Dsg1. Student's *t*-test *P* values are shown. **C:** Western blot of monolayer lysates showed that Δ381-Dsg1WT again reduced Dsc3 expression, but Δ381-Dsg1AAA did not. All other junctional markers (PG, DP, E-cadherin [E-cad], and β-catenin [β-cat]) and GAPDH were unaffected. RV, retrovirus.

localization (Figure 4B), consistent with its inability to coprecipitate PG. Of importance, Δ381-Dsg1AAA did not significantly alter the localization of other desmosomal components (DP and Dsc3) at intercellular borders compared with GFP-transduced cells, whereas Δ381-Dsg1 drastically impaired both DP and Dsc3 organization, but neither construct affected E-cadherin. Quantification of the intensity of Dsc3 and DP at intercellular borders (Figure 4E and data not shown) revealed a significant difference only for cells expressing Δ381-Dsg1WT, not Δ381-Dsg1AAA. Together these findings indicate that uncoupling the truncated cadherin from PG eliminates the potential of Δ381-Dsg1 to disrupt desmosomal organization at the cellular level.

To determine whether these cellular findings translated to similar results for functional adhesion, keratinocytes were transduced with similar amounts of Δ381-Dsg1WT, Δ381-Dsg1AAA, Δ569-Dsg1, or GFP as a negative control and then were differentiated as monolayers and subjected to mechanical stress. Although monolayers expressing Δ381-Dsg1WT were extremely fragile, those expressing Δ381-Dsg1AAA did not fragment significantly more than monolayers transduced with GFP or Δ569-Dsg1 (Figure 5, A and B). Consistent with this finding, expression of Δ381-Dsg1WT again reduced the levels of Dsc3, whereas Δ381-Dsg1AAA did not appreciably alter Dsc3 expression (Figure 5C). PG levels remained relatively constant despite introduction of the ectopic cadherins.

Given these results, we hypothesized that ectopic introduction of Δ381-Dsg1WT leads to sequestration of PG,

a limited stabilizing factor for desmosomal cadherins, in complexes unable to mediate adhesion, away from other adhesion-competent cadherins. Interestingly, Δ569-Dsg1, which possesses an intact PG-binding region, did not significantly affect the levels of endogenous cadherins. However, our previous studies indicated that Δ569-Dsg1 exhibits markedly reduced affinity for PG compared with that for the full-length cadherin,²⁹ which may explain its inability to compromise desmosomal organization or adhesion (Figures 2 and 3). Thus, our results remain consistent with a model in which ectodomain-truncated Dsg1 sequesters PG to destabilize other desmosomal cadherins and reduce intercellular adhesion.

Elevation of Plakoglobin Levels Restores Adhesion Despite Truncation of Dsg1 or ETA Treatment

Because our data suggested that Δ381-Dsg1 competes with endogenous cadherins for a limited pool of PG, we hypothesized that elevating the cellular levels of PG could ameliorate the effects of Δ381-Dsg1. To test this hypothesis, we generated another retrovirus to deliver a GFP-tagged version of PG (PG-GFP) along with Flag-tagged truncated Dsg1 (Δ381-Dsg1) to keratinocytes. As before, the truncated cadherin caused a profound loss of Dsc3 and DP from intercellular borders in neighboring cells expressing Δ381-Dsg1 compared with nearby control cells that were not transduced; however, ectopic expression of PG, which localized largely to intercellular

Figure 6. Ectopic plakoglobin restores desmosomal organization in cells expressing $\Delta 381$ -Dsg1. NHEKs were cotransduced with $\Delta 381$ -Dsg1 and PG-GFP, seeded as monolayers, and allowed to differentiate for 24 hours in 1.2 mmol/L calcium before fixation (**A** and **C**). Triple immunostaining was used to visualize ectopic Dsg1 (blue), PG (green), and endogenous Dsc3 or DP (red). Two fields are pictured for Dsc3 (**A**) and DP (**C**). **A** and **C**, Zoom: Magnified images show examples of a nonrescued (ie, PG-negative) intercellular border (blue dashed box) versus a rescued (ie, PG-positive) intercellular border (yellow dashed box). **B** and **D**: Quantification of immunofluorescence intensity shows again that Dsc3 and DP are significantly reduced at intercellular borders between pairs of cells expressing $\Delta 381$ -Dsg1 without PG-GFP. However, coexpression of PG-GFP along with $\Delta 381$ -Dsg1 allowed for significant recovery of both Dsc3 and DP at intercellular borders. Student's *t*-test *P* values are shown.

borders along with $\Delta 381$ -Dsg1, was sufficient to rescue desmosomal organization (Figure 6, **A** and **C**). By comparing pairs of cells expressing $\Delta 381$ -Dsg1, we were able to visualize a recovery of both Dsc3 and DP in adjacent keratinocyte pairs expressing PG-GFP along with $\Delta 381$ -Dsg1 (Figure 6A, Zoom, yellow dashed boxes) compared with those that expressed $\Delta 381$ -Dsg1 alone (blue dashed boxes); coexpression of only GFP along with $\Delta 381$ -Dsg1 did not rescue DP or Dsc3 localization (data not shown). Moreover, the mean intensity of Dsc3 and DP localized at intercellular borders revealed a highly significant difference between PG-rescued versus nonrescued keratinocyte pairs (Figure 6, **B** and **D**). These data indicate that increasing PG was sufficient to relieve the negative impact of $\Delta 381$ -Dsg1 on desmosome organization, supporting the notion that sequestration of PG by $\Delta 381$ -Dsg1 is responsible for the destabilization of desmosomes caused by the truncated cadherin.

To extend these cellular findings to the functional adhesion of keratinocytes, we tested whether coexpression of ectopic PG could restore the adhesive strength of monolayers expressing $\Delta 381$ -Dsg1. Again, $\Delta 381$ -Dsg1 markedly increased fragmentation compared with that of

control monolayers expressing the nonmembrane-tethered cytodomain of Dsg1 ($\Delta 569$ -Dsg1); however, ectopic PG-GFP (but not GFP alone) significantly reduced fragmentation of monolayers expressing $\Delta 381$ -Dsg1 (Figure 7, **A** and **C**). Although mechanical integrity was not completely restored to baseline, this result is consistent with the observation that some $\Delta 381$ -Dsg1-expressing cells were not transduced with ectopic PG (Figure 6, **A** and **C**) and would fail to be rescued in their desmosomal organization. Biochemically, ectopic expression of PG (but not GFP alone) together with $\Delta 381$ -Dsg1 was able to significantly restore expression of endogenous desmosomal cadherins (Dsg1 FL and Dsc3) but did not alter the level of endogenous PG or adherens junction components (Figure 7B). These results are consistent with previous reports indicating that restoration of PG expression in PG-null keratinocytes and PG-deficient bladder carcinoma cells can increase the levels of desmosomal cadherins.^{35,40}

Our findings indicate that increasing the level of cellular PG can overcome the sequestering effect of $\Delta 381$ -Dsg1 and is sufficient to stabilize endogenous cadherins, rescue desmosome organization, and restore the adhe-

Figure 7. Plakoglobin rescues intercellular adhesion in keratinocyte sheets despite Dsg1 truncation or ETA treatment. NHEKs were transduced with $\Delta 569\text{-Dsg1}$ or $\Delta 381\text{-Dsg1}$ and subjected to a second infection with either GFP or PG-GFP. Cells were seeded as monolayers and differentiated for 48 hours in 1.2 mmol/L calcium before lysis or fragmentation. **A:** Fragmented monolayers demonstrate that $\Delta 381\text{-Dsg1}$ reduced the integrity of keratinocyte sheets compared with cells expressing $\Delta 569\text{-Dsg1}$, but cotransduction with PG-GFP reduced fragmentation. **B:** Western blot of monolayer lysates showed that $\Delta 381\text{-Dsg1}$ again reduced endogenous Dsg1 and Dsc3 expression; coexpression of PG-GFP along with $\Delta 381\text{-Dsg1}$ restored Dsg1 FL and Dsc3 levels. All other junctional markers (DP, E-cadherin [E-cad], and β -catenin [$\beta\text{-cat}$]) and GAPDH were grossly unaffected. **C:** Triplicate monolayers were fragmented and counted to show a reduction in integrity caused by $\Delta 381\text{-Dsg1}$, but a significant restoration of adhesive strength when PG-GFP was coexpressed. Student's *t*-test *P* values are shown. **D:** NHEKs expressing GFP or PG-GFP were seeded as monolayers and differentiated for 24 hours in 1.2 mmol/L calcium; half were treated with 1 $\mu\text{g/ml}$ ETA. Fragmented monolayers show that ectopic PG was sufficient to prevent the fragmentation induced by toxin-mediated cleavage of endogenous Dsg1. **E:** Western blot of lysates from monolayers revealed that endogenous Dsg1 was fully cleaved in both GFP- and PG-transduced monolayers. Ectopic PG-GFP expression was sufficient to increase Dsc3 expression, whereas DP and GAPDH levels were unchanged. **F:** Triplicate monolayers were fragmented and counted to show that although ETA greatly decreased adhesion in GFP-transduced monolayers, ectopic expression of PG prevented toxin-induced fragmentation. Student's *t*-test *P* values are shown (not significant [NS]; >0.05). RV, retrovirus.

sive capacity of keratinocyte sheets. To verify the physiological relevance of our findings using ectopic truncated Dsg1 ($\Delta 381\text{-Dsg1}$), we tested whether ectopic PG could similarly rescue adhesion in keratinocyte monolayers after ETA-mediated cleavage of endogenous Dsg1. As expected, in GFP-transduced monolayers, ETA treatment resulted in a significant increase in fragmentation due to cleavage of endogenous Dsg1 (Figure 7, D and F). However, in monolayers expressing ectopic PG, the effect of ETA was abolished, suggesting that the increase in PG expression is sufficient to halt the negative effects of toxin-mediated cleavage of endogenous Dsg1. Immunoblotting confirmed that endogenous Dsg1 was fully cleaved by ETA in both control and PG-transduced monolayers (Figure 7E). Moreover, the expression of Dsc3, which was slightly reduced by ETA, was greatly increased by ectopic expression of PG, which probably explains the restoration of intercellular adhesion observed.

Histone Deacetylase Inhibition Ameliorates the Effect of Dsg1 Truncation or ETA on Intercellular Adhesion

Whereas ectopic expression of PG by retroviral transduction was sufficient to promote adhesion in keratinocytes, a pharmacological means to achieve the same outcome might offer therapeutic potential. Previous studies have indicated that PG expression is regulated by the activity of HDACs.³⁸ In fact, multiple HDAC inhibitors have been shown to up-regulate PG expression at the mRNA and protein levels in multiple epithelial cell types, including bladder and lung carcinoma cells.^{39,55} Whether these inhibitors would up-regulate PG in keratinocytes had not been determined, but we hypothesized that such an outcome might overcome the disruptive effect of Dsg1 truncation. Interestingly, TSA treatment (1 $\mu\text{mol/L}$ for 24 hours) of control keratinocytes transduced with GFP did not significantly up-regulate PG expression (Fig-

Figure 8. Histone deacetylase inhibition ameliorates the effect of truncated Dsg1 or ETA treatment on intercellular adhesion. NHEKs were transduced with Δ 381-Dsg1 or GFP as a control. Cells were seeded as monolayers and differentiated for 48 hours in 1.2 mmol/L calcium. At that time, monolayers were treated with 1 μ mol/L TSA or DMSO for 24 hours before fragmentation. **A:** Fragmented monolayers demonstrate that Δ 381-Dsg1 impaired the integrity of keratinocyte sheets compared with that of cells expressing GFP, but treatment with TSA significantly reduced fragmentation in both conditions. **B:** Triplicate monolayers were fragmented and counted to show a reduction in integrity caused by Δ 381-Dsg1, but a significant restoration of adhesive strength by TSA treatment. Student's *t*-test *P* values are shown (not significant [NS]; >0.05). **C:** Western blot of monolayer lysates shows that although TSA treatment may only slightly augment PG expression, it robustly increases the levels of endogenous desmosomal cadherins (Dsg1 and Dsc3) despite expression of Δ 381-Dsg1. No changes were seen in DP or GAPDH levels. **D:** NHEKs were seeded as monolayers and treated with either DMSO or TSA (1 μ mol/L) for 24 hours and treated with ETA (1 μ g/ml) for an additional 24 hours. Western blot of monolayer lysates revealed cleavage of endogenous Dsg1 by ETA and robust up-regulation of Dsc3 expression by TSA treatment. **E:** Triplicate monolayers were fragmented and counted to show that although ETA reduced the integrity of DMSO-treated monolayers, TSA-treated monolayers were resistant to ETA-induced fragmentation. Student's *t*-test *P* values are shown (not significant [NS]; >0.05).

ure 8C). Nevertheless, TSA-treated keratinocytes showed marked induction of endogenous Dsc3, which was destabilized by Δ 381-Dsg1 in previous experiments. Therefore, HDAC inhibition significantly increased intercellular adhesive strength as shown by reduced fragmentation of TSA-treated monolayers compared with that of DMSO-treated controls (Figure 8, A and B). These results are consistent with previous reports of TSA inhibiting invasive behavior and increasing Dsg2 expression in bladder carcinoma cells lacking PG,⁴⁰ but this is, to our knowledge, the first demonstration that HDAC inhibition promotes intercellular adhesion in epidermal keratinocytes.

To test whether TSA treatment could reverse the negative effect of Δ 381-Dsg1 on Dsc3 expression and restore functional adhesion, keratinocytes expressing Δ 381-Dsg1 were similarly treated with 1 μ mol/L TSA for 24 hours before the application of mechanical stress. As shown by a significant reduction in the fragmentation of TSA-treated sheets, HDAC inhibition was sufficient to promote adhesion despite expression of truncated Dsg1 (Figure 8, A and B). To determine whether TSA treatment promoted adhesion by up-regulation of desmosomal ad-

hesion, we analyzed monolayer lysates for expression of desmosome components (Figure 8C). Immunoblotting revealed that TSA did, in fact, augment the expression of endogenous Dsc3 in cells transduced with Δ 381-Dsg1, bringing Dsc3 levels back toward those in DMSO-treated control cells expressing GFP. Extending our results to ETA-induced keratinocyte sheet fragmentation, we found that 1 μ mol/L TSA was also able to ameliorate the loss of adhesion caused by toxin-mediated cleavage of endogenous Dsg1 (Figure 8E) with a concomitant increase in Dsc3 expression (Figure 8D). These findings indicated that HDAC inhibition was sufficient to up-regulate desmosomal cadherin expression in keratinocyte monolayers, overcoming the destructive effect of truncated Dsg1 on intercellular adhesion and could potentially serve as a novel therapeutic strategy to restore adhesion in patients with blistering diseases targeting the desmosome.

Discussion

The essential role of desmosomal cadherins in supporting cutaneous development has been demonstrated by

genetic targeting in mouse models, which can precipitate epidermal fragility, cutaneous barrier dysfunction, and hair follicle defects.^{1,3} Furthermore, their relevance to human disease has been bolstered by the demonstration that desmosomal cadherins are targeted in genetic disorders affecting the skin, hair, and heart as well as in the human autoimmune blistering disease called pemphigus.^{4,5,10} In addition to being a target for autoantibodies, Dsg1 is cleaved by ETA, a staphylococcal protease, which precipitates superficial epidermal blistering.^{13,14} Because the fate of toxin-cleaved Dsg1 and its effect on keratinocyte adhesion had not been thoroughly investigated, in our studies we examined the cellular consequences precipitated by the membrane-tethered Dsg1 cleavage product of ETA. We found that an ectodomain-truncated mutant of Dsg1 (Δ 381-Dsg1) mimics toxin-cleaved Dsg1 and induces similar pathological effects in keratinocytes. Interestingly, we demonstrated that the ability of truncated or ETA-cleaved Dsg1 to disrupt desmosomal organization and reduce adhesive capacity depends on its associated cadherin-stabilizing catenin protein, PG.

Previously, other groups have expressed different desmoglein mutants lacking a normal ectodomain and reported similar destructive effects.^{54,56} In mice, tissue-specific expression of ectodomain-truncated Dsg3 led to disruption of epidermal desmosomes and caused hyperproliferation.⁵⁷ In Madin-Darby canine kidney and A431 cells, expression of Dsg1 lacking its normal ectodomain could disrupt desmosomes and de-stabilize endogenous desmosomal cadherins.^{52,53} These cell types do not normally express the differentiation-dependent cadherin, and even full-length Dsg1 has been shown to disrupt desmosomes under these circumstances.^{52,58} Moreover, it has been shown that a crude deletion of the catenin-binding region could nullify the negative impact of truncated Dsg1.³⁰ Our results are entirely consistent with these previous reports; however, this is the first report of a Dsg1 truncation mutant expressed in primary human keratinocytes that exactly mimics the product of exfoliative toxin-mediated cleavage with clear implications for disease pathogenesis. In addition, by uncoupling PG via point mutation and, further, by rescuing functional adhesion with ectopic PG in epidermal keratinocyte monolayers, we provide strong evidence for the role of catenin binding in the ability of truncated Dsg1 to destabilize other desmosomal cadherins and compromise epidermal integrity.

Other studies of classic cadherins have indicated that the shed portion of the adhesive ectodomains after cleavage by matrix metalloproteinases can affect adhesion of epithelial cells.⁵⁹ For desmosomes in particular, it has been shown that small peptides interfering with the N-terminal cell adhesion recognition sites of desmogleins and desmocollins, required for mediating trans-adhesion, can disrupt cellular sorting and promote invasive behavior.^{60,61} The results reported here indicate that the membrane-tethered end-product of Dsg1 cleavage by the staphylococcal protease, ETA, is sufficient to cause significant pathological changes on its own, but we have not ruled out a contribution of the shed ectodomain of

Dsg1, which could interfere with cadherin dimerization and contribute to the pathogenesis of BI and SSSS. Previous studies from our laboratory demonstrated that cleavage of Dsg2 by matrix metalloproteinases produces a shed ectodomain and a retained fragment of the cadherin, leading to compromised adhesion in epithelial cancer cells.^{62,63} Whether a PG-dependent mechanism similar to that which drives the loss of adhesion upon ETA cleavage of Dsg1 might operate in the down-regulation of desmosomal adhesion in carcinogenesis remains to be determined.

Of interest, the specificity of the interaction between ETA and the ectodomain of human Dsg1 was originally demonstrated by substituting several human residues for the corresponding amino acids found in canine Dsg1, which is bound by the toxin but is resistant to cleavage.¹⁴ This manipulation generated a hybrid canine Dsg1 ectodomain that was susceptible to ETA hydrolysis. Thus, it might be possible to produce a small peptide corresponding to the region of human Dsg1 bound by ETA, but harboring a few crucial amino acid substitutions, as found in canine Dsg1, to prevent its proteolysis. Similar to the suggested use of toxin-inactivating antibodies,⁶⁴ administration of such a decoy peptide in life-threatening situations, such as in patients with SSSS, could perhaps halt toxin-induced blistering long enough to allow antibiotic treatment to eliminate the underlying infection and permit reestablishment of the cutaneous barrier.

The blistering observed in exfoliative toxin-mediated disease is histologically similar to that seen in PF, although these two diseases target Dsg1 in different ways. Interestingly, the pattern of Dsg1 internalization observed in our ETA-treated organotypic cultures (Figure 1D) was consistent with a previous observation of Dsg1 relocation in canine PF.⁶⁵ Thus, we suggest that a common mechanism may underlie both toxin- and antibody-induced blistering. In pemphigus, IgG antibodies against desmogleins and desmocollins have been isolated from patients and the former are sufficient to induce pathology in mice or human epidermal explants.^{21,66–70} Various models have been proposed to explain how pemphigus IgG binding to the desmosomal cadherins leads to loss of adhesion:^{71,72} direct steric hindrance of cadherin trans-dimerization;^{11,73} outside-in induction of pathogenic signaling, including via PG;^{37,74,75} or internalization and degradation of cadherins.^{36,76,77} In fact, evidence exists for all these mechanisms of antibody-induced blistering.

We propose that IgG-bound desmogleins may function similarly to ETA-cleaved Dsg1 because both targeted proteins are compromised in their ability to mediate trans-adhesion. The dominant-negative effects of these adhesion-deficient cadherins could explain the localization of blistering in pemphigus and BI and SSSS. Lesions occur where the targeted cadherins are expressed in sufficient amounts to grossly compromise adhesion, namely in the basal layer for Dsg3 in pemphigus vulgaris or the upper granular layer for Dsg1 in pemphigus foliaceus, BI and SSSS. Whether internalization and degradation of IgG-bound desmoglein or ETA-cleaved Dsg1 in epidermis is an attempt by the cell to rid itself of the dominant-negative effects of an adhesion-deficient cad-

herin remains to be determined but may suggest an alternative therapeutic strategy to reduce blister formation. Intriguingly, PG has been shown to co-internalize with antibody-bound Dsg3 in cells during desmosome disassembly after treatment with pemphigus vulgaris autoantibodies.³⁶ Perhaps antibody-bound Dsg3, which is routed for degradation along with PG, functions similarly to ectodomain-truncated Dsg1 to sequester PG in non-adhesive cadherin complexes. Further implicating its catenin partner in the cellular mechanisms driving epidermal blistering due to desmosome disruption, PG-null keratinocytes are markedly impaired in their responsiveness to pemphigus vulgaris antibodies.³⁷ Thus, we suggest that both autoantibody- and exfoliative toxin-induced blistering may rely on a common mechanism involving PG.

In summary, this study provides insight into the pathogenesis of exfoliative toxin-mediated disease by examining the cellular mechanism by which ectodomain-truncated Dsg1 precipitates epidermal blistering. The aim of current therapy for BI and SSSS is solely to rid patients of the underlying staphylococcal infection, which secretes the pathogenic toxin.^{15–17,78,79} Unfortunately, increasing resistance among both nosocomial and environmental strains of *S. aureus* promises to make treatment of BI and SSSS much more challenging.^{80–82} No current therapy exists to directly target the toxin nor its downstream effects on epidermal keratinocytes. This study has revealed a dominant-negative effect of an N-terminal truncation of Dsg1 mimicking the toxin's cleavage product, which occurs through its ability to bind PG. Importantly, the effects of either ectodomain-truncated or toxin-cleaved Dsg1 could be overcome by increasing the amount of PG in cells, which stabilized other cadherins, restored desmosomal organization, and rescued adhesive strength of keratinocyte monolayers. Whether prevention of ETA-induced epidermal blistering could be achieved *in vivo* via modulation of PG remains to be determined. However, it was recently shown that ectopic expression of Dsg2 could prevent ETA-induced blistering in mice,⁸³ suggesting that pharmacological agents capable of up-regulating other desmosomal cadherins might prove therapeutic for BI and SSSS. Consistent with this suggestion, we have shown that an HDAC inhibitor augmented Dsc3 expression in keratinocytes and inhibited the effects of ETA on intercellular adhesion. Thus, it seems possible that HDAC inhibitors, which are already in therapeutic use,^{84–86} could represent a novel treatment approach for blistering diseases targeting the desmosome.

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