Desmoglein Endocytosis and Desmosome Disassembly Are Coordinated Responses to Pemphigus Autoantibodies*

Received for publication, November 21, 2005, and in revised form, December 22, 2005  Published, JBC Papers in Press, December 23, 2005, DOI 10.1074/jbc.M512447200

Cathérine C. Calkins†1, Shannon V. Setzer†1,2, Jean Marie Jennings1, Susan Summers§, Kazuyuki Tsunoda‡, Masayuki Amagai§, and Andrew P. Kowalczyk†3

From the †Departments of Dermatology and Cell Biology, Emory University School of Medicine, Atlanta, Georgia 30322 and §Department of Dermatology, Keio University School of Medicine, Shinjuku-ku 160-8582, Japan

Desmosomes are adhesive intercellular junctions prominent in the skin and heart. Loss of desmosome function is associated with severe congenital and acquired disorders characterized by tissue fragility. Pemphigus vulgaris (PV) is an autoimmune disorder in which antibodies are directed against the desmosomal adhesion molecule Dsg3, resulting in severe mucosal erosions and epidermal blistering. To define the mechanisms by which Dsg3 autoantibodies disrupt keratinocyte adhesion, the fate of PV IgG and various desmosomal components was monitored in primary human keratinocytes exposed to PV patient IgG. PV IgG initially bound to keratinocyte cell surfaces and colocalized with desmosomal markers. Within 6 h after PV IgG binding to Dsg3, electron microscopy revealed that desmosomes were dramatically disrupted and keratinocyte adhesion was severely compromised. Immunofluorescence analysis indicated that PV IgG and Dsg3 were rapidly internalized from the cell surface in a complex with plakoglobin but not desmoplakin. Dsg3 internalization was associated with retraction of keratin filament networks from cell–cell borders. Furthermore, the internalized PV IgG-Dsg3 complex colocalized with markers for both endosomes and lysosomes, suggesting that Dsg3 was targeted for degradation. Consistent with this possibility, biotinylation experiments demonstrated that soluble Dsg3 cell surface pools were rapidly depleted followed by loss of detergent-insoluble Dsg3. These findings demonstrate that Dsg3 endocytosis, keratin filament retraction, and the loss of keratinocyte cell–cell adhesion are coordinated responses to PV IgG.

Desmosomes are adhesive intercellular junctions that mediate tight adhesion between epithelial cells (1, 2). Desmosomes are particularly prominent in tissues that experience mechanical stress, such as the skin and heart, and function as plasma membrane attachment sites for intermediate filaments. The importance of desmosomes in tissue function and integrity has been revealed by numerous genetic and autoimmune disorders that impact desmosomal components (3, 4). The desmosomal cadherins, the desmogleins and desmocollins, are the transmembrane components of desmosomes responsible for mediating cell–cell adhesion (5). The tails of the desmosomal cadherins interact with the cytoplasmic protein plakoglobin along with other related proteins to couple the cadherins to desmoplakin and the intermediate filament cytoskeleton (2, 6). A number of desmoglein and desmocollin isoforms have been identified, and the genes encoding these proteins are expressed in a tissue- and differentiation-specific manner (5, 7). Mutations in genes encoding desmosomal components lead to heart and skin disorders (3, 8, 9). Similarly, autoantibodies directed against the desmosomal cadherins lead to a class of severe epidermal blistering disorders termed pemphigus (10). These disorders underscore the importance of understanding how desmosomes assemble, disassemble, and contribute to tissue architecture and function.

The mechanisms by which intercellular junctions assemble and disassemble are not fully understood. However, recent work has focused attention on cadherin endocytosis as a means by which cells maintain a dynamic state of cell-cell contact (11–13). E-cadherin has been shown to be internalized and recycled back to the plasma membrane in a constitutive manner (14, 15). The decision to recycle cadherins back to the plasma membrane for engagement in additional rounds of adhesion or to target the cadherin for degradation appears to be coordinated with cellular pathways that regulate tumorigenic transformation, such as Src activation (16). In the case of both E-cadherin and VE-cadherin, the armadillo family protein p120-catenin appears to play a key role in cadherin membrane trafficking (11, 17, 18). Specifically, the loss of p120 leads to cadherin endocytosis and degradation in the lysosome, revealing a key role for p120 in modulating the presentation of classical cadherins at the plasma membrane and in the modulation of cadherin expression levels (19, 20). Nonetheless, it is currently unclear how changes in cadherin membrane trafficking are integrated into the pathophysiology of various epithelial tissues and how the balance between junction assembly and turnover is modulated during disease. Furthermore, virtually nothing is known about the cellular pathways that regulate cell surface presentation of the desmosomal cadherins. Because of the severe consequences of genetic or acquired disorders of the desmosome, it is important to understand how desmosomal cadherin membrane trafficking might be altered during disease states characterized by the loss of cell–cell adhesion and tissue fragility.

Pemphigus vulgaris (PV) is an autoimmune disease characterized by the loss of adhesion (acantholysis) between epithelial cells in the oral mucosa (4, 10). A series of studies has demonstrated that antibodies directed against the desmosomal cadherin desmoglein-3 (Dsg3) are both necessary and sufficient to cause blistering characteristic of PV patients (21). Dsg3-specific IgG purified from patient sera has been shown to cause acantholysis when injected into mice (22). Likewise, recombinant Dsg3 can be used to deplete patient sera of disease-causing antibodies, demonstrating that autoantibodies directed against Dsg3 are the cause of blistering and oral erosions observed in PV patients (23).

The abbreviations used are: PV, pemphigus vulgaris; Dsg3, desmoglein-3; NHK, normal human keratinocytes; KGM, keratinocyte growth medium; NH, normal human; PBS +, phosphate-buffered saline containing Ca2+ + Mg2+ ; PIPES, 1,4-piperazinediethanesulfonic acid; NHS, normal human serum.

© 2006 by The American Society for Biochemistry and Molecular Biology, Inc. Printed in the U.S.A.
**Pemphigus IgG Endocytosis**

Structural studies of classical cadherins (E- and N-cadherin) indicate that the amino-terminal domain of the cadherins forms trans dimers with cadherins from adjacent cells (24). Interestingly, pemphigus IgGs typically recognize the amino-terminal domain of the desmogleins, suggesting that pemphigus autoantibodies may target the adhesive interface of the desmosomal cadherins (22, 25, 26). This possibility is supported further by the experimental generation of Dsg3 antibodies in pemphigus model mice by adoptive transfer of Dsg3 \(^{-/-}\) lymphocytes (27). Antibodies generated by these mice were found to cause PV-like blistering. Furthermore, one antibody, AK23, which was isolated from PV model mice, was able to induce the PV phenotype and recognized sequences likely to be involved in the formation of the adhesive interface of Dsg3. These and numerous other studies provide strong evidence that PV IgG targets Dsg3 and causes blistering by disrupting adhesive interactions dependent upon Dsg3 function (4).

Interestingly, several studies have suggested that PV autoantibodies are internalized from the cell surface and that endocytosis of the antibodies may be important in PV pathogenesis (28–32). Recent advances in membrane trafficking pathways have resulted in the identification of molecular markers for specific endocytic compartments (33, 34), thereby providing a means to define the fate of PV IgG and desmosomal proteins after ligation of Dsg3 at the cell surface. To begin an investigation of the cellular and molecular pathways modulated upon PV IgG binding to Dsg3, primary cultures of human keratinocytes were used to investigate the effects of PV IgG on desmosome structure and keratinocyte adhesion. Our results suggest that PV autoantibodies trigger two major events after ligation of Dsg3 at the cell surface. First, PV IgG cause the disassembly of desmosomes at or near the cell surface and the retraction of keratin filaments from lateral cell-cell borders. Secondly, PV IgG trigger the co-endocytosis of Dsg3 and plakoglobin, leading to delivery of Dsg3 to lysosomal compartments and a dramatic decrease in Dsg3 protein levels. These findings suggest a coordinated cellular response to PV IgG in which Dsg3 endocytosis, the disassembly of the desmosomal plaque, and keratin filament retraction from the cell surface are mechanistically coupled to the loss of keratinocyte adhesion in response to pemphigus patient antibodies.

**EXPERIMENTAL PROCEDURES**

**Cells and Culture Conditions**—Normal human keratinocytes (NHKs) isolated from neonatal foreskin (Emory Skin Disease Research Center) were cultured in keratinocyte growth medium (KGM, Cambrex Corp., East Rutherford, NJ). Before experimentation, keratinocytes at passage 3 or 4 were seeded into tissue culture dishes, and calcium was switched to KGM medium containing 0.5 mM calcium 16–18 h before treatments and remained in this medium for the remainder of experimentation.

**Immunofluorescence**—NHKs cultured on glass coverslips were switched to KGM containing 0.5 mM calcium 16–18 h before treatment with either normal human serum (Irvine Scientific, Santa Ana, CA), sera from PV patients, or affinity-purified IgG from either normal human (NH) or PV patient sera. Under the culture conditions used throughout this study, the keratinocytes express predominantly Dsg3 and detectable but low levels of Dsg1. For our studies, sera from three different PV patients were tested and found to have similar effects. No differences were noted between IgG derived from patients with mucosal PV with autoantibodies against Dsg3 versus patients with mucocutaneous PV harboring autoantibodies against both Dsg3 and Dsg1.

For most experiments IgG was allowed to bind to cells by incubating cultures on ice for 30 min to 1 h at a concentration of 1 mg/ml with serum diluted in KGM containing 0.5 mM calcium and then returned to 37 °C for indicated times. Cells were fixed on ice using either −20 °C methanol for 5 min or 3.7% paraformaldehyde for 10 min followed by extraction in 0.5% Triton X-100 for 7 min. For monitoring cell surface desmoglein 3, cells were fixed on ice for 10 min in 3.7% paraformaldehyde. The localization of desmosomal and adherens junction components were monitored using the following antibodies: mouse anti-desmoglein 3, AK15 (27), mouse anti-desmoplakin 1 and 2 (Transduction Laboratories, San Diego, CA), mouse anti-plakoglobin (Transduction Laboratories), or mouse anti-B-catenin (Transduction Laboratories). Cytokeratin was detected using a mouse anti-cytokeratin antibody (Immunotech, Marseille, France). Either a polyclonal antibody against cathepsin D (Upstate, Lake Placid, NY) or a monoclonal antibody H5C6 directed against CD63 (Developmental Studies Hybridoma Bank at the University of Iowa) was used to detect late endosomes/lysosomes. The localization of early endosomes was monitored using a mouse monoclonal EEA-1 antibody (Transduction Laboratories) or a rabbit polyclonal EEA-1 antibody (Affinity Bioreagents, Golden, CO). Appropriate species cross-absorbed secondary antibodies conjugated to various Alexa Fluors (Molecular Probes, Eugene, OR) were used for dual-label immunofluorescence. A Leica DMR-E fluorescence microscope equipped with narrow band-pass filters and a Hamamatsu Orca camera was used for image acquisition. Images were captured and processed using either Open Lab software (Improvision, Inc., Lexington, MA) or Simple PCI (Compix, Inc., Cranberry Township, PA).

**Immunoelectron Microscopy**—NHKs were prepared as above and treated with either normal human serum or pemphigus serum. Cells were fixed for 30 min at room temperature in 4% paraformaldehyde with 0.1% glutaraldehyde followed by overnight fixation of 2% paraformaldehyde at 4 °C. Cells were then permeabilized with 0.05% Triton X-100 for 15 min followed by a 30-min block in goat serum. Human IgG were monitored by incubating cells with goat anti-human 5-nm gold-conjugated secondary antibodies (Aurion, Wageningen, The Netherlands) followed by silver enhancement. Samples were then processed for conventional electron microscopy.

**Dispase-based Dissociation Assay**—A dispase dissociation assay was performed as described previously (35). Briefly, NHK cultures were seeded in triplicate onto 35-mm dishes and allowed to grow to confluence. 24 h after reaching confluency, cultures were then switched to 0.5 mM calcium containing KGM for 18 h. The cells were treated with normal human serum or PV serum diluted 1:4 in KGM media containing 0.5 mM calcium and incubated on ice for 30 min to 1 h. Cells were then supplemented with additional KGM media containing 0.5 mM calcium and incubated at 37 °C for various amounts of time. After incubation, cells were washed three times in PBS + and then incubated in 1 unit/ml dispase in PBS + (Roche Diagnostics) for more than 30 min. Released monolayers were subjected to mechanical stress by transferring the cell sheets to 15-ml conical tubes containing 11 ml final volume of PBS +. The tubes were subjected to 30 inversion cycles on a rocker panel. Fragments were counted using a dissecting microscope.

**Internalization Assay**—IgG internalization was performed as previously described (19). NHKs were cultured on glass coverslips for experimentation. 1 mg/ml concentrations of either NH IgG or PV IgG was incubated with cells at 4 °C on ice for 30 min in KGM media containing a final concentration of 0.5 mM calcium. Cells were then washed 3 times with PBS + followed by incubation at 37 °C for 0–6 h in 0.5 mM calcium containing KGM media. The cells were rinsed, fixed, and processed for dual label immunofluorescence as described above. For the 24-h time points, the cells were prepared identically as the 6-h incubation with the exception that the PV IgG and NH IgG were not removed after incubation on ice.
Cell Surface Biotinylation—NHKs were grown to 70% confluency in 60-mm dishes in KGM media. 18 h before biotinylation the cell culture media was changed to that of media containing 0.5 mM calcium. Cultures were washed three times in cold PBS and labeled with EZ-Link Sulfo NHS-SS-Biotin (Pierce) at 1 mg/ml in PBS on ice for 1 h. Excess biotin was quenched with 3 washes on ice for 5 min each in PBS containing 50 mM ammonium chloride and followed by 4 washes in cold PBS. Cells were treated with either NH or PV IgG at a final concentration 1 mg/ml in KGM media containing 0.5 mM calcium on ice for 1 h followed by incubation at 37 °C for the indicated times. Samples were lysed using Cytoskeleton buffer (50 mM NaCl, 10 mM PIPES, 3 mM MgCl2, 300 mM sucrose, 1% Triton X-100, 10 mM NaF, 10 mM NaP2O7, pH 6.8) containing protease inhibitor mixture. After centrifugation at 14,000 g, the supernatant was recovered and incubated with Ultra-Link immobilized streptavidin (Pierce) for 2 h at 4 °C. Cell surface biotin-labeled proteins were captured by centrifugation, and the beads were washed four times in Cytoskeleton lysis buffer. Biotin-labeled proteins were released in 2× Laemmli buffer containing β-mercaptoethanol at 95 °C and then resolved by 7.5% SDS-PAGE and transferred to nitrocellulose according to standard protocols. The membranes were analyzed for both desmoglein 3 and E-cadherin using either an anti-Dsg3 mixture containing monoclonal antibodies AK15 and anti-Dsg3 (Zymed Laboratories Inc., South San Francisco, CA) or mouse anti-E-cadherin (BD Biosciences).

Sequential Detergent Extraction and Western Blot Analysis—Sequential detergent extraction was performed as described (36) with the following exceptions. Cells were grown on 60-mm plates and extracted for 10 min on iced Triton buffer (1% Triton X-100, 10 mM Tris, pH 7.5, 140 mM NaCl, 5 mM EDTA, 2 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml pepstatin A). Cells were scraped using a rubber policeman, vortexed for 30 s, and centrifuged at ~14,000 × g for 30 min at 4 °C. After centrifugation, the Triton-soluble pool was transferred to a fresh tube. The Triton-insoluble proteins were solubilized in SDS-urea buffer (1% SDS, 8 M urea, 10 mM NaF, 10 mM NaP2O7, pH 7.5, 5 mM EDTA). The samples were analyzed by SDS-PAGE followed by immunoblot analysis using chemiluminescence (ECL, Amersham Biosciences).

RESULTS

PV IgG Disrupt Desmosomes and Compromise Keratinocyte Adhesive Strength in Vitro—The role of Dsg3 autoantibodies in the pathogenesis of PV has been well established, but the precise mechanism by which these antibodies disrupt adhesion and lead to acantholysis in vivo remains unclear. To investigate the mechanism by which Dsg3 antibodies cause acantholysis, we sought to establish an in vitro system to model the disease and to determine how keratinocytes respond to pathogenic antibodies. The effect of PV IgG on desmosome assembly in primary human keratinocytes was evaluated by immunofluorescence microscopy. Cells exposed to purified NH IgG for 24 h exhibited well developed desmosomes with extensive desmoplakin staining at cell-cell contacts (Fig. 1, A and B). IgG binding to the cell layer was detected in some cells, but no junctional localization of the IgG was detected. In contrast,
keratinocytes exposed to IgG from PV patients for 24 h exhibited dramatic alterations in desmoplakin organization (Fig. 1, C and D). In control cells punctate desmoplakin staining was prominent at cell borders throughout the keratinocyte culture. However, junctional desmoplakin staining was discontinuous, and desmoplakin was found redistributed to interior regions of the cytoplasm in keratinocytes exposed to PV IgG.

FIGURE 2. PV IgG disrupt desmosomes and enter perinuclear vesicular compartments. Keratinocytes were incubated with NHS or PV sera, and the localization of human IgG was determined by pre-embedding immunogold electron microscopy using silver enhancement. PV IgG-decorated desmosomes in keratinocytes incubated with PV sera at 4 °C (A and B). Note the large number of well developed desmosomes along lateral borders and the numerous keratin filament bundles that extend out toward the cell periphery and attach to desmosomal plaques. After a 6-h exposure to PV IgG at 37 °C, desmosomes were absent from many cell borders, and keratin filaments were retracted (C). PV IgG were often detected in perinuclear vesicles (D and E), but these vesicular pools of gold labeling did not exhibit electron dense desmosomal plaques. Furthermore, PV IgG detected at the cell surface were often associated desmosomes that appeared to be split and partially disassembled (F, arrow). Control keratinocytes incubated in NHS for 6 h at 37 °C exhibited extensive desmosomes (G and H). To determine whether keratinocytes exposed to PV IgG exhibited decreased adhesive strength, confluent cultures of keratinocytes were incubated in the presence of NHS or PVs for 24 h. The cells were then released from the substrate by dispase treatment and subjected to mechanical stress. The cells were then photographed, and the number of cellular particles was counted (I). Results are representative of at least three independently conducted experiments using multiple culture wells.
The alterations in desmoplakin localization were associated with keratin filament retraction from the cell surface in keratinocytes treated with PV IgG. In control keratinocytes treated with NH IgG, keratin filaments extended to the cell periphery and were clearly visible at cell-cell boundaries (Fig. 1, E, F, and G). In contrast, keratin filaments were retracted from cell-cell junctions in keratinocytes exposed to PV IgG for 24 h (Fig. 1, H, I, and J). DIC imaging indicated that although the cells exposed to PV IgG exhibited disrupted desmosomes, the cells remained in close proximity. Similarly, E-cadherin distribution was largely unchanged in keratinocytes exposed to PV IgG for 24 h (not shown).

The effects of PV IgG on desmosomes were further characterized by pre-embedding immunoelectron microscopy and by functional assays to assess keratinocyte adhesive strength. PV IgG bound to desmosomes and the cell surface of keratinocytes incubated at 4 °C (Fig. 2, A and B). Desmosomes were prominent, and keratin filaments extended to the cell periphery and were visible at virtually every desmosomal junction. However, in keratinocytes incubated at 37 °C for 6 h in the presence of PV IgG, desmosomes were disrupted, and keratin filaments were retracted from the plasma membrane (Fig. 2, C, D, and E). Vesicular structures containing immunogold-labeled IgG were routinely observed (Fig. 2, D and E). Interestingly, PV IgG internalized into these cytoplasmic vesicles did not appear to colocalize with internalized desmosomes or half-desmosomes. In addition, PV IgG that remained at the cell surface was often associated with desmosomes that appeared to be splitting and partially disassembled (Fig. 2F, arrow). Control keratinocytes exposed to NHS for 6 h at 37 °C exhibited extensive desmosomes that were often associated with keratin filament bundles (Fig. 2, G and H). These results indicate that PV IgG specifically disrupt desmosomes and cause keratin filament retraction from cell-cell borders.

To determine whether the disruption of desmosomes in response to PV IgG resulted in a reduction in keratinocyte cell-cell adhesive strength, a dispase-based assay was used to assess the strength of cell-cell adhesion (35, 37). Dispase disrupts cell-matrix interactions but not cell-cell adhesion. Therefore, keratinocytes were released from the substrate using dispase and then placed in conical tubes and rotated to expose the keratinocyte cell layer to mechanical stress. Under these conditions keratinocytes incubated with normal human sera remained as an intact sheet of cells. In contrast, keratinocytes exposed to PV sera dispersed into numerous multicellular aggregates (Fig. 2). Identical results were obtained with purified IgG fractions from multiple patients (not shown). These results demonstrate that PV IgG causes changes in desmoplakin and keratin organization and disrupt desmosome morphology, as assessed by electron microscopy. These changes are associated with a dramatic reduction in the strength of keratinocyte cell-cell adhesion.

The mechanism by which pemphigus IgG disrupts desmosomes may involve steric hindrance of desmoglein adhesive interactions. However, several studies have suggested that keratinocyte responses are required after IgG ligation of desmogleins in order for pemphigus IgG to disrupt adhesion (38, 39). The latter possibility predicts that incubation of keratinocytes with PV IgG at low temperature should prevent the disruption of adhesion and that the loss of adhesion would occur over time for cells incubated at 37 °C. Therefore, keratinocytes were incubated with PV IgG or normal human IgG for 1 h at 4 °C. Under these conditions, PV IgG bound to keratinocyte cell surfaces and decorated Dsg3 located on the cell surface and at cell-cell borders (Fig. 3E). Desmoplakin remained localized at the cell-cell borders (Fig. 3A) and appeared indistinguishable from desmoplakin in control cells incubated with NH IgG (not shown). Note that these same conditions were also used for the immunoelectron microscopy studies shown in Fig. 2, A and B. Furthermore, in cells incubated on ice, PV IgG caused no change in keratinocyte adhesive strength relative to control cells incubated in NH IgG under the same conditions (Fig. 3D). These data indicate that desmosomes are not disrupted by PV IgG binding to cell surface Dsg3 if cells are metabolically unable to respond. In contrast, incubation at 37 °C led to substantial alterations in desmoplakin localization (Fig. 3, A–D) that correlated with a loss of adhesive strength over time (Fig. 3I). However, these changes occurred over a time course of several hours. In addition, the loss of adhesion correlated with the loss of PV IgG localization at cell-cell borders and with the appearance of PV IgG within intracellular vesicles. Together, these data indicate that cellular responses are
required for PV IgG to disrupt adhesion and that internalization of PV IgG is temporally associated with the disruption of desmosomes and the loss of keratinocyte adhesion.

**PV IgG Causes Co-internalization of Dsg3 and Plakoglobin and Delivery of the Complex to Degradative Endocytic Compartments—**As discussed above, PV IgG was often detected in vesicular compartments (Fig. 2, D and E, and Fig. 3, E–H), suggesting that endocytosis of the PV IgG complex is an early event in the disruption of desmosomes. Therefore, the fate of PV IgG relative to other desmosomal components was examined using deconvolution fluorescence microscopy. For these experiments, PV IgG was allowed to bind to the surface of keratinocytes incubated at 4 °C. In some cases keratinocytes were then transferred to 37 °C to determine the fate of PV IgG and various desmosomal components. In keratinocytes incubated at 4 °C, NH IgG did not exhibit extensive binding to keratinocytes or colocalization with Dsg3 (Fig. 4, A–C). In contrast, PV IgG bound to keratinocyte cell surfaces and colocalized extensively with Dsg3 (D–F). After 6 h at 37 °C, PV IgG was detected both at the cell surface and in vesicular pools. Both pools of Dsg3 exhibited colocalization with PV IgG (G–I). The internalized PV IgG also colocalized with plakoglobin (J–L) but not the adherens junction protein β-catenin (M–O). The PV IgG-Dsg3 complex colocalized with desmoplakin at cell-cell borders (P–R and arrows in R), but the internalized pool of PV IgG did not co-localize with desmoplakin. Bar (F) = 25 μm.

**FIGURE 4. Dsg3 and plakoglobin are co-internalized with PV IgG.** The localization of PV IgG was determined after 6 h of exposure to keratinocytes using immunofluorescence microscopy followed by deconvolution of z-series images. Dsg3 was localized using the Dsg3 monoclonal antibody AK15. Dsg3 was localized to desmosomes in keratinocytes exposed to NH IgG at 4 °C (A–C). PV IgG bound to keratinocyte cell surfaces and colocalized extensively with Dsg3 (D–F). After 6 h at 37 °C, PV IgG was detected both at the cell surface and in vesicular pools. Both pools of Dsg3 exhibited colocalization with PV IgG (G–I). The internalized PV IgG also colocalized with plakoglobin (J–L) but not the adherens junction protein β-catenin (M–O). The PV IgG-Dsg3 complex colocalized with desmoplakin at cell-cell borders (P–R and arrows in R), but the internalized pool of PV IgG did not co-localize with desmoplakin. Bar (F) = 25 μm.
desmoplakin at desmosomes (Fig. 4R, arrows), but the internalized pool of PV IgG did not colocalize with desmoplakin (Fig. 4, P–R). The fact that the internalized PV IgG complex colocalized with Dsg3 and plakoglobin, but not desmoplakin, suggests that interactions between desmosomal plaque components are disrupted during PV IgG-Dsg3 internalization.

To determine the subcellular localization of the internalized PV IgG-Dsg3 complex, molecular markers for endocytic compartments were used to determine the fate of the internalized Dsg3. PV IgG bound to keratinocyte cell surfaces but did not co-localize with the early endosome marker EEA-1 in keratinocytes incubated at 4 °C (Fig. 5, A–C). After 1 h at 37 °C, internalized PV IgG exhibited extensive colocalization with the early endosome marker EEA-1 (D–F). By 3 h, numerous vesicular pools of PV IgG colocalized with the late endosome/lysosome marker CD63 (G–I) and the lysosomal enzyme cathepsin D (J–L).

![FIGURE 5. PV IgG are internalized and delivered to degradative endocytic compartments. Deconvolution microscopy was used to determine the fate of internalized PV IgG using specific markers for subcellular compartments. PV IgG bound to the cell surface and did not co-localize with EEA-1 in cells incubated at 4 °C (A–C). After 1 h at 37 °C, internalized PV IgG exhibited extensive colocalization with the early endosome marker EEA-1 (D–F). By 3 h, numerous vesicular pools of PV IgG colocalized with the late endosome/lysosome marker CD63 (G–I) and the lysosomal enzyme cathepsin D (J–L).](image)

**FIGURE 6. Plakoglobin is delivered to endosomal compartments upon PV IgG treatment.** Deconvolution microscopy was used to determine the fate of plakoglobin upon PV IgG exposure using specific markers for early (EEA-1) and late (CD63) endosomal compartments. The localization of plakoglobin was determined after 3 h of exposure to PV IgG at 37 °C. Plakoglobin exhibited extensive colocalization with the early endosome marker EEA-1 (A–C) and late endosomes/lysosomes (D–F). Plakoglobin exhibited little or no localization with these markers in keratinocytes treated with NH IgG (not shown). The adherens junction protein β-catenin (β-cat; G–I) and the intermediate filament binding protein desmplakin (J–L) failed to colocalize with EEA-1.

![FIGURE 6. Plakoglobin is delivered to endosomal compartments upon PV IgG treatment.](image)
against the extracellular domain of Dsg3 (Fig. 8). The antibody used, AK15, has been characterized previously and found to bind to the central region of the Dsg3 extracellular domain (27). Therefore, AK15 was used to detect total cell surface Dsg3 levels by conducting immunofluorescence analysis of unpermeabilized keratinocytes exposed to NH IgG or PV IgG for various amounts of time. As shown in Fig. 8, keratinocytes incubated at 4 °C with PV IgG or NH IgG exhibited similar amounts of cell surface Dsg3. However, in keratinocytes exposed to PV IgG followed by incubation at 37 °C for various amounts of time, total Dsg3 cell surface levels were rapidly depleted compared with control cells incubated with NH IgG. These studies indicate that both non-desmosomal and desmosomal pools of cell surface Dsg3 are depleted by PV IgG.

The loss of cell surface Dsg3 and the colocalization of the PV IgG complex with lysosomal markers suggested that Dsg3 may be internalized and degraded upon PV IgG binding. To test this possibility, keratinocytes were exposed to PV IgG for 24 h, and the levels of various desmosomal and adherens junction components were examined by Western blot analysis. In virtually every experiment performed, PV IgG caused a decrease in steady state levels of Dsg3 (Fig. 9). Interestingly, desmoplakin levels were often decreased, although not substantially (not shown). Furthermore, no change was detected in either plakoglobin or E-cadherin (Fig. 9). These findings demonstrate that PV IgG cause Dsg3 internalization, delivery to late endosomal/lysosomal compartments, the destabilization of cell surface pools of Dsg3, and ultimately the depletion of total cellular levels of Dsg3.

DISCUSSION

The results of this study indicate that PV autoantibodies trigger a coordinated cellular response that includes disassembly of desmosomes, retraction of keratin intermediate filaments, endocytosis of Dsg3, and the loss of cell-cell adhesion. After internalization from the cell surface, the PV IgG/Dsg3 complex is targeted for lysosomal degradation, leading to the depletion of Dsg3 protein levels. The PV IgG-Dsg3 complex is co-internalized with plakoglobin, whereas desmoplakin appears to dissociate from the internalized cadherin-plakoglobin complex. These observations suggest that pemphigus autoantibodies may not only disrupt desmosomal mediated adhesion but also may trigger the disruption of protein complexes within the desmosomal plaque. Thus, pemphigus IgG binding to Dsg3 triggers a cascade of events leading to the disruption of desmosomes and the loss of adhesion molecules on the cell surface.

Previous studies have indicated that PV IgG are internalized by keratinocytes (28–32), suggesting that PV IgG internalization may be
related to pemphigus pathogenicity. In a human squamous cell carcinoma cell line Dsg3 levels were reportedly decreased by PV IgG treatment (40). However, none of these previous studies examined the fate of the PV IgG with respect to other desmosomal components and to molecular markers for membrane trafficking pathways. In the present study we systematically examined this issue in primary cultures of human keratinocytes using several biochemical and immunofluorescence-based approaches to define the fate of Dsg3 after PV IgG ligation. Our results demonstrate that Dsg3 is internalized from the plasma membrane and targeted for degradation via an endolysosomal pathway. Interestingly, plakoglobin, but not desmoplakin, appears to be co-internalized with the PV-IgG-Dsg3 complex (Fig. 4). These results raise the possibility that plakoglobin may play some role in cadherin internalization. In fact, plakoglobin has been implicated in PV pathogenesis (41). Using keratinocytes derived from plakoglobin null mice, Müller and co-workers (41) found that plakoglobin was required for keratin filament retraction and the loss of adhesion observed in cultured keratinocytes exposed to PV IgG. It will be important to further define the

FIGURE 8. Total cell surface Dsg3 levels are depleted in response to PV IgG. Keratinocytes cultured on glass coverslips were incubated with NH IgG or PV IgG at 4 °C and then switched to 37 °C for various amounts of time. To detect cell surface Dsg3 keratinocytes were processed for immunofluorescence analysis at each time point by fixing cells in paraformaldehyde without permeabilization. The Dsg3 monoclonal antibody AK15, which binds to the Dsg3 extracellular domain, was used to detect cell surface Dsg3. Total fluorescence per cell surface area was quantified using a digital imaging system and Simple PCI software. Seven independent fields were measured for each condition, and error bars represent S.E.
Pemphigus IgG Endocytosis

molecular mechanisms of Dsg3 endocytosis and the potential role of plakoglobin in targeting Dsg3 for internalization and degradation.

It is likely that Dsg3 internalization and degradation are central events leading to the loss of adhesion after PV IgG binding. Upon the addition of PV IgG to keratinocyte cultures, PV antibodies and Dsg3 are co-internalized (Fig. 4), thereby depleting cell surface Dsg3 levels (Figs. 7 and 8). However, down-regulation of Dsg3 occurs over several hours in our in vitro model system. Although some PV IgG and Dsg3 internalization can be detected within 30 min of switching keratinocytes from 4 to 37 °C (not shown), high levels of internalization and loss of Dsg3 from the cells surface were not apparent until 1–3 h after the addition of the antibodies (Figs. 7 and 8). Similarly, time course experiments indicate that disruption of keratinocyte adhesion does not occur in cells incubated at 4 °C but, rather, takes several hours after cells are switched to 37 °C (Fig. 3). In fact, the kinetics of the loss of adhesion (Fig. 3) and the destabilization of surface Dsg3 (Fig. 7) are strikingly similar. For example, notable disruption of desmoplakin localization and loss of adhesive strength are not apparent until ~3 h after incubation with PV IgG (Fig. 3). This time frame correlates with the loss of surface levels of Dsg3 (Figs. 7 and 8). The fact that adhesion is not notably disrupted after a 1-h incubation of keratinocytes with PV IgG at 4 °C (Fig. 3) strongly supports the notion that cellular responses to PV IgG are required for the autoantibodies to disrupt adhesion. The relative kinetics of Dsg3 internalization, the depletion of surface levels of Dsg3, and the time course over which adhesive interactions become compromised provides compelling evidence that the loss of surface levels of Dsg3 are causally related to the loss of adhesion.

A key question is whether endocytosis is a mechanism to clear plasma membrane of disrupted junctional complexes in response to PV IgG disruption of desmosomes or if the pathogenic antibodies trigger an
endocytic response that then drives the disruption of desmosomes. In model systems for epithelial-mesenchymal transitions, activation of ARF6 appears to drive E-cadherin internalization and the disruption of intercellular junctions (42, 43). These and other findings suggest that activation of membrane trafficking machinery may play a primary role in the dissolution of intercellular junctions (12, 13). Testing this hypothesis in the context of our PV model system will require a detailed understanding of the mechanism by which desmogleins are internalized and the development of reagents suitable to inhibit Dsg3 internalization in primary cultures of human keratinocytes. Regardless of whether endocytosis of Dsg3 is a primary or secondary event after Dsg3 ligation by pathogenic antibodies, the dramatic reduction in Dsg3 protein levels would undoubtedly exacerbate the effects of PV antibodies on Dsg3 adhesive function at the cell surface. For example, the loss of cell surface Dsg3 in response to PV IgG is mimicked by the loss of adhesion and pemphigus-like fragility observed in Dsg3 null mice (44). Similarly, corticosteroids, which are important clinically in the treatment of PV, increase Dsg3 levels in cultured keratinocytes (45). Collectively, these studies suggest that the design of therapeutic agents that regulate Dsg3 cell surface levels in vivo may be useful in PV treatment regimens.

A prominent feature of the keratinocyte response to PV IgG in our model system is the retraction of keratin filaments from lateral cell borders (Figs. 1 and 2). Previous studies using mouse keratinocytes also indicate that keratin filaments retract from desmosomes in response to PV autoantibodies (41). Furthermore, recent studies using a mouse model system and high resolution electron microscopy indicate that keratin filaments retract from lateral borders of basal keratinocytes (46). However, in this in vivo model system, keratin filaments did not retract from the apical surfaces of keratinocytes associated with the suprabasal cell layer. In the present study electron microscopic analysis indicated that keratin filaments were retracted toward the nucleus in cultured keratinocytes exposed to PV IgG (Figs. 1 and 2). Interestingly, we found no evidence for the internalization of half desmosomes associated with the retracted filament network. Rather, PV IgG that remained at the cell surface after 6 h were associated with remnants of desmosomes on the retracted filament network. Rather, PV IgG that remained at the cell surface after 6 h were associated with remnants of desmosomes on the retracted filament network. Rather, PV IgG that remained at the cell surface after 6 h were associated with remnants of desmosomes on the retracted filament network.

Taken together, the results of this study suggest a model in which the PV autoantibodies impact multiple cellular pathways that lead to the loss of desmosomes and compromised cell adhesion (Fig. 10). It is likely that the first event is the disruption of the adhesive interface between Dsg3 molecules within the desmosome. This initial binding event is then followed by the internalization of Dsg3 and the disruption of interactions between desmosomal plaque components. We favor the hypothesis that the plaque begins to disassemble at the cell surface, thereby releasing Dsg3 into a membrane domain that favors endocytosis and delivery to lysosomes. This reasoning is based on the fact that recent studies of E-cadherin endocytosis suggest that adhesive interactions between cadherins as well as cytoskeletal associations prevent cadherin internalization (47). An alternative possibility is that a soluble pool of Dsg3 present at the membrane is depleted by endocytosis (40). If this soluble pool is an important reservoir for desmosome assembly, then PV IgG may deplete this pool of desmoglein and thereby disrupt desmosomes. In fact, both possibilities are supported by our observations, and these hypotheses are not mutually exclusive (Fig. 10). Regardless of which Dsg3 pool is affected, cell surface Dsg3 is internalized and targeted to the lysosome (Fig. 5). Nonetheless, it will be important to determine whether PV IgGs impact the kinetics of Dsg3 incorporation into desmosomes or the rate of Dsg3 turnover after it has assembled into desmosomes. Recent studies have demonstrated that desmosomal cadherins exhibit exchange into and out of preexisting desmosomes (48, 49). Thus, PV IgG may impact one or more of the Dsg3 kinetics that cells utilize to maintain steady state levels of desmosomal adhesion.

It is interesting that plakoglobin appears to traverse the pathway with Dsg3, but yet, plakoglobin steady state levels are unaltered by PV IgG treatment. It is possible that plakoglobin is delivered to the lysosome with Dsg3 but dissociates from the cadherin before degradation. Alternatively, plakoglobin has numerous other binding partners, and the pool of plakoglobin bound to Dsg3 may be relatively small compared with total plakoglobin levels. Although some Dsg3 may be recycled back to the plasma membrane to re-engage in adhesive interactions, Western blot (Fig. 9) and quantitative immunofluorescence microscopy studies (Fig. 8) indicate that a substantial decrease in steady state levels of Dsg3 occurs in response to PV autoantibodies. Thus, the results of the present study demonstrate that pemphigus antibodies not only disrupt adhesion but also trigger the loss of cell surface desmoglein protein levels, which would further compromise epidermal integrity in the context of the human disease.

Acknowledgments—We are grateful for the reagents and helpful insights provided by Drs. Robert Swerlick, Kathleen J. Green, and Victor Faundez. We are appreciative of the assistance Hong Yi and the Emory School of Medicine Microscope Core.

REFERENCES
1. Getsios, S., Huen, A. C., and Green, K. J. (2004) Nat. Rev. Mol. Cell Biol. 5, 271–281
2. Yin, T., and Green, K. J. (2004) Semin. Cell Dev. Biol. 15, 665–677
3. Cheng, X., and Koch, P. J. (2004) J. Dermatol. 31, 171–187
4. Payne, A. S., Hanakawa, Y., Amagai, M., and Stanley, J. R. (2004) Curr. Opin. Cell Biol. 16, 536–543
5. Garrod, D. R., Merritt, A. J., and Nie, Z. (2002) Curr. Opin. Cell Biol. 14, 537–545
6. Kowalczyk, A. P., Bornslaeger, E. A., Norvell, S. P., Balka, H. L., and Green, K. J. (1999) Int. Rev. Cytol. 185, 237–302
7. Angst, B. D., Marcozzi, C., and Magee, A. I. (2001) J. Cell Sci. 114, 629–641
8. Protonotarios, N., and Tsatsopoulou, A. (2004) Cardiovasc. Pathol. 13, 185–194
9. Chidgey, M. (2002) Histol. Histopathol. 17, 1179–1192
10. Anhah, G. J., and Diaz, L. A. (2004) J. Am. Acad. Dermatol. 51, 520–521
11. Kowalczyk, A. P., and Reynolds, A. B. (2004) Curr. Opin. Cell Biol. 16, 522–527
12. D’Souza-Schorey, C. (2005) Trends Cell Biol. 15, 19–26
13. Bryant, D. M., and Stow, J. L. (2004) Trends Cell Biol. 14, 427–434
14. Le, T. L., Yap, A. S., and Stow, J. L. (1999) J. Cell Biol. 146, 219–232
15. Le, T. L., Joseph, S. R., Yap, A. S., and Stow, J. L. (2002) Am. J. Physiol. Cell Physiol. 283, 489–499
16. Palacios, F., Tushir, J. S., Fujita, Y., and D’Souza-Schorey, C. (2005) Mol. Cell. Biol. 25, 389–402
17. Vincent, P. A., Xiao, K., Buckley, K. M., and Kowalczyk, A. P. (2004) Am. J. Physiol. Cell Physiol. 286, 987–997
18. Reynolds, A. B., and Carnahan, R. H. (2004) Semin. Cell Dev. Biol., 15, 657–663
19. Xiao, K., Allison, D. F., Buckley, K. M., Kottke, M. D., Vincent, P. A., Faundez, V., and Kowalczyk, A. P. (2003) J. Cell Biol. 163, 535–545
20. Davis, M. A., Ireton, R. C., and Reynolds, A. B. (2003) J. Cell Biol. 163, 525–534
21. Stanley, J. R., Nishikawa, T., Diaz, L. A., and Amagai, M. (2001) J. Investig. Dermatol. 116, 489–490
22. Amagai, M., Karpati, S., Prussick, R., Klaus-Kortun, V., and Stanley, J. R. (1992) J. Clin. Investig. 90, 919–926
23. Amagai, M., Hashimoto, T., Shimizu, N., and Nishikawa, T. (1994) J. Investig. Dermatol. 94, 59–67
24. Koch, A. W., Manzur, K. L., and Shan, W. (2004) Cell. Mol. Life Sci. 61, 1884–1895
25. Amagai, M., Ishii, K., Hashimoto, T., Gamou, S., Shimizu, N., and Nishikawa, T. (1995) J. Investig. Dermatol. 105, 243–247
26. Kowalczyk, A. P., Anderson, J. E., Borgwardt, J. E., Hashimoto, T., Stanley, J. R., and Green, K. J. (1995) J. Investig. Dermatol. 105, 147–152
Pemphigus IgG Endocytosis

27. Tsunoda, K., Ota, T., Aoki, M., Yamada, T., Nagai, T., Nakagawa, T., Koyasu, S., Nishikawa, T., and Amagai, M. (2003) J. Immunol. 170, 2170–2178
28. Iwatsuki, K., Takigawa, M., Imaizumi, S., and Yamada, M. (1989) J. Am. Acad. Dermatol. 20, 578–582
29. Iwatsuki, K., Han, G. W., Fukutani, R., Ohtsuka, M., Kikuchi, S., Akiba, H., and Kaneko, F. (1999) Br. J. Dermatol. 140, 35–43
30. Milner, Y., Sagi, E., Timberg, R., Michel, B., Meotezoua, P., and Goldberg, M. (1989) J. Cell. Physiol. 139, 441–454
31. Patel, H. P., Diaz, L. A., Anhalt, G. J., Labih, R. S., and Takahashi, Y. (1984) J. Investig. Dermatol. 83, 409–415
32. Sato, M., Aoyama, Y., and Kitajima, Y. (2000) Lab. Invest. 80, 1583–1592
33. Robinson, M. S. (2004) Trends Cell Biol. 14, 167–174
34. Gruenberg, J., and Stenmark, H. (2004) Nat. Rev. Mol. Cell Biol. 5, 317–323
35. Huen, A. C., Park, J. K., Godsel, L. M., Chen, X., Bannon, L. J., Amargo, E. V., Hudson, T. Y., Mongiu, A. K., Leigh, I. M., Kelsell, D. P., Gumbiner, B. M., and Green, K. J. (2002) J. Cell Biol. 159, 1005–1017
36. Palka, H. L., and Green, K. J. (1997) J. Cell Science 110, 2359–2371
37. Muller, E., Caldelari, R., de Bruin, A., Baumann, D., Bierkamp, C., Balmer, V., V, and Suter, M. M. (2000) J. Investig. Dermatol. 115, 332
38. Kitajima, Y. (2002) Clin. Exp. Dermatol. 27, 684–690
39. Waschke, J., Bruggeman, P., Baumgartner, W., Zillikens, D., and Drenckhahn, D. (2005) J. Clin. Investig. 115, 3157–3165
40. Aoyama, Y., and Kitajima, Y. (1999) J. Investig. Dermatol. 112, 67–71
41. Caldelari, R., de Bruin, A., Baumann, D., Suter, M. M., Bierkamp, C., Balmer, V., and Muller, E. (2001) J. Cell Biol. 153, 823–834
42. Palacios, F., Price, L., Schweitzer, J., Collard, J. G., and D’Souza-Schorey, C. (2001) EMBO J. 20, 4973–4986
43. Palacios, F., Schweitzer, J. K., Boshans, R. L., and D’Souza-Schorey, C. (2002) Nat. Cell Biol. 4, 929–936
44. Koch, P. J., Mahoney, M. G., Ishikawa, H., Pulkkinen, L., Uitto, J., Shultz, L., Murphy, G., Whitaker-Menozes, D., and Stanley, J. R. (1997) J. Cell Biol. 137, 1091–1102
45. Nguyen, V. T., Arredondo, J., Chernyavsky, A. I., Kitajima, Y., Pittelkow, M., and Grando, S. A. (2004) J. Biol. Chem. 279, 2135–2146
46. Shimizu, A., Ishiko, A., Ota, T., Tsunoda, K., Amagai, M., and Nishikawa, T. (2004) J. Investig. Dermatol. 122, 1145–1153
47. Izumi, G., Sakisaka, T., Baba, T., Tanaka, S., Morimoto, K., and Takai, Y. (2004) J. Cell Biol. 166, 237–248
48. Windoffer, R., Borcher-Stuhlreger, M., and Leube, R. E. (2002) J. Cell Sci. 115, 1717–1732
49. Gloushankova, N. A., Wakatsuki, T., Troyanova, R. B., Elson, E., and Troyanosky, S. M. (2003) Cell Tissue Res. 314, 399–410