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Determining the Cellular Mechanism Involved in Pemphigus Vulgaris-Induced Desmoglein 3 Internalization and Desmosomal Disassembly

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By

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B.S., University of Richmond, 2002

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Abstract

Determining the Cellular Mechanism Involved in Pemphigus Vulgaris-Induced Desmoglein 3 Internalization and Desmosomal Disassembly

By Emmanuella Delva

Desmosomes are electron-dense intercellular adhesive complexes, responsible for maintaining tissue integrity in organs subjected to mechanical stress. They are composed of three families of proteins: the desmosomal cadherins, desmogleins and desmocollins; the armadillo plaque proteins, plakoglobin and plakophilin; and the plakin family of proteins, such as desmoplakin. Together, these proteins tether keratin intermediate filaments to the plasma membrane.

In the autoimmune epidermal blistering disease, pemphigus vulgaris (PV), the autoantibodies primarily target one of the desmosomal cadherins, desmoglein 3 (Dsg3) and in some cases desmoglein 1 (Dsg1). As a result, Dsg3 is internalized, where it enters an endo-lysosomal pathway for degradation. This degradation is associated with retraction of the keratin filaments, mislocalization of desmoplakin, and overall loss of cell-cell adhesion. The first part of the thesis focuses on determining the endocytic machinery involved in PV-induced Dsg3 internalization. The work reported here illustrates that Dsg3 internalization is mediated through a non-classical pathway, independent of clathrin and dynamin. Furthermore, inhibition of Dsg3 endocytosis prevents desmosomal disassembly. These findings, along with previous studies, demonstrate that the internalization of Dsg3 is tightly coupled to desmosomal disassembly in response to PV IgG.

The second part of the thesis addresses the role of the cytoskeletal network, in particular the actin filaments and microtubules, in regulating PV-induced Dsg3 internalization, as studies have implicated a role for the cytoskeleton in vesicular transport. Our preliminary findings suggest that depolymerizing actin results in increased accumulation of Dsg3 in the cytoplasm. Furthermore, depolymerizing microtubules prevents the initial entry of Dsg3 into the cell. These findings suggest that microtubules and actin provide tracks by which Dsg3-containing vesicles are transported from the plasma membrane to early endosomes and from early endosomes to lysosomes, respectively.

Altogether, these studies shed light on the cellular mechanism involved in the desmosomal disassembly and epidermal blistering in response to PV. Moreover, these data underscore the importance of regulating the intricate balance between desmosomal assembly and disassembly, which is important in both the context of pathologies affected by compromised cell-cell adhesion, such as PV, and in normal cellular processes, such as cellular motility, cell morphology, proliferation and differentiation.

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My journey to this point did not come easy and I could not have done this alone. I have a number of individuals to thank for providing me with the opportunity to work towards this goal – a goal that I have set for myself since I was in the seventh grade, in Mrs. Valerie Thompson class at St. Jerome's Elementary School.

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Chapter 1: Introduction to the Epidermis and the Desmosome

1.1 The Epidermis

The skin consists of three layers: the epidermis, dermis, and subcutaneous tissue (fat). The outermost layer, the epidermis, protects the body against foreign agents, mechanical stress, UV radiation from the sun, and prevents water loss. The epidermis is further categorized into 4 layers - the basal layer, the spinous layer, the granular layer, and the outermost stratum corneum (**Figure 1.1**). Within the various layers are 4 distinct cell types: keratinocytes, melanocytes, Langerhans cells, and Merkel cells. The keratinocyte is the principal cell of the epidermis, making up 95% of this stratifying epithelium. The difference in layer composition is attributed to the difference in keratinocyte function as keratinocytes proliferate in the basal layer and eventually differentiate as they move upward towards the stratum corneum.

The ability of the epidermis to act as a barrier against foreign agents and mechanical stress is due to the expression of adhesive junctions and intermediate filaments throughout the epidermis, namely desmosomes and keratin filaments, respectively.

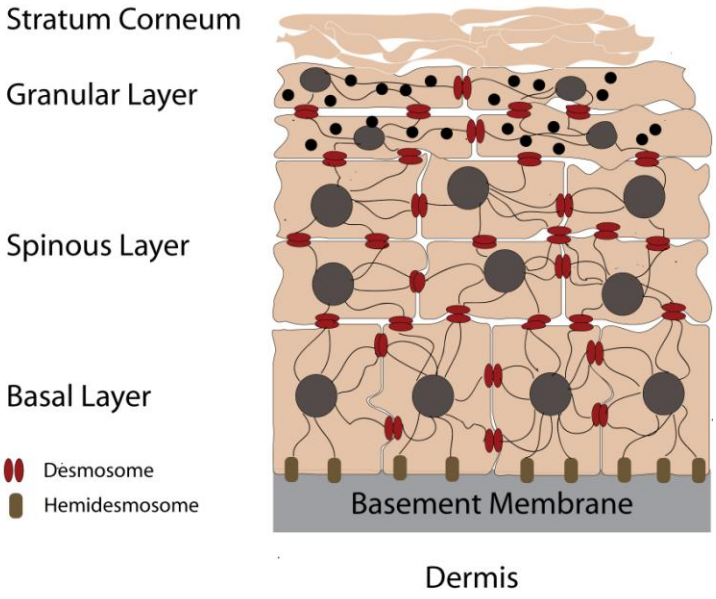


Figure 1.1 The various layers of the epidermis. The human epidermis is comprised of four distinct layers – the basal layer, spinous layer, granular layer, and the stratum corneum. As the keratinocytes proliferate/differentiate throughout the epidermis, the size and number of desmosomes change, to assist in maintaining stable, functional cell-cell adhesion between keratinocytes. Altogether, these layers contribute to protecting the body from foreign agents, mechanical stress, and UV radiation.

1.2 Cell-Cell Adhesion via Desmosomes

Desmosomes are adhesive junctions that provide adhesion strength between epithelial cells (Getsios et al., 2004; Kitajima, 2002). They are found primarily in tissues that experience mechanical stress, such as the myocardium, bladder, gastrointestinal mucosa, and skin (Getsios et al., 2004; Yin and Green, 2004). These adhesive junctions are important in providing structure and integrity to these organs. Furthermore, desmosomes contribute greatly to various dynamic biological processes, playing a pivotal role in skin cell differentiation, morphogenesis and tissue patterning, and epithelial-mesenchymal transitions (Getsios et al., 2004).

Desmosomes regulate cell-cell interactions between keratinocytes and mediate attachment of keratin intermediate filaments to the plasma membrane (Amagai, 1995; Amagai et al., 1991; Koch and Franke, 1994). Ultrastructurally, the desmosome is divided into three morphologically identifiable zones: the extracellular core region (desmoglea), the outer dense plaque and the inner dense plaque (**Figure 1.2A**) (Garrod and Chidgey, 2008; Green and Jones, 1996; Kowalczyk et al., 1994; North et al., 1999; Schmidt et al., 1994). The extracellular domain of the two primary desmosomal transmembrane adhesion molecules, desmogleins (Dsg) and desmocollins (Dsc), make up the desmoglea (Garrod and Chidgey, 2008; Kowalczyk et al., 1994). The interaction between these Type I transmembrane proteins occurs in a Ca^{++} dependent manner (Angst et al., 2001; Troyanovsky, 1999). The outer dense plaque consists of the cytoplasmic tails of the cadherins, which bind to members of the armadillo and plakin family of linker proteins (Garrod and Chidgey, 2008; Getsios et al., 2004; Kowalczyk et al., 1994). Plakoglobin (PG), a member of the armadillo family, interacts with portions of the desmosomal cadherins through domains on both its amino- and carboxy-termini (Wahl et al., 1996; Witcher et al., 1996). Desmoplakin (DP), a member of the plakin family, interacts with both PG and plakophilins (another member of the armadillo family), via the amino-terminus of the plakin protein. DP also binds to the keratin intermediate filament network via its carboxy-terminus (Cowin and Burke, 1996). The interaction between DP and the keratin filaments forms the inner dense plaque, tethering the cytoskeletal network to the adhesion complex (**Figure 1.2B**) (Garrod and Chidgey, 2008; Getsios et al., 2004; Kowalczyk et al., 1994).

The following sections illustrate the importance of understanding the functional role of the various desmosomal components, as they relate to establishing and maintaining cell-cell adhesive strength.

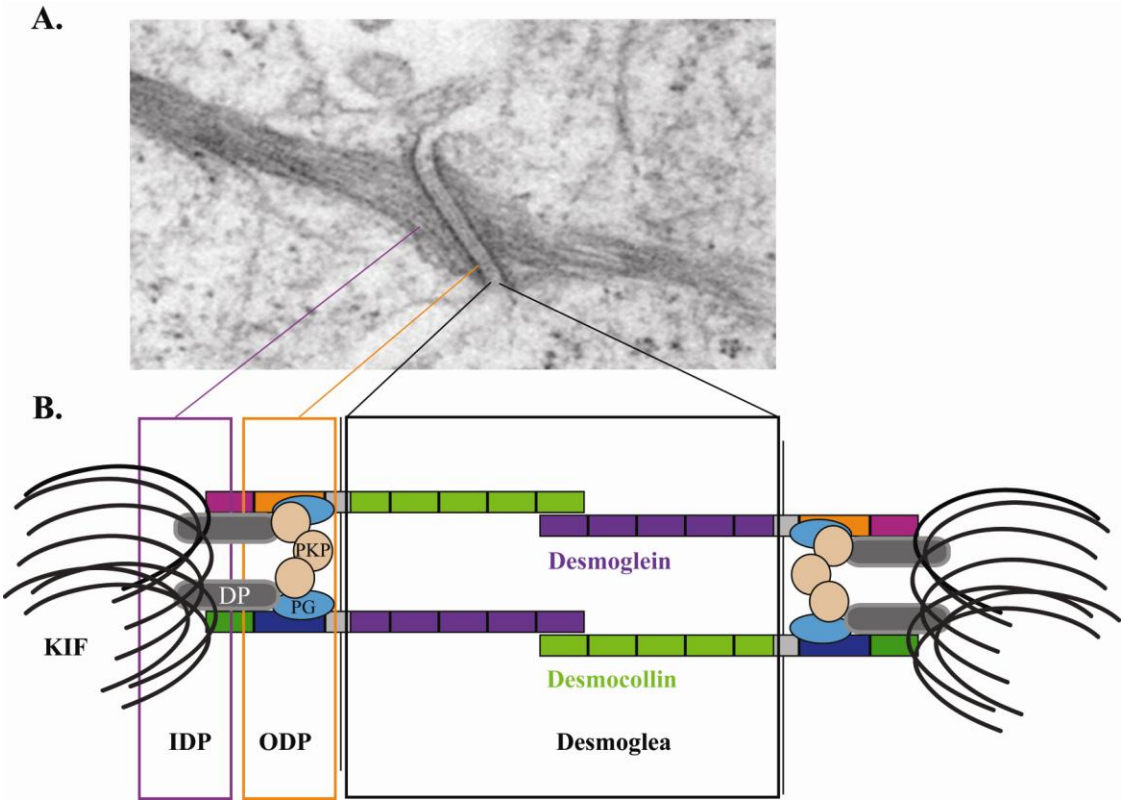


Figure 1.2 Structure of the Desmosome. The interaction between keratinocytes is partly maintained by interactions between the desmosomal cadherins, the desmogleins and desmocollins through a Ca^{++}-dependent manner. The cadherins are further associated with the linker proteins, plakoglobin (PG), the plakophilins (PKP) and desmoplakin (DP). DP binds to the keratin intermediate filaments (KIF), serving to tether the intermediate filaments to the plasma membrane. ODP, outer dense plaque; IDP, inner dense plaque.

Desmosomal Cadherins – Desmogleins and Desmocollins

Desmogleins (Dsgs) and desmocollins (Dscs) are the two major types of desmosomal cadherins (Chitaev and Troyanovsky, 1997). There are three isoforms of desmocollins (Dsc 1-3) and four isoforms of desmogleins (Dsg 1-4) (Cheng and Koch, 2004; Kljuic et al., 2003; Whittock and Bower, 2003). Furthermore, all three desmocollin genes undergo alternative splicing, resulting in the generation of the Dsc "a" form and a shorter Dsc "b" form which differ in the length of their respective C-termini (**Figure 1.3**) (Collins et al., 1991; North et al., 1999; Parker et al., 1991). Dscs are more structurally similar to the classical cadherins (i.e. E- and N-cadherin), than they are to the desmoglein cadherins.

Similar to the structurally related adherens junctions, desmosomes are formed through Ca^{++}-dependent, homo- and heterophilic interactions between the two groups of desmosomal cadherins. However, heterophilic interactions, which were first observed in human fibrosarcoma (HT-1080) cells expressing Dsg2 and Dsc1a (Chitaev and Troyanovsky, 1997) are the more likely interactions to form, based on in vitro experiments, using recombinant proteins encoding for the first two extracellular domain of Dsg2 and Dsc2 (Syed et al., 2002). Furthermore, while single adhesion-peptides are sufficient to block adhesion mediated by classical cadherins, using both Dsg and Dsc specific peptides are necessary to prevent desmosomal cadherin-mediated adhesion (Runswick et al., 2001; Tselepis et al., 1998).

Both desmogleins and desmocollins contain four extracellular (EC) domains, with EC1 containing the cell adhesion recognition (CAR) site, found necessary for trans-interactions between cells (Garrod et al., 2002; Tselepis et al., 1998).

The extracellular anchor (EA) and the intracellular anchor (IA) flank the transmembrane domain of the desmosomal cadherins. (Nollet et al., 2000) (Kowalczyk et al., 1999a). Desmogleins and the longer Dsc "a" form also contain an intracellular cadherin-like sequence (Kurzen et al.) (Kurzen et al.) domain allowing for binding of other members of the desmosome (in particular plakoglobin) to the cadherin (Troyanovsky et al., 1994). Furthermore, only the desmogleins contain the intracellular proline-rich linker (IPL) domain, a repeat unit domain (Freedman et al.) (Freedman et al.), and a glycine-rich desmoglein terminal domain (DTD) (**Figure 1.3**) (Garrod and Chidgey, 2008).

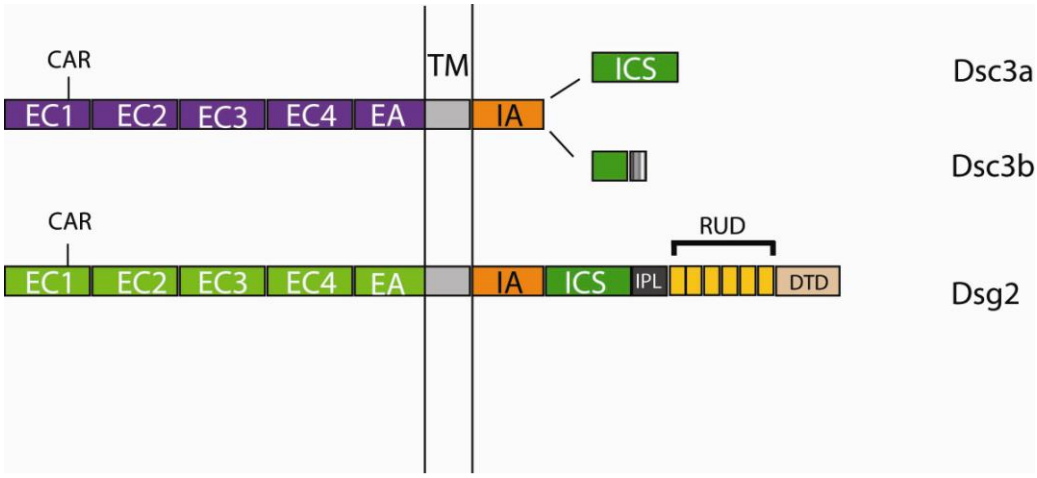


Figure 1.3 Domains of the Desmosomal Cadherins. All desmosomal cadherins are synthesized with N-terminal signals and pro-peptides (not shown) that are cleaved during protein maturation. Desmogleins and desmocollins contain five extracellular (EC) domains, with the cell adhesion recognition (CAR) site in EC1 domain (responsible for the adhesive function of the cadherin). They also contain a transmembrane domain, flanked by an extracellular anchor (EA) and intracellular anchor (IA) domain. Desmocollin "a" proteins and desmogleins contain an intracellular cadherin-like sequence (Kurzen et al.). Only desmogleins contain the additional intracellular proline-rich linker (IPL), repeat unit domain (Freedman et al.), and the desmoglein terminal domain (DTD).

The desmosomal cadherins are expressed throughout the body in a tissue specific pattern. All desmosomal cadherins, with the exception of Dsc2 and Dsg2, are solely expressed in stratified epithelial cells. Dsc2 and Dsg2 are also expressed in other desmosome-containing tissues, such as the colon and cardiac muscle. In the epidermis, all seven desmosomal cadherins are differentially expressed throughout the various layers (**Figure 1.4**) (Kottke et al., 2006). Dsg2 is mainly distributed throughout the lower layers of the epidermis, along with Dsg3. Conversely, Dsg1 is expressed mostly in the upper layers, and Dsg4 is mainly expressed in the hair follicle (although it is also expressed in the granular layer).

Dsc2 and Dsc3 are present in the basal and spinous layers, whereas Dsc1 is expressed in the granular layer. This expression profile may contribute greatly to the varying size and functionality of desmosomes as the keratinocytes proliferate and eventually differentiate throughout the epidermis.

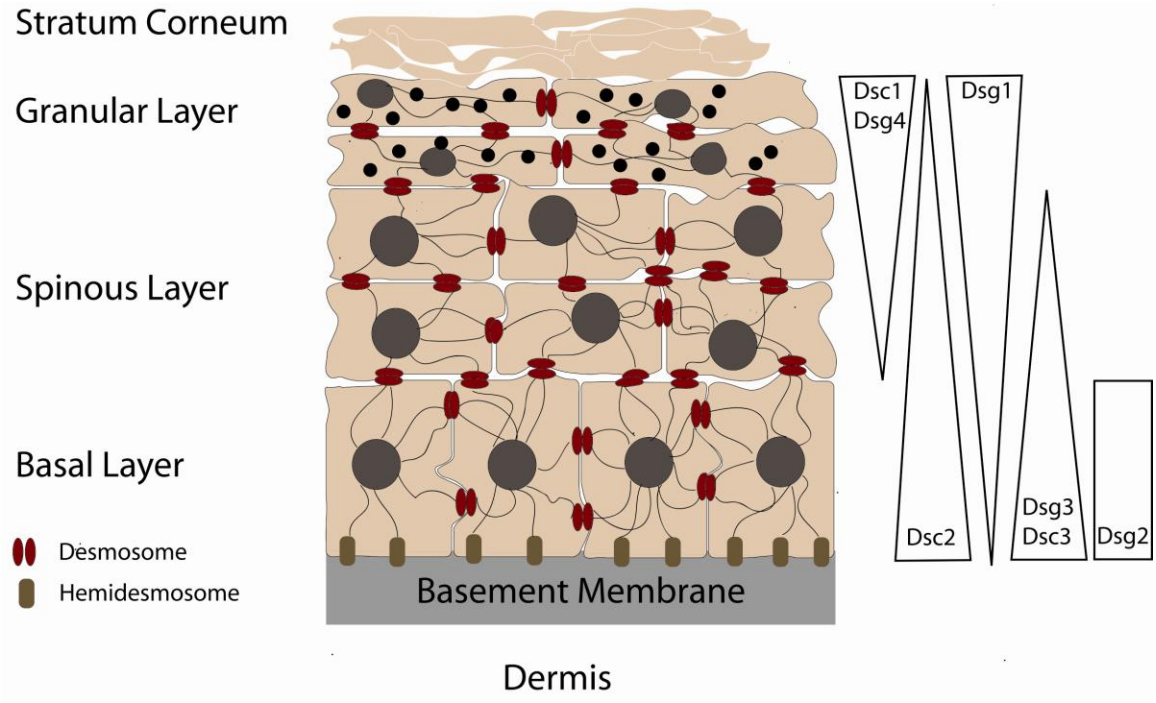


Figure 1.4 Expression Patterns of the Desmosomal Cadherins in the Epidermis. Keratin filaments are shown connecting to desmosomes at sites of cell-cell contact and to hemidesmosomes at the basement membrane. The profiles and relative expression levels of various desmosomal cadherins in the epidermal layers are depicted above.

Although the relationship between cadherin expression and desmosome function/size is not clearly understood, studies involving manipulation of desmosomal cadherin expression suggests that their localization is important in regulating tissue morphogenesis. For example, Dsg3 overexpression, using the keratin 1 promoter to drive expression in the suprabasal layers in transgenic mice resulted in hyperproliferation, and abnormal differentiation, including misexpression of keratin 1 and 14 (Merritt et al., 2002). Furthermore, overexpression of Dsg3 in the upper layers, driven by the involucrin promoter, resulted in a more severe phenotype, including a resemblance to the oral mucosa, a reduction in epidermal barrier function, and early post-natal lethality, due to extensive water loss (Elias et al., 2001).

These studies, and others, underscore the importance of desmosomal cadherin expression in regulating tissue proliferation/differentiation. Mutations in various desmoglein isoforms also contribute to several inherited disorders. Loss of Dsg4 is associated with defective hair-follicle differentiation (Kljuic et al., 2003), while Dsg1 mutations, due to an amino-terminal deletion resulting in haploinsufficiency, lead to striate palmoplantar keratoderma, an epidermal-thickening disease (Rickman et al., 1999). Mutations in conserved residues throughout Dsg2 result in arrhythmogenic right ventricular cardiomyopathy (ARVC) (Awad et al., 2006; Pilichou et al., 2006).

Desmocollins have also been shown to play an important role in structural integrity of the epidermis and proliferation/differentiation. Mice lacking Dsc1 (a desmosomal cadherin expressed in the upper layers of the epidermis) display loss of cell-cell adhesion in the granular layers of the epidermis, as well as hyperproliferation, hair follicle degeneration, and increased expression of the wound keratins, 6 and 16 (Chidgey et al., 2001). In contrast, overexpression of a basal cadherin (Dsc3) in the suprabasal layers, results in hyperproliferation and abnormal differentiation. Furthermore, these findings were due to stability of the proliferation signaling molecule, β-catenin (Hardman et al., 2005). Similar to studies involving Dsg expression, these results suggest that the expression pattern of Dscs is important in tightly regulating proliferation and differentiation within the epidermis, with a role for Dsc3 in regulating proliferation and a role for Dsc1 in maintaining proper differentiation. Moreover, recent work by Simpson and others reported the first case of a mutation in a desmocollin (Dsc2) and its association with a human disease, autosomal recessive ARVC (Simpson et al., 2008).

Altogether, these studies and others underscore the importance of proper desmosomal cadherin expression and function in providing strong cell-cell adhesion and possibly regulating tissue patterning, as mis-regulation of desmosomal cadherins results in devastating effects (as described in Chapter 2).

Armadillo Family of Proteins

Armadillo was initially discovered as a regulator of segment polarity in *Drosophila*, and later found to be the homolog of the junctional/signaling protein, β-catenin (Peifer et al., 1992). The armadillo proteins are characterized by the presence of a central domain containing repeating units of a 45 amino acid sequence homology domain (arm repeats) (Garrod and Chidgey, 2008). These proteins include plakoglobin (γ-catenin) and plakophilins 1-3 (PKP 1-3) (**Figure 1.5**). Their role in the desmosome appears to be to facilitate in the tethering of the keratin intermediate filaments and desmoplakin to the desmosome (as in the case of plakoglobin) and regulate clustering of the plaque proteins (as in the case of plakophilins). In addition to serving as linkers within the desmosome, they also possess non-junctional roles, as regulators of transcription (similar to β-catenin).

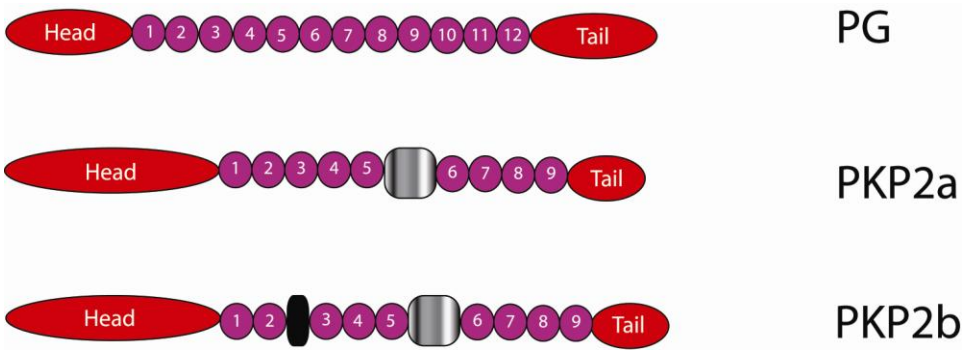


Figure 1.5 Members of the armadillo protein family. Plakoglobin contains 12 arm repeats (similar to β-catenin), while the plakophilins contain 9 arm repeats with an insert between repeats 5 and 6, which introduces a bend into the overall structure.

Plakoglobin

Plakoglobin (PG), the most studied armadillo protein in the desmosome, contains 12 arm repeats, flanked by distinct N- and C-termini (Huber et al., 1997; Peifer et al., 1992) (Garrod and Chidgey, 2008). Portions of the central arm repeats bind to the classical cadherins, whereas the desmosomal cadherins interact with domains within the PG amino-terminus, in conjunction with arm repeats found near the carboxy-terminus, particularly in the case of Dscs (Chitaev et al., 1996; Wahl et al., 1996; Witcher et al., 1996). Although it has been found to be localized to desmosomes and adherens junctions, plakoglobin's affinity for desmosomal cadherins is several times greater than its affinity for E-cadherin (Chitaev et al., 1996). The armadillo domain also allows for interaction between plakoglobin and desmoplakin, which tethers the intermediate filaments to the desmosomal plaque (Bornslaeger et al., 2001; Kowalczyk et al., 1997; Smith and Fuchs, 1998).

Plakoglobin is thought to be involved in regulating the association of desmoplakin and the keratin intermediate filaments with the outer dense plaque and desmosome size. While deletion of the plakoglobin N-terminus did not affect desmosome morphology, deletion of the C-terminus of plakoglobin causes formation of large desmosomes (Palka and Green, 1997). It is thought that plakoglobin's interaction with desmosomal cadherins regulates plakoglobin's signaling function. Co-expression of the Dsg1 cytoplasmic tail along with plakoglobin in *Xenopus* embryos prevented plakoglobin translocation to the nucleus and suppressed plakoglobin's ability to induce anterior axis duplication (Karnovsky and Klymkowsky, 1995).

Not surprisingly, mutations in PG lead to various conditions developed as a result of compromised desmosome integrity (Kottke et al., 2006). PG-knockout studies revealed that mice die due to fragility of the myocardium (Bierkamp et al., 1996; Ruiz et al., 1996) . These mice also exhibit acantholysis (loss of cell-cell adhesion), indicative of disrupted desmosomes. In some genetic backgrounds, mouse pups are viable but exhibit serious epidermal fragility, heart defects, and early postnatal lethality. Consistent with these findings, Naxos disease, an autosomal recessive disease characterized by ARVC, woolly hair, and palmoplantar keratoderma, develops as a result of a mutation leading to the truncation of the C-terminus of PG (McKoy et al., 2000). The heart defect occurs as a result of fragility of the myocyte syncytium which leads to its degradation and fibrofatty replacement (Kottke et al., 2006). Although the palmoplantar keratoderma phenotype may appear to result from weakened cell adhesion, Naxos patients do not display acantholysis and the epidermal manifestations generated are not as severe as the PG-null mice (Kottke et al., 2006). These patients also exhibit woolly hair, in which the hair is often lighter and finer, than unaffected individuals. Although the mechanism by which this occurs is unknown, PG (as well as β-catenin) has been implicated in hair formation. Similar to PG, β-catenin also functions as a regulator of signaling pathways, and binds to members of the T-cell factor (TCF) family of transcription factors (Huelsken and Behrens, 2002). Furthermore, it is believed that PG modulates TCF-signaling (Miravet et al., 2002; Yin and Green, 2004). While PG overexpression in mouse epidermis decreases keratinocyte proliferation and shortens the anagen phase of the hair cycle (Charpentier et al., 2000), β-catenin overexpression causes hyperproliferation and hair follicle differentiation (Gat et al., 1998).

This suggests that either the balance between PG and β-catenin signaling are misregulated in Naxos patients, or the phenotype is due to loss of proliferation/cell adhesion in the hair follicle/shaft.

Plakophilins

Plakophilins are a member of the prototypic p120-catenin sub-family of armadillo proteins. Plakophilins 1-3 share approximately 55% sequence similarity among each other in the arm domains and approximately 50% sequence similarity with the p120 arm domain (Hatzfeld, 2007). In contrast to p120, plakophilins are predominantly found at desmosomes instead of adherens junctions, with plakophilins 1 and 2 also localized to the nucleus.

Plakophilin 1 is primarily expressed in the suprabasal layers of stratified epithelia, whereas plakophilin 2 is expressed in simple epithelia, a portion of stratified epithelia, and in non-epithelial tissues (i.e. the cardiac muscle and lymph nodes) (Franke et al., 2007; Heid et al., 1994; Mertens et al., 1996; Mertens et al., 1999; Schmidt et al., 1997). Plakophilin 3 resides in simple and stratified epithelia, exhibiting uniform expression throughout the epidermis (Bonne et al., 1999; Schmidt et al., 1999). It also resides in the dendritic reticulum cells of lymph nodes (Schmidt and Jager, 2005).

There are no isoforms of plakophilin 3. However, both plakophilins 1 and 2 exist as 2 isoforms, each generating a short "a" form and a long "b" form, differing in the addition of 21 amino acids in arm repeat 3 (plakophilin 1) and the addition of 44 amino acids in arm repeat four (plakophilin 2) (Mertens et al., 1996; Schmidt et al., 1997) .

Based on structural analysis, the plakophilins contain 9 arm repeat domains (Hatzfeld, 2007). Although there is a prominent basic patch on the protein surface that may facilitate protein-protein interactions, all protein-protein interactions that have been identified are found at the N-terminus domain of the plakophilins.

While plakoglobin has been shown to be important in the linking desmosomes to the intermediate filaments, recent work has illustrated that plakophilins also play pivotal roles in the establishment and maintenance of desmosome structure/integrity. Plakophilin 1 is thought to be important in regulating desmosome size and strength in the epidermis, mediated by its interaction with desmoglein 1, desmoplakin, and keratin intermediate filaments (Hatzfeld et al., 2000). Further studies demonstrated that while plakophilin 1 interferes with PG for binding to desmoplakin, both armadillo proteins work in concert to promote clustering of the desmosomal plaque proteins (Bornslaeger et al., 2001). Furthermore, the head domain of plakophilin-1 is necessary for its binding to desmoplakin, as observed in yeast-two hybrid assays, enhancing recruitment of desmoplakin to the desmosome (Kowalczyk et al., 1999b). Based on these interactions, a model has been proposed where plakophilins may play a role in the lateral interactions between desmosomal plaque complexes. In support of this model, plakophilin 1 is largely expressed in the suprabasal layers of the epidermis, which may be necessary to stabilize desmosomes and cell adhesion in the layers most subjected to mechanical stress.

Plakophilin 2 appears to be less efficient at recruiting desmoplakin and other desmosomal proteins to the plasma membrane, compared to plakophilin 1 (Chen et al., 2002), suggesting differences in plakophilin function in regulating desmosomal assembly/maintenance. However, studies infer that plakophilin 2 is necessary for transport of desmoplakin to the plasma membrane during desmosome assembly. Desmoplakin is transported to the plasma membrane in particles, containing plakophilin 2, in close association with keratin intermediate filaments (Godsel et al., 2005). Similar to plakophilin 2, plakophilin 3 overexpression does not result in larger desmosomes or an increase in desmosome number (Bonne et al., 2003) (Bonne et al., 1999; Schmidt et al., 1999). Plakophilin 3 also appears to interact with the largest number of desmosomal proteins, including desmoplakin, plakoglobin, all three desmoglein proteins, Dsc 3a and 3b, and Dsc 1a and 2a (Hatzfeld, 2007).

The importance of plakophilin in maintaining desmosomal integrity is underscored by various diseases and/or defective cellular processes that are associated with mutations of each of the plakophilins. For example, patients who suffer from a severe autosomal recessive ectodermal dysplasia and skin fragility syndrome were found to have mutations in plakophilin 1 (more specifically, mutations caused by premature stop codons, leading to complete ablation of the protein) (McGrath et al., 1997) (McGrath et al., 1999). These phenotypes suggest that in addition to its role in regulating desmosome stability, plakophilin 1 may also regulate epidermal morphogenesis. Because plakophilin 2 is the only isoform expressed in the heart, mutations associated with this particular isoform cause arrhythmogenic right-ventricular cardiomyopathy (ARVC) (Gerull et al., 2004).

In most cases the mutations are found within the C-terminus of the gene, although other mutations have been observed. Based on experiments in which the gene encoding plakophilin 2 is ablated in mice, the effects seen in ARVC patients are likely the result of mechanical fragility. These mice display mid-gestational embryonic lethality due to cardiac patterning defects and fragility of the myocardium (Grossmann et al., 2004). Plakophilin 2-null mice also display retraction of the intermediate filaments from the plasma membrane, illustrating the importance of desmoplakin/IF tethering to the desmosome plaque in maintaining stable and functionally sound desmosomes in the heart.

Although no human pathologies are associated with mutations in plakophilin 3, recent work by Sklyarova and others have demonstrated its importance in hair follicle formation and skin development. Plakophilin 3 conditionally ablated out in mouse epidermis resulted in defective hair follicle morphogenesis, increased keratinocyte proliferation, and desmoplakin mislocalization. These mice are also more susceptible to dermatitis (skin inflammation) and secondary alopecia (hair loss) (Sklyarova et al., 2008).

Desmoplakin

Desmoplakin (DP) is the most abundant component of the desmosome (Mueller and Franke, 1983) and serves as the linker between the intermediate filaments and the plasma membrane (Bornslaeger et al., 1996; Leung et al., 2002; Leung et al., 2001). DP contains globular N- and C-termini, connected by a central, α-helical coiled-coil rod domain. The N-terminal head domain provides binding sites for PG.

The C-terminal tail contains three plakin repeat domains (PRDs; A, B, C), as well as a glycine-serine-arginine rich domain thought to regulate desmoplakin binding to intermediate filaments, via phosphorylation of the serine residue (**Figure 1.6**) (Getsios et al., 2004; Yin and Green, 2004) .

There are two DP isoforms (DP I and II), differing in the length of the central rod, with DP II missing approximately 2/3 of in the rod domain (Green et al., 1992). Similar to the various desmocollin proteins, these two desmoplakin proteins are produced as a result of alternative RNA splicing. Although both isoforms of DP are widely expressed in numerous tissues, DP II is absent from the heart and its expression is reduced in simple epithelia (Angst et al., 1990).

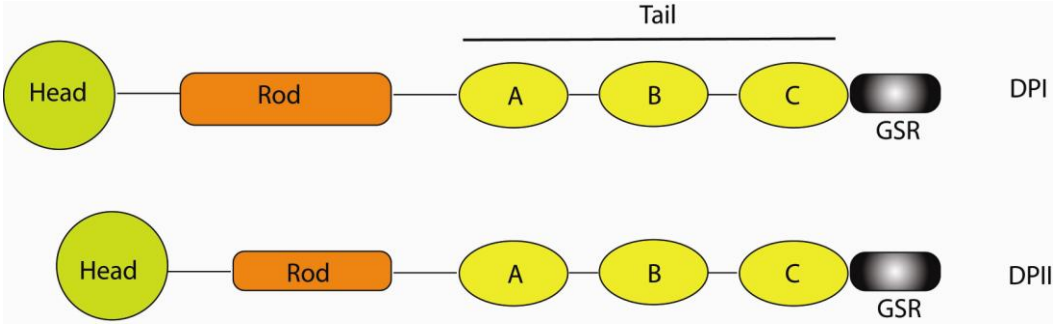


Figure 1.6 Structure of the plakin family member, desmoplakin. Shown above is the general structure of the two desmoplakin isoforms, DP I and II. A, B, and C are the plakin repeat domains. GSR represents the glycine-serine-arginine rich domain.

Several studies highlight the importance of DP in strengthening desmosome structure and function. Mice lacking DP die shortly after implantation at day E6.5 and exhibit fewer desmosomes, compared to wild-type mice (Gallicano et al., 1998). Furthermore, desmosomes that are seen do not attach to intermediate filaments and the absence of DP affected the tissue integrity and shaping of the growing embryo, supporting in vitro studies illustrating the importance of DP in linking intermediate filaments to the plasma membrane and desmosome assembly/stabilization (Bornslaeger et al., 1996). There is also support for DP playing a role in tissue morphogenesis. DP-null mouse embryos also fail to undergo the massive increase in cell proliferation as normally observed in E5-6 and other studies have illustrated that DP is pivotal in the development of the epidermis, neuroepithelium, heart and blood vessels (Gallicano et al., 2001; Vasioukhin et al., 2001).

There are a number of diseases associated with DP mutations, which vary in severity (Lai Cheong et al., 2005). DP haploinsufficiency causes striate palmoplantar keratoderma (Armstrong et al., 1999; Whittock et al., 1999). However, compound heterozygosity with N-terminal missense mutations and C-terminal nonsense mutations leads to a more severe keratoderma, skin fragility, and woolly hair/alopecia (Whittock et al., 2002). One patient who displayed compound heterozygous mutations which truncated the C-terminus, developed lethal acantholytic epidermolysis bullosa, in which the patient exhibited complete alopecia, neonatal teeth, nail loss, and death 10 days postpartum due to transcutaneous fluid loss as a result of extensive skin erosion (Jonkman et al., 2005).

Because the C-terminus is the portion of DP that interacts with intermediate filaments, this finding illustrates that attachment of intermediate filaments to the desmosome is necessary for providing desmosomal integrity.

The desmosome is an important intracellular adhesion complex, providing a supracellular network of adhesive strength. The importance of the desmosome as an adhesive complex has been highlighted by the numerous studies observed, in which misregulation of any of the desmosomal components results in dramatic loss of adhesion strength. Moreover, these studies have stressed that the desmosomal proteins may also play pivotal roles in other cellular processes, such as cell proliferation and differentiation. Therefore, it is not surprising that disruption of desmosome architecture is associated with a number of pathologies. Desmosomal disruption may occur as a result of genetic defects of desmosomal proteins, desmosomal cadherin cleavage by bacterial toxins (i.e. exfoliative toxins (Amagai et al., 2000)), or the binding of autoantibodies to various desmosomal cadherin isoforms. The following chapter will focus on the latter phenomenon and highlight a particular autoimmune epidermal disease, pemphigus vulgaris, which will be used as a model to understand the cellular mechanisms involved in the regulation of desmosomal dynamics and maintenance of epidermal integrity.

Chapter 2: Pemphigus Vulgaris

The term “pemphigus” is derived from the Greek word “pemphix”, which means blister. Pemphigus describes a group of chronic blistering diseases in which the common thread lies in the manner by which these diseases manifest themselves – due to autoantibody binding to certain desmosomal proteins on the surface of the keratinocytes, resulting in acantholysis (or loss of cell-cell adhesion). These pathologies include pemphigus foliaceus, fogo selvagem, and pemphigus vulgaris. Pemphigus vulgaris is the most common subtype of pemphigus worldwide (Prajapati and Mydlarski, 2008).

2.1 Etiology

Pemphigus vulgaris (PV) is a potentially fatal autoimmune skin disease, in which a person generates autoantibodies against the desmosomal cadherins, typically Desmoglein 3 (Dsg3) and sometimes Desmoglein 1 (Dsg1) (Amagai, 1996; Amagai, 1999; Amagai, 2002; Amagai et al., 1991; Payne et al., 2004). Dsg3, a 130kd glycoprotein, is found primarily in the suprabasal layer of the epidermis. As a result, PV is histologically characterized by suprabasal acantholysis and clinically by blistering of mucous membranes and/or the skin (**Figure 2.1**) (Amagai, 2002; Amagai et al., 1991; Payne et al., 2004). In over 50% of the cases, patients initially develop oral lesions, which may be only the manifestation that occurs (mucosal PV). In other cases of PV, the oral lesions are followed by cutaneous lesions (mucocutaneous PV) (Dick and Werth, 2006; Fantasia and Damm, 2002). These oral erosions are usually painful due to their ability to break easily in response to local physiological trauma. The areas most affected are the mucosa of the cheeks and palate (although other mucosal surfaces are subjected to blister formation).

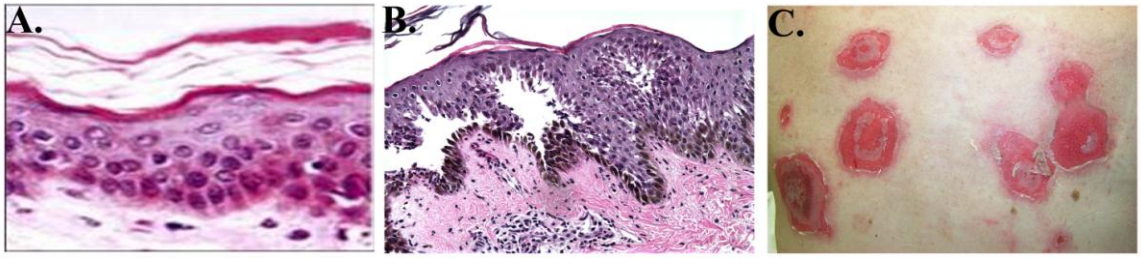


Figure 2.1 Hallmarks of pemphigus vulgaris. Normal epidermis is shown in panel *A*. However, in the presence of PV IgG, the epidermis undergoes suprabasal acantholysis, as a result of disruption of desmosomal adhesion (*B*). This loss of cell-cell adhesion is responsible for the oral erosions and epidermal blistering manifested in PV patients (*C*).

2.2 Prevalence

PV occurs with equal incidence in men and women, usually in their 5^{th} and 6^{th} decades. While the worldwide incidence of PV is approximately 0.1-0.5 per 100,000 people, the incidence of PV increases from 1.6-3.2 cases per 100,000 within the Ashkenazi Jewish population (Femiano, 2007; Prajapati and Mydlarski, 2008).

2.3 Treatment

Prior to the advent of corticosteroids, the mortality rate for untreated PV patients ranged from 60% to 90% (Prajapati and Mydlarski, 2008). Use of steroids and other therapies have reduced the rate to 10%. However, as with all therapies, there are associated side effects. Currently, the goal of managing PV is to induce and maintain remission, using the lowest dose of medication possible, so as to minimize these potentially fatal side effects.

Corticosteroids remain the primary treatment for PV patients, due to their ability to rapidly and effectively induce remission. The side effects associated with steroid use include weight gain, diabetes, hypertension, immunosuppression, sepsis, and extensive loss of bodily fluids (Buchman, 2001). Therefore, adjuvant therapies are provided in conjunction with corticosteroids to alleviate the effects of the primary agents.

In addition to conventional methods of therapy, there are a number of emerging therapies that have been developed over the years, including intravenous immunoglobulin, plasmapheresis, extracorporal photochemotherapy, and cholinergic agonists (Prajapati and Mydlarski, 2008). Furthermore, there have been many case reports suggesting the use of rituximab, a monoclonal anti-CD20 antibody and TNF-α antagonists (i.e. infliximab and etanercept) in suppressing PV (Berookhim et al., 2004; Jacobi et al., 2005; Pardo et al., 2005; Pfutze et al., 2008; Schmidt et al., 2006).

2.4 Mechanism of Acantholysis

The mechanism by which acantholysis occurs remains a major focus of PV research. Many mechanisms of PV pathogenesis have been proposed including steric hindrance of the Dsg3 adhesive interface, cell signaling which down-regulates cell-cell adhesion, and destabilization of the various desmosomal and non-desmosomal proteins. These models are discussed below.

Steric Hindrance Model

One of the earliest theories to explain the acantholysis involved in PV is that PV IgG binding to the N-terminal region of its antigen can directly prevent the desmosomal cadherin from forming stable cell-cell adhesion complexes with neighboring keratinocytes. Jones et al was the first to suggest that PV IgG binding interfered with desmosome function (Jones et al., 1986). Since this initial finding, a number of studies have been performed, which favor the idea that PV IgG binding to the adhesive interface of Dsg3 perturbs its trans and cis interactions. Mouse studies were performed in which PV IgG was affinity purified on columns containing β-galactosidase fusion proteins encoding either the amino EC1-2 domains or the more carboxyl EC3-5 domains of the "PV antigen". The purified antibody from the various columns were then injected into mice (Amagai et al., 1992). These studies revealed that the antibodies that were pathogenic and caused suprabasal acantholysis were directed against the amino terminus of Dsg3, thought to be responsible for adhesive binding. Mouse monoclonal antibodies directed against various extracellular domains of Dsg3 were found to have varying degrees of pathogenicity (as measured by induction of blister formation) (Tsunoda et al., 2003). The results illustrated that the antibodies that were pathogenic and caused blisters recognized a calcium-dependent conformational epitope on Dsg3, composed of specific residues believed to form the adhesive interface (predicted from structural studies of classical cadherins). However, the antibodies that failed to induce blisters recognized epitopes mapped to the middle or carboxyl-terminal extracellular region of Dsg3, where no direct intermolecular interaction is thought to occur.

Furthermore, Dsg3 -/- mice exhibited characteristics resembling PV, including suprabasal acantholysis and mucosal erosions (Koch et al., 1997). Lastly, recent studies in both a cell-free and cell-based system (using atomic force microscopy and laser tweezer measurements, respectively) illustrated that PV IgG directly blocks Dsg3 transinteraction and that peptides targeting the adhesive interface of Dsg3 can prevent PV IgG-induced acantholysis (Heupel et al., 2009; Heupel et al., 2008)

Other evidence argues against the steric hindrance hypothesis. First, PV IgG does not induce acantholysis in PG-null cells, which maintain fully functional desmosomal cadherins (Caldelari et al., 2001). Work from our laboratory also argues against the steric hindrance hypothesis. Neonatal human keratinocytes treated with PV IgG at 4°C do not cause loss of cell-cell adhesion when subjected to mechanical stress (Calkins et al., 2006). These data, along with others mentioned in the following sections, suggest that steric hindrance alone is not sufficient to induce the histological characteristic loss of cell-cell adhesion during PV.

Intracellular Signaling Model

More recently, several studies have implicated a role for signal transduction in mediating PV-induced acantholysis. Pemphigus IgG binding has been shown to cause activation of numerous cell signaling proteins, including phosphotidylcholine-specific phospholipase C (PC-PLC), 1,4,5-triphosphate (IP_3) and protein kinase C (PKC). The following pathways discussed below are not exclusive, but provide evidence from both earlier and recent studies that signaling pathways also contribute to PV pathogenesis.

Work by Rubenstein has illustrated that PV IgG binding induced phosphorylation of heat shock protein 27 (HSP27) via p38 mitogen-activating protein kinase (p38MAPK). MAPK has also been shown to be involved in phosphorylation of Dsg3 itself in response to PV IgG (Kawasaki et al., 2006). Furthermore, inhibition of MAPK activity prevents keratin retraction, actin reorganization, and formation of epidermal blisters in a mouse model (Berkowitz et al., 2005) (Berkowitz et al., 2006).

As with all other signaling pathways, the one question raised by these studies is whether or not p38MAPK activation is a direct result of PV IgG binding or if it is a phenomenon that occurs in response to acantholysis. There are other independent cellular processes that are associated with p38MAPK activation, including loss of cell-cell adhesion in rat intestinal epithelial cells (Rosen et al., 2002) and keratinocyte migration (Li et al., 2001). While inhibition of p38MAPK prevented keratinocyte migration, activation of the kinase pathway did not increase migration, suggesting that it is necessary but not sufficient for the migratory behavior.

The plasminogen activator system has also been implicated in the pathogenesis of PV. Plasminogen activators are serine proteases which convert the zymogen plasminogen to its active form, plasmin (a trypsin-like proteinase). Plasmin is generated and localized to the pericellular space, thus serving as a regulator of cell adhesion and migration (Mondino et al., 1999). One hypothesis provided to explain PV pathogenesis was the release of non-lysosomal proteases upon PV IgG binding. PV IgG induces the activation of PKC which is known to regulate secretion of the urokinase-type plasminogen activator (UPA) and expression of its receptor (uPAR), thus resulting in activation of plasminogen and subsequent degradation of pre-existing desmosomes.

There have been a number of observations which support the hypothesis including detection of uPA and uPAR in the epidermis of PV patients but not healthy individuals (Schaefer et al., 1996); induction of uPA and uPAR in cultured keratinocytes treated with PV IgG (Hashimoto et al., 1983; Seishima et al., 1997); and inhibition of acantholysis through use of both uPA and proteinase inhibitors (Dobrev et al., 1996; Hashimoto et al., 1989; Morioka et al., 1987; Xue et al., 1998).

However, there are reasons to believe that the uPA/uPAR system is not a major contributor to PV pathogenesis. Under normal conditions, plasminogen is activated and released to the pericellular space to assist in regulating cell-cell adhesion (Mondino et al., 1999). In the case of wound healing, uPAR is highly expressed at the front of regenerative epithelial outgrowths during re-epithelialization (Katsuta et al., 2003; Romer et al., 1994). Therefore, this increase in uPAR expression may very well be the result of the epidermis responding to the damage caused by PV IgG and is actually working to combat the damage done.

In addition, the role of plasminogen activators in PV pathogenesis has been examined in plasminogen knock-out mice. In both uPA and tissue-specific plasminogen activator (tPA) knock-out mice, PV IgG, as well as PF IgG, caused epidermal blistering and suprabasal and superficial acantholysis, respectively, to the same degree as control mice (Mahoney et al., 1999). Furthermore, the same results were observed in the uPA and tPA double knock-out mice. Altogether these results imply that the uPA/uPAR system is not a major contributor to PV-induced desmosomal disassembly and acantholysis.

PV IgG and Dsg3 Trafficking

Lastly, studies have shown that PV IgG binding may interfere with the normal turnover of Dsg3 and alter its trafficking. Initially, Dsg3 polypeptides are translocated to the plasma membrane where they form soluble clusters, not associated to intermediate filaments (Sato et al., 2000). These clusters then attach to the cytoskeleton and become incorporated into desmosomes. Recent work has illustrated that PV IgG interferes with both the assembly and disassembly of desmosomes, causing an imbalance in the maintenance proper cell-cell adhesion. The half-life of Dsg3 is reduced in the presence of PV IgG, from 24 hours to 18 hours and perturbs recruitment of soluble Dsg3 into pre-formed desmosomes (Cirillo et al., 2006; Sato et al., 2000). Early PV studies illustrated that internalization of PV IgG and its cell surface antigen on murine epidermal cell monolayers, as a phenomenon specific to PV IgG and not normal human control IgG (Patel et al., 1984). This finding was further supported by work in which the fate of PV IgG was monitored on keratinocytes from human PV patients and ultrastructural analysis revealed that PV IgG-antigen complexes were internalized (Iwatsuki et al., 1989). Furthermore, Sato and others demonstrated that PV IgG treatment on human squamous cell carcinomas (DJM-1) caused internalization of the non-desmosomal pool of Dsg3 into endosomes, through both immunofluorescence and immunoelectron microscopy (Sato et al., 2000). Subsequent studies by Calkins et al., illustrated that PV IgG binding to Dsg3 causes internalization of the antigen, and its degradation through an endo-lysosomal pathway (Calkins et al., 2006). Internalized Dsg3 co-localized with markers for early endosomes (early endosomal antigen -1), as well as markers for late endosomes and lysosomes (CD63 and cathepsin D, respectively).

In addition, PV IgG binding led to an increase in cell surface Dsg3 turnover, as monitored via biotinylation assays. This increase in turnover correlated with loss of cell surface levels of Dsg3, as well as loss in steady state levels of both the soluble and insoluble pools of Dsg3 after 24 hours of incubation. Lastly, Dsg3 internalization/degradation was tightly temporally associated with loss of cell-cell adhesion, characterized by mislocalization of desmoplakin and compromised cell-cell adhesion when keratinocytes treated with PV IgG were subjected to mechanical stress. This acantholysis required keratinocyte responses, as keratinocytes treated with PV IgG at 4°C did not exhibit acantholysis when subjected to mechanical stress. More recently, Jennings and others have demonstrated that over-expression of Dsg3 in human keratinocytes treated with PV IgG counteracts the effects of PV IgG on Dsg3 endocytosis, desmosomal disassembly, and loss of cell-cell adhesion (Jennings, Kottke, and Kowalczyk, unpublished). These findings and others suggest that endocytosis and degradation of Dsg3 play a causative role in PV pathogenesis, and highlight the importance of cadherin stability in maintaining tissue integrity in adult organisms.

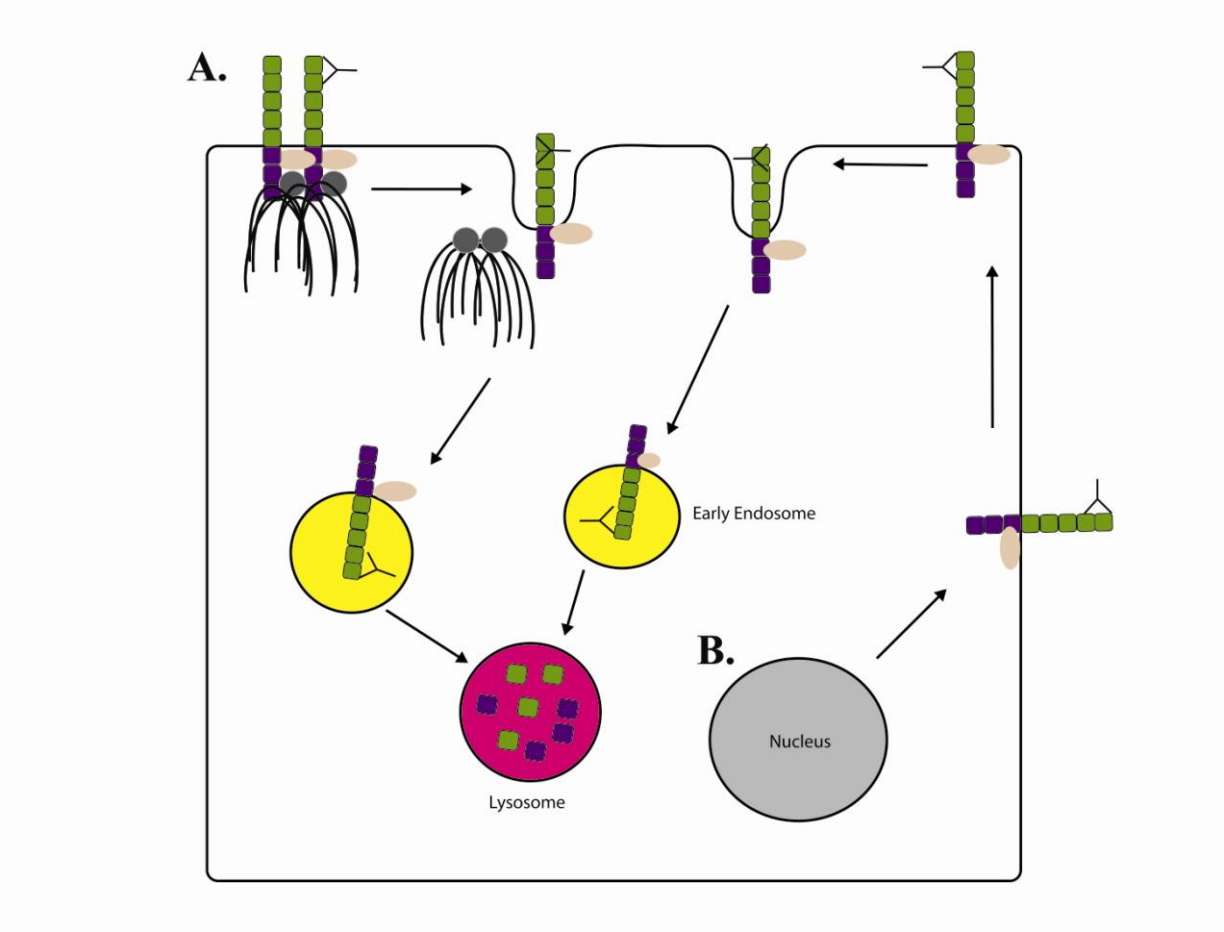


Figure 2.2 Model of desmosomal dynamics in response to PV IgG. Based on previous studies, we propose that PV IgG binding to Dsg3 causes an increase in Dsg3 internalization, ultimately resulting in its degradation in lysosomes. PV IgG can affect (*A*) the turnover of Dsg3 associated with the desmosome or (*B*) newly synthesized Dsg3.

The following section will delve into the current understanding of how cadherins are routed and how the interface between cadherins and membrane trafficking pathways regulate cell surface adhesive potential.

Chapter 3: Regulation of Cell-Cell Adhesion via Cadherin Internalization

Adapted from Delva E and Kowalczyk AP. Traffic 2009 Mar;10(3):259-67

3.1 General Routing of Cadherin Trafficking

In order to understand if PV IgG-induced Dsg3 endocytosis and desmosomal disassembly are causally related, we need to first consider the various pathways by which cadherins are generally internalized. Early studies revealed that cadherins could maintain adhesion only in the presence of calcium, and that upon calcium depletion cadherins become metabolically unstable. This observation led to the realization that cadherins can be retrieved from the plasma membrane to modulate the adhesive state of the cell surface (Kartenbeck et al., 1991; Mattey and Garrod, 1986; Troyanovsky et al., 2007; Troyanovsky et al., 2006; Windoffer et al., 2002). Like other receptors, cadherins are internalized by selective recruitment into specific endocytic pathways. These cadherin entry points include clathrin-mediated endocytosis, caveolae-mediated endocytosis, and internalization routes that are independent of both clathrin and caveolae, including lipid raft-mediated endocytosis and macropinocytosis.

Several classical cadherins, including E-cadherin and VE-cadherin, are internalized via the clathrin-mediated pathway (Ivanov et al., 2004; Izumi et al., 2004; Le et al., 1999; Xiao et al., 2005). Recruitment into the clathrin pathway typically requires an association of the cargo protein, in this case cadherin, with an adaptor complex that couples the cargo to clathrin during coated pit assembly. Thus, a key and emerging issue being addressed in cadherin biology is the identification of adaptors that bind cadherin tails and mediate recruitment of these adhesion molecules to endocytic invaginations for internalization. In the case of VE-cadherin, Gavard and colleagues reported an interaction with β-arrestin upon treatment of endothelial cells with the growth factor VEGF (Gavard and Gutkind, 2006). β-arrestin interaction with VE-cadherin is

dependent upon the presence of a serine residue in the cadherin tail that is a target for p21-activated kinase (Llorente et al.), downstream of Rac activation by VEGF. Furthermore, VE-cadherin endocytosis was shown to be coupled to the regulation of endothelial barrier function in response to VEGF. In addition, our laboratory has uncovered associations between the adaptor complex AP-2 and the VE-cadherin cytoplasmic domain (Chiasson et al., 2009), suggesting that multiple clathrin adaptors may cooperate during VE-cadherin internalization or, alternatively, that different adaptors are engaged selectively depending upon cell signaling activities. Consistent with this latter possibility, E-cadherin also associates with several endocytic adaptors, including disabled-2 (Dab2) and AP-1B (Ling et al., 2007; Yang et al., 2007). Dab2 is implicated in the regulation of epithelial cell polarity by selectively recruiting apical surface E-cadherin into a clathrin-mediated endocytic pathway. In a separate study, E-cadherin was shown to associate with the clathrin adaptor, AP-1B, indirectly through type Iγ phosphatidylinositol phosphate kinase (PIPKIγ) (Ling et al., 2007). This interaction appears to regulate both E-cadherin endocytosis and recycling. Remarkably, the binding site for PIPKIγ in the E-cadherin tail is mutated in patients suffering from gastric carcinogenesis (Ling et al., 2007; Yabuta et al., 2002). Collectively, these findings reveal an interface between membrane trafficking pathways and cadherin function in vascular biology, the development and maintenance of epithelial polarity, and in human tumorigenesis.

In addition to clathrin-mediated endocytosis, cadherins are also internalized through engagement of other types of endocytic machinery. For example, E-cadherin is internalized through a caveolae-mediated pathway in A431 human epidermoid carcinoma

cells (Lu et al., 2003). Similarly, Rac activation triggers E-cadherin internalization through a caveolae-mediated pathway in keratinocytes (Akhtar and Hotchin, 2001). Finally, cadherins are also internalized by macropinocytosis, a pathway that appears to be associated predominantly with the retrieval of non-junctional pools of cadherins (Bryant et al., 2007; Paterson et al., 2003). Thus, cadherins have been found to undergo endocytic processing by all of the major routes for internalization from the plasma membrane (**Table 3.1**). As discussed below, it appears that the pathway chosen for internalization is dictated by the cellular environment, particularly with respect to the growth factor milieu and the migratory needs of the cell.

Table 3.1: Different Routes and Regulators of Cadherin Internalization

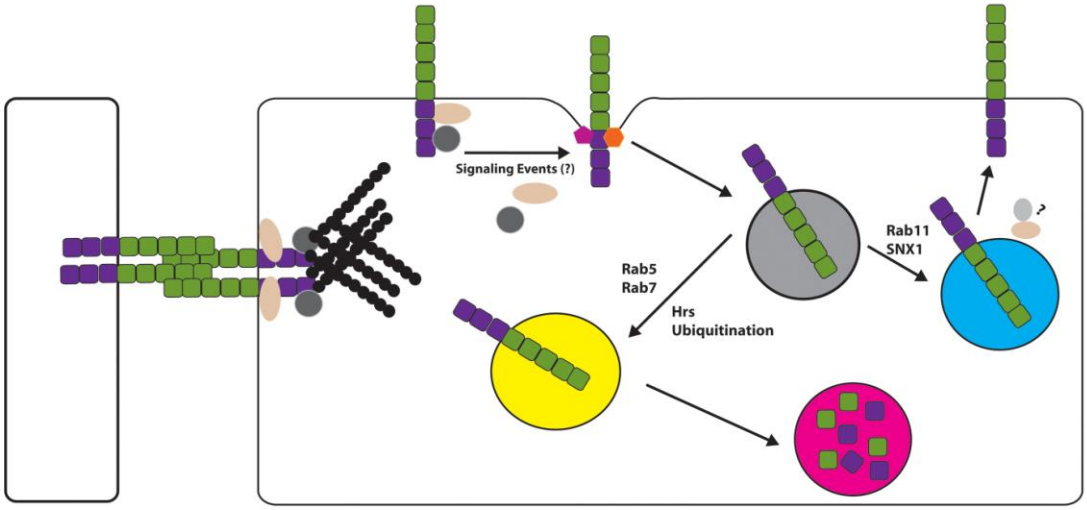
Internalization Route	Cadherin	Adaptors and Regulators
Clathrin-Mediated	**VE-cadherin**	p120 (Xiao et al., 2003; Xiao et al., 2005), β-arrestin (Gavard and Gutkind, 2006), VEGF (Gavard and Gutkind, 2006), Ang1 (Gavard et al., 2008), AP-2 (Chiasson et al., 2009)
	E-cadherin	p120 (Davis et al., 2003; Ireton et al., 2002), Dab2 (Yang et al., 2007), β-catenin (Dupre-Crochet et al., 2007), PIPKIγ (Ling et al., 2007), Rac-1/IQGAP1 (Izumi et al., 2004), FGF (Bryant et al., 2005), HGF (Kamei et al., 1999)
	N-cadherin	NMDAR (Tai et al., 2007), β-catenin (Tai et al., 2007)
Caveolae-Mediated	**E-cadherin**	Caveolin-1 (Akhtar and Hotchin, 2001), Rac-1 (Akhtar and Hotchin, 2001)
Lipid Raft-Mediated	**E-cadherin**	Arf6 (Palacios et al., 2001; Palacios et al., 2005; Paterson et al., 2003)
Macropinocytosis	**E-cadherin**	EGF (Bryant et al., 2007), Rac-1 (Bryant et al., 2007)
	N-cadherin	β-catenin (Sharma and Henderson, 2007), RAC-1/IQGAP1 (Sharma and Henderson, 2007)

3.2 Regulation of Cadherin Fate

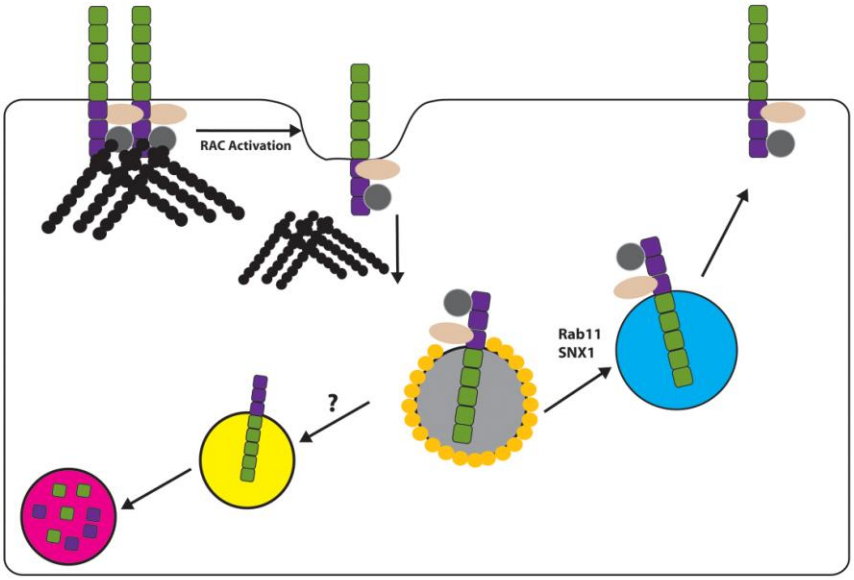
Once a cadherin is internalized, additional sorting machinery regulates routing of the receptor. Differences in post endocytic fates of the cadherin can have either short or long term consequences on the cell's adhesive potential, depending on whether the adhesion molecule is sorted to the lysosome for degradation or recycled back to the cell surface. Interestingly, a number of studies have implicated cadherin recycling and degradation in various developmental and morphogenic events. Very little is currently known about how cadherins are selected from sorting endosomes for trafficking to lysosomes. Ubiquitin tagging and association with the hepatocyte growth receptor substrate, Hrs, as well as Rab 5 and 7 GTPases, have been found to be essential in directing E-cadherin to the lysosome upon internalization(Palacios et al., 2005; Toyoshima et al., 2007). Conversely, several endosome proteins have been implicated in cadherin recycling, including Rab11 and the sorting nexin, SNX1, with SNX1 implicated in re-routing of E-cadherin back to the plasma membrane for the maintenance of cell-cell adhesion (**Figure 3.1**) (Bryant et al., 2007; Lock et al., 2005).

Based on these findings and other studies investigating the endocytic machinery involved in the internalization of various transmembrane proteins, we sought to test how Dsg3 internalization occurs in the presence of PV IgG and to determine if its internalization is relevant to PV-induced desmosomal disassembly.

A. Clathrin-Mediated Endocytosis



B. Macropinocytosis



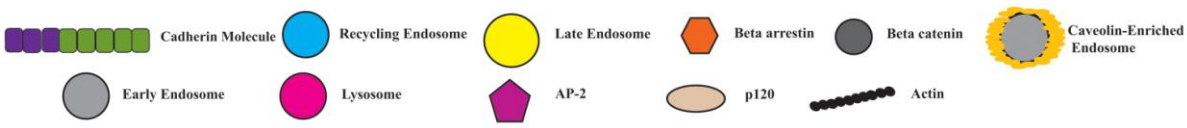


Figure 3.1 Different Endocytic Pathways Utilized by Cadherins. **(A)** Numerous studies have illustrated that classical cadherins, such as E-cadherin, can undergo various types of endocytic processing. Furthermore, the route taken by E-cadherin seems to be dependent on the presence of its cytoplasmic binding partner, β-catenin or p120. In the case of clathrin-mediated endocytosis, binding of β-catenin and p120 maintains E-cadherin's association with the actin cytoskeleton network and prevents E-cadherin endocytosis. However, signaling events may cause disassociation of the cadherin and the catenins, thereby allowing adaptors, such as AP-2 and β-arrestin, to interact with the cytoplasmic tail and recruit clathrin and other accessory proteins that promote internalization. Once internalized, E-cadherin enters sorting/early endosomes in which other molecules regulate the fate of the cadherin. GTPases, such as Rabs 5 and 7, as well as post-translational modification of the cadherin cytoplasmic tail (i.e. ubiquitination), promote E-cadherin entry into a lysosomal pathway for degradation. However, Rab 11 and the sorting nexin, SNX1, are essential for routing E-cadherin to recycling endosomes and ultimately back to the plasma membrane. **(B)** When E-cadherin undergoes clathrin-independent endocytosis, signaling events, such as Rac1 activation, are thought to rearrange the actin cytoskeleton network, allowing for internalization of the complex into early endosomes. This complex is internalized into a caveolin-enriched endosome and does not colocalize with markers for late endosomes or lysosomes, suggesting that the cadherin-catenin complex is then recycled back to the plasma membrane, with the assistance of the sorting nexin, SNX1.

3.3 Central Hypothesis of Thesis

My central hypothesis is that Dsg3 endocytosis is mechanistically coupled to desmosome disassembly in response to PV IgG binding to Dsg3. The goal of this research was to determine the molecular players and mechanism(s) involved in PV IgG induced-Dsg3 internalization and desmosomal disassembly. Although previous work demonstrated a correlation between Dsg3 internalization/degradation and desmosomal disassembly, these findings didn't establish a cause/effect relationship between the two processes. One could hypothesize that Dsg3 internalization is necessary to cause subsequent desmosomal disassembly and acatholysis. However, it is also possible that PV IgG binding causes disruption of the desmosome, which in turn allows for internalization of Dsg3, no longer associated with the adhesive complex. Therefore, in order to understand the cause/effect relationship between Dsg3 internalization and desmosomal disassembly, my primary goals were to (1) determine the cellular mechanism by which Dsg3 in internalized in response to PV IgG and (2) inhibit internalization of Dsg3 and assess if PV-induced desmosomal disassembly is prevented as a result of the inhibition. Part I of my dissertation focuses on determining the endocytic machinery involved in PV-IgG induced Dsg3 internalization. Results from this study indicate that the PV IgG-Dsg3 complex failed to colocalize with clathrin, and inhibitors of clathrin and dynamin-dependent pathways had little or no effect on Dsg3 internalization. In contrast, cholesterol-binding agents such as filipin and nystatin, and the tyrosine kinase inhibitor, genistein, dramatically inhibited Dsg3 internalization. Furthermore, the Dsg3 cytoplasmic tail specified sensitivity to these inhibitors. Moreover, inhibition of Dsg3 endocytosis with genistein prevented disruption of

desmosomes and loss of adhesion in the presence of PV IgG. Altogether, these results suggest that PV IgG-induced Dsg3 internalization is mediated through a clathrin and dynamin-independent pathway, and that Dsg3 endocytosis is tightly coupled to the pathogenic activity of PV IgG.

Part II of my dissertation involved understanding the role of the cytoskeletal networks, in particular the actin and microtubule filaments, in regulating Dsg3 internalization and desmosomal disassembly. Through the use of pharmacological agents which depolymerize actin and microtubules (latrunculin A and nocodazole, respectively), we found that depolymerizing microtubules protects against Dsg3 internalization and DP mislocalization in the presence of PV IgG, while actin depolymerization enhances these processes. These initial findings suggest that Dsg3 is initially internalized through a route that involves microtubules, afterwhich the vesicles then move along actin filaments, from early endosomes to the lysosome for degradation.

Although a rare disease, PV serves as a model for understanding the fundamental biology involved in desmosome assembly and disassembly, a process that is important in a number of biological processes, including skin cell differentiation, morphogenesis and tissue patterning, and epithelial-mesenchymal transition. Studying this rare disease will serve as a paradigm to understanding the mechanism involved in desmosome disassembly/assembly, as well as provide us with novel targets for treatment of the disease.

Chapter 4: Pemphigus vulgaris induced desmoglein 3 internalization and desmosomal disassembly is mediated by a clathrin and dynamin independent mechanism

Adapted from Delva E, Jennings JM, Calkins CC, Kottke MD, Faundez V, Kowalczyk AP. Journal of Biological Chemistry 2008 Jun 27;283(26):18303-13.

4.1 Introduction

Desmosomes are adhesive junctions that provide robust adhesion between epithelial cells (Getsios et al., 2004; Yin and Green, 2004). These organelles are prominent in tissues that experience substantial mechanical stress such as the heart, bladder, gastrointestinal mucosa, and skin. Desmosomes are comprised primarily of proteins from three major families: the desmosomal cadherins desmogleins and desmocollins; armadillo proteins, such as plakoglobin and the plakophilins; and members of the plakin family of cytolinkers such as desmoplakin (Garrod et al., 2002; Getsios et al., 2004; Yin and Green, 2004). Together, these proteins contribute to tissue integrity by coupling adhesive interactions mediated by the desmosomal cadherins to the keratin intermediate filament cytoskeleton, thereby integrating adhesive and cytoskeletal networks throughout the cells in a tissue. Although critical for tissue integrity, desmosomes are often remodeled and contribute to dynamic processes during development and wound healing. Furthermore, desmosomal components may also play pivotal roles in keratinocyte differentiation, morphogenesis and tissue patterning, as well as epithelial-mesenchymal transitions (Kottke et al., 2006; Presland and Dale, 2000).

Pemphigus vulgaris (PV) is a potentially fatal autoimmune skin disease in which autoantibodies are generated against the desmosomal cadherin, desmoglein-3 (Dsg3) (Amagai et al., 1991; Anhalt and Diaz, 2004; Payne et al., 2004). Dsg3, a 130kd glycoprotein, is found primarily in the spinous and basal layers of the epidermis and throughout the oral mucosa (Amagai et al., 1996).

As a result, PV is characterized histologically by suprabasal loss of cell-cell adhesion (*acantholysis*) and clinically by blistering of the skin and erosion of mucous membranes (Anhalt and Diaz, 2004; Payne et al., 2004). A wide range of approaches have demonstrated that Dsg3 is the key target of PV IgG (Amagai et al., 1994; Stanley et al., 2001). In addition, experimentally generated mice in which the Dsg3 gene has been ablated exhibit histopathological characteristics of PV patients (Koch et al., 1997). However, the precise cellular mechanism by which acantholysis occurs in response to PV IgG remains controversial.

The epitopes on Dsg3 that are recognized by PV patient IgG and pathogenic monoclonal Dsg3 antibodies have been mapped and reside predominantly in the amino-terminal domain of Dsg3, a region of cadherins that structural studies have implicated in forming the adhesive interface (Amagai et al., 1995; Amagai et al., 1992; Koch et al., 2004; Kowalczyk et al., 1995; Tsunoda et al., 2003). These types of studies argue strongly that PV IgG may cause loss of adhesion by steric hindrance of Dsg3 ectodomain interactions (Payne et al., 2004). However, other studies suggest that keratinocyte responses are needed for cells to lose adhesion (Freedman et al., 1999)For example, desmosomes remain intact and keratinocytes remain adherent when incubated in the presence of PV IgG at 4 degrees, even though PV IgG are bound to Dsg3 (Calkins et al., 2006). In fact, keratinocytes must be incubated at 37 degrees for several hours in order to detect substantial loss of adhesive strength. These and other data favor the hypothesis that keratinocyte responses are required for the loss of adhesion caused by PV IgG.

These responses may include alterations in p38 MAPK pathways (Berkowitz, 2006 #85), Rho GTPase activity (Waschke et al., 2006), activation of c-myc through plakoglobin-mediated signaling mechanisms (Williamson et al., 2006), and other cellular responses that influence cell-cell adhesive interactions (Lanza et al., 2006; Sharma et al., 2007).

Early studies demonstrated that PV IgG are internalized and targeted to lysosomes, and investigators postulated that PV IgG endocytosis might be coupled to the loss of adhesion characteristic of this disease (Iwatsuki et al., 1989; Patel et al., 1984). In fact, several studies indicate that Dsg3 internalization and destabilization of desmosome integrity may be a key keratinocyte response to PV IgG. For example, PV IgG disrupts the assembly of functional desmosomes and causes rapid internalization and degradation of Dsg3 (Aoyama and Kitajima, 1999; Calkins et al., 2006; Kottke et al., 2006).

Clathrin-dependent and clathrin-independent pathways represent the two major routes for internalization of cell surface receptors (Conner and Schmid, 2003; Kirkham and Parton, 2005; Parton and Richards, 2003). Clathrin-mediated endocytosis, the most thoroughly studied endocytic pathway, is characterized by the formation of clathrin-coated pits at the plasma membrane. Clathrin dependent endocytosis requires the GTPase dynamin (Damke et al., 1994; Hill et al., 2001; Sever et al., 2000), which participates in the budding of clathrin coated vesicles that are then destined for endosomal compartments. Clathrin-independent endocytosis, on the other hand, is less well-understood. However, many endocytic pathways fall under this category and include caveolae-mediated endocytosis, micro and macropinocytosis, as well as pathways that are both clathrin and caveolae independent (Kirkham and Parton, 2005).

Defining how specific receptors are internalized from the plasma membrane is critical for understanding how cells control the presentation of the receptor on the cell surface.

In the current study, a series of approaches were used to selectively manipulate various endocytic pathways and thereby reveal the mechanism of PV IgG induced-Dsg3 internalization. The results indicate that the Dsg3 cytoplasmic tail mediates Dsg3 internalization in response to PV IgG through a clathrin and dynamin independent endocytic pathway. Furthermore, in cells treated with PV patient IgG, inhibition of Dsg3 endocytosis prevents Dsg3 down-regulation, desmoplakin mislocalization, and loss of adhesion in functional assays. These findings provide evidence that destabilization of Dsg3 through clathrin independent endocytic pathways is functionally coupled to the loss of keratinocyte adhesion strength in response to PV IgG.

4.2 Materials and Methods

Cells and Culture Conditions. Normal human keratinocytes (NHKs) were isolated and cultured as described previously (Calkins et al., 2006). Briefly, NHKs were isolated from neonatal foreskin and cultured in keratinocyte growth medium (KGM, Cambrex Corp., East Rutherford, NJ). NHKs were used for experimentation at passage 2 or 3. For experiments, cells were shifted to media containing 0.5mM calcium 16–18 h before treatments and remained in this media throughout the duration of the experiments.

Antibodies and Ligands. Alexa Fluor EGF-488 and Cholera Toxin B (CTB)-488 were obtained from Invitrogen (Carlsbad, CA). The CD59 FITC-Conjugate monoclonal antibody was purchased from Millipore (Billerica, MA). The monoclonal clathrin antibody was purchased from BD Transduction Laboratories (San Jose, CA).

Monoclonal IL2-R was obtained from R&D Systems. Appropriate species cross-absorbed secondary antibodies conjugated to various Alexa Fluors (Molecular Probes, Eugene, OR) were used for dual-label immunofluorescence. Normal human serum was obtained from Irvine Scientific (Santa Ana, CA). PV IgG was a kind gift from Dr. Robert Swerlick (Emory University, Atlanta) and Dr. Masayuki Amagai (Keio University School of Medicine, Tokyo) . Monoclonal anti-Dsg3 antibodies, AK15 and AK23 (Tsunoda et al., 2003), were kind gifts from Dr. Masayuki Amagai.

Adenoviruses. A chimeric protein comprising the IL-2R extracellular domain and the VE-cadherin cytoplasmic tail was generated as described previously (Xiao et al., 2003). A construct encoding the extracellular domain of the IL-2 receptor (IL-2R) (LaFlamme et al., 1994) was used to construct a chimeric cDNA with the IL-2R extracellular domain, the entire Dsg3 cytoplasmic tail, and a carboxyl-terminal FLAG epitope tag. The Dsg3 cytoplasmic domain construct was generated by PCR using the following primers: 5-primer: 5'GCCATGACTAGTAGTGTGACTGTGGGGCAGGTTCTACT and the 3-primer: 5'CCGGATATCCTACTTATCGTCGTCATCCTTGTAATCTATTAGACGGGAGCAA GGATCCTCTGTACA. The 3-primer includes an in-frame FLAG epitope tag followed by a stop codon. The resulting PCR product was ligated into pKS followed by subcloning into the pAD-Track-CMV vector. All constructs were characterized fully by DNA sequence analysis, western blot, and immunofluorescence analysis. Adenoviruses carrying the IL-2R-Dsg3$_{cyto}$-FLAG chimeric construct were produced using the pAdeasy adenovirus-packaging system as described previously (Setzer et al., 2004; Xiao et al., 2003).

HA-dyn2WT and HA-dyn2K44A were kind gifts from Dr. Sandra Schmid (The Scripps Institute, La Jolla). The caveolin-1 Y14F virus was a kind gift from Dr. Masuko Ushio-Fukai (University of Illinois at Chicago).

Immunofluorescence. NHKs were prepared for immunofluorescence as described previously (Calkins et al., 2006). Briefly, cells were cultured on glass coverslips and shifted to media containing 0.5mM calcium 16–18 h before treatment. Under the culture conditions used throughout this study, the keratinocytes predominantly expressed Dsg3 and low but detectable levels of Dsg1. For most experiments, keratinocytes were incubated with affinity-purified human or mouse PV IgG to ice for 30 min to 1 h at a concentration of up to 1 mg/ml (diluted in media containing 0.5mM calcium) to label the cell surface. Both human and mouse IgG were left on the cells throughout the duration of the experiment. Cells were then transferred to 37 °C for indicated internalization time. Following incubation at 37°C, cells were returned to ice and treated with acid wash solution (3% BSA, 25mM glycine, pH 2.7) to remove cell-surface bound antibody. Cells were then fixed on ice using either -20 °C methanol for 5 min or 3.7% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) for 10 min followed by extraction in 0.5% Triton X-100 (Roche Diagnostics, Indianapolis, IN) for 7 min. In order to detect cell surface levels of Dsg3, cells were fixed on ice for 10 min in 3.7% paraformaldehyde. Following paraformaldehyde fixation, monoclonal anti-Dsg3 antibody, AK15 (Tsunoda et al., 2003), was used to stain for cell surface Dsg3. 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 Simple PCI (Compix, Inc., Cranberry Township, PA).

Manipulation of Endocytic Pathways. IgG internalization was performed as previously described (Calkins et al., 2006). NHKs were pretreated with 5μM Filipin III (Sigma, St. Louis, MO), 40mM genistein (Sigma), 10μM Nystatin (Sigma), 10μg/ml chlorpromazine (Sigma), hypertonic sucrose (0.4M sucrose) for 1 hour at 37°C. For K^+ depletion, cells were first incubated in hypotonic media (50% K^+ depletion solution, 50% H_2O) for 5 minutes at 37°C, followed by incubation in K^+ depletion solution (20mM Hepes, 140mM NaCl, 1mM $CaCl_2$, 1mM $MgCl_2$, pH 7.4) for 30 minutes at 37°C. Cells were then incubated with a pathogenic mouse monoclonal antibody against the extracellular domain of Dsg3, AK23 (Tsunoda et al., 2003) (diluted in media containing 0.5mM calcium) on ice for 30 min. Cells were then washed 3 times with PBS^+ (or K^+ depletion solution) followed by incubation at 37°C for various times in media containing 0.5mM calcium. Following incubation at 37°C, cells were returned to ice and treated with acid wash solution (3% BSA, 25mM glycine, pH 2.7) to remove cell-surface bound antibody. The cells were rinsed, fixed, and processed for dual label immunofluorescence as described above. In some cases, endocytosis of CTB was assessed by monitoring CTB delivery to the perinuclear region of the cell as reported previously (Le and Nabi, 2003; Yao et al., 2005).

Time-Lapse Microscopy. Primary keratinocytes were cultured on chambered coverglass plates (Lab-Tek/Nunc, Rochester, NY) and shifted to media containing 0.5mM calcium 16–18 h before treatment. Cells were then incubated with a fluorescently- tagged pathogenic mouse monoclonal antibody against the extracellular domain of Dsg3, AK23 (Tsunoda et al., 2003), to label the cell surface. Cells were then transferred to 37 °C for indicated internalization time.

An inverted Leica DMIRE2 microscope equipped with narrow band-pass filters and a Hamamatsu Electron Multiplier, back thinned and deep cooled CCD camera (C9100-12) was used for image acquisition. Temperature control was achieved using an environmental control chamber (Pecon Incubator ML) and heated stage insert (Pecon Heating Insert P). The camera, fully motorized microscope, and automated stage were driven by Simple PCI software.

Dispase Cell Dissociation Assay. A dispase dissociation assay was performed as described previously (Calkins et al., 2006). Briefly, NHK cultures were seeded in triplicate onto 35-mm dishes, containing KGM and allowed to grow to confluence. 24 h after reaching confluence, cultures were switched to media containing 0.5mM calcium for 16-18 h. The cells were then pretreated with 40mM genistein for 1 h at 37 °C (Sigma) and treated with either normal human IgG or PV IgG (diluted media containing 0.5 mM calcium) and incubated on ice for 30 min to 1 h. After incubation, cells were incubated in 1unit/ml dispase (diluted in PBS^{+}) (Roche Diagnostics, Indianapolis, IN) for more than 30 min. Released monolayers were subjected to mechanical stress by transferring the cell sheets to 15-ml conical tubes. The tubes were then subjected to 50 inversion cycles on a rocker panel. Fragments were counted using a dissecting microscope.

4.3 Results

The mechanism by which membrane receptors are internalized can be delineated by selectively inhibiting endocytic pathways using well established procedures. These approaches include manipulating the cellular ionic environment (Idkowiak-Baldys et al., 2006; Liu et al., 2004), altering membrane cholesterol availability (Orlandi and Fishman, 1998; Ros-Baro et al., 2001; Singh et al., 2003), expressing dominant negative mutants, and by inhibiting tyrosine kinase activity (Parton et al., 1994; Shajahan et al., 2004) (**Table 4.1**). In the current study, we utilized this matrix of approaches to define the mechanism of Dsg3 endocytosis and to examine the functional relationships between Dsg3 internalization and the loss of adhesion in response to pathogenic PV IgG.

Table 4.1: Requirements necessary for internalization via distinct endocytic pathways.

Endocytic Pathway	Clathrin Mediated	Caveolae Mediated	Non-Caveolar Raft Dependent
Dependent on Clathrin	X		
Dependent on Caveolin		X	
Sensitive to Hypertonic Sucrose, K+ Depletion, and Chlorpromazine	X		
Sensitive to Cholesterol Perturbing Agents i.e. Filipin and Nystatin		X	X
Dependent on Dynamin Activity	X	X	
Dependent on Tyrosine Kinase Activity	X	X	X

PV IgG-induced Dsg3 internalization is mediated by a clathrin-independent pathway. PV IgG binding causes Dsg3 internalization, dramatically accelerated degradation of Dsg3, and the loss of cell-cell adhesion strength (Calkins et al., 2006). To determine if PV IgG induced Dsg3 internalization is mediated through a clathrin-dependent pathway, hypertonic sucrose and K^+ depletion were employed to inhibit clathrin-mediated endocytosis. For these experiments, keratinocytes were incubated with either EGF (as a control) or a pathogenic antibody directed against Dsg3 (mAb AK23) and placed at 37°C to allow for internalization. A low pH wash was then used to remove cell surface bound ligand without removing internalized ligands. At 37°C, both AK23-Dsg3 and EGF-EGFR were internalized, as expected (**Figure 4.1, A, B, and G**). K^+ depletion and hypertonic sucrose dramatically inhibited EGF internalization, a ligand internalized via a clathrin-mediated pathway (**Figure 4.1, D, F, and G**). In contrast, substantial Dsg3 endocytosis was still observed when clathrin-mediated endocytosis was blocked (**Figure 4.1, C, E, and G**). These initial results suggest that Dsg3 internalization is mediated through a clathrin-independent pathway.

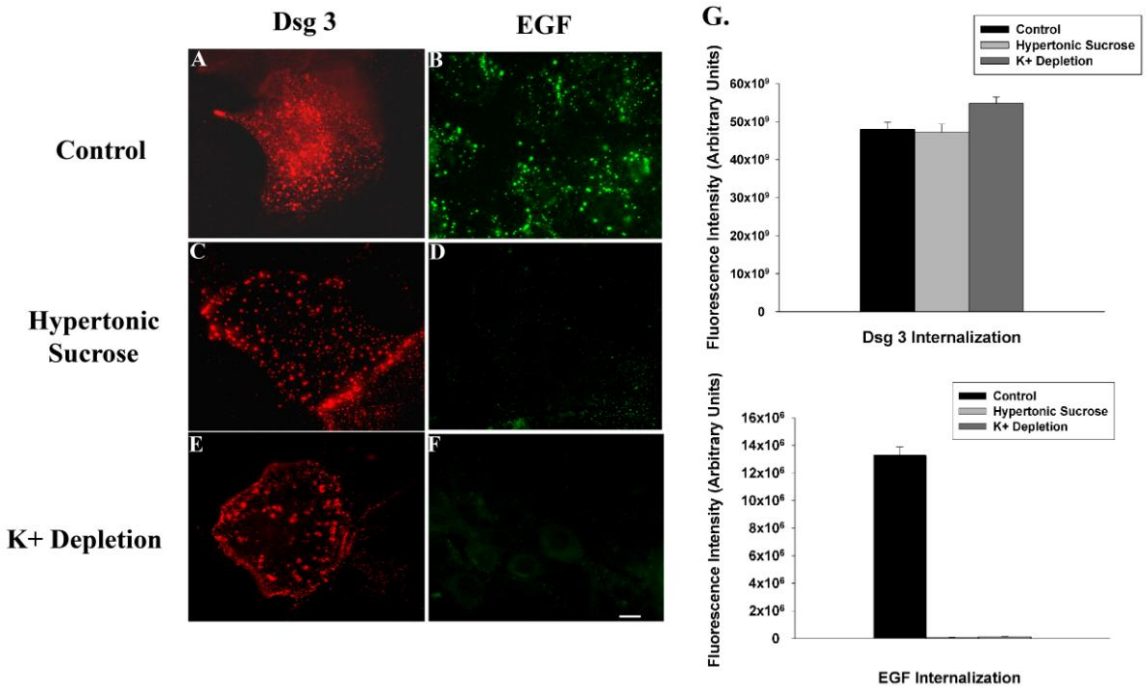


Figure 4.1: PV IgG-induced Dsg3 internalization is mediated through a clathrin-independent mechanism. Primary keratinocytes were either untreated, pretreated with hypertonic sucrose, or subjected to K^+ depletion at 37° C. Cells were then transferred to 4° C and incubated with either Alexa 555-labeled AK23 directed against Dsg3 (*A,C,E)* or fluorescently tagged EGF (*B,D,F*). Excess ligand was removed and keratinocytes were shifted to 37° C for 2 hours to allow internalization. A low pH wash was used to remove cell surface bound ligands to visualize internalized EGF or Dsg3. Total intracellular fluorescence was quantified using a digital imaging system and Simple PCI software (*G*). Error bars represent the standard error of the mean, where n = 15 fields of view. Bar, 30μm.

To further elucidate the mechanism of Dsg3 internalization, the localization of the PV-IgG-Dsg3 complex was compared to the distribution of clathrin and caveolin-1. As shown in Figure 4.2, we were unable to demonstrate colocalization between Dsg3 and clathrin (**A-C**). However, the PV IgG-Dsg3 complex colocalized with caveolin-1 at the cell surface and in intracellular vesicular pools (**Figure 4.2, D-F**). To determine if the PV IgG-Dsg3 complex is internalized through the use of lipid rafts, co-internalization assays were performed to monitor Dsg3 and CD59, a glycosylphosphatidyl inositol-anchored protein (GPI-AP) known to be internalized through a lipid raft-dependent pathway (Nichols et al., 2001; Ricci et al., 2000; Sabharanjak et al., 2002; Skretting et al., 1999). Dsg3 and CD59 colocalized extensively at both the cell surface and in vesicular pools (**Figure 4.2, G-I**). These results further suggest that Dsg3 is internalized through a clathrin-independent mechanism.

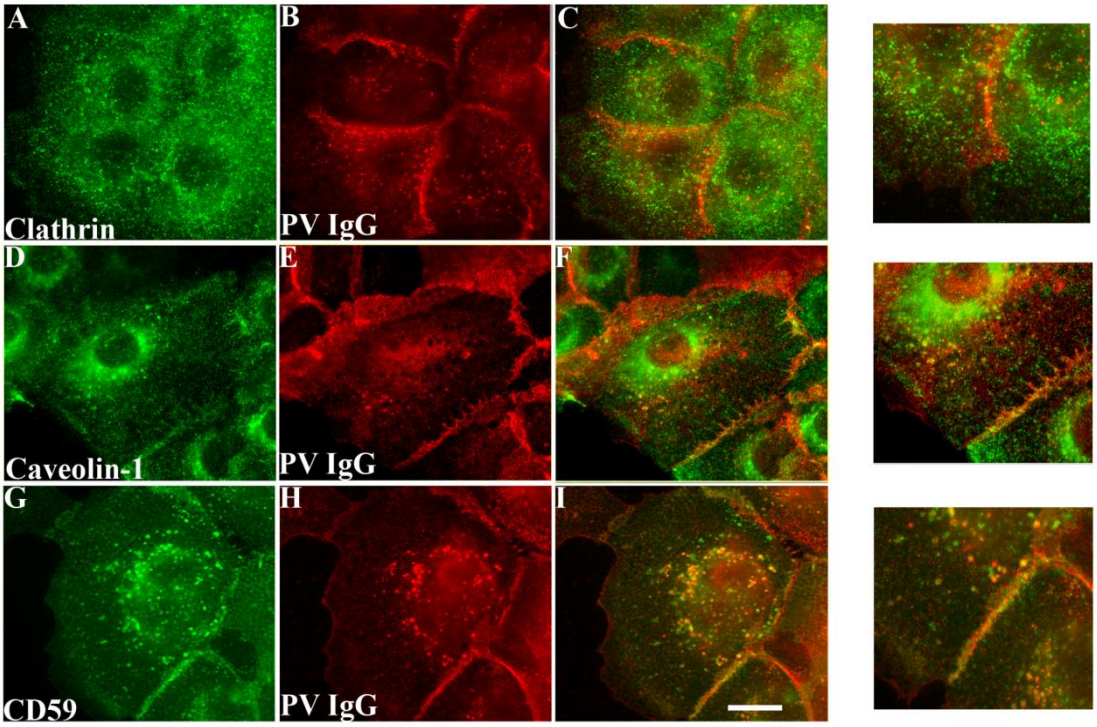


Figure 4.2: Internalized Dsg3 colocalizes with caveolin-1 and CD59 but not clathrin. Primary human keratinocytes were incubated with PV IgG for 1 hr at 37°C, fixed and stained to observe localization of PV IgG with respect to either clathrin (*A-C*) or caveolin-1 (*D-F*) (markers of clathrin-dependent and clathrin-independent pathways, respectively). PV IgG colocalizes with caveolin-1 at both the cell surface and in intracellular vesicles, whereas PV IgG and clathrin do not colocalize. To determine if Dsg3 internalization is mediated through lipid rafts, primary keratinocytes were incubated with both AK23 and a FITC-labeled antibody directed against CD59 (a marker of lipid-raft dependent endocytosis), to monitor internalization of Dsg3 and CD59 after 1 hr at 37°C (*G-I*). Notice the extensive colocalization between the two proteins at both cell borders and in the intracellular vesicles. Bar, 10μm.

The cytoplasmic tail of Dsg3 specifies the mechanism of internalization. While the extracellular domain of Dsg3 is the target of PV IgG binding, the cytoplasmic portion contains a number of domains which may play a role in mediating Dsg3 internalization. In previous studies we found that the VE-cadherin cytoplasmic tail mediates clathrin dependent endocytosis in microvascular endothelial cells (Xiao et al., 2005). These findings raise the possibility that different cell types internalize cadherins through different mechanisms, or alternatively, that different cadherins harbor determinants within their domain structure that dictates the mode of endocytosis. To distinguish these possibilities, the VE-cadherin and Dsg3 cytoplasmic tails were fused to the extracellular domain of the IL-2 receptor (IL-2R-VE-cad$_{cyto}$ and IL-2R-Dsg3$_{cyto}$, respectively). These chimeric proteins were then expressed in keratinocytes for use in internalization assays in the presence or absence of agents that selectively inhibit clathrin mediated endocytosis. As previously reported (Xiao et al., 2005) , internalization of the IL-2R-VE-cad$_{cyto}$ chimera is completely eliminated upon inhibition of clathrin- dependent endocytosis *(***Figure 4.3, D-G***)*. However, similar to endogenous Dsg3, the IL-2R-Dsg3$_{cyto}$ was largely refractory to inhibitors of clathrin-dependent internalization, including chlorpromazine and K^+ depletion *(***Figure 4.3, A-C, and G***)*. These findings demonstrate that the Dsg3 cytoplasmic tail exhibits specificity for a clathrin independent pathway.

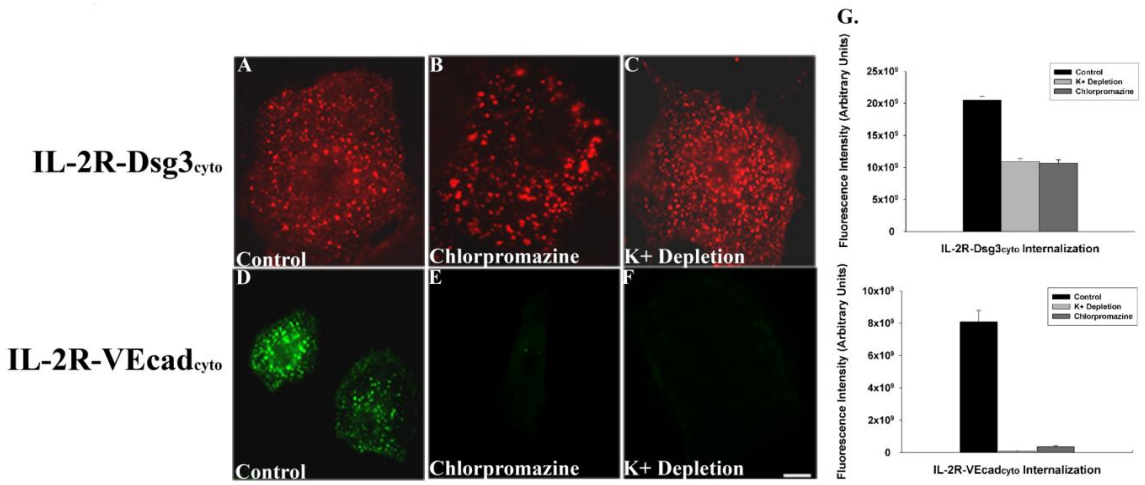


Figure 4.3: The Dsg3 cytoplasmic tail exhibits specificity for clathrin-independent internalization. Primary keratinocytes were infected with adenoviruses carrying the extracellular domain of IL-2R fused to either the Dsg3 cytoplasmic domain (IL-2R-Dsg3$_{cyto}$, *A-C*) or VE-cadherin cytoplasmic domain (IL-2R-VE-cad$_{cyto}$, *D-F*). Eighteen hours after infection, keratinocytes were treated with either chlorpromazine or K^+ depletion for 1hr at 37°C. Cells were then transferred to 4°C and incubated with an antibody against IL-2R. Excess ligand was removed and keratinocytes were shifted to 37°C for 30 minutes to allow internalization. A low pH media was used to remove cell surface bound antibodies to visualize the internalized chimera. Total fluorescence was quantified using a digital imaging system and Simple PCI software (*G*). Error bars represent the standard error of the mean, where n = 15 fields of view. Bar, 30μm.

Dsg3 internalization is sensitive to cholesterol-perturbing agents. Cholesterol is an important component of clathrin-independent endocytosis and numerous studies have demonstrated that cholesterol binding agents block internalization through clathrin independent endocytic pathways (Lamaze et al., 2001; Nichols, 2003; Nichols and Lippincott-Schwartz, 2001; Puri et al., 2001; Sabharanjak et al., 2002). Therefore, keratinocytes were pretreated with the cholesterol binding agents filipin and nystatin to determine their effects on Dsg3 internalization. Internalization of Dsg3 was dramatically inhibited by both of these cholesterol binding agents, as compared to untreated cells (**Figure 4.4, A, C, E, and G**). Filipin and nystatin also inhibited CTB internalization, a control ligand known to be internalized via a clathrin-independent pathway (**Figure 4.4, B, D, F, and G**). Together, these results suggest that PV IgG internalization is mediated in a clathrin-independent and cholesterol-dependent manner.

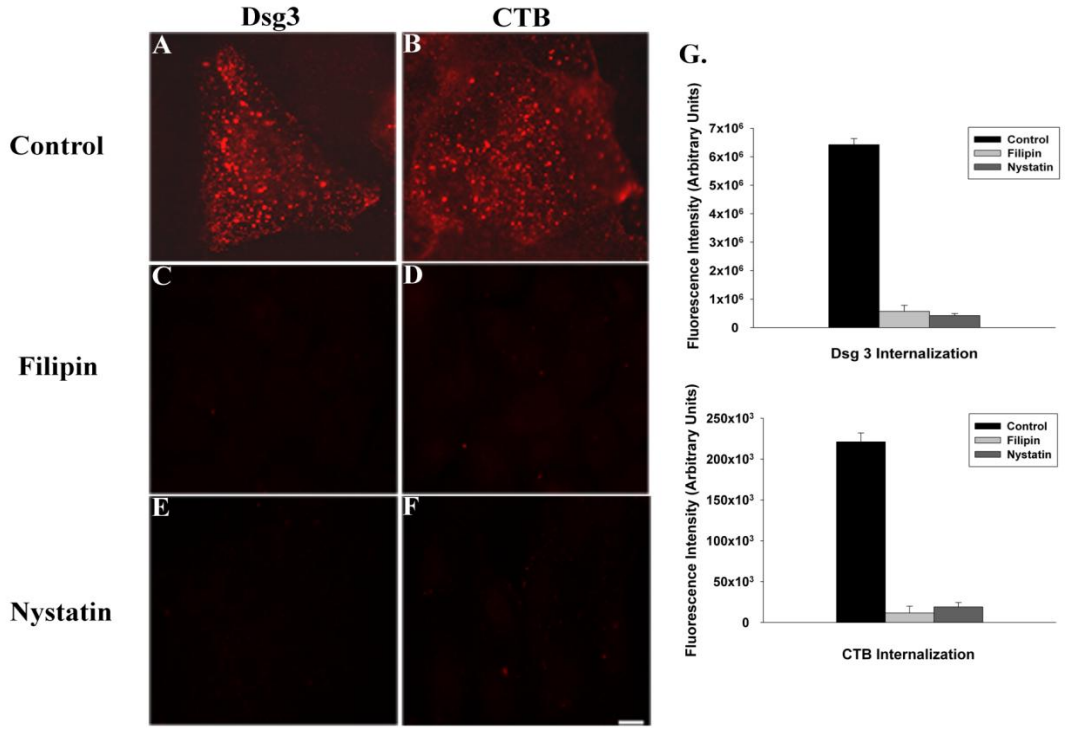


Figure 4.4: PV IgG induced Dsg3 internalization is sensitive to cholesterol-binding agents. Primary keratinocytes were either untreated, pretreated with 5uM filipin or 10uM nystatin for 1 hour at 37°C. Cells were then transferred to 4°C and incubated with either Alexa 555- labeled AK23 directed against Dsg3(*A,C,E)* or fluorescently tagged cholera toxin (CTB) (*B,D,F*), which is known to be internalized in a clathrin-independent manner. Excess ligand was removed and keratinocytes were shifted to 37°C for 2 hours to allow internalization. A low pH wash was used to remove cell surface bound ligands to visualize internalized CTB or Dsg3. Total intracellular fluorescence was quantified using a digital imaging system and Simple PCI software. Error bars represent the standard error of the mean, where n = 15 fields of view. Bar, 30μm.

Previous studies have demonstrated that PV IgG binding causes loss of cell surface levels of Dsg3, which correlates with an increase in Dsg3 turnover and Dsg3 internalization (Calkins et al., 2006). To test whether inhibiting Dsg3 internalization stabilizes cell surface levels of Dsg3 in the presence of PV IgG, keratinocytes were either incubated with affinity-purified normal human IgG (NH IgG), PV IgG, or were pretreated with filipin prior to PV IgG incubation. Cell surface levels of Dsg3 were then monitored using a fluorescence based approach as described previously (Calkins et al., 2006). Keratinocytes incubated with NH IgG exhibited a slight decrease in cell surface levels of Dsg3 over a 6 hour time course, representing a baseline loss of cell surface Dsg3 (**Figure 4.5, A, D, G, J, and M**). In contrast, cell surface levels of Dsg3 in keratinocytes treated with PV IgG were dramatically reduced (**Figure 4.5, B, E, H, K, and M**). Importantly, treating the cells with filipin prevented loss of Dsg3 cell surface levels in the presence of PV IgG (**Figure 4.5, C, F, I, L, and M**). Similar results were observed in cells treated with NH IgG (data not shown). These results further demonstrate that cholesterol perturbation inhibits Dsg3 internalization, resulting in the retention of Dsg3 at the cell surface even in the presence of PV IgG.

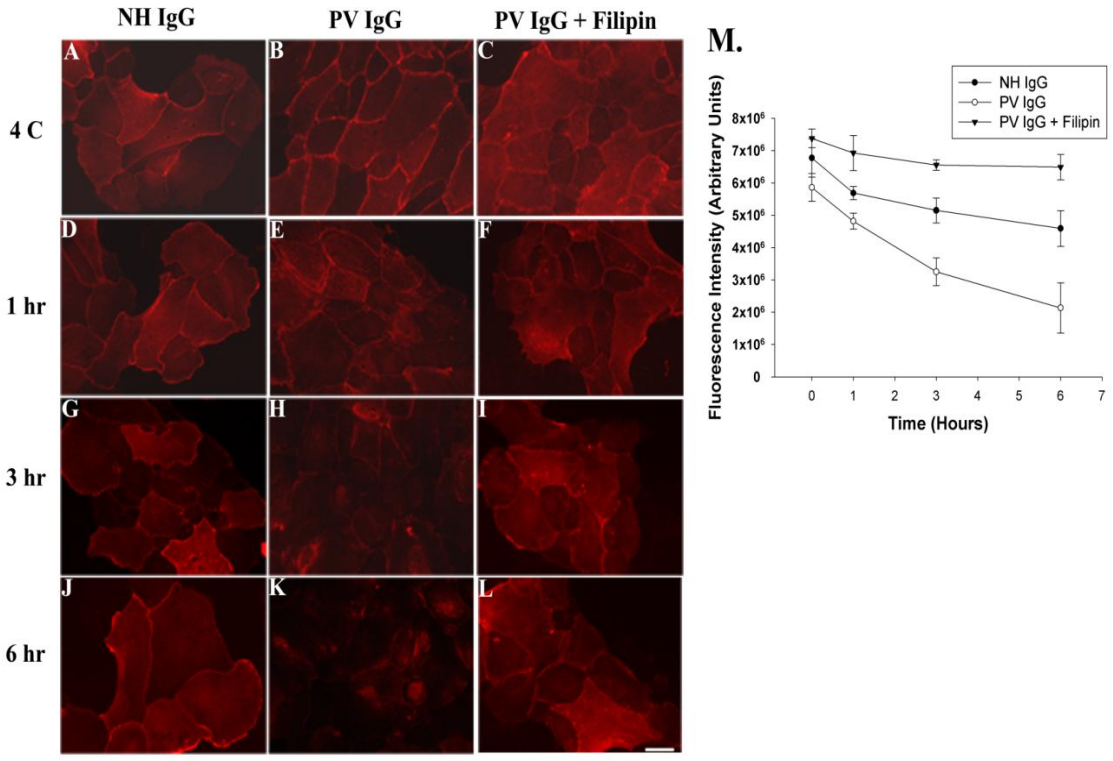


Figure 4.5: Cholesterol perturbation prevents loss of Dsg3 cell surface levels. Primary keratinocytes were either untreated or pretreated with 5uM filipin for 1 hour at 37° C. Cells were then transferred to 4° C and incubated with either normal human IgG (*A,D,G,J*) or PV IgG (*B-C,E-F,H-I,K-L*). Keratinocytes were then shifted to 37° C for various amounts of time. To measure cell surface levels of Dsg3, keratinocytes were processed for immunofluorescence analysis at each time point by fixing cells with paraformaldehyde without permeabilization. AK15, a monoclonal antibody which binds to the extracellular domain of Dsg3, was used to detect cell surface Dsg3. Total surface fluorescence was quantified using a digital imaging system and Simple PCI software (*M*). Error bars represent the standard error of the mean, where n = 15 fields of view. Bar, 40μm.

Dsg3 internalization is dynamin and caveolin independent. Clathrin dependent and a subset of clathrin independent endocytosis require the activity of dynamin, a GTPase responsible for pinching vesicles from the plasma membrane and thereby driving cargo internalization into carrier vesicles (Damke et al., 1994). To determine if Dsg3 internalization is dynamin dependent, wild type dynamin II (Dyn II WT) or a dominant negative dynamin II mutant (Dyn II K44A) were expressed in keratinocytes using an adenoviral delivery system. Expression of wild type Dyn II had no effect on either Dsg3 or EGF internalization (**Figure 4.6, A-C, G-I, and M**). As anticipated, EGF internalization was dramatically reduced in keratinocytes expressing the dominant negative Dyn II mutant (**Figure 4.6, J-L, and M**). However, Dsg3 internalization was not affected by Dyn II K44A, indicating that Dsg3 internalization is dynamin independent (**Figure 4.6, D-F, and M**). These results are consistent with the findings above and indicate that Dsg3 endocytosis occurs through a pathway that does not require clathrin-related mechanisms.

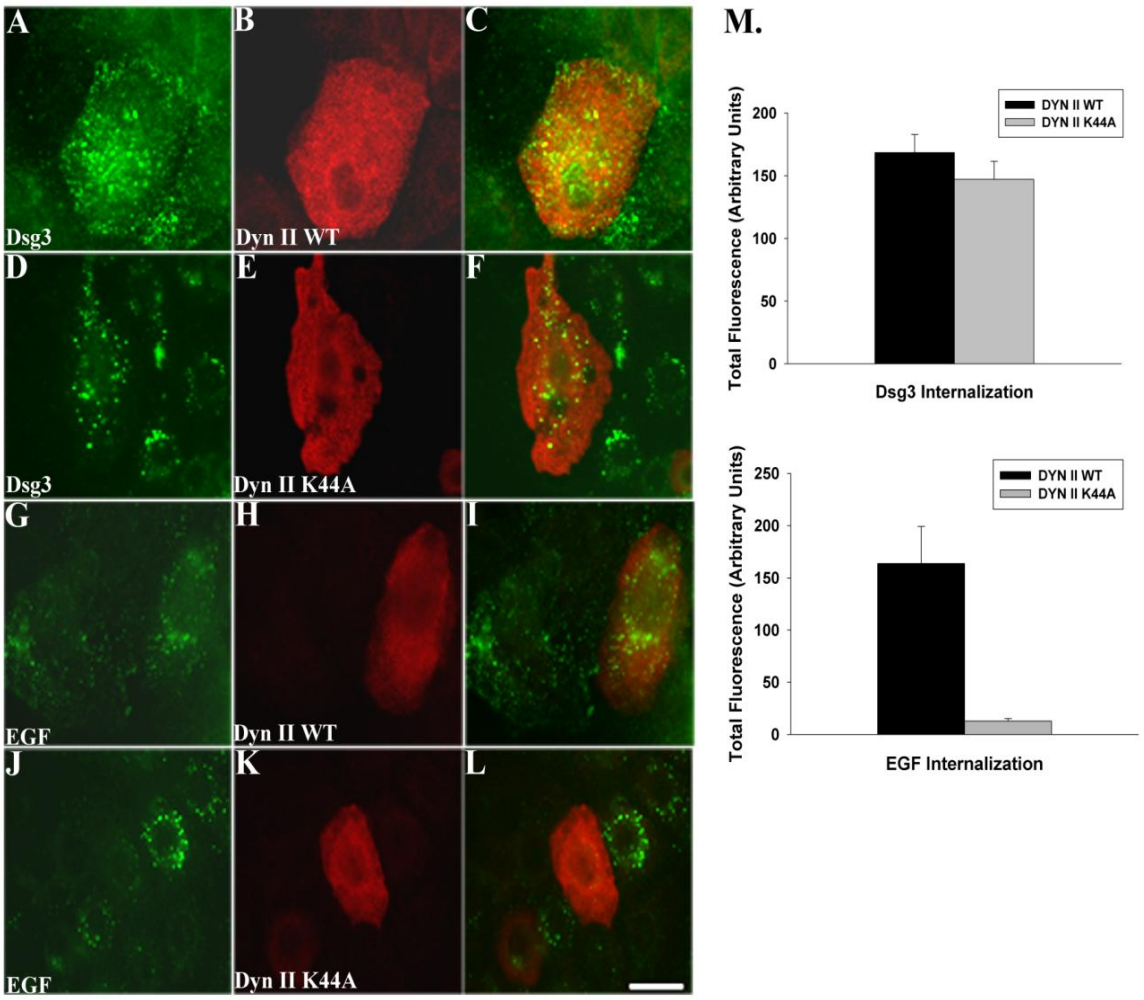


Figure 4.6: Dsg3 internalization is mediated through a dynamin-independent pathway. Primary keratinocytes were infected with adenoviruses carrying either HA tagged wildtype dynamin II (Dyn II WT) or a dominant negative mutant of dynamin II (Dyn II K44A). After 18 hours, cells were transferred to 4°C and incubated with either an antibody against the extracellular domain of Dsg3, AK23 (*A-F*) or fluorescently tagged EGF (*G-L*). Excess ligand was removed and keratinocytes were shifted to 37°C for 3 hours to allow internalization. Acid wash media was used to remove cell surface bound ligands to visualize internalized EGF or Dsg3. Total fluorescence was quantified using a digital imaging system and Simple PCI software (*M*). Error bars represent the standard error of the mean, where n = 20 fields of view. Bar, 30μm.

To further investigate the mechanisms of Dsg3 endocytosis, we examined a possible role for caveolin in Dsg3 internalization. For these experiments, a dominant negative caveolin-1 mutant (Y14F) was expressed in keratinocytes treated with PV IgG. The caveolin-1 Y14F mutant has been shown previously to disrupt caveolae and prevent caveolin mediated endocytosis (Minshall et al., 2003; Orlichenko et al., 2006). To verify functional inhibition of caveolin mediated internalization, endocytosis of CTB into perinuclear compartments was monitored as described previously (Le and Nabi, 2003; Yao et al., 2005). CTB internalization was dramatically inhibited in keratinocytes expressing the caveolin-1Y14F mutant (**Figure 4.7, A-F**). In contrast, EGF endocytosis was not inhibited (**Figure 4.7, G-L***)*, consistent with our findings that EGF internalization is mediated primarily through a clathrin dependent pathway in our model system (**Figure 4.1**). Furthermore, Dsg3 endocytosis also was unaffected by the caveolin-1 mutant (**Figure 4.7 M-R**). Together with the results shown in figure 6, these findings suggest that Dsg3 endocytosis is both dynamin and caveolin independent.

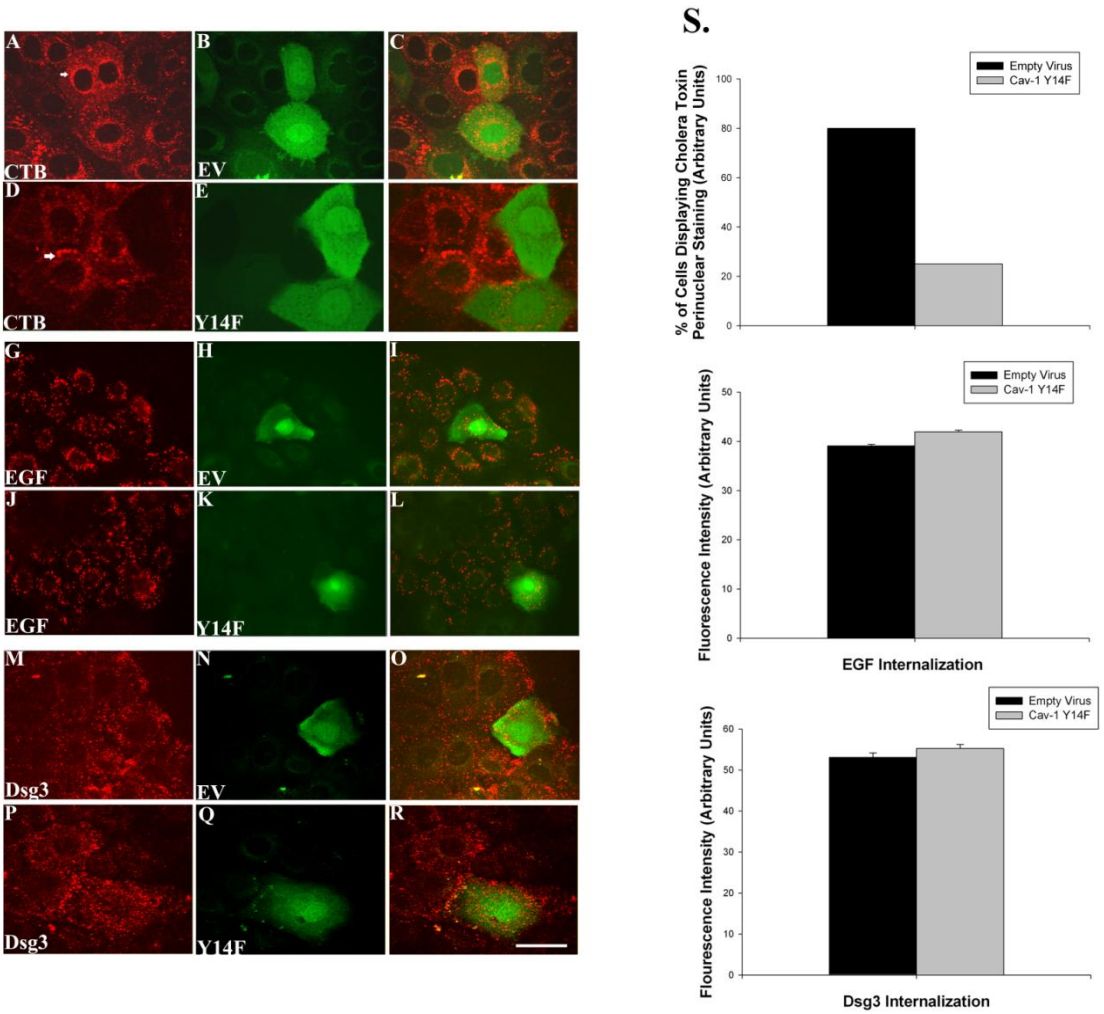


Figure 4.7: Dsg3 internalization is caveolin independent. Primary keratinocytes were infected with adenoviruses carrying either GFP (Empty Virus) or the caveolin mutant CavY14F (Y14F). After 18 hours, cells were transferred to 4°C and incubated with either fluorescently tagged cholera toxin (CTB) (*A-F*), fluorescently tagged EGF (*G-L*), or an antibody against the extracellular domain of Dsg3, AK23 (*M-R*). Excess ligand was removed and keratinocytes were shifted to 37°C for up to 2 hours to allow internalization. Acid wash media was used to remove cell surface bound ligands to visualize internalized EGF or Dsg3. Total fluorescence was quantified using a digital imaging system and Simple PCI software (*S*). In the case of cholera toxin, internalization and delivery to perinuclear compartments (see arrow) was used to quantify inhibition of CTB endocytosis, as reported previously (Le and Nabi, 2003; Yao et al., 2005). Error bars represent the standard error of the mean, where n = 15 fields of view or greater. Bar, 20μm.

Tyrosine kinase activity is required for PV IgG-induced Dsg3 internalization. Clathrin-independent endocytic pathways require tyrosine kinase activity for ligand internalization (Damm et al., 2005; Minshall et al., 2000; Sharma et al., 2004). Genistein is a tyrosine kinase inhibitor shown previously to inhibit clathrin-independent internalization of a variety of ligands, including CTB (Le and Nabi, 2003). Therefore, keratinocytes were treated with genistein and the internalization of Dsg3 and CTB was monitored. Extensive internalization of both Dsg3 and CTB were observed in untreated keratinocytes (**Figure 4.8, A, B, and E**). In contrast, keratinocytes treated with genistein exhibited a dramatic reduction in both Dsg3 and CTB internalization (**Figure 4.8, C, D, and E**). These results were confirmed using time lapse microscopy by following the fate of fluorescently tagged AK23 mAb in living keratinocytes. In untreated cells, AK23 exhibited extensive redistribution from cell borders followed by internalization (**Figure 4.8, F-I; Supplemental Movie 1**). In contrast, internalization of AK23 was inhibited in genistein treated cells (**Figure 4.8, J-M; Supplemental Movie 2**). These results indicate that in addition to cholesterol, tyrosine kinase activity also plays a role in regulating PV IgG-induced Dsg3 internalization. Both of these findings are consistent with the interpretation that Dsg3 internalization is mediated by a clathrin independent mechanism.

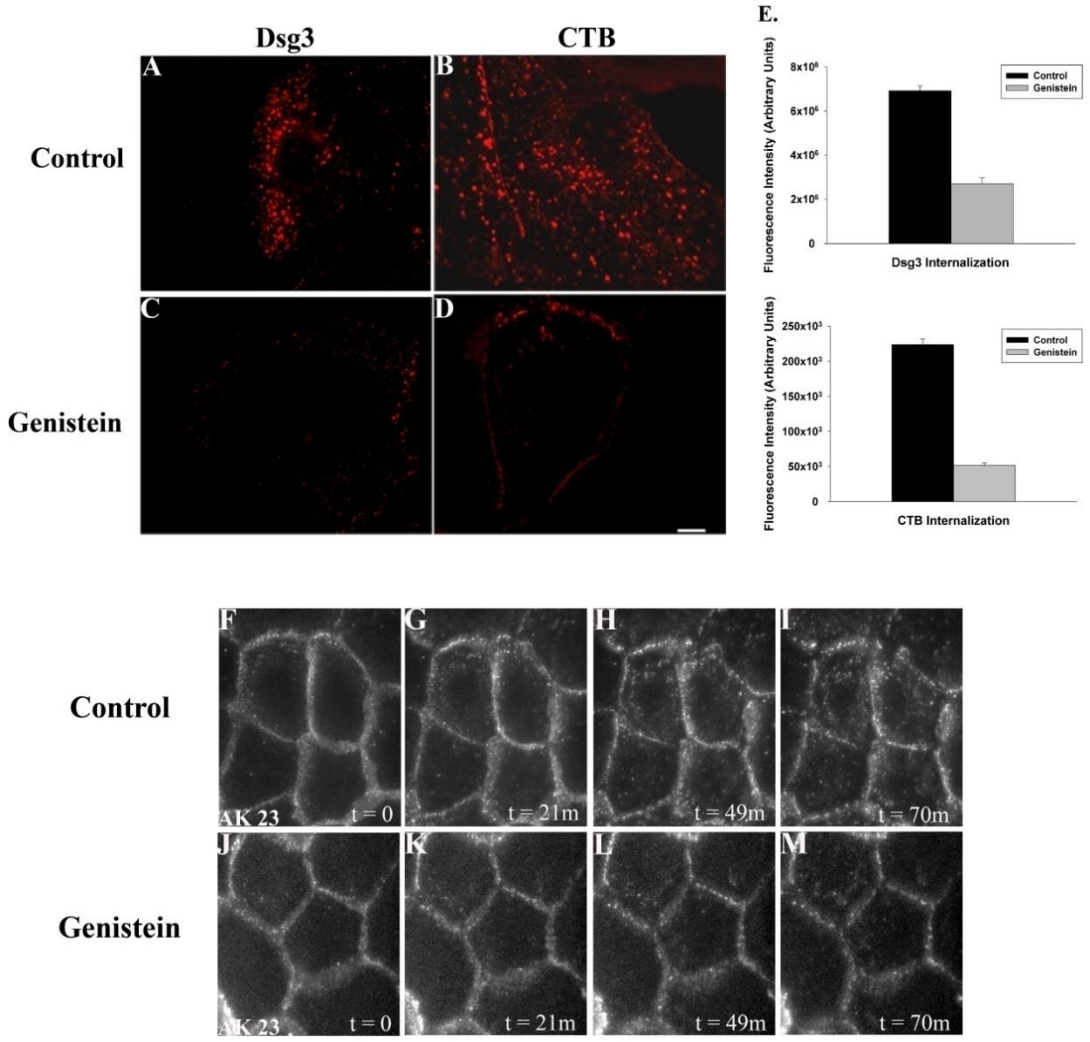


Figure 4.8: Genistein, a tyrosine kinase inhibitor, prevents PV-induced Dsg3 internalization. Primary keratinocytes were either untreated *(A, B)* or treated with 40uM genistein *(C, D)* for 1 hour at 37° C. Cells were then transferred to 4°C and incubated with either Alexa 555-labeled AK23 directed against Dsg3 or fluorescently tagged cholera toxin (CTB). Excess ligand was removed and keratinocytes were shifted to 37° C for 2 hours to allow internalization. A low pH wash was used at 4° C to remove cell surface-bound ligands to visualize internalized Cholera toxin-B or Dsg3. Total intracellular fluorescence was quantified using a digital imaging system and Simple PCI software (*E*). Error bars represent the standard error of the mean, where n = 15 fields of view. Bar, 30μm. For time lapse experiments, living keratinocytes were incubated with fluorescently-tagged AK23 at 4°C and then transferred to 37°C to monitor internalization of Dsg3 in either the absence (*F-I*) or presence of genistein (*J-M*). Supplemental Movies 1 and 2 correspond to panels F-I and J-M, respectively.

Dsg3 endocytosis is coupled to depletion of Dsg3 steady state levels and loss of adhesion. Previous studies from our lab and others indicate that PV IgG cause a down-regulation of Dsg3 steady state levels in cultured keratinocytes, in mouse models of disease, and in PV patient skin (Altschuler et al., 1998; Aoyama and Kitajima, 1999; Calkins et al., 2006). To determine if this down-regulation of Dsg3 can be prevented by agents that block Dsg3 endocytosis, keratinocytes were incubated for 24 hrs in the presence or absence of genistein and Dsg3 steady state levels were monitored by western blot analysis. As expected, PV IgG caused a down-regulation of Dsg3 in both the triton-soluble and insoluble pools (**Figure 4.9, A**) in untreated keratinocytes. However, genistein treatment prevented this loss of Dsg3. These data indicate that Dsg3 down-regulation occurs through an endocytic pathway that is blocked by genistein pre-treatment.

The observation that genistein blocked both Dsg3 endocytosis and depletion of steady state Dsg3 protein levels raised the possibility that inhibition of Dsg3 endocytosis might also prevent subsequent steps in disassembly and loss of adhesion. Therefore, the distribution of desmosomal components was monitored in keratinocytes pretreated with genistein in the presence of PV IgG. Normal human IgG (**Figure 4.9, B-D**) and genistein treatment alone (**Figure 4.9, E-G**) caused little or no change in Dsg3 and desmoplakin distribution. In contrast, PV IgG caused Dsg3 internalization and dramatic mislocalization of desmoplakin (**Figure 4.9, H-J**). However, genistein treatment dramatically reduced Dsg3 internalization and prevented disruption of Dsg3 and desmoplakin localization at cell junctions (**Figure 4.9, K-M**).

Therefore, we assessed whether genistein could prevent loss of adhesion in functional assays in which mechanical stress is used to disrupt keratinocyte cell sheets incubated in suspension. In this assay, the loss of keratinocyte adhesion strength leads to the disruption of the cell sheet into numerous fragments (Calkins et al., 2006; Huen et al., 2002). Very few fragments were observed for keratinocytes incubated with either normal human IgG or genistein alone. However, numerous particles were observed when keratinocytes were incubated with PV IgG for 24hrs (**Figure 4.9, N**). Importantly, genistein treatment prevented the loss of adhesion caused by PV IgG. Collectively, these findings support a model in which PV IgG cause Dsg3 endocytosis, which in turn leads to loss of Dsg3 steady state levels followed by disruption of desmosomal components and the loss of adhesion.

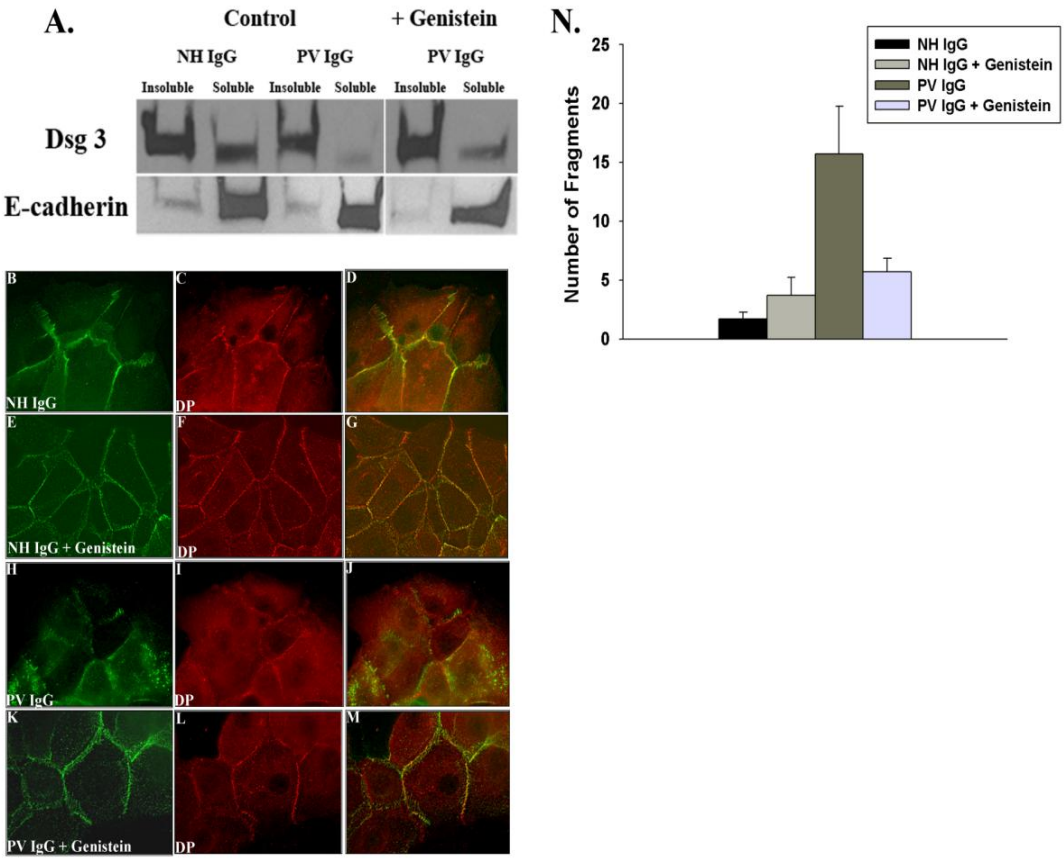


Figure 4.9: Genistein inhibits PV IgG induced desmosomal disassembly. Steady state levels of Dsg3 were monitored via western blot analysis. After 24 hours, Dsg3 levels were unchanged in the presence of normal human IgG, whereas Dsg3 levels were dramatically reduced in the presence of PV IgG. Treatment with genistein prevented this down-regulation of steady state Dsg3 levels (*A*). To determine the effects of genistein on displacement of desmosomal components, primary keratinocytes were either untreated (*B-D, H-J*) or treated with 40uM genistein (*E-G, K-M*) for 1 hour at 37° C. Cells were then transferred to 4° C and incubated with either normal human IgG or PV IgG. Keratinocytes were then shifted to 37° C for 4 hours. Cells were fixed in cold methanol and processed for immunofluorescence to monitor Dsg3 and desmoplakin (DP) localization. To determine the effects of genistein in a functional assay, keratinocytes were grown to 100% confluency, treated for 24 hrs with normal human or PV IgG, and released from the substrate using dispase, an enzyme which disrupts interactions between cells and the extracellular matrix but not cell-cell adhesion. The cell sheets were placed in conical tubes and subjected to mechanical stress; the number of fragments generated directly correlates to the magnitude of the loss of cell adhesive strength. Genistein treatment dramatically reduced the number of fragments in PV IgG treated cultures (*N*). Bar, 30μm.

4.4 Discussion

The results presented here suggest that PV IgG-induced Dsg3 internalization is mediated by a dynamin and clathrin-independent mechanism. Furthermore, the Dsg3 cytoplasmic tail confers specificity for this unusual internalization pathway. Lastly, the results suggest that inhibiting Dsg3 internalization also prevents desmosome disassembly, decreased steady state levels of Dsg3, and loss of adhesion strength that are caused by PV IgG.

Previous studies demonstrated that upon PV IgG binding, Dsg3 is internalized and enters an endo-lysosomal pathway, leading to degradation and loss of Dsg3 steady state levels (Calkins et al., 2006). The turnover rate of cell surface Dsg3 is dramatically increased in the presence of PV IgG and this increased turnover is associated with desmosome disassembly, keratin filament retraction, and significantly compromised ability of keratinocyte cell sheets to resist mechanical stress (Calkins et al., 2006). Recently, Kitajima and colleagues found that loss of Dsg3 steady state levels in cultured keratinocytes was caused specifically by Dsg3 monoclonal antibodies that are pathogenic in animal models of disease (Yamamoto et al., 2007). Furthermore, studies in mouse models and analysis of human patient epidermis indicate that depletion of keratinocyte Dsg3 also occurs in vivo in response to pathogenic antibodies (Shu et al., 2007). Along with the results presented here, these findings suggest a tight coupling between Dsg3 internalization, desmosome disassembly, and the loss of adhesion during PV pathogenesis.

The data presented here suggest that Dsg3 is internalized through a lipid raft mediated pathway upon PV IgG binding. Dsg3 internalization was dramatically inhibited by cholesterol-binding agents (filipin and nystatin; **Figure 4.4**), but largely insensitive to inhibitors of clathrin mediated pathways (**Figure 4.1**). Furthermore, Dsg3 endocytosis was insensitive to the K44A mutant of dynamin II (Altschuler et al., 1998) (**Figure 4.6**). Based on a wide range of other studies defining the characteristics of various endocytic pathways (Idkowiak-Baldys et al., 2006; Orlandi and Fishman, 1998; Parton et al., 1994; Ros-Baro et al., 2001; Shajahan et al., 2004; Singh et al., 2003) (**Table 4.1**), this sensitivity profile suggests that Dsg3 is internalized through a lipid raft mediated process. Consistent with this interpretation, Dsg3 co-localized extensively with the lipid raft marker CD59 at both the cell surface and in intracellular vesicular compartments (**Figure 4.2**). Furthermore, Dsg2 was recently shown to partition into lipid rafts, raising the possibility that these detergent insoluble membrane domains play a pertinent role in desmosome assembly and disassembly . It is formally possible that some pools of Dsg3 are internalized by clathrin dependent mechanisms, and partial inhibition of IL2R-$Dsg3_{cyto}$ chimera endocytosis was observed when the clathrin endocytic pathway was blocked (**Figure 4.3**). One possibility is that over-expression of the IL2R-$Dsg3_{cyto}$ chimera causes an influx of the protein through a clathrin-mediated pathway. Alternatively, the transmembrane or extracellular domains of Dsg3 may also contain motifs responsible for conferring its specificity through a clathrin and dynamin independent mechanism. However, the Dsg3-PV IgG complex did not colocalize with clathrin (**Figure 4.2**).

In addition, Dsg3 internalization was dramatically inhibited by cholesterol sequestration and the tyrosine kinase inhibitor genistein, but insensitive to disruption of either dynamin or caveolin function (**Figures 4.6 and 4.7**). These characteristics are hallmarks of lipid raft-mediated endocytosis (**Table 4.1**).

Cadherins have been reported to be internalized through both clathrin dependent and independent mechanisms, depending upon the cell type. However, no detailed analysis of desmosomal cadherin endocytosis has been reported to date. In previous studies we found that VE-cadherin is internalized in a clathrin dependent manner. Interestingly, IL2R chimeras with the VE-cadherin tail appear to be internalized in both keratinocytes and endothelial cells through the same clathrin dependent mechanism. In contrast, the IL2R-Dsg3$_{cyto}$ chimera is internalized in a clathrin independent manner (**Figure 4.3**). These results resemble the internalization profile of endogenous VE-cadherin and Dsg3 under the same conditions (**Figure 4.1** and unpublished data), demonstrating that the cytoplasmic domains of cadherins play key roles in dictating the mechanism of endocytosis independently of the cell type. The cytoplasmic tails of the Dsgs are evolutionarily divergent from the classical cadherins, such as VE-cadherin. Current studies in our lab are addressing which domains mediate Dsg3 endocytosis and confer specificity for clathrin independent internalization pathways.

The results of this study support a model in which there is a functional relationship between membrane trafficking activity and desmosome disassembly in the context of PV pathophysiology. In this regard, inhibition of Dsg3 internalization and/or desmosomal disassembly may serve as potential targets for PV therapeutics. In fact, genistein was recently reported to prevent blistering in a mouse model of PV.

Although genistein may exert effects on the desmosome independent from the regulation of Dsg3 endocytosis, these and other studies highlight the possibility that PV blistering in patients might be treated by pharmacological strategies that target keratinocyte responses to PV IgG. Likewise, Dsg3 endocytosis may be important in other aspects of keratinocyte biology. For example, desmosome assembly and desmosomal cadherin turnover may be balanced to accommodate needs for tissue integrity on the one hand, and keratinocyte motility and plasticity on the other. Understanding the mechanism involved in Dsg3 turnover and desmosomal disassembly will provide novel insight into the regulation of desmosomal dynamics during PV, and perhaps other conditions where desmosomes are rapidly remodeled.

Chapter 5: Regulation of PV IgG-Induced Dsg3 Internalization and Desmosome Disassembly by the Actin and Microtubule Cytoskeletal Network

5.1 Introduction

Based on previous data generated in our laboratory, along with the findings described in the previous chapter, we propose that Dsg3 internalization and desmosomal disassembly are tightly coupled in contributing to the pathogenesis of PV. Furthermore, recent work has illustrated that desmosomal disassembly in response to PV occurs in three distinct but overlapping phases. In Phase 1 the soluble pool of Dsg3 is internalized, without depleting the desmosomal pool of the cadherin. Phase 2 involves the arrangement of the desmosomes into linear arrays on the cell surface. Formation of the linear arrays is followed by Phase 3, which includes depletion of the insoluble pool of Dgs3 and overall loss of cell-cell adhesion.

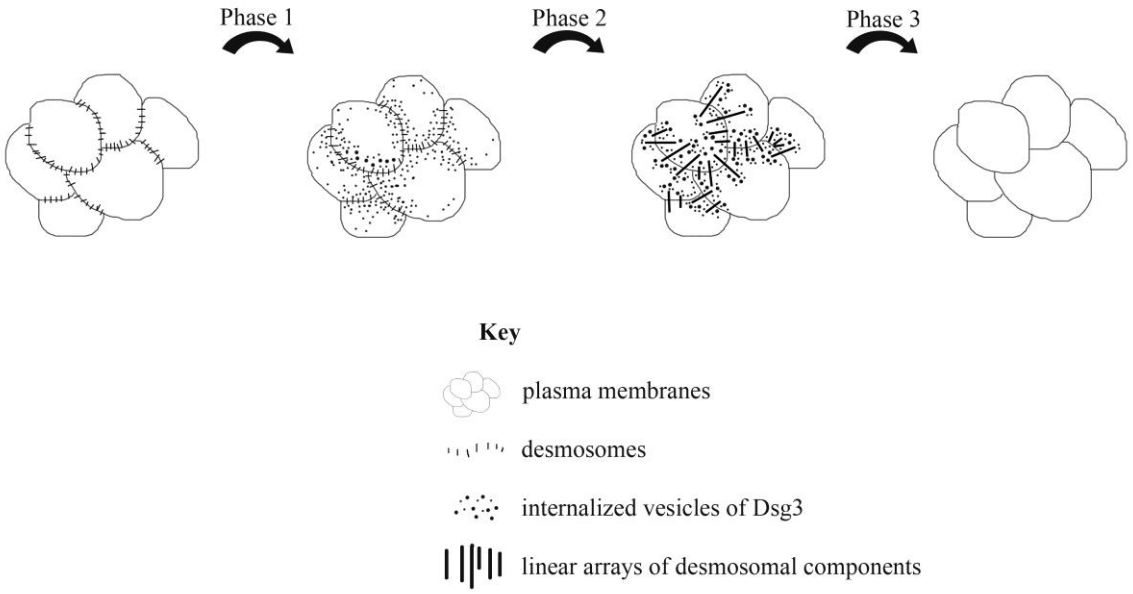


Figure 5.1: Three phase-model of desmosomal disassembly in response to PV IgG. In our model, we propose that PV IgG induced-desmosomal disassembly occurs in three distinct but overlapping phases. In Phase 1, PV IgG binds to the non-desmosomal pool of Dsg3, resulting in its internalization via a pathway independent of clathrin and dynamin. Phase 2 involves the rearrangement of desmosomes into linear arrays on the cell surface. Vesicles enriched in Dsg3 bud off from these arrays and enter the cytoplasm for degradation. At later timepoints, Phase 3 occurs, in which we observe depletion of both the soluble and insoluble pools of Dsg3 and overall loss of cell-cell adhesion. *Adapted from Jennings, Kottke, and Kowalczyk (in preparation).*

The linear arrays generated in Phase 2 comprise of Dsg3, as well as various desmosomal components, including DP, PG, Dsc2, and plakophilin-2 (Jennings, Kottke, and Kowalczyk, unpublished). We also observed colocalization between Dsg3 and the actin cytoskeletal network in these arrays. Therefore, we sought to investigate the role of the cytoskeletal network (in particular, actin and microtubules) in regulating PV-induced Dsg3 internalization and desmosomal disassembly.

The vertebrate cytoskeleton is composed of actin (microfilaments), microtubules, and intermediate filaments. The cytoskeleton is a complex network of polymers, designed to assist in a number of important cellular processes, including mitosis, cell motility, muscle contraction, and maintenance of cell morphology (Brown and Stow, 1996; Kelly, 1990; Mays et al., 1994) . Actin and intermediate filaments provide structural integrity to adherens junctions and desmosomes, respectively, as they are tethered to the plasma membrane at points of cell-cell contact. In addition, recent studies have demonstrated that the cytoskeletal network, particular microfilaments and microtubules, play pivotal roles in the transport of endocytic vesicles throughout the cell (Apodaca, 2001; Musch, 2004; Soldati and Schliwa, 2006).

Microtubules are long filaments involved in organelle localization and transport. Transport along microtubules is relatively rapid, with movement occurring at approximately 1um/sec over long distances. One family of motor proteins responsible for movement along the microtubules are kinesins, which generally move towards the fast growing microtubule plus-end, although there are minus-directed kinesins. Dyneins, another set of microtubule motor proteins, move towards the minus-end of microtubules. Microfilaments are also thought to be important in vesicular transport throughout the cell.

Movement along these filaments is slower (approximately 0.1um/sec) and vesicles are usually carried over shorter distances. Myosin motors are responsible for transport along actin filaments. Although there are thirteen classes of myosin proteins, class I, V, VI, and VII have been implicated in vesicular transport.

In the previous section, data were presented which suggested that PV-induced Dsg3 internalization in Phase 1 is mediated by a process that is devoid of clathrin, caveolin, and dynamin. Furthermore, Dsg3 internalization is sensitive to cholesterol disruption and inhibition of tyrosine kinase activation. The linear arrays formed in Phase 2 consist of Dsg3, as well as other components of the desmosome (i.e. DP, PKP-2, and PG) (Jennings, Kottke, and Kowalczyk, unpublished). In addition, the PV IgG-Dsg3 complex also colocalizes with the actin cytoskeletal network. Based on these findings, we sought to determine if the cytoskeleton may also provide another level of regulation of desmosomal disassembly. More specifically, we examined the role (if any) of the actin and microtubule network in Dsg3 internalization and desmosomal disassembly.

5.2 Materials and Methods

Cells and Culture Conditions. Normal human keratinocytes (NHKs) were isolated and cultured as described previously (Calkins et al., 2006). Briefly, NHKs were isolated from neonatal foreskin and cultured in keratinocyte growth medium (Cascade Biologics, Portland, OR). NHKs were used for experimentation at passage 2 or 3. For experiments, cells were shifted to media containing 0.55mM calcium 16–18 h before treatments and remained in this media throughout the duration of the experiments.

Antibodies and Ligands. Normal human serum was obtained from Irvine Scientific (Santa Ana, CA). PV IgG was a kind gift from Dr. Robert Swerlick (Emory University, Atlanta) and Dr. Masayuki Amagai (Keio University School of Medicine, Tokyo). Monoclonal anti-Dsg3 antibodies, AK15 and AK23 (Tsunoda et al., 2003), were kind gifts from Dr. Masayuki Amagai. The localization of desmoplakin was processed using monoclonal anti-desmoplakin (R&D Systems, Minneapolis, MN). Appropriate species cross-absorbed secondary antibodies conjugated to various Alexa Fluors (Molecular Probes, Eugene, OR) were used for dual-label immunofluorescence.

Immunofluorescence. NHKs were prepared for immunofluorescence as described previously (Calkins et al., 2006). Briefly, cells were cultured on glass coverslips and shifted to media containing 0.5mM calcium 16–18 h before treatment. Under the culture conditions used throughout this study, the keratinocytes predominantly expressed Dsg3 and low but detectable levels of Dsg1. NHKs were pretreated with 250nM Latrunculin A (Molecular Probes, Carlsbad, CA) or 30uM Nocodazole (Sigma, St. Louis, MO), for 1 hour at 37°C. Cells were then incubated with a tracer amount of pathogenic mouse monoclonal antibody against the extracellular domain of Dsg3, AK23 (Tsunoda et al., 2003) (diluted in media containing 0.55mM calcium) on ice for 30 min. For most experiments, keratinocytes were then incubated with either affinity-purified normal human or human PV IgG at a concentration of up to 1 mg/ml (diluted in media containing 0.55mM calcium) and transferred to 37 °C for indicated internalization time. Following incubation at 37°C, cells were returned to ice and treated with acid wash solution (3% BSA, 25mM glycine, pH 2.7) to remove cell-surface bound antibody.

Cells were then fixed on ice using either -20 °C methanol for 5 min or 3.7% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) for 10 min followed by extraction in 0.5% Triton X-100 (Roche Diagnostics, Indianapolis, IN) for 7 min. 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 Simple PCI (Compix, Inc., Cranberry Township, PA).

Manipulation of Cytoskeletal Organization. IgG internalization was performed as previously described (Calkins et al., 2006). NHKs were pretreated with 250nM Latrunculin A or 30uM Nocodazole, for 1 hour at 37°C. Cells were then incubated with a tracer amount of pathogenic mouse monoclonal antibody against the extracellular domain of Dsg3, AK23 (Tsunoda et al., 2003) (diluted in media containing 0.55mM calcium) on ice for 30 min. Cells were then washed 3 times with PBS^{+} followed by incubation, with either normal human of PV IgG at 37°C for various times in media containing 0.55mM calcium. Following incubation at 37°C, cells were returned to ice and treated with acid wash solution (3% BSA, 25mM glycine, pH 2.7) to remove cell-surface bound antibody. The cells were rinsed, fixed, and processed for dual label immunofluorescence as described above.

Time-Lapse Microscopy. Primary keratinocytes were cultured on chambered coverglass plates (Lab-Tek/Nunc, Rochester, NY) and shifted to media containing 0.5mM calcium 16–18 h before treatment. NHKs were pretreated with 250nM Latrunculin A or 30uM Nocodazole, for 1 hour at 37°C. Cells were then incubated with a tracer amount of fluorescently-tagged pathogenic mouse monoclonal antibody against the extracellular domain of Dsg3, AK15 (Tsunoda et al., 2003), to label cell surface Dsg3.

Excess antibody was rinsed off and cells were incubated with affinity-purified human PV IgG at a concentration of up to 1 mg/ml (diluted in media containing 0.55mM calcium) and transferred to 37°C for indicated internalization times. Confocal time-lapse microscopy was carried out using two solid state lasers (491nm and 561 nm) and a VT Infinity 2D array scanner. An inverted Leica DMIRE2 microscope equipped with a Hamamatsu electron multiplier deep cooled CCD camera (C9100-12) was used for image acquisition. Time lapse images and movies represent projection images of the entire cell z-plane of the cell. Temperature control was achieved using an environmental control chamber (Pecon Incubator ML) and heated stage insert (Pecon Heating Insert P). The camera, fully motorized microscope, and automated stage were driven by Simple PCI software.

5.3 Results

In order to determine if actin and/or microtubules are involved in regulating PV-induced Dsg3 internalization, we took advantage of well characterized pharmacological inhibitors of both cytoskeletal networks. Latrunculin A is an actin depolymerizing agent, originally shown to disrupt actin filaments in mammalian tissue culture cells (Spector et al., 1983). It directly interacts with monomeric actin in a 1:1 ratio, preventing incorporation of G-actin into filaments (Coue et al., 1987). Nocodazole is a microtubule disrupting agent, found to directly associate with tubulin, interfering with microtubule polymerization (Hoebeke et al., 1976). We first wanted to ensure that the concentration at which we were using nocodazole and latrunculin A were effective in disrupting the cytoskeletal network. As shown in Figure 5.1, treatment of keratinocytes with the pharmacological agents did depolymerize actin and microtubules.

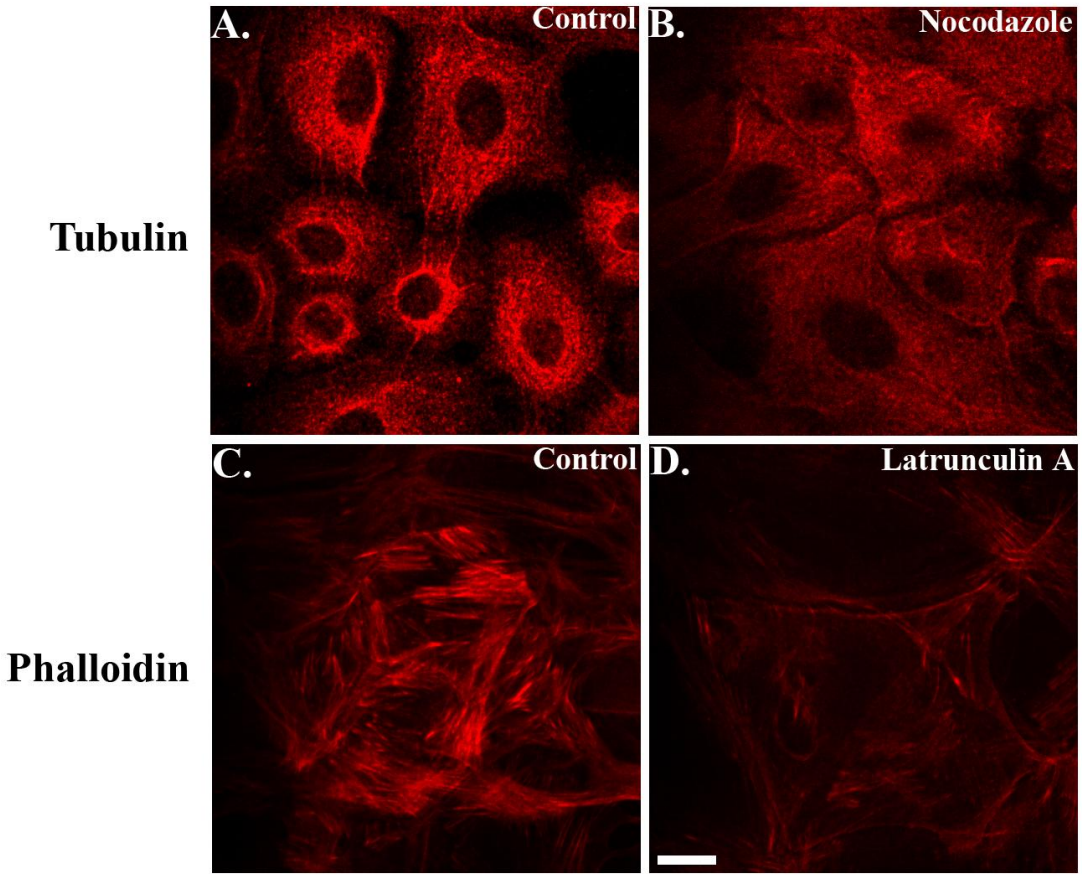
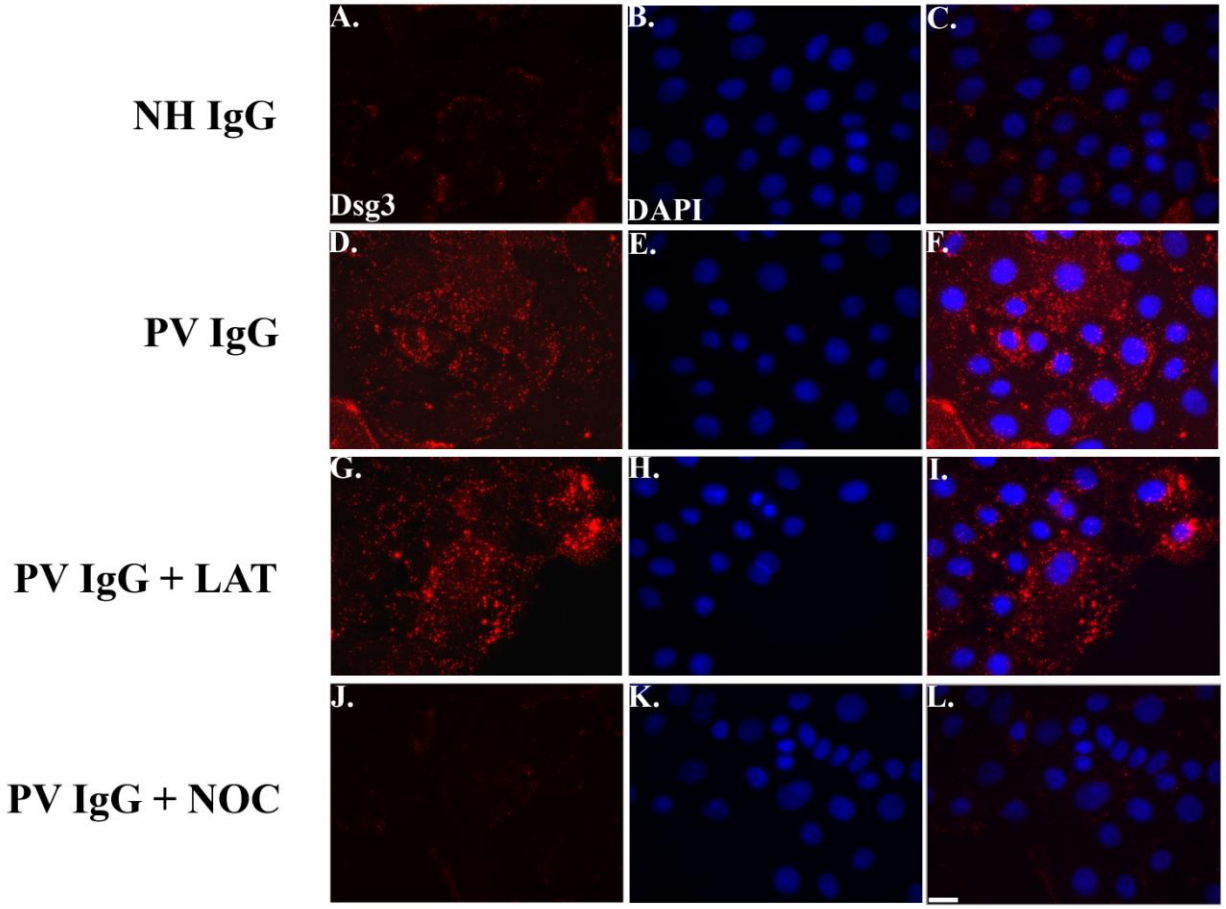


Figure 5.2: Latrunculin A and nocodazole disrupt microfilaments and microtubules, respectively. Actin and microtubules are highlighted, staining for phalloidin and β-tubulin (*A, C*). Keratinocytes were treated with latrunculin A and nocodazole for 3 hours at 37 °C (*B, D*). Bar, 10μm.

Depolymerizing actin enhancing Dsg3 internalization while depolymerizing microtubules blunts the effects of PV IgG-induced Dsg3 internalization. Numerous studies lend support to the idea that the cytoskeleton plays an important role in endocytosis of various proteins. Therefore, keratinocytes subjected to PV IgG were pretreated with latrunculin A and nocodazole to determine if depolymerizing the actin or microtubule cytoskeleton would affect Dsg3 internalization. As expected, keratinocytes incubated with NH IgG exhibited very little Dsg3 internalization, while keratinocytes incubated with PV IgG displayed increased Dsg3 internalization (**Figure 5.2, A-F, M-Q; Supplemental Movie 3**). When keratinocytes were pretreated with latrunculin A, we observed a dramatic increase in internalized Dsg3 (**Figure 5.2, G-I, R-U; Supplement Movie 4**). In contrast, keratinocytes treated with nocodazole showed a marked decrease in Dsg3 internalization (**Figure 5.2, J-M, V-Y; Supplement Movie 5**). Altogether, these results illustrate that actin and microtubules are both playing important roles in PV IgG-induced Dsg3 internalization.


NH IgG
PV IgG
PV IgG + LAT
PV IgG + NOC
A.
B.
C.
D.
E.
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G.
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I.
J.
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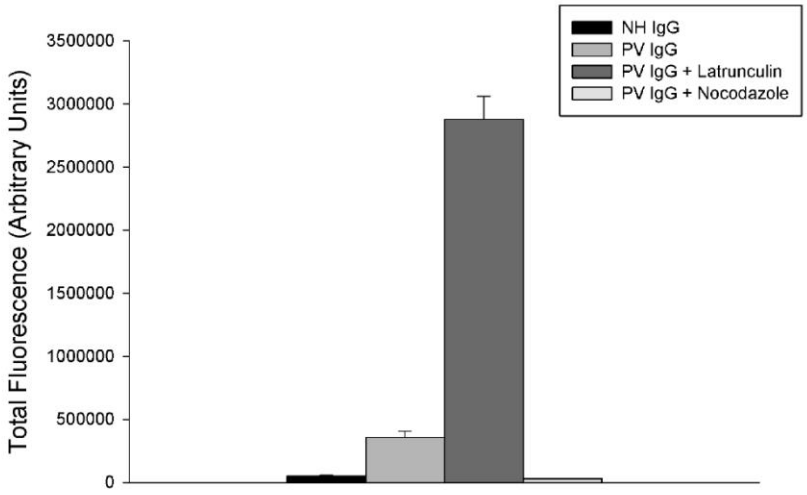
L.
Dsg3
DAPI

M.


Total Fluorescence (Arbitrary Units)
3500000
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2500000
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NH IgG
PV IgG
PV IgG + Latrunculin
PV IgG + Nocodazole

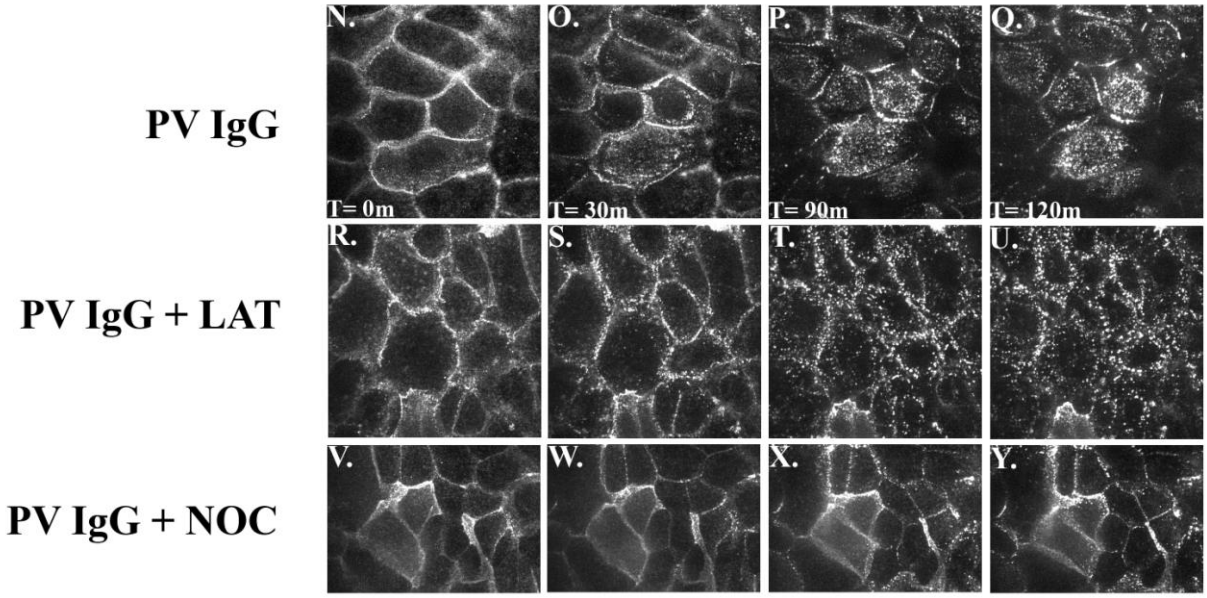

Figure 5.3: Latrunculin A and nocodazole treatment have opposing effects on PV-induced Dsg3 internalization. Primary keratinocytes were either untreated or pretreated with latrunculin A or nocodazole at 37° C. Cells were then transferred to 4° C and incubated with a tracer amount of AK23 directed against Dsg3. Excess ligand was removed and keratinocytes were incubated with either NH IgG (*A-C*) or PV IgG (*D-L*) and shifted to 37° C for 4 hours to allow internalization. A low pH wash was used to remove cell surface bound AK23 to visualize internalized Dsg3. Total intracellular fluorescence was quantified using a digital imaging system and Simple PCI software (*M*). Error bars represent the standard error of the mean, where n = 100 cells. Bar, 10μm. For time lapse experiments, living keratinocytes were incubated with fluorescently-tagged AK23 and PV IgG at 4°C and then transferred to 37°C to monitor internalization of Dsg3 in either the absence (*N-Q*) or presence of latrunculin (*R-U*) or nocodazole (*V-Y*). Supplemental Movies 3, 4, and 5 correspond to panels N-Q, R-U and V-Y, respectively.

Depolymerizing actin exacerbates the effects of PV on DP localization whereas depolymerizing microtubules prevents DP mislocalization from occurring. Previous studies have illustrated the tightly coupled relationship between Dsg3 internalization and desmosomal disassembly. Dsg3 endocytosis is associated with DP mislocalization, keratin retraction, and compromised cell-cell adhesion strength (Calkins et al., 2006). Therefore, we set out to observe the effects of latrunculin and nocodazole on DP mislocalization. Based on the Dsg3 internalization results, we predicted that depolymerizing actin would augment the effects of PV-induced DP mislocalization, while nocodazole would have opposite effects. Keratinocytes incubated with NH IgG displayed normal DP localization along cell-cell borders (**Figure 5.3, A-C**). As anticipated, DP mislocalization was observed in keratinocytes incubated with PV IgG (**Figure 5.3, D-F**). Pretreatment with latrunculin A resulted in a more dramatic loss of DP localization (**Figure 5.3, G-I**). In contrast, nocodazole treatment blunted the effects of PV IgG (**Figure 5.3, J-L**). These observations correlate with the results obtained in the Dsg3 internalization assay, further illustrating the opposing roles of actin and microtubules in regulating Dsg3 internalization and desmosomal disassembly and further supports the tightly-coupled relationship between Dsg3 endocytosis and desmosomal disassembly.

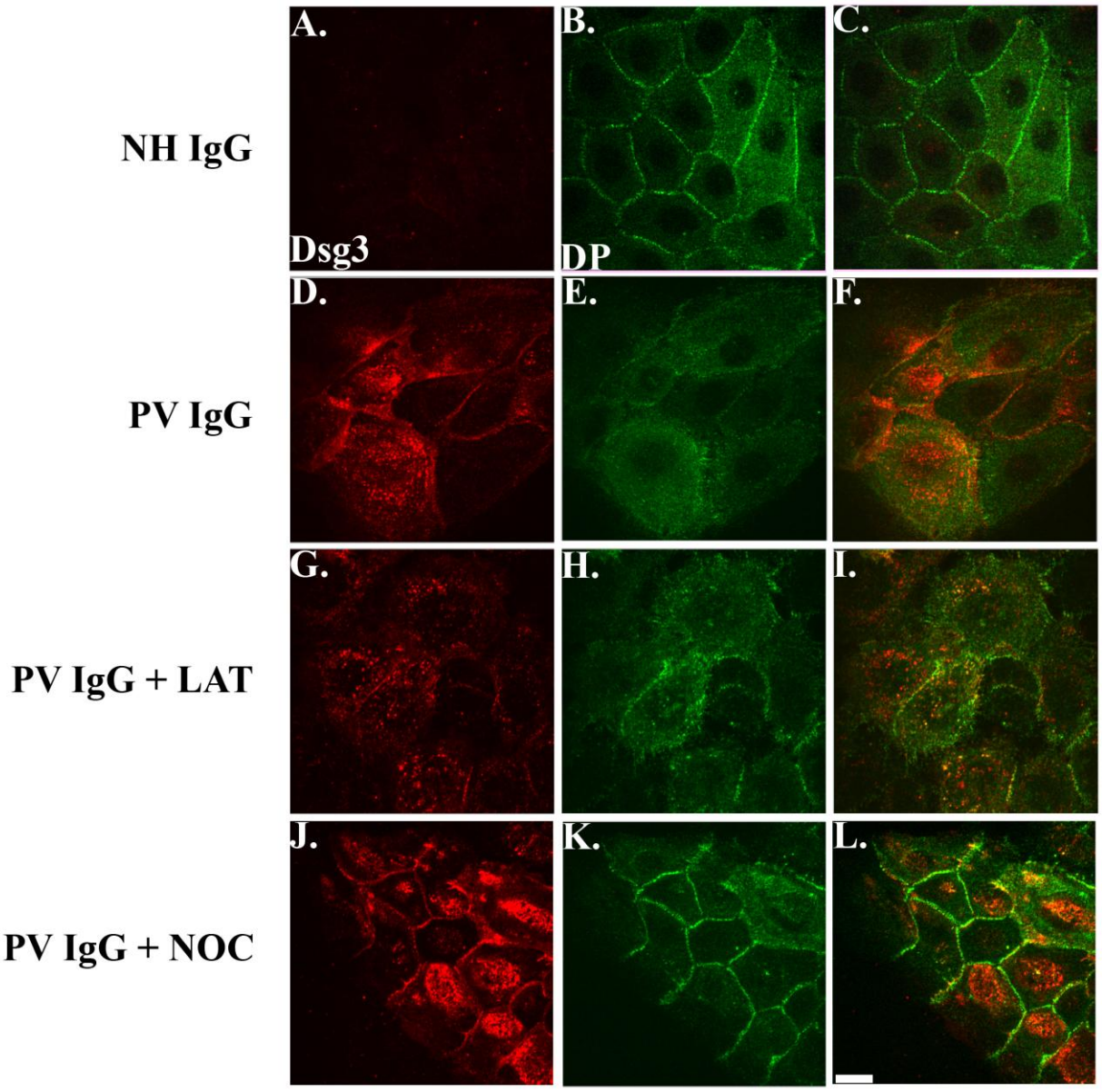

Figure 5.4: Latrunculin A enhances whereas nocodazole treatment protects against DP mislocalization. To determine the effects of depolymerizing actin and microtubules on displacement of desmosomal components, primary keratinocytes were either untreated (*A-F*), treated with latrunculin A (*G-I*), or nocodazole (*J-L*) for 1 hour at 37° C. Cells were then transferred to 4° C and incubated with either normal human IgG or PV IgG. Keratinocytes were then shifted to 37° C for 4 hours. Cells were fixed in cold methanol and processed for immunofluorescence to monitor Dsg3 and desmoplakin (DP) localization. Bar, 10μm.

5.4 Discussion

The preliminary results presented here suggest that both microtubules and microfilaments play a role in regulating PV-induced Dsg3 internalization and desmosomal disassembly. These findings provide further insight into the manner by which desmosome disassembly is regulated, and provide new evidence that microtubules play a key role in Dsg3 endocytic trafficking and in maintaining a balance between soluble desmosomal cadherins and association of cadherins with desmosomes.

Latrunculin A, an actin depolymerizing agent, enhanced Dsg3 internalization (**Figure 5.2**) and DP mislocalization (**Figure 5.3**) whereas, nocodazole, a microtubule depolymerizing agent, had opposing effects. Our results correlate very well with studies in which microtubule disruption in keratinocytes actually induce cell-cell adhesion in non- and early differentiating cells, through redistribution of E-cadherin to the cell periphery (Kee and Steinert, 2001). Furthermore, this induction of cell-cell adhesion is accompanied by actin polymerization, as observed by an increase in stress fibers in nocodazole-treated cells. Work done by Vasioukhin and others has also illustrated that actin polymerization is necessary for epithelial cell-cell adhesion (Vasioukhin et al., 2000). These studies underscore the importance of the cytoskeletal network in establishing and maintaining adhesive complexes as cell proliferate and differentiate.

A number of studies have illustrated the importance of microtubules in regulating cell-cell adhesion. One mode of regulation may involve interactions between microtubules and DP at the plasma membrane. DP has been shown to recruit ninein, a microtubule anchoring protein to the plasma membrane, as keratinocytes differentiate (Lechler and Fuchs, 2007).

Localization of another microtubule-binding protein, pp170, has also been monitored in MDCK cells and it is found in close proximity to areas of cell-cell contact. Through use of confocal and immunoelectron microscopy, pp170 was found to accumulate at desmosomes (Wacker et al., 1992) . One can speculate that linking microtubules to the plasma membrane, via DP, allows from internalization of Dsg3 and transport to the early endosomes; therefore, either depolymerizing microtubules or inhibiting anchoring of the microtubules to the plasma membrane prevents PV-induced Dsg3 internalization and disassembly.

Work in *D. discoideum* suggests that microtubules may also regulate endocytosis by serving as tracks for vesicles to travel along, once proteins have been internalized. Clarke and others observed that microtubules facilitate the fusion of macropinosomes with pre-existing endosomes, through a process that is dynein-dependent (Clarke et al., 2002). Earlier studies have illustrated the importance of microtubules in maintaining fluid-phase endocytosis in macrophages. Use of another microtubule depolymerizing agent, cholchicine, has reduced fluid-phase uptake of exogenous markers, horseradish peroxidase and colloidal gold in rat peritoneal macrophages (Piasek and Thyberg, 1980; Pratten and Lloyd, 1979). Disruption of microtubules with nocodazole increased cell surface levels of invaginated caveolae and abolished movement of caveolae vesicles from the plasma membrane (Mundy et al., 2002). Furthermore, studies have implicated a role for dynein light chain-1 in the initial steps of pinosome formation and trafficking, suggesting a role for microtubule motors in early stages of clathrin-independent endocytic vesicle transport (Yang et al., 2005).

Our findings, implicating actin disruption and its contribution to PV pathogenesis, lend support to previous studies that have examined the role of microfilaments in cell-cell adhesive potential. The Rho family of GTPases is largely responsible for regulating the dynamics of the actin cytoskeleton.

Rac and cdc42 activation result in formation of lamellipodia and filopodia, respectively. RhoA activation regulates stress fiber formation. In keratinocytes, inactivation of RhoA led to actin disruption and mislocalization of E-cadherin complexes from adherens junctions (Braga et al., 1999; Braga et al., 1997; Takaishi et al., 1997). Furthermore, RhoA inactivation in an ex vivo human skin model resulted in pemphigus-induced epidermal blistering (Waschke et al., 2006). Altogether, these studies, along with our findings, suggest that actin polymerization (through RhoA activation) plays an active role in blunting the effects of PV and maintaining stable, functional desmosomal complexes.

Similar to microtubules, actin has been widely recognized as playing an important role in vesicle trafficking, in particular transport of late endosomes to lysosomes (Apodaca, 2001). Previous studies have demonstrated that internalized Dsg3 is transported to early and late endosomes before it is transported to lysosomes for degradation (Calkins et al., 2006). The increase in Dsg3 internalization caused by latrunculin A may very well be the result of accumulation of Dsg3 in the cell, due to its inability to move along actin from late endosomes to the lysosome for degradation. It will be interesting to examine the redistribution (if any) of Dsg3 and various endocytic markers. If our hypothesis is correct, we would expect latrunculin A to increase Dsg3 colocalization with CD63 (a late endosomal marker) and less association with the

lysosomal marker Cathepsin D. We may also observe increased Dsg3 internalization but less Dsg3 degradation.

Altogether, our results suggest that actin and microtubules work in concert in regulating Dsg3 internalization in response to PV IgG (**Figure 5.5**). Our results suggest that microtubules may provide tracks by which non-junctional Dsg3 vesicles travel along from the plasma membrane to early endosomes (**Figure 5.5 A**). Disruption of microtubules prevents Phase 1 disassembly, consistent with the idea that endocytosis from the non-junctional pool is required. Actin's involvement in regulating desmosomal dynamics is two-fold. Once Dsg3 vesicles fuse with early endosomes, Dsg3 vesicles may then travel along actin filaments to late endosomes and lysosomes for degradation, as actin dynamics have been implicated in post-endocytic transport from the late endosomes to lysosomes (**Figure 5.5 B**). Therefore, disrupting actin filaments prevents this transport, perturbs Dsg3 degradation, and leads to accumulation of Dsg3 vesicles in the cytoplasm. Actin polymerization is also important at sites of cell-cell contact, in stabilizing desmosomes at the plasma membrane (**Figure 5.5 C**). It is also possible that the actin cytoskeleton may play a more structural role in regulating desmosomal dynamics. Previous studies suggest a synergistic relationship between actin and intermediate filaments and the importance of this relationship on desmosome stability and adhesive strength. A431 cells infected with DPNTP (a mutant form of DP lacking the rod and IF-binding domains) and treated with latrunculin A were significantly more affected when subjected to mechanical stress, compared to cells treated with either latrunculin A or DPNTP alone (Huen et al., 2002) . These results suggest that the actin network and intermediate filaments work together to regulate adhesion strength. Therefore, disruption

of actin may also contribute to PV pathogenesis by increasing the movement of Dsg3 out of the desmosome, thereby increasing autoantibody binding to the soluble pool of Dsg3 and subsequent internalization.

Altogether, these results further implicate the importance of Dsg3 endocytosis in contributing to PV pathogenesis. Furthermore, there are multiple ways by which the cytoskeletal network may play a role in regulating desmosomal dynamics and we recognize that these various roles may not be mutually exclusive.

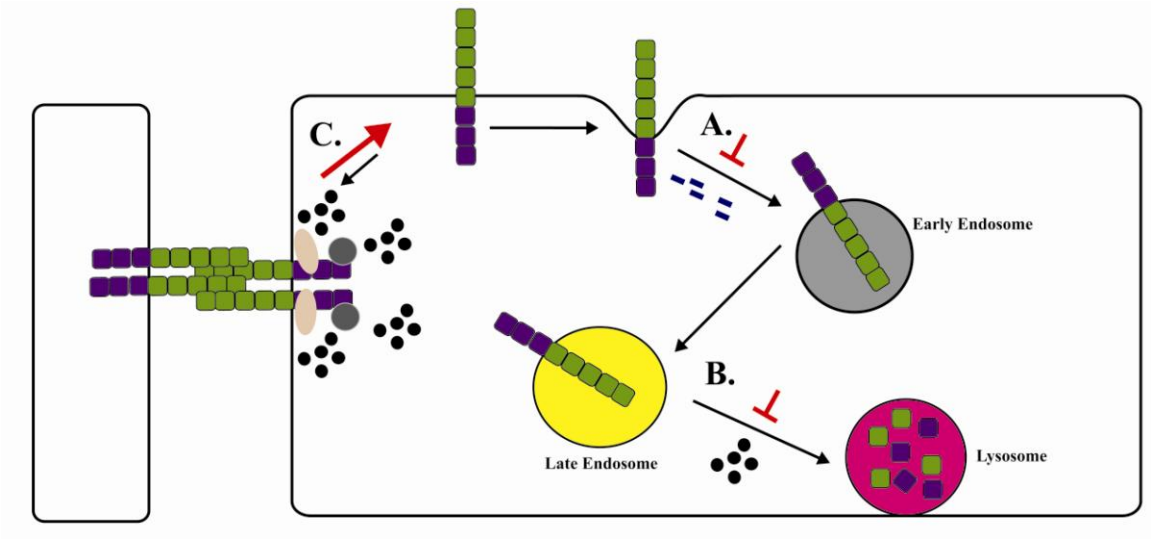

Figure 5.5: Model for regulation of desmosomal dynamics by actin and microtubules. Once internalized, the non-junctional pool of Dsg travels along microtubules from the plasma membrane to early endosomes (A). Once Dsg3 vesicles fuse with early endosomes, Dsg3 vesicles then travel along actin filaments from late endosomes to lysosomes for degradation (B). Actin disruption prevents this latter transport, perturbing Dsg3 degradation. Actin polymerization is also important at sites of cell-cell contact, in stabilizing desmosomes at the plasma membrane (C). Therefore, disruption of actin may also contribute to PV pathogenesis by further increasing the rate by which Dsg3 moves out of desmosomes, thereby increasing autoantibody binding to the soluble pool of Dsg3 and subsequent internalization.

Although preliminary, the aforementioned results provide exciting, novel evidence for regulation of PV-induced Dsg3 internalization and desmosomal disassembly by both microtubules and actin. Work is currently underway to study the effects of cytoskeletal disruption on the kinetics of Dsg3 internalization as well as functional assessment through the use of our cell-cell disassociation assay. Once we have confirmed that latrunculin and nocodazole have opposing effects on PV-induced desmosomal disassembly, we can examine the role(s) of the various motor proteins that may be responsible for the movement of Dsg3-containing vesicles along the different cytoskeletal tracks, including kinesin, dynein, and myosin. Use of more specific inhibitors of these motor proteins will be necessary to further support that transport of Dsg3 is regulated through microtubules and actin filaments. These findings, and others, lend support to the use of microtubule-disrupting agents as a therapeutic target for PV patients. Future studies should examine the use of microtubule perturbing agents in a PV mouse model to determine if nocodazole treatment can blunt the epidermal blistering effects of PV.

Chapter 6: Future Directions and Concluding Remarks

The results presented here indicate that PV is an epidermal blistering disease which compromises cell adhesion strength by interfering with the regulation of desmosomal dynamics. Previous studies have demonstrated that PV IgG causes increased turnover of Dsg3 and loss of cell-cell adhesion. Therefore, we set out to determine (1) the endocytic machinery involved in PV IgG induced Dsg3 internalization and (2) if inhibition of Dsg3 internalization would thereby prevent desmosomal disassembly.

Recent studies in our laboratory suggest that the relationship between Dsg3 internalization and desmosomal disassembly is tightly coupled. Through the use of biochemical, cell biological, and imaging approaches, we've determined that in our model system, PV-induced desmosomal disassembly occurs in 3 distinct but overlapping phases (Jennings, Kottke, and Kowalczyk, unpublished). Initially, we observe internalization of the non-desmosomal pool of Dsg3, with virtually no effect on the desmosomal pool. At later timepoints, keratinocytes exhibit rearrangements of desmosomes into linear arrays, which are composed of all of the desmosomal components, and the actin cytoskeleton. These linear arrays seem to serve as a site for further internalization of Dsg3 and other desmosomal plaque proteins. Lastly, we see depletion of both the soluble and insoluble pools of the Dsg3 as well as an overall loss of cell-cell adhesion. Interestingly, not only can we prevent desmosomal disassembly by preventing Dsg3 internalization, but we can also prevent desmosomal disassembly by exogenously expressing Dgs3 in keratinocytes, resulting in robust biosynthesis of the cadherin. Altogether, these results demonstrate that Dsg3 internalization plays a causal role in PV pathogenesis and is not a secondary response to PV IgG binding.

Future work regarding Dsg3 internalization will delve into the role of other desmosomal components in regulating desmosome dynamics, including the armadillo proteins and desmosomal cadherins in PV-induced desmosomal disassembly. Recently, we overexpressed Dsg3 in keratinocytes subjected to PV IgG and found that increasing the biosynthesis of Dsg3 counteracts the effects caused by PV IgG (Jennings, Kottke, and Kowalczyk, unpublished). Current studies in our laboratory are examining the role of plakophilins in PV-induced desmosomal disassembly.

Furthermore, it will be important to understand if the route by which Dsg3 is internalized in response to PV is different from how it is internalization under normal physiological conditions. It is widely assumed that under normal conditions, there is a state of equilibrium between desmosomal cadherins entering and leaving the desmosome, as a way to regulate the plasticity of cell adhesion. As part of this equilibrium, desmosomal cadherins, including Dsg3, are constantly internalized only to be recycled and brought back to the plasma membrane. The presence of PV IgG dramatically shifts the equilibrium, causing the increased internalization and subsequent degradation of Dsg3. If this is the case, what is the signaling pathway responsible for this dramatic shift in the equilibrium? Answering this question will not only provide more targets for combating PV but it will also provide insight into how desmosomes are regulated to maintain cell-cell adhesion during normal cellular processes.

Genistein, as a general tyrosine kinase inhibitor, inhibits PV IgG-induced Dsg3 internalization and desmosomal disassembly and illustrates the significance of “inside-out” signaling in PV pathogenesis. In various PV model systems, several phosphorylation targets have been proposed, including the Dsg3 , MAPK, and HSP70 .

In addition, recent work has suggested that there are at least three different proteins whose phosphorylation in response to PV contributes to acantholysis. Using more specific kinase inhibitors in the presence of PV IgG will help further elucidate the pathways/proteins that are causally involved in PV pathogenesis. Furthermore, use of these kinase inhibitors in a mouse model will not only support the pivotal role of signaling pathways in PV pathogenesis but will also provide a new avenue by which PV patients can be treated, without suppressing the immune system.

Recent work has shown that monovalent antibodies against Dsg3 can also cause epidermal blistering in a mouse model and similar results have been shown for Fab fragments produced from endemic pemphigus foliaceus autoantibodies. In the case of endemic PF (Hilario-Vargas et al.), on a molar basis, the Fab fragments were more pathogenic than the intact IgG (Rock et al., 1990) . These results lend support to the idea that Fab fragments may be able to produce epidermal blistering due to increased accessibility to the antigen. Moreover, work done by Mao and others have shown that monovalent PV antibodies disrupt the assembly of desmosomes, causing internalization of newly synthesized Dsg3, depletion of Dsg3 from preformed desmosomes, and loss of cell-surface Dgs3 and DP during assembly (Mao et al., 2008). Furthermore, recent observations in the Amagai laboratory have monitored comparisons between different Dsg3 antibody isotypes in causing PV in mouse models. Preliminary observations suggest that Dsg3 IgM (as a pentamer, which can bind up to 10 epitopes) is *less* effective at causing epidermal blistering in mice, in comparison to Dsg3 IgG (a monomer).

These initial results may be due to the IgM's inability to access Dsg3, due to its size. Current work in our laboratory is ongoing, examining the role of antibody isotype in causing Dsg3 endocytosis and acantholysis.

Increased interest in the basic cellular mechanisms of cadherin membrane trafficking has been paralleled by new insights into how these pathways are usurped in human pathologies. A number of disease states are associated with defects in cadherin endocytosis and misregulation of cadherin cell surface levels. In particular, studies have shed light on the role of cadherin destabilization in tumor cell metastasis. One of the hallmarks of the transition from benign overgrowth to a metastatic tumor is the aforementioned Epithelial to Mesenchymal Transition (EMT). Hakai, an ubiquitin ligase is thought to mediate ubiquitin tagging of the E-cadherin tail, leading to endocytosis and degradation in the lysosome (Fujita et al., 2002). MDM-2, another E3 ubiquitin ligase misregulated in numerous metastatic tumors, also downregulates E-cadherin and promotes cell motility and invasiveness in breast cancer cells (Yang et al., 2006). HIV-1 internalization and infection in polarized trophoblastic cells is mediated by a pathway, similar to Dsg3, whereby neither clathrin, caveolae, nor dynamin is required (Vidricaire and Tremblay, 2007). These studies and others underscore the importance of membrane trafficking pathways in providing critical regulatory oversight of cadherin function. Understanding how cadherin adhesion and dynamics are integrated will provide significant insights into a range of developmental processes and disease pathologies associated with alterations in cadherin dynamics.

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