# A Central Role for the Armadillo Protein Plakoglobin in the Autoimmune Disease Pemphigus Vulgaris

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***Abstract.*** In pemphigus vulgaris (PV), autoantibody binding to desmoglein (Dsg) 3 induces loss of intercellular adhesion in skin and mucous membranes. Two hypotheses are currently favored to explain the underlying molecular mechanisms: (a) disruption of adhesion through steric hindrance, and (b) interference of desmosomal cadherin-bound antibody with intracellular events, which we speculated to involve plakoglobin. To investigate the second hypothesis we established keratinocyte cultures from plakoglobin knockout ($PG^{-/-}$) embryos and $PG^{+/+}$ control mice. Although both cell types exhibited desmosomal cadherin-mediated adhesion during calcium-induced differentiation and bound PV immunoglobin (IgG) at their cell surface, only $PG^{+/+}$ keratinocytes responded with keratin retraction and loss of adhesion. When full-length plakoglobin was reintroduced into $PG^{-/-}$ cells, responsiveness to PV IgG was restored. Moreover, in these cells like in $PG^{+/+}$ keratinocytes, PV IgG binding severely affected the linear distribution of plakoglobin at the plasma membrane. Taken together, the establishment of an in vitro model using $PG^{+/+}$ and $PG^{-/-}$ keratinocytes allowed us (a) to exclude the steric hindrance only hypothesis, and (b) to demonstrate for the first time that plakoglobin plays a central role in PV, a finding that will provide a novel direction for investigations of the molecular mechanisms leading to PV, and on the function of plakoglobin in differentiating keratinocytes.

Key words: catenins • desmosomes • mouse keratinocytes • epithelial differentiation • blistering disease

## *Introduction*

Pemphigus vulgaris (PV)[1] is a severe autoimmune disease characterized by suprabasal blisters in the skin and mucous membranes of a wide range of mammals, including humans (Amagai, 1995; Stanley, 1995; Suter et al., 1998). Passive transfer of PV IgG, affinity purified on the recombinant adhesion molecule desmoglein (Dsg) 3, was found to induce suprabasal blisters in the skin of neonatal mice, similar to those seen in PV patients (Amagai et al., 1992). This observation provided compelling evidence that antibodies directed against Dsg 3 are responsible for and sufficient to induce lesions. Although it has been hypothesized that antibody binding provokes lesion formation only by steric hindrance of Dsg 3–mediated adhesion (Futei et al., 2000), examination of classical signaling pathways indicated that pemphigus antibody binding interferes with intracellular events (Kitajima et al., 1999). This topic was first discussed many years ago (Jones et al., 1984). The molecular mechanism leading to PV antibody-induced blister formation remains unresolved.

Dsg 3 is part of a higher order intercellular adhesion structure, the desmosome (Koch and Franke, 1994; Amagai, 1995; Garrod et al., 1996; Kowalczyk et al., 1999). Within this structure, different transmembrane desmosomal cadherins, including Dsg 3, are associated with submembranous plaque proteins that anchor to the keratin network. One of these plaque proteins is plakoglobin. It is the only major component known to be shared between desmosomes and adherens junctions (Cowin et al., 1986), and was thus suggested to coordinate the assembly of these two major intercellular junctions (Lewis et al., 1997). In spite of this important role, plakoglobin knockout embryos die by embryonic day E12–16 only due to heart failure (sometimes by embryonic day E18 [Bierkamp et al., 1996]). This is long after establishment of the first desmosomes which occurs at embryonic day E3.5 (Ducibella et al., 1975). Together with the fact that the epidermal architecture of plakoglobin knockout mice appeared intact

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[1]*Abbreviations used in this paper:* Dsg, desmoglein; E-cad, E-cadherin; GFP, green fluorescent protein; IF, immunofluorescence; nhIgG, normal human IgG; PV, pemphigus vulgaris; WB, Western blot.

(Ruiz et al., 1996) or only affected superficially (Bierkamp et al., 1996), these findings indicated that other plaque proteins such as plakophilin 1 may substitute for plakoglobin in constituting desmosome-mediated adhesion (Ruiz et al., 1996). The late embryonic death of plakoglobin knockout embryos further suggested that plakoglobin is not significantly involved in morphoregulatory signaling cascades during early embryogenesis, as is the case for its close relative β-catenin (Miller and Moon, 1996; Barker et al., 2000; Zhurinsky et al., 2000). β-Catenin resides in the plaque of adherens junctions, but can also translocate to the nucleus and transactivate the transcription of target genes. Aside from many structural homologies, plakoglobin shows similar transcriptional transactivation capacities as β-catenin in ectopic expression studies (Simcha et al., 1998; Hecht et al., 1999; Williams et al., 2000). Emerging data indicate that whereas plakoglobin does not exert this signaling role in the developing embryo, it may well function as such postnatally in tissues that undergo significant renewal, such as epithelia (Charpentier et al., 2000; Kolligs et al., 2000).

The suggested role for plakoglobin in adhesion and signaling (Kowalczyk et al., 1999), together with the fact that plakoglobin is associated with the PV IgG–targeted antigenic complex (Korman et al., 1989), led us to speculate that binding of PV antibodies could interfere with the proper functioning of this protein. To test this hypothesis, we made use of the recently established plakoglobin knockout embryos (Bierkamp et al., 1996). Although these embryos suffer embryonic death and are therefore not suitable for passive transfer studies of patient sera in vivo (Anhalt et al., 1982), their suprabasal architecture appears to be intact (Bierkamp et al., 1996). Thus, we set up keratinocyte cultures ($PG^{-/-}$) from the epidermis of these mice in parallel with cultures from wild-type mice. This allowed us to establish a reliable in vitro system to study PV and to demonstrate, for the first time, that keratin retraction and loss of adhesion in response to PV IgG is plakoglobin dependent.

## Materials and Methods

### Establishment of Keratinocyte Cultures from $PG^{-/-}$ and $PG^{+/+}$ Mice and Culture Conditions

Keratinocytes from 17.5-d-old $PG^{-/-}$ embryos (Bierkamp et al., 1996) and $PG^{+/+}$ littermates were isolated, and long term cultures were established as described previously (Caldelari et al., 2000). For all experiments, culture conditions were held strictly identical. Namely, cells were expanded in low calcium ($LowCa^{2+}$) medium (0.07 mM; supplemented defined keratinocyte-SFM), and passaged 4–5 d before analyses. At confluency (which was evaluated optically), cultures were either left in low calcium, or differentiation was induced by increasing the calcium concentration in the medium to 1.2 mM (Hennings et al., 1980). This concentration corresponds to physiological calcium concentrations, and will hereafter be termed "high calcium" as compared with "low calcium" concentrations. Cells were maintained in high calcium medium during stimulation with PV IgG.

### Antibodies

Three PV sera (PV IgG 1, 2, and 3) and normal human IgG (nhIgG) combined from different healthy volunteers (T. Hunziker; University Hospital, Bern, Switzerland) were affinity purified over a protein A–Sepharose column, and used at 1 mg/ml for PV IgG 1 and PV IgG 2, and at 2 mg/ml for PV IgG 3 for stimulation. Serum titers were PV IgG 1 (1:640), PV IgG 2 (1:320), and PV IgG 3 (1:160). As tested on epidermal extracts and recombinant baculovirus protein (M. Amagai, Keio University School of Medicine, Tokyo, Japan [Amagai et al., 1994]), all sera contained antibodies against Dsg 3 and no detectable antibodies against Dsg 1 (see Fig. 3, a and b, shown for PV IgG 1). This finding was further confirmed for PV IgG 1 by ELISA on recombinant Dsg 1 and Dsg 3 (performed by M. Amagai [Amagai et al., 1999]). The defined indices of Dsg 1 and Dsg 3 antibodies were 16.4 and 248.8, respectively. An index >20 is considered significant. As a control, nhIgG was used in the concentration corresponding with PV IgG. Polyclonal rabbit antibodies against the extracellular domain of Dsg 3 were generated using a recombinant baculovirus-encoded fusion protein consisting of the extracellular region of human Dsg 3 and the constant region of human $IgG_1$ (Amagai et al., 1994). For immunofluorescence (IF) studies and/or Western blot (WB) analyses we used antibodies against Dsg 3 (IF; developed in our laboratory), and antibodies against the extracellular domain EC5 of Dsg 3 (WB; J. Stanley, University of Philadelphia, Philadelphia, PA [Koch et al., 1998]); Dsg 1/2 (WB; DG 3.10; Progen); plakoglobin (WB; C26 200; Transduction Laboratories); plakoglobin (IF; PG 5.1), plakophilin 1 (WB; 11C6), and β-catenin (WB; 10C4) from P. Wheelock (University of Toledo, Toledo, Ohio); desmoplakin I + II (WB; NW161; K. Green, Northwestern University, Chicago, IL); E-cadherin (E-cad) (WB; DECMA; R. Kemler, Max-Planck Institute für Immunobiologie, Freiburg, Germany); keratin 14 (WB; 146M; Biogenex); pankeratin (IF; LP34; DAKO); and green fluorescent protein (GFP) (IF; 8363; CLONTECH Laboratories, Inc.).

### Adhesion Assay

The assay was done essentially according to Calautti et al. (1998). In brief, keratinocytes were seeded in triplicate in 6-well plates and grown to confluency. After the indicated treatments, they were incubated in situ with 2 ml Dispase II (>2.4 U/ml; Roche Diagnostics) for 30 min to detach the epithelial sheets. These sheets were washed in PBS containing 0.9 mM calcium and transferred to PBS without calcium. Mechanical stress was then applied with a programmable, semiautomatic Eppendorf Response 4850 1-ml pipette, by pipetting 15 times up and down (setting: medium speed, 120 μl). Aggregates were left to sediment exactly 1 min at 1 *g* and released single cells were evaluated by counting a 10-μl aliquot of the supernatant in a hemocytometer. The cell pellet was digested with trypsin to count total cells. The ratio of total over single cells is indicative of intercellular adhesive strength. Statistical analyses were done using a Bonferroni-corrected unpaired Kruskal-Wallis test.

### IF Microscopy

Keratinocytes were grown to confluency on coverslips (LAB-TEK®; Nalge Nunc). After indicated treatments cells were fixed and permeabilized with precooled 100% methanol for 7 min at −20°C and 0.5% Triton X-100, 2 mM PMSF, 2 mM *N*-tosyl-L-phenylalanin chloromethyl ketone (Sigma-Aldrich) for 10 min. Alternatively, for in vivo labeling with PV IgG, cells were incubated with PV IgG 1 (1 mg/ml) for 1 h at 4°C, and fixed (see Fig. 4 a) or analyzed without fixation (see Fig. 6 a).

### Ectopic Expression of Plakoglobin

Full length human plakoglobin (W. Franke, Max-Planck Institute, Heidelberg, Germany [Franke et al., 1989]) was cloned into the mammalian expression vector pEGFP-N1 (CLONTECH Laboratories, Inc.) under control of a constitutive cytomegalo virus promoter. Cells were electroporated with 30 μg expression vector construct or the vector alone. Green fluorescent cells were sorted with a FACS Vantage™ cell sorter (Becton Dickinson) and used without subcloning. Retroviral infection with recombinant plakoglobin-expressing virus was done according to Gandarillas and Watt (1997) with some modifications. In brief, using the cationic liposomal agent FuGENE6™ (Boehringer), the retroviral packaging cells Bosc23 (G.P. Nolan, Stanford University School of Medicine, Stanford, CA) were transfected with the transfer vector pBabe puro (B. Amati, Institut Suisse de Recherche Expérimentale sur le Cancer, Epalinges, Switzerland) in which we had cloned human plakoglobin (Franke et al., 1989) in frame with GFP-sg25. Stably transfected cultures were obtained by selection with 2.5 μg/ml puromycin, and subsequently cocultured for infection in the same ratio with either $PG^{-/-}$ or $PG^{+/+}$ keratinocytes in 25% DMEM/75% defined keratinocyte-SFM. After 24 h, 5 μg/ml polybrene was added to the culture medium for 24 h. The medium was then changed to 100% defined keratinocyte-SFM and cultures were allowed to proliferate for 1 wk. Subsequently, cultures were partially trypsinized to remove the Bosc23 cells and keratinocytes were incubated with 2.5 μg/ml puromy-

cin in defined keratinocyte-SFM for 1 wk before use. Keratin staining was used to confirm that all Bosc23 cells, which are of fibroblastic origin, had been removed.

### *Protein Extraction and Immunoprecipitation Protocol*

Human epidermal extracts were obtained according to Sugi et al. (1989). Recombinant baculovirus-encoded desmoglein proteins were harvested from the supernatant of infected high five insect cells as described (Amagai et al., 1994). Total cell lysates were obtained by scraping cells directly into Laemmli sample loading buffer. To extract fully assembled desmosomal proteins that are not soluble in mild anionic detergents (Cowin et al., 1986) we used RIPA buffer. Fractionation of 1% Triton X-100 soluble and cytoskeleton-associated proteins was done according to Pasdar et al. (Pasdar and Nelson, 1988; Pasdar et al., 1991). All immunoprecipitations were done using protein G–Sepharose (Amersham Pharmacia Biotech) and the protocol that was developed for desmosomal proteins by Pasdar et al. (Pasdar and Nelson, 1988; Pasdar et al., 1991).

## *Results*

### *Characterization of PG$^{-/-}$ and PG$^{+/+}$ Keratinocytes*

To investigate the role of plakoglobin in PV we established cell cultures from the skin of PG$^{-/-}$ mouse embryos and PG$^{+/+}$ littermates (Bierkamp et al., 1996) according to our protocol for the isolation of differentiating long-term mouse keratinocyte cultures (Caldelari et al., 2000). First we defined the capacity of the established PG$^{-/-}$ and PG$^{+/+}$ cultures to form desmosome-mediated intercellular adhesion in response to increased extracellular calcium (1.2 mM), a process described to mimic epithelial differentiation (Hennings et al., 1980). After 6 h of culture in high calcium medium, distribution of Dsg 3 to cell–cell borders and the formation of a continuous epithelial sheet were similar in PG$^{-/-}$ and PG$^{+/+}$ cells, as shown in IF studies (Fig. 1 a). To assess whether the membrane-tethered Dsg 3 was anchored to the keratin network at that time point, we coprecipitated cellular proteins with keratin 14–specific antibodies using RIPA buffer extracts. As shown by WB, the keratin 14 coprecipitates contained low but comparable amounts of Dsg 3 and plakophilin 1 in PG$^{-/-}$ and PG$^{+/+}$ cells (Fig. 1 b). As expected, plakoglobin could only be coprecipitated from PG$^{+/+}$ cell extracts. Together these findings demonstrated that Dsg 3, the target of PV IgG, was anchored to the keratin network in both cell types to a similar extent. This is consistent with the subsequent observation that Dsg 3 is contained, together with plaque proteins, in the cytoskeletal fraction in both cell types (see Fig. 5, SDS). The capacity of these keratin-anchored desmosomal structures to confer adhesive strength was tested in an adhesion assay (Calautti et al., 1998). In brief, isolated epithelial sheets were subjected to substantial mechanical stress and the ratio of total cells over released single cells was calculated. Compared with cell cultures held in low calcium medium, intercellular adhesiveness of PG$^{-/-}$ cells increased during calcium-induced differentiation, although

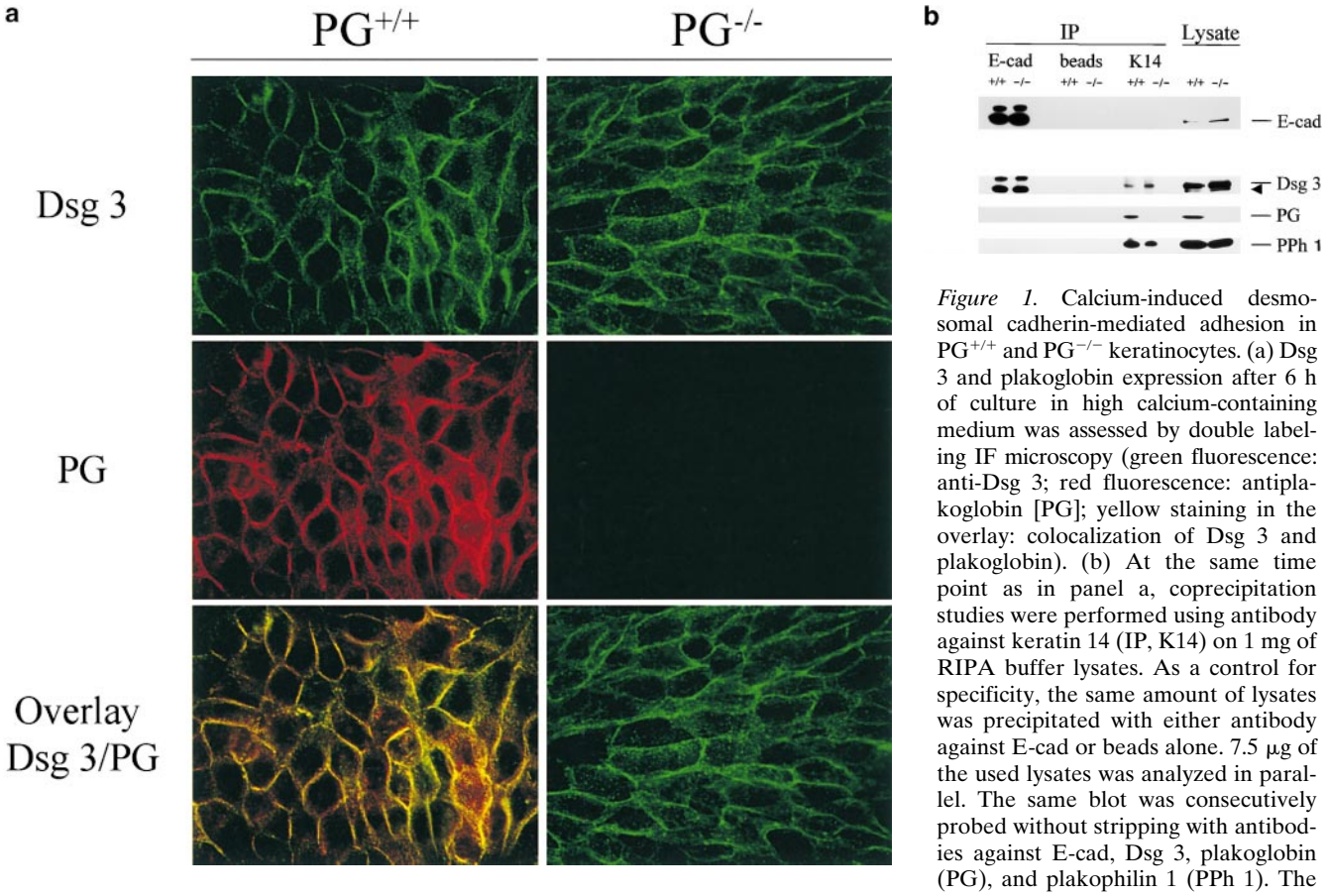


*Figure 1.* Calcium-induced desmosomal cadherin-mediated adhesion in PG$^{+/+}$ and PG$^{-/-}$ keratinocytes. (a) Dsg 3 and plakoglobin expression after 6 h of culture in high calcium-containing medium was assessed by double labeling IF microscopy (green fluorescence: anti-Dsg 3; red fluorescence: antiplakoglobin [PG]; yellow staining in the overlay: colocalization of Dsg 3 and plakoglobin). (b) At the same time point as in panel a, coprecipitation studies were performed using antibody against keratin 14 (IP, K14) on 1 mg of RIPA buffer lysates. As a control for specificity, the same amount of lysates was precipitated with either antibody against E-cad or beads alone. 7.5 μg of the used lysates was analyzed in parallel. The same blot was consecutively probed without stripping with antibodies against E-cad, Dsg 3, plakoglobin (PG), and plakophilin 1 (PPh 1). The upper band in the E-cad immunoprecipitates is likely to represent unprocessed E-cad (Posthaus et al., 1998). The arrowhead points at remaining E-cad signal in the blot probed with Dsg 3. Low amounts of plakoglobin in the E-cad precipitate were detected after extended exposure (data not shown).

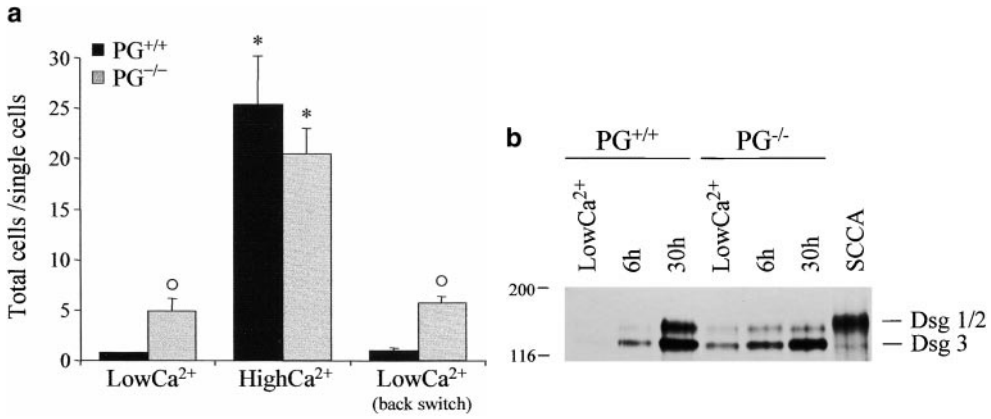


*Figure 2.* Calcium-induced adhesive strength in $PG^{+/+}$ and $PG^{-/-}$ keratinocytes. (a) The intercellular adhesive strength between keratinocytes in low calcium ($LowCa^{2+}$) medium, after 30 h in high calcium ($HighCa^{2+}$) medium, or reversed to low calcium medium (back switch: 6 h high calcium, 24 h low calcium) was quantified by the adhesion assay. Time points corresponded with subsequent incubation times used in experiments with PV IgG (see Fig. 4 c). Results of two experiments done in triplicate are presented as the counts of total over single cells, indicative of intercellular adhesive strength. $PG^{+/+}$, $*P = 0.003$; $PG^{-/-}$, $*P = 0.004$; $PG^{+/+}$ vs. $PG^{-/-}$ in low calcium, $°P = 0.008$. Bars represent standard deviations. (b) 15 μg of total cell lysates from $PG^{+/+}$ and $PG^{-/-}$ keratinocytes grown under low calcium or high calcium concentrations (chosen time points are relevant for PV IgG stimulations) was assessed for Dsg 3 and Dsg 1 expression using WB. The same amount of total cell lysate from a canine squamous cell carcinoma cell line (SCCA) overexpressing Dsg 1 (de Bruin et al., 1999) was used as a control to illustrate Dsg 1 steady-state level in a cell that does not respond to PV IgG with keratin retraction (data not shown). Note that the Dsg 3 antibody raised against mouse EC5 domain has low avidity for canine Dsg 3.

to a lesser extent than in $PG^{+/+}$ cells (Fig. 2 a, shown for 30 h of high calcium incubation). When returned to medium with low calcium concentration, the adhesion structures that had formed in both cell types during 6 h of culture in high calcium medium disassembled within 24 h. Together, these findings indicated that the calcium-induced, de novo–assembled adhesion junctions conferred adhesiveness, and that this was reversible in $PG^{+/+}$ and $PG^{-/-}$ cells.

Both stabilization of Dsg 3 at the plasma membrane (Fig. 1 a) and improvements in adhesive strength in response to raised extracellular calcium (Fig. 2 a) were paralleled by an increase in the steady-state levels of Dsg 3 protein in $PG^{+/+}$ and $PG^{-/-}$ cells, as demonstrated in WB analysis on total protein extracts (Fig. 2 b). Interestingly, under low calcium conditions Dsg 3 protein was substantially higher in $PG^{-/-}$ cells than in $PG^{+/+}$ cells. Dsg 3 in $PG^{-/-}$ cells then increased more gradually in high calcium medium to reach a similar level in both cell types at 30 h. The steady-state level of Dsg 1 protein in $PG^{-/-}$ keratinocytes increased only slightly during 6 h of high calcium treatment and then remained at a low level, whereas a substantial increase could be observed in $PG^{+/+}$ cells. The difference in adhesion molecule expression may account for variations in adhesiveness between $PG^{-/-}$ and $PG^{+/+}$ cells (Fig. 2 a). For example, the low Dsg 1 level together with a slightly reduced level of Dsg 3 protein in the cytoskeleton fraction (see Fig. 5, SDS) is likely to be the reason for the weaker adhesiveness seen in $PG^{-/-}$ cells in high calcium medium (Fig. 2 a).

In conclusion, the increase in the steady-state level of Dsg 3 protein (Fig. 2 b), its localization at the plasma membrane (Fig. 1 a), its anchoring to the cytoskeleton (Fig. 1 b), and its containment with plaque proteins in the insoluble protein fraction (see Fig. 5, SDS) indicate that desmosomal cadherin-mediated adhesion is formed and that it contributes to the increase in adhesiveness observed during calcium-induced differentiation in $PG^{-/-}$and $PG^{+/+}$ cells. The fact that $PG^{-/-}$ cells establish desmosome-mediated adhesion similar to that of $PG^{+/+}$ cells made them an ideal tool to assess whether the presence of plakoglobin is required for, and contributes to, the pathogenesis of PV.

### *PV IgG–induced Keratin Retraction and Loss of Adhesion*

For our studies we chose three PV sera (PV IgG 1, PV IgG 2, and PV IgG 3) from human patients satisfying typical PV criteria (Amagai, 1995). The protein A–purified IgG were directed against Dsg 3 as determined by WB analysis on epidermal extracts and on recombinant proteins, and contained no significant level of antibodies against Dsg 1 (Fig. 3, a and b, shown for PV IgG 1). This finding was confirmed by ELISA (Amagai et al., 1999). In addition, these IgG fractions caused suprabasal cleft formation in an "in vitro skin model," mimicking lesions seen in PV patients (Müller et al., 2000). As demonstrated by IF studies, PV IgG bound to a similar extent to the surface of nonpermeabilized $PG^{-/-}$ and $PG^{+/+}$ keratinocytes, which had been incubated in high calcium medium for 6 h (Fig. 4 a, shown for PV IgG 1). Stimulation of the cells with PV IgG for 24 h led to a visible retraction of the keratin filaments from the cell–cell borders in $PG^{+/+}$ cells (Fig. 4 b, left). This phenomenon was accompanied by a sixfold loss in adhesive strength as demonstrated by the adhesion assay (Fig. 4 c). In contrast, keratin retraction (Fig. 4 b, right) and loss of adhesion (Fig. 4 c), the hallmark of PV lesions in the patient, did not occur in $PG^{-/-}$ cells despite the binding of PV IgG to the cell surface (Fig. 4 a). The normal human control IgG (nhIgG) which did not bind to the cells (Fig. 4 a) had no effect (Fig. 4, b and c), and is thus representative of unstimulated differentiating keratinocytes.

It has been shown that the primary target of PV antibodies is membrane-associated, Triton X-100–soluble Dsg 3 (Aoyama and Kitajima, 1999) which is not yet associated with desmoplakin and anchored to the keratin network (Pasdar and Nelson, 1988). To define the molecular composition of the targeted Dsg 3 complexes at the time point of PV IgG stimulation, Triton X-100–soluble and insoluble

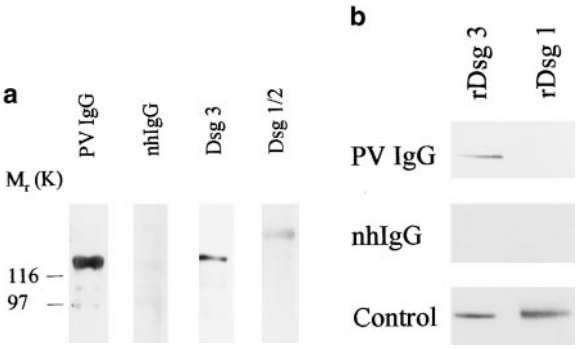


*Figure 3.* Characterization of PV IgG. (a) WB strips containing 20 μg of human epidermal extract were probed with PV IgG 1 (0.375 mg/ml), the same concentration of control nhIgG or antibodies directed against Dsg 3 or Dsg 1/2. (b) The main antigenic target of PV IgG was identified by immunoblots with recombinant baculovirus-expressed human Dsg 3-$IgG_1$ or Dsg 1-$IgG_1$ (rDsg 3, rDsg 1) incubated with PV IgG 1 (0.375 mg/ml), or nhIgG followed by alkaline phosphatase–coupled anti–human $IgG_4$ (Southern Biotechnology Associates, Inc.). As control for equal loading, the same quantity of recombinant protein was assessed in parallel with anti–human IgG (H+L; Bio-Rad Laboratories). Note that PV IgG 1 recognizes recombinant Dsg 3 but not Dsg 1, which is consistent with the antibody profile detected by ELISA (see Materials and Methods). Reproduced from Müller et al., 2000, with the permission of Verlag Hans Hüber, Bern, Switzerland.

proteins were harvested from $PG^{-/-}$ and $PG^{+/+}$ cells, and the soluble fraction was precipitated with PV IgG (Fig. 5). Dsg 3 was coprecipitated with plakophilin 1 from both cell extracts and, as expected, also with plakoglobin from $PG^{+/+}$ extracts. This indicated that in $PG^{+/+}$ and $PG^{-/-}$ cells, PV IgG bind to Dsg 3 complexes which are associated with the plaque proteins investigated here. Analysis of the steady-state levels of proteins from soluble and insoluble fractions provided some additional information about the distribution of desmosomal proteins in both cell types. Dsg 3 in the soluble fraction was consistently higher in $PG^{-/-}$ cells than in $PG^{+/+}$ cells (Fig. 5). Note that higher amounts of soluble Dsg 3 may give rise to the more granular IF pattern we observed using cell surface labeling of $PG^{-/-}$ cells (Fig. 4 a). Conversely, the amount of Dsg 3 extracted from the corresponding Triton X-100–insoluble fraction, which is thought to contain keratin-anchored desmosomal complexes (Pasdar and Nelson, 1988), appeared slightly lower in $PG^{-/-}$ cells. Accordingly, plaque proteins, though abundant in this fraction, were also slightly reduced compared with $PG^{+/+}$ cells (Fig. 5, SDS). Containment of Dsg 3 and plaque proteins in the keratin cytoskeletal fraction of both $PG^{-/-}$ and $PG^{+/+}$ cells parallels our finding that Dsg 3 anchors to keratin 14 (see Fig. 1 b), and further indicates that $PG^{-/-}$ and $PG^{+/+}$ cells contained adhesion complexes that are fully assembled and anchored to keratin at the time of PV IgG stimulation.

### *Reversal of Keratin Retraction in $PG^{-/-}$ Keratinocytes*

To demonstrate that the lack of responsiveness of $PG^{-/-}$ keratinocytes to PV IgG was solely due to the absence of plakoglobin, we reintroduced this protein into the $PG^{-/-}$ cells. Green fluorescent cells, constitutively expressing recombinant human plakoglobin fused to GFP ($PG_{GFP}$), or as a control GFP alone, were sorted with a FACS Vantage™ cell sorter (Becton Dickinson) and analyzed without further subcloning. Using immunofluorescence studies we demonstrated that ectopically expressed plakoglobin distributed to cell–cell borders and colocalized with PV antigen in the $PG_{GFP}$-expressing $PG^{-/-}$ cells (Fig. 6 a, left). Progressive association of $PG_{GFP}$ with keratin-anchored proteins during calcium-induced differentiation was confirmed by WB analysis on fractionated proteins (Fig. 6 b). The membrane-anchored, ectopically expressed plakoglobin enhanced the intercellular adhesiveness in $PG_{GFP}$-transfected $PG^{-/-}$ cells compared with GFP-transfected control cells, as demonstrated by the adhesion assay (Fig. 7 b, nhIgG). Collectively, these findings demonstrate that the ectopically expressed plakoglobin codistributed with desmosomal components to the plasma membrane, was incorporated into cytoskeleton-anchored structures and was functional as it conferred enhanced adhesive strength.

In response to PV IgG stimulation, the strong adhesion in $PG_{GFP}$-transfected $PG^{-/-}$ cells was severely affected (Fig. 7 b, PV IgG 1). Reestablishment of responsiveness to PV IgG was also confirmed by immunofluorescence studies, demonstrating that ectopic plakoglobin expression fully restored keratin retraction in PV IgG1-treated $PG^{-/-}{}_{PGGFP}$ cells (Fig. 7 a, right). Moreover, loss of adhesion was paralleled by perturbance of the $PG_{GFP}$ distribution pattern at the plasma membrane. $PG_{GFP}$ formed aggregates rather than exhibiting the regular distribution observed in nhIgG-stimulated cells, and the signal generated by the $PG_{GFP}$ fusion protein appeared reduced in its intensity. Consistent with our results for untransfected $PG^{-/-}$ cells (Fig. 4 c), adhesion in the GFP-expressing control cells, which also lack plakoglobin, was not affected (Fig. 7, a, bottom, and b).

### *PV IgG Binding Perturbs the Localization of Plakoglobin at the Membrane*

The distribution pattern of ectopically expressed $PG_{GFP}$ was altered upon PV IgG binding to $PG_{GFP}$-expressing $PG^{-/-}$ keratinocytes (Fig. 7 a). To assess whether this feature was a result of the knockout phenotype or the $PG_{GFP}$ construct, we investigated the localization of retrovirally expressed $PG_{GFP}$ in $PG^{-/-}$ and $PG^{+/+}$ cells, and that of endogenous plakoglobin in $PG^{+/+}$ cells. In all three experimental setups the distribution of plakoglobin was altered after 24 h of stimulation with PV IgG. The pattern became discontinuous, showing aggregate formation, and did not compare with the linear arrangement along the membrane observed in nhIgG-treated cells (Fig. 8, a and b, shows $PG^{+/-}{}_{PGGFP}$ and $PG^{+/+}$ cells, respectively). Furthermore, the intensity of the fluorescence signal was reduced. In summary, disturbed plakoglobin distribution was observed in all here investigated PV IgG–responsive mouse keratinocytes.

## *Discussion*

### *$PG^{-/-}$ Keratinocytes Establish Desmosome-mediated Adhesion in Early Calcium-induced Differentiation*

Despite good evidence for an important role of plakoglobin in intercellular adhesion formation (Cowin and Burke, 1996; Kowalczyk et al., 1999), plakoglobin knockout em-

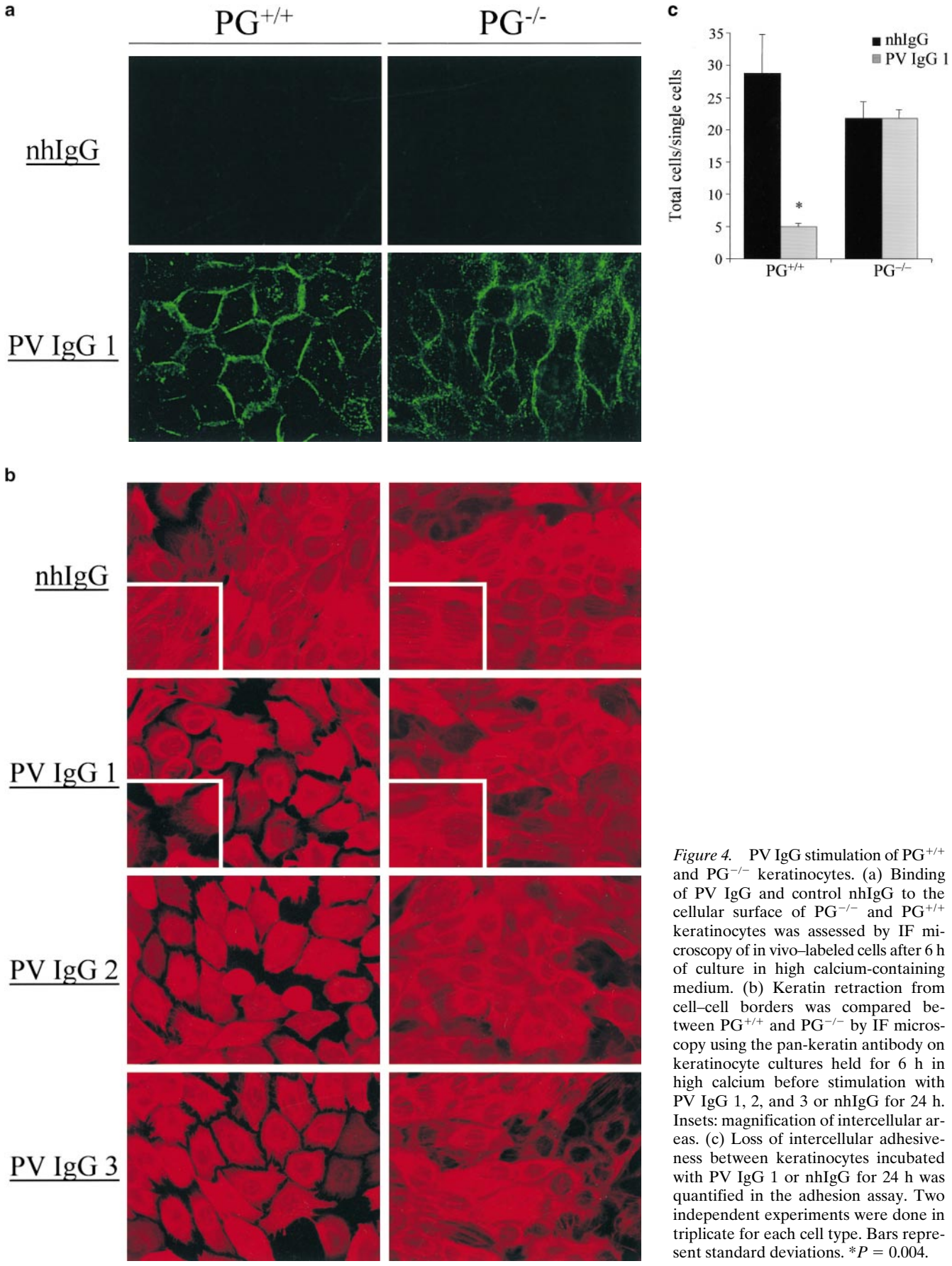


*Figure 4.* PV IgG stimulation of $PG^{+/+}$ and $PG^{-/-}$ keratinocytes. (a) Binding of PV IgG and control nhIgG to the cellular surface of $PG^{-/-}$ and $PG^{+/+}$ keratinocytes was assessed by IF microscopy of in vivo–labeled cells after 6 h of culture in high calcium-containing medium. (b) Keratin retraction from cell–cell borders was compared between $PG^{+/+}$ and $PG^{-/-}$ by IF microscopy using the pan-keratin antibody on keratinocyte cultures held for 6 h in high calcium before stimulation with PV IgG 1, 2, and 3 or nhIgG for 24 h. Insets: magnification of intercellular areas. (c) Loss of intercellular adhesiveness between keratinocytes incubated with PV IgG 1 or nhIgG for 24 h was quantified in the adhesion assay. Two independent experiments were done in triplicate for each cell type. Bars represent standard deviations. $*P = 0.004$.

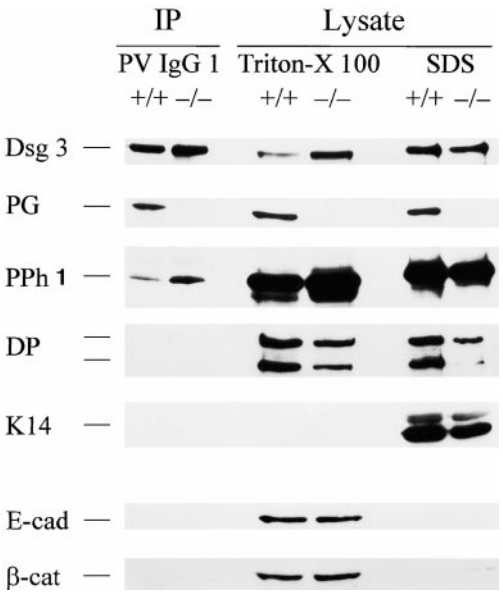


*Figure 5.* Dsg 3 complexes and the distribution of soluble and insoluble proteins in $PG^{+/+}$ and $PG^{-/-}$ cells. 1 mg of Triton X-100–soluble cell lysate was coprecipitated with 75 μg PV IgG 1 (IP PV IgG 1) and analyzed by immunoblotting. 20 μg of the lysates used for immunoprecipitation was loaded as control for protein composition (Triton X-100) and compared with the same portion of the corresponding cytoskeletal fraction (SDS). The same blot was probed consecutively with the indicated antibodies. PPh1, plakophilin 1; DP, Desmoplakin I + II; K14, keratin 14; β-cat, β-catenin. Note that the specificity of the coprecipitation reaction and the purity of the SDS fraction are demonstrated by the absence of E-cad and β-catenin from the immunoprecipitation reaction and the SDS lysates.

bryos were found to survive far beyond assembly of the first desmosomes (Bierkamp et al., 1996; Ruiz et al., 1996). This is in clear contrast with mice deficient for desmoplakin which do not survive E6.5 (Gallicano et al., 1998). Together these findings suggested that, despite the absence of plakoglobin, desmosome-mediated adhesion is established and alternative partners, such as plakophilin family members, can substitute for the missing plakoglobin (Ruiz et al., 1996). Our findings in differentiating $PG^{-/-}$ cells support these notions. $PG^{-/-}$ cells did not demonstrate either retracted keratin filaments from cell–cell borders, or spontaneous loss of adhesion in early stages of cal-

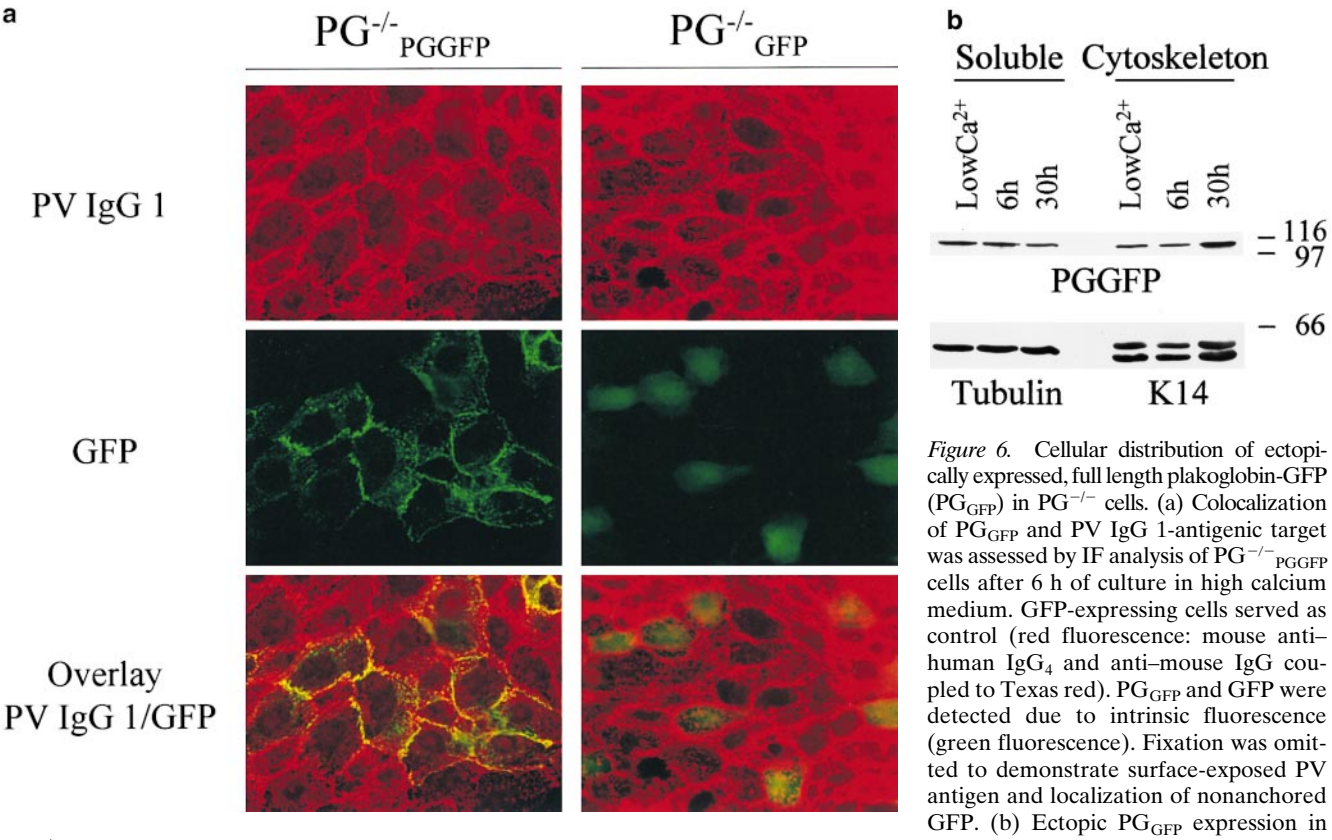


*Figure 6.* Cellular distribution of ectopically expressed, full length plakoglobin-GFP ($PG_{GFP}$) in $PG^{-/-}$ cells. (a) Colocalization of $PG_{GFP}$ and PV IgG 1-antigenic target was assessed by IF analysis of $PG^{-/-}{}_{PGGFP}$ cells after 6 h of culture in high calcium medium. GFP-expressing cells served as control (red fluorescence: mouse anti–human $IgG_4$ and anti–mouse IgG coupled to Texas red). $PG_{GFP}$ and GFP were detected due to intrinsic fluorescence (green fluorescence). Fixation was omitted to demonstrate surface-exposed PV antigen and localization of nonanchored GFP. (b) Ectopic $PG_{GFP}$ expression in $PG^{-/-}$ cells was assessed at the indicated time points during calcium-induced differentiation using WB of the soluble and corresponding cytoskeletal fraction. The top panel depicts steady-state levels of $PG_{GFP}$ protein revealed with plakoglobin antibody and the bottom panel incubations of the same blot with tubulin or keratin 14 (K14) antibodies used as loading controls. Bars indicate the molecular weight markers (in kD).

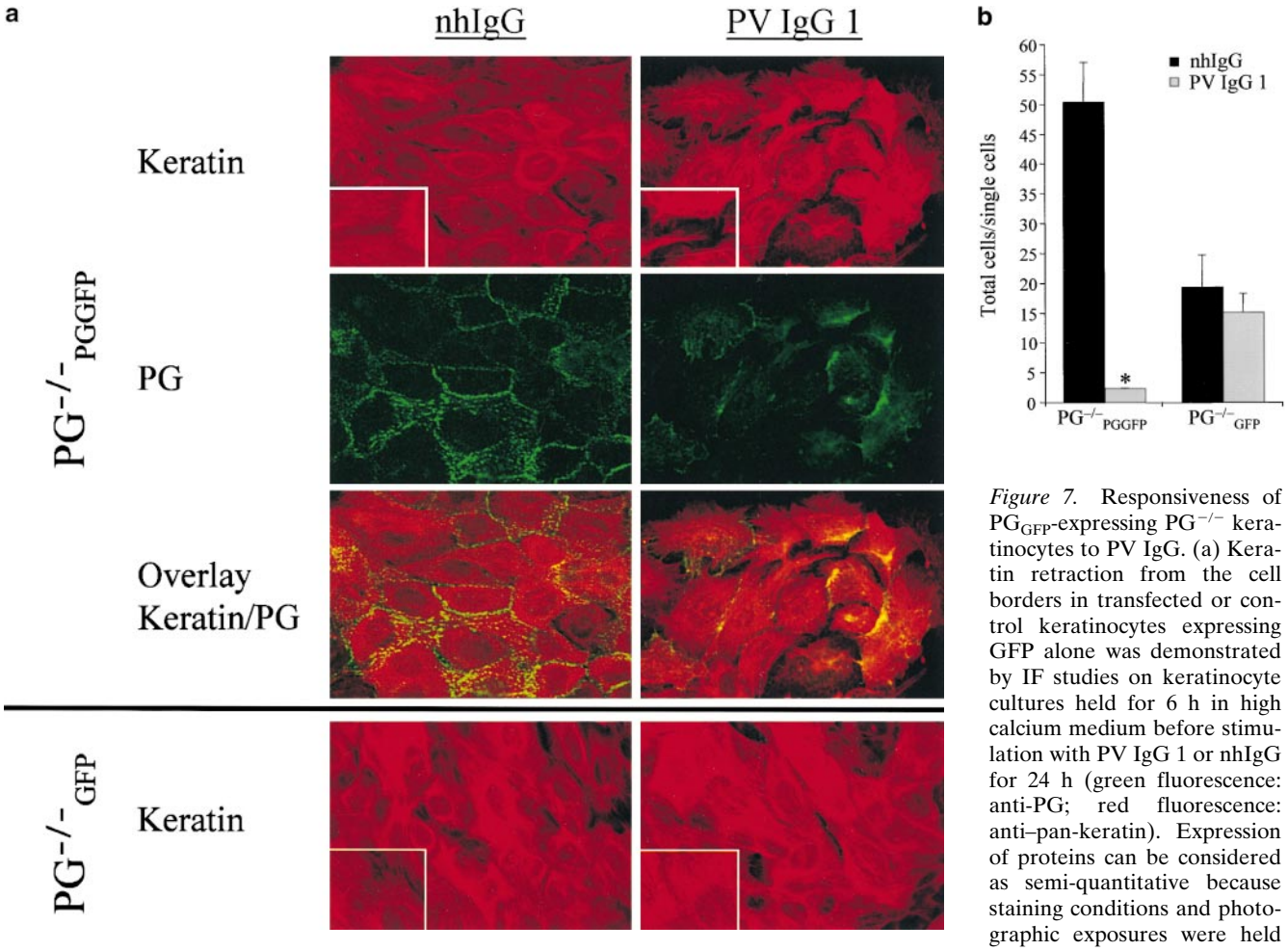


*Figure 7.* Responsiveness of $PG_{GFP}$-expressing $PG^{-/-}$ keratinocytes to PV IgG. (a) Keratin retraction from the cell borders in transfected or control keratinocytes expressing GFP alone was demonstrated by IF studies on keratinocyte cultures held for 6 h in high calcium medium before stimulation with PV IgG 1 or nhIgG for 24 h (green fluorescence: anti-PG; red fluorescence: anti–pan-keratin). Expression of proteins can be considered as semi-quantitative because staining conditions and photographic exposures were held unchanged. Insets: magnification of intercellular areas. (b) Loss of intercellular adhesiveness in PV IgG 1– or nhIgG-treated cells for 24 h was quantified by the adhesion assay. Each experiment was done in triplicate. Bars represent standard deviations. $*P = 0.004$.

cium-induced differentiation. Furthermore, biochemical and immunohistological investigations demonstrated a similar overall composition and distribution of desmosomal components in the cytoskeletal fraction of $PG^{-/-}$ and $PG^{+/+}$ cells. Whereas these results support establishment of desmosomal adhesion, our finding that a substantial amount of plakophilin 1 coprecipitated with Triton X-100–soluble Dsg 3 in differentiating $PG^{-/-}$ cells is compatible with an effective substitution of plakoglobin by plakophilins during initiation of desmosome formation. It is noteworthy that in a yeast two-hybrid approach, plakophilin 1 was found to associate with Dsg 1, but not with Dsg 3 (Hatzfeld et al., 2000). It is conceivable that other factors are required to allow Dsg 3–plakophilin 1 association, and that these factors are only present in cells that normally assemble desmosomes, like the keratinocytes used in this study.

Although the absence of plakoglobin does not abolish assembly of desmosomal components at the plasma membrane, the presence of this plaque protein contributes to the strengthening of intercellular adhesion. The substantial increase in resistance to mechanical stress seen in our $PG^{-/-}$ cells after ectopic expression of plakoglobin confirms this ability. Under circumstances where desmosome-mediated adhesion is already established, such as in $PG^{-/-}$ cells, ectopic plakoglobin may improve adhesive strength through increased lateral interconnection between desmosomal plaque proteins (Smith and Fuchs, 1998), and/or through more effective clustering of desmosomal cadherins (Palka and Green, 1997). Lack of efficient interconnection/clustering is likely to account for the observation in electron microscopical studies that desmosomes are less prominent in primary $PG^{-/-}$ keratinocytes (Bierkamp et al., 1999). That plakoglobin contributes to establishing strong adhesion is also seen in those plakoglobin knockout embryos that survive longer (Bierkamp et al., 1996). Whereas the epidermal architecture of early embryos appears intact (Ruiz et al., 1996), the epidermis of older individuals develops acantholysis and necrosis in the superficial layers. Together with the knowledge that embryogenesis does not proceed in the absence of desmosomes (as observed in desmoplakin null mutant mice [Gallicano et al., 1998]), this suggests that desmosomal cadherin-mediated adhesion is established during development and is sufficiently strong; however, it becomes limiting in the upper epidermis exposed to increased mechanical stress (Bierkamp et al., 1996). Mislocalization of β-catenin to Dsg 1/2 which is strongly expressed in the upper epidermis (Mahoney et al., 1999), was also hypothesized to affect the epidermal integrity in these mice (Bierkamp et al., 1999;

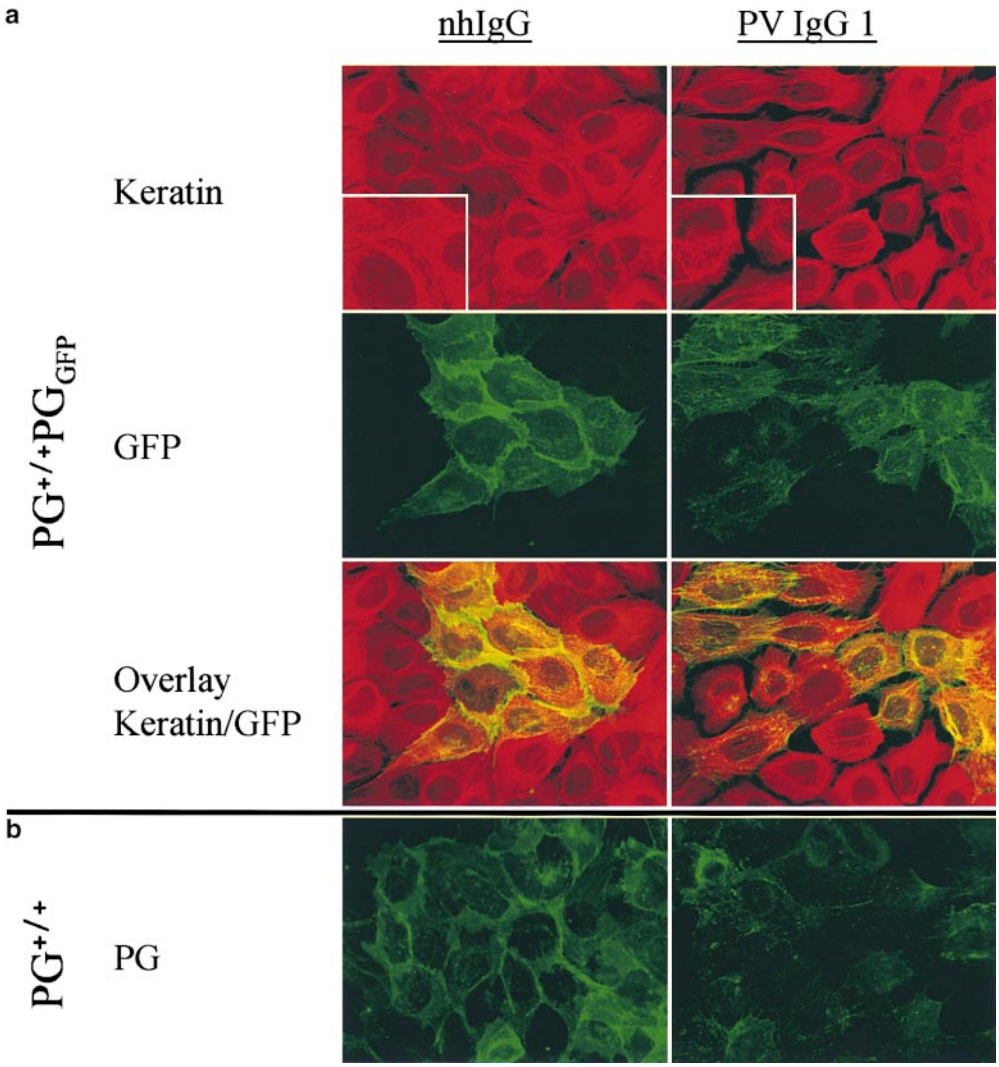


*Figure 8.* Ectopic and endogenous plakoglobin distribution in PV IgG–treated $PG^{+/+}$ cells. (a) Retrovirus-encoded $PG_{GFP}$ was assessed in IF studies after 24 h stimulation with PV IgG 1 (green fluorescence: anti-GFP; red fluorescence: anti–pan-keratin). (b) Using a plakoglobin antibody distribution of endogenous plakoglobin (PG) including loss of the fluorescence signal was demonstrated in normal $PG^{+/+}$ cells after 24 h of PV IgG 1 stimulation. Staining conditions and photographic exposures were held unchanged for all micrographs.

therein Dsg 1/2 was confused with Dsg 3). Dsg-bound β-catenin presumably impairs keratin anchorage to the desmosomal plaque, which in turn weakens adhesiveness. In early calcium-induced differentiation analyzed in our study, Dsg 1 expression is very low in $PG^{-/-}$ cells. This may explain our finding that β-catenin does not colocalize to desmosomal components (see Fig. 5). Absence of β-catenin interference with desmosomal adhesion in deep epidermis at the site where mainly Dsg 3 is expressed (Mahoney et al., 1999) is consistent with the intact suprabasal architecture seen in $PG^{-/-}$ mice. It is also consistent with the substantial adhesion that is established in $PG^{-/-}$ keratinocyte cultures early in differentiation.

### *Perturbed Localization of Plakoglobin Accompanies Loss of Adhesion*

The finding that $PG^{-/-}$cells establish desmosome-mediated adhesion during calcium-induced differentiation provided us with the necessary tool to assess the involvement of plakoglobin in PV. Our observations that $PG^{-/-}$ cells do not exhibit keratin retraction or loss of adhesion despite PV IgG binding, and that this responsiveness is restored after ectopic plakoglobin expression, demonstrate that steric hindrance alone is not sufficient to induce loss of adhesion. Taken together, our results provide strong evidence for an indispensable role of plakoglobin in the pathogenesis of PV. The involvement of plakoglobin in the disease process is further emphasized by our concurrent observation that the linear organization of plakoglobin, but not β-catenin (data not shown), is completely disrupted in PV IgG–stimulated $PG^{+/+}$ and plakoglobin-transfected $PG^{-/-}$ cells. This particular finding, though described in some PV patients (Burge et al., 1993), contradicts observations made in the squamous cell carcinoma cell line DJM-1 (Aoyama and Kitajima, 1999). Although PV IgG binding to these cells provoked rapid depletion of Dsg 3 from the membrane, the linear pattern of plakoglobin at cell–cell borders remained unaffected even after prolonged incubation times. Significantly, keratin retraction, the hallmark of PV, did not occur in these cells. The most likely explanation for the lack of keratin retraction in this neoplastic cell line is the strong and persistent Dsg 1 expression described by the authors. Dsg 1 was recently suggested to compensate for the loss of Dsg 3–mediated adhesion in the presence of PV IgG (Mahoney et al., 1999). Accordingly, in these DJM-1 cells Dsg1 could con-

tinue to anchor plakoglobin at the plasma membrane. We made a similar observation (unpublished data) in a canine squamous cell carcinoma cell line overexpressing Dsg 1 (de Bruin et al., 1999). Conversely, in the $PG^{+/+}$ mouse keratinocytes which demonstrate keratin retraction, Dsg 1 expression is very low at the time point of PV IgG stimulation, corresponding well with levels in the human patient's stratified squamous epithelium at the site where blisters occur (Shirakata et al., 1998; Mahoney et al., 1999). Dsg 1 expression is also very low in the $PG^{-/-}$cells at the time of PV IgG stimulation, and is not upregulated as differentiation progresses. These findings, together with the fact that Dsg 1 expression in $PG^{-/-}$ cells is at any time point lower than that in our SCCA cell line (Fig. 2 b), exclude the possibility that $PG^{-/-}$ cells are not responsive to PV IgG because of Dsg 1 expression. Collectively, these data corroborate our finding that plakoglobin is required for keratin retraction and loss of adhesion to proceed in differentiating keratinocytes, and extend the significance of perturbance in its localization for the disease process.

### *PV IgG–induced Loss of Adhesion: Known and Speculated Mechanisms*

The molecular mechanisms regulating adhesion and dysadhesion remain inadequately understood (Kowalczyk et al., 1999; Gumbiner, 2000). Accordingly, analysis of the impaired adhesion seen in PV may provide a novel perspective on these mechanisms. Analogous to a ligand/receptor activation, binding of PV IgG to Dsg 3 may induce a conformational change of the targeted antigen, which then provokes the serine phosphorylation of Triton X-100–soluble Dsg 3 described to occur early in the pathogenesis of PV (Aoyama et al., 1999). It is conceivable that this phosphorylation induces the abnormal conformation of plakoglobin which was suggested to affect the availability of cadherin-binding sites within the plakoglobin moiety (Chitaev et al., 1996), and which ultimately leads to the observed dissociation of plakoglobin from Dsg 3 (Aoyama et al., 1999). Loss of plakoglobin plasma membrane staining seen in our IF studies is consistent with the notion that this plaque protein dissociates from its membrane-tethered localization. The consequence of this event may be the subsequent rapid depletion of Triton X-100–soluble Dsg 3 in the DJM-1 cells (Aoyama et al., 1999), and which was also observed in our mouse keratinocytes (Caldelari, R., unpublished observation). Desmosome formation requires the concomitant presence of Dsg and desmocollin isoforms (Chitaev and Troyanovsky, 1997). Continuous depletion of detergent-soluble Dsg 3, the main desmosomal cadherin at the early stage of differentiation (Garrod et al., 1996), has thus been suggested to prevent new desmosome formation (Aoyama and Kitajima, 1999). The importance of Dsg 3 depletion in the deep epidermis was underscored by the finding that Dsg 3 knockout mice exhibit spontaneous blister formation in the suprabasal location of stratified squamous epithelia where little or no compensating Dsg 1 is expressed (Koch et al., 1997). In the $PG^{-/-}$ cells where the expression level of Dsg 1 is very low and barely upregulated during calcium-induced differentiation, a PV IgG–induced depletion of Dsg 3 would be expected to have a drastic effect. That these cells in contrast lack responsiveness to PV IgG, together with our preliminary results demonstrating their unaffected turnover of Dsg 3 in response to PV IgG (Caldelari, R., unpublished observation), strongly indicate that plakoglobin somehow contributes to the depletion of Dsg 3 in early PV.

### *PV IgG–induced Loss of Adhesion: The Alternate Role of Plakoglobin*

According to our findings, plakoglobin has a pivotal role in PV which is not exerted by plakophilin in $PG^{-/-}$ cells. Such a role of plakoglobin cannot be discussed without including very recent findings on the signaling role of this protein in the proliferation/differentiation of adult epithelial tissue. Namely, moderate overexpression of plakoglobin targeted to the basal layer of the epidermis by a keratin 14 promoter–driven transgene, was found to suppress epithelial proliferation independently of its role in intercellular adhesion (Charpentier et al., 2000). The capacity of plakoglobin to directly interfere with proliferation/differentiation was subsequently confirmed in colon and kidney epithelial cells, where ectopically expressed plakoglobin mediated cellular transformation through direct transcriptional transactivation of c-*myc* (Kolligs et al., 2000). C-*myc* plays a key role in cell proliferation, differentiation, and apoptosis. On the basis of the previous findings, the results we have obtained with PV IgG demonstrating dependence of disease development on plakoglobin may receive a different significance. It is conceivable that upon PV IgG binding modulation of available plakoglobin at the plasma membrane at first supports rapid degradation of Dsg 3, and subsequently affects the ongoing differentiation process. This in turn could result in impaired differentiation-dependant upregulation of desmosomal components, for example Dsg 1 (Garrod et al., 1996), thus exacerbating the events at the plasma membrane. Even though we found no alterations in c-*myc* expression in preliminary results with PV IgG–stimulated cells (data not shown), additional evidence for a correlation between altered proliferation/differentiation and disease development based on impaired plakoglobin signaling may be provided by the recent identification of the homozygous plakoglobin mutation leading to Naxos disease (McKoy et al., 2000). In this disease, the mutated protein lacks the COOH-terminal portion that bears transcriptional transactivation capacity (Simcha et al., 1998; Hecht et al., 1999; Williams et al., 2000) but is not required for binding to either Dsg, desmocallin, or E-cad (Troyanovsky et al., 1996). Even though deletion of this domain results in formation of longer desmosomes (Palka and Green, 1997), the features of disturbed epidermal turnover without blister formation as seen in Naxos patients suggests that the disease is provoked by abrogation of plakoglobin's signaling function, rather than by a deleterious effect on adhesion. Even though this hypothesis has yet to be confirmed, it might indicate that the signaling function of plakoglobin in adult epithelium is more important than originally anticipated. Although many mechanistic questions remain unresolved within plakoglobin's dual function in adhesion and signaling, the $PG^{-/-}$ keratinocyte cell line we have developed will provide the necessary tool for addressing these questions.

It is noteworthy that two papers that raise some doubt about the pathogenicity of PV antibodies directed against Dsg 3 have very recently been published (Nguyen et al., 2000a,b). Because the study herein focused on downstream effects of PV IgG in a well defined system lacking plakoglobin, and not on the antigenic target itself, this finding was not further addressed. For a specific discussion of this controversial topic, refer to Stanley et al. (2001).

In summary, by establishing a calcium-dependent differentiation model to study the molecular pathogenesis of PV using $PG^{+/+}$ and $PG^{-/-}$ keratinocytes, we have discovered a central role for plakoglobin in the disease process. The absence of keratin retraction after binding of PV IgG to $PG^{-/-}$ keratinocytes demonstrates that steric hindrance alone is not sufficient to induce PV lesions, and that this process is dependent on plakoglobin. Taken together, these results provide new directions for investigators of PV, and will also have an impact on keratinocyte proliferation/differentiation and armadillo protein research.

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