INTRODUCTION

Epithelial sheets consist of highly specialized polarized cells tightly bound together by characteristic cell junctions at many points of cell-cell and cell-matrix contact (Simons and Fuller, 1985). Functionally, cell junctions can be classified as (i) occluding, e.g. tight junctions, (ii) communicating, e.g. gap junctions, and (iii) anchoring junctions, which mechanically attach cells and cytoskeleton to neighbouring cells or to the extracellular matrix and are thus most abundant in solid tissues. Anchoring junctions include two structurally and functionally distinct types of cell-cell junctions, the adherens junctions, where actin filaments anchor, and desmosomes, which are spot-like junctions connected to the intermediate filament (IF) system. Adherens junctions are formed by Ca\(^{2+}\)-dependent cell-cell adhesion molecules called cadherins, which are connected with the actin network in a complex with intracellular attachment proteins, including α-catenin, β-catenin, γ-catenin, p120\(\text{ctn}\), vinculin and α-actinin (Ozawa et al., 1989; Reynolds et al., 1994; Knudsen et al., 1995; Aberle et al., 1996; Rimm et al., 1995). Desmosomes exhibit an electron-dense cytoplasmic plaque (Green and Jones, 1996; Cowin and Burke, 1996; Garrod et al., 1996) consisting of an outer dense plaque subadjacent to the plasma membrane and an inner dense plaque through which IFs appear to loop. Through desmosomes, IFs of adjacent cells are indirectly connected to form a continuous network of great tensile strength throughout a tissue, e.g. an epithelial sheet. The transmembrane linker proteins involved in this network are the desmosomal cadherins, a subgroup of the cadherin superfamily.

Plakoglobin (also termed γ-catenin), initially identified as a constitutive component of the cytoplasmic plaque of desmosomes (Cowin et al., 1986; Franke et al., 1989), is also part of the cadherin-catenin complex in adherens junctions (Aberle et al., 1994; Butz and Kemler, 1994; Jou et al., 1995; Knudsen and Wheelock, 1992; Peifer et al., 1992; Piepenhagen and Nelson, 1993; Sacco et al., 1995; Lewis et al., 1997). In desmosomes, plakoglobin (Pg) interacts with the cytoplasmic domains of desmosomal cadherins, called desmocollins (Dsc) and desmogleins (Dsg), and with the N terminus of desmoplakin (Dp), which in turn binds to IFs (Korman et al., 1989; Kowalczyk et al., 1994; Mathur et al., 1994; Allen et al., 1996; Roh and Stanley, 1995; Troyanovsky 1994a,b; Kouklis et al., 1994; Bornslaeger et al., 1996; Kowalczyk et al., 1997). The hierarchy of molecular interactions that couple desmosomal cadherins with IFs in the assembly of desmosomes has been studied largely in cultured cells (Chitaev...
et al., 1996). Deletion of the plakoglobin-binding domain in Dsc abrogates its ability to anchor IFs at the plaque (Troyanovsky et al., 1994a). Chimeric connexin-Dsc-tail proteins recruit Pg and other plaque proteins, leading to anchorage of cytoskeleton filaments, thus suggesting a role of Pg in clustering desmosomal cadherins and attaching of IFs (Troyanovsky et al., 1993). Interaction partners of Pg in both the desmosomal plaque (Lewis et al., 1997) and the adherens junction have been analysed in detail using recombinant proteins (Mathur et al., 1994; Palka and Green, 1997; Kowalczyk et al., 1997; Chitaev and Troyanovsky, 1997; Marcozzi et al., 1998).

Plakoglobin and β-catenin, as members of the Armadillo- or Arm-repeat family, have multiple repeats of a 42 amino acid sequence first identified in the product of the Drosophila segment polarity gene armadillo (Peifer and Wieschaus, 1990; Peifer et al., 1994; Peifer, 1995; Huber et al., 1996a). Arm-repeat sequences are believed to represent functional motifs regulating protein-protein interactions. Other recently identified members of this family are also localized in the adhesive plaques of desmosomes and adherens junctions. These include band 6 protein/plakophilin1, plakophilin2a and 2b, p0071 and p120ctn (Hatzfeld et al., 1994; Hatzfeld and Nachtsheim, 1996; Heid et al., 1996). Their homology to Armadillo suggested a similar signaling function for β-catenin and plakoglobin now supported by experimental results (Behrens et al., 1996; Huber et al., 1996b; Karnovsky and Klymkowsky, 1995).

Gene targeting revealed that Pg null-mutant (Pg−/−) embryos are embryonic lethal at embryonic days E12-16 due to severe defects in heart structure, resulting in ventricle burst and blood flooding the pericardium (Bierkamp et al., 1996; Ruiz et al., 1996). Beside this primary embryonic lethal phenotype, on some genetic backgrounds a few Pg null-mutant embryos survived until late gestation, exhibiting a skin phenotype with blistering and subcorneal acantholysis (Bierkamp et al., 1996). Here we have analysed this skin phenotype in more detail, intrigued by the fact that it first appears rather late during development and primarily in tissues subjected to mechanical stress. Embryonic skin differentiation is of particular interest as it represents the transition of a simple epithelium to a multilayer epidermis consisting of basal, spinous and granular layers.

MATERIALS AND METHODS

Genotypic and histological analysis of mutant embryos

Mice and embryos were genotyped by Southern and PCR analysis as previously described (Bierkamp et al., 1996). Plakoglobin heterozygous mice were kept on a 129/Sv and a C57Bl/6 background. For histology, embryos were fixed in 4% paraformaldehyde (Sigma) in PBS at 4°C overnight, washed 1 hour in PBS at room temperature, dehydrated in graded alcohols, embedded in paraffin (Histowachs, Reichert-Jung), sectioned at 7 µm, and stained with hematoxylin and eosin.

Antibodies and indirect immunofluorescence staining

Affinity-purified antibodies directed against the extracellular domain of mouse E-cadherin (anti-gp84) and anti-peptide antibodies against the N terminus of plakoglobin (anti-D15A) were used as previously described (Butz and Larue, 1995). Monoclonal antibodies against α-catenin (C2081, Sigma) and β-catenin (C19220, Transduction Laboratories; C2206, Sigma) were diluted 1:125. Mouse monoclonal antibodies against desmoplakin (11-5F, used at 1:20) and polyclonal rabbit antibodies against desmocollins 1, 2 and 3 (Amber, 1:100), were kindly provided by Allison North and David Garrod. Polyclonal guinea-pig antiserum against plakophilin1 (1:4000) was a kind gift of Werner Franke. Antibodies to desmoglein 1 and 3 (DG3.10, Biotrend), cytokeratins 5 and 14 (C2562, Sigma), 1 and 10 (C7284, Sigma), loricrin (PRB-145P, BAbCO), and filaggrin (PRB-417P, BAbCO) were used according to the manufacturer’s instructions. Secondary antibodies conjugated with DTAF (dichlorotriazinyl aminofluorescein), FITC (fluorescein-isothiocyanate) or TR (Texas red) were from Dianova and Sigma.

For sectioning, organs of E17.5 embryos and intact E15.5 and E12.5 postimplantation embryos were washed in PBS, embedded in Tissue-Tek OCT compound (Miles, Elkhart, IN, USA) and frozen on dry ice. Cryostat sections were prepared as previously described (Butz and Larue, 1995). Tissues were exposed at room temperature to primary antibody overnight and for 60 minutes to secondary antibodies. After washing twice, nuclei were stained for 5 minutes with DAPI (4’-6-diamino-2-phenylindole) (Sigma) (1:2000) in PBS. Indirect immunofluorescence was observed under a fluorescence microscope (Axioskop, Zeiss) or a confocal krypton-argon laser scanning microscope (Leica). Digital images were printed on a Fujix Pictography 3000 printer (Fuji Photo Film).

Immunofluorescence on ultrathin sections of methacrylate-embedded samples was done basically as described for immunoelectron microscopy (see below and Schwarz et al., 1993). Bound rabbit anti-β-catenin antibodies were detected with Cy3-conjugated goat anti-rabbit antibodies (Jackson/Dianova, Hamburg) and nucleic acids were counterstained with propidium iodide. Both signals were visualized simultaneously using a Cy3-band-pass filter (#F41-007, AHF analysentechnik, Tübingen).

Generation and differentiation of primary keratinocytes

E17.5 embryos were killed and the whole skin dissected in PBS. Skin was digested in dispase (3.2 U/ml) (Gibco-BRL #17105) in PBS overnight on ice; epidermis was separated from dermis, minced and incubated in 5 ml trypsin (0.25%)/EDTA (0.04%), in saline buffer at 37°C with gentle rocking. After addition of 5 ml DMEM/10% FCS, cells were centrifuged (5 minutes at 1000 revs/minute). For culturing, 2x10⁶ cells were seeded on a 3.5 cm dish and fed with KSFM (Gibco-BRL #10744). To induce differentiation, cells were incubated for 24 hours in medium with high (1.2 mM) Ca²⁺ (Hennings et al., 1980). For immunohistochemistry, keratinocytes were grown on coverslips, washed in PBS, fixed for 5 minutes in 1% PFA in PBS, washed again, refixed for 20 minutes in ice-cold methanol and further processed as for cryosectioning.

TUNEL assay for apoptosis

To look for apoptotic cells in situ, cells were washed in PBS, fixed in 4% PFA in PBS, washed again in PBS and permeabilized in 0.3% Triton X-100. The TUNEL enzymatic reaction was carried out by incubating with 12.5 units of terminal-deoxynucleotide-transferase (TdT) (Gibco BRL) in TdT buffer (Gibco BRL), including 4 mM CaCl₂, 0.3% Triton X-100, 0.5 mmol BODIPY-conjugated-DUTP (Molecular Probes), at 37°C for 3 hours. The reaction was stopped by adding 15 mM EDTA, pH 8.0 and washing in PBS, 0.3% Triton X-100.

Immunoprecipitations and immunoblots

Skin from E17.5 Pg+/− and Pg−/− mouse embryos was dissected and washed in PBS, lysed in ice-cold lysis buffer (10 mM Tris-HCl, pH 7.5; 150 mM NaCl, 1 mM EDTA; 0.5% Triton X-100; 2mM CaCl₂) and homogenized in a douncer at 4°C. Insoluble material was removed by centrifugation for 10 minutes at 4°C. In vitro
differentiated keratinocytes were washed 3× with PBS, 1 mM MgCl₂, 1 mM CaCl₂, lysed in 250 µl lysis buffer for 10 minutes at 4°C and homogenized in a douncer. For metabolic labelling, cells pregrown 2 hours in methionine- and cysteine-free DMEM were cultured 10 hours with 50 µCi/ml [³⁵S]methionine/[³⁵S]cysteine (3000 Ci/mmol, Amersham-Buchler, Braunschweig).

Immunoprecipitation and immunoblot experiments were carried out as described (Butz and Kemler, 1994). For each immunoprecipitation 2.5 µg anti-desmoglein 1 and 3 (clone DG3.10, Biotrend), 2 µg anti-plakoglobin or anti-β-catenin (Signal Transduction) antibodies and 40 µl of a suspension of Protein A-Sepharose (Pharmacia) beads were gently agitated at 4°C for 2 hours.

**Electron microscopic analysis**

Embryos were dissected and fixed with 3% paraformaldehyde in 200 mM Hepes, pH 7.5, for 10 minutes at room temperature and then overnight with 8% paraformaldehyde on ice, as described by Griffiths (1993). For immunocytochemistry, fixed samples were dehydrated in ethanol at progressively lower temperatures (Carlemalm et al. 1982), embedded in the polar methacrylate resin Lowicryl K11M (Polysciences, Eppelheim, Germany) and polymerized by UV irradiation at −35°C.

For ultrastructural studies, samples were postfixed with 1% osmium tetroxide in PBS, pH 7.2, for 1 hour on ice, block stained with 1% aqueous uranyl acetate for 1 hour and dehydrated in ethanol at progressively lower temperatures. Samples were transferred into an acetone-Epon mixture at −35°C, infiltrated at room temperature in Epon and polymerized at 60°C for 48 hours.

For immunogold labelling experiments, unspecific binding sites on ultrathin sections were blocked with 0.5% bovine serum albumin, 0.2% gelatin in PBS, incubated with rabbit antibodies against β-catenin (Schneider et al. 1993) or against all three isoforms of

**Fig. 1.** Embryonic wild-type and Pg⁻/⁻ E17.5 epidermis. (A,B) Toluidine blue-stained sections. Pg⁻/⁻ epidermis (B) contained large gaps between cells of the spinous and basal layers where blisters occasionally form. Granular cells were detached (asterisks) and the stratum corneum was absent. (C-H) Cryostat sections of wild-type (C,E,G) and Pg⁻/⁻ epidermis (D,F,H) were stained for desmosomal proteins as indicated, Pg, plakoglobin; DP, desmplakin; Dsg1,3, desmoglein 1 and 3. No plakoglobin was detected in Pg⁻/⁻ epidermis (compare D to C) and desmosomal proteins were diffusely localized along this membrane and throughout the cytoplasm (F,H). bl, basal cell layer. Bar in A corresponds to 25 µm, same for B-H.
desmocollins, and subsequently labelled with protein A-15 nm gold complexes (Schwarz et al., 1993). The labelled sections were stained with uranyl acetate and lead citrate and photographed in a Philips CM10 electron microscope at 60 kV using a 30 m objective aperture.

RESULTS

Blister formation and subcorneal acantholysis appear in \( \text{Pg}^-/^- \) embryonic epidermis after the onset of stratification

Since the phenotype of our \( \text{Pg} \) knock-out mice (Bierkamp et al., 1996) differed somewhat from that described by Ruiz and co-workers (1996), we have analysed our targeted allele in more detail to exclude the possibility that a truncated \( \text{Pg} \) mRNA or protein was generated. For this, embryonic stem (ES) cells lines mutant for \( \text{Pg} \) were established from heterozygous (\( \text{Pg}^{+/-} \)) intercrosses and analysed by northern, immunoblot and immunoprecipitation experiments. These mRNA and protein analyses clearly demonstrated that the homologous recombination event had completely inactivated the plakoglobin gene (not shown).

To analyse the appearance of skin defects, individually genotyped embryos from \( \text{Pg}^{+/-} \) intercrosses were examined microscopically at E12.5, E15.5 and E17.5. The epidermis of \( \text{Pg}^-/^- \) embryos showed no differences to that of \( \text{Pg}^{+/-} \) or wild-type littermates at E12.5 when epidermis consisted a single-layered periderm, or at E15.5 after the early onset of stratification. However, at E17.5, a markedly fragile skin became apparent all over the body in \( \text{Pg}^-/^- \) embryos, with detaching skin in regions of the face, footpads, armpits and on the trunk. The time of appearance of this phenotype correlates with the onset of the adult pattern of epidermal stratification, including basal cell proliferation and suprabasal differentiation of cells as well as with the definitive restriction of the expression patterns of Dsc3 and Dsc1 into basal and suprabasal layers, respectively (Chidgey et al., 1997). Histological sections revealed the dissociation, or acanthokeratolysis, of cells of the granular layer and the loss of the stratum corneum (Fig. 1A,B). In addition, the intercellular spaces in the upper and lower spinous and basal layers were larger (Fig. 1B). Hair follicles seemed normal. Stratified epithelia in mouth, tongue and nasal pits were affected similarly (not shown).

To examine whether the lack of plakoglobin affected the expression pattern or localization of junctional proteins, genotyped embryos at E12.5, E15.5 and E17.5 were processed for cryosectioning and immunofluorescence tests with antibodies against proteins of adherens and desmosomal junctions. In the early stages of skin differentiation (E12.5 and E15.5), no differences between wild-type and \( \text{Pg}^-/^- \) embryos were observed in the staining patterns of adherens junction proteins (E-cadherin, \( \beta \)-catenin) and the desmosomal proteins, Dsc1, Dsc2 and Dsc3 (not shown). In E15.5 skin, Dsc expression was very strong suprabasally (Dsc1) and weaker basally (Dsc3), thus differing from the adult expression patterns previously described (Chidgey et al., 1997). More importantly, the distribution of all desmosomal proteins was...
obtained from wild-type embryos (3·10^6 versus 6·10^6 cells/embryo). These cells were used to prepare cultures of keratinocytes, which were then seeded at a density of 2-3·10^6 cells/3.5 cm dish and cultured with low (0.08 mM) Ca^2+ , mutant keratinocytes proliferated at only half the rate of wild-type cells. Thus Pg^-/- keratinocytes proliferated without forming cell-cell contacts and appeared morphologically similar (not shown).

When primary keratinocyte cultures were prepared from embryos of wild-type and Pg^-/- E17.5 embryos, the yield of Pg^-/- cells was consistently only half the cell number obtained from wild-type embryos (3·10^6 versus 6·10^6 cells/embryo). Seeded at the same density (2·3·10^6 cells/3.5 cm dish) and cultured with low (0.08 mM) Ca^2+ , mutant keratinocytes proliferated at only half the rate of wild-type cells. Thus Pg^-/- epidermis contained either fewer or more slowly proliferating stem cells. At low Ca^2+ , both wild-type and Pg^-/- keratinocytes proliferated without forming cell-cell contacts and appeared morphologically similar (not shown).

At 80% confluence, the Ca^2+ concentration was raised to 1.2 mM and left for 24 hours to induce differentiation and stratification (Hennings et al., 1980). Wild-type keratinocytes changed shape, becoming flat with numerous cell-cell contacts and formed epithelial sheets with neat borders as differentiation proceeded (Fig. 3A). In contrast, Pg^-/-

A similar difference in the membrane localization of desmosomal proteins, desmoplakin and desmocollin 2, was found in the intestine at E17.5. In sections from wild-type embryos, staining was punctate at apical junctions and less pronounced than normal along the basolateral membranes (Fig. 3A) while, in Pg^-/- embryos, staining was diffuse and greatly reduced in the entire villus. However, the characteristic apical staining pattern was still visible in crypt cells (Fig. 3B). We conclude that the absence of plakoglobin strongly affects the distribution and membrane localization of desmosomal proteins. Nonetheless, no obvious morphological abnormalities were observed in the mutant intestine in histological sections of Pg^-/- embryos (not shown).

Immunostaining for the adherens junction proteins (E-cadherin, a-catenin and b-catenin) in mutant and wild-type E17.5 epidermis revealed that, for these proteins, expression and localization were less affected by the absence of plakoglobin. Interestingly, in the wild type, b-catenin, but not E-cadherin or a-catenin clearly exhibited a graded expression pattern, being high in the basal cell layers and greatly reduced or absent in the granular layer and stratum corneum. Thus, normally this adherens junction protein is less expressed in those cell layers that in Pg^-/- skin showed clear acanthokeratolysis (Fig. 2G,H).

To ask whether the epidermal differentiation programme was altered in Pg^-/- embryonic epidermis, E17.5 skin cryosections were stained with antibodies for skin differentiation markers: anti-filaggrin for the stratum corneum (not shown); anti-loricrin for the granular layer (Fig. 2A,B); anti-keratin 1,10 (K1,10) for all suprabasal layers (Fig. 2C,D) and anti-keratin 5, 14 (K5,14) for the basal layer (Fig. 2E,F). All antibodies recognized the same specific epidermal layers in sections of both wild-type and Pg^-/- embryos, so there were no changes in the programme of epidermis differentiation in mutant embryos. Acanthokeratolysis was clearly confined to the granular layer, which was positive for loricrin and keratin 1,10.

**Primary keratinocyte cell cultures from Pg^-/- embryos**

When primary keratinocyte cultures were prepared from epidermis of wild-type and Pg^-/- E17.5 embryos, the yield of Pg^-/- cells was consistently only half the cell number obtained from wild-type embryos (3·10^6 versus 6·10^6 cells/embryo). Seeded at the same density (2·3·10^6 cells/3.5 cm dish) and cultured with low (0.08 mM) Ca^2+ , mutant keratinocytes proliferated at only half the rate of wild-type cells. Thus Pg^-/- epidermis contained either fewer or more slowly proliferating stem cells. At low Ca^2+ , both wild-type and Pg^-/- keratinocytes proliferated without forming cell-cell contacts and appeared morphologically similar (not shown). At 80% confluence, the Ca^2+ concentration was raised to 1.2 mM and left for 24 hours to induce differentiation and stratification (Hennings et al., 1980). Wild-type keratinocytes changed shape, becoming flat with numerous cell-cell contacts and formed epithelial sheets with neat borders as differentiation proceeded (Fig. 3A). In contrast, Pg^-/-
keratinocytes formed smaller epithelial cell sheets, with scattered borders often interrupted by regions of cells with visible gaps and few cell-cell contacts (Fig. 3B). Staining for cytokeratins in wild-type cells decorated an elaborate filament network oriented to cell-cell contacts (Fig. 3C); by double-labelling, co-localization of K1,10 and Dsc in dots along the membrane was observed (Fig. 3C). In \( P_g^{-/-} \) cells, the anchorage of cytokeratins at the cell membrane was irregular and less prominent (Fig. 3D). Here double-labelling revealed the loss of double-positive integration sites, with Dsc staining being uniform along the membrane and K1,10 being less contact-oriented and more perinuclear (Fig. 3D and not shown).

Mutant cell cultures contained a high proportion of round and detached cells with perinuclear K1,10 filament staining. To see if the number of apoptotic cells was increased in \( P_g^{-/-} \) cultures, TUNEL assays were made. In wild-type keratinocyte cultures, very few apoptotic cells (on the order of 1/100-1/500) were identified (Fig. 3E). In mutant cultures, this frequency was approx. 50-fold higher (Fig. 3F).

**Fig. 4.** Ultrastructure of epidermis from E17.5 embryos. (A,C,E,G) Wild-type cells. (A) A cell from the granular layer (gl) containing keratohyalin granules (kh) localized next to the plasma membrane and numerous desmosomes (arrows) with connected keratin or tonofilaments (tf) at contact sites to neighbouring cells. (C) Spinous layer (sl) cells again share numerous desmosomes with attached tonofilaments (arrowheads). (E) Contacts between basal layer cells (bl), one nucleus (nu), and spinous layer cells (sl) with desmosomes and tonofilaments (arrowheads) along the membrane. (G) Higher magnification of a desmosome consisting of desmoglea (dg), membrane, and desmosomal plaque (arrowheads) with inserted tonofilaments (tf). (B,D,F,H) \( P_g^{-/-} \) epidermis cells. (B) Overview showing acanthokeratolysis in the granular cell layer (gl), loss of cell adhesion, and perinuclear keratin filament retraction in the spinous cell layers (sl), and reduced cell contacts in the basal cell layer (bl). (D) Acantholytic cells in the granular cell layer with keratin tonofilaments (tf) collapsed around the keratohyalin granules (kh). Dying cells with desmosomes (arrow) left attached to the neighbouring cell, with retracted filaments (tf). The intercellular space is filled with amorphous material (asterisks). (F) Spinous layer cells have fewer and ultrastructurally altered desmosomes (arrow) with few filaments attached and large intercellular spaces (asterisks). (H) Higher magnification of a mutant desmosome with cytoplasmic plaque missing (arrowheads) and reduced numbers of filaments inserted adjacent to the membrane; note the desmoglea (dg) and retracted tono-filaments (tf). Bar, 10 \( \mu \text{m} \) (B), 1 \( \mu \text{m} \) (A,C-F) and 0.5 \( \mu \text{m} \) (G,H).
Plakoglobin is required for correct desmosomal assembly in skin and intestine

Here, beside extending our ultrastructural analysis of plakoglobin mutant versus wild-type epidermis, we have compared epidermal desmosomes with those of another epithelium, the intestine. In E17.5 PgL−/− embryonic epidermis, acanthokeratolysis was observed in the granular cell layer, resulting in detached cells, signs of collapsing cytoskeleton and necrosis (Fig. 4). In the upper and lower spinous and basal cell layers, gaps between cells were filled with amorphous material and membranes (Fig. 4D,F). The number of desmosomes was about 20-fold lower (relative to wild-type epidermis) in all cell layers of PgL−/− epidermis, and those desmosomes remaining were altered in structure. The inner electron-dense cytoplasmic plaques were missing and only few tonofilaments were seen inserted directly at the outer cytoplasmic plaque adjacent to the membrane (Fig. 4G versus H). Frequently, collapsed filaments were observed around the nucleus, as well as keratohyalin granules in the perinuclear space. The acanthokeratolytic cells of the granular layer often had broken desmosomes, with fragments of the desmosomes left attached to the neighbouring cell. The intercellular space, or desmoglea, which mostly contains glycosylated extracellular domains of desmosomal cadherins, appeared unchanged (Fig. 4H).

In the intestine of PgL−/− E17.5 embryos, a roughly 20-fold reduction in the number of desmosomes versus wild type was also observed and the remaining desmosomes appeared altered here, with missing inner cytoplasmic plaques and fewer attached IFs (Fig. 5C,D). However, the pathological alterations in the epidermis described above (Fig. 1) were not found in the intestinal epithelium. Occluding junctions and adherens junctions were unaffected in PgL−/− intestine (Fig. 5C,D).

Fig. 5. Ultrastructure of intestinal epithelial cells from E17.5 embryos.

(A,B) Cryostat sections from wild-type (A) and PgL−/− (B) intestine stained for desmoplakin revealed a reduced and cytoplasmic staining in mutant villi (vi) with some residual staining on the membrane of crypt cells (cr). (C,D) Electron microscopy of wild-type (C) and PgL−/− (D) E17.5 embryonic intestine. Occluding junctions (asterisk) and adherens junctions (arrow) appeared structurally normal in mutant cells, but the number of desmosomes was reduced and the ultrastructure (arrowheads) changed. (E,F) Immunogold labelling of β-catenin in wild-type (E) and PgL−/− (F) intestinal cells. Note that in PgL−/− but not in wild-type cells β-catenin localizes to both adherens junctions (arrows) and desmosomes (arrowheads). A desmosomal localization of β-catenin is seen in cells of E17.5 facial skin in PgL−/− (K), but not in wild-type embryos (I). Arrowheads indicate desmosomes; arrows show adherens junctions. Immunogold labelling with anti-desmocollin antibodies revealed localization of Dsc in desmosomes (arrowheads) of wild-type (G) and PgL−/− (H) cells, but in mutant cells also some membrane localization of Dsc outside of desmosomes (H). Bars, 25 μm (A, B), and 1 μm (C–K).
Association of $\beta$-catenin with desmosomal cadherins in the absence of plakoglobin

Double-immunofluorescence labelling suggested a co-localization of desmocollin and $\beta$-catenin in primary keratinocyte cultures derived from $Pg^{-/-}$ embryos (not shown). To demonstrate directly and unambiguously this desmosomal localization of $\beta$-catenin and its association with desmosomal cadherins, both immunogold-labelling and immunoprecipitation experiments were performed with epidermal and with intestinal cells from both $Pg^{-/-}$ and wild type.

In wild-type epidermis and intestine, $\beta$-catenin was found exclusively localized in adherens junctions as reported earlier (Fig. 5E,1). Remarkably, in $Pg^{-/-}$ basal and spinous layer cells, $\beta$-catenin was also found in desmosomes, where desmocollin also localizes (Fig. 5E,H,K). Concordant with the immunogold-labelling results, an association of $\beta$-catenin with desmosomal cadherins was demonstrated by immunoprecipitation (IP) of cell lysates from $Pg^{-/-}$ epidermis, using antibodies against Dsg, $\beta$-catenin and plakoglobin. In IPs from wild-type epidermis Dsg was only co-precipitated with anti-plakoglobin antibodies (Fig. 6A, lanes 1-3). With $Pg^{-/-}$ epidermis, as expected, Dsg was no longer co-precipitated with anti-plakoglobin antibodies, but here, unlike with wild-type extracts, Dsg now co-precipitated with anti-$\beta$-catenin antibodies (Fig. 6, lanes 4-6). The interaction between $\beta$-catenin and desmoglein was confirmed by reciprocal IPs using Dsg1 and Dsg3 antibodies on cell lysates from in vitro-differentiated wild-type and $Pg^{-/-}$ keratinocytes. Again, $\beta$-catenin was co-precipitated with Dsg1 and Dsg3 antibodies only with $Pg^{-/-}$ cell lysates (Fig. 6B, lane 4 versus lane 3), confirming that $\beta$-catenin is indeed specifically present in the desmosomal protein complex of $Pg^{-/-}$ epidermis.

**DISCUSSION**

In our original description of the phenotype of plakoglobin-deficient embryos, we reported that the phenotype differs depending on the genetic background (Bierkamp et al., 1996). While, in the 129/Sv strain, the embryos died because of heart defects (Ruiz et al., 1996; Bierkamp et al., 1996), for C57Bl/6 mice, a few per cent of mutant embryos survived longer and showed an additional skin phenotype. Both the heart and skin defects were consistent with the known role of plakoglobin in the structure and function of desmosomes.

Here we have concentrated on the skin defect of $Pg^{-/-}$ embryos, with two major objectives. First, to characterize further the mutant defects during embryonic skin differentiation; second, to investigate why the phenotype first appears so late during embryogenesis, although desmosomes are already assembled early on during mouse preimplantation development. More generally, the formation of epithelia is a very early morphogenetic event in development and desmosomes play a significant role functionally in epithelial cells.

**Lack of plakoglobin during embryonic skin differentiation**

No differences between wild-type and $Pg^{-/-}$ embryos were detected at E12.5, when the epidermis consists of a single-layered periderm or, at E15.5, when the first signs of stratification can be observed. The appearance of morphological defects of $Pg^{-/-}$ embryos at E17.5 corresponded with the initiation of epidermal differentiation and the restriction of Dsc1 and Dsc3 expression domains (Chidgey et al., 1977), which leads to the adult pattern of cell layers with proliferating stem cells restricted to the basal layer and differentiated cells located suprabasally. In the differentiating $Pg^{-/-}$ epithelium, the presence of cell breakage and necrosis correlates with increased mechanical stress. Concordant with this interpretation, no cell breakage or necrosis was observed in $Pg^{-/-}$ keratinocyte cultures, which are probably not subjected to mechanical stress, but here increased apoptosis was seen.

Further, in the E17.5 $Pg^{-/-}$ intestine, we did not find any
morphological aberrations as seen in the skin, although desmosomes were visibly affected. Since the intestine is not fully differentiated until 3 weeks postnatally, any phenotype here might well appear only later (Schmidt et al., 1988).

The skin phenotype in Pg<sup>-/-</sup> embryos, e.g. subcorneal acantholysis, is reminiscent of human disorders of the cytokeratin filament network such as epidermolytic hyperkeratosis, in which an autosomal dominant mutation in the gene coding for K1 and K10 results in cytolysis and keratin hyperkeratosis, in which an autosomal dominant mutation in

The phenotypes in humans and in the skin of Pg<sup>-/-</sup> embryos, e.g. subcorneal acantholysis, is reminiscent of human disorders of the cytokeratin filament network such as epidermolytic hyperkeratosis, in which an autosomal dominant mutation in the gene coding for K1 and K10 results in cytolysis and keratin hyperkeratosis, in which an autosomal dominant mutation in

The lack of plakoglobin clearly reduced the number of desmosomes, and those formed in the absence of plakoglobin were structurally altered. This suggested some partial compensatory mechanisms involving other homologues of plakoglobin, such as plakophilin1, known to bind Dsg and IFs (Heid et al., 1994, Smith and Fuchs, 1998), and/or a direct interaction of the cytoplasmic domain of Dsc with desmoplakin (Troyanovsky et al., 1994b). We do not favour this explanation, although it is formally possible. First, both plakophilin 1 and Dp were diffusely distributed in Pg<sup>-/-</sup> cells indicating that their desmosomal function requires plakoglobin. More importantly, we found β-catenin, the closest homologue of plakoglobin, present as a desmosomal component in Pg<sup>-/-</sup> skin and intestine. Furthermore, an association of β-catenin with Dsg was observed by co-immunoprecipitation, and a desmosomal localization of β-catenin was further substantiated by immunogold labelling and electron microscopic analysis. Consequently, we propose that β-catenin can to some extent replace plakoglobin in desmosomes. Hereby, desmosomes could assemble to a certain degree, perhaps less tightly but sufficiently to participate in the formation of epithelia during early stages of development. However, as shown here, β-catenin cannot completely assemble all desmosomal proteins or ensure all the correct molecular interactions, which becomes particularly apparent when cells are subjected to normal mechanical stress. It will be of future interest to see whether β-catenin can bind as strongly as plakoglobin to desmoplakin and other desmosomal cadherins, or perhaps exhibits a more restricted binding affinity to desmosomal proteins. Alternatively, the amount of β-catenin available may simply be too limited to substitute fully for the lack of plakoglobin since its major role in epithelial cells is in adherens junctions. Indeed, we do not observe any upregulation of β-catenin synthesis in Pg<sup>-/-</sup> cells. Analysis of β-catenin-plakoglobin chimeric proteins in Pg<sup>-/-</sup> cells should permit the mapping of the protein sequences of plakoglobin necessary for correct desmosomal assembly and function.

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Desmosomal localization of β-catenin


