Plakoglobin Suppresses Epithelial Proliferation and Hair Growth In Vivo

Emmanuelle Charpentier,* Robert M. Lavker,‡ Elizabeth Acquista,* and Pamela Cowin*

*Departments of Cell Biology and Dermatology, New York University Medical School, New York 10016; and ‡Department of Dermatology, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104

Abstract. Plakoglobin regulates cell adhesion by providing a modulatable connection between both classical and desmosomal cadherins and their respective cytoskeletal linker proteins. Both plakoglobin and the related protein β-catenin are posttranscriptionally upregulated in response to *Wnt-1* in cultured cells. Upregulation of β-catenin has been implicated in potentiating hyperproliferation and tumor formation. To investigate the role of plakoglobin in these functions we expressed a full-length (PG) and an NH_2-terminally truncated form of plakoglobin (ΔN80PG) in mouse epidermis and hair follicles, tissues which undergo continuous and easily observed postnatal renewal and remodeling. Expression of these constructs results in stunted hair growth, a phenotype that has also been observed in transgenic mice expressing *Wnt3* and *Dvl2* (Millar et al., 1999). Hair follicles from PG and ΔN80PG mice show premature termination of the growth phase (anagen) of the hair cycle, an event that is regulated in part by FGF5 (Hebert et al., 1994). The proliferative rate of the epidermal cells was reduced and apoptotic changes, which are associated with entry into the regressive phase of the hair follicle cycle (catagen), occurred earlier than usual.

Key words: plakoglobin • β-catenin • *Wnt* • cadherin • proliferation

Introduction

Plakoglobin, β-catenin, and *Drosophila* Armadillo are closely related multifunctional proteins that regulate cell adhesion and participate in signal transduction cascades. All three proteins provide modulatory links in a chain of proteins that connect cadherin cell adhesion molecules to the actin filaments of adherens junctions. Plakoglobin differs from β-catenin and Armadillo in its additional role in desmosomes, where it binds strongly to desmosomal cadherins and weakly to desmoplakins and intermediate filaments (for reviews, see Cowin and Burke, 1996; Kowalczyk et al., 1997; Smith and Fuchs, 1998; Cowin, 1999).

Plakoglobin, β-catenin, and Armadillo are also found in cytoplasmic and nuclear complexes that integrate signals from *Wnt/wingless* (*wg*)[1] proto-oncogenes and the *adenomatous polyposis coli* (APC) tumor suppressor protein to direct cell fate and govern aspects of cell proliferation (Miller and Moon, 1996; Ben-Ze'ev and Geiger, 1998; Gumbiner, 1998; Cowin, 1999). By analogy to the *wg* pathway in flies, one model for vertebrate *Wnt* signaling posits that secreted Wnts bind to specific members of the Frizzled transmembrane receptor family, resulting in recruitment of cytosolic disheveled (Dvl) proteins to the plasma membrane and inactivation of glycogen synthase kinase (GSK-3β; Bhanot et al., 1996; Wang et al., 1996; He et al., 1997). GSK-3β normally acts as part of a protein complex that promotes a series of posttranslational modifications that target cytoplasmic β-catenin for proteosomal degradation (Rubinfeld et al., 1996; Yost et al., 1996; Aberle et al., 1997; Orford et al., 1997; Zeng et al., 1997). Thus, Wnt inactivation of GSK-3β causes β-catenin to accumulate, translocate to the nucleus, bind to Tcf/Lef transcription factors and regulate target genes such as *c-myc* and *cyclin D* (Funayama et al., 1995; Karnovsky and Klymkowsky, 1995; Love et al., 1995; Behrens et al., 1996; Molenaar et al., 1996; Van de Wetering et al., 1997; Bauer et al., 1998; Cavallo et al., 1998; He et al., 1998; Roose et al., 1998; Waltzer and Bienz, 1998; Tetsu and McCormick, 1999).

Experiments in rodent mammary and neuropheochromocytoma cells have shown that *Wnt-1* expression upregulates both plakoglobin and β-catenin (Bradley et al., 1993; Hinck et al., 1994). In addition, injection of either β-catenin or plakoglobin mRNAs into *Xenopus* embryos results

Address correspondence to Dr. Pam Cowin, Department of Cell Biology, New York University Medical School, 550 First Ave., New York, NY 10016. Tel.: (212) 263-8715. Fax: (212) 263-8139/8752. E-mail: cowinp01@med.nyu.edu

[1]*Abbreviations used in this paper:* βG, β-globin sequence; APC, *adenomatous polyposis coli*; Dvl, disheveled; K14, keratin 14; KGM, keratinocyte growth medium; TdR, [^{3}H]thymidine; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling; TX-100, Triton X-100; *wg*, *Wnt/wingless.*

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in the identical *Wnt-1* phenotype, axis duplication (McMahon and Moon, 1989; McCrea et al., 1993; Karnovsky and Klymkowsky, 1995). These results implicate both plakoglobin and β-catenin as effectors of *Wnt* signals. Whether plakoglobin directly transduces *Wnt* signals or acts to modulate β-catenin's functionality is currently an area of debate (Merriam et al., 1997; Miller and Moon, 1997; Williams et al., 1998). The late embryonic-lethal phenotype of plakoglobin null mice and the inability of endogenous plakoglobin to rescue the early embryonic-lethal phenotype of β-catenin null mice suggest that plakoglobin does not play a significant role in *Wnt* pathways governing early development (Haegel et al., 1995; Bierkamp et al., 1996). However, the participation of plakoglobin as a mediator of Wnts in tissues that undergo significant postnatal development and renewal has not been investigated. Formation of the epidermal appendages of hair, feathers, and mammary gland are therefore excellent models in which to study this.

In adults, inappropriate activation of elements in this type of *Wnt* signaling cascade has been linked to a number of cancers and several studies implicate β-catenin in this process. Mutations in the β-catenin gene that result in a stabilized protein product occur in colonic, gastric, hepatocellular, and hair follicle tumors and melanomas (Munemitsu et al., 1995; Rubinfeld et al., 1997; Chan et al., 1999). Overexpression of β-catenin in vivo increases the proliferative rate of crypt cells and induces polyp formation in intestine and induces hair follicle formation and benign tumors in skin (Gat et al., 1998; Wong et al., 1998; Harada et al., 1999). The role of plakoglobin in proliferation and cancer is less well documented. However, several facts suggest that plakoglobin may act as a tumor suppressor. For example, the plakoglobin gene lies in the 17q-21 locus, which is subject to loss of heterozygosity in human breast tumors (Aberle et al., 1995). Plakoglobin is absent in a number of tumor cell lines, and experimentally induced overexpression of plakoglobin in highly transformed cells decreases their tumorigenicity (Navarro et al., 1991; Sommers et al., 1994; Aberle et al., 1995; Simcha et al., 1996).

To address the questions of whether plakoglobin acts to transmit *Wnt* signals in adult tissues, and whether it promotes or suppresses proliferation and tumor formation in normal cells, we expressed two forms of plakoglobin in the basal cells of mouse epidermis and hair follicles under the control of the keratin 14 (K14) promoter. Our results show that even modest expression of these transgenes reduces the proliferative potential of the epidermal cells and significantly decreases the length of the growth phase of the hair follicle cycle, shortening hairs by 30%. This effect suggests that plakoglobin participates in the transduction of growth-suppressive signals to normal epithelial cells.

Materials and Methods

Plasmids and Transgene

Flag-tagged human plakoglobin cDNA (p163/7-PG) was produced by PCR using hPG Ca 2.1 as template (GenBank accession number Z68228; Franke et al., 1989). 20 cycles of standard PCR conditions of 1 min at 94°C, 1 min at 54°C, and 3 min at 72°C were carried out with forward oligonucleotide (5′-ccggaattcgccgcc*ATGGACTACAAGGACGACATGACAAG*ATGGAGGTGATGAACCTG-3′), which contains an EcoRI restriction site, Kozac's sequence, a sequence encoding the Flag epitope (*MDYKDDDDK*) and the first five amino acids of hPG, and reverse oligonucleotide (5′-ccggaattcCTAGGCCAGCATGTGGTC-3′) that contains an EcoRI restriction site and the last 18 bases of the hPG Ca 2.1 coding sequence including the stop codon. The PCR product was subcloned into the EcoRI restriction site of the p163/7 expression vector (Niehrs et al., 1992) containing the MHC class I H-2 promoter, a polylinker embedded in rabbit β-globin sequence (βG; last 20 bp of exon 2, entire intron 2 and the polylinker dividing exon 3) and both βG and SV-40 polyadenylation signals, generating p163/7-PG. The transgene construct, pK14-βG-Flag-PG (see Fig. 1) was generated by introducing a BamHI linker into the NotI site of the p163/7 polylinker downstream of Flag-hPG. This permitted excision and insertion of a 2,935-bp BamHI fragment containing 5′ βG sequence and Flag-hPG into the BamHI site of a keratin 14 cassette, which was kindly provided to us by Dr. Elaine Fuchs (University of Chicago; see Fig. 1).

A cDNA encoding a myc-tagged stabilized form of plakoglobin deleted in the first 80 amino acids was generated by PCR. A 531-bp DNA fragment was amplified using hPG Ca 2.1 as template and forward oligonucleotide (5′-ctcgcggccgcggatccgaattcgccgcc*ATGGAGCAAAAGCTTATTCTGAAGAGGACTTG*AGGGCCAAACGGGTGCGGAG-3′), which contains NotI, BamHI, and EcoRI restriction sites, Kozac's sequence, sequence encoding the myc epitope (*MEQKLISEEDL)* and amino acids 81–87 of hPG, and the reverse oligonucleotide 817 Rev (5′-GCTGAGCATGCGGACCAGAGC-3′) encompassing the internal SphI at base 698 of hPG Ca 2.1. The 863-bp NotI-SphI fragment of hPG Ca 2.1 was replaced by the 519-bp NotI-SphI PCR fragment to generate pBS-ΔN80PG from which the 3,192-bp EcoRI fragment was excised and subcloned into p163/7 generating p163/7-ΔN80PG. A 2,798-bp BamHI fragment containing βG-ΔN80PG was excised from the latter construct and subcloned into the keratin 14 transgene vector generating pK14-ΔN80PG. The PCR generated regions and newly generated cloning sites of all clones were fully sequenced by the dideoxy termination method (Sanger et al., 1977).

Plasmid DNA was prepared by alkaline lysis followed by double banding on CsCl gradients. The K14-PG and K14-ΔN80PG transgenes (see Fig. 1 a) were excised from the pGEM 3Z backbone with KpnI, which cuts in the 5′ polylinker and NdeI, which cuts at base 2.083 within the K14 3′ noncoding sequence, leaving a 327-bp segment after the polyadenylation signal provided by the hGH sequences.

Southern Blot Analysis of Genomic DNAs

Genomic DNA was prepared by digesting 0.5 cm of mouse tail in 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 20 mM NaCl, 1% SDS, and 1 mg/ml of proteinase K for 12 h at 55°C followed by two ethanol precipitations. For Southern blot analysis, 10–20 μg of genomic DNA samples were digested with 80 units of BamHI and separated by electrophoresis in an 1× TAE-0.8% agarose gel at 3 V/cm. After UV cleavage, denaturation, and neutralization, samples were transferred to Nytran membranes (Schleicher and Schuell). Blots were prehybridized for 2 h at 68°C in a solution of 6× SSC, 1% SDS, 1× Denhardt's, and 125 μg/ml of denatured tRNA and hybridized for 12 h in the same buffer containing 50 ng of random primed α-^{32}P-plakoglobin cDNA probe, specific activity 10^9 cpm/ug (ICN). Blots were washed twice in 2× SSC, 0.1% SDS, for 20 min each at 68°C and exposed for 12 h to PhosphorImager screens (Molecular Dynamics).

Transfection, Immunoprecipitation, and Western Blot Analysis

Human kidney cells transformed with SV-40 T-antigen (293T) were maintained, transfected, and immunoprecipitated as described (Witcher et al., 1996). Forty 30-μm cryosections of tail from 25-d F1 transgenic and normal sex-matched littermates were boiled in 200 μl of 25 mM Tris-HCl, pH 7.4, 3 mM EDTA, 150 mM NaCl, 1% SDS, 10 μg/ml aprotinin, and 1 mM PMSF and diluted in 1 ml of 2% Triton X-100 (TX-100) in 50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 150 mM NaCl, and 10 μg/ml aprotinin, 1 mM PMSF. Insoluble debris was pelleted for 15 min at 10,000 *g* and processed for immunoprecipitation as described (Witcher et al., 1996). Protein concentration was determined by the method of Bradford using a BCA (bicinchronic acid) protein assay kit (Pierce). Protein samples and immunoprecipitations were separated by electrophoresis through 7.5% SDS-PAGE, electrophoretically transferred to Protran membranes (Schleicher and Shuell) and blocked by incubation in PBS containing 0.1% Tween 20 and 5% nonfat dried milk. The blots were incubated with the following mouse monoclonal antibodies: M2 anti-flag (Sigma) 9E10 anti-myc (a kind gift of Dr. Harold Varmus, NIH, Bethesda, MD), PG5172 anti-plakoglobin (Cowin et al., 1986); anti–β-catenin, anti–GSK-3β, anti–E-cadherin

(Transduction Laboratories), anti-ribophorin antibody (a kind gift of Dr. Gert Kreibich, NYU Medical School, NY). The primary antibody incubations were performed overnight at a dilution of 1:4,000 in PBS containing 0.1% Tween 20 and 5% nonfat dried milk followed by 1 h incubation with peroxidase-coupled goat anti–mouse IgG diluted 1:6,000 in the same buffer (Kirkegaard and Perry Laboratories Inc.). Blots were washed three times for 15 min in PBS-Tween after each antibody and the immune complexes were detected by ECL chemiluminescence (Amersham International).

Reporter Assays

293T cells were transfected as described (Witcher et al., 1996) with combinations of 2 μg PG, ΔN80Pg, and Lef1 cloned into the pCDNA3 vector, together with 1 μg of pGL3-Topflash Tcf/Lef-responsive luciferase reporter gene or a control pGL3-Fopflash in which the Tcf-responsive elements have been mutated (Van de Wetering et al., 1997). 1 μg of CMV-β-gal was included as a control for transfection efficiency. All samples were made up to a total of 6 μg DNA by addition of empty vector DNA. HaCaT keratinocytes (Boukamp et al., 1988) were transfected with one quarter of the above using Lipofectamine plus according to the manufacturer's instructions (GIBCO BRL). Cells were extracted using a kit that permitted luciferase and β-galactosidase measurements to be made on the same extracts according to the manufacturer's instructions (Promega).

Indirect Immunofluorescence

Tail and back skin biopsies, respectively, from F1 transgenic and normal sex-matched littermates were snap frozen in liquid nitrogen and stored at −70°C. 5-μm frozen sections were attached to multiwell slides and allowed to air dry overnight at room temperature. Primary cultured keratinocytes were fixed for 5 min in acetone/methanol (1:1) at –20°C then allowed to air dry. The sections and cells were incubated with primary antibodies diluted 1:50 in PBS, washed three times for 10 min each in PBS then incubated with Alexa-conjugated goat anti–mouse or anti–rabbit IgG diluted 1:200 (Molecular Probes). After three washes in PBS, the slides were mounted in Gelvatol and the sections examined by epifluorescence with a Zeiss axiophot photomicroscope III. The primary antibodies used were DP2.15 anti-desmoplakin (Cowin et al., 1985); PG5172 anti-plakoglobin (Cowin et al., 1986); anti–β-catenin and anti–γ-catenin (H80) NH_2-terminal plakoglobin antibody (Santa Cruz Biotechnology), anti–E-cadherin (Transduction Laboratories), and 9E10 anti-myc. Individual rabbit anti–mouse K14, K5, K1, K6, and loricrin were used at dilutions of 1:100 (kindly provided by Dr. Stuart Yuspa, NCI, Bethesda, MD). Mouse monoclonal antibody AE14 anti-high sulfur protein was the gift of Dr. Michael O'Guin (NYU School of Medicine, NY).

Tissue Histology and Measurement of Hair Follicles and Hairs

Biopsies of mouse skin taken from the middle of the back were fixed overnight or longer in 10% phosphate-buffered formalin, progressively dehydrated through gradients of alcohol, and embedded in paraffin. 5-μm sections were cut, deparaffinized in xylene, rehydrated, and then stained with hematoxylin and eosin. The lengths of five hair follicles were measured on sections from two to three pairs of F1 transgenic and normal sex-matched mice per line taken at various time intervals over the 41-d period covering the first two hair cycles (Wilson et al., 1994). Histological sections were viewed and photographed on an Olympus Vanox X microscope using Kodachrome II, ASA 25 film. Pelage hairs were plucked from the heads of three pairs of 25-d F1 transgenic and control sex-matched littermates, and individually attached to microscope slides with double-sided tape. 100 hairs were examined to determine the percentage of each hair type. 5–10 hairs of each type from 3–6 pairs of normal and transgenic littermates per line were scanned. The length of each hair was measured with the aid of Adobe Photoshop and NIH programs. The average length and standard deviation were calculated and the Student's *t* test was performed.

Keratinocyte Cell Culture

Primary epidermal keratinocytes were established in vitro as follows using modifications of the method of Hennings (Hennings et al., 1980; Dlugosz et al., 1995). Newborn mice were killed, then washed in a 1:10 solution of Betadine, rinsed twice in sterile dH_2O and twice in 70% alcohol. The skin was removed and floated overnight on 2 ml of dispase (Dispase II, 25 U/ml; Boehringer Mannheim). The epidermis was detached from the dermis, minced and placed in 2 ml of 0.05% trypsin and 0.01% EDTA for 15 min at 37°C. DNase I (100 units) was added and the cells were mechanically dissociated by vigorous pipetting followed by filtration through two layers of sterile gauze. The cells were washed in an equal volume of DME containing 10% FCS to inhibit the trypsin and plated in low Ca^{2+} keratinocyte growth medium (KGM; Clonetics-Biowhittaker) adjusted to 0.05 mM Ca^{2+} by addition of Ca^{2+}-free KGM.

Sequential Detergent Extraction of Epidermal Proteins

Primary keratinocytes were grown to confluence on 60-mm dishes under low Ca^{2+} conditions (0.05 mM) to maintain a basal cell phenotype then switched to high (2 mM) Ca^{2+} for 24 h to permit cell junction formation (Hennings and Holbrook, 1983). Cellular proteins were extracted sequentially in saponin buffer (0.01% saponin, 10 mM Tris, pH 7.5, 140 mM NaCl, 5 mM EDTA, 2 mM EGTA, 1 mM PMSF, and 10 μg/ml aprotinin) and then in TX-100 buffer (1% TX-100, 10 mM Tris-HCl, pH 7.5, 140 mM NaCl, 5 mM EDTA, 2 mM EGTA, 1 mM PMSF, and 10 μg/ml aprotinin) as described (Palka and Green, 1997), with the exception that extracts were centrifuged for 1 h at 100,000 *g*.

Pulse–Chase

100-mm dishes of transgenic keratinocytes grown as described above were starved for 1 h in methionine/cysteine-free DME then metabolically labeled with methionine/cysteine-free DME containing 0.5 mCi ^{35}S-methionine/cysteine/ml for 20 min. The cultures were rinsed with DMEM containing 1,000-fold excess of cold methionine and incubated in this medium for the time periods indicated. The cells were harvested, boiled in 1% SDS, and immunoprecipitated as described above.

Determination of Single and Double Pulse-labeled Keratinocytes

The method was carried out as described (Lehrer et al., 1998). 4-d-old mice were injected subcutaneously with 50 μg of BrdU per gram body weight. After a 24-h chase period, long enough for the BrdU-labeled cells to traverse into a second S phase, the same animals were injected with 10 μCi/g of [^{3}H]thymidine (TdR) and killed 60 min later. Backskin was removed, fixed in 70% ethanol, 50 mM glycine for 24 h, embedded in paraffin and sectioned at 5 μm. After deparaffination and rehydration, tissue sections were processed for immunochemical staining with anti-BrdU antibody and alkaline phosphatase-conjugated goat anti–mouse IgG (5-bromo-2′-deoxy-uridine labeling and detection kit II, Boehringer Mannheim Corp.), followed by visualization with the Vector® Red alkaline phosphatase substrate kit I (Vector Laboratories Inc.) according to the manufacturer's instructions. Slides were air dried overnight, coated in nuclear track emulsion (Ilford K-2; Ilford) diluted 1:3 in distilled water at 40°C, air dried again for 2 h, and exposed for 14 d at 4°C in light tight boxes containing anhydrous $CaSO_4$ (Hamond Drierite). Autoradiographs were developed as described previously (Lehrer et al., 1998). Slides were lightly counterstained with hematoxylin. All slides were examined under oil immersion (×63) with a Zeiss Axiophot light microscope. A cell was considered double labeled if it contained greater than five silver grains per nucleus over a bright nuclear pattern of red staining. The epidermal labeling index was determined by counting the number of ^{3}H-TdR–stained nuclei per 1,000 basal nuclei. The percentage of BrdU-labeled cells that were additionally labeled with ^{3}H-TdR was also determined (Lehrer et al., 1998).

Terminal Deoxynucleotidyl Transferase-mediated dUTP Nick End Labeling Analysis

The terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) analysis was performed on 5-μm formalin fixed paraffin-embedded mouse backskin sections using the Promega Fluorescein Apoptosis Detection System.

Results

Generation of Transgenic Mice

Previous studies on the signaling function of β-catenin employed full-length and NH_2-terminally deleted forms of

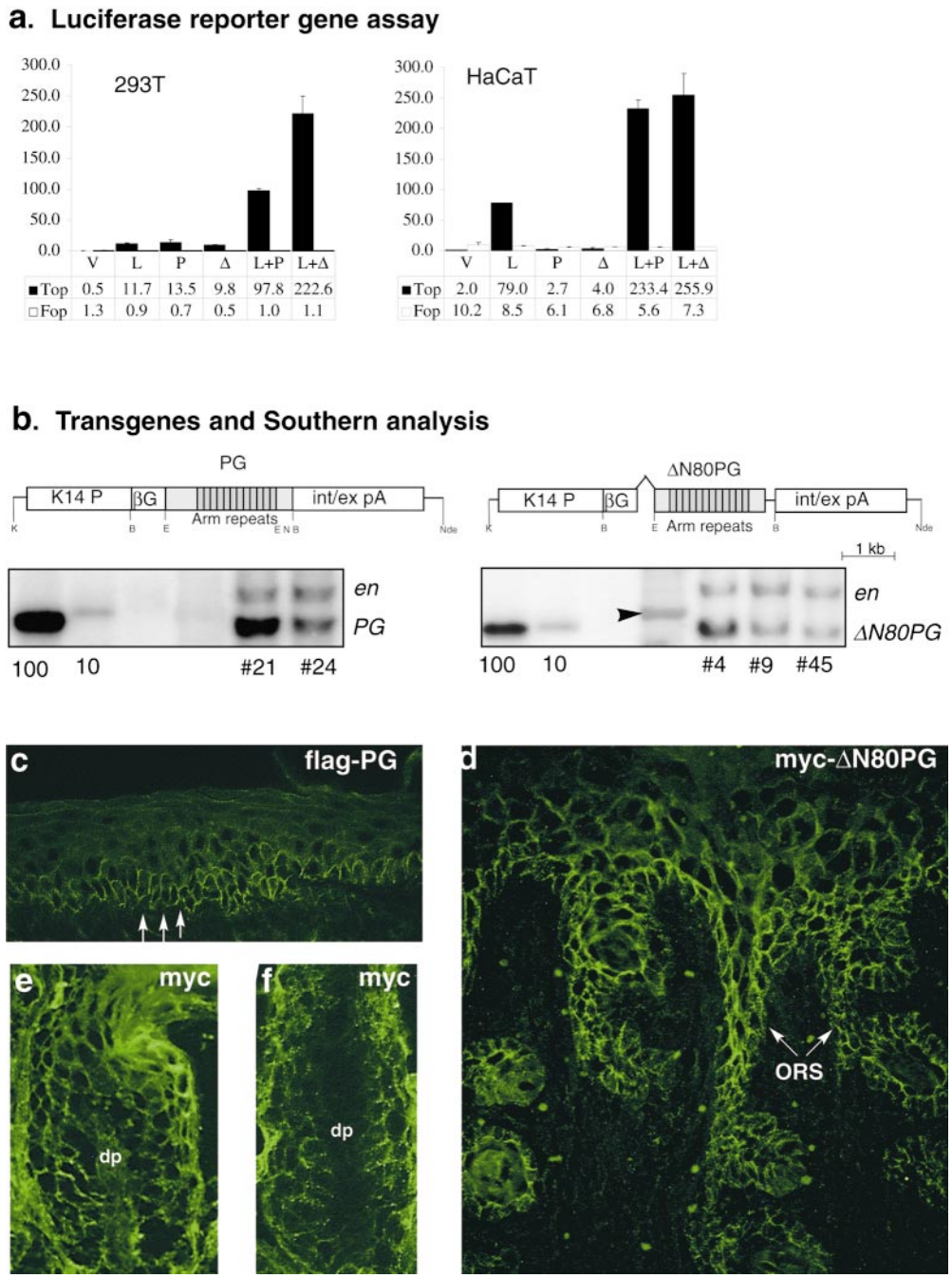

Figure 1. PG and ΔN80PG transgenes. (a) Luciferase reporter assays. 293T cells and HaCat keratinocyte cultures were transfected with 1 μg pTOPFLASH (Top) or 1 μg pFOPFLASH (Fop) 1 μg CMV-Lac Z with or without 2 μg PG (P), ΔN80PG (Δ), Lef-1 (L) cloned in pCDNA3 or pCDNA3 (V). Luciferase activity expressed in light units represents an average of three samples, with standard deviations shown in bars after correction for transfection efficiency as measured by β-galactosidase activity. (b) Diagram of the K14-PG and K14-ΔN80PG transgene constructs comprising the K14 promoter (K14P), hGH intron/exon/polyadenylation sequences (int/ex pA) and K14 3′ noncoding sequence. Sequence encoding the NH_2- and COOH-terminal domains and 13 central armadillo (arm) repeats of the PG and ΔN80PG cDNA are represented by shaded rectangles. B, BamHI; E, EcoRI, K, KpnI; N, Not1; Nde, NdeI. Southern blot detection of K14-PG and K14-ΔN80PG transgenes in the genome of transgenic lines hybridized with the ^{32}P-labeled PG insert and mouse *engrailed* gene (*en*) fragments. Lanes marked 100 and 10 show respective copy equivalents of PG and ΔN80PG transgene plasmid. The arrow points to a 3-kb DNA marker. Lanes marked nos. 21, 24, 4, 9, and 45 show DNA from these respective transgenic mouse lines. Expression of the transgene products detected by indirect immunofluorescence. Frozen sections of 5-d (c) or newborn tail skin (d) and 5-d backskin (e–f) stained with anti-flag (c) or anti-myc (d–f) antibody detect the PG (c) and ΔN80PG (d–f) transgene products. Strongest staining is seen at the intercellular borders of the basal layer of transgenic epidermis and continuous layers of the outer root sheath (ORS) of developing hair follicles (c–d) where the K14 promoter is active. Note the horseshoe staining of the basal cells due to absence of reaction on hemidesmosomes (arrows). Staining, representing the persistence of the transgene product in progeny of former K14-expressing cells, is also observed in suprabasal layers of the epidermis (c–d) and in cells within the hair follicle bulbs lying next to the dermal papilla (DP) (e–f) where the K14 promoter is not active.

β-catenin, which show enhanced stability (Munemitsu et al., 1995). We therefore constructed analogous cDNAs encoding an epitope-tagged full-length (PG) and NH_2-terminally deleted (ΔN80PG) form of plakoglobin and tested if the products of these constructs retained transcriptional activity by performing a luciferase reporter assay (Van de Wetering et al., 1997). The products of both plakoglobin constructs transactivated ~100–200-fold a Topflash luciferase reporter in the presence of a putative transcriptional partner Lef-1 (Fig. 1 a). This effect was observed in both 293T cells that make few intercellular junctions and HaCaT keratinocytes that contain many desmosomes (Boukamp et al., 1988). Low levels of transactivation ~10-fold of the Topflash reporter occurred in the absence of Lef-1 and presumably resulted from activation of endogenous Lef/Tcf transcriptional partners. Neither form of plakoglobin transactivated

the control Fopflash luciferase reporter construct which is mutated in the Tcf/lef-responsive elements (Fig. 1 a).

To test the potential role of plakoglobin in regulating cell proliferation, in vivo, we employed a K14 transgene cassette, kindly provided to us by Dr. Elaine Fuchs, which directs expression of transgenes under the control of the K14 promoter to the basal layer of the interfollicular epidermis and the outer root sheath of hair follicles (Guo et al., 1993; Vassar et al., 1989). Both full-length K14-PG and NH_2-terminally deleted K14-ΔN80PG (Fig. 1 b) were used to generate transgenic mice in the Swiss Webster strain.

Two mice (nos. 21 and 24) tested positive by Southern analysis of tail genomic DNA for integration of the K14-PG transgene and gave Mendelian transmission to the F1 generation. Seven mice tested positive for integration of the K14-ΔN80PG transgene. Of these, nos. 1, 4, 45, 46 showed Mendelian transmission of the transgene to the F1 generation, nos. 5 and 9 showed <10% transmission, suggesting mosaicism, and no. 31 gave 75% transmission to the F1 progeny, suggesting that the transgene inserted at multiple sites in the genome. Immunofluorescence detection of the epitope tags on frozen sections of tail epidermis indicated that expression of the transgene protein products occurred in one K14-PG founder (no. 21) and three K14-ΔN80PG founders (nos. 4, 9, and 45). Significantly only these four mice displayed hair phenotypes. Their F1 progeny were studied further in the experiments described below.

Expression of the ΔN80PG Transgene

Southern analysis of tail genomic DNA from F1 mice with probes derived from plakoglobin cDNA and a fragment of the *engrailed* gene showed that lines nos. 21, 4, 9, and 45

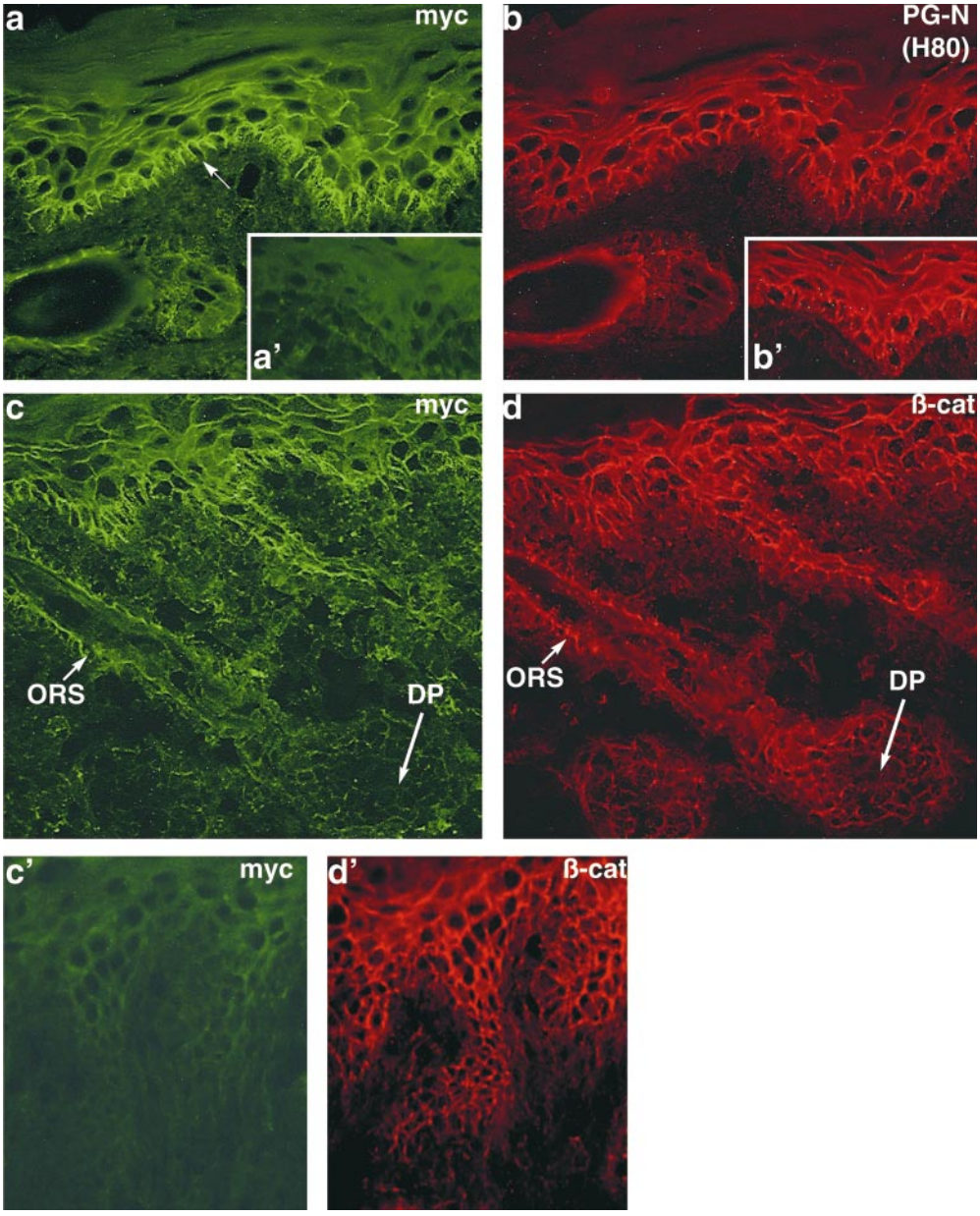

Figure 2. Expression of the transgene products detected by indirect immunofluorescence. Frozen sections of tail skin from newborn sex-matched ΔN80PG transgenic (a–d) and control (a′ and d′) F1 littermates. Anti-myc antibody detects the transgene product in transgenic skin (a and c) but gives no reaction on normal skin (a′ and c′). Strong staining is seen at the intercellular borders of the basal layer of transgenic epidermis and contiguous outer root sheath (ORS) of the hair follicle (a and c) where the K14 promoter is active. Note the horseshoe staining of the basal cells due to absence of reaction on hemidesmosomes (arrows). Staining representing the persistence of the transgene product in progeny of former K14-expressing cells is observed in suprabasal layers of the epidermis (a and c) where the K14 promoter is not active. Note that the cellular localization of endogenous plakoglobin (b and b′), detected with an antibody H-80 directed against NH_2-terminal epitopes not present in the transgene, or of endogenous β-catenin (d and d′) is the same in transgenic (b and d) skin as in normal skin (b′ and d′). DP, dermal papilla.

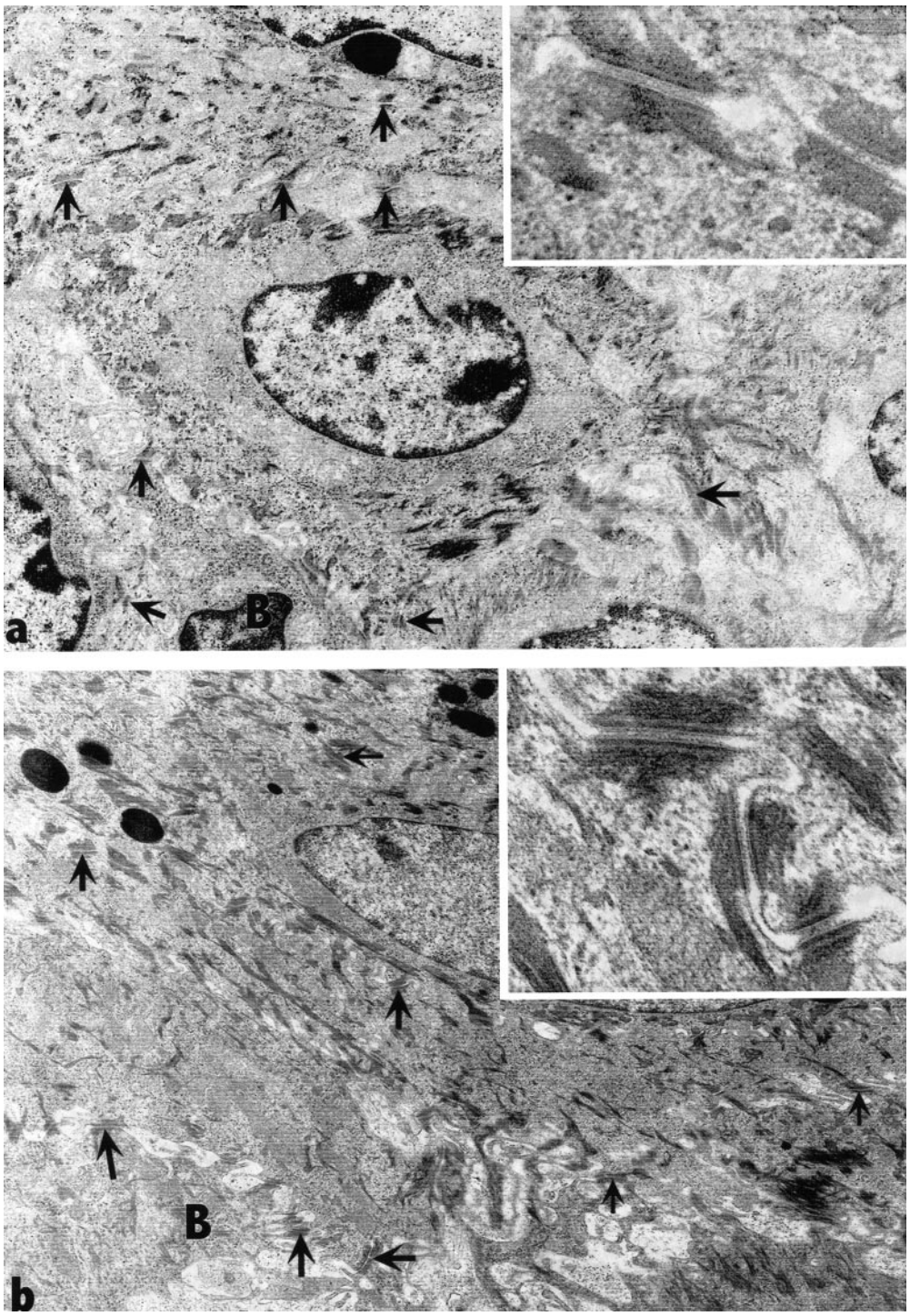

Figure 3 (continues on facing page).

carried 50, 40, 10, and 15 copies, respectively, of the transgene, based on normalization and calibration to a series of titrated plasmid controls (Fig. 1 b). Immunofluorescence microscopy with anti-flag and anti-myc antibody on frozen sections of F1 tails detected the epitope tags in transgenic epidermis (Fig. 1, c and d) but not normal littermate epidermis showing the strongest expression within the basal layer. Staining was most obvious at the intercellular borders of keratinocytes reflecting the distribution of desmosomes and adherens junctions but was absent from the basal borders where the cells contact the basal lamina via hemidesmosomes (see arrows in Fig. 1, c and d). In transgenic mice from all four lines, expression was also seen strongly in outer root sheath cells of the hair follicles (Figs. 1 d and 2 c). Staining was also observed in layers of the epidermis and hair follicle, which do not express the K14 promoter. For example, staining was seen in suprabasal epidermal cells (Figs. 1 d and 2, a and c), and in epithelial cells of the hair follicle bulb including those surrounding the dermal papilla (Fig. 1, e and f). This likely reflects per-

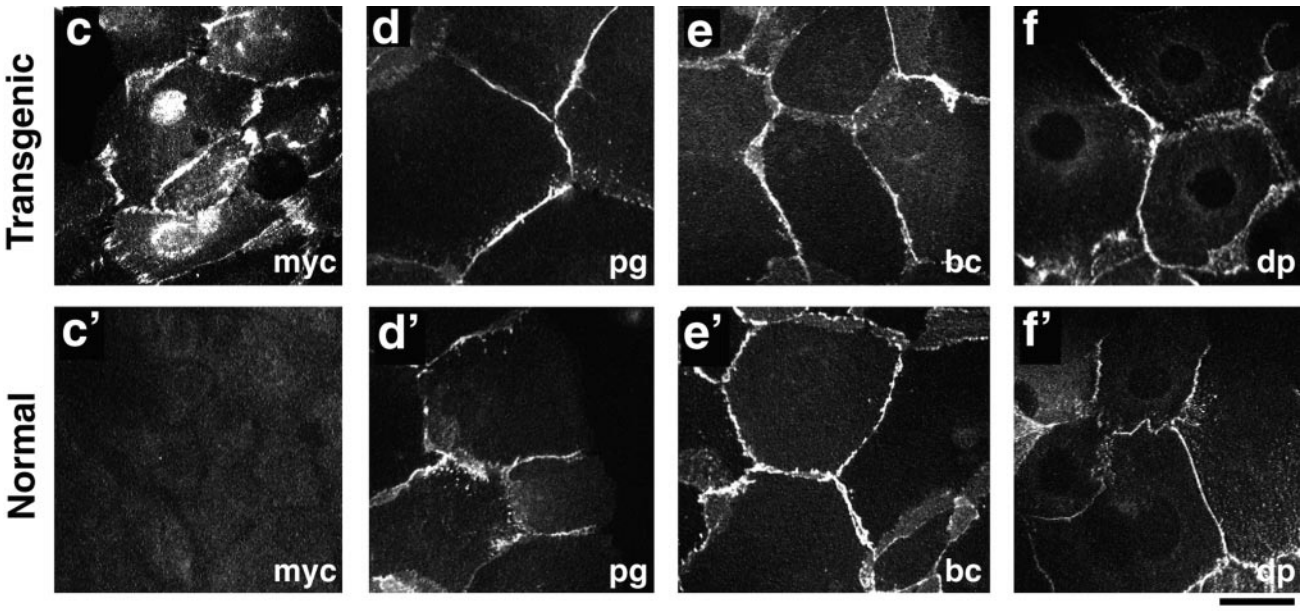

Figure 3. Electron micrographs show that desmosomes (arrows) occur with normal frequency and structural appearance in the basal layers B, and superficial layers of normal (a) and transgenic (b) epidermis. Primary keratinocytes from newborn transgenic ΔN80PG (c–f) and control (c′–f′) littermates were processed for indirect immunofluorescence microscopy. Anti-myc (myc) (c and c′), anti-plakoglobin (pg) (d and d′), anti–β-catenin (bc) (e and e′), and anti-desmoplakin (dp) (f and f′) were used as primary antibodies. Note that the presence of the transgene product (c–f) causes no alterations in the localization of other junctional components (compare c–f with c′–f′). Bars: (a and b) 0.5 μm; (c–f and c′–f′) 25 μm; (insets) 0.1 μm.

sistence of the transgene protein product in progeny of previously K14 positive stem cells. Weak staining was seen in the cytoplasm of all positive cells, nuclear localization was not generally observed in the transgenic tissue sections.

It has been argued in other systems that plakoglobin signaling may be an indirect result of competitive displacement of β-catenin from the membrane. To address this possibility we compared the localization of endogenous β-catenin plakoglobin in transgenic (Fig. 2, a–d) and normal skin (Fig. 2, a′–d′). Staining for endogenous plakoglobin with an NH_2-terminal antibody (Fig. 2, b and b′) and for endogenous β-catenin with a COOH-terminal antibody (Fig. 2, d and d′) showed essentially similar patterns in transgenic (Fig. 2, b and d) and normal (Fig. 2, b′ and d′) epidermis and hair follicles.

Examination of mouse skin under the electron microscope showed that transgenic skin contained desmosomes with normal appearance and frequency demonstrating that the transgene expression had no deleterious effect on formation or stability of these cell junctions (Fig. 3, a and b). Immunofluorescent staining of primary keratinocyte cultures from the epidermis of transgenic mice showed strong localization of the epitope tag at cell–cell borders and faint cytoplasmic staining (Fig. 3 c). Strong reaction was also observed in the nucleus of some but not all transgenic cultured keratinocytes (Fig. 3 c). These staining patterns were specific for transgenic keratinocytes and were not observed in cells derived from normal littermates (Fig. 3 c′). As predicted from previous studies, which showed that the first 80 amino acids of plakoglobin are dispensable for its interactions with all known partners (desmoglein, desmocollin, E-cadherin, α-catenin, APC, and Lef/Tcf), no perturbations were seen in the patterns of localization of several major junctional proteins such as desmoplakins, β-catenin, endogenous plakoglobin or E-cadherin (Fig. 3, d–f and d′–f′) (for review, see Cowin and Burke, 1996).

Confirmation that the epitopes recognized by the antibodies in the immunofluorescence assays above represented the PG and Δ80PG products encoded by the transgene was obtained by cross-blotting anti-plakoglobin immunoprecipitates with anti-flag or anti-myc antibody (Fig. 4 a). In each case, a protein of the appropriate mass, ~86 kD for PG and 75 kD for Δ80PG, could be detected in transgenic animals (lanes 2, 4, 6, and 8) but not in their normal littermates (lanes 1, 3, 5, and 7). Primary keratinocytes from normal and transgenic F1 mice were cultured under low Ca^{2+} conditions, which enrich for basal cells and hence for cells expressing the K14 promoter (Hennings et al., 1980; Dotto, 1998). After 2–3 d in culture the cells were switched for 12 h to high Ca^{2+} to permit cell-junction formation (Hennings and Holbrook, 1983; Dotto, 1998). Under these conditions, 100% of the cells express the transgene product as judged by immunofluorescence microscopy. Equal amounts of protein from normal and transgenic keratinocytes, as judged by protein estimation, were processed for immunoblotting. Protein loading was controlled by monitoring the expression level of ribophorin, a resident protein of the endoplasmic reticulum (Fig. 4 b). The level of transgene expression was found to be ~50% that of the endogenous protein in line no. 4. No significant downregulation of the endogenous pool of plakoglobin or β-catenin was observed in the transgenic keratinocytes when compared with normal keratinocytes.

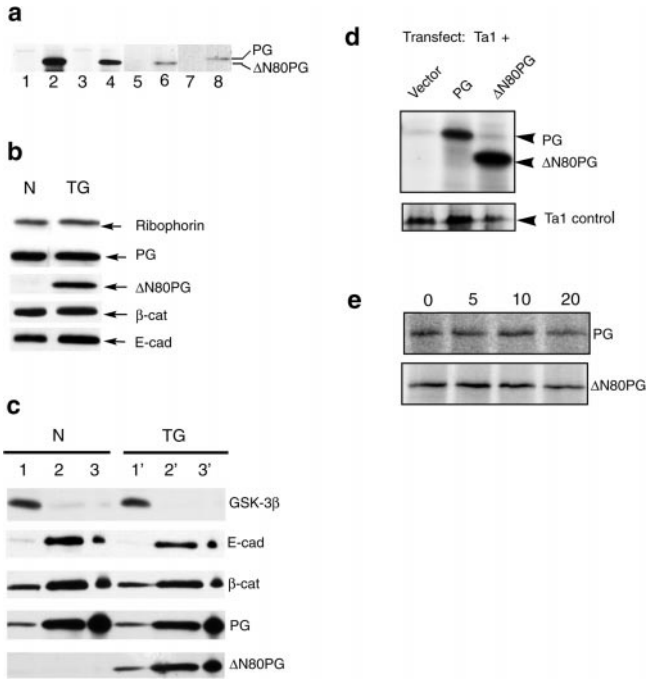

Figure 4. Western blot analysis of PG and ΔN80PG protein expression in skin and keratinocytes. (a) Proteins from tail skin of 25-d sex-matched F1 normal mice (N) nos. 4, 9, 45, 21 (lanes 1, 3, 5, and 7, respectively), and their transgenic littermates (lanes 2, 4, 6, and 8, respectively) were immunoprecipitated then immunoblotted with anti-myc (lanes 1–6) and anti-flag (lanes 7 and 8) antibodies. The arrowheads denote the migration of the 86-kD PG and 75-kD ΔN80PG and transgene products. (b) Equal amounts of total proteins, extracted from primary keratinocytes were blotted with anti-ribophorin antibody as a loading control; anti-plakoglobin, anti–β-catenin, and anti–E-cadherin antibodies were used to detect the endogenous proteins and anti-myc antibody to detect the ΔN80PG as indicated. Expression of endogenous plakoglobin and β-catenin are not affected by expression of the transgene but E-cadherin is slightly elevated. (c) Primary keratinocytes were sequentially detergent extracted. Proteins soluble in saponin (lanes 1 and 1′) or TX-100 (lanes 2 and 2′) and those in the TX-100 insoluble pellet (lanes 3 and 3′) were blotted with anti–GSK-3β and anti–E-cadherin antibodies to control the fractionation procedure. Anti-myc antibody detected the transgene product ΔN80PG in the same relative proportions in all three fractions as the endogenous plakoglobin. No differences were detected in the relative partitioning of endogenous plakoglobin and β-catenin as a result of transgene expression. (d) DN80PG shows slightly higher levels of expression compared with PG when expressed in 293T cells. Total proteins extracted from metabolically labeled 293T cells cotransfected with myc-Ta1 (transfection control) and either empty p163/7 vector (lane 1), p163/7-PG (lane 2), or p163/7-ΔN80PG (lane 3), were divided in half and immunoprecipitated with anti-plakoglobin antibody (top panel) or anti-myc antibody (bottom panel). (e) Pulse–chase experiments. Transgenic PG and ΔN80PG keratinocytes were metabolically labeled with 0.5 mCi ^{35}S-methionine/cysteine/ml for 20 min then chased for the hours indicated above the lanes. Note that in primary keratinocytes both the full-length and NH_2-terminally deleted forms of plakoglobin are stable.

However, we observed a slight increase in E-cadherin levels in transgenics (Fig. 4 b). To determine the biochemical partitioning of ΔN80PG protein, keratinocytes were sequentially extracted first in saponin and then in TX-100 containing buffers. GSK-3β and E-cadherin were used, respectively, as markers of cytosolic and membrane-bound fractions (Fig. 4 c). ΔN80PG was found in all three fractions in the same relative proportions as endogenous plakoglobin. No changes were seen in the relative distributions of endogenous plakoglobin or β-catenin among these subcellular fractions when normal mice were compared with transgenics (Fig. 4 c).

In other studies, NH_2-terminally deleted ΔN89 forms of β-catenin have been shown to have increased stability due to removal of critical phosphoserines that are required for targeting for degradation (Munemitsu et al., 1995). In transient expression experiments in 293T cells, we consistently saw higher steady-state expression of ΔN80PG than PG, suggesting that removal of this analogous domain may increase the steady-state level of plakoglobin in a similar fashion (Fig. 4 d). We examined the stability of the two forms of plakoglobin by pulse–chase experiments on cultured transgenic keratinocytes. However, in this cell type we saw no obvious difference in the stability of the full-length product as compared with the ΔN80PG product (Fig. 4 e). While ΔN89-β-catenin has been expressed in epidermal keratinocytes, its stability relative to full-length β-catenin has not been assessed in this cell type (Gat et al., 1998). Thus, at the present time it is not clear if there is a fundamental difference in the mechanisms regulating turnover of plakoglobin and β-catenin, or if keratinocytes differ from colonic and 293T cells in regulating the activity of GSK-3β or other elements in the putative GSK-3β–dependent degradation pathway. Regulated rather than constitutive activity of GSK-3β has recently been reported to occur in *Dictyostelium discoideum* (Kim et al., 1999).

Transgenic Mice Have Stunted Hair Growth

The striking feature that distinguished transgenic animals of PG line no. 21 and all three ΔN80PG lines from their normal littermates was a pronounced short hair phenotype giving the mice a less fluffy appearance to their coat and a pronounced pink hue (Fig. 5, a and b). Three founders (nos. 21, 4, and 45) and the 50% of their offspring that carried the transgene had dramatically shorter hair uniformly throughout their pelt. Founder no. 9, however, showed both short and long hair in banded and patchy fashion but the 10% of F1 progeny from this line that were transgenic showed the uniform short hair phenotype. These observations are consistent with the presence of the transgene causing the shortening of the hair and with founder no. 9 having mosaic expression of the transgene within its epidermis.

Transgenic F1 mice had a taut facial appearance and prominent ears, features resulting in part from the shortness of the hair tufts found abundantly in the cheeks and around the ears of normal mice. It was possible to accurately identify transgenic mice from day 11 on the basis of coat morphology. The short hair phenotype was most obvious once the coat was well formed, between days 15–40, the time span during which the first two highly synchronized hair cycles occur in the mouse. Adult transgenic mice remained distinguishable from wild-type littermates, having a close-cut smooth coat, although the phenotype became less obvious in the older mice probably due to the

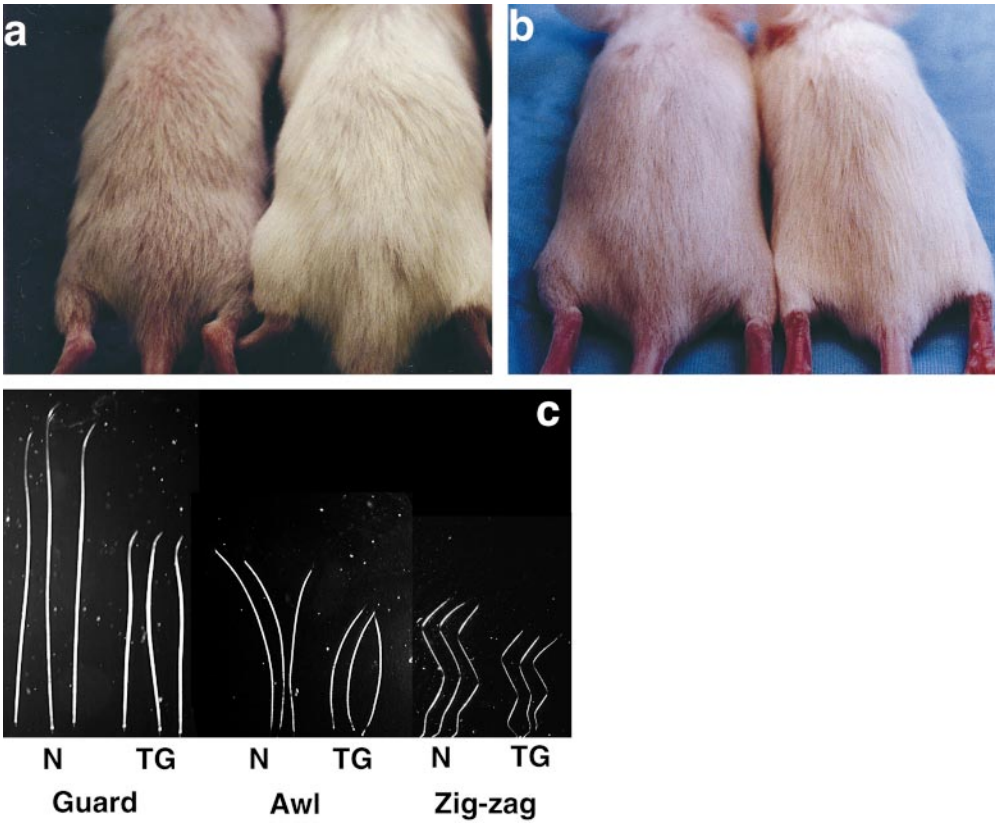

Figure 5. ΔN80PG (a) and 21-d PG (b) transgenic mouse (left) compared with their normal littermates (right). Note the short hair and pink hue of the transgenics. (c) Hair from 25-d normal (N) and ΔN80PG (TG) mice.

asynchrony of subsequent hair cycles. Heterozygous and homozygous transgenic mice showed no increase in morbidity or mortality or impairment of fertility up to one year of age. Heterozygous and homozygous pups often looked smaller than their normal littermates, but, as there was no consistent correlation in weight differences between these groups, this observation reflected a difference in coat appearance. The identical phenotype elicited by both the full-length and the NH_2-terminally deleted form of plakoglobin argues strongly that the latter acts as an active form of plakoglobin and not by interfering with the function of endogenous plakoglobin.

Analysis of Hair

Hairs plucked from the heads of 25-d sex-matched F1 transgenic and control littermates were examined under a dissecting microscope. We first asked whether the appearance of the transgenic mice resulted from lack of the longer outer coat hairs. The percentage of each of three major types of hair (guard, awl, and zigzag), however, were represented to the same degree in both the transgenic animals and their normal littermates. All hair types of the transgenic mice showed a striking reduction in size, being 30–40% shorter than hairs of normal littermates of the same age (Fig. 5 c and Table I). The root and tapered ends of the hairs were clearly visible, ruling out the possibility that the hairs were shorter due to fragility and breakage. In zigzag hairs, all four segments were present but each segment was shorter in the transgenic than in the wild-type hairs. Moreover, the segments became progressively more affected, with segments that formed first being reduced in length by ~20% and those formed last being reduced by ~50%. By scanning electron microscopy, the structure of the cuticle of the coat hairs appeared normal. However, transgenic hairs were composed of ~20% fewer cells than normal hairs and the cells were 14% smaller in diameter. No significant differences were found between the length of hairs in the different lines, suggesting that the transgene dose was irrelevant beyond a critical threshold. This was also suggested by the fact that homozygotes could not be phenotypically distinguished from heterozygotes.

Mechanistic Studies: PG and ΔN80PG Expression Results in Early Withdrawal from the Growing Phase (Anagen) of the Hair Follicle Cycle, Reduction in Cycling Cells, Premature Apoptosis, and Follicle Degeneration but Not from Changes in the Differentiation Program

Histology. To search for a basis for the unusual length of hairs from the transgenic mice, we examined skin from the mid-back region of two to three pairs of normal and transgenic littermates from each line at multiple time intervals throughout the first 41 d encompassing the first two hair cycles. Up to day 11 there was no discernible difference in the histological appearance of the transgenic epidermis or primordial follicles from those of normal littermates. The length and width of the follicular bulb, number, spacing,

Table I. Lengths (mm) ± Standard Deviations of Hairs

Line	No. of mice	Awl		Guard		Zigzag	
		N	TG	N	TG	N	TG
ΔNPG no. 4	6	5.6 ± 0.43	3.6 ± 0.45	8.1 ± 0.99	5.8 ± 0.68	5.0 ± 0.32	3.3 ± 0.29
ΔNPG no. 9	6	5.7 ± 0.46	3.6 ± 0.44	8.4 ± 0.93	6.0 ± 0.80	4.9 ± 0.45	3.3 ± 0.29
ΔNPG no. 45	3	5.5 ± 0.42	3.7 ± 0.34	8.2 ± 1.16	5.6 ± 0.61	4.9 ± 0.37	3.3 ± 0.30
PG no. 21	3	5.5 ± 0.34	4.3 ± 0.66	7.7 ± 0.58	7.1 ± 0.79	4.9 ± 0.34	4.0 ± 0.53

Pelage hairs of three (line nos. 45 and 21) to six (lines nos. 4 and 9) pairs of 25-d sex-matched F1 normal littermate (N) and transgenic mice (TG). Values represent means ± standard deviations of the total lengths of five hairs of guard type and ten hairs of awl and zigzag types from 3–6 mice. The P value was <0.001 for awl and zigzag hairs and $0.002 < P < 0.01$ for guard hairs. Each type of hair from transgenic mice was 30–40% shorter compared with the normal littermates.

and angling of the hair follicles was similar and in cross-sections of epidermis the follicle density appeared identical in both groups. However, remarkable differences were observed during later stages of anagen. In this strain of mice (Swiss Webster) anagen begins late in gestation ~E17 and peaks around day 14 as estimated by follicle length (see below). At day 13 normal mouse skin shows the classical features of the anagen phase of the hair cycle with the epithelial component of the follicle bulb containing large numbers of cells in mitotis (see arrows in Fig. 6 a). In contrast, by day 13 transgenic hair follicles have already entered into catagen and display large numbers of involuting follicles trailing compact dermal papilla (Fig. 6 b). As a result the dermis of the transgenic skin becomes much thinner than the normal. Hair follicles from normal skin do not enter catagen until day 15 (Fig. 6 c) at which time the follicles of the transgenic skin have already fully regressed and entered the quiescent telogen phase (Fig. 6 d). Follicle lengths were measured, from the base of the follicle bulb to the epidermis, to quantify the first two hair cycles (Fig. 7). A similar but more attenuated scenario is seen in the second cycle. Transgenic mice enter the second cycle slightly ahead of their normal counterparts but again leave earlier (Fig. 7). All transgenic lines showed this trend.

Proliferation. To determine the effects of ΔN80PG expression on cellular proliferation in epidermis and hair follicles we undertook a double (pulse) labeling procedure. Double (pulse) labeling gives information both on the number of cells cycling at any one time and the number of cells that have undergone two rounds of DNA synthesis (Lehrer et al., 1998). Mice were first injected with BrdU then 24 h later injected with ^{3}H-TdR. A statistically significant decrease was observed in the number of labeled cells in the transgenic group of mice (8.9 ± 4.5) as compared with their wild-type littermates (11.6 ± 3.1; $P < 0.001$

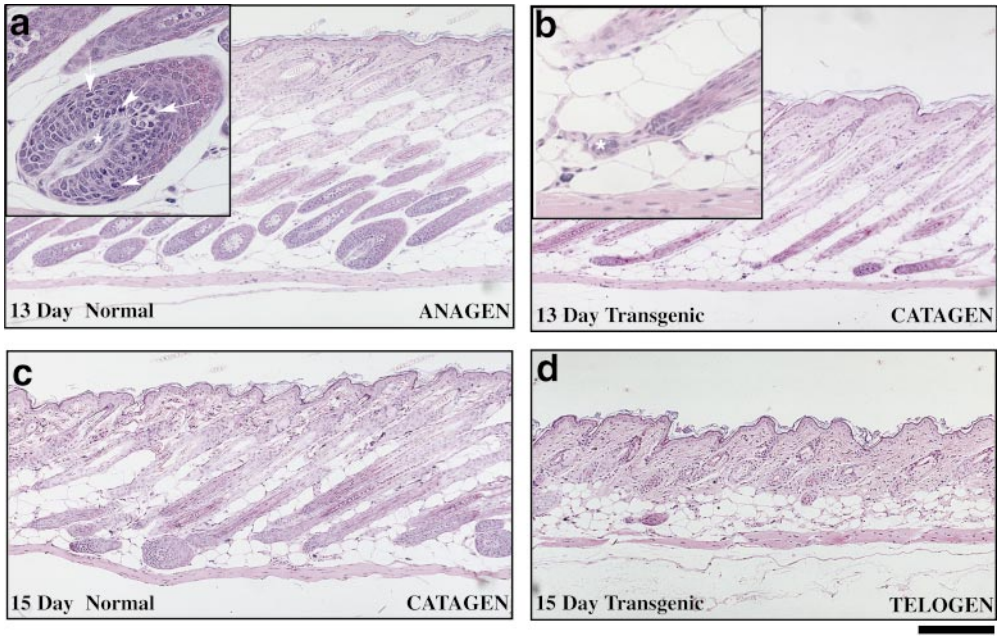

Figure 6. Histopathology of transgenic mice. 5-μm paraffin-embedded sections of backskin from sex-matched control and ΔN80PG transgenic F1 littermates were stained with hematoxylin and eosin (a–d). (a) 13-d control hair follicles show the classical features of anagen, large numbers of well-formed hair follicle bulbs surrounding dermal papilla. Many matrix cells in the follicular bulbs contain mitoses (see arrows in a inset). In contrast 13-d transgenic hair follicles (b) have already entered catagen typified by the degenerative appearance of the involuting hair follicles trailing compact dermal papillae (b inset). Normal mouse hair follicles begin to enter catagen at day 15 (c), whereas hair follicles of 15-d transgenic mice have already completed the involution process and entered telogen (d). Bar: (a–d) 250 μm; (insets) 80 μm.

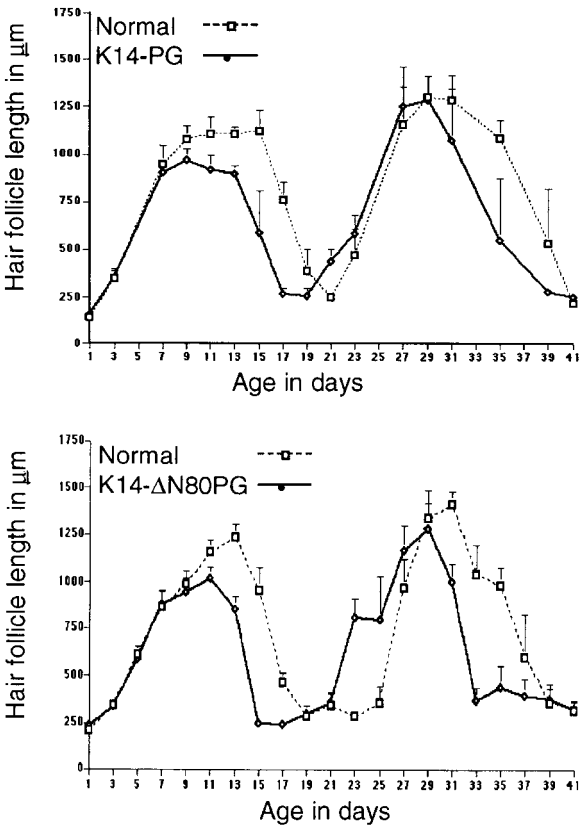

Figure 7. Comparison of the first two hair follicle growth cycles in K14-PG and K14-ΔN80PG transgenic mice and their normal littermates. Hair follicle length corresponds to the length in μm from the base of the hair follicle bulb to the epidermal surface. Data shown represent the mean of measurements of five hair follicles from each of three pairs of control and transgenic Swiss Webster mice. Standard errors are indicated by bars. Notice in both hair cycles that the hair follicles in transgenic mice enter into catagen earlier compared with the normal control.

where the number [n] of mice in each group = 10). Moreover a highly significant difference was seen in the number of cells undergoing two S phases within a 24-h period (Fig. 8). In transgenic epidermis, 8.5 ± 4.5% of cells initially labeled with BrdU were also labeled with ^{3}H-TdR indicating they had undergone two rounds of DNA synthesis as compared with 13.4 ± 4.8% of cells in normal epidermis ($P <$ 0.001 where $n = 10$ mice in each group). Thus, a significant decrease in cycling cells results from ΔN80PG expression.

Apoptosis. The factors governing the extremely rapid and synchronous involution of hair follicles during catagen are poorly understood, but are thought to involve an apoptotic pathway (Lindner et al., 1997). We therefore investigated whether the early involution observed by day 13 in the hair follicles of mice expressing ΔN80PG results from or is associated with premature onset of such a mechanism. TUNEL analysis was performed to detect DNA fragmentation as a marker of apoptotic nuclei in sections of three pairs of transgenic and normal littermate epidermis. Representative pictures are shown (Fig. 9). In normal epidermis, no apoptotic nuclei were observed until day 13 and at this stage they were restricted to a few clusters of cells within the inner root sheath of the hair follicle. Apoptotic nuclei were not observed in normal matrix keratinocytes of the hair follicle until day 15 when the earliest signs of catagen became obvious. TUNEL positive nuclei reached a maximum at day 17 and were clustered around the dermal papilla cells next to the hair matrix and in the bulge isthmus. These results are similar to previous observations on normal epidermis (Lindner et al., 1997). In contrast we observed a small number of apoptotic nuclei at day 11 in the inner root sheath of the transgenic hair follicles, a few apoptotic nuclei were seen in the matrix keratinocytes at day 13 reaching a peak at day 15. Apoptotic changes occur two days earlier in the transgenic than in normal hair follicles, with a time course in both groups that reflects the stage of involution. In both groups apoptotic nuclei first appear in the early stages of catagen as the lower portion of the follicles begin to degenerate and reach a maximum at mid catagen. Thus, in both groups apoptotic changes detected by TUNEL accompany but do not precede the changes observed histologically in the hair follicles.

Differentiation. Changes in K10 and K16 expression have recently been reported to lead to altered proliferative potential of keratinocytes and hair follicles in vivo and in vitro (Paladini and Coulombe, 1998; Paramio et al., 1999). To determine whether the transgenic mice exhibited changes in the normal differentiation program of the epidermis or hair follicles frozen sections of epidermis were stained with a panel of antibodies. These included antibodies against: keratins 5 and 14, which are synthesized in the basal layers; keratin 1, which is expressed in the suprabasal layers; keratin 6, which is expressed under conditions of hyperproliferation; high sulfur hair proteins, which are restricted to cells of the hair cuticle and loricrin which is expressed in the granular layer. No detectable changes were observed in response to transgene expression (Fig. 10).

Discussion

Our results show that modest expression of either PG or a truncated ΔN80 form of plakoglobin in the basal cells of the epidermis and outer root sheath of the hair follicle stunts hair growth. The principal findings are that hair follicles of these mice leave anagen prematurely, proliferation of epidermal and hair follicle keratinocytes is reduced and apoptotic changes occur earlier than usual. These results are consistent with the hypothesis that plakoglobin regulates aspects of cell proliferation as well as cell adhesion in normal cells in vivo and raises the interesting possibility that plakoglobin may assist in coordinating these cellular functions.

The identity of the PG and ΔN80PG phenotype argues that the latter acts as an active rather than dominant negative form of plakoglobin. This is reminiscent of the situation with β-catenin where the full-length and NH_2-terminally deleted forms act similarly in many experimental signaling assays. The lack of dose dependency in the phenotype between four independent transgenic lines strongly suggests that plakoglobin exerts its full effect on proliferation at a critical threshold and that expression above that threshold is irrelevant. Such a feature suggests that there

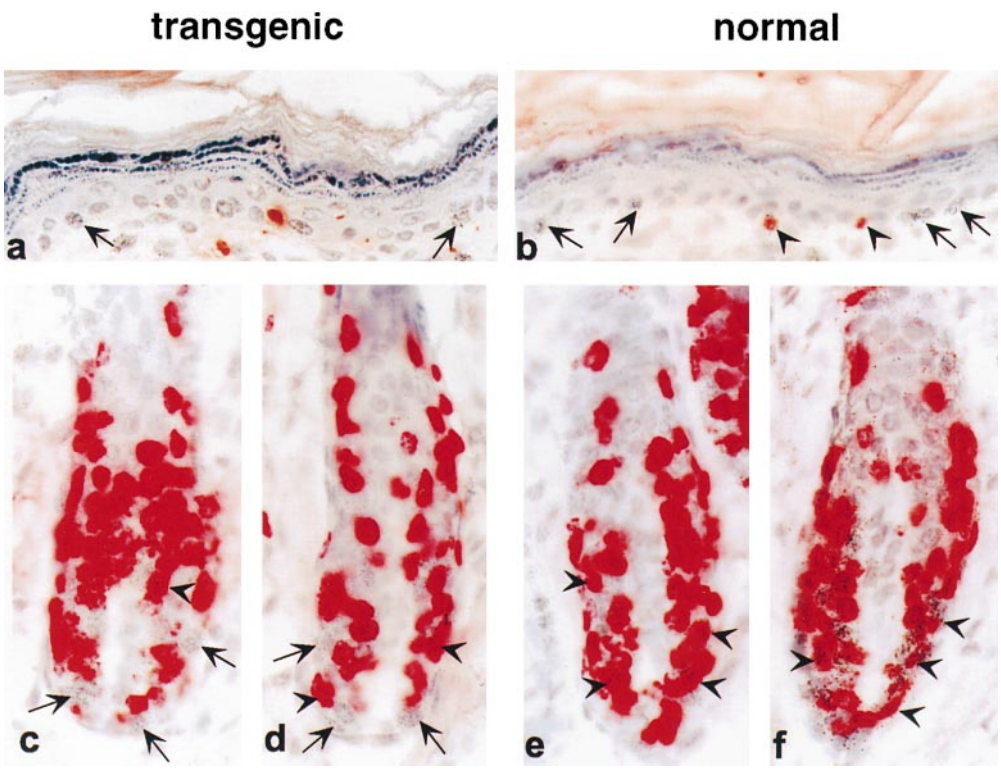

Figure 8. Comparison of BrdU- and ^{3}H-TdR–labeled nuclei of ΔN80PG transgenic and normal epidermis. 4-d ΔN80PG transgenic (left) and normal littermates (right) were subjected to subcutaneous injection of BrdU followed 24 h later by a similar injection of ^{3}H-TdR. Arrowheads indicate double-labeled cells; arrows indicate ^{3}H-TdR–labeled nuclei. (a and b) epidermis; (c–d and e–f) hair follicle in the anagen phase of the hair growth cycle. Few if any double pulse-labeled cells are seen in the TG epidermis (a) compared with the normal littermates (b). Likewise matrix keratinocytes in transgenic hair follicles (c and d) contain fewer double pulse-labeled cells than normal littermates (e and f). Overall, there was a decrease in cycling transit amplifying cells in epidermis of transgenic mice (a) compared with normal control (b).

are a limited number of binding sites to be occupied on downstream effectors. This would be expected if plakoglobin is acting, in a manner similar, or perhaps antagonistic to the transcriptional role played by its close relative β-catenin, by saturating the binding sites on promoters of a limited set of target genes. That plakoglobin is capable of this type of role is suggested by its ability to transactivate a luciferase reporter gene. This transactivation capability was observed even in keratinocytes, which make numerous desmosomes that sequester the majority of plakoglobin at the membrane.

Plakoglobin and Proliferation

Follicle stem cells reside in the bulge, a structure that lies at the bottom of the permanent part of the hair follicle and is continuous with the outer root sheath and basal layer of epidermis (Cotsarelis et al., 1990; Morris and Potten, 1999). Bulge stem cells have been postulated to respond to signals from the closely adjacent telogen dermal papilla by transiently proliferating and producing a population of transit amplifying cells that migrate downwards to regenerate the hair follicle (Cotsarelis et al., 1990; Sun et al., 1991). Thus progenitor cells residing in the epidermal basal layer and in the bulge structure and their immediate transit amplifying progeny express the K14 promoter and the transgene product. The principal effect of plakoglobin expression is seen in the hair follicles and hairs rather than the epidermis. This can be explained by the fact that follicular cells divide at six times the rate of epidermal cells (12 h for follicular keratinocytes versus 3 d for epidermal keratinocytes), and so factors that regulate proliferation will be more apparent in these structures (Lehrer et al., 1998). It should be noted, however, that the proliferative capacity of the basal epidermal keratinocytes was also reduced. Due to the much slower cell cycle time a morphological effect on the epidermis was not observed. However, we noted that the transgenic mice had taut skin suggesting that the transgene effect on the proliferative rate of epidermal cells might have restricted the surface area of the epidermis rather than the vertical thickness, which in haired animals consists essentially of only 2–3 layers of cells. The reduction in both the single labeling as well as in the double labeling index demonstrates that the transgene expression has reduced the number of transit amplifying cells cycling at any given time and slowed their rate of their proliferation (Lehrer et al., 1998). We propose that this reduction in proliferative capacity of transit amplifying cells limits their capacity to regenerate full size hair follicles or full-length hairs, which is seen most obviously at later stages of anagen due to the exponential nature of the proliferative process. This is entirely consistent with studies showing that overexpression of plakoglobin reduced the tumorigenicity and growth rate of highly transformed cell lines (Simcha et al., 1996). Our results extend these observations to show that even modest levels of expression of either full-length or NH_2-terminally deleted forms of plakoglobin restricts the proliferative potential of normal cells in vivo. This suggests that negative regulation of proliferation may be a generalized function of plakoglobin. These results contrast strikingly to the effects of overexpressing ΔN89-β-catenin in the same cells under the

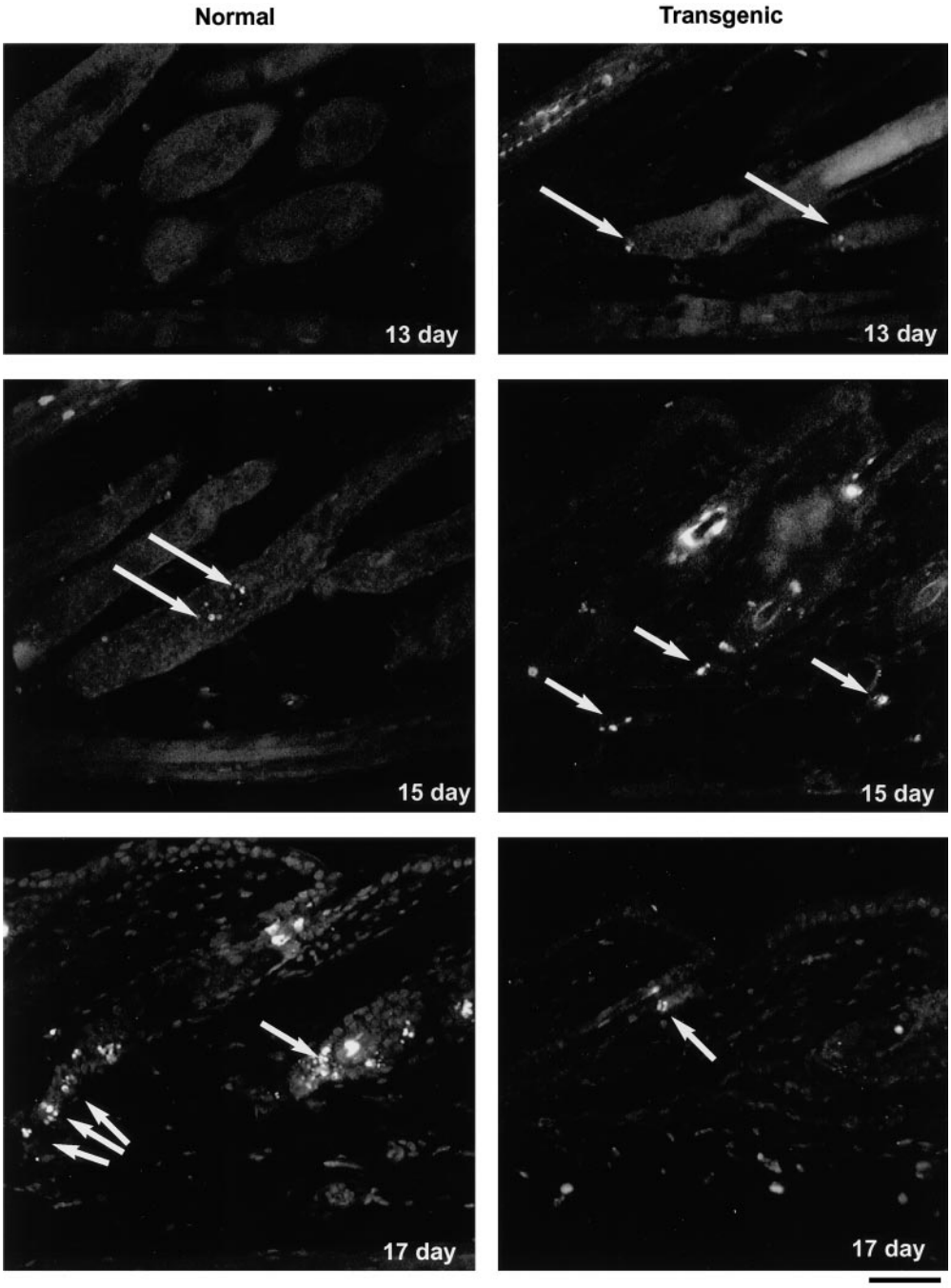

Figure 9. Apoptotic changes detected by TUNEL staining occur earlier in the matrix cells of transgenic hair follicles. Paraffin embedded sections from the backskin of K14-ΔN80PG and control littermates were subjected to TUNEL staining to determine DNA fragmentation during apoptotic cell death. No apoptotic nuclei are seen in the matrix keratinocytes of the wild-type day 13 hair follicles which are at the peak of anagen. In transgenic littermates of the same age, the hair follicles are in catagen and a few apoptotic nuclei can be seen (arrows). By day 15 wild-type hair follicles begin to enter catagen and a few apoptotic nuclei are found close to the trailing dermal papilla. Day 15 transgenic hair follicles are in the last stages of catagen and contain many apoptotic nuclei. Apoptotic nuclei reach a maximum by day 15 in the transgenic and by day 17 in normal mice. Bar, 25 μm.

same promoter (Gat et al., 1998). Expression of a ΔN89-β-catenin induces de novo hair follicles in mature haired skin, suggesting that it may cause epidermal cells to retain pluripotent stem cell potential and promote their ability to proliferate in response to unknown factors, present in haired regions of skin, that induce hair follicle formation (Gat et al., 1998). Furthermore, ΔN89-β-catenin expression eventually induces two types of follicular tumor in epidermis (Chan et al., 1999; Gat et al., 1998). Plakoglobin appears therefore to act in the opposite fashion to ΔN89-β-catenin by restricting the hair follicle's potential for proliferation.

ΔN80PG Does Not Act by Downregulating β-Catenin

Two studies have suggested that plakoglobin expression modulates β-catenin levels and that plakoglobin's signaling capabilities relate to this action. In *Xenopus*, plakoglobin expression was proposed to sequester APC and, thus, protect β-catenin from degradation and promote its signaling function (Miller and Moon, 1997). This is clearly not the case in epidermis, as full-length plakoglobin and ΔN80PG do not produce a β-catenin phenotype. In the second study, plakoglobin overexpression in HT1080 cells downregulated β-catenin levels, and was hypothesized to do this by competitively displacing β-catenin from the low

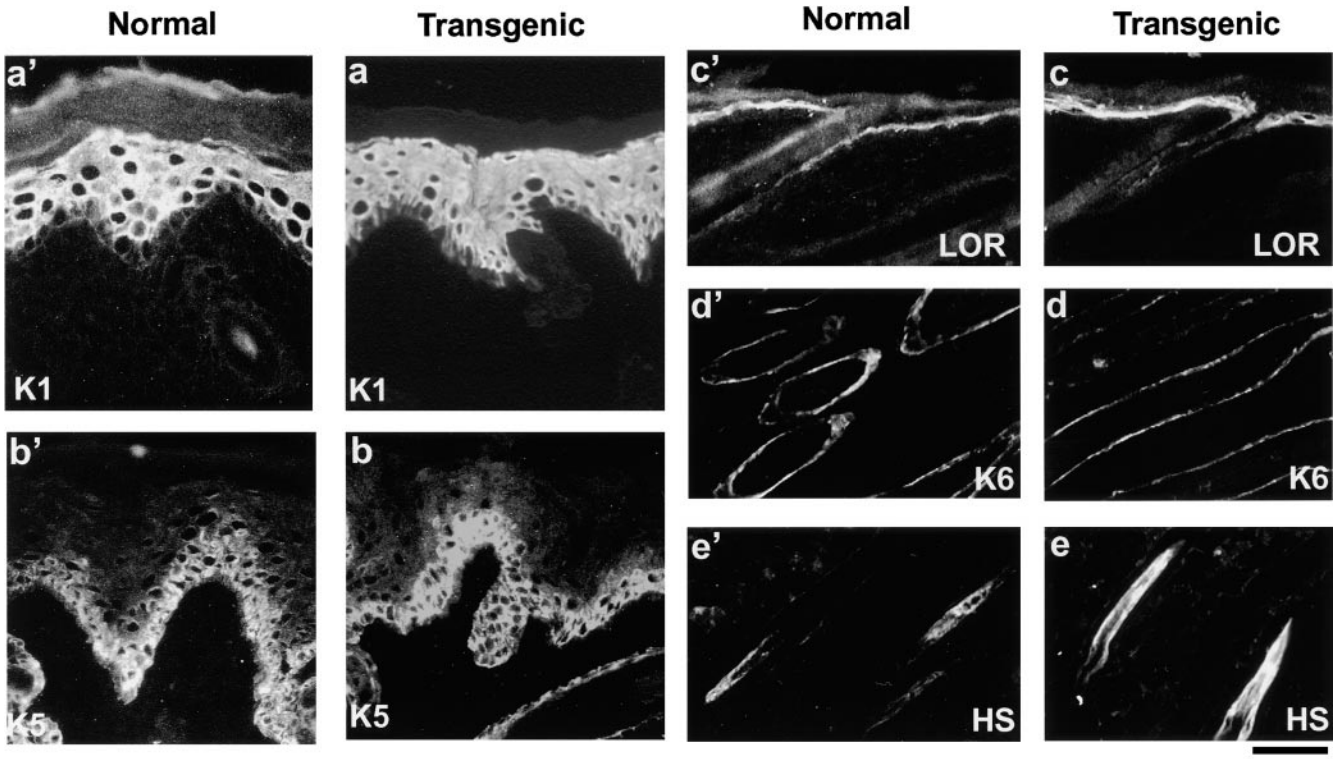

Figure 10. Expression of differentiation-specific markers in epidermis of transgenic compared with normal mice. 5-μm frozen sections of newborn tail skin (a, b, a′ and b′) or 7-d backskin (c–e and c′–e′) from sex-matched normal and K14-ΔN80PG transgenic F1 littermates were stained with anti-K1 (K1) and anti-K5 (K5); anti-desmoplakin (DP), anti-loricrin (LOR), anti-K6 (K6) and anti-high sulphur protein (HS). Note that the differentiation markers tested are expressed normally in the transgenic epidermis. Bar: (a′–b) 30 μm; (c′–e) 50 μm.

levels of cadherin present in these cells thereby exposing the β-catenin to the cytosolic degradation machinery (Salomon et al., 1997). However, in keratinocytes ΔN80PG does not appreciably decrease the levels of endogenous β-catenin or plakoglobin leading us to conclude that the effect of ΔN80PG on proliferation does not involve down-regulation of β-catenin. This raises the possibility that plakoglobin and ΔN80PG may antagonize β-catenin's ability to transactivate/derepress genes such as *myc* and *cyclin D* that are involved in growth promotion, and/or modulate the cell's response to β-catenin signaling by stimulating intersecting cadherin and/or growth factor mediated pathways at the membrane.

Plakoglobin and Wnt

The dramatic difference between the effects of plakoglobin/ΔN80PG and ΔN89-β-catenin expression raises the question as to which of these Armadillo homologues is the downstream effector of *Wnts* in epidermis and its appendages. This is a complex issue as many *Wnt* genes (Wnt-3, 4, 7a, 10a, 10b and 11) as well as proteins involved in *Wnt*-signal transduction (Dvl-1, 2, Lef-1, and Tcf-3) have been described in embryonic or adult epidermis and its appendages (Bitgood and McMahon, 1995; Cygan et al., 1997; Gat et al., 1998; Millar, 1997; Millar et al., 1999; Wang and Shackleford, 1996). Some of these Wnts are known, in other systems, to act through alternative and possibly antagonistic pathways, and, *Wnt* cascades are subject to a highly complex system of negative regulation at each step (van Genderen et al., 1994; Wong et al., 1994; Du et al., 1995; Zhou et al., 1995; Kratochwil et al., 1996; Brown and Moon, 1998). Significantly, the K14-PG and K14-ΔN80PG mice show a strikingly similar phenotype to that observed in mice expressing K14-Wnt-3 or K14-Dvl-2 (see Millar et al., 1999; Fig. 4). Specifically, all four mice display an identical short hair phenotype. K14-Wnt-3 mice display additional cyclical balding that is not seen in K14-Dvl-2, K14-PG, or K14-ΔN80PG mice. This additional phenotype results from paracrine activation of *Wnt* cascades in more distant cells of the hair follicle that produce hair shaft structural defects. The short hair phenotype, however, that is common to all four mice, is independent of this process, and, as Dvl2, PG, and ΔN80PG are, by their cytoplasmic nature, restricted to the cells that express them must result from an initial effect on cells of the outer root sheath. Taken together this suggests that plakoglobin may function downstream of Dvl-2 in an endogenous *Wnt* pathway operating in outer root sheath cells, a cell type that has previously been shown to play a role in limiting hair growth (Hebert et al., 1994). While premature withdrawal from anagen and changes in proliferative rate were not observed in the K14-Wnt-3 or K14-Dvl-2 mice it is not clear that a sufficient sample size of mice were investigated in order to detect the subtle changes seen in the K14-PG mice (Millar et al., 1999).

Potential Effects of ΔN80PG at the Plasma Membrane

Our EM, immunofluorescence and biochemical studies found no evidence that cell junctions were deleteriously affected by expression of PG or ΔN80PG. However, as the transgene products are found in all three subcellular fractions of transgenic keratinocytes, we cannot rule out that plakoglobin may additionally, or alternatively, exert its effects via enhancing the function of a transmembrane or cytoskeletal partner protein. Cytokeratin 10 and 16 have been shown to lead to changes in keratinocyte proliferation and reduced number of hair follicles (Paladini and Coulombe, 1998; Paramio et al., 1999). No significant changes were seen in the patterns of intermediate filaments ruling out their possible involvement in the plakoglobin phenotype. No changes were seen in desmosomes or in the expression levels of junctional proteins beyond a slight upregulation of E-cadherin again suggesting that the plakoglobin phenotype does not result from a deleterious effect on cell adhesion. The marginal changes in E-cadherin expression may contribute to the proliferative and apoptotic changes that are elements of the plakoglobin phenotype: E-cadherin has been shown to decrease cell proliferation (St. Croix et al., 1998). However, E-cadherin has been variously reported to promote and prevent apoptosis (Wong et al., 1998; Carmeiet et al., 1999; Hermiston et al., 1996; Pece et al., 1999). Moreover, genetic experiments in flies show that apoptosis is induced by the transcriptional functions rather than the adhesive functions of Armadillo (Ahmed et al., 1998). Transgenic expression of several cytokines and growth factors in skin produce shortened hair as part of their phenotype and both EGFR and FGF5 are known to regulate the timing of the end of anagen (Vassar and Fuchs, 1991; Turksen et al., 1992; Hebert et al., 1994; Murillas et al., 1995). Both plakoglobin and β-catenin are known to bind to and be substrates for the EGF receptor (Hoschuetzky et al., 1994). However, as the EGFR is only transiently expressed during the early stages of mouse hair development and the plakoglobin phenotype is persistent a connection between these proteins is unlikely to account for the plakoglobin phenotype (Vassar and Fuchs, 1991). There is no reported connection between plakoglobin and FGF5 expression. However, as cadherins have been shown to stimulate FGFR activity in other systems (Doherty et al., 1991; Doherty and Walsh, 1996), the possibility that plakoglobin enhancement of E-cadherin expression might simulate an FGF5 pathway must be considered. Development of ΔN80PG mice and their derived keratinocytes now permits these potential models to be tested both genetically and biochemically.

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