

Targeted Mutation of Plakoglobin in Mice Reveals Essential Functions of Desmosomes in the Embryonic Heart

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Abstract. Plakoglobin (γ -catenin), a member of the *armadillo* family of proteins, is a constituent of the cytoplasmic plaque of desmosomes, as well as of other adhering cell junctions, and is involved in anchorage of cytoskeletal filaments to specific cadherins. We have generated a *null* mutation of the plakoglobin gene in mice. Homozygous $-/-$ mutant animals die between days 12–16 of embryogenesis due to defects in heart function. Often, heart ventricles burst and blood floods the pericard. This tissue instability correlates with the

absence of desmosomes in heart, but not in epithelial organs. Instead, extended adherens junctions are formed in the heart, which contain desmosomal proteins, i.e., desmoplakin. Thus, plakoglobin is an essential component of myocardial desmosomes and seems to play a crucial role in the sorting out of desmosomal and adherens junction components, and consequently in the architecture of intercalated discs and the stabilization of heart tissue.

PLAKOGLOBIN (γ -catenin) is the first member discovered of the *armadillo* family of proteins (Cowin et al., 1986; Franke et al., 1989), which are characterized by domains composed of variable numbers of *arm* repeats. These repeats, 42 amino acids in length, have been identified in the gene product of *Drosophila armadillo*, a segment polarity gene involved in *wingless* signaling (Peifer and Wieschaus, 1990; Peifer et al., 1994; Peifer, 1995). Other prominent members of this family are β -catenin, α -importin, p120^{CAS} and the product of the adenomatous polyposis coli (APC)¹ gene; they have such different functions as control of development, cell–cell interactions, tumor progression, nuclear import, and signal transduction (Hülsken et al., 1994a; Kussel and Frasch, 1995; Török et al., 1995; Peifer, 1996).

Plakoglobin consists of thirteen *arm* repeats flanked by unique NH₂- and COOH-terminal sequences, and is a constitutive component of plaques associated with diverse adhering junctions. These include (a) desmosomes, which anchor intermediate-sized filaments (IF), (b) various microfilament anchoring junctions, such as the *zonulae*

adhaerentes of epithelia and the belt plaques of endothelial adhering junctions, and (c) junctions without detectable filaments, such as in lymphatic endothelia and granule cells of cerebellar glomeruli (Franke et al., 1987; Garrod, 1993; Schmidt et al., 1994; Rose et al., 1995). Plakoglobin exists in a regulated equilibrium between a diffusible cytosolic form and the plaque-assembled form, which is specifically bound to a defined domain in the COOH-terminal part of certain cadherins such as desmogleins, desmocollins, and classical cadherins (Cowin et al., 1986; Korman et al., 1989; Knudsen and Wheelock, 1992; Troyanovsky et al., 1993; Kowalczyk et al., 1994; Mathur et al., 1994; Plott et al., 1994; Troyanovsky et al., 1994; Hülsken et al., 1994b; Ozawa et al., 1995; Sacco et al., 1995; Chitaev et al., 1996). β -Catenin, the closest relative of plakoglobin, associates with classical cadherins only (e.g., E-, N- and VE-cadherin) and mediates the interaction to the microfilament-associated component α -catenin (Rubinfeld et al., 1993; Su et al., 1993; Aberle et al., 1994; Kowalczyk et al., 1994; Näthke et al., 1994; Plott et al., 1994; Hülsken et al., 1994b). The domains of plakoglobin and β -catenin, which associate with cadherins and with the cytoskeleton-associated α -catenin, have recently been mapped: while the central *arm* repeats interact with cadherins, the NH₂-terminal region as well as the first *arm* repeat bind to α -catenin (Cowin et al., 1986; Aberle et al., 1994; Hoschuetzky et al., 1994; Näthke et al., 1994; Hülsken et al., 1994b; Sacco et al., 1995; Chitaev et al., 1996; Witcher et al., 1996). Thus,

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1. *Abbreviations used in this paper:* APC, adenomatous polyposis coli; IF, intermediate-sized filament.

two distinct adhesion complexes can be defined in vitro, one containing E-cadherin, β -, and α -catenin, and the other containing E-cadherin, plakoglobin, and α -catenin. Both, plakoglobin and β -catenin can be tyrosine phosphorylated by several receptor and nonreceptor tyrosine kinases (Matsuyoshi et al., 1992; Behrens et al., 1993; Aberle et al., 1994; Hoschuetzky et al., 1994; Kanai et al., 1995). Plakoglobin and β -catenin can also interact with the tumor suppressor gene product APC (Rubinfeld et al., 1993; Su et al., 1993; Hülsken et al., 1994b; Ozawa et al., 1995; Rubinfeld et al., 1995, 1996) and APC and the cadherins compete for binding to the *arm* repeats (Hülsken et al., 1994b; Rubinfeld et al., 1995). Recent gene ablations of β -catenin, as well as of E-cadherin, have demonstrated the essential role of these molecules in the function of adherens junctions and in tissue formation (Larue et al., 1994; Haegel et al., 1995; Riethmacher et al., 1995).

Desmosomes are intercellular junctions characteristic of epithelial cells, where they anchor IF of the cytokeratin type, as well as of myocardial cells for which IF containing desmin are typical. Desmosomes contain cell type-specific isoforms of the desmosomal cadherins, i.e., the desmogleins Dsg 1-3 and the desmocollins Dsc 1-3 (Koch et al., 1992; Buxton et al., 1993; for review see Garrod, 1993; Koch and Franke, 1994), the cytoplasmic domains of which contribute to plaque formation.

The desmosomal cadherins are associated with plakoglobin and form a plaque containing desmoplakin I (Franke et al., 1982; Cowin et al., 1986; Stappenbeck et al., 1993; Kowalczyk et al., 1994; Mathur et al., 1994; Troyanovsky et al., 1994) and certain cell type-specific accessory proteins such as desmoplakin II, plakophilins 1 and 2, and others (Schwarz et al., 1990; Garrod, 1993; Hatzfeld et al., 1994; Schmidt et al., 1994). Plakoglobin is thus the only known member of the *armadillo* family, which can be a component of two distinct major types of intercellular junctions: adherens junctions and desmosomes.

Drosophila armadillo, the plakoglobin and β -catenin homologue, has been shown to function in cell adhesion and to play a role in the signaling of *wingless*: the secreted *wingless* protein mediates a signal to the cell surface receptor *frizzled* (Bhanot et al., 1996) as well as to the cytoplasmic proteins *dishevelled*, *zeste white-3*, and *armadillo* (Peifer and Wieschaus, 1990; Noordermeer et al., 1994; Siegfried et al., 1994; Peifer et al., 1994; Peifer, 1995; Yanagawa et al., 1995). When activated by *wingless*, *armadillo* translocates to the nucleus and affects gene expression (Peifer et al., 1994; Peifer, 1995; see also Behrens et al., 1996). There is evidence that the *wingless* signaling pathway is conserved in vertebrates: in *Xenopus*, modulation of β -catenin and plakoglobin expression affects dorsal mesoderm formation, a process regulated by *wnt/wingless* (McMahon and Moon, 1989; Smith and Harland, 1991; Heasman et al., 1994; He et al., 1995; Karnovsky and Klymkowsky, 1995; cf. also Bradley et al., 1993; Hinck et al., 1994; Yost et al., 1996). It has also been demonstrated that β -catenin and plakoglobin bind to the transcription factor lymphocyte enhancer binding factor-1 (LEF-1) and that this interaction modulates the function of LEF-1 (Behrens et al., 1996).

Here, we describe the targeted mutation of the plakoglobin gene in mice using homologous recombination and embryonic stem cell technology. We have found that dur-

ing embryonal development, plakoglobin is an essential component of cardiac but not of epithelial desmosomes: its absence leads to embryonal death between days 12–16 of development due to defects in heart histology, stability, and function. In the hearts of mice lacking plakoglobin, desmosomes are no longer detected and the remaining junctional structures and their compositions are drastically altered, resulting in a different distribution of desmoplakin and desmoglein. Surprisingly, typical desmosomes are present in embryonal epithelia, from intestine to epidermis, suggesting that in these structures the absence of plakoglobin may be compensated by (an)other molecule(s), which link desmocollins and desmogleins to the cytoskeleton. Thus, our study demonstrates an essential role for plakoglobin in the assembly of intercalated discs, which prove to be key elements in the stabilization of heart tissue and consequently, heart function.

Materials and Methods

Plakoglobin Targeting Vector and Generation of Mutant Mice

A plakoglobin genomic clone was isolated from a 129/Sv mouse genomic library (Stratagene, La Jolla, CA), and a targeting construct was assembled from a 11-kb genomic fragment. In this vector, a large part of exon 3, the following intronic sequences and the 5' region of exon 4 (encoding amino acids 70-160) were replaced by a neomycin gene cassette (neo) inserted in the same transcriptional orientation. A herpes simplex virus thymidine kinase gene cassette (HSV-TK) was inserted at the 3' end of the construct (Mansour et al., 1988). The linearized targeting vector was electroporated into murine E14.1 ES-cells, which were selected using G418 and gancyclovir.

Unique integration and appropriate recombination of the targeting construct were verified by Southern blotting: cells were treated at 55°C in lysis buffer (100 mM Tris-HCl pH 8.5, 5 mM EDTA, 200 mM NaCl, 0.2% SDS) with 0.1 mg/ml proteinase K, and genomic DNA was precipitated. After digestion with XhoI and XbaI, the fragments were separated by electrophoresis on 0.8% agarose gels and transferred to Hybond-N⁺ membranes (Amersham Corp., Arlington Heights, IL). ³²P-labeled probes were made from genomic plakoglobin sequences used for constructing the targeting vector: after digestion with BspEI and XbaI (1.1 kb 3' external probe) and with BstEII and NdeI (1.3 kb internal probe), fragments were isolated and labeled using the multiprime labeling system (Amersham). Hybridizations were carried out at 65°C overnight in hybridization buffer (5 × SSC, 0.5% SDS, 5 × Denhardt's solution, 10% dextran sulfate, and 0.1 mg/ml denatured sonicated herring sperm DNA), and the blots were exposed to X-ray film for 24 h. ES-cell clones harboring the desired integration event were injected into C57BL/6 recipient blastocysts and these were transferred into pseudopregnant NMRI females to generate chimeric mice (Mansour et al., 1988). Male offspring exhibiting extensive coat color chimerism were mated to C57BL/6 females, and genotypes were identified first by PCR and then by Southern blot analysis of DNA isolated from tails. The day of vaginal plug formation was considered as day 0.5 of gestation.

For PCR genotyping, embryonic yolk sacs and mouse tails were digested with 0.1 mg/ml proteinase K at 55°C in lysis buffer, and genomic DNA was precipitated. PCR was carried out in 15- μ l aliquots containing 2 μ l of DNA in PCR buffer, 2 mM of each nucleotide, 1.3 μ g/ml of each primer and 1 U of Taq polymerase (Roche, Perkin-Elmer, Norwalk, CT). The following primers were used: plakoglobin sense strand 5'-CGGC-CATCGTCCATCTCATC; plakoglobin antisense strand 5'-CCTCC-TTCTTGACAGCTGG; neo-sense strand 5'-CTTCTATCGCCTTCT-TGACG (to detect a 300-bp fragment in the wild-type and a 150-bp fragment in the mutant allele). Samples were amplified for 30 cycles (96°C for 5 s; 60°C for 20 s; 72°C for 60 s) using a Biometra thermocycler, and reaction products were visualized by ethidium bromide staining in 1.2% agarose gels.

For Northern blot analysis, poly(A)⁺ RNA was isolated from 11.5-d-old mouse embryos. Tissues were immediately frozen in liquid nitrogen

and stored at -80°C . For RNA preparation, tissues were ground in a mortar under liquid nitrogen and the powdered tissue mixed with hot phenol (80°C) saturated with TNE (20 mM Tris-HCl pH 7.5, 1 mM EDTA, 100 mM NaCl). Poly(A)⁺RNA was isolated from oligo-dT cellulose. After gel electrophoresis, blots were hybridized as outlined above with a 372-bp mouse plakoglobin cDNA (67–439 bp) or a 1.3-kb mouse actin cDNA.

For immunoblot analysis, proteins from 11.5-d mouse embryos were resolved on 10% SDS-PAGE. Blots were incubated with polyclonal antibodies directed against human plakoglobin amino acids 731–745 (E1021; Aberle et al., 1994) and against β -catenin (Hülsken et al., 1994b), followed by horseradish peroxidase-labeled secondary antibodies and chemiluminescence detection (ECL kit, Amersham, UK).

Histology and Immunocytochemistry

For histological analysis, embryos were fixed in 4% formaldehyde at 4°C , dehydrated in a graded EtOH series, and embedded in Technovit 7100 (Heareus Kulzer, Wehrheim, FRG). 6- μm sections were stained with haematoxylin-eosin and photographed using a Zeiss Axiophot light microscope.

For analysis with the confocal microscope, frozen sections were fixed with acetone for 10 min and incubated with primary antibodies at 4°C in PBS containing 1% BSA. Antibody concentrations used were as follows: E1021, 1 $\mu\text{g}/\text{ml}$ (affinity-purified anti-plakoglobin antibody); anti- β -catenin antiserum, 1:400 (see Hülsken et al., 1994b); monoclonal anti- β -catenin (Transduction Labs); 1 $\mu\text{g}/\text{ml}$; monoclonal anti-desmoplakin 1 and 2 antibodies, undiluted cocktail of hybridoma supernatants (Trojanovsky et al., 1993); anti-desmoglein 2 antiserum (Dsg2-I, 1:100). After washing, sections were incubated with either Texas red-conjugated goat anti-guinea pig or Cy3-conjugated goat anti-mouse antibodies, together with Cy5-conjugated goat anti-rabbit antibodies (1:200; Jackson ImmunoResearch Labs Inc., West Grove, PA) and FITC-conjugated phalloidin (1:100; Sigma). Sections were mounted in Mowiol (Calbiochem-Novabiochem Corp., La Jolla, CA) and analyzed using a confocal microscope (Leica TCS, Bensheim, FRG).

Electron and Immunoelectron Microscopy

For electron microscopy, tissue specimens were fixed in 2.5% glutaraldehyde, postfixed with OsO_4 and contrasted with tannic acid and uranyl acetate. Specimens were dehydrated in a graded ethanol series and embedded in Epon 812. Ultrathin sections were contrasted with lead citrate and analyzed, using a Zeiss electron microscope EM 10.

Pre-embedding immunoelectron microscopy was essentially carried out as described by Rose et al. (1995): Before antibody incubation, frozen sections mounted on coverslips were fixed with 2% formaldehyde in PBS for 15 min followed by permeabilization with 0.1% saponin in PBS for 10 min. Monoclonal antibodies against desmoplakin (DP 2.15, 2.17, 2.19; Progen Biotechnik, Heidelberg, FRG) were applied for 2 h and the secondary anti-mouse IgG coupled to "Nanogold" (Bio Trend, Cologne, FRG) for 4 h. After fixation with 2.5% glutaraldehyde and postfixation with 2% OsO_4 , the bound gold was enhanced using the silver-enhancement kit "HQ-Silver" (Bio Trend) for 4 min in the dark. Dehydration and embedding was as previously described (Franke et al., 1987).

Functional Analyses

For measurement of the amplitude of heart contraction and heart rate, we used a high resolution echo-tracking device, a highly sensitive angiometer equipped with a noninvasive ultrasound beam (NIUS 02; see Girerd et al., 1994). 11.5-d-old embryos were dissected from the uterus and placed in PBS on a rubber dish. Single embryos were evaluated 6–8 times over a period of 5 min, and data are given as mean μm (amplitude) and beats per minute, respectively.

Results

Generation of Plakoglobin Mutant Mice

We generated a *null* mutation of the plakoglobin gene by homologous recombination in embryonic stem cells (Fig.

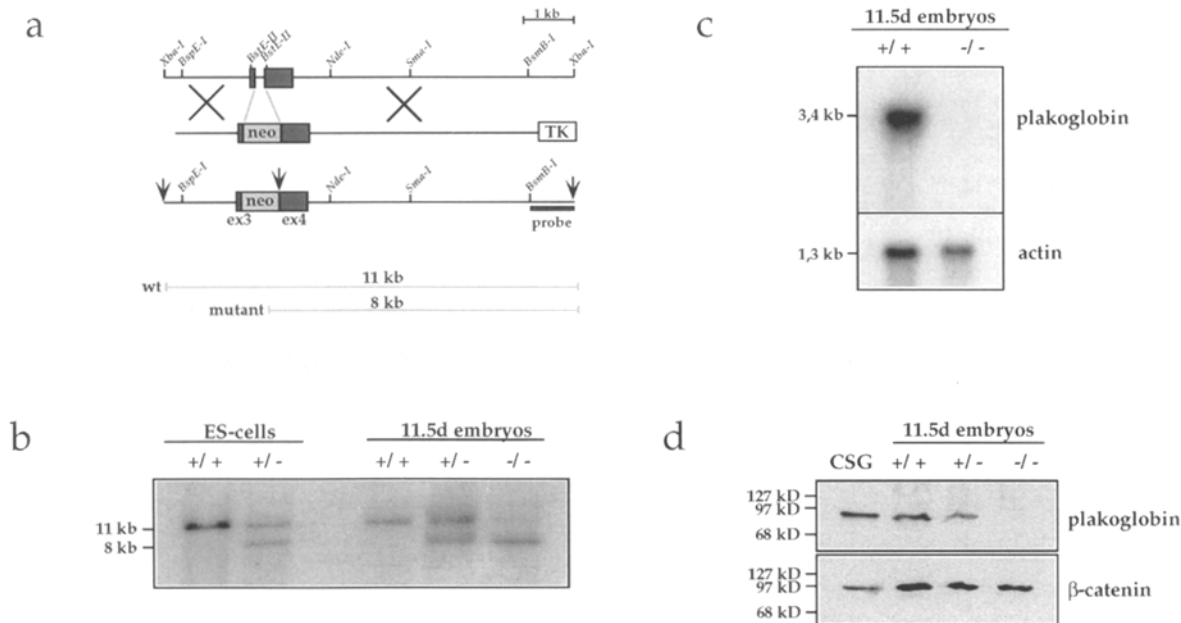


Figure 1. Targeting vector and Southern, Northern, and immunoblot analyses of ES-cells and embryos for plakoglobin. (a) Restriction map of the genomic plakoglobin clone used for vector construction (*top*) of the targeting vector used for gene ablation (*middle*), and of the targeted locus (*bottom*). Exons 3 and 4 are represented by boxes (ex3 and ex4). The 1.1-kb long 3' external probe used for Southern blot analysis is shown as a black bar (*probe*). Arrows indicate restriction sites for XbaI and XhoI. (b) Southern blot analysis of genomic DNA after restriction digest with XbaI and XhoI from ES-cells and 11.5-d-old embryos. The 3' external probe detected restriction fragments of 11 kb (wild-type allele) and 8 kb (targeted allele). (c) Northern blot analysis of poly(A)⁺RNA from 11.5-d-old embryos: blots were hybridized with mouse plakoglobin (*top*) or actin cDNA probes (*bottom*). (d) Western blot analysis of total proteins from 11.5-d-old control and $-/-$ embryos (mouse salivary gland carcinoma cells, CSG, served as control). Plakoglobin was probed with antibody E1021 (*top*) and β -catenin as a control with a specific antiserum (*bottom*). Molecular mass references are indicated in kilodaltons (kD).

Table I. Viability of Embryos Resulting from Heterozygous Intercrosses*

Stage	Number of litters	+/+	+/-	-/-‡
10.5	3	4	19	8
11.5–12.5	34	73	161	43 (21)
14.5–15.5	11	24	53	7 (13)
Adult	18	30	70	0

*Genotyping was performed by PCR.

‡In parentheses, the numbers of dead homozygous mutant embryos are shown.

1 *a*). ES-cells carrying the mutant allele were injected into C57BL/6 blastocysts, chimeric mice were produced and heterozygous animals were bred. We found that only wild-type (+/+) and heterozygous (+/-), but not homozygous (-/-) animals were born, indicating that plakoglobin -/- animals die during embryogenesis (Fig. 1 *b* and Table I). We therefore isolated embryos at different stages of development and observed that most plakoglobin -/- embryos died between days 12 and 16 of gestation. To assess whether our strategy had resulted in fact in a null mutation of the plakoglobin gene, we conducted Northern and Western blot analysis on 11.5-d-old embryos. While wild-type and heterozygous embryos expressed both plakoglobin mRNA (Fig. 1 *c*) and protein (Fig. 1 *d*), neither were detected in homozygous mutant animals.

Plakoglobin -/- Mice Develop Heart Defects during Embryogenesis

To determine the cause of lethality in plakoglobin -/- mice, we closely examined embryos between days 10 and 16 of development. Heterozygous animals could not be distinguished in overall morphology from wt animals. However, plakoglobin -/- embryos were slightly growth-retarded at day 12 of gestation, and blood supply, particularly of liver and placenta, was reduced (Fig. 2, *a* and *b*). We also observed that the pericardial cavities of mutant embryos were frequently swollen and filled with blood (see arrow in Fig. 2 *d*). The hearts of these embryos were often found to be ruptured, although they were still beating. Histological analysis revealed that heart rupture occurred in the ventricles (see short arrow in Fig. 2 *f*; a blood clot in the pericard is marked with the long arrow). We also analyzed heart function in 11.5-d-old embryos, using a high resolution ultrasonic echo-tracking device: the amplitude of heart contraction was strongly reduced in plakoglobin -/- animals ($28 \pm 8 \mu\text{m}$) when compared to the control animals ($53 \pm 15 \mu\text{m}$). Furthermore, the heart rate was increased (88 ± 20 beats per min) when compared to the control animals (64 ± 17 beats per min). We conclude from these observations that plakoglobin -/- embryos die of defects in heart function at mid-gestation.

Desmosomes Are Absent in the Intercalated Discs of Plakoglobin -/- Mice

Immunofluorescence microscopy showed extensive expression of plakoglobin in the embryonic heart at day 10.5 (Fig. 2, *g* and *i*; the absence of plakoglobin in the heart of homozygous mutant embryos is shown in Fig. 2 *h*). Ultrastructurally, cardiomyocytes at this stage contain well-organized sarcomeres anchored at the cytoplasmic plaques

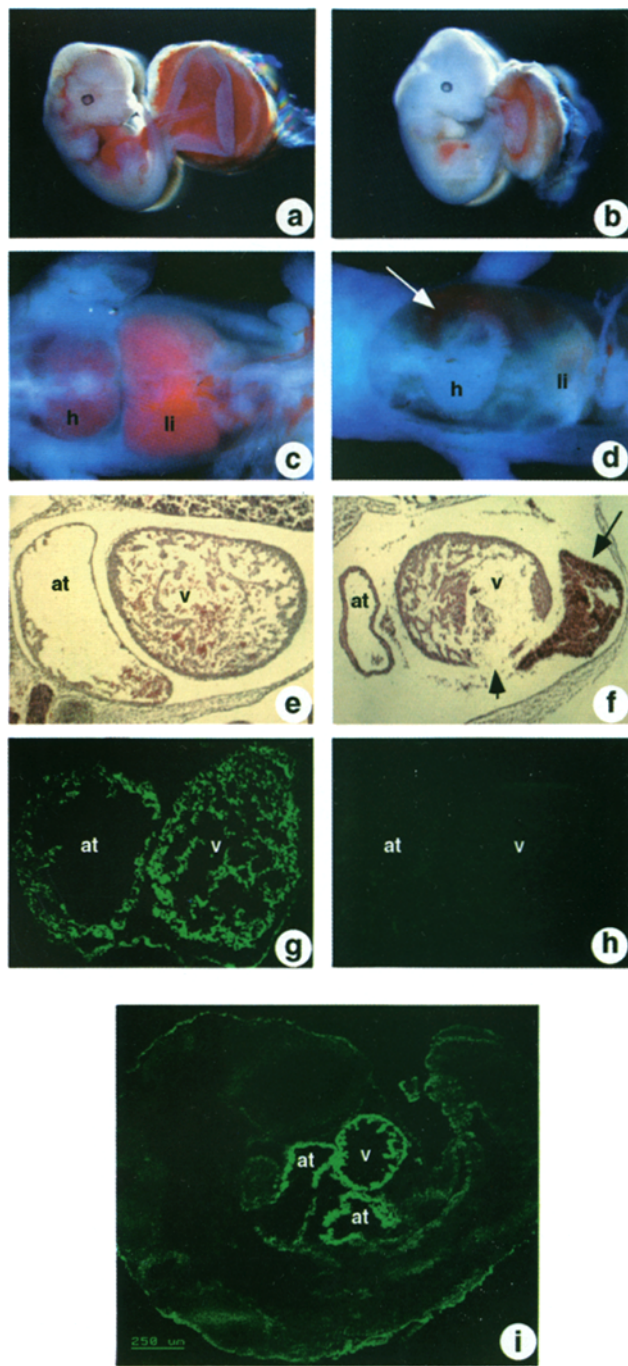


Figure 2. Phenotype of plakoglobin -/- and control embryos at day 12 of gestation. External and histological appearance of wild-type (*a*, *c*, and *e*) and plakoglobin -/- (*b*, *d*, and *f*) embryos. Note the reduced blood supply of plakoglobin -/- embryos, compared with control littermates, particularly in liver and placenta (compare *a* and *c* with *b* and *d*). The white arrow in *d* and the large arrow in *f* indicate blood flooding the pericardial cavity; the small arrow in *f* points to a rupture in the left heart ventricle in a plakoglobin -/- embryo. (*g*) Immunofluorescence microscopy showing plakoglobin (by anti-plakoglobin antibody staining) in cryostat sections through heart tissue of a wild-type embryo and a homozygous plakoglobin -/- mutant (*h*). (*i*) Shows a section through a whole embryo stained for plakoglobin. *h* and *li* (in *c* and *d*) indicate heart and liver, respectively; *v* and *at* (in *e*-*i*) indicate ventricle and atrium.

of adherens junctions, which were readily identified in the intercalated discs of heart tissue from wt and plakoglobin $+/-$ embryos (arrows in Fig. 3 *a*). Note that desmosomes in the intercalated discs are not connected to sarcomeric fibrils (arrowhead in Fig. 3 *a*). The general morphology of cardiac structures was unaltered in plakoglobin $-/-$ embryos: atrial and ventricular chambers were present, endocardial cushions and ventricular trabeculae were well developed (Fig. 2, *e* and *f*). However, the morphology of the intercalated discs was grossly altered as they did not show differentiation of desmosomes and adherens junctions. Typical desmosomes were no longer detectable, and instead adherens junctions with particularly prominent plaques were seen (arrow in Fig. 3 *b*). Desmosomes were absent already at embryonal day 8.0, i.e., when the heart begins to beat, but mechanical stress is still low (not shown). Remarkably, in various epithelia of plakoglobin $-/-$ mice, as for example in epidermis and the gastrointestinal tract, desmosomes were readily detected and were similar in morphology to desmosomes of the same tissue in control animals (arrowheads in Fig. 3, *c-f*).

Molecular Composition of Wild-Type and Mutant Junctions

The molecular composition of the intercalated discs in control and mutant animals was examined by confocal laser scanning immunofluorescence and by immunoelectron microscopy. In hearts of control embryos, desmoplakin (yellow spots in Fig. 4 *a*) and desmoglein (yellow spots in Fig. 4 *c*) were largely segregated from β -catenin (blue spots in Fig. 4, *a* and *c*), as expected for desmosomal and adherens junction proteins, respectively. Surprisingly, in plakoglobin $-/-$ embryos desmoplakin colocalized in many places with β -catenin (pink color in Fig. 4 *b*; note the increased size of the junctions). Desmoglein was not clustered in these new desmoplakin- and β -catenin-positive junctions of the heart, but the signal was rather weak and diffusely scattered over the cell surface (Fig. 4 *d*). We could not analyze the distribution of desmocollins yet, since our antibodies are directed against human and bovine, but not mouse tissues. In polarized epithelia such as in the gut, the adherens junction protein β -catenin and the desmosomal protein desmoplakin were largely segregated in both control and plakoglobin $-/-$ embryos (data not shown).

By immunoelectron microscopy of hearts of control animals, we found the normal pattern of typically structured, desmoplakin-positive desmosomes of 0.2–0.6 μm diameters (labeled *D* in Fig. 5, *a-c*) next to desmoplakin-negative adherens junctions (arrows in Fig. 5 *c*) at which the sarcomeric actomyosin fibrils insert. By contrast, in hearts of plakoglobin $-/-$ mice typical desmosomes were absent and desmoplakin occurred in all plaque-bearing structures, i.e., junctions resembling adherens junctions with myofibrillar bundles attached (arrows in Fig. 5, *d-f*). This new form of junctions varied in sizes, including some extremely large ones with axes of up to 4.5 μm (see Fig. 5 *d*).

Additional Features of Plakoglobin $-/-$ Mice

At day 12 of embryogenesis, heart rupture occurs frequently and represents the primary defect found in plakog-

lobin $-/-$ animals. As a consequence, blood flow to liver and placenta is not sufficiently maintained, resulting in a characteristic paleness of the embryos. When we examined such embryos between days 13 and 14 of development by histological analysis, we found that the spongiotrophoblast of the placenta was reduced in size and poorly supplied with blood. At day 14, the liver parenchyme showed initial signs of deterioration (e.g., nuclear fragmentation and swelling of mitochondria; data not shown). A few plakoglobin $-/-$ embryos were found to be alive at day 16–18 of gestation; these were swollen and covered with many edemas and showed a strong blood retention in kidney and liver (data not shown).

Discussion

We report here the functional analysis of plakoglobin (γ -catenin) in the mouse, which was achieved by the introduction of a *null* mutation into the gene. Plakoglobin was found to be essential for the segregation of desmosomes and adherens junctions during the formation of intercalated discs in the embryonic heart. In the hearts of plakoglobin $-/-$ mice, desmosomes are not present, instead extended adherens junctions develop, which contain desmosomal proteins such as desmoplakin. Consequently, the architectural stability and function of the embryonic heart is impaired. In the absence of plakoglobin, ventricles rupture at midgestation and blood floods the pericardial cavity. Surprisingly, desmosomes in epithelial cells, for instance in skin and gut, do form appropriately in mutant mice. Our data demonstrate that functional heart desmosomes and proper sorting of adherens junction proteins are required during the second half of gestation, when the embryo becomes dependent on its own blood circulation and therefore on a functional cardiovascular system.

Absence of Plakoglobin Results in Drastic Changes in the Architecture of Intercalated Discs

The intercalated discs of the heart are extended regions of contact between cardiomyocytes in which different types of junctions occur side by side. These are, besides gap junctions, typical desmosomes which anchor desmin-containing IF and *fascia adhaerentes* at which the bundles of sarcomeric myofilaments attach. Distinct desmosomes have been observed already in very early stages of cardiac development of diverse vertebrate species, as well as in cardiomyocyte cultures (Kartenbeck et al., 1983; Atherton et al., 1986; Kuruc and Franke, 1988; Shiozaki and Shimada, 1992; Viragh et al., 1993; Hertig et al., 1996). It has previously been shown that the myocardial desmosomes contain desmoplakin I (Franke et al., 1982; Kartenbeck et al., 1983), plakoglobin (Cowin et al., 1986; Franke et al., 1987), plakophilin 2 (Mertens et al., 1996), and the cadherins desmoglein Dsg2 (Schäfer et al., 1994) and desmocollin Dsc2 (Theis et al., 1993; Legan et al., 1994; Lorimer et al., 1994; Nuber et al., 1995). By contrast, the *fasciae adhaerentes* contain N-cadherin in association with plakoglobin, β -catenin and α -catenin, vinculin, and other actin-binding proteins (Tokuyasu et al., 1981; Kartenbeck et al., 1983; Hertig et al., 1996).

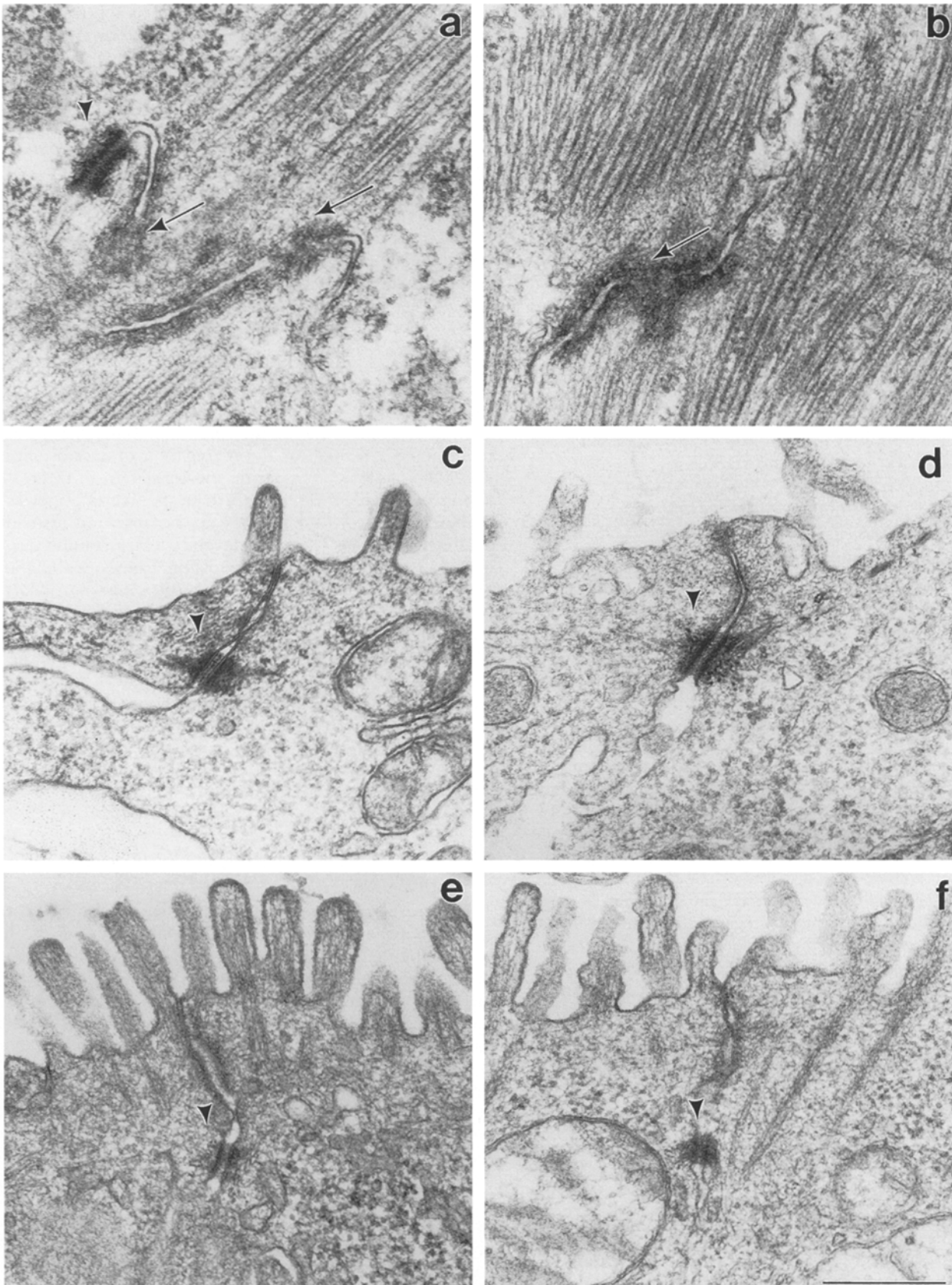


Figure 3. Transmission electron microscopy of cardiac (*a* and *b*) and skin tissues (*c* and *d*) of 11.5-d embryos, and of small intestine (*e* and *f*) of 16-d embryos. In heterozygous plakoglobin $+/-$ animals (*a*, *c*, *e*), desmosomes (*arrowheads*) as well as adherens junctions (*arrows*) are abundant. Plakoglobin $-/-$ mice lack typical desmosomes in myocardium (*b*), but not in the epidermis (*d*) and gut (*f*). Bar, 0.5 μ m.

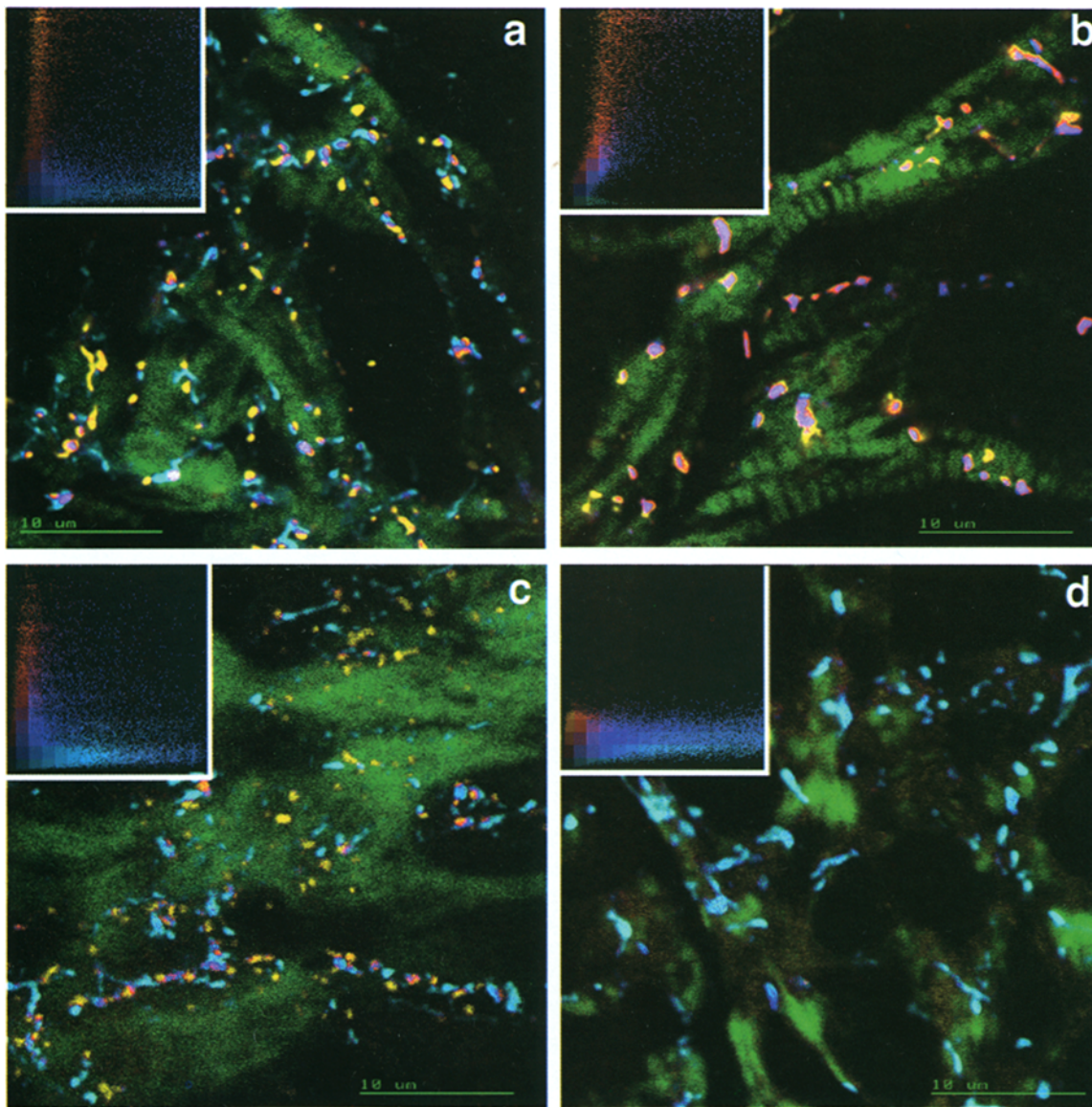


Figure 4. Confocal laser immunofluorescence microscopy of desmoplakin, desmoglein and β -catenin in heart tissue. In wt embryos, desmoplakin (a) and desmoglein (c) are largely separated (both in yellow) from β -catenin (a and c, blue), resulting in distinct signals. In plakoglobin $-/-$ embryos (b and d), desmoplakin (yellow) and β -catenin (blue) are colocalized in numerous places appearing as pink signals (b). Note the increased sizes of the extended junctions in the plakoglobin $-/-$ embryos. In contrast, desmoglein (d, yellow) is expressed, but diffusely distributed over the specimen and therefore not readily seen. F-actin appears in green, as detected by FITC-conjugated phalloidin. Inserts show the densitographs of the two independent photomultipliers.

We demonstrate here that the absence of plakoglobin in fetal heart of mice does not only affect the desmosomal junction, but also results in several drastic changes of the architecture of the intercalated disc: (a) distinct desmosomes are no longer seen; (b) the normal segregation of desmosomal and adherens junction constituents is lost, i.e., junctional molecules of both sets are mixed, resulting in the appearance of a novel, amalgamated junction characterized by the coexistence of desmoplakin with β -catenin, which anchors the sarcomeric myofilament bundles; (c) the new “mixed type” adhering junction occurs in a wide range of sizes, including extremely large ones, that can be traced for up to $4.5 \mu\text{m}$; (d) the desmosomal cadherin Dsg2 is no longer clustered and coassembled with

desmoplakins into distinct junctional structures, but is rather diffusibly spread over the cell surface. Apparently, plakoglobin is thus not only essential for the formation of stable cardiac desmosomes, but is also critically involved in the segregation and/or sorting of the two sets of molecules into desmosomes on the one hand and *fasciae adherentes* on the other. Such a specific structure-forming and sorting function of plakoglobin has also been previously indicated (Trojanovsky et al., 1993, 1994), since the plakoglobin-binding site in desmoglein and desmocollin chimeras is necessary to nucleate the formation of a plaque and to cluster desmoglein. Since desmosomes were already absent in the heart of plakoglobin deficient embryos at day 8.0 of development, it is likely that this heart-

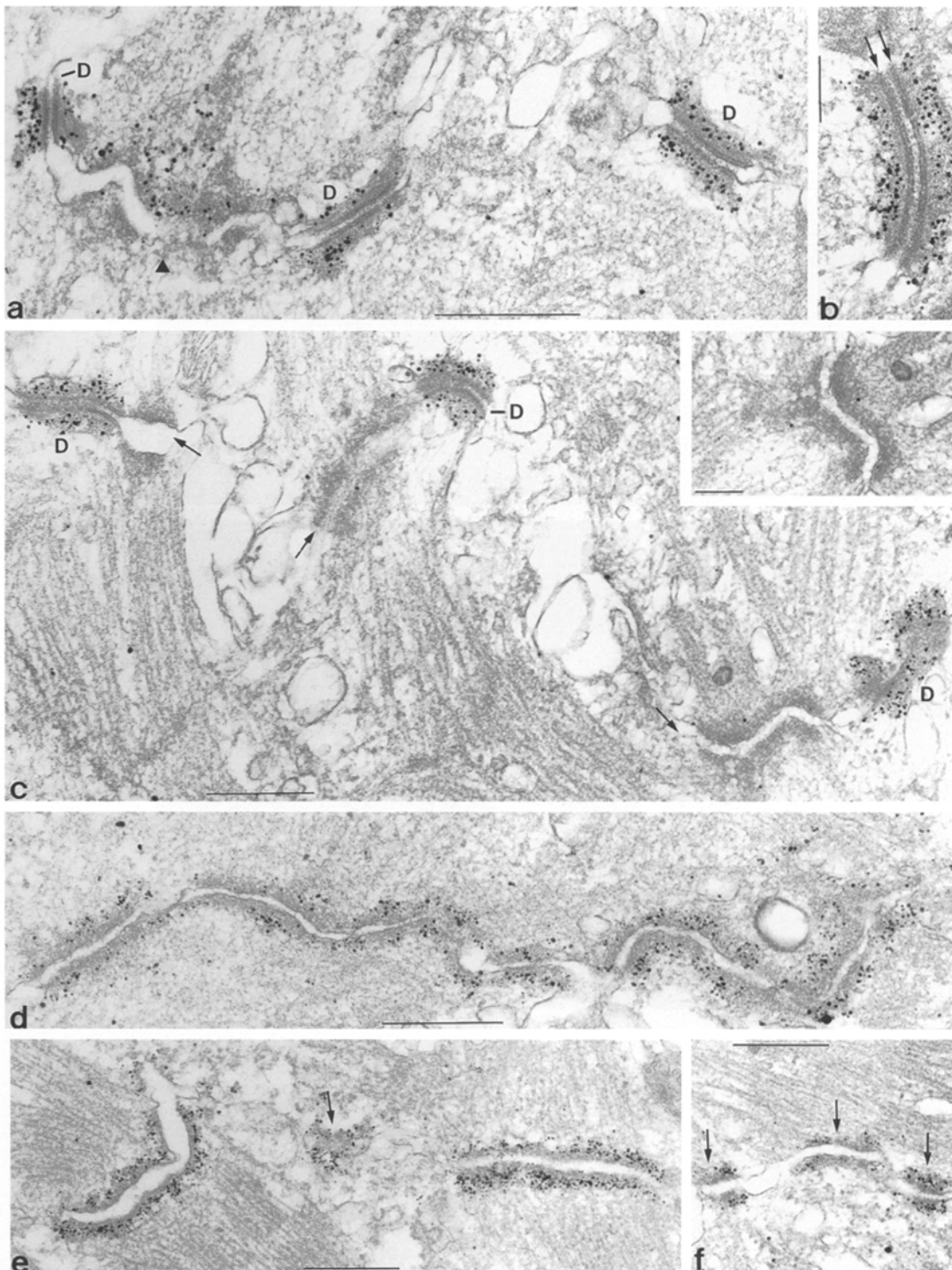


Figure 5. Immunoelectron microscopy showing immunogold labeling for desmoplakin in intercalated discs of unfixed, freeze-sectioned myocardial tissue of wt (*a-c*) and plakoglobin $-/-$ mice (*d-f*). Note that in the wt mice the desmosomes (*D*) display their typical ultrastructural organization and intense desmoplakin labeling. Higher resolution is presented in *b*, showing that the antibody-label is restricted to the plaque, whereas the membrane lipid bilayer structure (*short arrows*) and the mesoglea are free of label. By contrast, adherens junctions (*arrows* in *c* and *insert*) interspersed between the desmosomes, which anchor the bundles of sarcomeric microfilaments, are not significantly labeled. The triangle in *a* denotes one of the less frequent junctions in hearts of wt animals positive for desmoplakin but lacking typical desmosomal morphology. In the embryos lacking plakoglobin, the organization of the plaques in the intercalated discs is grossly altered. Here, extended junctional regions are frequent, which are almost entirely covered by a dense cytoplasmic plaque intensely labeled for desmoplakin (*d*). In these mutants, desmoplakin-positive plaques are also seen at junctions with fascia adherens-like morphology that anchor sarcomeric myofilament bundles (in the left and in the right of *e*), as well as on tiny junctions not yet classified (*arrows* in *e* and *f*). Bars: (*b*, *insert* in *c*) 0.2 μm ; (*a*, *c-f*) 0.5 μm .

specific embryonal defect is not due to mechanical stress resulting in the modification of junctions, but due to an intrinsic compositional difference of junctional complexes. While our results demonstrate the indispensability of plakoglobin in myocardial structure and function, as well as in the formation of myocardial desmosomes, they do not yet allow the definitive conclusion that these two effects are interdependent. At present, it cannot be excluded that it actually may not be the absence of desmosomes, but rather the simultaneous loss of distinct *fasciae adherentes*, the appearance of a new "mixed" type of adhering junction or the loss of other, nonjunctional plakoglobin functions, that is causal for the fatal heart defects.

Difference between Heart and Epithelial Desmosomes

The absence of plakoglobin in mutant animals significantly affects formation of desmosomes in the heart, but not in epithelial tissues as epidermis and gut. Recent evidence indicates that in heart, only a subset of desmosome-specific proteins is expressed, including desmoglein-2, desmocollin-2, and plakophilin 2, whereas in epidermal keratinocytes three desmogleins (Dsg1-3), three desmocollins (Dsc1-3), and plakophilin 1 have been described (see Garrod, 1993; Koch and Franke 1994; Schmidt et al., 1994; Mertens et al., 1996). Plakophilin 1 (formerly referred to as band-6 protein) is a recently characterized member of the *armadillo* family of proteins, which is absent from cardiac desmosomes (Kapprell et al., 1988; Hatzfeld et al., 1994; Heid et al., 1994; Schmidt et al., 1994). Its next relative is p120^{CAS} (Reynolds et al., 1994), a ubiquitous protein that binds to cadherins, but does not mediate interaction with α -catenin and with the actin cytoskeleton (Daniel and Reynolds, 1995). Specific plakophilins or other related molecules may thus contribute to desmosomal formation in the mutant animals, compensating for the absence of plakoglobin in epithelial desmosomes.

Previously, human skin blistering diseases such as *Pemphigus vulgaris* and *Pemphigus foliaceus* were found to be caused by auto-antibodies against desmosomal cadherins, Dsg-1 and Dsg-3, respectively (Amagai et al., 1991; Stanley, 1995). Interestingly, rupture within the skin in these diseases occurs exactly in the cell layers in which Dsg-1 or Dsg-3 are most highly expressed. These data suggest a crucial role of desmosomes in the organization and stability of skin tissue. Hereditary human cardiomyopathies are characterized by impaired myocardial contractility and ventricular dilatation, and frequently affect myofibril function (Carter and Rubin, 1994). Mutations in genes coding for components of the cytoskeletal network, i.e., tropomyosin, myosin, and troponin, have been identified in hypertrophic cardiomyopathy (Thierfelder et al., 1994; Marian and Roberts, 1995). The human plakoglobin gene is located on chromosome 17q21 (Aberle et al., 1995), a region not yet identified in human cardiomyopathy patients. However, alterations of desmosomes and other junctions within the intercalated discs have been reported in damaged myocardial tissue as a result of toxic effects (Hull and Lockwood, 1986), in certain cardiomyopathies or in ischaemia (for review see Bullock, 1986). It is therefore tempting to speculate that more subtle alterations of the

plakoglobin gene than the *null* mutation reported here or other types of interferences with plakoglobin might impair heart function and play a role in human heart disease.

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