Plakophilin 1 interferes with plakoglobin binding to desmoplakin, yet together with plakoglobin promotes clustering of desmosomal plaque complexes at cell-cell borders

Elayne A. Bornslaeger[1], Lisa M. Godsel[1], Connie M. Corcoran[1], Jung K. Park[1], Mechthild Hatzfeld[2], Andrew P. Kowalczyk[3,*] and Kathleen J. Green[1,3,‡]

[1]Department of Pathology, Dermatology and the Robert H. Lurie Cancer Center, Northwestern University Medical School, Chicago, IL 60611, USA
[2]Molecular Biology Group of the Medical Faculty, Martin-Luther-University of Halle, Magdeburger Strasse 18, 06097 Halle/Saale, Germany
[3]Department of Dermatology and the Robert H. Lurie Cancer Center, Northwestern University Medical School, Chicago, IL 60611, USA
*Present address: Department of Dermatology, Emory University School of Medicine, Woodruff Memorial Building, Rm 5007, 1639 Pierce Drive, Atlanta, GA 30322, USA
‡Author for correspondence (e-mail: kgreen@northwestern.edu)

Accepted 12 December 2000
Journal of Cell Science 114, 727-738 © The Company of Biologists Ltd

SUMMARY

Desmosomes are adhesive junctions that link intermediate filament networks to sites of strong intercellular adhesion. These junctions play an important role in providing strength to tissues that experience mechanical stress such as heart and epidermis. The basic structural elements of desmosomes are similar to those of the better-characterized adherens junctions, which anchor actin-containing microfilaments to cadherins at the plasma membrane. This linkage of actin to classic cadherins is thought to occur through an indirect mechanism requiring the associated proteins, α- and β-catenin. In the case of desmosomes, both linear and lateral interactions have been proposed as playing an important role in formation of the plaque and linkage to the cytoskeleton. However, the precise nature of these interactions and how they cooperate in desmosome assembly are poorly understood.

Here we employ a reconstitution system to examine the assembly of macromolecular complexes from components found in desmosomes of the differentiated layers of complex tissues. We demonstrate the existence of a Triton-soluble complex of proteins containing full length desmoplakin (DP), the arm protein plakoglobin, and the cytoplasmic domain of the desmosomal cadherin, desmoglein 1 (Dsg1). In addition, full length DP, but not an N-terminal plakoglobin binding domain of DP, co-immunoprecipitated with the Dsg1 tail in the absence of plakoglobin in HT1080 cells. The relative roles of the arm proteins plakoglobin and plakophilin 1 (PKP1) were also investigated. Our results suggest that, in the Triton soluble pool, PKP1 interferes with binding of plakoglobin to full length DP when these proteins are co-expressed. Nevertheless, both plakoglobin and PKP1 are required for the formation of clustered structures containing DP and the Dsg1 tail that ultrastructurally appear similar to desmosomal plaques found in the epidermis. These findings suggest that more than one armadillo family member is required for normal assembly and clustering of the desmosomal plaque in the upper layers of the epidermis.

Key words: Desmosome, Desmoplakin, Plakophilin, Plakoglobin, Desmosomal cadherin

INTRODUCTION

Adherens junctions and desmosomes are intercellular adhesive junctions specialized to provide strong but dynamic cell-cell adhesion in a variety of cell types and tissues (Cowin and Burke, 1996; Garrod et al., 1996; Green and Gaudry, 2000; Kowalczyk et al., 1999a; Schmidt et al., 1994). Members of the cadherin superfamily of adhesion molecules form the adhesive interface of these junctions in each case (Koch et al., 1999; Koch and Franke, 1994; Magee and Buxton, 1991). In addition to mediating adhesion, the cadherins also link the plasma membrane to the underlying cytoskeleton through interactions between their cytoplasmic tails and associated proteins that include catenins (Aberle et al., 1996). For desmosomes, it has been suggested that these indirect linkages may occur through both linear and lateral assemblies of proteins that form a macromolecular complex called the junctional plaque (Kowalczyk et al., 1997; Kowalczyk et al., 1999b; Smith and Fuchs, 1998). However, the mechanism of assembly of the junctional plaque is still only poorly understood.

Desmosomes arose relatively late in evolution, after the appearance of adherens junctions and as organisms increased in size and complexity. This fact is reflected by the abundance of desmosomes in tissues that experience mechanical stress. The experimental ablation or mutation of desmosomal plaque and transmembrane components results in defects in adhesion and tissue integrity in mice (Allen et al., 1996; Bierkamp et al., 1996; Gallicano et al., 1998; Koch et al., 1997; Ruiz et al., 1996). In addition, human autoimmune and inherited diseases

that target the desmosomal cadherins lead to similar defects in tissue integrity (Rickman et al., 1999; Stanley, 1995). It has been shown that mutations in the desmosomal plaque protein plakophilin 1 (PKP1) result in a skin fragility syndrome (McGrath et al., 1997) and that haploinsufficiency in desmoplakin (DP) leads to a form of striate palmoplantar keratoderma (SPPK) (Armstrong et al., 1999). More recently, truncating mutations of plakoglobin and DP have been shown to cause cardiac and skin defects in humans (McKoy et al., 2000; Norgett et al., 2000).

Based on these findings, there is ample evidence to support the importance of desmosomes in tissue integrity. However, we are only now beginning to appreciate the true extent of desmosomal structural and possible functional complexity. Desmosomes are commonly expressed in all types of epithelia, but are also found in a restricted number of other specialized tissues such as the myocardium and Purkinje fiber cells of the heart, meningeal cells and the follicular dendritic cells of lymph nodes (Schwarz et al., 1990). These junctions are particularly abundant in complex epithelia such as the epidermis; however, their number, composition and structure vary depending on tissue type and during terminal differentiation (Kowalczyk et al., 1999a). Six desmosomal cadherin genes have been identified and divided into two subclasses called desmogleins and desmocollins (Buxton et al., 1994; Koch and Franke, 1994; Kowalczyk and Green, 1996). These cadherins are each expressed in a tissue- and differentiation-specific pattern. Other proteins that contribute to plaque assembly and linkage of intermediate filaments (IF) to the desmosomal cadherin tails are members of the armadillo gene family, found in intercellular junctions and the nucleus. The desmosomal arm proteins include plakoglobin and plakophilins 1-3 (Anastasiadis and Reynolds, 2000; Gelderloos et al., 1997; Hatzfeld, 1999). The plakophilins also exhibit tissue-specific and differentiation-specific distributions that may translate into differences in desmosome function in different body sites. Recent work from our lab and others has shown that two arm family members, PKP1 and plakoglobin, both interact with the obligate desmosomal component and IF-associated protein, DP (Hatzfeld et al., 2000; Hofmann et al., 2000; Kowalczyk et al., 1997; Kowalczyk et al., 1999a; Smith and Fuchs, 1998), and that this interaction is mediated by the DP N terminus, the domain that targets this IF linker to the desmosomal plaque (Hatzfeld et al., 2000; Kowalczyk et al., 1999a).

In the present work we explore the assembly properties of proteins found in desmosomes of the differentiated layers of complex epithelia. To overcome difficulties inherent in studying these proteins in situ, where their insolubility hampers the examination of protein-protein interactions, we have employed a reconstitution approach. Using this approach we demonstrate here the existence of a complex containing DP, plakoglobin and the desmoglein 1 (Dsg1) tail. Although plakoglobin is required for the formation of triple complexes containing the first 584 amino acids of the DP N terminus, full length DP associates with the Dsg1 tail in the presence and absence of plakoglobin in HT1080 cells. This observation raises the possibility that plakoglobin-independent interactions between downstream sequences in DP and the Dsg1 tail may occur. We also examine the relative abilities of PKP1 and plakoglobin to interact with full length DP. When both PKP1 and plakoglobin are present, Triton-soluble full-length DP associates preferentially with PKP1, whether in the absence or presence of the Dsg1 tail. In spite of this difference in binding preference for DP, our results indicate that plakoglobin and PKP1 are both required for the organization of components into clustered structures appearing ultrastructurally similar to the desmosomal plaque. These results suggest that the repertoire of arm family members and associated cadherin tails expressed in a particular desmosome will have an important impact on the assembly, morphology and function of junctions in different cells and stages of differentiation.

MATERIALS AND METHODS

Generation of expression constructs

The E-cadherin/Dsg1 chimera (E-Dsg1), comprising the extracellular domain of mouse E-cadherin and the cytoplasmic domain of human desmoglein 1, in the expression plasmid LK444 under the control of the β-actin promoter was described previously (Kowalczyk et al., 1997). As previously reported, the presence of the E-cadherin extracellular domain serves to stabilize the Dsg1 tail at cell-cell borders, which happens only inefficiently in the case of Dsg1 alone, possibly due to the absence of a desmosomal cadherin adhesion partner (Kowalczyk et al., 1997). Also described previously were constructs encoding full-length human plakoglobin in LK444 (Kowalczyk et al., 1994; Palka and Green, 1997) and a CMV construct encoding a C-terminally FLAG-epitope tagged polypeptide comprising the first 584 amino acids in DP, called DPNTP (DPNTP.FLAG) (Kowalczyk et al., 1999b). The full-length ‘a’ form of PKP-1 was assembled and cloned into pCMV script as described (Kowalczyk et al., 1999b).

Full length C-terminally FLAG-tagged DP (DP.FLAG; p806) was generated as follows. A 3.1 kb *Not*I-*Eco*RV fragment corresponding to nucleotides –332 to 3671 of DP was isolated from p611, a pSK.DPΔC construct. This fragment was ligated to *Not*I-*Eco*RV pCMV5c (SIGMA) to generate p794. An 8.6 kb *Sac*II-*Pml*I insert corresponding to nucleotides 28-8616 was isolated from p613, pCMV.DP.myc, and ligated to p794 digested with *Sac*II and *Eco*RV to generate p806.

Cell culture and transfections

HT1080 human fibrosarcoma cells, a kind gift from Dr Noel Bouck, Northwestern University, and COS-7 cells were cultured in DMEM containing 10% fetal bovine serum and penicillin/streptomycin (Kowalczyk et al., 1997). Transient transfections of HT1080 cells were performed using Fugene 6 reagent (Roche-Boeringer Mannheim) and were assayed 24 hours following transfection. Transient transfections of COS-7 cells were performed using either Fugene 6 or calcium phosphate as previously described (Stappenbeck and Green, 1992).

Immunofluorescence

Cells were grown on glass coverslips, rinsed in phosphate buffered saline and fixed in methanol at –20°C for two minutes. E-Dsg1 was detected by the rat monoclonal antibody DECMA-1 directed against the extracellular domain of E-cadherin (a gift from Dr Stephen Byers, Georgetown University). Plakoglobin was detected by the mouse monoclonal antibody 11E4 (a gift from Dr M. Wheelock, University of Toledo; Kowalczyk et al., 1994) or chicken antibody 1407 (C. A. Gaudry et al., unpublished). A rabbit antibody #667 against the PKP1 head domain was used to detect PKP1 (Hatzfeld et al., 2000). DPNTP.FLAG or full length DP.FLAG were detected by the rabbit polyclonal antibody NW161 (Bornslaeger et al., 1996), directed against a bacterially expressed polypeptide comprising the first 189 amino acids of DP, by PC28, a mouse monoclonal antibody directed

against the DP central rod domain (a gift from Dr Pamela Cowin), NW6, directed against the DP C terminus (Angst et al., 1990) or by M2 (Sigma Chemicals, St Louis, MO), a mouse monoclonal antibody directed against the FLAG tag. It should be noted that due to the high ratio of ectopic to endogenous protein, similar results were obtained with either M2 or DP-specific antibodies in transient transfections. Primary antibodies were detected using Alexa conjugates (Molecular Probes, Eugene, Oregon). Coverslips were examined using a Leica DMB microscope and images captured using a Hamamatsu Orca digital camera and Improvision Openlab software.

Electron microscopy

Transiently transfected COS 7 cells grown on Permanox culture dishes (Electron Microscopy Sciences, Ft. Washington, PA) were processed for conventional electron microscopical analysis as previously described (Jones and Goldman, 1985) and stained sections were viewed and photographed using a JEOL 100CX electron microscope. Parallel immunofluorescence experiments were carried out for each transfection combination to ensure that ultrastructural analysis was carried out on populations exhibiting high co-transfection efficiencies.

Immunoprecipitation, Triton solubility and Immunoblot analysis

HT1080 or COS-7 cells transiently expressing junctional components were lysed in Tris buffered saline (TBS) containing 0.5% Triton X-100; lysates were vortexed and cleared by centrifugation at 14,000 ***g*** for 30 minutes. To immunoprecipitate FLAG-tagged DP polypeptides and associated proteins, lysates were incubated with M2 agarose beads (Sigma, St Louis, MO) for 1 hour at 4°C. Beads were captured by centrifugation at 11,000 ***g*** for 2 minutes and washed 4 times by vortexing for 30 seconds each in TBS-0.5% Triton at 4°C. Proteins were released by incubation with reducing SDS-containing sample buffer and analyzed by SDS PAGE and immunoblot analysis. For fractionation of transfected cells into Triton soluble and insoluble pools, cells were lysed in 0.5% Triton X-100 containing buffer. Lysates were vortexed, centrifuged, and the cytoskeletal-associated fraction was subsequently solubilized in urea sample buffer as described (Jones and Goldman, 1985). The E-Dsg1 chimera was detected with DECMA or ECCD-2 (a gift from M. Takeichi, Kyoto University, Kyoto Japan). Plakoglobin was detected either by the rabbit polyclonal antibody VB3 (a gift from F. Watt, Imperial Cancer Research Fund, London) or a polyclonal chicken antibody 1407, developed against human plakoglobin (C. A. Gaudry et al., unpublished). β-Catenin was detected using the mAb C2206 (Sigma, St Louis, MO). PKP1 was detected with polyclonal antibody 667 (Kowalczyk et al., 1999b). DP and DPNTP were detected using the polyclonal antibody NW161 (Bornslaeger et al., 1996). After incubation with HRP conjugated goat anti-rabbit or goat anti-chicken IgG (Jackson ImmunoResearch, West Grove, PA or Aves Labs, Tigard, Oregon, respectively), immunoreactive proteins were detected by enhanced chemiluminescence (ECL; Amersham Pharmacia Biotech). Proteins present in the soluble and insoluble fractions were quantified by scanning densitometry using Molecular Analyst software.

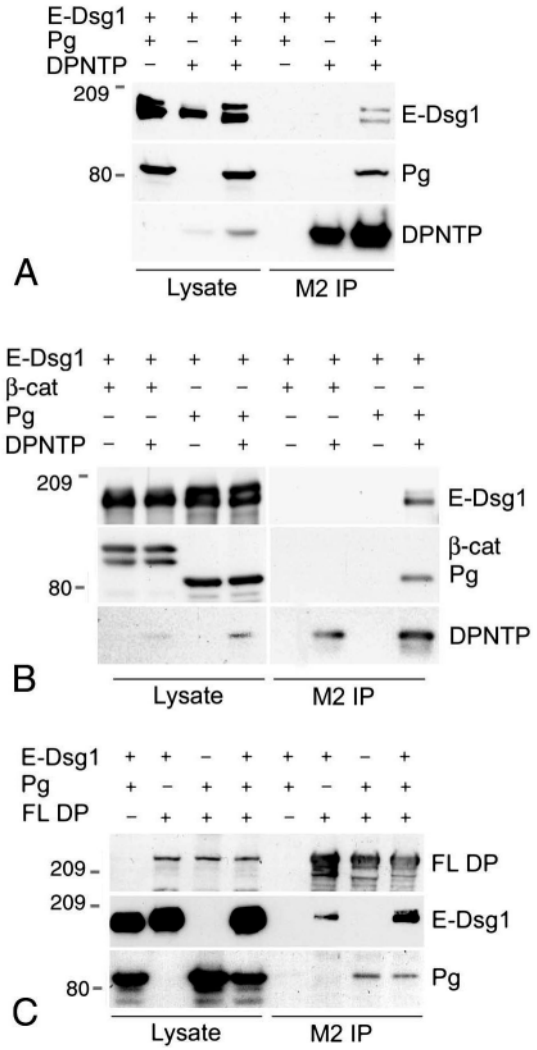

Fig. 1. Formation of complexes containing E-Dsg1 and desmoplakin. HT1080 cells were transfected with combinations of cDNAs as indicated above. M2 agarose was used to immunoprecipitate FLAG-tagged DPNTP (A,B) or full length DP (C), and complexes were analyzed by SDS-PAGE/immunoblotting using antibodies directed against individual components in the complex. The identities of the proteins in the complex are noted at the right, and molecular masses at the left. Note that the presence of E-Dsg1 in the DPNTP complex requires plakoglobin (Pg, A), but some E-Dsg1 is seen associated with FL DP in the absence of Pg (C). β-Catenin cannot substitute for Pg in this assay (B). In the absence of FLAG-tagged DP, neither plakoglobin or E-Dsg1 non-specifically associate with M2-agarose. The two bands recognized by DECMA represent processed and unprocessed forms of the cadherin. In C, these forms are not as well resolved.

RESULTS

The DP N terminus forms a triple complex containing the desmoglein 1 tail which requires plakoglobin

We previously demonstrated that an N-terminal polypeptide comprising 584 amino acids of DP, DPNTP, is recruited to a chimeric cadherin with the cytoplasmic domain of human Dsg1 and the extracellular domain of mouse E-cadherin (Kowalczyk et al., 1997). Recruitment of DPNTP to the Dsg1 tail was dependent on the co-expression of plakoglobin in COS-7 epithelial cells and mouse L cell fibroblasts (Kowalczyk et al., 1997). This observation, in conjunction with data showing that plakoglobin associates directly with both DP (Kowalczyk et al., 1997; Smith and Fuchs, 1998), and desmoglein 1 (Mathur et al., 1994; Witcher et al., 1996), suggested the existence of a triple complex in which DP's association with the Dsg1 tail is mediated indirectly by the arm protein plakoglobin. However, to date, a cellular complex containing DP and desmosomal

cadherins has not been detected. This is likely due to the fact that DP, once assembled into junctions, is largely insoluble, making it difficult to assay for DP-containing protein complexes.

To examine whether the N terminus of DP forms a triple complex with plakoglobin and the Dsg1 tail, we transiently expressed DPNTP.FLAG, with or without plakoglobin and E-Dsg, into COS cells, which have very few desmosomes (Kowalczyk et al., 1997) or HT1080 fibrosarcoma cells, which do not have DP and do not assemble desmosomes (Chitaev and Troyanovsky, 1997). To assess whether the previously observed recruitment of DPNTP to cell-cell borders reflected the formation of complexes containing all three proteins, analysis of the Triton-soluble lysate was carried out (Fig. 1, and data not shown). Because DP is largely insoluble (see Fig. 9), we concentrated the limited pool of soluble DP by immunoprecipitation. M2 agarose was used to precipitate DPNTP.FLAG, complexes were separated by SDS-PAGE and transferred to nitrocellulose, and the presence of plakoglobin and the E-Dsg1 chimera was assessed by immunoblotting. As shown in Fig. 1A, both plakoglobin and the chimeric cadherin were identified in a complex with DPNTP.FLAG, and this complex was only observed in the presence of plakoglobin. The E-Dsg1 chimera was not observed in a complex with DPNTP when co-expressed with the related arm protein β-catenin instead of plakoglobin, providing further evidence that plakoglobin is specifically required for this association (Fig. 1B). Similar experiments demonstrated that a triple complex containing the Dsc2 tail also forms under these conditions (not shown). These results suggest that the 584 residue N-terminal region of DP interacts indirectly with desmosomal cadherin tails by virtue of its association with plakoglobin.

Full length DP associates with E-Dsg1 and is recruited to E-Dsg1:plakoglobin at cell-cell borders

To examine whether full length DP behaves similarly to DPNTP, recruitment and immunoprecipitation assays were carried out using DP.FLAG. In the absence of a co-expressed cadherin, full length DP colocalized mostly with IF networks, as expected from its known association with IF polypeptides (Kouklis et al., 1994; Meng et al., 1997; Stappenbeck et al., 1993; Stappenbeck and Green, 1992) (Fig. 2B). Plakoglobin appeared largely diffuse in the cytoplasm in methanol-fixed cells (Fig. 2A), although some co-localization with IF networks can be seen in detergent-extracted cells (not shown). It is important to note that both plakoglobin and full length DP are capable of incorporating into desmosomes on their own (Palka and Green, 1997; Smith and Fuchs, 1998; Stappenbeck et al., 1993); this localization is more apparent in cells with abundant junctions and in cells expressing lower levels of protein. To test whether a desmosomal cadherin will recruit plakoglobin and DP.FLAG to the cell-cell borders, these proteins were co-expressed with the E-Dsg chimera. In these cells, both plakoglobin (not shown) and DP colocalized at cell-cell borders (Fig. 2C-D, arrows).

To examine whether, like DPNTP, full length DP forms a complex with the Dsg1 tail that is dependent on the presence of plakoglobin, M2 agarose was used to precipitate DP.FLAG and the presence of protein binding partners was assessed as described above. As shown in Fig. 1C, full length DP was observed in a complex with plakoglobin and E-Dsg1. Interestingly, E-Dsg1 was also detected in a complex with DP.FLAG in the absence of plakoglobin (Fig. 1B), raising the possibility that sequences downstream in full length DP that are not present in DPNTP may contribute to interactions with the E-Dsg1 tail.

Full length desmoplakin binds preferentially to the arm protein PKP1 in the presence of plakoglobin

The results described above support the idea that DP is linked

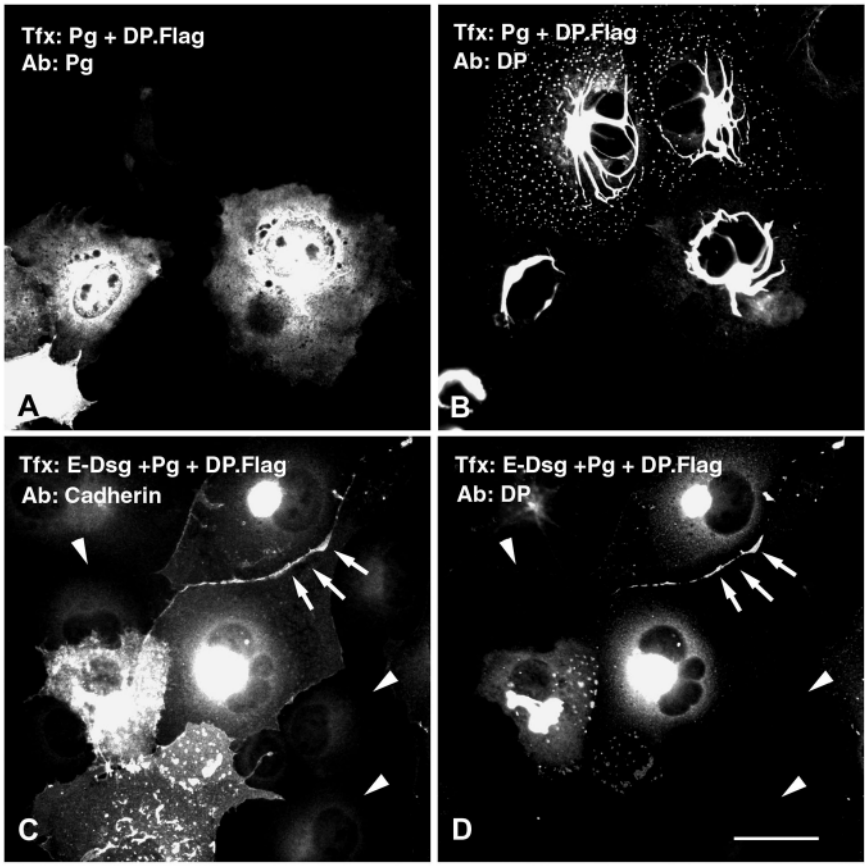

Fig. 2. Full length DP is recruited to the Dsg1 tail in COS cells in the presence of plakoglobin. C-terminally FLAG-tagged desmoplakin (DP.FLAG) was co-transfected with plakoglobin (Pg) alone (A and B) or together with a chimeric cadherin containing the E-cadherin extracellular domain and the Dsg1 cytoplasmic domain (E-Dsg) into COS-7 cells (C and D). Double label immunofluorescence was carried out using antibodies against plakoglobin (A), DP (B,D) and E-cadherin (C). In the absence of a cadherin, plakoglobin and DP is localized primarily in the cytoplasm. In cells expressing the Dsg1 tail and plakoglobin, full length DP is recruited to the plasma membrane where it co-localizes with Pg (not shown) and E-Dsg (arrows in C and D) Similar results were obtained when an M2 antibody against the FLAG tag on DP was used. The E-cadherin antibody used in this experiment does not recognize untransfected cells, denoted by arrowheads. Bar, 20 μm.

to the plasma membrane through plakoglobin and the desmosomal cadherin tails. However, it has also been proposed that lateral interactions play an important role in plaque assembly. We reported that PKP1 may be a candidate for mediating lateral interactions by binding to DP and enhancing its recruitment to cell borders in the most differentiated layers of the epidermis (Kowalczyk et al., 1999b). However, PKP1 is expressed in these same layers along with plakoglobin; furthermore, both PKP1 and plakoglobin have been shown to interact with the same 584 amino acid region of the DP N terminus (Kowalczyk et al., 1997; Kowalczyk et al., 1999b). Thus the question arises as to whether PKP1 binding influences binding of plakoglobin to this domain, and if so, what the impact will be for desmosome plaque assembly and function.

We previously showed that PKP1 recruits DPNTP to cell-cell borders in transient transfection assays (Kowalczyk et al., 1999b). To address whether full length DP is also recruited to borders by PKP1, DP.FLAG was expressed with or without PKP1 in COS cells. Immunofluorescence analysis showed that like DPNTP, full length DP is recruited to plasma membranes in the presence of PKP1 (Fig. 3E,F). Likewise, an obvious increase in continuous staining for endogenous DP was observed in COS cells singly transfected with PKP1 (Fig. 3C,D).

Next, to examine the relative ability of DP to associate with PKP1 and plakoglobin, full length DP.FLAG was transfected along with PKP1, plakoglobin or both PKP1 and plakoglobin in COS cells. Triton soluble lysates were isolated, DP.FLAG was precipitated with M2 agarose and complexes were analyzed by SDS-PAGE and immunoblotting. As shown in Fig. 4 when expressed separately, both PKP1 and plakoglobin interacted with full length DP. When DP, plakoglobin and PKP1 were expressed together, only PKP1 was clearly seen in a complex with DP (Fig. 4). Plakoglobin was undetectable under these conditions, and only barely detectable at higher exposures (Fig. 4, right lane).

Roles of PKP1 and plakoglobin in organization of desmosomal plaque components

To further explore the contribution of PKP1 and plakoglobin to desmosome plaque assembly, we carried out a series of co-transfections, followed by localization of ectopically expressed proteins in COS cells. As shown in Fig. 5, when E-Dsg1 was co-expressed with PKP1 and DP.FLAG, the protein distributions shifted relative to those observed when PKP1 and DP (Fig. 3) were co-expressed. Some cell border staining was observed in cells co-expressing PKP1, DP and E-Dsg (Fig. 5A-D, arrows), but the majority of staining appeared in cytoplasmic linear arrays co-localizing with keratin IF (data not shown). The appearance of this particular pattern was dependent on the co-expression of all three components, as cells within the same population, expressing only one or two of the proteins, displayed different patterns of localization. (See, for instance, the cell in 5C marked by arrowheads which exhibits a more diffuse, cell surface pattern and expresses E-Dsg1 but not PKP1).

However, when all four proteins, PKP1, plakoglobin, DP.FLAG and E-Dsg1, were co-expressed in COS cells, many of the cells present in the population exhibited a pattern of dots in the cytoplasm (arrowheads in Fig. 7) and at cell borders (arrows in Figs 6 and 7). The extent of cell-cell border staining varied depending on expression levels within individual cells in the population, ranging from more widely-spaced dots to more heavily stained but punctuated borders. In high expressers the dots frequently appeared stitched together into a more continuous but bumpy pattern that was distinct in character from

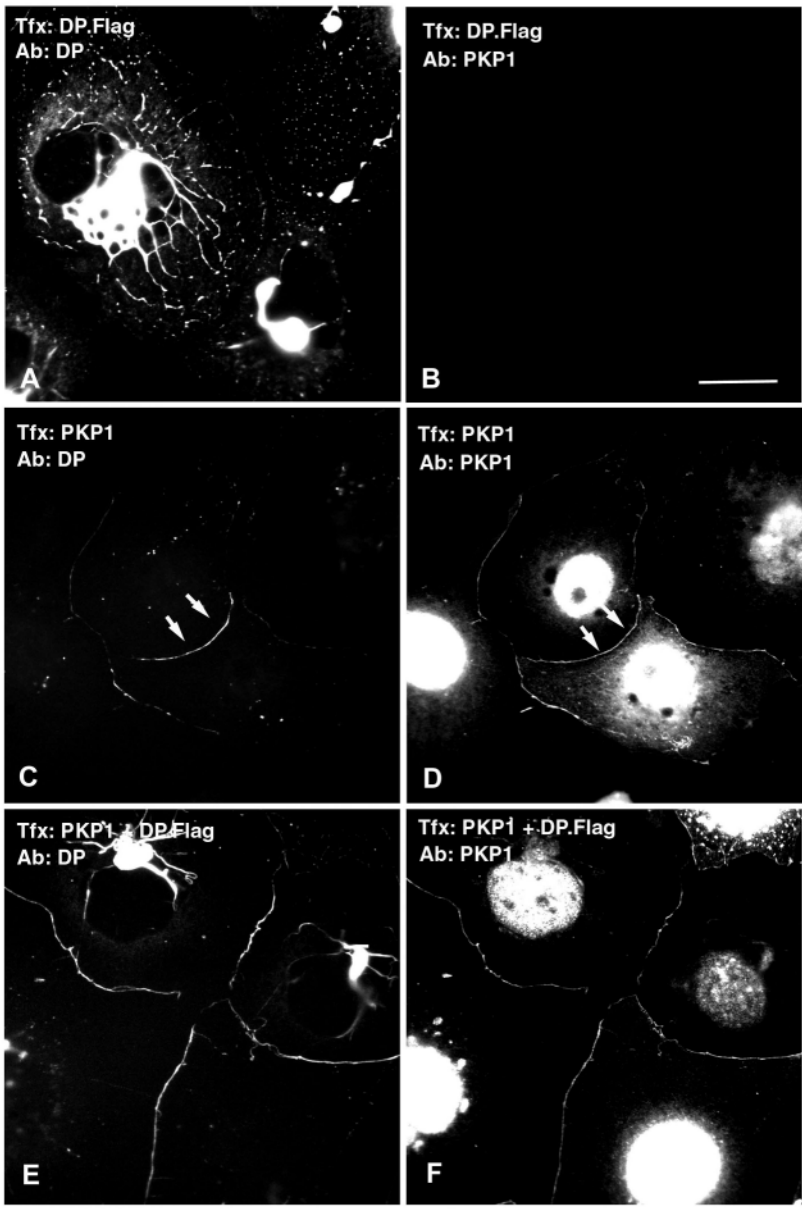

Fig. 3. PKP1 enhances recruitment of full length DP to cell-cell borders. COS-7 cells were transfected with full length DP.FLAG only (A,B), PKP1 only (C,D), or both DP.FLAG and PKP1 (E,F) and processed for dual label fluorescence using antibodies against DP (left panels) or PKP1 (right panels). Note enhanced, continuous, staining for the limited amount of endogenous DP denoted by arrows at cell borders in C, and efficient recruitment and co-localization of DP and PKP1 in a continuous pattern at cell-cell interfaces in E and F. Bar, 20 μm.

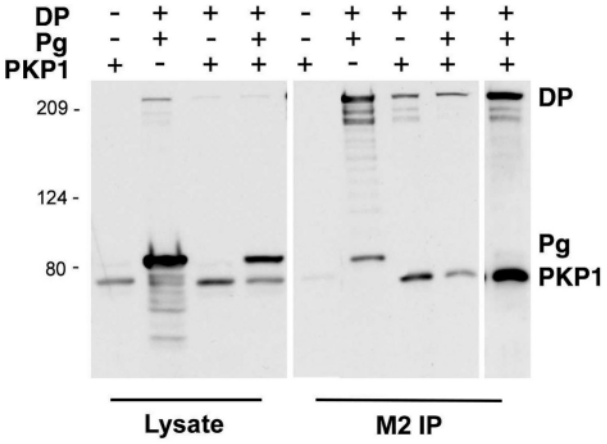

Fig. 4. Preferential association of full length DP with PKP1 in the presence of plakoglobin. COS-7 cells were transfected with DP.FLAG and plakoglobin, DP.FLAG and PKP1 or all three cDNAs, followed by immunoprecipitation of DP as described in Materials and Methods. Immunoblots were reacted with a pool of antibodies directed against all three components. Both plakoglobin and PKP1 interact with DP when expressed as pairs. In the presence of all three proteins, only PKP1:DP complexes are detected in the Triton soluble pool. Note that only a very small amount of non-specific binding of PKP1 is seen in the beads only control lane. The lane on the far right is a longer exposure of the lane to its left.

the smoother swathlike borders observed in cells expressing plakoglobin, DP and E-Dsg1 (compare Figs 6A-D and 7A-B with Fig. 2C,D) or PKP1 and DP only (compare Figs 6A-D and 7A-B with Fig. 3E,F). The borders seen within the population of cells ectopically expressing PKP1, DP and the E-Dsg1 chimera also tended to be more continuous in nature than those in quadruply transfected cells (compare Figs 6A-D and Fig. 7A-B with Fig. 5A-D). Thus, in this reconstitution system, ectopic PKP1 and plakoglobin appeared to be required to efficiently cluster desmosomal plaque components into punctate structures.

PKP1 is required for the formation of plaque-containing structures ultrastructurally resembling desmosomes in the COS cell reconstitution system

In order to more closely examine differences in cytoplasmic and border staining among transfected populations and to compare the appearance of assembled structures to desmosomal plaques, ultrastructural analysis was performed. Due to the presence of some endogenous plakoglobin in COS cells, particular focus was placed on a comparison of triple transfectants expressing E-Dsg1, plakoglobin and DP with quadruple transfectants expressing E-Dsg1, plakoglobin, PKP1 and DP. Cells in triply transfected cell populations exhibited extended plaque structures at cell-cell borders not found in untransfected or vector only controls (compare Fig. 8A to D). These plaques had a fine filamentous substructure which appeared similar to meshworks previously seen in the cytoplasm of COS-7 cells transiently transfected with DP constructs lacking the plasma membrane targeting domain (Stappenbeck and Green, 1992). The plaques lacked an electron dense region subjacent to the plasma membrane (compare Fig. 8A to D).

Transfected cells in populations expressing E-Dsg1, plakoglobin, PKP1 and DP were readily identified due to the abundance of membrane-associated, multi-layered, paired and unpaired plaques grouped together in the cytoplasm. Nothing resembling these structures was seen in the triply transfected cell populations lacking PKP1 or in control cells. Thus it seems likely that these membrane-associated structures corresponded to the dots seen in the cytoplasm of quadruply transfected cells (Figs 6 and 7) which co-stained in triple label immunofluorescence experiments (Fig. 7, arrowheads). The plaques were often semicircular or arc-shaped, apparently associated with membrane vesicles (Fig. 8B,C,E,F). A thinner

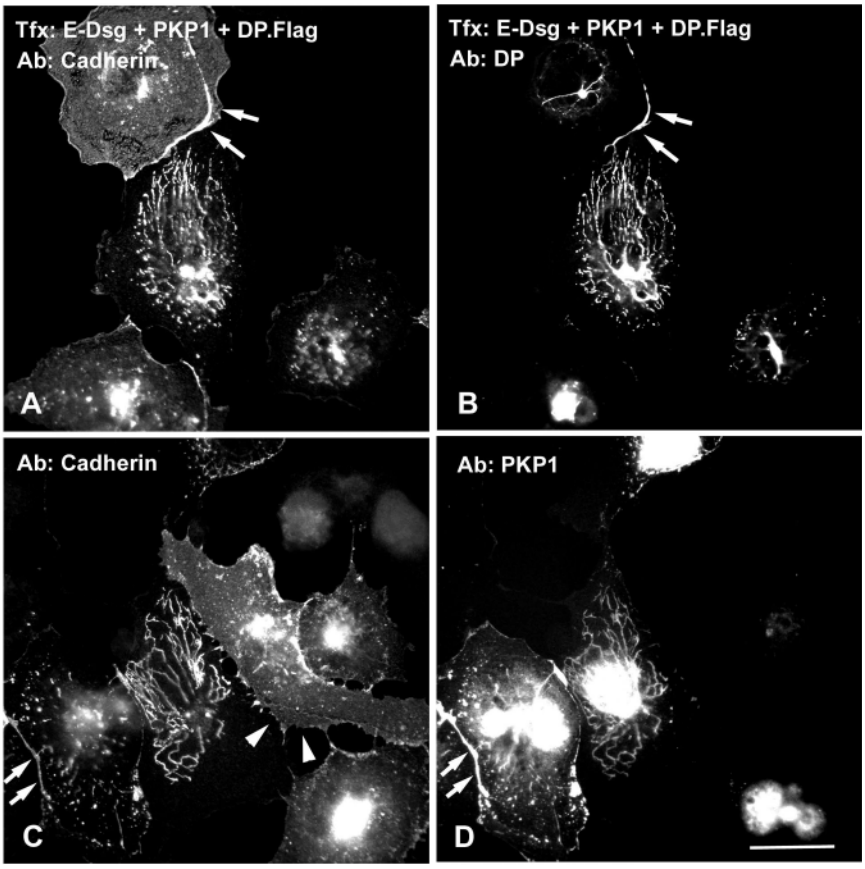

Fig. 5. Localization of E-Dsg1, PKP1 and full length DP in transiently transfected COS cells. COS-7 cells were transfected with full length DP.FLAG, E-Dsg1, and PKP1 and processed for dual label fluorescence with antibodies directed against the extracellular domain of E-cadherin (A,C), DP (B) or PKP1 (D). The cytoplasmic fibrous, semi-punctate arrays characteristic of this combination of transfected cDNAs align with keratin filaments (not shown). Note also cell-cell border staining is continuous in nature (arrows A-D) compared with the interrupted staining often seen in quadruply transfected cells (compare with Figs 6 and 7). Bar, 20 μm.

approximately 20 nm electron dense region resembling the outer dense plaque of desmosomes, was subjacent to a less electron dense, fibrous region measuring ~75 nm. This fibrous region of the plaque was thicker than that associated with the smaller desmosomes in control COS cells (compare control in Fig. 8D with E). In many areas, the plaque structures were associated with, and even connected by, surrounding 10 nm IF (Fig. 8B′,C,F, arrow). A proportion of these intracellular structures in each transfected cell exhibited paired mirror-image plaques (Fig. 8B′ and C). Electron dense plaques were

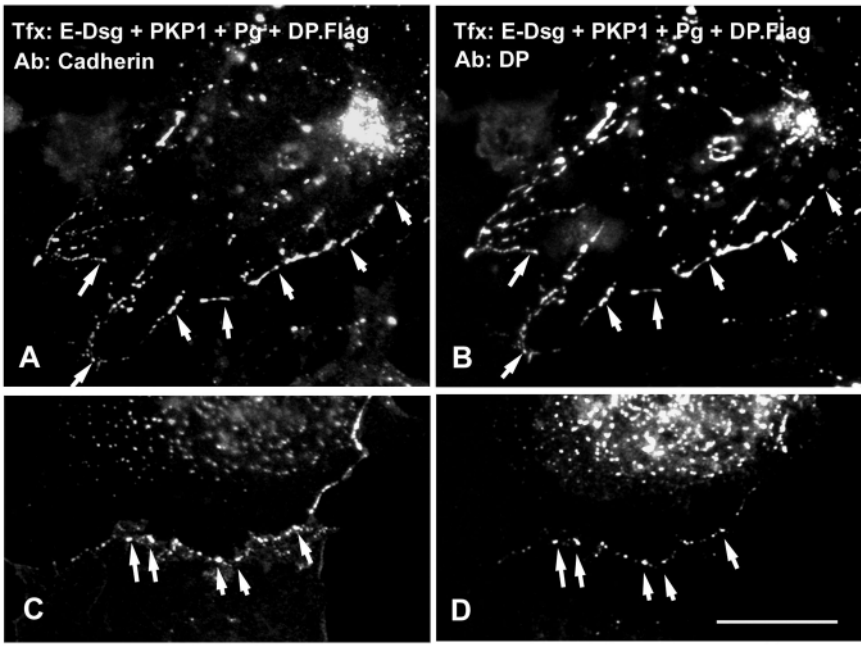

Fig. 6. Co-expression of E-Dsg1, PKP1, plakoglobin and full length DP lead to the formation of punctate structures in the cytoplasm and at cell-cell borders. COS-7 cells were transfected with full length DP.FLAG, E-Dsg1, plakoglobin and PKP1 and processed for dual label fluorescence with antibodies directed against E-cadherin (A,C) and DP (B,D). Co-expression with plakoglobin results in the disappearance of most of the fibrous arrays observed in its absence. Note the presence of punctate structures at cell-cell borders (arrows). Bar, 20 μm.

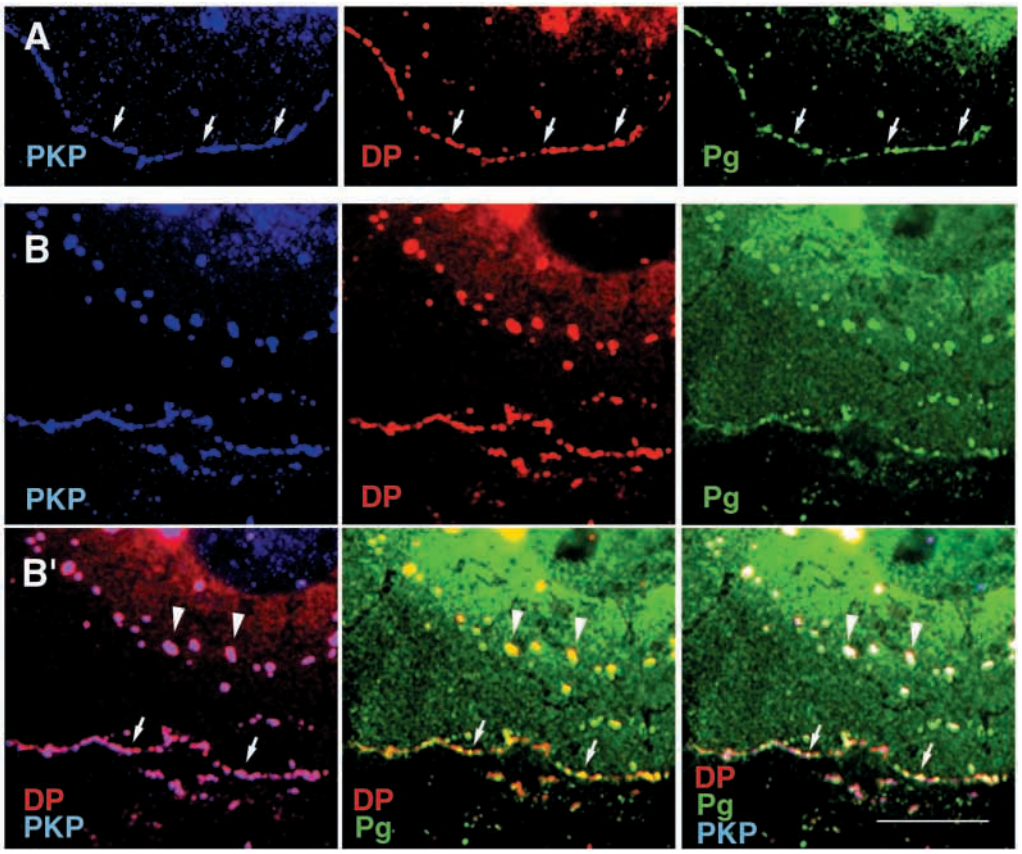

Fig. 7. Co-localization of DP, PKP1 and plakoglobin in punctate structures in cells transfected with full length DP.FLAG, E-Dsg1, plakoglobin and PKP1. COS-7 cells were quadruply transfected and processed for triple label immunofluorescence with antibodies directed against desmoplakin (DP, red), plakophilin 1 (PKP1, blue) and plakoglobin (Pg, green). (A) Independent staining patterns for each protein which co-localize extensively in punctate structures as denoted by arrows. (B) A second cell border, again showing the localization of each protein independently. (B′) Three sets of overlays for the cell border shown in B. On the left, DP and PKP are shown, with the pink color indicating overlap of the blue and red staining patterns. Note that DP and PKP1 overlap almost completely. In the middle panel, DP and Pg are shown, with the yellow color seen in cytoplasmic dots and at cell borders indicating co-localization and overlap of the green and red staining patterns. In this particular example, the overlap is not as complete as that exhibited by PKP1 and DP, consistent with biochemical data showing that PKP1 is DP's preferred binding partner. The right panel shows a triple overlay, with the white staining corresponding to areas where all three proteins co-localize. Arrows in each panel denote examples of puncta where all three proteins localize. Bar, 10 μm.

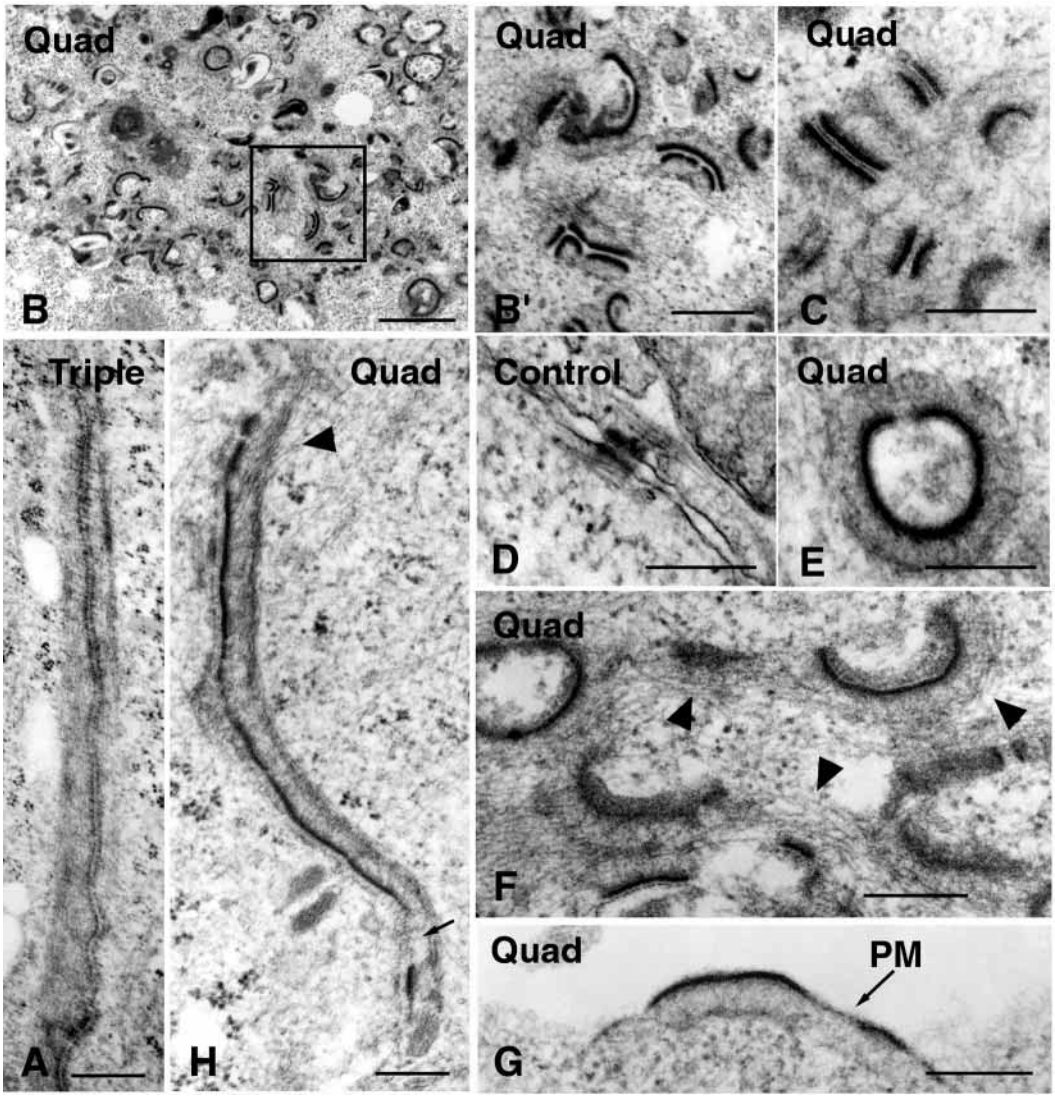

Fig. 8. Formation of desmosome-like plaques in transfected COS cells requires PKP1 expression. Comparative ultrastructural analysis of COS cells transiently transfected with E-Dsg1, PKP1, and full length DP ('Triple', A), or quadruply transfected with E-Dsg1, PKP1, plakoglobin and full length DP ('Quad', panels B,B′,C,E,F,G) and processed for conventional EM. A desmosome from a vector only control COS cell population is shown in D. Note distinctive multi-layered plaques associated with structures in the cytoplasm and cell borders found only in quadruply transfected populations. These plaques were composed of a 20 nm electron dense outer layer and a 75 nm fibrous inner layer associated with IF (arrowheads in F,H). B′ is a higher magnification of the boxed in portion of B, showing several examples of paired plaques found within the group of cytoplasmic membrane-associated structures. A and H provide a comparison of plaques at cell borders in triply (A) and quadruply (H) transfected cells. G shows an example of an unpaired plaque on the cell surface of a quadruply transfected cell (PM=plasma membrane). Bars: 1 μm (B); 0.5 μm (B′,A,H); 0.25 μm (C,D,E,F,G).

also present on plasma membranes, both in the presence (Fig. 8H) and absence (Fig. 8G) of neighboring cells. Plaques at cell-cell borders occupied varying lengths of plasma membrane, and frequently appeared to be composed of shorter structures fused in linear arrays and interrupted by less electron dense regions (e.g. arrow in Fig. 8H).

Solubility profiles of desmosomal components correlates with their assembly into plaque structures

During desmosome assembly precursor proteins are recruited from a Triton-soluble to a Triton-insoluble pool, which is thought to reflect assembly into intercellular junctions and association with the IF cytoskeleton (Pasdar and Nelson, 1988; Pasdar and Nelson, 1989; Penn et al., 1987). To assess the impact of co-expression of specific desmosomal proteins on their solubility, a series of co-transfections was carried out, followed by fractionation into Triton-soluble and Triton insoluble pools. As shown in Fig. 9, the E-Dsg1 chimera is largely soluble when expressed on its own, or co-expressed with plakoglobin, PKP1 or both arm proteins. Co-expression of E-Dsg1 and full length DP shifts the cadherin from a soluble:insoluble ratio of ~75:15 to ~50:50. Co-expression of E-Dsg1 with both arm proteins and DP leads to an additional decrease in E-Dsg1 solubility to ~30:70. The majority of protein in the quadruply tranfected cells is found in the Triton-

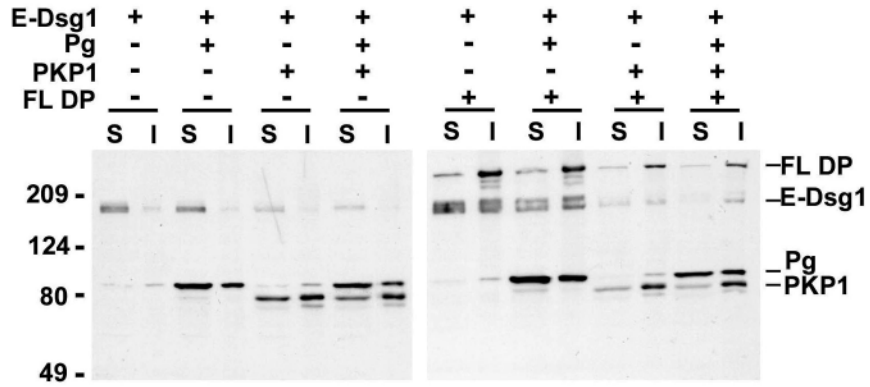

Fig. 9. Solubility of reconstituted desmosomal complexes. COS-7 cells were transfected with various combinations of E-Dsg1, plakoglobin, PKP1 and DP.FLAG as indicated above the blots. 24 hours after transfection, cells were fractionated into 0.5% Triton-soluble and insoluble pools, which were subsequently analyzed by SDS-PAGE and immunoblotting with pooled antibodies against all components as described in Materials and Methods. The position of each protein is noted at the right. Both the processed and unprocessed form of E-Dsg1 are visible in some lanes. The majority of full length DP is insoluble in each case. E-Dsg1 solubility is shifted from a soluble to insoluble ratio of ~75:15 when expressed alone, to ~50:50 when co-expressed with DP, to ~30:70 when co-expressed with a combination of DP and arm proteins.

insoluble pool, likely representing the immunofluorescent structures seen in Figs 6 and 7 and plaque-containing structures seen in Fig. 8.

DISCUSSION

In this study we have investigated the contribution of components found in desmosomes of the differentiated layers of complex tissues to the assembly of the desmosomal plaque. In particular our results shed light on the relative roles played by two members of the desmosomal arm family, plakoglobin and PKP1.

Association of DP with the Dsg1 tail in the presence and absence of plakoglobin

It is now possible to make a comparison between the protein complexes in which classic versus desmosomal cadherins participate. In the case of classic cadherins, it has been shown that the E-cadherin tail binds directly to β-catenin which in turn binds to α-catenin (Jou et al., 1995). Here we have demonstrated that there exists a triple complex containing DP, plakoglobin, and a desmosomal cadherin tail. The presence of E-Dsg1 in a complex with the N-terminal DPNTP domain is dependent on the presence of plakoglobin. This observation is consistent with previous data showing that the Dsg1 tail did not recruit the N-terminal DPNTP region of DP to COS cell borders in the absence of ectopically expressed plakoglobin (Kowalczyk et al., 1997). In addition, E-Dsg1 is not observed in the complex when plakoglobin is replaced with β-catenin, consistent with previously reported yeast two hybrid data showing that DP is unable to bind directly to β-catenin (Kowalczyk et al., 1998).

In addition, in contrast to α-catenin, which interacts only indirectly with E-cadherin through β-catenin (Jou et al., 1995), full length DP was associated with the Dsg1 tail in the absence of ectopically expressed plakoglobin, albeit at a somewhat reduced level. This experiment was done in HT1080 cells which express extremely low levels of plakoglobin that are undetectable in this experiment, suggesting the possibility that sequences in full length DP, downstream of the first 584 amino acids, can interact under certain conditions with the Dsg1 tail in a plakoglobin-independent fashion. Although it seems likely that in desmosome-containing cells desmosomal cadherins are usually associated with an arm family member, interactions outside the arm binding domain could provide another mechanism for stabilizing the complex and promoting lateral interactions in the plaque. We cannot rule out the presence of endogenous HT1080 proteins in the observed FL DP: E-Dsg1 complex; however, it seems likely that in this transient overexpression system such proteins would be present in minor amounts.

Supporting the idea that plakoglobin-independent interactions between DP and desmosomal cadherins can occur, an interaction between DP and the desmocollin 1 tail was reported (Troyanovsky et al., 1994). More recent studies have suggested the Dsc1 tail can interact directly with DP, and that the first 176 amino acids of DP is sufficient for this interaction (Smith and Fuchs, 1998). It is not known whether the ability to bind to cadherins through both plakoglobin-dependent and independent interactions is a unique property of Dsg1 and Dsc1, or whether it is a common trait held by other desmosomal cadherins. However, it is interesting to note that both Dsc1 and Dsg1 are differentiation-specific cadherins, expressed together in the superficial layers of complex epithelia (King et al., 1996; Nuber et al., 1996). It seems plausible that the existence of multiple sites for DP on the Dsc1/Dsg1-catenin complex could confer additional mechanical strength to the superficial cells of the epidermis, enabling these tissues to better withstand mechanical stress.

Assembly of the desmosomal plaque: role of arm family members

The continued growth of the plakophilin family of desmosomal molecules highlights the fact that the desmosomal plaque is much more complex than once appreciated (Bonne et al., 1999; Hatzfeld et al., 1994; Heid et al., 1994; Mertens et al., 1996; Schmidt et al., 1999; Schmidt et al., 1997). Previous reports describing a skin fragility syndrome in patients lacking PKP1, suggested that in desmosomes of the superficial epidermis, this arm protein plays a critical role in maintaining tissue integrity (McGrath et al., 1999; McGrath et al., 1997). The work described here which showed that PKP1 efficiently concentrates endogenous and ectopically expressed full length DP at the plasma membrane is consistent with previous studies (Hatzfeld et al., 2000; Kowalczyk et al., 1999b; Smith and Fuchs, 1998). In addition, the abundance of 10 nm IF seen associated with the plaque structures in quadruply transfected

cells (Fig. 8) highlights the role of PKP in recruiting IF to the desmosomal plaques. Together, these observations support our proposed model that PKP1 increases the number of IF binding sites in desmosomes of the superficial epidermis by recruiting its preferred binding partner, DP, to the membrane (Kowalczyk et al., 1999b). A contribution for PKP1 in directly anchoring IF to the desmosomal plaque, supported by in vitro binding studies (Kapprell et al., 1988; Hofman et al., 2000; Hatzfeld et al., 1994), cannot be ruled out in our study, but has recently been questioned in light of PKP1's position immediately proximal to the plasma membrane (North et al., 1999). A structural role for PKP1 in this membrane-proximal region is also suggested by the increased electron density of the outermost plaque in quadruply transfected cells compared with triply transfected cells lacking PKP1 (Fig. 8).

Most plakoglobin null mice die early in embryogenesis, probably due to the inability of the heart to withstand mechanical stress, and those that survive exhibit epidermal defects. Thus, like PKP1, plakoglobin also plays an important role in tissue integrity (Bierkamp et al., 1996; Ruiz et al., 1996). However, it must do so in the context of plakophilins, as it is now clear that all desmosomes are likely to have one or more plakophilins in addition to plakoglobin in the cytoplasmic plaque. Here we investigated the relative roles played by plakoglobin and PKP1 in the formation of desmosome-like structures.

In quadruple transfections when both plakoglobin and PKP1 were introduced into cells with E-Dsg1 and DP a pattern of dots in the cytoplasm and at cell-cell borders was observed. These cytoplasmic dots and punctuated borders differed from the continuous staining visible at cell-cell borders in cells transfected with all other combinations of plasmids. Furthermore, the solubility profile of components in the quadruple transfectants was what might be predicted based on studies of normal epithelial cells. That is, the majority of DP (~80%), PKP1 (~75%) and E-Dsg1 (70%) were present in a Triton-insoluble pool of proteins when all four proteins were co-expressed. These light microscope observations were corroborated by ultrastructural analysis of transfected cells which revealed structures in the cytoplasm and on cell surfaces with multi-layered, membrane-associated electron dense plaques tethered to IF. These plaques appeared more robust than the smaller, more rarely seen desmosomes in untransfected or vector transfected COS cells (compare Fig. 8D with B′ and C), and more closely resembled desmosomes in stratified epithelia.

Plasma membrane-associated plaques were frequently seen in the absence of cell-cell contact. A previous report from Demlehner et al., suggested that in normal cultured epithelial cells desmosomal half plaques are continuously synthesized and subsequently endocytosed in the absence of cell-cell contact to stabilize the structure (Demlehner et al., 1995). Although these authors could not exclude the possibility that the vesicles they observed formed in the cytoplasm without first assembling at the plasma membrane, they suggested that most formed by endocytosis. Whether the intracellular vesicles we observed in transfected cells formed first in the cytoplasm and were then delivered to the plasma membrane, or formed first at the plasma membrane and were subsequently endocytosed is not known. However, in these highly expressing transfected cells, it seems possible that both could occur.

Together, the observations presented here are consistent with the idea that a desmosomal cadherin tail, a plakophilin, plakoglobin and DP together assemble a clustered plaque structure with cytoplasmic features similar to that of a desmosome. Although plaque lengths in quadruply transfected cells varied at cell-cell borders, there was a general trend that expression of both arm proteins together led to more clustered borders (Figs 6, 7, arrows). A role for plakoglobin in limiting the size of desmosomes is suggested by the increased size of desmosomes in the hearts of plakoglobin null mice (Ruiz et al., 1996) and in A431 cells expressing C-terminally truncated plakoglobin molecules (Palka and Green, 1997). These observations are consistent with the transformation of the continuous border staining described above into interrupted cell borders when both PKP1 and plakoglobin are present. Based on the sequestration of E-Dsg1/PKP1/DP complexes along filament arrays in the absence of co-expressed plakoglobin it is also possible that plakoglobin ensures the proper behavior and transport of the desmosomal cadherins to the plasma membrane. Such a role was recently suggested for β-catenin in the transport of E-cadherin to basolateral membranes of MDCK cells (Chen et al., 1999). Interestingly, plakoglobin by itself does not appear to be sufficient to limit the size of the plaque, as cells expressing E-Dsg1, DP and plakoglobin exhibited more continuous border staining as shown in Fig. 2. It was only when plakoglobin and PKP1 were both expressed that plaques of desmosome-like appearance and dimension were generated, suggesting that this event requires the cooperative activity of both proteins.

These observations raise an apparent paradox. That is, how does this cooperation occur when PKP1 binds to DP at the expense of plakoglobin? The DP-PKP1 complexes analyzed in Fig. 5 represent interactions in the soluble pool only. It seems likely that DP complexes containing both PKP1 and plakoglobin do exist in the insoluble pool representing the cytoplasmic dots and puncta containing all three proteins shown in Figs 6 and 7 and plaque structures in Fig. 8. The data suggest furthermore that the presence of PKP1-DP and E-Dsg1-Pg-DP complexes favors the assembly of multiple plaques of more limited size. Precisely how this occurs at a molecular level is unclear. One contributing factor is likely to be the repertoire and affinity of protein-protein interactions that can occur. So, for instance, PKP1 and plakoglobin do not interact (X.-T. Chen and K. Green, unpublished). Thus, a possible scenario could be that insertion of a particular component, such as PKP1, might inhibit further lateral outgrowth of plaques via plakoglobin when both are present. The final size of desmosomes and extent of clustering would be dependent on the subcellular localization and availability of plakoglobin and PKP1 for assembly and incorporation into the growing plaque. In addition, the stoichiometry of components, something that is difficult to regulate in the transient approach used here, is also likely to play an important role in regulating the size and dimensions of the plaque. Thus it is not surprising that there is substantial variation in border lengths observed in quadruply transfected cells, which could be explained by varying ratios of plakoglobin and PKP1 expressed in these cells. Finally, desmocollins are normally a major desmosomal constituent. In the current work, we have bypassed the requirement for desmocollins at the adhesive interface by replacing the Dsg1 extracellular domain with the E-cadherin

extracellular domain. However, both the longer 'a' and shorter 'b' form of the desmocollins most likely make important contributions to the normal plaque structure of desmosomes.

Our results provide several critical insights into the rules that govern desmosome assembly. They suggest that the repertoire of arm family members, in concert with the cadherin tails, plays an important role in determining the structure and dimensions of the plaque, as well as the number of IF binding sites along the length of the plaque. The overlapping expression patterns of desmosomal components in stratified tissues would thus provide an opportunity for tailoring desmosome structure and function in response to different functional requirements that occur during epithelial differentiation.

The authors thank Cheryl Olson for her generous assistance with preparation of materials for EM. Thanks go also to those who provided antibody reagents including Drs Peggy Wheelock and Keith Johnson, Pam Cowin, Stephen Byers, Masatoshi Takeichi, and Fiona Watt. Members of the Green lab provided helpful discussion and critical reading of the manuscript. This work was supported primarily by grants RO1 AR43380 and project #4 of PO1 DE12328 to K. Green, with additional support from AR41836.

REFERENCES

Aberle, H., Schwarz, H. and Kemler, R. (1996). Cadherin-catenin complex: protein interactions and their implications for cadherin function. *J. Cell. Biochem.* **61**, 514-523.

Allen, E., Yu, Q.-C. and Fuchs, E. (1996). Mice expressing a mutant desmosomal cadherin exhibit abnormalities in desmosomes, proliferation, and epidermal differentiation. *J. Biol. Chem.* **133**, 1367-1382.

Anastasiadis, P. Z. and Reynolds, A. B. (2000). The p120 catenin family: complex roles in adhesion, signaling and cancer. *J. Cell Sci.* **113**, 1319-1334.

Angst, B. D., Nilles, L. A. and Green, K. J. (1990). Desmoplakin II expression is not restricted to stratified epithelia. *J. Cell Sci.* **97**, 247-257.

Armstrong, D. K. B., McKenna, K. E., Purkis, P. E., Green, K. J., Eady, R. A. J., Leigh, I. M. and Hughes, A. E. (1999). Haploinsufficiency of desmoplakin causes a striate subtype of palmoplantar keratoderma. *Hum. Mol. Genet.* **8**, 143-148.

Bierkamp, C., McLaughlin, K. J., Schwarz, H., Huber, O. and Kemler, R. (1996). Embryonic heart and skin defects in mice lacking plakoglobin. *Dev. Biol.* **180**, 780-785.

Bonne, S., van Hengel, J., Nollet, F., Kools, P. and van Roy, F. (1999). Plakophilin-3, a novel armadillo-like protein present in nuclei and desmosomes of epithelial cells. *J. Cell Sci.* **112**, 2265-2276.

Bornslaeger, E. B., Corcoran, C. M., Stappenbeck, T. S. and Green, K. J. (1996). Breaking the connection: Displacement of the desmosomal plaque protein desmoplakin from cell-cell interfaces disrupts anchorage of intermediate filament bundles and alters intercellular junction assembly. *J. Biol. Chem.* **134**, 985-1002.

Buxton, R. S., Magee, A. I., King, I. A. and Arnemann, J. (1994). Desmosomal genes. In *Molecular Biology of Desmosomes and Hemidesmosomes* (ed. J. E. Collins and D. R. Garrod), pp. 1-131. Austin: R.G. Landes Co.

Chen, Y.-T., Stewart, D. B. and Nelson, W. J. (1999). Coupling assembly of the E-cadherin/β-catenin complex to efficient endoplasmic reticulum exit and basal-lateral membrane targeting of E-cadherin in polarized MDCK cells. *J. Biol. Chem.* **144**, 687-699.

Chitaev, N. A. and Troyanovsky, S. M. (1997). Direct Ca^{2+}-dependent heterophilic interaction between desmosomal cadherins, desmoglein and desmocollin, contributes to cell-cell adhesion. *J. Biol. Chem.* **138**, 193-201.

Cowin, P. and Burke, B. (1996). Cytoskeleton-membrane interactions. *Curr. Opin. Cell Biol.* **8**, 56-65.

Demlehner, M. P., Schafer, S., Grund, C. and Franke, W. W. (1995). Continual assembly of half-desmosomal structures in the absence of cell contacts and their frustrated endocytosis: a coordinated Sisyphus cycle. *J. Biol. Chem.* **131**, 745-760.

Gallicano, G. I., Kouklis, P., Bauer, C., Yin, M., Vasioukhin, V., Degenstein, L. and Fuchs, E. (1998). Desmoplakin is required early in development for assembly of desmosomes and cytoskeletal linkage. *J. Biol. Chem.* **143**, 2009-2022.

Garrod, D., Chidgey, M. and North, A. (1996). Desmosomes: differentiation, development, dynamics and disease. *Curr. Opin. Cell Biol.* **8**, 670-678.

Gelderloos, J. A., Witcher, L., Cowin, P. and Klymkowsky, M. W. (1997). Plakoglobin, the other 'Arm' of vertebrates. In *Cytoskeletal-Membrane Interactions and Signal Transduction* (ed. P. Cowin and M. Klymkowsky), pp. 12-30. Austin: Landes Bioscience.

Green, K. J. and Gaudry, C. A. (2000). Are desmosomes more than tethers for intermediate filaments? *Nature Reviews Mol. Cell Biol.* **1**, 208-216.

Hatzfeld, M., Kristjansson, G. I., Plessmann, U. and Weber, K. (1994). Band 6 protein, a major constituent of desmosomes from stratified epithelia, is a novel member of the armadillo multigene family. *J. Cell Sci.* **107**, 2259-2270.

Hatzfeld, M. (1999). The armadillo family of structural proteins. *Int. Rev. Cytol.* **186**, 179-224.

Hatzfeld, M., Haffner, C., Schulze, K. and Vinzens, U. (2000). The function of plakophilin 1 in desmosome assembly and actin filament organization. *J. Cell Biol.* **149**, 209-222.

Heid, H. W., Schmidt, A., Zimbelmann, R., Schafer, S., Winter-Simanowski, S., Stumpp, S., Keith, M., Figge, U., Schnolzer, M. and Franke, W. W. (1994). Cell type-specific desmosomal plaque proteins of the plakoglobin family: plakophilin 1 (band 6 protein). *Differentiation* **58**, 113-131.

Hofmann, I., Mertens, C., Brettel, M., Nimmrich, V., Schnolzer, N. and Herrmann, H. (2000). Interaction of plakophilins with desmoplakin and intermediate filament proteins: an in vitro analysis. *J. Cell Sci.* **113**, 2471-2483.

Jones, J. C. R. and Goldman, R. D. (1985). Intermediate filaments and the initiation of desmosome assembly. *J. Biol. Chem.* **101**, 506-517.

Jou, T. S., Stewart, D. B., Stappert, J., Nelson, W. J. and Marrs, J. A. (1995). Genetic and biochemical dissection of protein linkages in the cadherin-catenin complex. *Proc. Nat. Acad. Sci. USA* **92**, 5067-5071.

Kapprell, H. P., Owaribe, K. and Franke, W. W. (1988). Identification of a basic protein of Mr 75,000 as an accessory desmosomal plaque protein in stratified and complex epithelia. *J. Cell Biol.* **106**, 1679-1691.

King, I. A., O'Brien, T. J. and Buxton, R. S. (1996). Expression of the 'skin-type' desmosomal cadherin DSC1 is closely linked to the keratinization of epithelial tissues during mouse development. *J. Invest. Derm.* **107**, 531-538.

Koch, A. W., Bozic, D., Pertz, O. and Engel, J. (1999). Homophilic adhesion by cadherins. *Curr. Opin. Struct. Biol.* **9**, 275-281.

Koch, P. J. and Franke, W. W. (1994). Desmosomal cadherins: another growing multigene family of adhesion molecules. *Curr. Opin. Cell Biol.* **6**, 682-687.

Koch, P. J., Mahoney, M. G., Ishikawa, H., Pulkkinen, L., Uitto, J., Shultz, L., Murphy, G. F., Whitaker-Menezes, D. and Stanley, J. R. (1997). Targeted disruption of the pemphigus vulgaris antigen (desmoglein 30 gene in mice causes loss of keratinocyte cell adhesion with a phenotype similar to pemphigus vulgaris. *J. Biol. Chem.* **137**, 1091-1102.

Kouklis, P. D., Hutton, E. and Fuchs, E. (1994). Making a connection: direct binding between keratin intermediate filaments and desmosomal proteins. *J. Biol. Chem.* **127**, 1049-1060.

Kowalczyk, A. P., Palka, H. L., Luu, H. H., Nilles, L. A., Anderson, J. E., Wheelock, M. J. and Green, K. J. (1994). Posttranslational regulation of plakoglobin expression: Influence of the desmosomal cadherins on plakoglobin metabolic stability. *J. Biol. Chem.* **269**, 31214-31223.

Kowalczyk, A. P. and Green, K. J. (1996). The desmosome: a component system for adhesion and intermediate filament attachment. *Curr. Topics Membr.* **43**, 187-209.

Kowalczyk, A. P., Bornslaeger, E. A., Borgwardt, J. E., Palka, H. L., Dhaliwal, A. S., Corcoran, C. M., Denning, M. F. and Green, K. J. (1997). The amino-terminal domain of desmoplakin binds to plakoglobin and clusters desmosomal cadherin-plakoglobin complexes. *J. Biol. Chem.* **139**, 773-784.

Kowalczyk, A. P., Navarro, P., Dejana, E., Bornslaeger, E. A., Green, K. J., Kopp, D. S. and Borgwardt, J. E. (1998). VE-cadherin and desmoplakin are assembled into dermal microvascular endothelial intercellular junctions: a pivotal role for plakoglobin in the recruitment of desmoplakin to intercellular junctions. *J. Cell Sci.* **111**, 3045-3057.

Kowalczyk, A. P., Bornslaeger, E. A., Norvell, S. M., Palka, H. L. and Green, K. J. (1999a). Desmosomes: intercellular adhesive junctions specialized for attachment of intermediate filaments. *Int. Rev. Cytol.* **185**, 237-302.

Kowalczyk, A. P., Hatzfeld, M., Bornslaeger, E. A., Kopp, D. S., Borgwardt,

J. E., Corcoran, C. M., Settler, A. and Green, K. J. (1999b). The head domain of plakophilin-1 binds to and enhances its recruitment to desmosomes: implications for cutaneous disease. *J. Biol. Chem.* **274**, 18145-18148.

Magee, A. I. and Buxton, R. S. (1991). Transmembrane molecular assemblies regulated by the greater cadherin family. *Curr. Opin. Cell Biol.* **3**, 854-861.

Mathur, M., Goodwin, L. and Cowin, P. (1994). Interactions of the cytoplasmic domain of the desmosomal cadherin Dsg1 with plakoglobin. *J. Biol. Chem.* **269**, 14075-14080.

McGrath, J. A., McMillan, J. R., Shemanko, C. S., Runswick, S. K., Leigh, I. M., Lane, E. B., Garrod, D. R. and Eady, R. A. J. (1997). Mutations in the plakophilin 1 gene result in ectodermal dysplasia/skin fragility syndrome. *Nature Genet.* **17**, 240-244.

McGrath, J. A., Hoeger, P. H., Christiano, A. M., McMillan, J. R., Mellerio, J. E., Ashton, G. H., Dopping-Hepenstal, P. J., Lake, B. D., Leigh, I. M., Harper, J. I. and Eady, R. A. (1999). Skin fragility and hypohidrotic ectodermal dysplasia resulting from ablation of plakophilin 1. *Br. J. Dermatol.* **140**, 297-307.

McKoy, G., Protonotarios, N., Crosby, A., Tsatsopoulou, A., Anastasakis, A., Coonar, A., Norman, M., Baboonian, C., Jeffery S. and McKenna W. J. (2000). Identification of a deletion in plakoglobin in arrhythmogenic right ventricular cardiomyopathy with palmoplantar keratoderma and woolly hair (Naxos disease). *Lancet* **355**, 2119-2124.

Meng, J.-J., Bornslaeger, E. A., Green, K. J., Steinert, P. M. and Ip, W. (1997). Two hybrid analysis reveals fundamental differences in direct interactions between desmoplakin and cell type specific intermediate filaments. *J. Biol. Chem.* **272**, 21495-21503.

Mertens, C., Kuhn, C. and Franke, W. W. (1996). Plakophilins 2a and 2b: constitutive proteins of dual location in the karyoplasm and the desmosomal plaque. *J. Biol. Chem.* **135**, 1009-1025.

Norgett, E. E., Hatsell, S. J., Carvajal-Huerta, L., Cabezas, J. C. R., Common, J., Purkis, P. E., Whittock, N., Leigh, I. M., Stevens, H. P. and Kelsell, D. P. (2000). Recessive mutation in desmoplakin disrupts desmoplakin-intermediate filament interactions and causes dilated cardiomyopathy, woolly hair and keratoderma. *Mol. Genetics* **9**, 2761-2766.

North, A. J., Bardsley, W. G., Hyam, J., Bornslaeger, E. A., Cordingley, H. C., Trinnaman, B., Hatzfeld, M., Green, K. J., Magee, A. I. and Garrod, D. R. (1999). Molecular map of the desmosomal plaque. *J. Cell Sci.* **112**, 4325-4336.

Nuber, U. A., Schafer, S., Stehr, S., Rackwitz, H.-R. and Franke, W. W. (1996). Patterns of desmocollin synthesis in human epithelia: immunolocalization of desmocollins 1 and 3 in special epithelia and in cultured cells. *Eur. J. Cell Biol.* **71**, 1-13.

Palka, H. L. and Green, K. J. (1997). Roles of plakoglobin end domains in desmosome assembly. *J. Cell Sci.* **110**, 2359-2371.

Pasdar, M. and Nelson, W. J. (1988). Kinetics of desmosome assembly in Madin-Darby canine kidney epithelial cells: temporal and spatial regulation of desmoplakin organization and stabilization upon cell-cell contact I. Biochemical analysis. *J. Biol. Chem.* **106**, 677-685.

Pasdar, M. and Nelson, W. J. (1989). Regulation of desmosome assembly in epithelial cells: kinetics of synthesis, transport, and stabilization of desmoglein I, a major protein of the membrane core domain. *J. Biol. Chem.* **109**, 163-177.

Penn, E. J., Burdett, I. D. J., Hobson, C., Magee, A. I. and Rees, D. A. (1987). Structure and assembly of desmosome junctions: biosynthesis and turnover of the major desmosome components of Madin-Darby canine kidney cells in low calcium medium. *J. Biol. Chem.* **105**, 2327-2334.

Rickman, L., Simrak, D., Stevens, H. P., Hunt, D. M., King, I. A., Bryant, S. P., Eady, R. A., Leigh, I. M., Arnemann, J., Magee, A. I. et al. (1999). N-terminal deletion in a desmosomal cadherin causes the autosomal dominant skin disease striate palmoplantar keratoderma. *Hum. Mol. Genet.* **8**, 971-976.

Ruiz, P., Brinkmann, V., Ledermann, B., Behrend, M., Grund, C., Thalhammer, C., Vogel, F., Birchmeier, C., Gunthert, U., Franke, W. W. et al. (1996). Targeted mutation of plakoglobin in mice reveals essential functions of desmosomes in the embryonic heart. *J. Biol. Chem.* **135**, 215-225.

Schmidt, A., Heid, H. w., Schafer, S., Nuber, U. A., Zimbelmann, R. and Franke, W. W. (1994). Desmosomes and cytoskeletal architecture in epithelial differentiation: cell type-specific plaque components and intermediate filament anchorage. *Eur. J. Cell Biol.* **65**, 229-245.

Schmidt, A., Langbein, L., Rode, M., Pratzel, S., Zimbelmann, R. and Franke, W. W. (1997). Plakophilins 1a and 1b: widespread nuclear proteins recruited in specific epithelial cells as desmosomal plaque components. *Cell Tiss. Res.* **290**, 481-499.

Schmidt, A., Langbein, L., Pratzel, S., Rode, M., Rackwitz, H.-R. and Franke, W. W. (1999). Plakophilin 3-a novel cell-type-specific desmosomal plaque protein. *Differentiation* **64**, 291-306.

Schwarz, M. A., Owaribe, K., Kartenbeck, J. and Franke, W. W. (1990). Desmosomes and hemidesmosomes: constitutive molecular components. *Annu. Rev. Cell Biol.* **6**, 461-491.

Smith, E. A. and Fuchs, E. (1998). Defining the interactions between intermediate filaments and desmosomes. *J. Biol. Chem.* **141**, 1229-1241.

Stanley, J. R. (1995). Autoantibodies against adhesion molecules and structures in blistering skin diseases. *J. Exp. Med.* **181**, 1-4.

Stappenbeck, T. S. and Green, K. J. (1992). The desmoplakin carboxyl terminus coaligns with and specifically disrupts intermediate filament networks when expressed in cultured cells. *J. Biol. Chem.* **116**, 1197-1209.

Stappenbeck, T. S., Bornslaeger, E. A., Corcoran, C. M., Luu, H. H., Virata, M. L. A. and Green, K. J. (1993). Functional analysis of desmoplakin domains: specification of the interaction with keratin versus vimentin intermediate filament networks. *J. Biol. Chem.* **123**, 691-705.

Troyanovsky, S. M., Troyanovsky, R. B., Eshkind, L. G., Leube, R. E. and Franke, W. W. (1994). Identification of amino acid sequence motifs in desmocollin, a desmosomal glycoprotein, that are required for plakoglobin binding and plaque formation. *Proc. Nat. Acad. Sci. USA* **91**, 10790-10794.

Witcher, L. L., Collins, R., Puttagunta, S., Mechanic, S. E., Munson, M., Gumbiner, B. and Cowin, P. (1996). Desmosomal cadherin binding domains of plakoglobin. *J. Biol. Chem.* **271**, 10904-10909.