The Function of Plakophilin 1 in Desmosome Assembly and Actin Filament Organization

Mechthild Hatzfeld, Christof Haffner, Katrin Schulze, and Ute Vinzens

Molecular Biology Group of the Medical Faculty, University of Halle, 06097 Halle/Saale, Germany

Abstract. Plakophilin 1, a member of the *armadillo* multigene family, is a protein with dual localization in the nucleus and in desmosomes. To elucidate its role in desmosome assembly and regulation, we have analyzed its localization and binding partners in vivo. When overexpressed in HaCaT keratinocytes, plakophilin 1 localized to the nucleus and to desmosomes, and dramatically enhanced the recruitment of desmosomal proteins to the plasma membrane. This effect was mediated by plakophilin 1's head domain, which interacted with desmoglein 1, desmoplakin, and keratins in the yeast two-hybrid system. Overexpression of the armadillo repeat domain induced a striking dominant negative phenotype with the formation of filopodia and long cellular protrusions, where plakophilin 1 colocalized with actin filaments. This phenotype was strictly dependent on a conserved motif in the center of the armadillo repeat domain. Our results demonstrate that plakophilin 1 contains two functionally distinct domains: the head domain, which could play a role in organizing the desmosomal plaque in suprabasal cells, and the armadillo repeat domain, which might be involved in regulating the dynamics of the actin cytoskeleton.

Key words: keratinocytes • desmoglein • armadillo • cell adhesion • cell motility

Introduction

Desmosomes are adhering junctions that anchor intermediate filaments to sites of cell–cell contact. Biochemically, they are distinct, but are related to the adherens junctions that anchor actin filaments. They contain two types of transmembrane proteins of the cadherin superfamily, the desmogleins (Dsgs)[1] and desmocollins (Dscs). There are at least three different desmogleins, and three different desmocollin genes (Dsg1-3 and Dsc1-3) that are differentially expressed (Koch and Franke, 1994; Schmidt et al., 1994). Whereas Dsg2 and Dsc2 are ubiquitously expressed in all cells that possess desmosomes, the expression of Dsgs1 and 3 and Dscs1 and 3 is restricted to stratified epithelia (Schmidt et al., 1994; Garrod et al., 1996). Expression patterns of isoforms of the desmosomal cadherins overlap, and individual desmosomes can contain more than one isoform (North et al., 1996). Clustering of desmosomal cadherins and desmosome formation depends on both Dsgs and Dscs (Chitaev and Troyanovsky, 1997).

The intracellular domains of the desmosomal cadherins associate with a number of plaque proteins that establish the link to the intermediate filament system (Troyanovsky et al., 1993, 1994a, 1996; Mathur et al., 1994; Chitaev et al., 1996; Kowalczyk et al., 1996; Witcher et al., 1996). Plakoglobin and desmoplakin are essential components of the plaque. Plakoglobin associates with both types of desmosomal cadherins and binds to several Dsg and Dsc isoforms (Mathur et al., 1994; Troyanovsky et al., 1994a,b, 1996; Chitaev et al., 1996; Wahl et al., 1996; Witcher et al., 1996). The binding of plakoglobin to E-cadherin is a prerequisite for desmosome formation, and its COOH terminus is involved in regulating desmosome size (Ruiz et al., 1996; Lewis et al., 1997; Palka and Green, 1997). Moreover, a signaling role in the Wnt pathway, which is similar to that of β-catenin and the *Drosophila* homologue *armadillo*, has been reported (Karnovsky and Klymkowsky, 1995; Rubenstein et al., 1997). Direct interactions between desmosomal cadherins and desmoplakin have been reported only in vitro (Smith and Fuchs, 1998), and it appears that plakoglobin is necessary to link these proteins in vivo (Kowalczyk et al., 1996, 1997). Desmoplakin binds to intermediate filaments through its COOH-terminal domain and connects desmosomes to the cytoskeleton (Stappenbeck et al., 1993, 1994; Kouklis et al., 1994; Bornslaeger et al., 1996; Meng et al., 1997). Thus, intermediate filaments seem to be linked to the plasma membrane

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Address correspondence to M. Hatzfeld, Molecular Biology Group of the Medical Faculty, University of Halle, Magdeburger Strasse 18, 06097 Halle/Saale, Germany. Tel.: 49-345-557-4422. Fax: 49-345-557-4421. E-mail: mechthild.hatzfeld@medizin.uni-halle.de

[1] *Abbreviations used in this paper:* arm, armadillo; DP-NTP, desmoplakin NH_2-terminal polypeptide; Dsc, desmocollin; Dsg, desmoglein; ONPG, o-nitrophenyl-β-D-galactopyranoside.

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through a linear sequence of interactions between keratins, desmoplakin, plakoglobin, and the cytoplasmic tail of cadherins.

Additional components of the desmosomal plaque are plakophilins 1, 2, and 3, and p0071 (Hatzfeld et al., 1994; Heid et al., 1994; Hatzfeld and Nachtsheim, 1996; Mertens et al., 1996; Bonne et al., 1999; Schmidt et al., 1999). These proteins contain a central domain that consists of a series of 45 amino acid repeats (arm repeats), and are members of the $p120^{ctn}$ family of armadillo (arm) -related proteins (Reynolds et al., 1994; Hatzfeld and Nachtsheim, 1996; Daniel and Reynolds, 1997). Plakophilin 1 is a major component of desmosomes from stratified and complex epithelia, and it is predominantly expressed in the suprabasal layers (Kapprell et al., 1988). It binds to keratins in vitro (Kapprell et al., 1988; Hatzfeld et al., 1994; Smith and Fuchs, 1998), but the significance of this interaction in vivo has not yet been established. More recently, plakophilin 1 has been described as a widespread nuclear protein that is also expressed in nondesmosome-bearing cells, where it accumulated in the nucleoplasm (Schmidt et al., 1997; Klymkowsky, 1999). The function of plakophilin 1 in the nucleus remains unknown so far. An essential role in desmosome organization and stability has been suggested recently on the basis of a genetic skin disease. Patients lacking plakophilin 1 suffer from a skin fragility syndrome. Desmosomes in their skin are small and poorly formed with widening of keratinocyte intercellular spaces and perturbed desmosome/keratin filament interactions (McGrath et al., 1997). Desmoplakin was found predominantly cytoplasmic in these patients, suggesting a role for plakophilin 1 in organizing suprabasal desmosomes. These findings point to an essential role of plakophilin 1 in establishing stable cell contacts, desmosomal plaque size, and organization. In a recent paper, a direct interaction between the desmoplakin NH_2 terminus and plakophilin 1 and a role of plakophilin 1 in recruiting desmoplakin to the membrane was described (Kowalczyk et al., 1999). It was proposed that this interaction may be important for clustering of desmosomal components through lateral interactions.

To learn more about the function of plakophilin 1, we have analyzed its function in desmosome assembly in more detail. Wild-type plakophilin 1 recruited endogenous desmosomal proteins into the plaque when overexpressed in keratinocytes. This function was mediated by its head domain, and we show that this domain interacts with Dsg1, desmoplakin, and keratins. In contrast, the arm repeat domain had a dominant negative phenotype: it promoted formation of filopodia and long cellular protrusions, where it colocalized with actin filaments. Deletion of a conserved motif in the center of the arm domain abolished the ability of plakophilin 1 to modulate cellular morphology and to associate with actin. Our data suggest that plakophilin 1 is involved in regulation of desmosome assembly as well as dynamics of the actin cytoskeleton.

Materials and Methods

RNA Isolation and Plasmid Constructs

RNA was prepared according to the LiCl/urea extraction method (Auffray and Rougeon, 1980), and cDNAs were synthesized by reverse transcriptase–PCR, with expand reverse transcriptase and expand high fidelity polymerase (Roche Diagnostics). Suitable restriction sites for cloning were included in the primer sequences. PCR products were either directly cloned into the expression vectors or first ligated into the PCRII vector using the TOPO TA cloning kit (Invitrogen BV). All PCR products were sequenced completely.

Prokaryotic expression was performed in the pRSET A vector, which includes an NH_2-terminal His tag (Invitrogen Corp.). Expression in eukaryotic cells was performed with the following vectors: pCMV5 (with NH_2-terminal T7 tag; Andersson et al., 1989), pCMVscript (without tag; Stratagene), pOPRSV (without tag; Stratagene), and pEGFP (with NH_2-terminal GFP tag; CLONTECH Laboratories). Vectors for the expression of GAL4 fusion proteins in yeast were the pBD and pAD vectors (Stratagene) and the pAS2-1 and pGAD424 vectors (CLONTECH Laboratories). Fig. 1 a gives an overview of the plakophilin 1 constructs used in this study. The intracellular domains of human Dsg1, Dsg2, and Dsg3, and Dsc1a, 1b, Dsc2a, 2b, and Dsc3a, 3b were amplified by reverse transcriptase–PCR from HaCaT cell RNA and cloned into the pGAD424 vector. Dsg1 domains IA (amino acids 568–643), CS (amino acids 644–764), and the Dsg-specific domain (Dsg; amino acids 765–1,049) were amplified by PCR and cloned into the pGAD424 vector. Human keratins 8, 18, 6, and 17 and their individual domains in pAD and pBD have been described elsewhere (Schnabel et al., 1998). The complete coding sequence of human β-actin was amplified by PCR from HeLa cell RNA and cloned into pGAD424.

In Vitro Mutagenesis

In vitro mutagenesis was performed to delete the 5–amino acid motif ENCMC (amino acids 452–456) in the plakophilin 1 arm repeat domain. The reaction was performed with the QuickChange site-directed mutagenesis kit (Stratagene). The deletion was verified by sequence analysis.

Two-Hybrid Assays

Plasmids were transformed into the yeast strain YRG2 (Stratagene) by electroporation. Double transformants were grown on plates lacking leucine and tryptophane. Expression of the His reporter gene was analyzed on plates lacking histidine in addition to leucine and tryptophane. lacZ reporter gene expression was analyzed in the colony lift filter assay and quantitated using the ONPG (o-nitrophenyl-β-D-galactopyranoside) substrate as described in the yeast protocols handbook (CLONTECH Laboratories).

Recombinant Protein Purification and Antibody Production

The plakophilin 1 head and arm repeat domains in pRSET were expressed in BL21 DE3 bacteria and purified under denaturing conditions on Ni-NTA resin (Qiagen). The purified protein fragments were used for immunization of rabbits.

Cell Lines, Wound Healing Assay, and Transfections

HeLa and HaCaT (Boukamp et al., 1988) cells were routinely cultured in DME supplemented with 10% FBS. Normal human epidermal keratinocytes cells and keratinocyte medium were obtained from Promocell. Wound healing assays were performed with HaCaT cells grown to confluency, and a wound was inserted by scraping. Cells were analyzed 24 h after wounding. For transient transfection experiments, cells were plated 12–16 h before transfection. Cells were transfected either by the calcium phosphate precipitation method (5prime→3prime, Inc.) or using the DOSPER liposomal transfection reagent (Roche Biochemicals). Cells were fixed and processed for immunofluorescence analysis after 20–44 h.

Antibodies and Immunofluorescence Microscopy

Cells grown on coverslips were rinsed in PBS and fixed in methanol at −20°C for 10 min, followed either by acetone treatment for 1 min or by treatment in 0.2% Triton X-100 in PBS for 20 min. Alternatively, cells were fixed in 3.7% formaldehyde in PBS freshly prepared from paraformaldehyde and permeabilized in 0.2% Triton in PBS. Cells were washed in PBS and incubated with 1% BSA in PBS before antibody application.

Plakophilin 1 and its fragments were detected by the polyclonal rabbit sera against the head and repeat domains. Alternatively, plakophilin 1 head and repeat domains, which were expressed in the pCMV5 vector, were detected with a T7 mAb (Novagen, Inc.). For double labeling the following antibodies were used: antidesmoplakin 1 and 2, DP1&2 2.15 or DP1&2 mix (2.15 + 2.17 + 2.20); anti-Dsg1 + 2 DG3.10, anti-Dsc3 Dsc3 U114 were obtained from Progen. Antiplakoglobin and anti–Pan-cadherin were from Sigma Chemical Co.; the keratin antibody RCK107 was from Dr. F. Ramaekers (University Hospital, Rotterdam, The Netherlands).

Secondary antibodies were donkey anti–rabbit or anti–mouse coupled to Cy2 or Cy3 (Jackson ImmunoResearch Laboratories, Inc., through Dianova) or Alexa 488 goat anti–mouse or goat anti–rabbit IgG (Molecular Probes, Inc.). Actin filaments were visualized by incubation with FITC- or TRITC-labeled phalloidin (Sigma Chemical Co.).

Microscopy was carried out with a Nikon Eclipse E600 microscope with narrow band filters.

Laser Scanning Microscopy

Cells processed for immunofluorescence microscopy were analyzed using a Zeiss LSM 510 laser scanning microscope equipped with a helium-neon and an argon laser and a Plan-Apochromat 63× objective. Excitation wavelengths were 488 nm for Alexa 488 and 543 nm for Cy3. The used detection filters were BP505-530 for Alexa 488 and LP560 for Cy3. Fluorescence was recorded using the multitracking procedure to get complete separation of the fluorescence signals.

Western Blot Analysis

Total protein extracts were prepared by adding SDS sample buffer heated to 100°C to the cell culture dishes. Yeast protein extracts were prepared according to the SDS-urea method in the presence of the complete protease inhibitor cocktail tablets (Roche Diagnostics), as described in the Yeast Protocols Handbook. Samples were separated on 8 or 10% acrylamide gels and transferred to nitrocellulose. Filters were blocked in 5% nonfat dry milk in TBS with 0.05% Tween 20. Primary antibodies were applied for 2 h at room temperature or overnight at 4°C. Filters were washed and incubated with alkaline phosphatase–coupled secondary antibodies, and bound antibodies were visualized either with the CDP-Star chemiluminescence reagent (Tropix) or with NBT/BCIP (Boehringer Ingelheim Bioproducts). In some experiments, the ECL detection system (Amersham Pharmacia Biotech) was used.

Results

Plakophilin 1 Constructs and Antibodies

To address the function of plakophilin 1 in desmosome assembly and structure, we studied targeting of its domains in epithelial cells and analyzed its direct binding partners in the yeast two-hybrid system. Fig. 1 a summarizes the plakophilin 1 constructs tested in transfection assays and in the yeast two-hybrid system. The GFP constructs of all domains were analyzed in parallel with nontagged or T7-tagged constructs to verify that the GFP tag did not interfere with intracellular sorting.

Rabbit polyclonal antibodies against the plakophilin 1 NH_2-terminal domain and the arm repeat domain were generated and tested for their specificity by Western blotting on total cellular extracts. Fig. 1 b shows that both antibodies reacted with a single band of 80 kD, demonstrating that they did not cross-react with related proteins, such as plakophilin 2 (96 kD) and 3 (86 kD), $p120^{ctn}$ (various isoforms of 96–115 kD), or p0071 (130 kD). The majority of the protein was detected in the insoluble protein fraction.

Wild-Type Plakophilin 1 and Its Head Domain Associate with Desmosomes and Enhance Recruitment of Desmosomal Proteins to the Plasma Membrane in HaCaT Cells

Since plakophilin 1 has been described as a protein with dual localization in desmosomes and in the nucleus (Schmidt et al., 1997), we analyzed intracellular targeting of the protein after overexpression. Attempts to obtain clonal cell lines that strongly overexpress plakophilin 1, or its head, or repeat domain, thus far, have been unsuccessful. This may be due to the phenotype that is caused by strong overexpression of plakophilin 1 or its fragments (see below). Therefore, we have used transient transfection studies to analyze the function of plakophilin 1 and its domains in a cellular context. Wild-type plakophilin 1, which was overexpressed in HaCaT keratinocytes, localized predominantly to the nucleus and to cell borders in confluent monolayers (Fig. 2 a), which is in agreement with the intracellular localization of the endogenous protein (Schmidt et al., 1997). The balance between nuclear localization and plasma membrane association appeared similar in transfected and nontransfected cells. Double la-

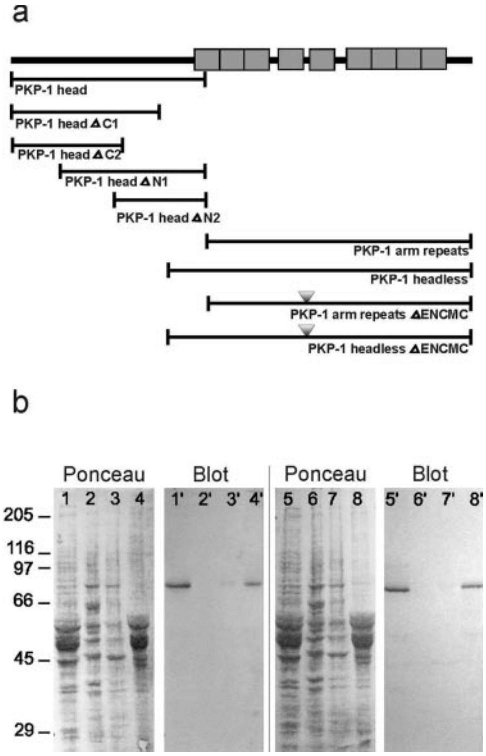

Figure 1. (a) Schematic representation of plakophilin 1 (PKP-1) and its deletion mutant constructs used in this study. The PKP-1 head comprises amino acids 1–286; PKP-1 ΔC1, amino acids 1–213; PKP-1 ΔC2, amino acids 1–168; PKP-1 ΔN1, amino acids 70–286; and PKP-1 ΔN2, amino acids 147–286. The PKP-1 arm repeat domain contains amino acids 287–726, and the PKP-1 headless fragment contains amino acids 224–726. The ΔENCMC fragments carry an additional internal deletion comprising amino acids 422–426. (b) Specificity of plakophilin head and arm repeat domain antibodies. Total protein extracts (lanes 1 and 5) as well as Triton-soluble (lanes 2 and 6), high salt soluble (lanes 3 and 7), and insoluble fractions (lanes 4 and 8) of HaCaT keratinocytes were separated on 8% SDS gels, transferred to nitrocellulose, and probed with the plakophilin head (lanes 1′–4′) and repeat domain antibodies (lanes 5′–8′). Both antibodies reacted with a single band of 80 kD in total cell extracts (lanes 1′ and 5′). The majority of the protein was in the insoluble fraction (lanes 4′ and 8′).

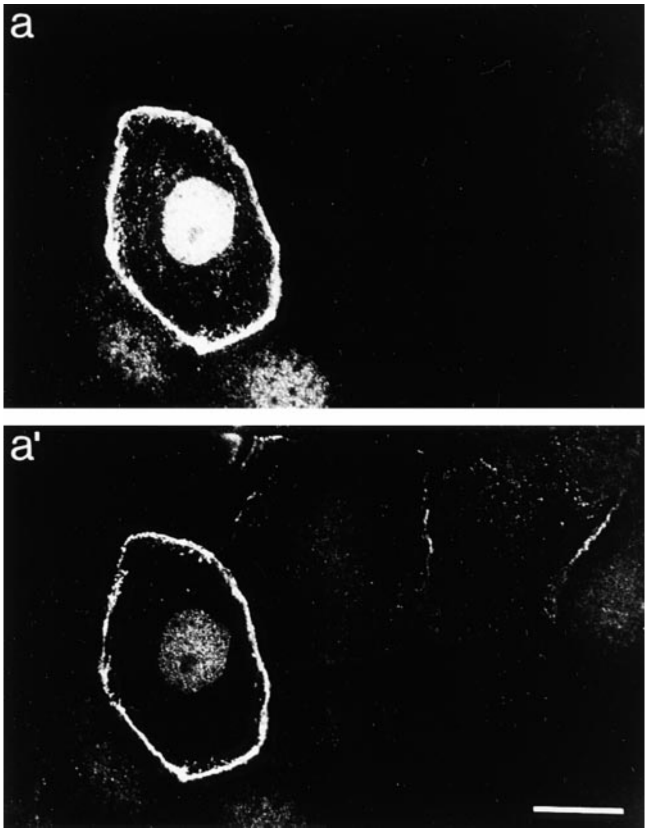

Figure 2. Expression of full-length plakophilin 1 in HaCaT cells. Cells were fixed in methanol 30 h after transfection, and double labeled with the plakophilin 1 head domain antibody (a) and the desmoplakin 2.15 antibody (a′). In confluent monolayers, plakophilin 1 accumulated in the nucleus and at the plasma membrane, and desmoplakin was recruited to the plasma membrane of the transfected cells. Labeling of endogenous desmoplakin in nontransfected adjacent cells was comparatively weak. Bar, 20 μm.

beling with desmoplakin antibodies revealed a strong increase of endogenous desmoplakin at the plasma membrane in the transfected cells compared with nontransfected cells (Fig. 2 a′).

To identify the domains that target plakophilin 1 to desmosomes and to the nucleus, the head and the arm repeat domains of plakophilin 1 were expressed separately. Whereas the arm repeat domain colocalized with the actin cytoskeleton (see below), the head domain, like the full-length protein, was detected in the nucleus and along the cell periphery (Fig. 3, a–e) and strongly enhanced recruitment of desmoplakin to the plasma membrane (Fig. 3 a′). Costaining for other desmosomal proteins revealed that recruitment of Dsg (Fig. 3 b′), Dsc (Fig. 3 c′) and, to a lesser extent, plakoglobin (Fig. 3 d′) was also enhanced. The amount of recruited protein roughly correlated with the size of the membrane pool of plakophilin 1. In cells with a large membrane pool of plakophilin 1, recruited proteins were detected continuously along the plasma membrane (Fig. 3, a–d’). In other cells, the typical punctate pattern of individual desmosomes was retained (Fig. 4). Costaining for keratins showed colocalization of a small pool of these proteins to plasma membrane patches enriched for plakophilin 1 (Fig. 3, e and e′). These data demonstrate that plakophilin 1 is able to recruit various desmosomal plaque proteins to the plasma membrane, and that this effect is mediated by its head domain.

In addition to its plasma membrane association, the head domain showed very strong nuclear localization. Surprisingly, some desmoplakin, Dsg, and Dsc were also detected in the nucleus, suggesting that plakophilin 1 coimported a fraction of these proteins into the nucleus.

To analyze the recruitment of desmosomal plaque proteins to the plasma membrane in more detail, we used laser scanning microscopy on HaCaT cells expressing the head domain of plakophilin 1. Whereas plakophilin 1 and E-cadherin staining overlapped only very little (Fig. 4 a), a high degree of overlap was found between plakophilin 1 and desmoplakin staining (Fig. 4 b), demonstrating that the major portion of overexpressed plakophilin 1 head domain does not localize to adherens junctions. These data indicate that plakophilin 1–mediated recruitment of proteins occurs primarily in desmosomes. To investigate the effect of the recruitment on desmosome size and number, we quantitated desmoplakin staining at cell borders by scanning along plasma membrane stretches (Fig. 4, b′ and b′′, arrows). The data are displayed as fluorescence intensity profiles below the corresponding image. The number and size of the peaks within these profiles were significantly higher when recorded along cell borders of two transfected cells (Fig. 4 b′), compared with the cell border between transfected and nontransfected cells (Fig. 4 b′′). Assuming that each peak represents a desmosome or a group of desmosomes, these data indicate that plakophilin 1–mediated recruitment of plaque proteins might result in the generation and enlargement of desmosomes.

To determine the region within the plakophilin 1 head domain responsible for desmosome association, several fragments were constructed (Fig. 1 a). Whereas all of them were still able to associate with desmosomes in HaCaT cells (Fig. 5), only the ΔN1, ΔN2, and ΔC1 fragments were capable of significantly enhancing the recruitment of endogenous desmoplakin (Fig. 5) and other desmosomal plaque proteins (data not shown) to the cell membrane. The ΔC2 fragment did not recruit endogenous desmosomal proteins, although it associated with desmosomes. A major portion of the ΔN2 and ΔC2 fragments remained cytoplasmic (Fig. 5). All fragments were still able to enter the nucleus, but nuclear targeting was more efficient with the ΔN2 and ΔC2 constructs. These experiments show that at least one region mediating plasma membrane targeting of plakophilin 1 as well as a signal directing nuclear localization is retained in all head deletion constructs.

Wild-Type Plakophilin 1 and Its Head Domain Accumulate in the Nucleus of HeLa Cells

When overexpressed in simple epithelial HeLa cells, plakophilin 1 accumulated in the nucleus, but was not recruited to the plasma membrane (Fig. 6 a), suggesting that the nuclear function of plakophilin 1 is conserved among all cells, whereas its function in stabilizing intercellular junctions is restricted to certain cell types. The lack of desmosome association of plakophilin 1 in HeLa cells may be due either to the lack of an appropriate binding partner

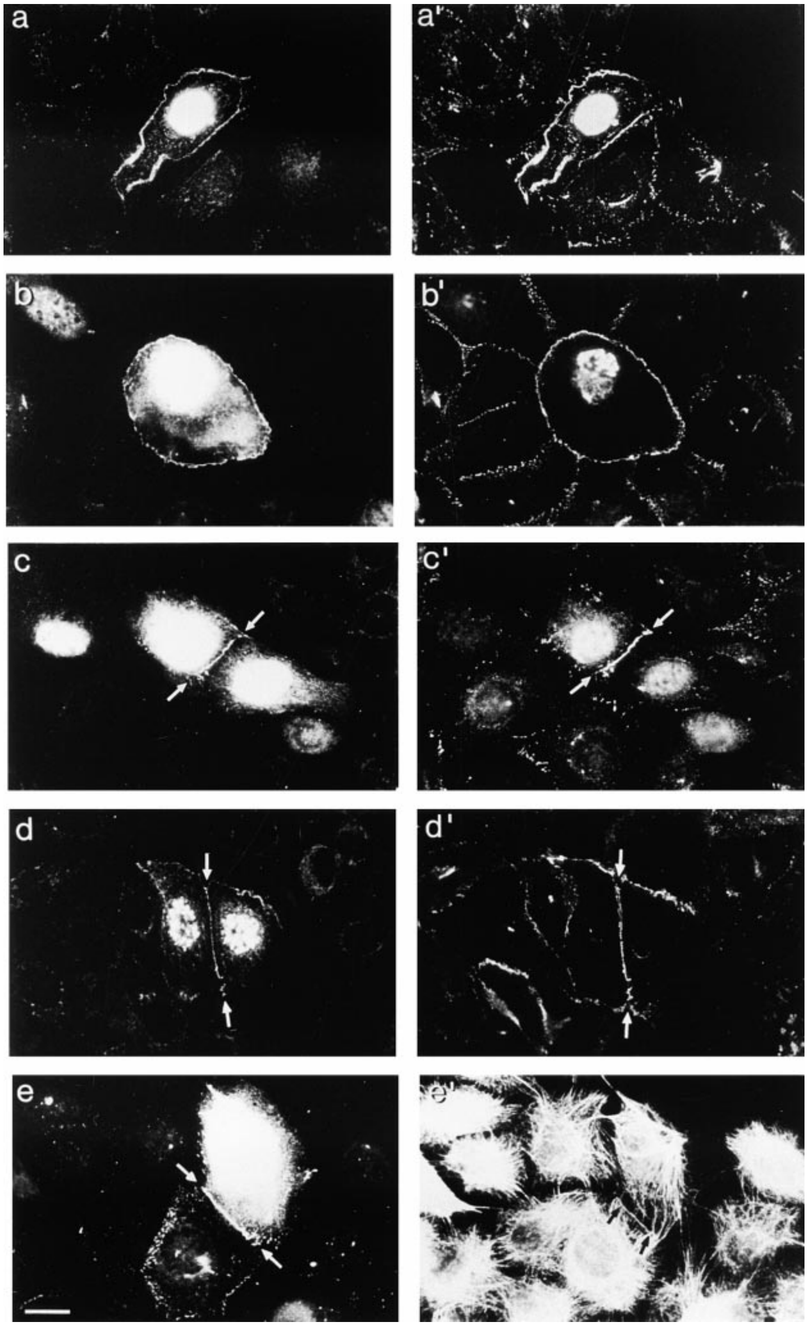

Figure 3. Expression of the plakophilin 1 head domain in HaCaT cells. Plasmid DNAs encoding the plakophilin 1 head domain in pCMV5 were transfected into HaCaT cells. Cells were fixed in methanol and extracted in Triton X-100 and double labeled with the plakophilin 1 head domain antibody (a–e) and antibodies against desmoplakin (a′), desmoglein (b′), desmocollin (c′), plakoglobin (d′), and keratin (e′). In a and b, single transfected cells are in the center; arrows in c–e denote the plasma membranes between two transfected cells. The plakophilin 1 head domain was found in the nucleus and at cell borders; it enhanced the recruitment of desmoplakin (a′), desmoglein (b′), desmocollin (arrows, c′) and, to a lesser extent, of plakoglobin (arrows, d′) to the plasma membrane. Keratins colocalized with plakophilin 1 at the borders of transfected cells (arrows, e′). Bar, 20 μm.

such as cell type–specific Dsg and/or Dsc isoforms, or to different regulatory mechanisms that control modification and/or assembly of desmosomal proteins in HeLa cells. In addition to its nuclear localization, plakophilin 1 was found along actin filaments, as demonstrated by double labeling with phalloidin (Fig. 6, a and a′).

Transfection studies with the plakophilin 1 head domain in HeLa cells showed almost exclusive nuclear localization of the fragment (Fig. 6 b). Decoration of actin filaments was not observed, suggesting that the binding site for direct or indirect actin filament association is located in the arm repeat domain (see below). Desmoplakin staining was strong in the transfected cells, but it appeared in a punctate pattern in the cytoplasm rather than in membranes (Fig. 6 b′). A similar distribution of desmoplakin was seen in mitotic cells (Fig. 6 b′, arrowheads), where desmosomal proteins have been internalized in vesicles. Nontransfected, nonmitotic cells revealed the punctate staining pattern along the plasma membrane, which is typical of desmosomes (Fig. 6 b′, arrows). The extent of cytoplasmic staining of desmoplakin seemed to correlate with plakophilin 1 expression levels. The cytoplasmic staining could be due to internalization of desmosomes and/or enhanced synthesis and assembly of desmosomal proteins in the cytoplasm (Demlehner et al., 1995). The ΔC1 (Fig. 6 c), ΔC2, ΔN1, and ΔN2 (not shown) constructs showed almost exclusive nuclear localization with the same effect on desmoplakin distribution as described above.

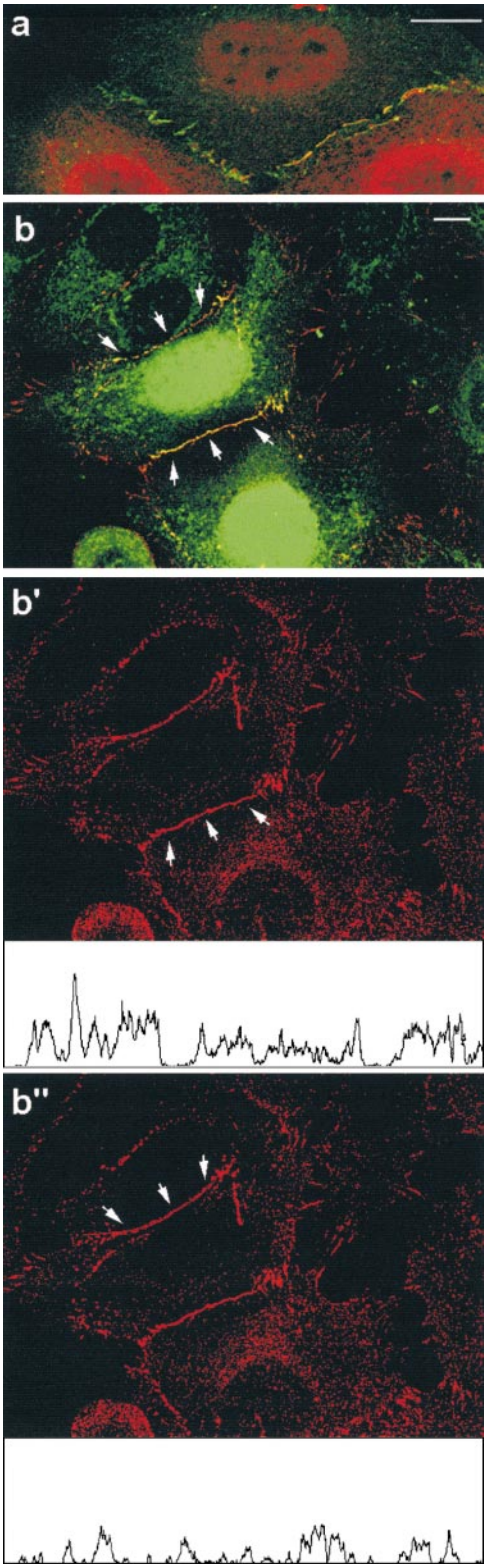

Figure 4. Laser scanning microscopy analysis of HaCaT cells expressing the plakophilin 1 head domain. (a) Cells were stained with the plakophilin 1 head domain antibody (red fluorescence) and the anti–Pan-cadherin antibody (green fluorescence). Overlay of both fluorescence signals showed only little overlap along the plasma membrane, demonstrating that the major portion of plakophilin 1 does not localize to adherens junctions. (b) Cells were stained with the plakophilin 1 head domain antibody (green fluorescence) and an antidesmoplakin antibody (red fluorescence). A high degree of colocalization is visible along cell borders of transfected cells (arrows), demonstrating that plakophilin 1 is recruited primarily to desmosomes. To test whether the size or the number of desmosomes is affected by this recruitment, we recorded fluorescence intensities in the desmoplakin channel by scanning along two defined plasma membrane stretches (arrows, b′ and b′′). The scan results are displayed as intensity profiles below the corresponding image. In the profile recorded along a cell border between two transfected cells (b′), the peak size and number are increased when compared with the profile recorded along a cell border between a transfected and a nontransfected cell (b′′). Note that photobleaching during the scan accounts for the slight differences in the two fluorescent pictures. Bars, 10 μm.

Since wild-type plakophilin 1 decorated actin filaments in transfected HeLa cells, we analyzed plakophilin 1 localization more carefully in nontransfected cells to distinguish whether this was an artifact due to heavy overexpression that disturbed the intracellular sorting mechanisms, or whether it was connected to a novel function of plakophilin 1. In a wound healing experiment with HaCaT cells, colocalization of actin filaments and plakophilin 1 was observed at the tips of cellular protrusions (Fig. 6, d and d′), suggesting a role for plakophilin 1 in regulating actin filament organization. Association with stress fibers was not observed.

The Plakophilin 1 Head Domain Binds to Desmoglein1, Desmoplakin, and Keratins in the Yeast Two-hybrid Assay

Plakophilin 1 has been shown to bind to Dsg1, Dsc1a, and desmoplakin in in vitro overlay assays (Smith and Fuchs, 1998), and to Dsg1 and desmoplakin in the two-hybrid system (Kowalczyk et al., 1999). To localize the binding sites of these proteins in plakophilin 1, the cytoplasmic domains of Dsgs1-3 and Dscs1a,b-3a,b and the NH_2 terminus of desmoplakin were tested in the yeast two-hybrid system. From all the desmosomal cadherins, only Dsg1 interacted with the plakophilin 1 head domain (Fig. 7 a) and with all head domain deletion constructs (Fig. 7, b and c), although the ΔN2 and ΔC2 constructs appeared somewhat less efficient in reporter gene activation, suggesting that the Dsg1 binding site was not completely retained in these constructs. Desmoplakin binding was retained in the ΔC1 and ΔC2 fragments (Fig. 7 b), but not in the ΔN1 and ΔN2 fragments (Fig. 7 c), demonstrating that desmoplakin binds close to the NH_2 terminus of plakophilin 1. These results suggest that desmoplakin and Dsg1 do not compete for the same binding site in the plakophilin 1 head.

Since plakophilin 1 and plakoglobin (Troyanovsky et al., 1993; Chitaev and Troyanovsky, 1997) both bind to desmoplakin and Dsg1, we wanted to analyze if these two proteins provide alternative links between the cadherins and the cytoskeleton, or if plakophilin 1 stabilizes the Dsg-plakoglobin-desmoplakin interaction through additional protein interactions. Therefore, we determined the plakophilin 1 binding site in the Dsg1 cytoplasmic domain. The plakophilin 1 head domain interacted with the intact Dsg1 cytoplasmic domain, the Dsg + CS domain and, although

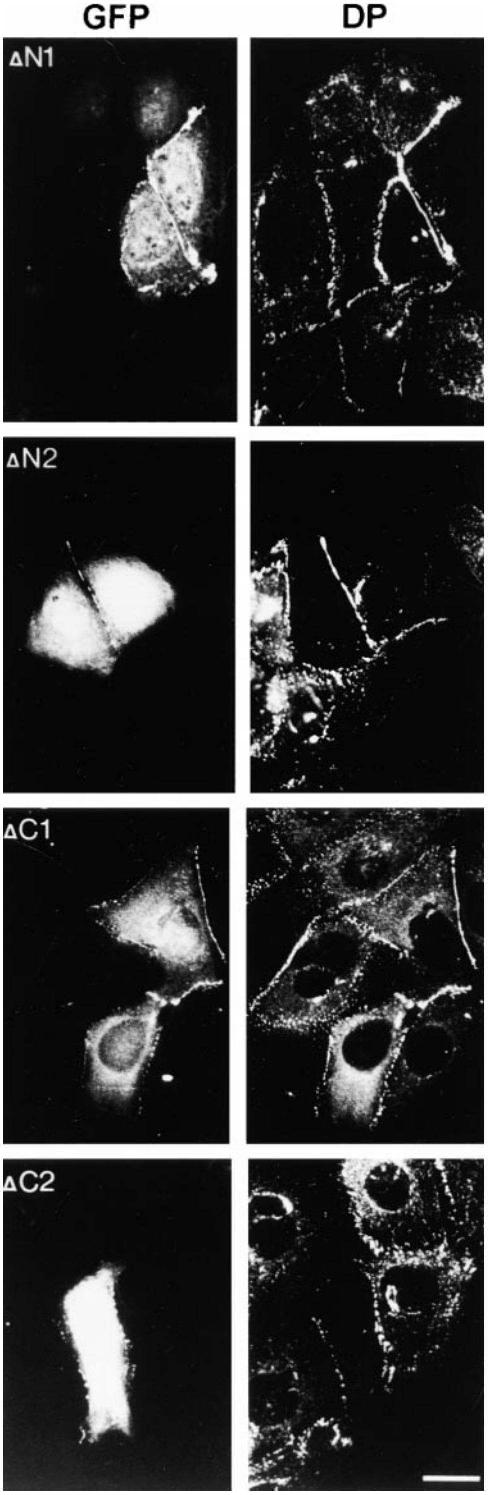

Figure 5. Expression of plakophilin 1 head domain fragments in HaCaT cells. Plasmid DNAs encoding the GFP-tagged plakophilin 1 head domain fragments were transfected into HaCaT cells, and their ability to recruit desmoplakin to the cell membrane was analyzed by immunofluorescence. Whereas desmosome localization was found with all fragments, nuclear staining was strong only with ΔN2 and ΔC2. ΔN1 and ΔC1 showed reduced nuclear staining. Desmoplakin recruitment to the plasma membrane, as revealed by continuous labeling along the cell periphery, was enhanced with ΔN1, ΔN2, and ΔC1. In ΔC2-overexpressing cells, desmoplakin staining showed no considerable increase. Bar, 20 μm.

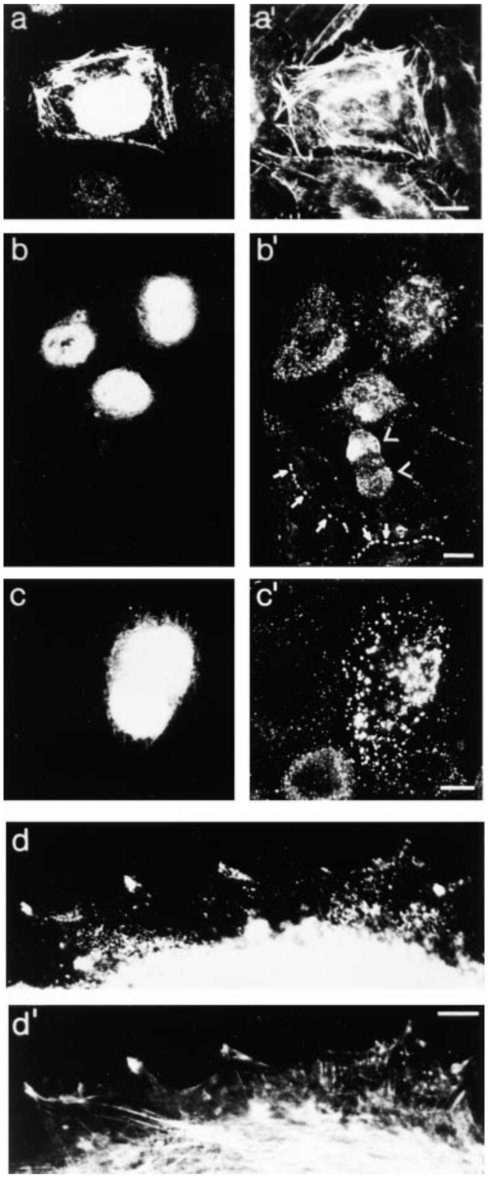

Figure 6. Plakophilin 1 associates with the actin cytoskeleton in HeLa (a–c) and HaCaT (d) cells. Plasmids encoding wild-type plakophilin 1 (a), the head domain (b), and the ΔC1-GFP fusion construct (c) were transfected into HeLa cells, and the cells were fixed in formaldehyde (a) or methanol (b and c) and processed for immunofluorescence. (a) Wild-type plakophilin 1 was predominantly in the nucleus and decorated actin filaments as revealed by double labeling with FITC-phalloidin (a′). (b) The head domain was almost exclusively in the nucleus. In transfected cells, desmoplakin revealed a punctate staining pattern in the cytoplasm. A similar distribution of desmoplakin was seen in mitotic cells (arrowheads). In contrast, desmoplakin showed the punctate staining pattern along the plasma membrane that is typical of desmosomes in other nontransfected cells (arrows, b′). (c) The ΔC1 construct showed a similar distribution as the head domain. It was almost exclusively nuclear, whereas desmoplakin labeling was cytoplasmic (c′). (d) A wound was inserted into a confluent monolayer of HaCaT cells, and cells were fixed in formaldehyde and processed for immunofluorescence after 24 h. Endogenous plakophilin 1 (d) colocalized with actin (d′) at the tips of cellular protrusions of cells next to the wound. Bars, 10 μm.

to a somewhat lesser extent, with the Dsg domain alone (Fig. 7 d), indicating that the plakophilin 1 binding site differs from the plakoglobin binding site in the CS domain. The requirement of the CS domain for strong binding suggests that the plakophilin 1 binding site is close to the plakoglobin binding site, and that simultaneous binding might be prevented because of steric hindrance.

Since plakophilin 1 has been shown to bind keratins in vitro (Kapprell et al., 1988; Hatzfeld et al., 1994; Smith and Fuchs, 1998), we also examined several keratin constructs for their interaction with the plakophilin 1 head domain. As shown in Fig. 7 e, the type I keratins K17 and K18 strongly interacted with the plakophilin 1 head, whereas of the type II keratins tested, only K8 showed a weak interaction. Interaction studies with the head fragments revealed binding of K17 and K18 to the ΔC1 and ΔC2 constructs, but not to ΔN1 and ΔN2. The K8 binding site appeared to differ since binding was retained in the ΔN1, ΔC1, and ΔC2 constructs, but was lost in the ΔN2 fragment (not shown).

Interactions between the plakophilin 1 head fragments and desmoplakin and Dsg1 were quantitated by measuring LacZ reporter gene activation with the ONPG substrate. As shown in Fig. 7 g, the desmoplakin–plakophilin 1 and the Dsg1–plakophilin 1 interactions were much stronger with the plakophilin 1 head in the pBD vector compared with the pAS2-1 vector, although this vector allows high protein expression levels as verified by Western blotting with Gal4 and plakophilin 1–specific antibodies (Fig. 8 a). The high protein expression could either interfere with correct folding of plakophilin 1, or the desmoplakin and Dsg1 binding sites are masked by inter- or intramolecular interactions after expression in the pAS vector. Dsg1 interacted most strongly with the ΔN1 construct, which lacks the desmoplakin binding site. Interaction with the ΔC1 construct was somewhat weaker. In contrast, the ΔC2 and ΔN2 constructs showed considerably reduced reporter gene activation, suggesting that these constructs did not contain the entire Dsg1 binding site. Desmoplakin interacted most strongly with the ΔC2 construct, which lacks part of the Dsg1 binding site. The interaction was lost with the ΔN1 and ΔN2 constructs, indicating that the binding site is in the NH_2-terminal region.

Since all previous experiments had shown interactions between cadherins and the arm repeat domains, but not the end domains of arm proteins, we also analyzed whether the headless plakophilin 1 or the repeat domain interacted with any of the desmosomal cadherins. As shown in Fig. 7 f, none of the desmosomal cadherins interacted with headless plakophilin 1. The same result was obtained with the arm domain construct. Expression of the headless fragment in yeast cells was verified by Western blotting with anti-Gal4 antibodies (Fig. 8 a, lane 7′). Here, the headless fragment gave the strongest signal, indicating that a lack of protein expression did not account for the lack of binding.

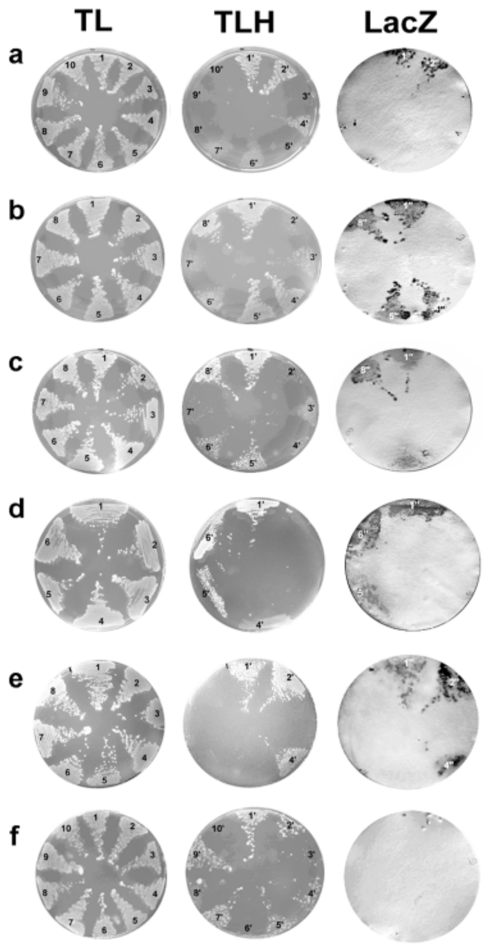

Figure 7 (continues on facing page).

The armadillo Repeat Domain of Plakophilin 1 Associates with Actin and Induces the Formation of Filopodia and Long Cellular Protrusions

The colocalization of wild-type plakophilin 1 with stress fibers as well as actin-rich structures at the tips of filopodia (Fig. 6) pointed to a possible role of plakophilin 1 in regulating the actin cytoskeleton. Therefore, we expressed the plakophilin 1 arm repeat and the headless domain in HeLa and HaCaT cells and verified expression by Western blotting (Fig. 8 b). Cells with high levels of these plakophilin 1 fragments displayed a highly unusual morphology with formation of filopodia, lamellipodia, or long protrusions (Fig. 9, a–d), which interfered with normal monolayer formation. The transfected cells often sat on top of the other cells. Cells with lower expression levels still displayed a normal cell morphology (Fig. 10, a–e). However, double labeling with desmoplakin antibodies showed that desmoplakin had been internalized, and disintegration of junctions had already begun (Fig. 10, e and e′). The plakophilin 1 arm repeat domain colocalized with actin in lamellipodia (Fig. 10, a, c, and d) and sometimes stress fibers (Fig. 10, c and c′), suggesting a role in regulating actin polymerization and filopodia formation. This phenotype was observed in HeLa and HaCaT cells.

The Phenotype Produced by the Plakophilin 1 Arm Domain and Its Capacity to Associate with Actin Filaments Critically Depend on a Conserved Motif

A similar phenotype, the formation of long dendritelike cellular protrusions, had been observed in transfection

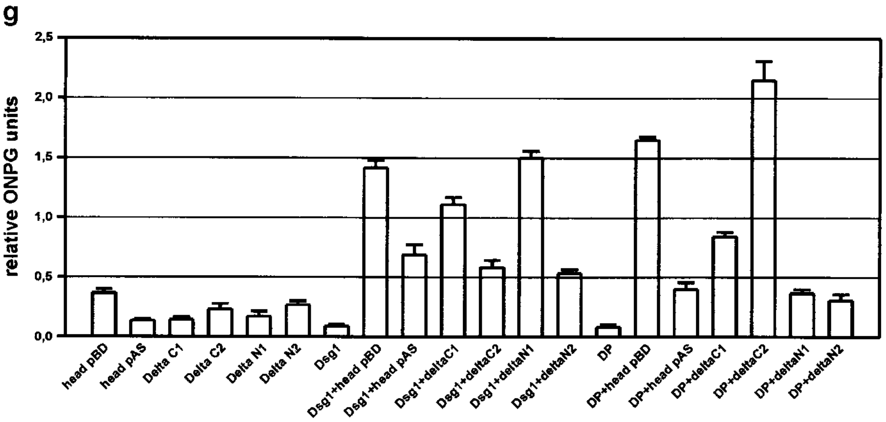

Figure 7. Two-hybrid analysis of protein–protein interactions. (a) YRG2 yeast cells were double transformed with the plakophilin 1 head in pAS2-1 and desmoplakin-NH_2 terminus (DP-NTP, 1), Dsg1 (2), Dsg2 (3), Dsg3 (4), Dsc1a (5), Dsc1b (6), Dsc2a (7), Dsc2b (8), Dsc3a (9), and Dsc3b (10) intracellular domains. All cells grew on selection plates lacking tryptophan and leucine (−TL), indicating that they contain both plasmids. Histidine reporter gene activation was analyzed on plates lacking histidine (−TLH) and LacZ reporter gene activation in a filter lift assay. DP-NTP and Dsg1 activated both reporter genes. (b) Double transformations of the ΔC1 construct in pAS2-1 and Dsg1 (1), Dsc1a (2), Dsc1b (3), and DP-NTP (4), and of ΔC2 with DP-NTP (5), Dsc1b (6), Dsc1a (7), and Dsg1 (8). DP-NTP and Dsg1 interacted with the ΔC1 and ΔC2 constructs, although LacZ reporter gene activation seemed weaker with Dsg1 + ΔC2. (c) Double transformations of the ΔN1 construct in pAS2-1 with Dsg1 (1), Dsc1a (2), Dsc1b (3), and DP-NTP (4) and of ΔN2 with DP-NTP (5), Dsc1b, (6), Dsc1a (7), and Dsg1 (8). ΔN1 and ΔN2 reacted with Dsg1, whereas DP-NTP did not interact with the ΔN1 and ΔN2 constructs. Dsc1a and b did not interact with any of the head domain fragments. (d) Double transformants of the head domain with Dsg1 deletion constructs containing the complete cytoplasmic domain (1), the IA domain (2), the CS domain (3), the IA and the CS domain (4), the Dsg domain (5), and the Dsg and CS domains (6). The head domain interacted strongly with the complete Dsg cytoplasmic domain and with the Dsg+CS domain. The interaction with the Dsg domain alone was weaker. (e) Double transformations of the head domain with K8 (1), K18 (2), K6 (3) and K17 (4) and of the arm domain with K8 (5), K18 (6), K6 (7) and K17 (8). The plakophilin 1 head domain interacted weakly with K8 and more strongly with K17 and K18. The arm repeats did not interact with any of the keratins tested. (f) Double transformations of headless plakophilin 1 with the following intracellular domains: DP-NTP (1), Dsg1 (2), Dsg2 (3), Dsg3 (4), Dsc1a (5), Dsc1b (6), Dsc2a (7), Dsc2b (8), Dsc3a (9), and Dsc3b (10). Although the His reporter gene was weakly activated by some constructs, the LacZ reporter gene was not activated. (g) Interactions between the cytoplasmic domain of Dsg1 and DP-NTP and the plakophilin 1 head domain fragments were quantitated using a β-galactosidase assay and the ONPG substrate. The bars represent three independent experiments each performed in triplet. None of the plakophilin 1 constructs activated the LacZ reporter gene on its own. Dsg1 interacted with all constructs tested. However, the ΔC2 and ΔN2 constructs showed a strong decrease in reporter gene activation suggesting that these constructs do not contain the entire Dsg1 binding site. DP-NTP interacted most strongly with the ΔC2 construct and revealed no interaction with the ΔN1 and ΔN2 constructs.

studies with full-length p120^{ctn} (Reynolds et al., 1996) and δ-catenin (Lu et al., 1999). This suggested that the phenotype is conserved among p120^{ctn} family members, and might depend on the interaction with a common binding partner that is involved in regulating actin filament organization. To characterize this binding site in plakophilin 1, we constructed a deletion mutant that lacks a central pentapeptide motif conserved among all p120^{ctn} family members. The motif (ENCM/VC) is specific for this family and not detected in other arm related proteins. Transfection studies with this mutant construct (plakophilin 1 arm ΔENCMC) showed that the mutant had lost its capacity to induce changes in cell morphology and no longer associated with actin filaments in filopodia (Fig. 10, f and f′). Instead, it accumulated in the cytoplasm, sometimes in an aggregated form (Fig. 10 f).

To analyze if the interaction between plakophilin 1 and actin is direct, we used the two-hybrid system. These experiments revealed no direct interaction between β-actin and the plakophilin 1 repeat domain (data not shown), suggesting that the interaction either depends on an intact microfilament or is mediated through an actin-associated protein in vivo.

Discussion

Plakophilin 1 was shown to localize to desmosomes and to the nucleus, raising the possibility for a dual function in cell adhesion and signal transduction (Schmidt et al., 1997). In the present study, we have determined the regions in plakophilin 1 responsible for binding of desmosomal proteins and provide a functional analysis of the plakophilin 1 domains.

The Head Domain of Plakophilin 1 Mediates Binding to Desmoplakin, Dsg1, and Keratins

Plakophilin 1 is a desmosome-associated protein and has been shown to bind Dsg1, Dsc1a, and desmoplakin in vitro (Smith and Fuchs, 1998) and desmoplakin in vivo (Kowalczyk et al., 1999). Using the yeast two-hybrid assay, we have mapped the binding sites of desmosomal proteins and keratins within plakophilin 1. We show that it is the head domain that mediates the interactions between plakophilin 1 and Dsg1, desmoplakin, as well as keratins. Whereas desmoplakin binds close to the NH_2 terminus between amino acids 1–70 of the head domain, the Dsg1 binding site is located between amino acids 70 and 213 (Fig. 11). Our data suggest that these two sites do not act independently. Dsg1 bound most strongly to the deletion construct lacking the desmoplakin binding site and vice versa. This could be due to reduced accessibility of the binding sites because of intramolecular interactions, which are similar to those described for vinculin and ERM family members (Winkler et al., 1996; Tsukita et al., 1997), or interactions with other proteins. A similar observation was

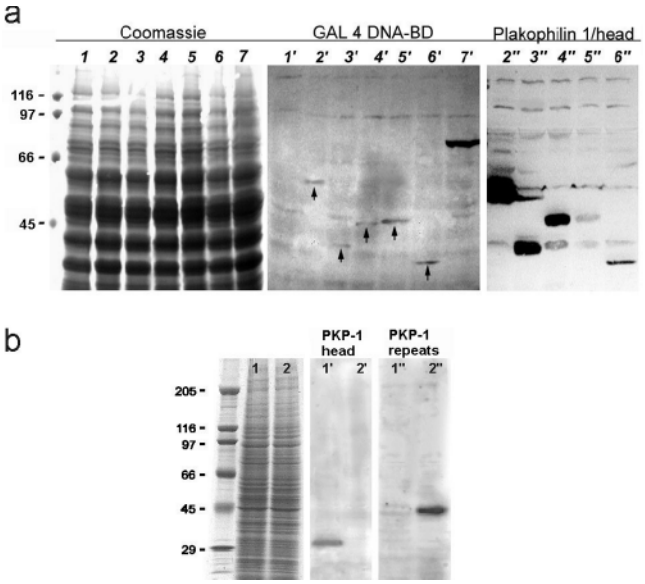

Figure 8. Expression of plakophilin 1 constructs in yeast (a) and HeLa cells (b). (a) Yeast cell extracts were prepared as described in Materials and Methods, and the cell extracts were stained with Coomassie (lanes 1–7) or blotted with anti-GAL4 (lanes 1′–7′) or plakophilin 1 head antibodies (lanes 2″–6″). (lane 1) YRG2 yeast cells without plasmid; (lane 2) plakophilin 1 head domain; (lane 3) ΔC2; (lane 4) ΔC1; (lane 5) ΔN1; (lane 6) ΔN2; and (lane 7) headless plakophilin 1. Arrows denote the plakophilin 1 head fragments reacting with the GAL4 antibody. (b) Total extracts from HeLa cells transfected with the plakophilin 1 head (lanes 1–1″) or the arm repeats (lanes 2–2″) were prepared in SDS sample buffer and probed with the plakophilin 1 head and arm repeat antibodies as indicated. Lanes 1 and 2 show the Ponceau red–stained protein.

made for plakoglobin, certain internal fragments of which bound better to E-cadherin than the entire molecule (Chitaev et al., 1996). Alternatively, high expression of plakophilin 1 could interfere with correct folding and thereby prevent the interaction in an unspecified manner.

The localization of the plakophilin 1 binding site within the Dsg1 cytoplasmic tail showed that it is distinct from the reported plakoglobin binding site (Mathur et al., 1994; Chitaev et al., 1996). However, the close proximity of the two binding sites could prevent simultaneous binding. In contrast to plakophilin 1, other arm family members including β-catenin, plakoglobin, and p120^{ctn} associate with classical or desmosomal cadherins through their arm repeat region (Hinck et al., 1994; Mathur et al., 1994; Aghib and McCrea, 1995; Daniel and Reynolds, 1995; Sacco et al., 1995; Shibamoto et al., 1995; Aberle et al., 1996; Chitaev et al., 1996; Reynolds et al., 1996; Troyanovsky et al., 1996; Wahl et al., 1996; Witcher et al., 1996). Moreover, mutational analysis has revealed that the arm repeat domains of β-catenin and plakoglobin were sufficient to direct nuclear localization (Funayama et al., 1995; Karnovsky and Klymkowsky, 1995). It is interesting that both characteristics, cell contact association as well as nuclear localization, are conserved between β-catenin, plakoglobin, and plakophilin 1, but the domains responsible for these functions are not in the conserved sequence region.

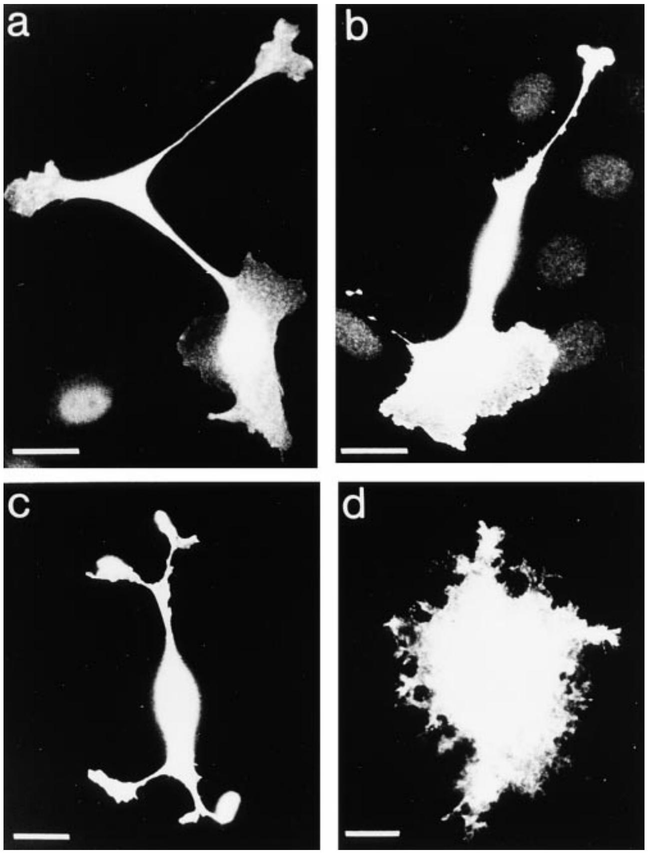

Figure 9. Expression of the arm repeat domain in HaCaT (a and b), L6 (c), and HeLa (d) cells. Cells were transfected with the arm repeat domain in pCMV5 (a, b, and d) or the headless construct in pEGFP (c). Transfected cells were visualized with the T7 antibody (a and b) or the plakophilin 1 repeat antibody (d) or by GFP fluorescence (c). Transfected cells showed changes in cellular morphology, with the development of filopodia and long cellular protrusions. Bars: (a and b) 40 μm; (c and d) 20 μm.

In our two-hybrid assay, we could not confirm the interaction between plakophilin 1 and Dsc1 reported by Smith and Fuchs (1998) using an overlay assay. Since most of the two-hybrid vectors allow only low protein expression we were unable to detect expression of the Dsg, Dsc and keratin fragments by Western blotting. Therefore, we cannot unequivocally rule out the possibility that we might have missed the plakophilin 1–Dsc1 interaction because of a lack of protein expression or a lack of nuclear import of Dsc 1. Alternatively, both proteins could associate in vitro after their denaturation, but not under physiological conditions.

We also detected interactions between plakophilin 1 and keratins. We found a weak binding of K8 and strong binding of K17 and K18, suggesting a preference for type I keratins that had also been proposed on the basis of in vitro overlay assays (Kapprell et al., 1988). In contrast, Smith and Fuchs (1998) have reported that plakophilin 1 binds preferentially to type II keratins. The controversial data may be either due to the analysis of different keratins (K5 and K14 versus K8, K18, K6, and K17) or the use of different assay systems (in vitro overlay versus in vivo assays). Since plakophilin 1 is expressed in suprabasal cells

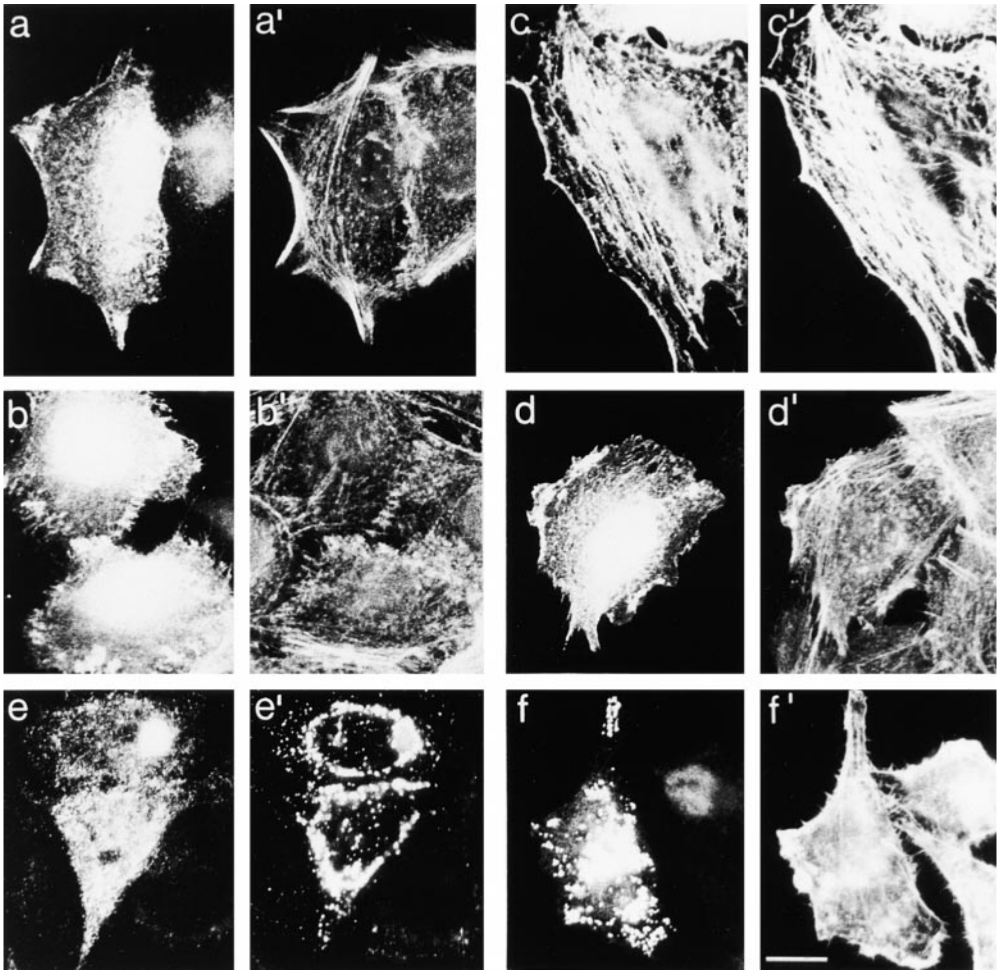

Figure 10. Expression of the arm repeat domain in HaCaT and HeLa cells. (a) HaCaT cells were transfected with plakophilin 1 arm repeats and processed for immunofluorescence after 20 h. Cells were stained with the plakophilin 1 repeat antibody (a) and FITC-phalloidin (a′). (b) HaCaT cells transfected with the GFP-tagged headless construct. Cells were fixed 20 h after transfection and labeled with FITC phalloidin (b′). (c) HeLa cells were transfected with the GFP-tagged headless construct. Cells were labeled with TRITC-phalloidin (c′). (d) HeLa cells transfected with the arm repeats were double stained with the arm repeat antibody (d) and FITC-phalloidin (d′). (e) HeLa cells transfected with the arm repeats were double stained with the arm repeat antibody (e) and the desmoplakin antibody (e′). (f) HeLa cells transfected with the repΔENCMC construct were double labeled with the T7 tag antibody and FITC-phalloidin (f′). Bar, 20 μm.

of stratified epithelia, its in vivo interaction partner is probably one of the keratins specifically expressed in differentiated keratinocytes such as K10.

Plakophilin 1 Enhances Recruitment of Desmosomal Proteins to the Plasma Membrane

We have analyzed intracellular targeting of plakophilin 1 after overexpression. We chose two different cell lines for our studies, HaCaT keratinocytes, which express endogenous plakophilin 1 and consequently all its essential interaction partners, and simple epithelial HeLa cells. These cells possess desmosomes and express the ubiquitous desmosomal proteins, but lack certain cell type–specific desmosomal proteins including Dsg1 and 3, Dsc1 and 3, and plakophilin 1 (Schmidt et al., 1994). Moreover, desmosomes are less abundant and smaller in HeLa cells.

In HaCaT cells, overexpressed plakophilin 1 was found in the nucleus as well as plasma membrane associated, in agreement with the intracellular localization of the endogenous protein (Schmidt et al., 1997). Using deletion clones of plakophilin 1, we have determined which domains target plakophilin 1 to desmosomes (Table I). Whereas cell contact association of other arm proteins including β-catenin, plakoglobin, and p120^{ctn} is mediated by their arm repeat domain (Hinck et al., 1994; Mathur et al., 1994; Aghib and McCrea, 1995; Daniel and Reynolds, 1995; Shibamoto et al., 1995; Aberle et al., 1996; Chitaev et al., 1996; Reynolds et al., 1996; Troyanovsky et al., 1996; Wahl et al., 1996; Witcher et al., 1996), we found that it is the head domain of plakophilin 1 that directs its localization to desmosomes as well as to the nucleus. This is consistent with the localization of the binding sites for desmosomal proteins determined in the two-hybrid system. The nuclear localization was observed in all cell types examined, indicating that the nuclear function is conserved among different cell types. In contrast, desmosome association was restricted to HaCaT cells, suggesting that binding to a cell type–specific desmosomal protein might be essential for targeting, or that regulatory mechanisms prevent the cell contact association of plakophilin 1 in simple epithelial HeLa cells. All the head domain fragments were still able to associate with desmosomes and to enter the nucleus. With the ΔC1 and ΔN1 fragments, desmosome association was preferred over the nuclear localization. This may be due to better accessibility of desmosomal binding sites in these constructs (see above).

In HeLa cells, full-length plakophilin 1 also decorated actin filaments. The head domain alone localized only to the nucleus, indicating that it is the arm repeat domain that mediates the association with the actin cytoskeleton. In HaCaT cells, we also found colocalization of the plakophilin 1 head domain with keratins along membrane patches. This could be due either to the direct interaction

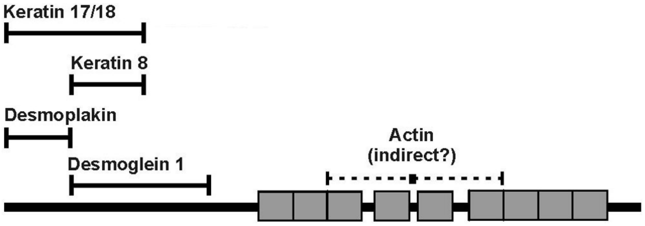

Figure 11. Binding sites of plakophilin 1–associated proteins.

between plakophilin 1 and keratins, as shown in the two-hybrid system, or to recruitment via the keratin-binding protein desmoplakin. Nevertheless, these data, together with the results of the two-hybrid assay, suggest that plakophilin 1 interacts with keratins in vivo. Recruitment of desmoplakin and keratins to the plasma membrane has also been described in cells overexpressing a plakoglobin-synaptophysin chimera (Chitaev et al., 1996).

In a recent report, Kowalczyk et al. (1999) showed that the desmoplakin NH_2 terminus was recruited to the membrane when overexpressed together with plakophilin 1 in COS cells. We extended these experiments and analyzed the recruitment of various endogenous desmosomal proteins in HaCaT cells overexpressing plakophilin 1. As judged by immunofluorescence recruitment of desmoplakin, Dsg and Dsc were strongly enhanced, and that of plakoglobin was slightly enhanced, suggesting a major role for plakophilin 1 in desmosome assembly. In cells with high plakophilin 1 expression, we observed nuclear localization of other desmosomal proteins including desmoplakin, probably due to coimport mediated by plakophilin 1. This conclusion is supported by the fact that the ΔN1 and ΔN2 constructs, which lack the desmoplakin binding site, never coimported desmoplakin into the nucleus (Fig. 5).

There are two ways in which additional desmosomal proteins could be recruited to the plasma membrane. First, plakophilin 1 could bind to endogenous desmosomal proteins, target them to the plasma membrane, and thereby dramatically increase their stability. This is consistent with the finding that desmosomal proteins are usually synthesized in excess, and their cytoplasmic pool is rapidly degraded (Pasdar and Nelson, 1988, 1989). In this model, plakophilin 1 plays a structural role in desmosome assembly. Second, plakophilin 1 might be directly involved in regulating the synthesis of desmosomal proteins in the nucleus. In this model, the induction of desmosomes would depend on a putative signaling function of plakophilin 1. Our experiments do not allow us to distinguish between these two models, since all fragments that were able to induce desmosome formation also revealed nuclear localization and, therefore, might combine the signaling and structural functions. Further, both mechanisms might contribute to the recruitment of endogenous desmosomal proteins. Plasma membrane association of desmoplakin and plakophilin 1 after combined overexpression in COS cells (Kowalczyk et al., 1999) argues for a contribution of the recruitment mechanism.

Using laser scanning microscopy, we demonstrate that plakophilin 1 preferentially associates with and recruits desmosomal proteins, and that the recruitment of desmosomal components might result in the generation and the enlargement of desmosomes. The possibility that expression of plakophilin 1 enhances desmosome formation in keratinocytes is consistent with the observation that desmosomes of suprabasal cells are larger than basal cell desmosomes, and that desmosomes are more numerous in suprabasal cells. Moreover, this finding explains why desmosomes were small and rare in a patient lacking plakophilin 1 (McGrath et al., 1997). Therefore, we propose that plakophilin 1 plays an essential role in regulating desmosome organization and size during keratinocyte differentiation.

The Arm Repeat Domain of Plakophilin 1 Associates with Actin Filaments and Induces Formation of Filopodia

In HeLa cells, overexpressed plakophilin 1 associated with actin filament, suggesting that it might be involved in regulating the actin cytoskeleton. This association was also observed in nontransfected cells, where plakophilin 1 colocalized with actin in normal cells at the tips of plasma membrane protrusions. A similar localization has been described for β-catenin, which interacts with the actin filament bundling protein fascin through its arm repeat domain (Tao et al., 1996). Actin filament association of plakophilin 1 also appeared to be mediated by its arm repeats. When overexpressed at high levels in HeLa and HaCaT cells, the arm repeat domain induced the formation of long cellular protrusions, supporting a possible role in the regulation of cell motility. This phenotype interfered with intercellular adhesion, and transfected cells were separated from the monolayer. Since full-length pla-

Table I. Intracellular Localization of Plakophilin 1 and Its Fragments

	Desmosome	Nucleus	Cytoplasm	IF-associated	Actin-associated
PKP1 head	++	++	(+)*	(+)*	−
PKP1 head ΔC1	++	+	(+)*	(+)*	−
PKP1 head ΔC2	+	++	+	+	−
PKP1 head ΔN1	++	+	(+)	(+)*	−
PKP1 head ΔN2	++	++	+	(+)*	−
PKP1 headless	n.d.[‡]	(+)*	++	−	++
PKP1 arm repeat	n.d.[‡]	(+)*	++	−	++
PKP1 arm repeat ΔENCMC	−	(+)*	++	−	−

*This localization is only seen in a few cells but not in the majority of transfected cells.

[‡] Cannot be determined due to the phenotype that is characterized by a loss of desmosomes.

kophilin 1 had no such effect, we conclude that desmosome association of the head domain is preferred. A similar phenotype has been described for p120^{ctn} (Reynolds et al., 1996) and δ-catenin (Lu et al., 1999) after expressing the full-length protein. In the case of p120^{ctn}, the arm domain was required for this effect (Reynolds et al., 1996), suggesting that this function is conserved in the arm domain of p120^{ctn} family members. We identified a 5–amino acid motif (ENCMC) that is conserved among p120^{ctn} family members. Deletion of this motif in the plakophilin 1 arm repeat domain abolished the ability of the mutant to associate with actin filaments and to induce the phenotype. This suggests that a protein–protein interaction mediated by this motif is responsible for this effect. Since we were unable to detect a direct interaction between the plakophilin 1 arm repeats and actin in the two-hybrid system, the interaction either requires an intact microfilament, as opposed to an actin monomer, or it is mediated by an actin-binding protein.

The phenotype in patients lacking plakophilin 1 suggested an important role for plakophilin 1 in stabilizing intercellular adhesion, although the lack of hair follicles and sweat glands suggests an additional role in certain differentiation processes (McGrath et al., 1997). Our results support the conclusion that plakophilin 1 has an important structural function and explain the role of plakophilin 1 in desmosome assembly at a molecular level. The localization of desmosomal binding sites to the head domain correlates with the finding that this domain recruits endogenous desmosomal proteins to sites of cell contact, whereas the arm repeat domain reduced cell contacts and induced the formation of motility-associated structures. In confluent keratinocytes, localization of plakophilin 1 to desmosomes is preferred over association with adherens junctions and actin filaments. This is consistent with strong intercellular adhesion in these cells. However, in cells that lack contact to adjacent cells, plakophilin 1 localizes to filopodia. Here, it may have a function in inducing junction formation as soon as the tip of the cell contacts an opposing cell. This idea is consistent with the finding that formation of actin-associated cell contacts precedes desmosome formation and is a prerequisite for desmosome formation (Lewis et al., 1994, 1997). Plakophilin 1 could play a role in recruiting desmosomal proteins from the cytoplasm to the plasma membrane at sites of newly formed cell contacts.

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