The Amino-terminal Domain of Desmoplakin Binds to Plakoglobin and Clusters Desmosomal Cadherin–Plakoglobin Complexes

Andrew P. Kowalczyk,‡§ Elayne A. Bornslaeger,*,§ Jeffrey E. Borgwardt,*,§ Helena L. Palka,*,§ Avninder S. Dhaliwal,*,§ Connie M. Corcoran,*,§ Mitchell F. Denning,*,§ and Kathleen J. Green*,‡§

*Department of Pathology; ‡Department of Dermatology; and §the Robert H. Lurie Cancer Center, Northwestern University Medical School, Chicago, Illinois 60611

Abstract. The desmosome is a highly organized plasma membrane domain that couples intermediate filaments to the plasma membrane at regions of cell–cell adhesion. Desmosomes contain two classes of cadherins, desmogleins, and desmocollins, that bind to the cytoplasmic protein plakoglobin. Desmoplakin is a desmosomal component that plays a critical role in linking intermediate filament networks to the desmosomal plaque, and the amino-terminal domain of desmoplakin targets desmoplakin to the desmosome. However, the desmosomal protein(s) that bind the amino-terminal domain of desmoplakin have not been identified. To determine if the desmosomal cadherins and plakoglobin interact with the amino-terminal domain of desmoplakin, these proteins were co-expressed in L-cell fibroblasts, cells that do not normally express desmosomal components. When expressed in L-cells, the desmosomal cadherins and plakoglobin exhibited a diffuse distribution. However, in the presence of an amino-terminal desmoplakin polypeptide (DP-NTP), the desmosomal cadherins and plakoglobin were observed in punctate clusters that also contained DP-NTP. In addition, plakoglobin and DP-NTP were recruited to cell–cell interfaces in L-cells co-expressing a chimeric cadherin with the E-cadherin extracellular domain and the desmoglein-1 cytoplasmic domain, and these cells formed structures that were ultrastructurally similar to the outer plaque of the desmosome. In transient expression experiments in COS cells, the recruitment of DP-NTP to cell borders by the chimera required co-expression of plakoglobin. Plakoglobin and DP-NTP co-immunoprecipitated when extracted from L-cells, and yeast two hybrid analysis indicated that DP-NTP binds directly to plakoglobin but not Dsg1. These results identify a role for desmoplakin in organizing the desmosomal cadherin–plakoglobin complex and provide new insights into the hierarchy of protein interactions that occur in the desmosomal plaque.

Desmosomes are highly organized adhesive intercellular junctions that couple intermediate filaments to the cell surface at sites of cell–cell adhesion (Farquhar and Palade, 1963; Staehelin, 1974; Schwarz et al., 1990; Garrod, 1993; Collins and Garrod, 1994; Cowin and Burke, 1996; Kowalczyk and Green, 1996). Desmosomes are prominent in tissues that experience mechanical stress, such as heart and epidermis, and the disruption of desmosomes or the intermediate filament system in these organs has devastating effects on tissue integrity (Steinert and Bale, 1993; Coulombe and Fuchs, 1994; Fuchs, 1994; McLean and Lane, 1995; Stanley, 1995; Bierkamp et al., 1996; Ruiz et al., 1996). Desmosomes are highly insoluble structures that can withstand harsh denaturing conditions.
mechanism by which the desmosomal cadherins mediate cell–cell adhesion remains elusive (Amagai et al., 1994; Chid-gey et al., 1996; Kowalczyk et al., 1996), although hetero-
philic interactions have recently been detected between desmogleins and desmocollins (Chitave and Troyanovsky, 1997). Both classes of the desmosomal cadherins associate with the cytoplasmic plaque protein plakoglobin (Kowal-
czyk et al., 1994; Mathur et al., 1994; Roh and Stanley, 1995b; Troyanovsky et al., 1994), which is part of a grow-
ing family of proteins that share a repeated motif first iden-
tified in the Drosophila protein Armadillo (Peifer and Wieschaus, 1990). This multigene family also includes the desmosomal proteins band 6/plakophilin 1, plakophilin 2a and 2b, and p0071, which are now considered to comprise a subclass of the armadillo family of proteins (Hatzfeld et al., 1994; Heid et al., 1994; Schmidt et al., 1994; Hatzfeld and Nachtsheim, 1996; Mertens et al., 1996).

The most abundant desmosomal plaque protein is des-
moplakin, which is predicted to be a homodimer contain-
ing two globular end domains joined by a central α-helical coiled-coil rod domain (O’Keefe et al., 1989; Green et al., 1990; Virata et al., 1992). Previous studies have demon-
strated that the carboxy-terminal domain of desmoplakin interacts with intermediate filaments (Stappenbeck and Green, 1992; Stappenbeck et al., 1993; Kouklis et al., 1994; Meng et al., 1997), and the amino-terminal domain of des-
moplakin is required for desmoplakin localization to the desmosomal plaque (Stappenbeck et al., 1993). Direct evi-
dence supporting a role for desmoplakin in intermediate filament attachment to desmosomes was provided recently when expression of an amino-terminal polypeptide of des-
moplakin was found to displace endogenous desmoplakin

1. Abbreviation used in this paper: DP-NTP, desmoplakin amino-terminal polypeptide.
A deletion of plakoglobin lacking the amino-terminal domain cDNA was isolated and subcloned into the mammalian expression vector LK-444. For each of the plakoglobin deletion constructs, PCR was performed using primers LN54 (5'-ATGGGCTATTCTACATGGGATCT-3') to generate a 3' HindIII restriction site in pBluescript and then into the mammalian expression vector LK-444. The resulting PCR product of plakoglobin cDNA and the 3' end of the E-cadherin were each subcloned into the RC4B expression vector (Evans and Scarpulla, 1988) for transfection into eukaryotic cells. The EcadPlsg chimera was subcloned into the mammalian expression vector LK-444, which uses the β-actin promoter as described previously (Kowalczyk et al., 1994).

**Plakoglobin and Plakoglobin Deletion Mutants.** A full length plakoglobin cDNA was isolated and subcloned into the mammalian expression vector pBluescript (Strategene, La Jolla, CA) and PCR-generated regions were replaced with cloned DNA or sequenced to verify that errors were not introduced into the coding sequence. The KpnI sites were used to ligate the cDNA encoding the extracellular domain of E-cadherin to the plasminogen domain of Dsg1. cDNAs encoding Dsg1, Dsc2a, and the Dsc1cend chimeras were each subcloned into the RC4B expression vector (Evans and Scarpulla, 1988) for transfection into eukaryotic cells. The EcadDsg1 chimera was subcloned into the mammalian expression vector LK-444, which uses the β-actin promoter as described previously (Kowalczyk et al., 1994).

**Immunoprecipitation and Immunoblot Analysis**

Immunoprecipitation was carried out as described (Kowalczyk et al., 1994) with the following changes. L-cells were scraped into Tris-buffered saline containing 0.5% Triton X-100, vortexed, and subjected to centrifugation at 14,000 g. Antibodies directed against Dsg1 (peptidomimetic peptide # 982), the desmoplakin amino terminus (NW161), or preimmune serum from antibody NW161 were incubated with the cell lysate for 2 h at 4°C. Immune complexes were released by incubation in reducing SDS-PAGE sample buffer and analyzed by immunoblot using Enhanced Chemiluminescence (Amersham Intl, Arlington Heights, IL). Plakoglobin was detected using mAb 11E4, which is directed against the amino-terminal domain of plakoglobin, or a polyclonal antibody directed against the carboxy-terminal domain (Hinck et al., 1994; Kowalczyk et al., 1994).

**Yeast Two Hybrid Constructs and Assays**

Yeast two hybrid vectors encoding the GAL4 DNA binding (pAS-CYH2; Harper et al., 1993) or transcription activation domain (pACTII; Bai and Elledge, 1995) were generously provided by Dr. Stephen Elledge (Baylor College of Medicine, Houston, TX). The amino-terminal deletion construct of human plakoglobin (pPgA) described above was subcloned from pBluescript into the activation domain construct pACTII. The cDNA in pBluescript was restriction digested with SalI and treated with Klenow fragment of DNA polymerase I to create a blunt 5' end. The DNA was subsequently digested with EcoRI to remove the pPgA insert and subcloned into the SalI and EcoRI sites of pACTII to generate a pPgA cDNA, which was inserted in frame with upstream sequences encoding the GAL4 transcription activation domain (p515). A construct comprising the cDNA sequences encoding the entire cytoplasmic tail of human Dsg1 in the pASI-CYH2 DNA binding domain vector was a generous gift from Dr. Michael Klymkowsky (University of Colorado, Boulder, CO). The cDNA sequences encoding Dsg1 were removed from pASI-CYH2 by digestion with EcoRI and BamHI and subcloned into the same sites of the Path 1 vector (p47), which contains a SalI restriction site 3' of the BAM HI site. The Dsg1 sequences were then removed with EcoRI and SalI and subcloned into the EcoRI and Xhol sites of pACTII to generate a construct encoding nucleotides 1,633–3,072 of human Dsg1 in frame with upstream sequences encoding the GAL4 activation domain (p504). The cDNA encoding DP-NTP in pBluescript (p552) was restriction digested with BamHI and SalI and subcloned into the BamHI and Xhol sites of pACTII (p597). This construct was subsequently digested with Nool and SmaI to subclone the DP-NTP sequences into the same restriction sites of the pASI-CYH2 vector (p601). The first 584 amino acids of human desmoplakin are encoded in both the pACTII and pAS1-CYH2 constructs, similar to the DP-NTP vector described above.

To assay interactions between proteins, yeast (strain HF7C) were transformed with the plasmids of interest and grown on synthetic defined media lacking either tryptophan (SD-try) for the pASI-CYH2 vector or leucine (SD- Leu) for the pACTII vector to select for transformed clones. Transformations and β-galactosidase assays were performed according to methods published in the Matchmaker™ Two Hybrid product protocol (Clontech Laboratories Inc., Palo Alto, CA). Briefly, isolated colonies were transformed with 5 to 10 μg plasmid DNA using a standard lithium acetate/polyethylene glycol method. Interactions between proteins were tested by streaking transformed colonies onto Whatman filter membranes and assaying β-galactosidase activity using the substrate X-Gal (5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside; Sigma Chemical Co.). As a sec-
ond reporter for interactions, colonies were also tested for growth on SD-leu-trp-his in the presence of 20 mM 3-aminotriazole (Sigma Chemical Co.). Materials for base media and agar were obtained from DIFCO Laboratories (Detroit, MI), and materials for synthetic defined media were purchased from Clontech Laboratories Inc.

Results

The Desmoplakin Amino-terminal Domain Clusters Desmosomal Cadherin–Plakoglobin Complexes

The amino-terminal domain of desmoplakin was previously demonstrated to target desmoplakin to the desmosomal plaque (Stappenbeck et al., 1993; Bornslaeger et al., 1996). We hypothesized that the amino-terminal region of desmoplakin may interact with the desmosomal cadherin-plakoglobin complex, thereby coupling the desmosomal cadherins to the intermediate filament cytoskeleton. To address this question, a series of stable L-cell fibroblast cell lines expressing various desmosomal molecules was established to reconstitute complexes of specific desmosomal components. A schematic diagram depicting the constructs used in this study is shown in Fig. 1. L-cell lines expressing Dsg1 and plakoglobin (L-Dsg1/Pg), Dsg1, plakoglobin and DP-NTP (L-Dsg1/Pg/DP-NTP), or the neomycin resistance marker (L-neo) were analyzed by immunoblot using antibodies directed against Dsg1 (pemphigus foliaceus sera # 982; Kowalczyk et al., 1994, 1995), plakoglobin (monoclonal antibody 11E4; Kowalczyk et al., 1994), or the amino-terminal domain of human desmoplakin (NY161; Bornslaeger et al., 1996). The arrow in the DP-NTP blot identifies the 70-kD DP-NTP polypeptide; two higher molecular weight bands are detected nonspecifically. A rabbit polyclonal antibody directed against vimentin (ICN Pharmaceuticals, Inc. [Costa Mesa, CA]) was used to demonstrate that similar amounts of total protein were loaded in each lane. Note that plakoglobin in the L-Dsg1/Pg line contains a carboxyl-terminal c-myc epitope tag and migrates more slowly than untagged plakoglobin in the L-Dsg1/Pg/DP-NTP cell line.
ence of DP-NTP. Out of a total of 84 L-cell lines expressing Dsg1 and plakoglobin that were generated, clustering was not observed in any of these cell lines (see also Kowalczyk et al., 1996). In contrast, 16 independent clones of L-cell lines expressing the desmoplakin polypeptide were generated, and clustering was observed in all 16 clones. Furthermore, in the representative Dsg1/Pg/DP-NTP cell line shown here, punctate Dsg1 staining was observed in 71 out of 111 cells. This is in comparison to 0 out of 133 cells for the Dsg1/Pg line, indicating that the clustering was due to the co-expression of the desmoplakin polypeptide.

Laser scanning confocal microscopy was used to further characterize the distribution of the punctate clusters in cell lines co-expressing Dsg1, plakoglobin, and DP-NTP. In many instances, punctate clusters of Dsg1 were visualized along the ventral cell surface, as shown in Fig. 4A, where the focal plane was set at the cell–substrate interface. These clusters colocalized with plakoglobin (not shown), consistent with the results shown in Fig. 3. Vertical sections of the cell in Fig. 4A reveals the presence of clusters on both the ventral and dorsal cell surfaces (Fig. 4B). This distribution was observed consistently, as demonstrated by the additional vertical sections shown in Fig. 4, C and D. In addition, treatment of cell lines expressing Dsg1, plakoglobin, and DP-NTP with trypsin demonstrated that the Dsg1...
was sensitive to trypsin degradation (Fig. 4 E). The antibody used to detect Dsg1 (Ab NW1) is directed against the Dsg1 cytoplasmic domain (Kowalczyk, A.P., unpublished observations), indicating that the generation of a Dsg1 fragment of \( \sim 100 \) kD in cells treated with trypsin is due to degradation of a portion of the Dsg1 extracellular domain. Together with the observations that the punctate clusters of Dsg1 were observed along the plasma membrane by confocal microscopy, the trypsinization results indicate that the vast majority of Dsg1 was present on the cell surface. This is consistent with a previous report demonstrating that both Dsg1 and Dsc2a are present on the cell surface when expressed in L-cells (Kowalczyk et al., 1995).

**DP-NTP Also Clusters Dsc2a–Plakoglobin Complexes**

In contrast to adherens junctions, desmosomes contain two distinct subclasses of cadherins, desmogleins and desmocollins. Therefore, the ability of DP-NTP to cause clustering of Dsc2a–plakoglobin complexes was also analyzed (Fig. 5). When expressed alone, Dsc2a (Fig. 5 A) and plakoglobin (Fig. 5 B) were distributed in a diffuse, largely uniform staining pattern along the membrane of the L-cells. However, in the presence of DP-NTP, both Dsc2a (Fig. 5 C) and plakoglobin (Fig. 5 D) were colocalized in distinct punctate clusters. In addition, DP-NTP (Fig. 5 E) and plakoglobin (Fig. 5 F) colocalized in these clusters, indicating that DP-NTP causes clustering of both classes of desmosomal cadherins.

![Figure 5](image5.png)

**A Chimeric Cadherin with the E-cadherin Extracellular Domain and the Dsg1 Cytoplasmic Domain (EcadDsg1) Recruits Plakoglobin and DP-NTP to Cell–Cell Borders**

A number of previous studies have demonstrated that the desmosomal cadherins do not mediate homophilic adhesion when expressed in L-cells (Amagai et al., 1994; Chidgey et al., 1996; Kowalczyk et al., 1996). A previous study demonstrated that a chimeric cadherin with the E-cadherin extracellular domain and the Dsg3 cytoplasmic domain mediates homophilic adhesion in L-cells (Roh and Stanley, 1995a). Therefore, a chimera containing the E-cadherin extracellular domain and Dsg1 cytoplasmic domain (EcadDsg1) was co-expressed with plakoglobin and DP-NTP to determine if the chimera could recruit plakoglobin and DP-NTP to regions of cell–cell contact. In the absence of DP-NTP, the EcadDsg1 chimera (A) and plakoglobin (B) colocalized at cell–cell interfaces. In the presence of DP-NTP, the EcadDsg1 chimera (C) and plakoglobin (D) were present in a punctate staining pattern at cell–cell borders. In addition, DP-NTP was also present at cell interfaces (E) and colocalized with plakoglobin (F) in punctate clusters, indicating that DP-NTP was recruited to cell–cell interfaces by the cadherin–plakoglobin complex. Bar, 10 \( \mu \)m.
trubuted in a punctate pattern. In addition, the EcadDsg1 chimera and plakoglobin were often localized at regions of cell–cell contact. Furthermore, DP-NTP (Fig. 6 E) was recruited to cell–cell contacts and colocalized with plakoglobin (Fig. 6 F). The punctate distribution of DP-NTP at cell–cell interfaces was observed only in L-cells expressing the EcadDsg1 chimera. The observation that the EcadDsg1 chimera, plakoglobin, and DP-NTP were all present at cell–cell borders suggests that all three proteins were present in the same complex and that these complexes were recruited to areas of intercellular contact by the homophilic interactions of the E-cadherin extracellular domain.

Cell lines expressing the EcadDsg1 chimera and plakoglobin in the presence or absence of DP-NTP were also examined by electron microscopy. In L-cell lines co-expressing DP-NTP, electron-dense regions subjacent to the plasma membrane were observed at regions of cell–cell contact (Fig. 7 B). Interestingly, these structures are similar to the junctions lacking association with intermediate filaments that were previously reported in A431 cell lines expressing DP-NTP (Fig. 7 C; Bornslaeger et al., 1996). Although the EcadDsg1 chimera and plakoglobin did colocalize at cell–cell borders in the absence of DP-NTP, the extensive electron-dense plaque-like structures such as those present in cells co-expressing DP-NTP (Fig. 7 B) were never observed in the absence of DP-NTP (Fig. 7 A). These data suggest that clustering of the cadherin–plakoglobin complex by DP-NTP leads to the formation of a submembrane plaque that can be identified ultrastructurally and which closely resembles the outer dense plaque of the desmosome.

**Plakoglobin Bound to the Desmoglein Cytoplasmic Domain Does Not Associate with α-Catenin**

In contrast to the desmosomal cadherins, E-cadherin is coupled to the actin cytoskeleton through interactions with α-catenin, which is able to bind directly to both β-catenin and plakoglobin. The ability of plakoglobin to bind to α-catenin and to associate with both desmosomal cadherins and classical cadherins raises the question as to how these two classes of cadherins are coupled to distinct cytoskeletal filament networks. Therefore, we used the EcadDsg1 chimera along with a Dsg1Ecad chimeric cadherin to determine if α-catenin could associate with plakoglobin that is bound to the Dsg1 cytoplasmic domain. The chimeric cadherins were immunoprecipitated from cell lysates using antibodies directed against the extracellular domain of either chimera. Immunoblot analysis of the immunoprecipitated proteins revealed that α-catenin co-immunoprecipitated only with the Dsg1Ecad chimera and did not co-immunoprecipitate with the EcadDsg1 chimera (Fig. 8 A). However, plakoglobin co-immunoprecipitated with both the Dsg1Ecad and the EcadDsg1 chimeras (Fig. 8 B). These data indicate that plakoglobin that is associated with the Dsg1 cytoplasmic domain does not bind to α-catenin.

**Plakoglobin Is Required for DP-NTP to Associate with the Dsg1 Cytoplasmic Domain**

In adherens junctions, β-catenin is thought to link E-cadherin to α-catenin by binding directly to both proteins. Due to the fact that plakoglobin and β-catenin are closely related, we reasoned that plakoglobin may couple desmoplakin to the desmosomal cadherin cytoplasmic domains. To test this hypothesis, the distribution of Dsg1 was monitored in L-cell lines expressing DP-NTP in the presence of plakoglobin deletion mutants or in the absence of exogenously expressed plakoglobin (Fig. 9). Similar to control cells expressing full length plakoglobin (see Fig. 3), Dsg1 was distributed in punctate clusters in L-cells co-expressing DP-NTP in the presence of plakoglobin deletion mutants lacking either the amino- (Fig. 9 A) or carboxyl-terminal (Fig. 9 B) domains (refer to Fig. 1 for deletion map). In the absence of exogenously expressed plakoglobin, Dsg1 (Fig. 9, C and E) was distributed in a diffuse pattern in cells co-expressing Dsg1 and DP-NTP. Very low levels of endogenous plakoglobin are expressed in L-cells (Fig. 9 D),
and only small amounts of endogenous plakoglobin can be detected in L-cells expressing Dsg1 (Kowalczyk et al., 1994). Although DP-NTP was occasionally observed in a punctate staining pattern, DP-NTP was more diffuse (Fig. 9 F) in the absence of exogenous plakoglobin than in cells co-expressing plakoglobin (see also Figs. 3, 5, and 6).

To further define the role of plakoglobin in the association of DP-NTP with the desmosomal cadherin cytoplasmic domains, the EcadDsg1 chimera, plakoglobin, and DP-NTP were transiently expressed in COS cells (Fig. 10). When co-expressed in the absence of the chimera, plakoglobin (Fig. 10 A) was distributed diffusely in the cytoplasm and DP-NTP (Fig. 10 B) was distributed in perinuclear aggregates. COS cells assemble some desmosomes, and low levels of both plakoglobin and desmoplakin staining at cell borders could be detected in transiently transfected cells, presumably reflecting incorporation of the transiently expressed proteins into existing desmosomes (Stappenbeck et al., 1993). However, the vast majority of transiently expressed DP-NTP was not recruited to cell borders by the EcadDsg1 chimera in the absence of plakoglobin (Fig. 10, A and B). In COS cells cotransfected with the EcadDsg1 chimera and DP-NTP, the EcadDsg1 chimera accumulated at cell–cell borders (Fig. 10 C), whereas DP-NTP remained in perinuclear aggregates (Fig. 10 D). In contrast, in cells transiently expressing plakoglobin, DP-NTP, and the EcadDsg1 chimera, the EcadDsg1 chimera (Fig. 10 E) colocalized at borders with both plakoglobin (not shown) and DP-NTP (Fig. 10 F). These findings are consistent with the results observed in L-cell lines and demonstrate that plakoglobin is required for the EcadDsg1 chimera to recruit DP-NTP to areas of cell–cell contact.

Plakoglobin Forms Complexes with DP-NTP in L-cells and Binds Directly to DP-NTP

Co-immunoprecipitation experiments were conducted to determine if protein complexes could be identified in L-cells that exhibited clustered cadherin–plakoglobin complexes. L-cells co-expressing Dsg1, DP-NTP, and plakoglobin or deletion mutants of plakoglobin were solubilized in 0.5% Triton X-100, and immunoprecipitations were carried out using an antibody directed against either Dsg1 or DP-NTP.
Plakoglobin or plakoglobin deletion mutants were then detected using antibodies directed against the amino-terminal (PgNT) or carboxyl-terminal domain (PgCT) of plakoglobin. The results indicate that plakoglobin and the plakoglobin deletion mutants co-immunoprecipitate with both Dsg1 and DP-NTP. Preimmune sera from antibody NW161 was used as a control to verify that plakoglobin was co-immunoprecipitated specifically with DP-NTP. These data demonstrate that plakoglobin is in a complex with DP-NTP in the L-cell lines. In addition, the fact that plakoglobin deletion mutants lacking either the amino- or carboxyl-terminal end domains of plakoglobin also co-immunoprecipitated with DP-NTP suggests that the central armadillo repeats of plakoglobin are important for association with both Dsg1 and DP-NTP.

To determine if plakoglobin and DP-NTP interact directly, these proteins were expressed in the yeast two-hybrid system and interactions were analyzed by monitoring β-galactosidase activity (Fig. 12, A–D). Cotransformation of plasmids containing the interacting domains from p53 and large T antigen (p53DB and LTA) were used as positive controls for transcription activation (Fig. 12 A). Control experiments were also conducted to verify that the DP-NTP (DP-NTPDB) and plakoglobin constructs (PGDN-TA) did not spontaneously activate transcription. No β-galactosidase activity was observed when PGDN-TA was tested for interaction with p53DB (Fig. 12 B). However, β-galactosidase activity was consistently observed when yeast were cotransformed with PGDN-TA and DP-NTPDB (Fig. 12 C), indicating that these proteins interact directly. Although DP-NTPDB was found to interact with PGDN-TA (Fig. 12 C), an interaction between DP-NTPDB and the Dsg1 cytoplasmic tail (Dsg1TA) was not detected (Fig. 12 D). Similar to previous studies (Witcher et al., 1996), the Dsg1 cytoplasmic tail exhibited strong interactions with a PGDN-DNA–binding domain fusion, indicating that the Dsg1 construct was functional (not shown). Although DP-NTP did not activate transcription in the DNA-binding domain vector, PGDN in the DNA-binding domain construct did exhibit low levels of transcription activation, precluding our ability to test PGDN interaction with DP-NTP in the reverse orientation. However, these results were confirmed using growth in the absence of histidine as a second, independent reporter for the activation of transcription. Yeast cotransformed with DP-NTPDB and PGDN-TA exhibited growth on agar plates lacking histidine in the presence of 20 mM 3-aminotriazole (Fig. 12 G), whereas no growth was detected in parallel experiments in which the Dsg1 tail was tested for interactions with DP-NTP (Fig. 12 H). These data indicate that DP-NTP binds directly to plakoglobin but not Dsg1.
Figure 12. Plakoglobin interacts directly with DP-NTP in the yeast two hybrid system. Colonies of yeast strain HF7c were transformed with the indicated constructs, and colonies were assayed for β-galactosidase activity (A–D) or the ability to grow in the absence of histidine (E–H). Positive control vectors encoding interacting domains of p53 in the DNA-binding domain vector (DB) and large T antigen in the transcription activation vector (TA) were cotransformed as controls for transcription activation. Yeast transformed with these vectors exhibited high levels of β-galactosidase activity (A) and large amounts of growth on histidine drop out medium (E). In contrast, cotransformation with p53DB and a plakoglobin deletion construct PGΔN TA did not exhibit β-galactosidase activity or growth in the absence of histidine. However, yeast cotransformed with PGΔN TA and DP-NTPDB exhibited both β-galactosidase activity (C) and growth on histidine drop out plates (G), indicating that these two proteins interact directly. In contrast, no activation of transcription was detected when DP-NTP was tested for interaction with the Dsg1 cytoplasmic domain (D and H).

Discussion

The molecular interactions that couple the desmosomal cadherins to the intermediate filament cytoskeleton have not been well characterized. Previous studies had shown that the amino-terminal domain of desmoplakin is important for localizing desmoplakin to the cytoplasmic plaque of the desmosome (Stappenbeck et al., 1993; Bornslaeger et al., 1996). In the current study, we sought to determine if desmosomal cadherin–plakoglobin complexes interact with the amino-terminal domain of desmoplakin. The results indicate that the amino-terminal domain of desmoplakin clusters desmosomal cadherin–plakoglobin complexes and binds directly to plakoglobin, but not desmoglein. These results provide new insights into the hierarchy of molecular interactions that occur in the cytoplasmic plaque of the desmosome.

In L-cell lines co-expressing DP-NTP, both Dsg1- (Fig. 3) and Dsc2a–plakoglobin (Fig. 5) complexes are clustered into punctate regions of staining, which appear to be at the plasma membrane. It is interesting to note that the desmosomal cadherins are clustered in L-cells co-expressing DP-NTP, even though these cells do not aggregate in suspension (not shown). The fact that the desmosomal cadherins are clustered but are not engaged in adhesion in L-cells indicates that clustering can occur independent of adhesion. These observations are consistent with the finding that desmosomal complexes resembling half desmosomes are assembled in HaCaT keratinocytes grown in low calcium medium (Demlehner et al., 1995). Clustering of desmosomal cadherins and plakoglobin represents only one aspect of desmosome assembly, and it will be interesting to determine if other desmosomal components such as pinin (Ouyang and Sugrue, 1992, 1996), envoplakin (Ruhberg et al., 1996), and the plakophilins require desmosomal cadherin-mediated adhesion for assembly into the plaque.

Co-immunoprecipitation experiments demonstrated that plakoglobin and DP-NTP are present in complexes that can be extracted from L-cell lysates (Fig. 11). Although we could not detect the desmosomal cadherins in these complexes, it is likely that the complexes that form between DP-NTP and plakoglobin do contain the cadherins. This is supported by the fact that the desmosomal cadherins are clustered by DP-NTP along with plakoglobin. Furthermore, in cell lines expressing the EcadDsg1 chimera, plakoglobin and DP-NTP are both redistributed to cell–cell interfaces and colocalize with the chimera. These observations strongly suggest that the desmosomal cadherins, plakoglobin, and DP-NTP exist in the same complex.

In addition to the observation that plakoglobin and DP-NTP co-immunoprecipitate, yeast two hybrid analysis demonstrated that DP-NTP binds directly to plakoglobin, but not Dsg1 (Fig. 12). The lack of DP-NTP binding to Dsg1 in the two hybrid system is consistent with the observation that Dsg1 clustering did not occur in L-cell lines in the absence of plakoglobin and with the observation that DP-NTP was not recruited to COS cell–cell borders with the EcadDsg1 chimera in the absence of plakoglobin (Fig. 10). These results lead us to propose that plakoglobin couples desmoplakin to the desmosomal cadherins, thereby linking these cadherins to the intermediate filament cytoskeleton. Previous studies by Troyanovsky et al. (1993, 1994) demonstrated that a connexin-Dsc2a chimera recruited endogenous desmoplakin to the plasma membrane, even when the plakoglobin-binding region of the Dsc2a tail was deleted. The data presented here do not rule out the possibility that desmoplakin binds to desmocollin directly, or that other proteins in addition to plakoglobin can facilitate desmoplakin–desmosomal cadherin interactions. However, our data indicate that plakoglobin plays an important role in linking desmoplakin to the desmoglein cytoplasmic domain. In the desmosome, therefore, plakoglobin may play a role analogous to the adherens junction protein β-catenin, which couples the classical cadherins to α-catenin (Aberle et al., 1994; Jou et al., 1995; Cowin and Burke, 1996).

Recently, transgenic mice with a null mutation in the plakoglobin gene were reported to have defects in desmosome assembly in the intercalated discs of the heart (Bierkamp et al., 1996; Ruiz et al., 1996). In these mice, Dsg2 no longer clustered into distinct junctional structures but exhibited a diffuse distribution on the cell surface. In addition, normal desmosomes were no longer detected in the
intercalated discs of the plakoglobin null mice, and adherens junctions and desmosomal components appeared to be mixed into the same junctional structures. Interestingly, desmoplakin localized in these structures, suggesting that the molecule can associate with other junctional proteins in addition to plakoglobin. A family of proteins related to plakoglobin, termed plakophilins, has been identified, and the desmosomal component originally termed band 6 is now known to be a plakophilin family member (Hatzfeld et al., 1994; Heid et al., 1994; Hatzfeld and Nachtsheim, 1996; Mertens et al., 1996). It is possible that plakophilin family members or other desmosomal components also play a role in linking the desmosomal cadherins to desmoplakin.

An interesting property of plakoglobin is that it binds to both classical and desmosomal cadherins. The observation that DP-NTP binds to plakoglobin and clusters desmosomal cadherin–plakoglobin complexes implies that desmoplakin binds to plakoglobin that is associated with the desmosomal cadherin cytoplasmic domain. In contrast, plakoglobin that is associated with the desmosomal cadherin cytoplasmic domain is unable to bind to α-catenin (Fig. 8; Platt et al., 1994; Roh and Stanley, 1995a). Recent studies have demonstrated that the α-catenin and Dsg1 binding sites on plakoglobin overlap in the amino-terminal armadillo repeats of plakoglobin, suggesting that Dsg1 and α-catenin cannot bind to the same plakoglobin molecule simultaneously (Sacco et al., 1995; Aberle et al., 1996; Chitaev et al., 1996; Troyanovsky et al., 1996; Witcher et al., 1996). However, the classical cadherins bind to the central armadillo repeats of plakoglobin, leaving the amino-terminal armadillo repeats of plakoglobin available to bind α-catenin. In the present study, deletion mutants of plakoglobin lacking either the amino- or carboxyl-terminal domain co-immunoprecipitated with DP-NTP and were clustered in L-cells co-expressing DP-NTP. These results, along with the ability of the amino-terminal plakoglobin deletion to interact with DP-NTP in the two hybrid system, suggest that desmoplakin may bind to the central armadillo repeats of plakoglobin. In A431 cells, the expression of plakoglobin deletion mutants lacking the amino- and carboxyl-terminal end domains promoted the assembly of elongated and fused desmosomes, suggesting that the central armadillo repeats of plakoglobin can promote interactions between desmosomal proteins, which may include desmoplakin (Palka and Green, 1997). Future studies will be directed at mapping precisely where desmoplakin binds to plakoglobin in relation to the sites on plakoglobin that bind to the desmosomal cadherins. Such studies should provide insight into the mechanisms by which the cadherin cytoplasmic tails govern the domains on plakoglobin that are available for interactions with either α-catenin or desmoplakin.

The authors would like to thank Drs. M. Wheelock, K. Johnson, J. Papkoff, M. Udey, M. Takeichi, K. Trevor, and J. Stanley for sharing antibody –and cDNA reagents that made this work possible. Thanks also to Drs. S. Elledge and M. Klymkowsky for generously providing yeast two hybrid reagents and expertise. We also would like to thank S. Norvell for assistance with the confocal microscopy, D. Kopp for assistance with the COS transfections, S. Guy for cDNA sequence analysis, and K. Myung and M. Moody for assistance preparing samples for electron microscopy.

This work was supported by the National Institutes of Health (ROIAR43380 and ROIAR41836) and a March of Dimes Birth Defects Foundation grant (1-FTY6-0146) to K.J. Green. A.P. Kowalczyk was supported by a postdoctoral fellowship from the Dermatology Foundation, and K.J. Green is a Faculty Research Award recipient of the American Cancer Society.

Received for publication 20 January 1997 and in revised form 8 August 1997.

References

Aberle, H., S. Butz, J. Stappert, H. Weissig, R. Kemler, and H. Hoschuetzky. 1994. Assembly of the cadherin-catenin complex in vitro with recombinant proteins. J. Cell Sci. 107:3655–3663.
Aberle, H., H. Schwartz, H. Hoschuetzky, and R. Kemler. 1996. Single amino acid substitutions in proteins of the armadillo gene family abolish their binding to α-catenin. J. Biol. Chem. 271:1520–1526.
Amagai, M., S. Karpati, V. Klaus-Kovtun, M.C. Udey, and J.R. Stanley. 1994. The extracellular domain of pemphigus vulgaris antigen (Desmoglein 3) mediates weak homophilic adhesion. J. Invest. Dermatol. 102:402–408.
Bai, C., and S.J. Elledge. 1995. Cloning using the two-hybrid system. Methods Enzymol. 273:331–347.
Bierkamp, C., K.J. McAulughlin, H. Schwarz, O. Huber, and R. Kemler. 1996. Embryonic heart and skin defects in mice lacking plakoglobin. Dev. Biol. 180:780–785.
Bornslaeger, E.A., C.M. Corcoran, T.S. Stappenbeck, and K.J. Green. 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. Cell Biol. 134:985–1001.
Buxton, R.S., P. Cowin, W.W. Franke, D.R. Garrod, K.J. Green, L.A. King, P.J. Koch, A.J. Magoz, D.A. Rees, J.R. Stanley, and M.S. Stemberg. 1993. Nomenclature of the desmosomal cadherins. J. Cell Biol. 121:481–483.
Chidgey, M.A.J., P.J. Clarke, and D.R. Garrod. 1996. Expression of full-length desmosomal glycoproteins (desmocollins) is not sufficient to confer strong adhesion on transfected L929 cells. J. Invest. Dermatol. 106:689–695.
Chitaev, N.A., and S.M. Troyanovsky. 1997. Direct Ca2+–dependent heterophilic interaction between desmosomal cadherins, desmoglein, and desmocollin contributes to cell-cell adhesion. J. Cell Biol. 138:193–201.
Chitaev, N.A., R.E. Leube, R.B. Troyanovsky, L.G. Eshkind, W.W. Franke, and S.M. Troyanovsky. 1996. The binding of plakoglobin to desmosomal cadherins: patterns of binding sites and topogenic potential. J. Cell Biol. 133:359–369.
Collins, J.E., and D.R. Garrod. 1994. Molecular biology of desmosomes and hemidesmosomes. R.G. Landes Co., Austin, Texas.
Coulombe, P.A., and E. Fuchs. 1994. Molecular mechanisms of keratin gene disorders and other bullous diseases of the skin. In Molecular Mechanisms of Epithelial Cell Junctions: From Development to Disease. S. Citi, editor. R.G. Landes Co., Austin, Texas. 259–285.
Cowin, P., and S. Mechanic. 1994. Desmosomal cadherins and their cytoplasmic interactions. In Molecular Mechanisms of Epithelial Cell Junctions: From Development to Disease. S. Citi, editor. R.G. Landes Co., Austin, Texas. 141–155.
Cowin, P., and B. Burke. 1996. Cytoskeleton-membrane interactions. Curr. Opin. Cell Biol. 8:56–65.
Demleuter, M.P., S. Schafer, C. Grund, and W.W. Franke. 1996. Continual assembly of half-desmosomal structures in the absence of cell contacts and their frustrated endocytosis: a coordinated syzygyous cycle. J. Cell Biol. 131:745–753.
Evans, M.J., and R.C. Scarpulla. 1988. Both upstream and intron elements are required for elevated expression of rat somatic cytochrome c gene expression in COS-1 cells. Mol. Cell. Biol. 8:35–41.
Farquhar, M.G., and G.E. Palade. 1963. Junctional complexes in various epithelia. J. Cell Biol. 17:375–412.
Franke, W.W., M.D. Goldschmidt, R. Zimbellmann, H.M. Mueller, D.L. Schiller, and P. Cowin. 1989. Molecular cloning and amino acid sequence of human plakoglobin, the common junctional plaque protein. Proc. Natl. Acad. Sci. USA 86:4027–4031.
Fuchs, E. 1994. Intermediate filaments and disease: mutations that cripple cell strength. J. Cell Biol. 125:511–516.
Garrod, D. 1993. Desmosomes and hemidesmosomes. Curr. Opin. Cell Biol. 5:30–40.
Gorbsky, G., and M.S. Steinberg. 1981. Isolation of the intercellular glycoproteins of desmosomes. J. Cell Biol. 90:243–248.
Green, K.J., D.A.D. Parry, P.M. Steinert, M.L.A. Virata, R.M. Wagner, B.D. Angst, and L.A. Nilles. 1990. Structure of the human desmoplakins. Implications for function in the desmosomal plaque. J. Biol. Chem. 265:2603–2612.
Green, K.J., T.S. Stappenbeck, S. Noguchi, R. Oyasu, and L.A. Nilles. 1991. Desmoplakin expression and distribution in cultured rat bladder epithelial cells of varying tumorigenic potential. Exp. Cell Res. 193:143–143.
Harper, J.W., G. Adami, N. Wei, K. Keyomarsi, and S.J. Elledge. 1993. The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. Cell 75:805–816.

Kowalczyk et al. Desmosomal Plaque Protein Interactions 783
Hatzfeld, M., and C. Nachtshiem. 1996. Cloning and characterization of a new family of desmosomal plaque proteins. J. Cell Biol. 131:267–277.

Hatzfeld, M., I.K. Gunnar, U. Plessmann, and K. Weber. 1994. Band 6 protein, a major constituent of desmosomes from stratified epithelia, is a novel mem-

Jones, S.M., J.R. Green, and R.D. Goldmann. 1998. Comparison of the polypeptide composition of desmosomes prepared from two bovine epidermal tis-

Koch, P.J., M.J. Walsh, M. Schmelz, M.D. Goldschmidt, R. Zimbelmann, and W.W. Franke. 1994. Cell-type-specific desmosomal plaque proteins of the plakoglobin family: plako-

Kowalczyk, A.P., H.L. Palka, H.H. Luu, L.A. Nilles, J.E. Anderson, M.J. Wheelock, and K.J. Green. 1995. Identification of α-actinin with the cadherin/catenin cell-cell adhesion complex via α-catenin. J. Cell Biol. 130:67–77.

Koch, P.J., M.J. Walsh, M. Schmelz, M.D. Goldschmidt, R. Zimbelmann, and W.W. Franke. 1990. Identification of desmoglein, a constitutive desmosomal glycoprotein, as a member of the cadherin family of cell adhesion receptors. Eur. J. Cell Biol. 53:1-12.

Kouklis, P.D., E. Hutton, and E. Fuchs. 1994. Making a connection: direct binding between keratin intermediate filaments and desmosomal proteins. J. Cell Biol. 127:1049–1060.

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

Kowalczyk, A.P., J.E. Anderson, J.E. Borgwardt, T. Hashimoto, J.R. Stanley, and K.J. Green. 1995. Pemphigus sera recognize conformationally sensitive epitopes in the amino-terminal region of desmoglein-1. J. Investig. Dermatol. 105:147–152.

Kowalczyk, A.P., J.E. Borgwardt, and K.J. Green. 1996. Analysis of desmosomal cadherin adhesion function and stoichiometry of the desmosomal cadherin/plakoglobin complex. J. Investig. Dermatol. 107:293–300.

Kowalczyk, A.P., and K.J. Green. 1996. The desmosome: a component system for adhesion and intermediate filament attachment. In Current Topics in Membranes. Vol. 43. W.J. Nelson, editor. Academic Press, Inc., San Diego. 187–209.

Lewis, J.E., III, J.K.W., K.M. Sass, P.J. Jensen, K.R. Johnson, and M.J. Wheelock. 1997. Cross-talk among adhesive junctions and desmosomes depends on plakoglobin. J. Cell Biol. 136:919–934.

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

McCrea, P.D., C.W. Turck and B. Gumbiner. 1991. A homologue of the armadillo E-cadherin cDNA. Science 251:159–161.

McLean, W.H.I., and E.B. Lane. 1995. Intermediate filaments in disease. Curr. Opin. Cell. Biol. 7:118–125.

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

Mertens, C., C. Kuhn, and W.W. Franke. 1996. Plakophilins 2a and 2b: constitutive protein cadherin adhesive function and stoichiometry of the desmosomal cadherin-intermediate filament complex. J. Cell Biol. 135:1009–1025.

Mori, S., H. Hirohashi. 1992. Cadherin dysfunction in a human cancer cell line: possibility of involvement of a cadherin expression in reduced cell-cell adhesiveness. Cancer Res. 52:5770–5774.

Ozawa, M., R. Collins, S. Puttagunta, S.E. Mechanic, M. Munson, B. Gumbiner, and P. Cowin. 1996. Desmosomal cadherin-binding domains of plakoglobin. J. Biol. Chem. 271:10904–10909.

Ozawa, M., M. Ringwald, and R. Kemler. 1990. Uvomorulin-catenin complex codes for a functionally modular protein that is the man plakoglobin. J. Biol. Chem. 265:849–857.

Roh, J.-Y., and J.R. Stanley. 1995a. Intracellular domain of desmoglein 3 (pemphigus vulgaris antigen) confers adhesive function on the extracellular domain of E-cadherin without binding cadherins. J. Cell Biol. 128:939–947.

Steinert, P.M., and S.J. Bale. 1993. Genetic skin diseases caused by mutations in Drosophila cadherin-like intracytoplasmic tail segment. J. Investig. Dermatol. 107:720–724.

Troyanovsky, S.M., L.G. Eskhrid, R.B. Troyanovsky, R.E. Leube, and W.W. Franke. 1996. Identification of amino acid sequence motifs in desmoplakin that are shared by proteins with diverse cellular functions. Cell 76:789–791.

Whitmore, L.T., R. Collins, S. Puttagunta, S.E. Mechanic, M. Munson, B. Gumbiner, and P. Cowin. 1996. Desmosomal cadherin-binding domains of plakoglobin. J. Biol. Chem. 271:10904–10909.

Yoshimura, Y., A. Nagafuchi, S. Fujita, M. Gotou, M. Takeuchi, S. Tsukita, and S. Hirohashi. 1992. Cadherin dysfunction in a human cancer cell line: possibility of involvement of loss of α-catenin expression in reduced cell-cell adhesiveness. Cancer Res. 52:5770–5774.

Yoshimura, Y., A. Nagafuchi, S. Fujita, M. Gotou, M. Takeuchi, S. Tsukita, and S. Hirohashi. 1992. Cadherin dysfunction in a human cancer cell line: possible involvement of loss of α-catenin expression in reduced cell-cell adhesiveness. Cancer Res. 52:5770–5774.

Yoshimura, Y., A. Nagafuchi, S. Fujita, M. Gotou, M. Takeuchi, S. Tsukita, and S. Hirohashi. 1992. Cadherin dysfunction in a human cancer cell line: possible involvement of loss of α-catenin expression in reduced cell-cell adhesiveness. Cancer Res. 52:5770–5774.

Yoshimura, Y., A. Nagafuchi, S. Fujita, M. Gotou, M. Takeuchi, S. Tsukita, and S. Hirohashi. 1992. Cadherin dysfunction in a human cancer cell line: possible involvement of loss of α-catenin expression in reduced cell-cell adhesiveness. Cancer Res. 52:5770–5774.