

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

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Abstract. The desmosomal plaque protein desmoplakin (DP), located at the juncture between the intermediate filament (IF) network and the cytoplasmic tails of the transmembrane desmosomal cadherins, has been proposed to link IF to the desmosomal plaque. Consistent with this hypothesis, previous studies of individual DP domains indicated that the DP COOH terminus associates with IF networks whereas NH₂-terminal sequences govern the association of DP with the desmosomal plaque. Nevertheless, it had not yet been demonstrated that DP is required for attaching IF to the desmosome. To test this proposal directly, we generated A431 cell lines stably expressing DP NH₂-terminal polypeptides, which were expected to compete with endogenous DP during desmosome assembly. As these polypeptides lacked the COOH-terminal IF-binding domain, this competition should result in the loss of IF anchorage if DP is required for linking IF to the desmosomal plaque.

In such cells, a 70-kD DP NH₂-terminal polypeptide (DP-NTP) colocalized at cell–cell interfaces with desmosomal proteins. As predicted, the distribution of endogenous DP was severely perturbed. At cell–cell borders where endogenous DP was undetectable by immunofluorescence, there was a striking absence of attached tonofibrils (IF bundles). Furthermore, DP-NTP assembled into ultrastructurally identifiable junctional structures lacking associated IF bundles. Surprisingly, immunofluorescence and immunogold electron microscopy indicated that adherens junction components were coassembled into these structures along with desmosomal components and DP-NTP. These results indicate that DP is required for anchoring IF networks to desmosomes and furthermore suggest that the DP–IF complex is important for governing the normal spatial segregation of adhesive junction components during their assembly into distinct structures.

THE potential structural importance of cytoskeletal–membrane interactions at cell–cell and cell–substrate interfaces has long been recognized, and a number of candidates for linking molecules have been identified (for reviews see Luna and Hitt, 1992; Cowin and Burke, 1996). However, since these linking proteins often reside in large macromolecular complexes within the cytoplasmic plaque regions of adhesive junctions, it has been difficult to define a specific role in cytoskeletal attachment for individual components at a molecular level.

Two major adhesive junctions mediate the attachment

of epithelial cells to one another: microfilament-associated adherens junctions (*zonula adherens*) and intermediate filament-associated desmosomes (*macula adherens*) (Farquhar and Palade, 1963; Staehelin, 1974; Geiger and Ginsberg, 1991; Jones and Green, 1991; Tsukita et al., 1992; Garrod, 1993; Citi, 1994; Collins and Garrod, 1994; Schmidt et al., 1994). The transcellular glycoprotein components of these junctions are the classic cadherins and the desmosomal cadherins, respectively (Magee and Buxton, 1991; Cowin and Mechanic, 1994; Koch and Franke, 1994). The cytoplasmic plaque protein plakoglobin, a member of the family of armadillo repeat proteins (Cowin et al., 1986; Cowin, 1994), binds to the cytoplasmic tails of either desmosomal or classic cadherins (Knudsen and Wheelock, 1992; Peifer et al., 1992; Kowalczyk et al., 1994; Mathur et al., 1994; Troyanovsky et al., 1994), although in the latter case, this association may be with lower affinity (Aberle et al., 1994; Kowalczyk et al., 1994; Nathke et al., 1994). With the exception of plakoglobin, the plaques of these junctions

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are made up of distinct components, which include α - and β -catenin in adherens junctions and desmoplakin in desmosomes. These junction-specific plaque proteins are thought to govern the attachment of specific cytoskeletal elements to the appropriate junction.

At the ultrastructural level, desmosomes appear as pairs of symmetrical cytoplasmic plaques abutting a plasma membrane-containing central core domain, composed largely of the extracellular domains of the desmosomal cadherins, desmoglein and desmocollin (Buxton and Magee, 1992; Buxton et al., 1993; Koch and Franke, 1994). The desmosomal plaque is made up predominantly of the cytoplasmic tails of these desmosomal cadherins, plakoglobin, and the most abundant desmosomal plaque proteins, desmoplakin I (DPI),¹ a constitutive desmosomal component, and the variably expressed DPII (Skerrow and Matoltsy, 1974; Mueller and Franke, 1983; Cowin et al., 1985; Kapprell et al., 1985; Angst et al., 1990). Desmosomes exhibit additional cell-type specificity; there are at least three genes encoding each of the desmoglein and desmocollin subfamilies that are expressed in a tissue- and differentiation-specific manner (Buxton et al., 1994; Garrod and Collins, 1994) as well as a number of tissue-specific and/or minor accessory proteins (Wiche, 1989; Ouyang and Sugrue, 1992; Hatzfeld et al., 1994; Heid et al., 1994; Skalli et al., 1994; Brakenhoff et al., 1995).

By serving as a cell surface attachment site for cytoplasmic intermediate filaments (IF), desmosomes facilitate the formation of a transcellular cytoskeletal network that plays a critical role in the maintenance of tissue integrity (Steinert, 1993; Fuchs and Weber, 1994; McLean and Lane, 1995; Roop, 1995). The abundance and localization of DP in the inner portion of the desmosomal plaque through which IF appear to loop (Kelly, 1966) led to the proposal that DP acts to link IF to the desmosome (Jones and Goldman, 1985; Miller et al., 1987; Steinberg et al., 1987). DPI and II are large proteins derived from a single gene (molecular masses from predicted amino acid sequence are \sim 330 and 260 kD, respectively) and each contain three major structural domains based on predicted amino acid sequence and rotary shadowing electron microscopy (O'Keefe et al., 1989; Green et al., 1990; Green et al., 1992a; Green et al., 1992b; Virata et al., 1992). Both DPI and DPII are predicted to be homodimers consisting of globular NH₂- and COOH-terminal domains flanking a central α -helical coiled coil rod domain, which is shorter in DPII than DPI due to alternative splicing (Virata et al., 1992).

The proposal that DP plays a role in linking IF to the desmosome is supported by previous studies from our laboratory, which demonstrated that DP is a modular protein with domains that facilitate interactions with both IF networks and the desmosomal plaque (Stappenbeck and Green, 1992; Stappenbeck et al., 1993; Stappenbeck et al., 1994). In these studies, DP functional domains were mapped by transiently expressing epitope-tagged DP polypeptides in cultured epithelial cells. The results indicated that the COOH-terminal domain of DP, either alone or in combination with the central rod domain, is capable of as-

sociating with vimentin or keratin IF networks. Recently, Kouklis et al. have demonstrated that the COOH-terminal domain of DP and type II epidermal keratins interact *in vitro*, suggesting that at least in certain cases, the interaction between DP and IF may be direct (1994). The DP COOH-terminal domain is apparently not required for the association of DP with the desmosome plaque, however, because an ectopically expressed DP polypeptide comprising the NH₂-terminal and rod domains but lacking the COOH-terminal domain colocalizes with desmosomes (Stappenbeck et al., 1993). In addition, this result indicates that the assembly of DP into desmosomes does not require an interaction between DP and IF networks, which supports previous observations (Mattey and Garrod, 1986; Bologna et al., 1986; Duden and Franke, 1988; Baribault and Oshima, 1991). However, the assembly of DP into desmosomes does require NH₂-terminal sequences, as ectopically expressed DP lacking the first 194 amino acid residues of the DP NH₂-terminal domain is unable to colocalize with desmosomes (Stappenbeck et al., 1993). While the results of these domain expression studies are consistent with the idea that DP could act to link IF to the desmosome, it nevertheless remained to be determined whether DP is in fact required for anchoring IF to the desmosomal plaque.

Here we have tested this hypothesis directly by generating stable A431 epithelial cell lines expressing DP NH₂-terminal polypeptides. We reasoned that such polypeptides, which lack the COOH-terminal IF binding domain, would act in a dominant negative manner and compete with endogenous DP during junction assembly. In these cell lines, a 70-kD DP NH₂-terminal polypeptide assembled along with desmosomal components into distinct junctions at cell-cell interfaces. As predicted, endogenous DP was lost from many cell-cell borders and aberrantly distributed at others. Keratin IF bundles were not attached at cell-cell borders lacking detectable endogenous DP, providing direct evidence that DP plays a central role in the assembly and/or anchorage of IF bundles at the desmosomal plaque. Surprisingly, adherens junction components, including the classic cadherin E-cadherin and α - and β -catenin, were dramatically redistributed in these cell lines, colocalizing with the DP NH₂-terminal polypeptide and endogenous desmosome components. These observations raise the possibility that DP is not only required for linking IF bundles to desmosomes but may also contribute to the normal segregation of desmosome and adherens junction components during the assembly of adhesive junctions in epithelial cells.

Materials and Methods

Construction of Vectors for Eukaryotic Expression of DP NH₂-terminal Polypeptides

Vectors for eukaryotic expression of DP NH₂-terminal polypeptides were generated by internal deletion from an expression vector containing a cDNA comprising the DP-coding sequence with 332 bp of predicted 5' untranslated sequence (GenBank accession M77830) and a 3' tandem array of seven copies of a ten amino acid c-myc epitope tag (pDP.7myc) (Stappenbeck et al., 1993). A construct encoding the entire predicted DP NH₂-terminal domain followed by the first 168 amino acid residues of the DPI rod domain (DP.NTRod168.7myc; base pairs 1-4003; see Fig. 1) was gen-

1. *Abbreviations used in this paper:* DP, desmoplakin; DP-NTP, 70-kD DP NH₂-terminal polypeptide; IF, intermediate filament.

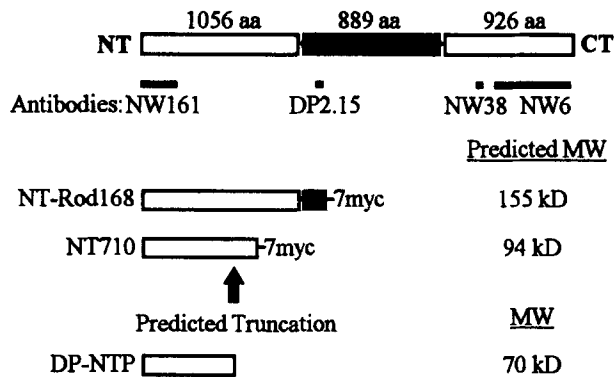


Figure 1. Schematic diagram of DP NH₂-terminal polypeptides. A diagram of DPI is shown at the top of the figure, with the approximate locations of the epitopes for the DP antibodies used in this study. Below this are diagrams of the two 7myc-tagged DP NH₂-terminal polypeptides as well as their predicted molecular weights. DP.NTRod168 consists of the entire NH₂-terminal domain of DP and the first 168 amino acid residues of the DPI rod domain, while DP.NT710 consists of the first 710 amino acid residues of the DP NH₂-terminal domain. Also depicted is a model for the generation of the 70-kD DP NH₂-terminal polypeptide (DP-NTP), detected in A431 cells expressing the 7myc-tagged DP polypeptides by immunoblotting with NW161 (see Fig. 2).

erated by digestion of pDP.7myc with SalI (which cuts just 5' of the 7myc tag), blunting with T4 DNA polymerase, digestion with EcoRV (which cuts within DPI), and religation of the plasmid. A construct encoding the first 710 predicted amino acid residues of the NH₂-terminal domain, DP.NT710.7myc (base pairs 1–2461), was generated in a similar manner, except that the site within DP, AfIII, was blunted prior to ligation.

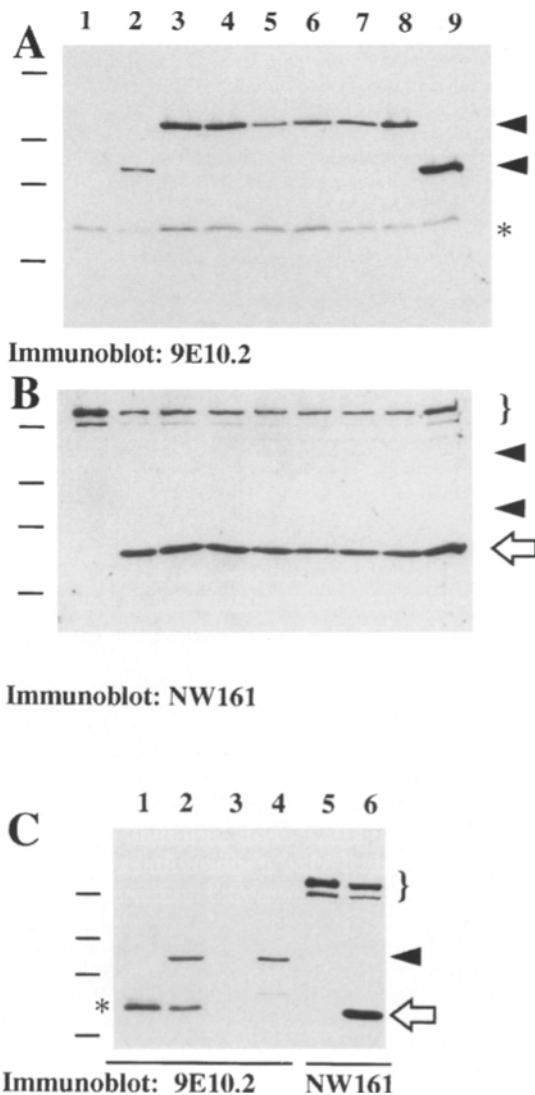
Construction of a Vector for Bacterial Expression of a DP NH₂-terminal Fusion Protein

PCR was used to amplify the first 567 nucleotides of DP cDNA following the predicted methionine start codon; to aid in subcloning, the PCR primers contained restriction sites. The resulting PCR product was digested with BamHI and HindIII and subcloned into the bacterial expression vector pQE-30 (Qiagen Inc., Chatsworth, CA), in frame with a 5' sequence encoding six tandem histidine residues. This construct was designated p6HIS.DP.NT189.

Cell Culture and Generation of Stable Cell Lines

A431 epithelial cells (a gift from M. Wheelock, University of Toledo) were cultured in DME containing 10% fetal calf serum, 100 U/ml penicillin, and 100 µg/ml streptomycin. For generation of stable cell lines, cells were cotransfected with calcium phosphate precipitates containing a DP vector and either pSV2neo or pSV2pacΔP (a gift of J. Ortin, University of Madrid and K. Johnson, University of Toledo); other cultures were trans-

Figure 2. Immunoblot and immunoprecipitation analysis of A431 cells expressing DP NH₂-terminal polypeptides. *A* and *B* represent duplicate loadings of whole cell lysates from a G418 resistant control cell line (lane 1; neo A) as well as a series of eight cell lines ectopically expressing the 7myc-tagged DP-NTP, DP.NTRod168 (lanes 3–8; lines 1, 29, 9, 11, 8, and 5, respectively) or DP.NT710 (lanes 2 and 9; lines C1 and D3, respectively; note that lysates of lines A1 and A2 are not shown). As described in Materials and Methods, the volumes of cell lysates loaded were adjusted to contain similar levels of keratin 18. Immunoblotting was performed with either 9E10.2 (*A*), a mouse monoclonal antibody that recognizes the c-myc epitope tag, or NW161 (*B*), a rabbit polyclonal antibody directed against the first 189 residues of DP (see Materials



and Methods). Closed arrowheads indicate the positions of the 155-kD polypeptide detected with 9E10.2 in cells expressing NTRod 168.7myc and the position of the 94-kD 9E10.2-reactive polypeptide detected in cells expressing NT710.7myc; the open arrow indicates the position of the 70-kD NW161-reactive polypeptide, DP-NTP, detected in both types of DP.NT cell lines. The asterisk indicates an unrelated polypeptide recognized by 9E10.2 in whole cell lysates or Triton X-100 soluble fractions of all A431 cells, whether experimental or control; this unrelated polypeptide is not detected in 9E10.2 immunoblots of NW161 immunoprecipitates, as shown in *C*, lane 4. The brackets indicate DPI and DP. Levels of endogenous DP were examined in four control cell lines in addition to neo A; the level of endogenous DP in the neo A line (*B*, lane 1) is representative. (*C*) Results of an experiment in which material from control puroC cells (lanes 1, 3, and 5) or NT710.C1 cells (lanes 2, 4, and 6) was immunoprecipitated with NW161 and immunoblotted with 9E10.2 (lanes 3 and 4); whole cell lysates immunoblotted with 9E10.2 (lanes 1 and 2) or NW161 (lanes 5 and 6) are shown for comparison. Markings are the same as for *A* and *B*. Note that the 9E10.2-reactive DP polypeptides (closed arrowheads) are not detected by immunoblotting with NW161. Also note that no additional myc-tagged polypeptides of 5 kD or greater molecular weight were detected by 9E10.2 immunoblot analysis of DP.NT710.C1 whole cell lysates subjected to 15% SDS-PAGE (data not shown). Molecular weight markers from top to bottom: 205, 121, 86, and 50 kD.

ected with only pSV2neo or pSV2pacΔP to generate control G418 resistant (neo) or puromycin resistant (puro) cell lines, respectively. 24 h after transfection, cells were split into medium for selection containing either 0.7 mg/ml (active concentration) G418 (GIBCO BRL, Grand Island, NY) or 1 μg/ml puromycin (Sigma Chemical Co., St. Louis, MO). Drug-resistant colonies were cloned and expanded, and whole cell lysates were prepared in urea sample buffer (Green et al., 1991), and then screened by immunoblot analysis using the monoclonal antibody 9E10.2 directed against the c-myc epitope tag (Evans et al., 1985).

Generation of Polyclonal Antibody NW161

A bacterial fusion protein containing six histidine residues followed by the first 189 predicted amino acids of DP was generated by inducing expression of p6HIS.DP.NT189 in JM109 bacteria and purified using nondenaturing conditions essentially according to protocols described in *The QIA expressionist* (Qiagen Inc.). 500 μg of 6HIS.DP.NT189 was used to inject a rabbit for the production of polyclonal serum; secondary and tertiary boosts of 380 and 100 μg, respectively, were also performed (HRP Inc., Denver, PA). The resulting polyclonal serum, NW161, recognized the NH₂-terminal domain of DP as judged by positive immunoblot reactions against the original bacterially expressed DP NH₂-terminal polypeptide and against DP present in a variety of cultured cells (e.g., see Fig. 2 B) and in a preparation from bovine tongue enriched in desmosomes (not shown). In addition, indirect immunofluorescence using NW161 gave rise to a typical desmosomal pattern in a variety of epithelial cells, including A431 cells (e.g., see Fig. 4). In all cases, appropriate controls using preimmune serum were performed in parallel.

Immunofluorescence Analysis

Cells plated on glass coverslips were rinsed in PBS and fixed in methanol for 2 min at -20°. Mouse monoclonal primary antibodies used were 9E10.2, directed against the c-myc epitope tag (Evans et al., 1985); DP2.15 (Boehringer-Mannheim, Indianapolis, IN), directed against the DPI/II rod domain; KSB17.2 (Sigma Chemical Co.), directed against keratin 18; E7 (Developmental Studies Hybridoma Bank Iowa City, IA), directed against tubulin; 11E4, directed against plakoglobin; 7G6, directed against desmocollin 2 (Kowalczyk et al., 1994); 6D8, directed against desmoglein (Wahl et al., 1996); 5H10, directed against β-catenin; and 1G5, directed against α-catenin (Johnson et al., 1993). Also used was E9, a rat monoclonal directed against E-cadherin. 11E4, 7G6, 6D8, 1G5, 5H10, and E9 were kindly provided by Dr. M. Wheelock. Rabbit polyclonal primary antibodies used were NW6 and NW38, both directed against the COOH-terminal domain of DP (Angst et al., 1990; Stappenbeck et al., 1993), and NW161, directed against the NH₂-terminal domain of DP (see above); a

rabbit polyclonal antibody directed against α-catenin (Aghib and McCrea, 1995) was kindly provided by Dr. P. McCrea, University of Texas, M.D. Anderson Cancer Center, Houston, TX. Rhodamine phalloidin (Molecular Probes, Inc., Eugene, OR) was used to detect F-actin. To visualize the primary antibodies, appropriate fluorescein or rhodamine conjugated anti-rabbit, anti-mouse, or anti-rat secondary antibodies (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD) were used. Controls included incubation of fixed cells with secondary antibodies alone or with preimmune rabbit serum as a primary antibody. In addition, single label immunofluorescence for each antibody used was performed as a control in double label immunofluorescence experiments.

Immunoblot and Immunoprecipitation Analysis

Cell lysates or immunoprecipitates were analyzed by SDS-PAGE followed by electrotransfer to nitrocellulose, performed as described previously (Angst et al., 1990). Immunodetection was performed by incubating blots with antibodies diluted in 5% powdered milk, 0.05% Tween, in PBS, followed by enhanced chemiluminescent detection (ECL; Amersham Corp., Arlington Heights, IL). To compare protein levels between cell lines, the volumes of lysates loaded were first normalized for keratin 18 content by immunoblotting with the monoclonal antibody KSB17.2. Protein levels were assessed by scanning densitometry. Immunoprecipitation analysis was carried out as described (Kowalczyk et al., 1994).

Electron Microscopy and Immunoelectron Microscopy

For both types of electron microscopy, experimental cell lines were used that exhibited many cell-cell borders with little endogenous DP staining by immunofluorescence analysis. For conventional electron microscopy, cells cultured on Permanox dishes (Electron Microscopy Sciences, Ft. Washington, PA) were processed as previously described (Green et al., 1991). For immunoelectron microscopy, cells cultured on Permanox dishes were fixed for 2–5 min in 2% paraformaldehyde in PBS at room temperature, rinsed thoroughly in PBS, and then permeabilized with 0.1% saponin for 15 min at room temperature. After extensive washing with PBS, cells were incubated with antibodies at room temperature overnight. Cells were then rinsed in PBS and incubated for 4 h at 37°C with a 1:5 dilution of AuroProbe EM goat anti-rabbit 10-nm and/or goat anti-mouse 5-nm gold-conjugated secondary antibodies (Amersham Corp.). Samples were then processed for conventional EM as described (Green et al., 1991). The en bloc counterstaining with uranyl acetate performed for conventional electron microscopy was omitted for immunoelectron microscopy samples. For double label immunoelectron microscopical analyses, controls included preimmune or nonimmune sera as well as single label immunoelectron microscopy for each of the antibodies.

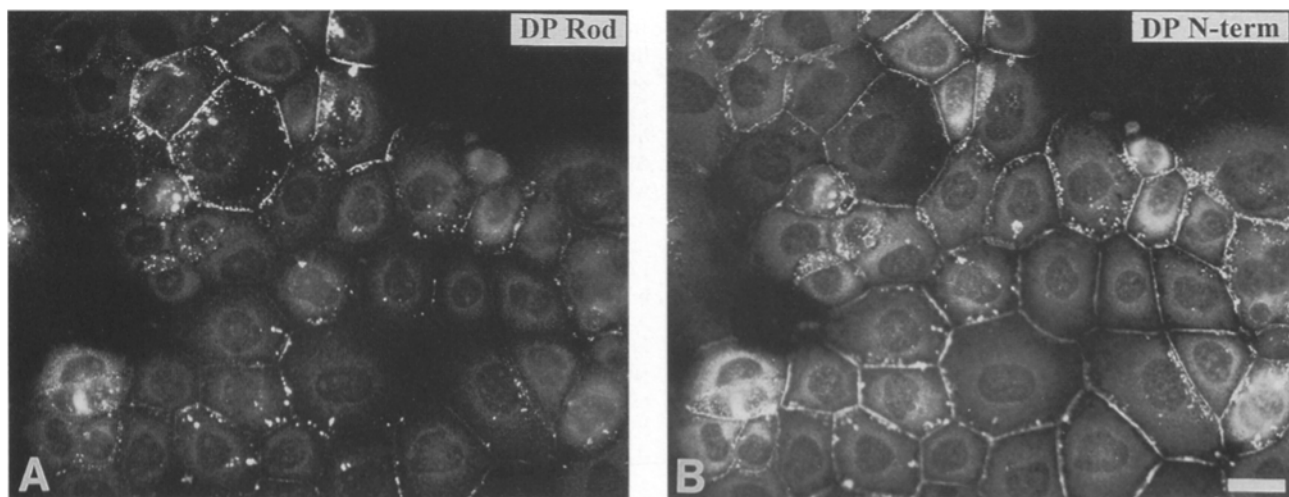


Figure 3. Indirect double label immunofluorescence to detect endogenous DP and DP-NTP. NT710.C1 cells were reacted with DP2.15 (A), which recognizes the DP rod domain of endogenous DP, or NW161 (B), directed against the DP NH₂ terminus. (Note that these and subsequent figures are labeled to indicate the detected antigen.) Some populations of cells exhibited a fairly substantial amount of endogenous DP, as shown here, although quite commonly other populations of cells exhibited even less immunoreactivity for endogenous DP. Bar, 25 μm.

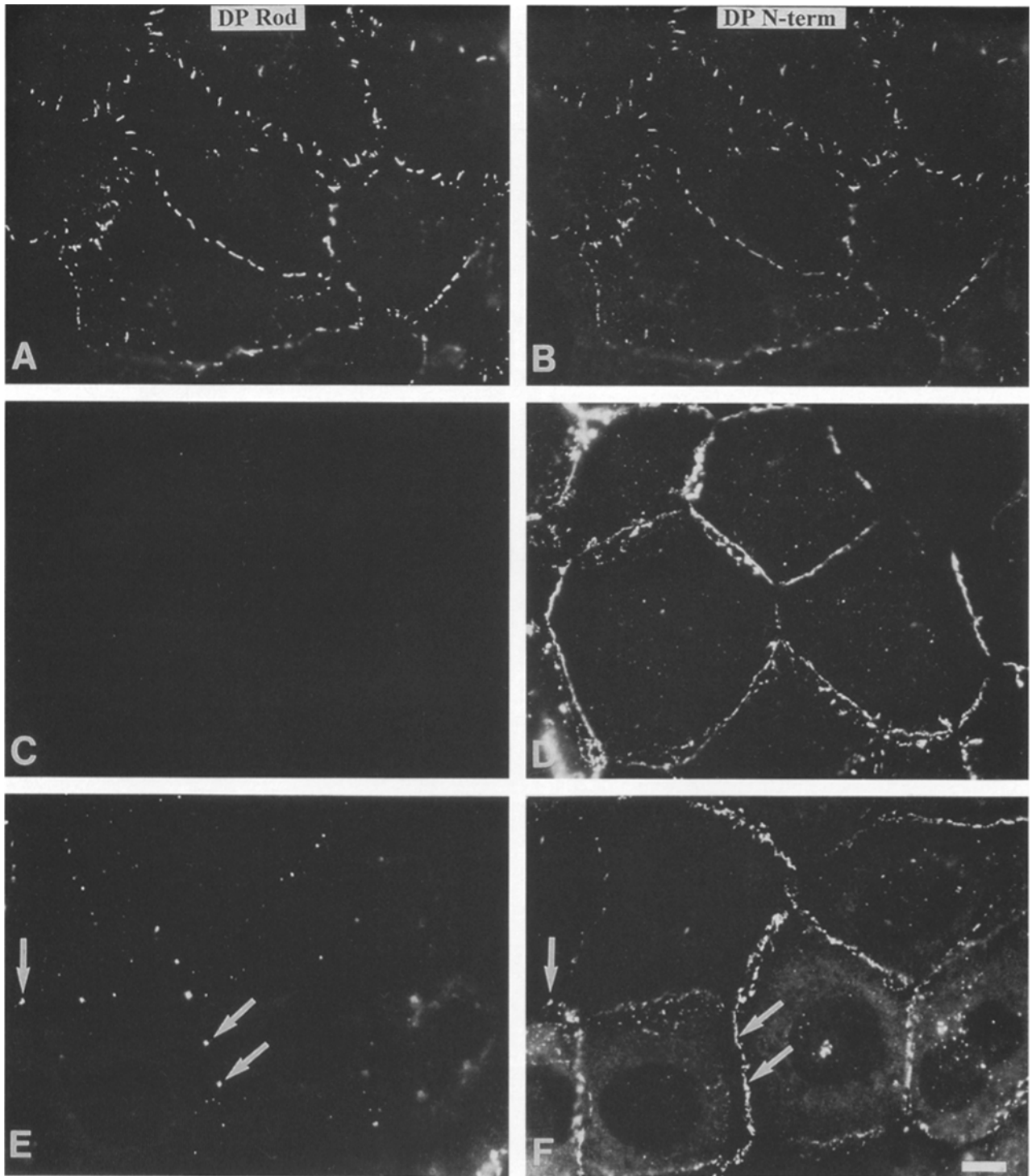
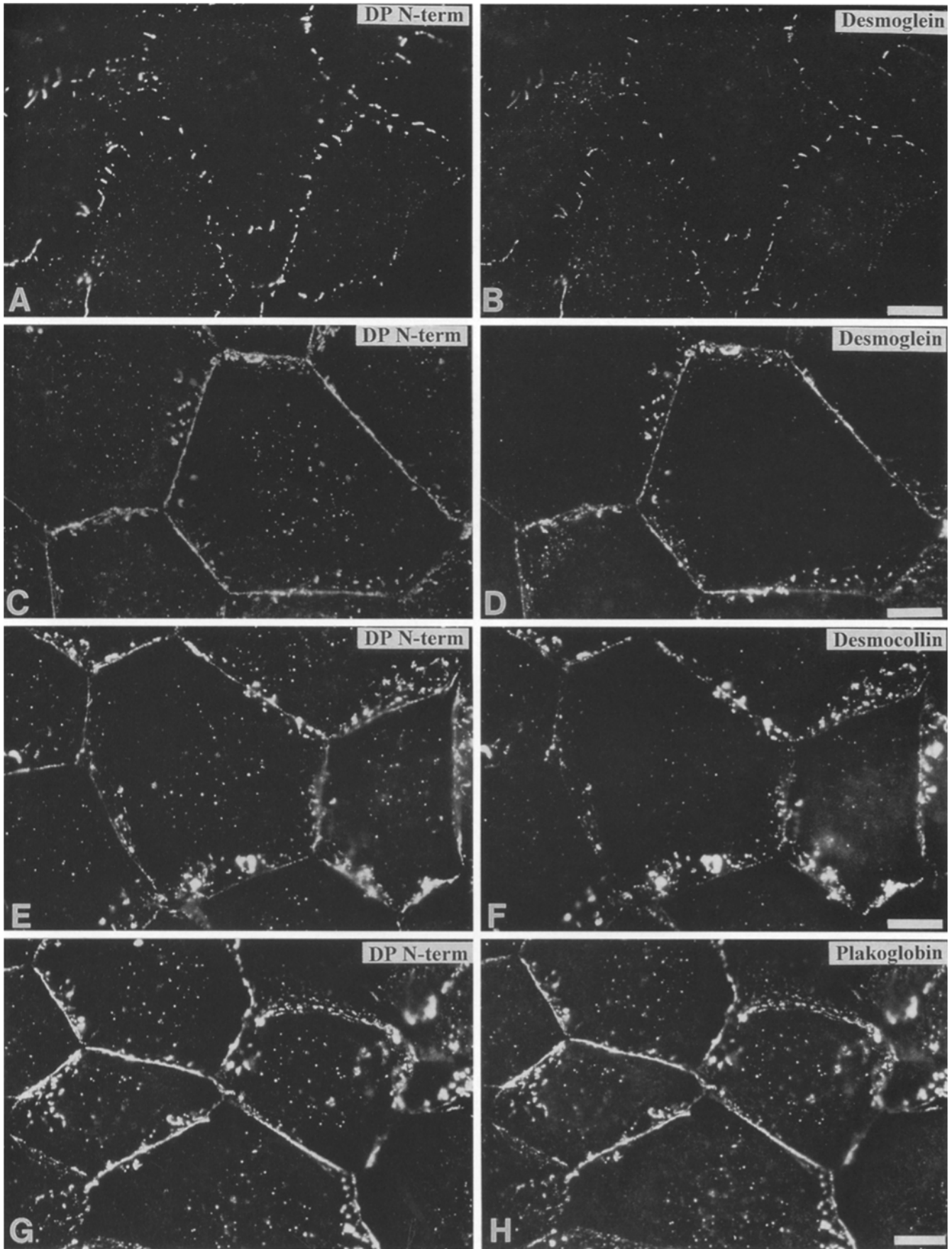


Figure 4. Indirect double label immunofluorescence to detect endogenous DP and DP-NTP. Control puromycin resistant (*A* and *B*), NT710.C1 (*C* and *D*), or NTRod168.1 (*E* and *F*) cells were reacted with DP2.15, which recognizes the DP rod domain of endogenous DP (*A*, *C*, and *E*) or NW161, directed against the DP amino terminus (*B*, *D*, and *F*). *C* and *D* depict cells with essentially no detectable endogenous DP; although such an area was quite typical, other areas of the culture appeared to have more endogenous DP (e.g., see Fig. 3). The arrows in *E* indicate endogenous DP at cell–cell borders; endogenous DP is also present in small cytoplasmic aggregates. These likely represent internalized desmosomes based on ultrastructural analysis (data not shown). Bar, 10 μ m.



Results

Generation of A431 Cell Lines Stably Expressing DP NH₂-terminal Polypeptides

A431 epithelial cell lines stably expressing DP NH₂-terminal polypeptides (Fig. 1) were generated to test whether these polypeptides would act in a dominant negative manner to interfere with the assembly of endogenous DP into desmosomes. One polypeptide, DP.NTRod168, contained the entire predicted DP NH₂-terminal domain as well as the first 168 residues of the rod domain (about one-fifth of the length of the predicted DPI rod). The other, DP.NT710, contained the first 710 predicted amino acid residues of the DP NH₂ terminus. Both polypeptides were tagged at their COOH termini with a tandem series of seven c-myc epitope tags (7myc; see Materials and Methods). The monoclonal antibody 9E10.2, generated against the single myc epitope (Evans et al., 1985), detects the 7myc tag with extremely high sensitivity (Stappenbeck et al., 1993).

From several independent transfections, six lines were generated expressing DP.NTRod168.7myc and four lines were generated expressing DP.NT710.7myc (see Materials and Methods; Fig. 2). Polypeptides of the predicted molecular masses of 155 and 94 kD, respectively, were detected by immunoblot analysis of cell lysates using the monoclonal antibody 9E10.2 (Fig. 2 A; note that the 68-kD band, denoted by the asterisk, was detected by 9E10.2 in whole cell lysates and Triton X-100 soluble material from all A431 cells, including control and parental cells). However, when immunoblot analysis was performed using the polyclonal antibody NW161, directed against the first 189 amino acid residues of the DP NH₂ terminus (see Materials and Methods), polypeptides of these molecular weights were not detected (Fig. 2 B; *closed arrowheads*). Likewise, although the epitope for the monoclonal antibody DP 2.15 was present within DP.NTRod168.7myc, a 155-kD polypeptide was never detected by DP 2.15 in cell lines in which this 7myc-tagged polypeptide was expressed (not shown). The 7myc-tagged DP NH₂-terminal polypeptides could be immunoprecipitated with NW161 and detected by immunoblotting with 9E10.2, indicating that they contained both the NW161 DP NH₂-terminal epitope and the COOH-terminal 7myc tag (Fig. 2 C, *closed arrowhead*; note that the unrelated 9E10-reactive protein denoted by the asterisk was not immunoprecipitated by NW161). Given the great sensitivity with which 9E10.2 detects the 7myc tag (Stappenbeck et al., 1993), it seemed likely that these 7myc-tagged polypeptides contained the NW161 DP NH₂-terminal epitope but were present at levels too low to be detected by immunoblotting with NW161.

Although these polypeptides could not be detected by immunoblotting with NW161, a polypeptide of ~70 kD was readily detected, specifically in cell lines expressing either of the 7myc-tagged polypeptides (Fig. 2, B or C, *open arrowhead*) or a construct encoding an untagged DP.NT710

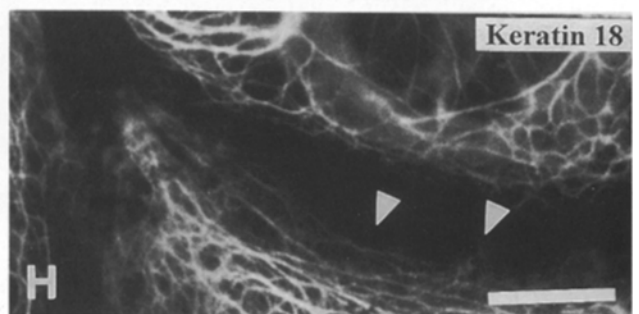
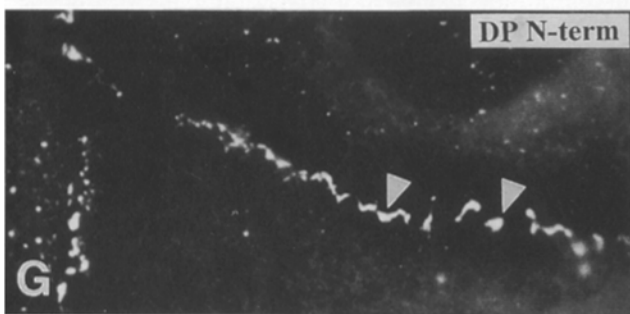
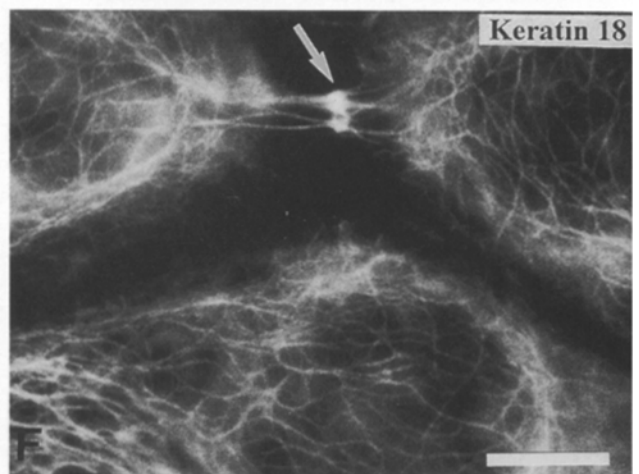
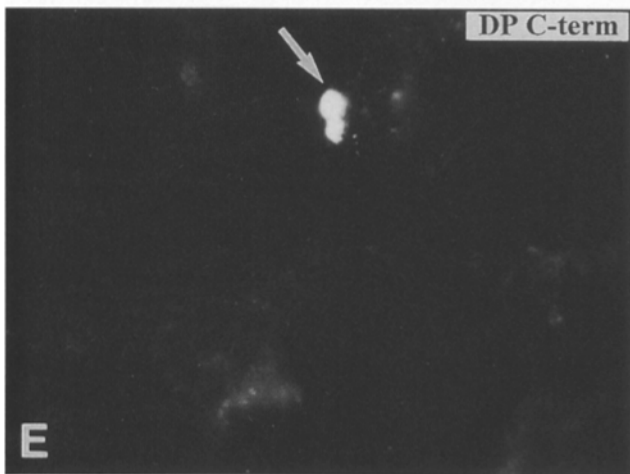
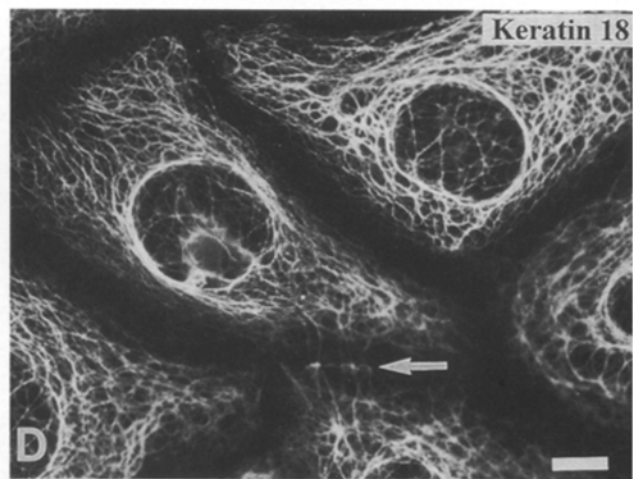
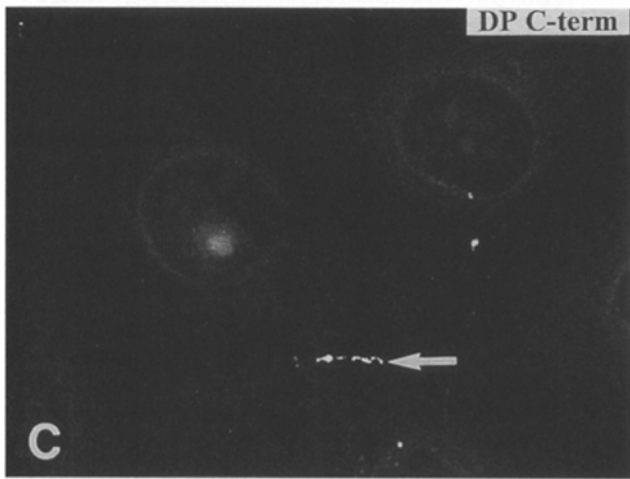
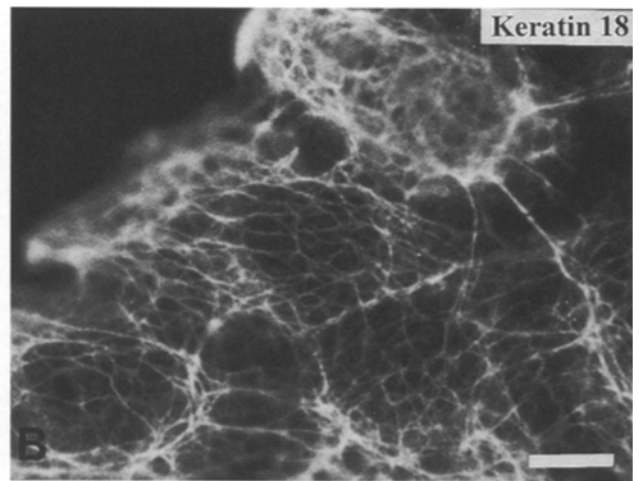
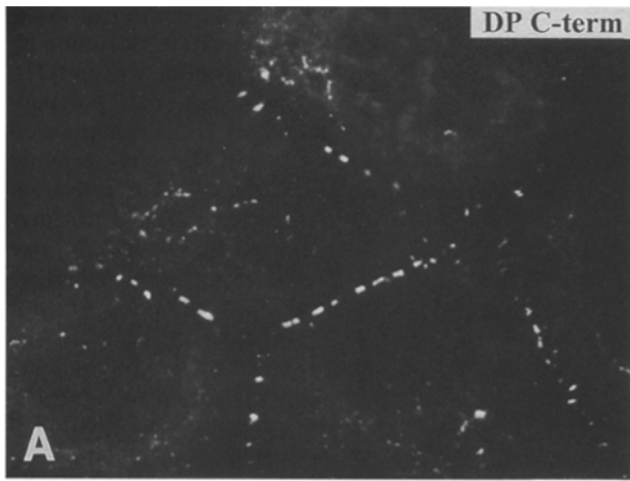
polypeptide (data not shown), but not in control A431 cells (Fig. 2 B, lane 1, and Fig. 2 C, lane 5). Its detection by NW161 suggested that the 70-kD polypeptide was an NH₂-terminal DP polypeptide related to the larger DP polypeptides, as depicted in Fig. 1. This idea was further supported by the observations that the 70-kD DP NH₂-terminal polypeptide (DP-NTP) was also detected in COS cells transiently expressing DP.NTRod168.7myc or DP.NT710.7myc and in L cell fibroblasts stably expressing DP.NT710.7myc (E. Bornslaeger, A. Kowalczyk, K. Green, unpublished results). The generation of this 70-kD polypeptide was not caused by the 7myc epitope tag, since in an A431 cell line expressing an untagged DP.NT710 construct, the only ectopically expressed DP polypeptide detected with NW161 was DP-NTP (data not shown). Furthermore, in coupled in vitro transcription/translation reactions in which the input construct encoded DP.NT710, the single metabolically labeled product detected comigrated precisely with DP-NTP from a whole cell extract of DP.NT710.C1 cells (A. Kowalczyk, J. Borgwardt, and K. Green, unpublished results); this evidence directly supports the contention that DP-NTP is derived specifically from the expression of the NH₂-terminal DP cDNA constructs transfected into the A431 cell lines.

The Localization of Endogenous DP at Cell-Cell Borders is Perturbed in Cells Expressing the 70-kD DP NH₂-terminal Polypeptide

Biochemical analysis indicated that DP-NTP was present at moderately (about threefold) higher levels than endogenous DP and that the level of endogenous DP in lines expressing DP-NTP was approximately half that typically seen in drug resistant or parental control lines (Fig. 2 B). Indirect immunofluorescence using the antibodies NW6, NW38, or DP 2.15 to specifically detect endogenous DP, not DP-NTP (Fig. 1), indicated that DP-NTP interfered with the assembly of endogenous DP into desmosomes. For three of the NT710 lines (lines A1, A2, and D3), two of the NTRod168 lines (lines 5 and 8), and a line expressing untagged NT710, some cell-cell borders exhibited the fine, regularly spaced, punctate pattern of DP staining typical of desmosomes, but many cell-cell borders exhibited an aberrant pattern of coarse, irregularly spaced aggregates of DP (data not shown).

The endogenous DP pattern was even more dramatically perturbed in one of the NT710 lines (line C1) and four of the NTRod168 lines (lines 1, 9, 11, and 29; Fig. 3 A; Fig. 4, C and E). Although the distribution of endogenous DP was also heterogenous in these latter cell lines, fewer cell-cell borders exhibited a typical DP pattern. Endogenous DP most often appeared as coarse, irregularly spaced aggregates, which sometimes occupied extensive stretches of cell-cell borders. In addition, small aggregates of endogenous DP were sometimes observed within cells (e.g., Fig. 4 E). Furthermore, many cell-cell borders had little

Figure 5. Indirect double label immunofluorescence to detect DP-NTP and desmosomal components. Control puromycin resistant (A and B) or NT710.C1 (C-H) cells were reacted with NW161, directed against the DP amino terminus (A, C, E, and G), and either 6D8 (B and D), directed against desmoglein, 7G6 (F), directed against desmocollin 2, or 11E4 (H), directed against plakoglobin. Bar, 10 μ m.



detectable endogenous DP; quite commonly, populations of cells exhibited even less immunoreactivity for endogenous DP than represented in Fig. 3 A.

It seemed likely that the aberrant distribution of endogenous DP in these cells was due to competition between ectopically expressed DP NH₂-terminal polypeptides and endogenous DP for association with desmosome assembly intermediates. If this were the case, these NH₂-terminal polypeptides would be predicted to localize at cell-cell borders. Not surprisingly, due to their low abundance (see above), no 7myc-tagged DP NH₂-terminal polypeptides could be detected by indirect immunofluorescence using the anti-c-myc antibody, 9E10.2 (data not shown). The localization of DP-NTP was therefore assessed by performing double label immunofluorescence using the monoclonal antibody DP2.15, to specifically detect endogenous DP, and NW161, directed against the DP NH₂ terminus. Cell-cell borders that had little or no endogenous DP exhibited abundant NW161-immunoreactivity (Fig. 3 B; Fig. 4, D and F), indicating that DP-NTP did in fact localize to cell-cell interfaces. It did so, however, in an atypical pattern that involved more cell surface area than that occupied by endogenous DP in control cells (compare Fig. 4 B and D or F). These observations indicate that DP-NTP has information necessary and sufficient to target it to the inner cell surface and suggest that it can compete with endogenous DP during junction assembly, resulting in the aberrant distribution and often loss of detectable endogenous DP from cell-cell borders. As discussed below, small amounts of DP detectable at an ultrastructural level may still be present at cell-cell borders.

Desmoglein, Desmocollin, and Plakoglobin Colocalize with DP-NTP at Cell-Cell Borders

In order to determine the spatial relationship of DP-NTP and desmosomal components with which DP-NTP would be predicted to associate, the distribution of the transmembrane desmosomal cadherins and associated protein plakoglobin was assessed. Double label immunofluorescence analysis to simultaneously detect endogenous DP (using NW6 or NW38) and the desmosomal proteins desmoglein, desmocollin, or plakoglobin revealed that cell borders lacking endogenous DP still exhibited immunoreactivity for these desmosomal proteins (data not shown). However, the distribution of desmoglein, desmocollin, and plakoglobin at cell-cell interfaces differed from the regularly spaced punctate pattern observed in control cells (Fig. 5, A and B). Instead, all three desmosomal proteins occupied a significantly larger surface area, colocalizing with DP-NTP at cell-cell borders (Fig. 5, C-H). These observations support the idea that DP-NTP competes with endogenous DP during junction assembly by associating directly or indirectly with these desmosomal components. These results also suggest that DP-NTP governs the distri-

bution of desmosomal cadherin/plakoglobin complexes in these cell lines.

Keratin Filament Bundles Are Not Attached to Inner Cell Surfaces Lacking Endogenous DP

If the attachment of IF to desmosomes requires DP, then keratin IF anchorage should be compromised at cell-cell borders lacking endogenous full-length DP. To test this idea, we examined the distribution of keratin 18-containing tonofibrils in control cells and in cell lines expressing DP-NTP. (The term "tonofibrils" denotes bundles formed by the lateral association of individual IF; such bundles are seen in the light microscope as fibers that converge upon desmosomes at cell-cell borders [Bloom and Fawcett, 1975]). In control cells, abundant tonofibrils converged on desmosomes (Fig. 6, A and B). In cells expressing DP-NTP, however, keratin 18-containing tonofibrils were not attached at cell-cell interfaces lacking detectable endogenous DP (Fig. 6, C-F), even though abundant DP-NTP was present (Fig. 6, G and H). Tonofibrils were always anchored at cell-cell interfaces where endogenous DP was localized, however (Fig. 6, C-F). More faintly immunofluorescent keratin 18 meshworks were sometimes observed abutting cell-cell margins; it was unclear at this level of resolution, however, whether such filaments were attached to the inner cell surface.

We performed an ultrastructural analysis to determine if observations made by immunofluorescence represented a loss of attachment of IF bundles to cell-cell interfaces and to examine whether the DP-NTP at cell-cell interfaces participated in the assembly of some type of junctional structure. In cells expressing DP-NTP, we occasionally observed oversized desmosomes with large plaques attached to IF bundles (not shown), probably corresponding to the aggregates of endogenous DP seen at the light microscopic level (Fig. 6, C-F). However, the prevalent junctional structures in these cells (Fig. 7, C and D) differed from the normal desmosomes seen in control cells (Fig. 7, A and B). Although these junctional structures did appear to have a central dense stratum and outer dense plaque, the fibrous mat of inner plaque material associated with normal desmosomes was missing. Furthermore, IF bundles were not attached to these novel junctional structures, which is consistent with the immunofluorescence data (Fig. 6) and indicates that DP is required for the desmosomal attachment of all types of cytoplasmic IF bundles present in A431 cells (Moll et al., 1982).

Some of these junctional structures were associated with sparse, loosely packed 10-nm filaments (Fig. 7 E). This, in conjunction with the observation that faintly immunofluorescent keratin filament meshworks occasionally abutted cell-cell margins at sites where endogenous DP was undetectable by immunofluorescence, suggested that small amounts of endogenous DP might still be present within

Figure 6. Indirect double label immunofluorescence to detect endogenous DP or DP-NTP as well as keratin IF. Control puromycin-resistant (A and B), NT710.C1 (C and D), or NTRod168.1 (E-H) cells were reacted with KSB17.2, directed against keratin 18 (B, D, F, and H), and either NW6 (A and C) or NW38 (E), both directed against the DP COOH terminus, to detect endogenous DP, or NW161, directed against the DP NH₂ terminus, to detect DP-NTP (G). Arrows (C-F) indicate areas along cell-cell borders where endogenous DP is localized. Arrowheads (G and H) indicate examples of areas where DP-NTP is localized at cell-cell borders. Bar, 10 μm.

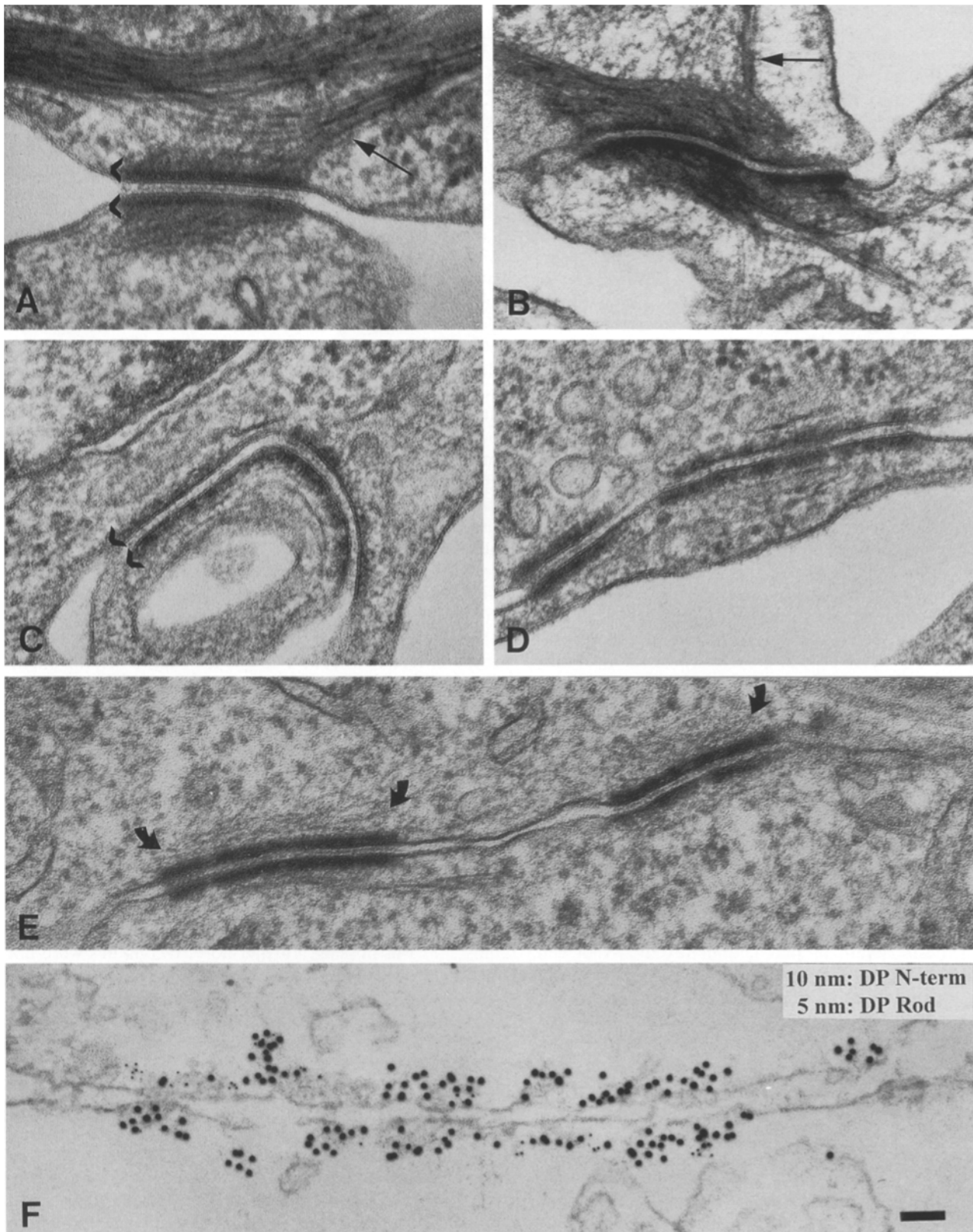


Figure 7. Ultrastructural analysis of junctional structures in cells expressing DP-NTP. Control puromycin resistant (*A* and *B*) or DP-NTP-expressing cells (*C–F*) were prepared for conventional (*A–E*) or immunogold (*F*) electron microscopy using antibodies directed against the NH₂ terminus of DP (NW161; large gold particles) or the rod domain of DP (DP2.15; small gold particles). Note that control desmosomes (*A* and *B*) as well as the DP-NTP-containing junctional structures (*C–E*) exhibit outer dense plaques (*brackets*). However, control desmosomes also exhibit an inner plaque region continuous with attached IF bundles (*A* and *B*; *straight arrows*). Occasionally, sparse arrays of 10-nm filaments are seen in association with junctional structures in DP-NTP-expressing cells (*E*; *curved arrows*), which

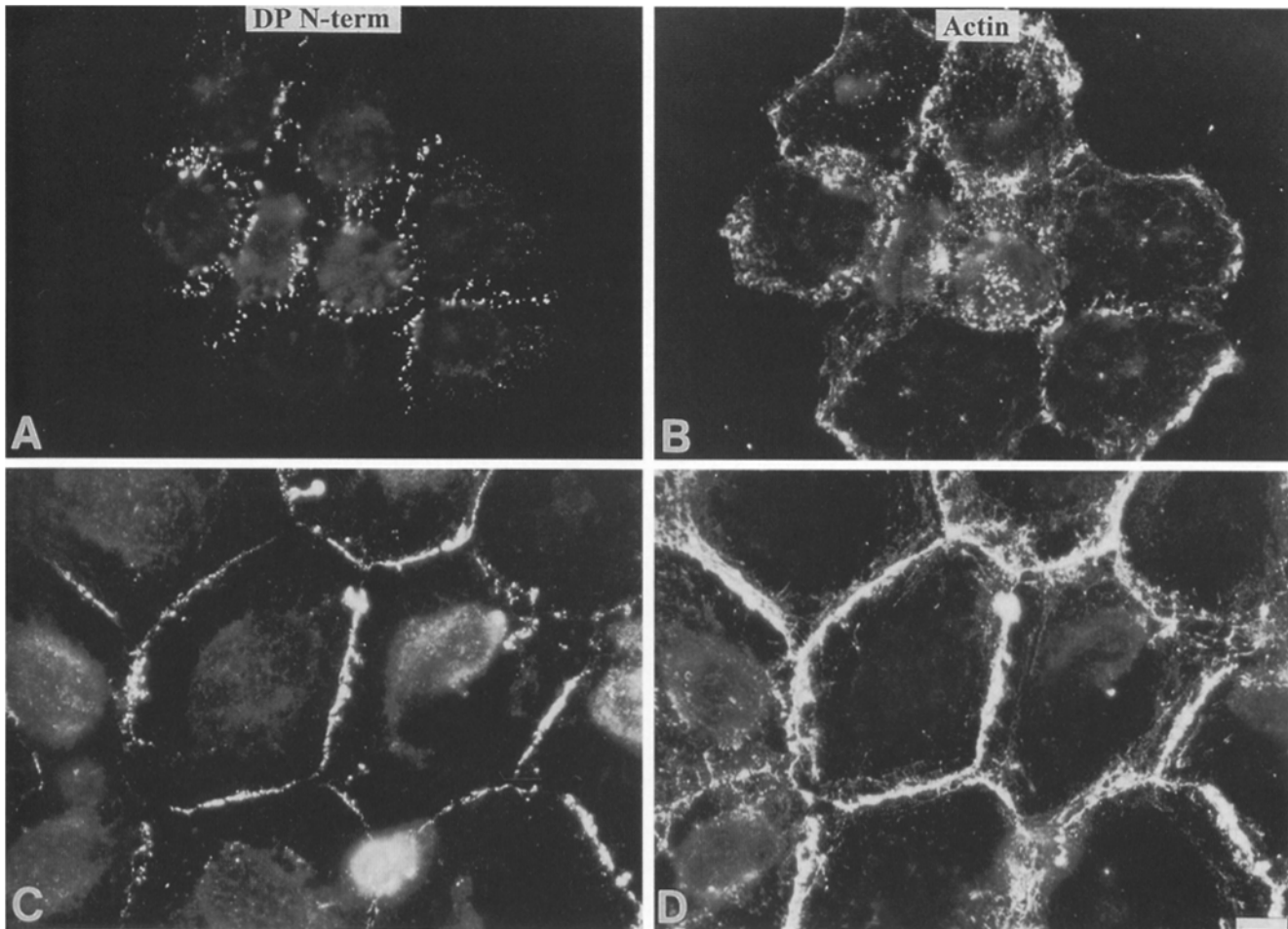


Figure 8. Analysis of actin distribution in control A431 and DP-NTP-expressing cell lines. Double label fluorescence was performed on either control puromycin-resistant (*A* and *B*) or DP.NT710.C1 cells (*C* and *D*) reacted with NW161 (*A* and *C*) to detect the DP NH₂ terminus or rhodamine phalloidin (*B* and *D*) to detect F-actin. The actin filament pattern is often not well developed in control cells, often taking on a punctate appearance rather than appearing as stress fibers, whereas the actin pattern in NT710.C1 cells is quite robust, frequently colocalizing at cell-cell borders with NW161. Bar, 10 μ m.

some of these junctional structures. This would not be unexpected given that we had employed a dominant negative approach. To test this idea, we performed immunogold electron microscopy using the antibody NW161 (Fig. 7 *F*; large gold particles) in conjunction with DP2.15 to specifically detect endogenous DP (Fig. 7 *F*; small gold particles). Small amounts of endogenous DP were indeed present within some of these junctions.

Together, these results suggest that a range of junctional structures coexist in the DP-NTP cell lines, from oversized desmosomes containing large amounts of endogenous DP and associated with IF bundles to structures containing DP-NTP but little endogenous DP and largely lacking associated IF.

Adherens Junction Components Colocalize with DP-NTP and Desmosomal Cadherins at Cell-Cell borders

The loss of IF bundle anchorage in DP-NTP cell lines did not reflect a nonspecific effect on cytoskeletal organization, as immunofluorescence analysis indicated that microtubule networks were largely undisturbed (data not shown). Furthermore, staining with rhodamine phalloidin indicated that there was not a loss of cortical actin microfilaments in these cell lines (Fig. 8). In fact, the cortical microfilament system of cells expressing DP-NTP was quite robust relative to that of control A431 cells, suggesting that actin may be enriched at cell-cell borders in DP-NTP-

may be accounted for by low levels of endogenous DP (*F*; small gold particles). Note that the sparsely packed filaments observed in conventionally fixed specimens were not seen in cells extracted for immunoelectron microscopy, even along areas containing small amounts of endogenous DP, although large attached IF bundles were preserved in control lines using this extraction procedure (not shown). This extraction was used to optimize accessibility to second antibody-conjugated gold particles. All micrographs are at the same magnification. Bar, 0.1 μ m.

expressing cells. The transmembrane adhesive glycoproteins of the microfilament-associated adherens junctions, the classical cadherins, can associate with plakoglobin, as can the desmosomal cadherins (Knudsen and Wheelock, 1992; Peifer et al., 1992; Butz and Kemler, 1994; Kowalczyk et al., 1994; Mathur et al., 1994; Nathke et al., 1994; Troyanovsky et al., 1994; Chitaev et al., 1996; Wahl et al., 1996; Witcher et al., 1996). Since plakoglobin colocalized with DP-NTP in a pattern differing from that of typical desmosomes (Fig. 5), we examined whether alterations in desmosome organization caused by ectopic expression of DP-NTP had any effect on adherens junction assembly by analyzing the distribution of adherens junction components, including the classic cadherin E-cadherin and the catenins.

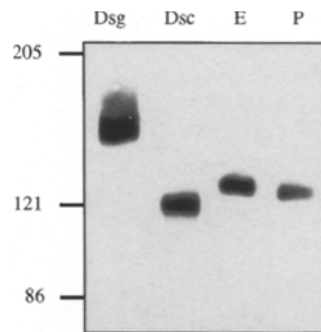
Immunoblotting demonstrated that the anticadherin antibodies used in this study were monospecific (Fig. 9). Double label immunofluorescence analysis revealed that the distributions of α - and β -catenin and E-cadherin were dramatically altered in cells expressing DP-NTP (Figs. 10 and 11). In control cells, adherens junction components were broadly distributed along cell-cell borders in a pattern that was more continuous than that of desmosomal components; although the patterns overlapped somewhat, they were quite distinctive (Fig. 10, *A* and *B*; Fig. 11, *A* and *C*). In cells expressing DP-NTP, however, both E-cadherin and α -catenin, and to a lesser extent β -catenin, colocalized extensively with both DP-NTP and the endogenous desmosomal proteins desmoglein and desmocollin (Fig. 10, *C-F*; Fig. 11, *B* and *D*, and data not shown), suggesting that these two types of junction components were intermingled in DP-NTP cell lines. For dual label immunofluorescence analysis, α -catenin was used as an adherens junction marker (Fig. 11) since the specific localization of E-cadherin in adherens junctions has been questioned (Jones, 1988; Horiguchi et al., 1994). Colocalization of α -catenin with DP-NTP or desmosomal cadherins was especially obvious within discrete punctate structures visible in en face views of lateral cell borders (Fig. 11, *B* and *D*; arrows).

To determine whether the prevalent DP-NTP-containing junctional structures identified at the ultrastructural level contained adherens junction components as well, immunoelectron microscopic analysis was performed. α -catenin and DP-NTP colocalized within such junctional structures (Fig. 10 *G*) as well as along other areas of the cell membrane (not shown). These structures therefore appeared to be composite junctions containing both desmosomal and adherens junction components, as well as DP-NTP.

Discussion

Ectopic Expression of an NH₂-terminal Desmoplakin Polypeptide Perturbs the Distribution of Endogenous Desmoplakin

Based on its abundance and location within the innermost



6A9, directed against P-cadherin (*P*). Note that each antibody recognized a single distinct band.

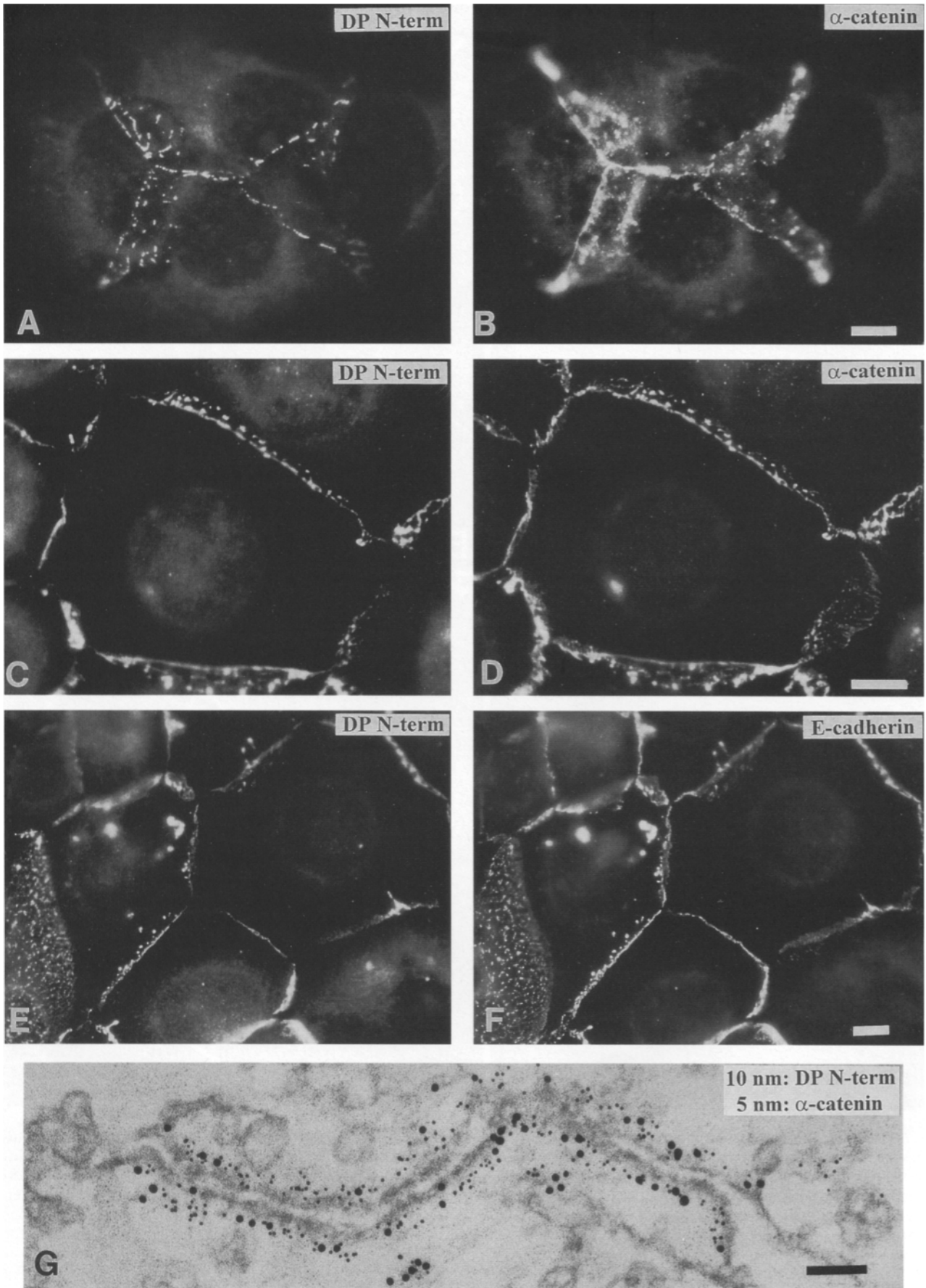
Figure 9. Immunoblot analysis of antibodies directed against classic and desmosomal cadherins. Whole cell extracts of A431 cells were subjected to SDS-PAGE (6.5% gel), transferred to nitrocellulose, and immunoblotted with the monoclonal antibodies 6D8, directed against desmoglein (*Dsg*); 7G6, directed against desmocollin (*Dsc*); E9, directed against E-cadherin (*E*); or

portion of the desmosomal plaque, DP was proposed to link cytoplasmic IF networks to the desmosome (Jones and Goldman, 1985; Miller et al., 1987; Steinberg et al., 1987). Although studies defining the functions of individual DP domains supported this idea (Stappenbeck and Green, 1992; Stappenbeck et al., 1993; Kouklis et al., 1994), they did not test directly whether IF anchorage to the desmosome requires DP. We reasoned that the assembly of endogenous DP into desmosomal plaques would be perturbed by the expression of DP NH₂-terminal polypeptides. The aberrant distribution and apparent loss of endogenous DP from cell-cell interfaces as well as the loss of IF anchorage were observed in a number of A431 cell lines stably expressing cDNAs encoding DP NH₂-terminal polypeptides.

In these cell lines, the full-length DP NH₂-terminal polypeptides were present at extremely low levels; however, a 70-kD DP NH₂-terminal polypeptide designated DP-NTP was expressed at levels about threefold higher than endogenous DP (Fig. 2 *B*). The mechanism by which DP-NTP arises remains to be elucidated, although it is independent of the presence of a COOH-terminal 7myc epitope tag (see results). DP-NTP was not detected in control A431 cells or in A431 cells stably expressing full-length DP.7myc (Fig. 2 *B*, and data not shown), suggesting that it may be generated in the absence of the DP COOH-terminal domain. However, DP-NTP was detected in COS cells transiently overexpressing DP.7myc (data not shown), suggesting that DP-NTP can accumulate when the expression of full-length DP exceeds a certain level.

Even though the exact mechanism by which DP-NTP arises is unclear, the observed dominant negative effects were most likely caused by this 70-kD polypeptide, based on several lines of evidence. First, the COOH-terminal 7myc tag is not responsible for these effects, since they were also observed in a cell line expressing a construct encoding an untagged DP.NT710 construct. Second, since DP-NTP was the only polypeptide detected by NW161 in

Figure 10. Colocalization of DP-NTP with adherens junction components. Double label immunofluorescence was performed on either control puromycin-resistant (*A* and *B*) or NT710.C1 cells (*C-F*) reacted with NW161, directed against the DP NH₂ terminus (*A*, *C*, and *E*) and either 1G5, directed against α -catenin (*B* and *D*) or E9, directed against E-cadherin (*F*). The degree of colocalization seen in *C-F* is typical. Note discrete punctate areas of colocalization at lateral cell-cell borders. *G* is an electron micrograph of NT710.C1 cells immunogold labeled as described in Materials and Methods using NW161 (large gold particles) and 1G5, directed against α -catenin (small gold particles). *A-F*, Bar, 10 μ m. *G*, Bar, 0.1 μ m.



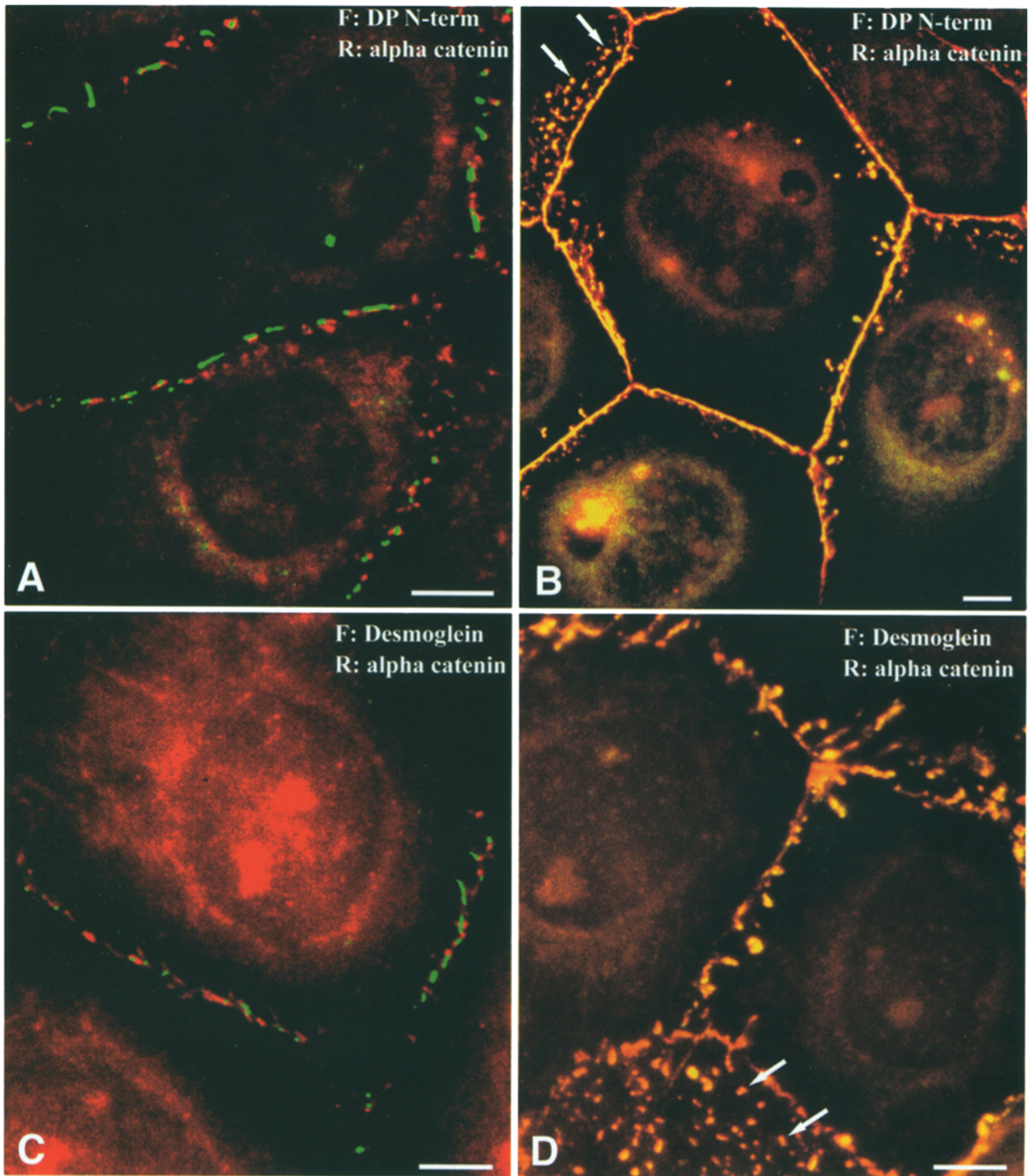


Figure 11. Dual color overlay double label immunofluorescence analysis of DP-NTP or desmoglein and α -catenin. Double label immunofluorescence was performed on either control A431 cells (*A* and *C*) or NT710.C1 cells (*B* and *D*) reacted with (*A* and *B*) NW161 (fluorescein), directed against the DP NH₂ terminus, and 1G5 (rhodamine), directed against α -catenin, or (*C* and *D*) 6D8 (fluorescein), directed against desmoglein, and a rabbit polyclonal antisera, directed against α -catenin (rhodamine). Note distinct areas of red and green fluorescence in control cells (*A* and *C*), whereas in NT710.C1 cells (*B* and *D*), a yellow signal is seen almost exclusively, indicating extensive colocalization of DP-NTP and desmoglein with α -catenin. Similar colocalization was obtained for desmocollin and α -catenin or for both of the desmosomal cadherins and E-cadherin (not shown). Arrows in *B* and *D* indicate discrete punctate areas of colocalization at lateral cell-cell borders. Bar, 5 μ m.

immunoblots other than endogenous DP, the abundant NW161-immunoreactive material at cell–cell borders that did not colocalize with endogenous DP must represent DP-NTP. Third, the colocalization of endogenous desmosomal components with DP-NTP at cell–cell borders, together with the aberrant distribution of endogenous DP, suggests that DP-NTP competes with endogenous DP during junction assembly.

The apparent local variation in the ratio of endogenous DP and DP-NTP observed along individual cell–cell borders (Fig. 3) is also consistent with the idea that these polypeptides are in competition for junctional binding partners. The range of junctional structures observed in these cells at an ultrastructural level is probably a reflection of such variation, as discussed below. In addition, the overall lower level of endogenous DP in cell lines expressing DP-NTP raises the possibility that endogenous DP not assembled into junctions is degraded.

Desmoplakin Is Required for Anchoring IF Bundles to Desmosomes

The consequence of the dramatic decrease in endogenous DP at many cell–cell interfaces is the loss of attachment of IF bundles. This is the best evidence to date that DP plays an essential role in the anchorage and organization of IF bundles at the desmosome.

The junctional structures prevalent in cells expressing DP-NTP (Fig. 7, C and D), presumably containing mostly DP-NTP and little or no endogenous DP, lacked a fibrous mat of inner plaque material and associated IF, supporting the idea that the DP rod domain plays a role in the assembly of the inner desmosome plaque, as previously suggested (Stappenbeck and Green, 1992). Some of the junctions in DP-NTP-expressing cells exhibited loosely packed 10-nm filaments (Fig. 7 E) that were likely to be associated with small amounts of endogenous DP, not detectable by immunofluorescence, but detectable by immunoelectron microscopy (Fig. 7 F). The majority of endogenous DP was apparently present within oversized desmosomes that were associated with IF bundles (not shown); such desmosomes presumably have a comparatively high level of endogenous DP relative to DP-NTP. One possible mechanism underlying the assembly of these oversized structures is that DP-NTP might promote enhanced clustering of desmosome assembly complexes containing endogenous DP.

Although our results clearly indicate that DP is required for anchoring IF bundles to desmosomes, we cannot rule out the possibility that linkage between IF and DP is indirect, or that other IF-associated proteins may also contribute to the establishment and/or maintenance of desmosome/IF linkage. For instance, two possible candidates, IFAP 300 and plectin, are not restricted to desmosomes but have been reported to be present at low levels in these junctions; both of these DP family members have been suggested to play a role in linking IF to sites of desmosomal as well as hemidesmosomal attachment (Wiche, 1989; Skalli et al., 1994). New members of this gene family may also prove to function in a similar manner (Fujiwara et al., 1996). Another candidate, plakophilin or band 6 protein (B6P), has been demonstrated by *in vitro* assays to associate with certain IF polypeptides (Kapprell et al., 1987;

Hatzfeld et al., 1994) as well as desmoglein 1 (Mathur et al., 1994). In addition, results of Troyanovsky and colleagues (1994) suggest that plakoglobin may play an accessory role along with DP in mediating IF anchorage.

Desmosomal Components and Adherens Junction Components Coassemble with DP-NTP into Distinct Junctional Structures

The colocalization at cell–cell borders of DP-NTP and desmosomal components, as well as the corresponding loss of endogenous DP from cell–cell borders, suggests that sequences necessary and sufficient to direct the association of DP with desmosomal plaque components during desmosome assembly are contained within DP-NTP. However, unlike endogenous DP, DP-NTP coassembles into junctions that contain adherens junction components such as E-cadherin and α -catenin, as assessed by both immunofluorescence and immunogold electron microscopy. Although DP and plakoglobin are localized along with nondesmosomal cadherins in certain cell type-specific junctions, such as syndesmos (Schmelz and Franke, 1993), the association of nondesmosomal cadherins with DP is not normally observed in epithelial cells such as A431 cells, which assemble both desmosomes and adherens junctions.

What is the mechanism for this unusual coassembly of desmosomal and adherens junction proteins in the presence of DP-NTP? It seems unlikely that α -catenin coassembles into these novel junctions by binding directly to desmosomal cadherin–plakoglobin complexes since although α -catenin has been shown to bind directly to plakoglobin (Aberle et al., 1994; Jou et al., 1995; Rubinfeld et al., 1995; Sacco et al., 1995), it does not appear to associate with plakoglobin bound to desmosomal cadherins (Plott et al., 1994; A. Kowalczyk and K. Green, unpublished data). Therefore, it seems most likely that this codistribution of adherens junction and desmosomal components represents the lateral association of transmembrane complexes containing desmosomal cadherins, classic cadherins, and their respective associated proteins, and that this lateral association is mediated by DP-NTP. Although a direct interaction between DP and plakoglobin has not yet been demonstrated, it seems possible that lateral alignment of desmosomal cadherin and E-cadherin complexes may be facilitated by interactions, whether direct or indirect, between DP-NTP and plakoglobin, which binds to both classic and desmosomal cadherins (Korman et al., 1989; Peifer et al., 1992; Knudsen and Wheelock, 1992; Kowalczyk et al., 1994; Mathur et al., 1994; Chipaev et al., 1996; Wahl et al., 1996; Witcher et al., 1996). Together with the moderate overexpression of DP-NTP, plakoglobin homodimerization (Kapprell et al., 1987) may further enhance coclustering of desmosomal cadherin and classical cadherin complexes. Since intermingling of adherens junction and desmosome components does not occur in normal epithelial cells, it seems most likely that sequences within endogenous DP but lacking from DP-NTP are somehow responsible for governing the normal spatial segregation of adherens junction and desmosomal components at cell–cell borders. It remains to be determined whether the critical sequences are to be found in the remainder of the DP NH₂-terminal domain, or whether the rod and COOH-terminal domains,

which are required for DP dimerization and the formation of a dense IF-associated inner plaque, are required for the normal spatial segregation of adhesive junction components.

Another important issue is whether the observed disruption of IF attachment has more global effects on cell morphology or behavior. So far, we have not observed any obvious effects on intercellular adhesion, cell growth, motility, or other aspects of cell morphology or behavior of these cells growing in culture. This is not surprising, however, in light of the fact that perturbation or loss of IF networks in cultured cells by antibody injection or expression of mutant keratin proteins also does not have obvious effects on cell morphology or behavior; it is only when keratins are disrupted in vivo, in embryos or in developing tissues, that their functional importance is revealed (Klymkowsky et al., 1983; Albers and Fuchs, 1987; Vassar et al., 1991; Klymkowsky et al., 1992). Thus, it seems reasonable that the functional importance of IF attachment to junctional contact sites might also be best determined by perturbation of this association within a complex tissue. For instance, abnormalities in mechanical integrity and cell migration were reported in the epidermis of mice in which the putative hemidesmosomal IF linker, bullous pemphigoid 230-kD antigen, was ablated by targeted homologous recombination (Guo et al., 1995). Likewise, the ablation of the DP gene, or the targeted expression of NH₂-terminal DP polypeptides to specific tissues such as mouse epidermis, may provide a more sensitive test of the function of DP in cell-cell adhesion, differentiation, and the maintenance of tissue integrity.

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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.
- Aghib, D.F., and P.D. McCrea. 1995. The E-cadherin complex contains the src substrate p120. *Exp. Cell Res.* 218:359-369.
- Albers, K., and E. Fuchs. 1987. The expression of mutant epidermal keratin cDNAs transfected in simple epithelial and squamous cell carcinoma lines. *J. Cell Biol.* 105:791-806.
- Angst, B.D., L.A. Nilles, and K.J. Green. 1990. Desmoplakin II expression is not restricted to stratified epithelia. *J. Cell Sci.* 97:247-257.
- Baribault, H., and R.G. Oshima. 1991. Polarized and functional epithelia can form after the targeted inactivation of both mouse keratin 8 alleles. *J. Cell Biol.* 115:1675-1684.
- Bloom, W., and D.W. Fawcett. 1975. *A Textbook of Histology*. W.B. Saunders Co., Philadelphia. 1033 pp.
- Bologna, M., R. Allen, and R. Dulbecco. 1986. Organization of cyokeratin bundles by desmosomes in rat mammary cells. *J. Cell Biol.* 102:560-567.

- Brakenhoff, R.H., M. Gerretsen, E.M.C. Knippels, M. van Dijk, H. van Essen, D.O. Weghuis, R.J. Sinke, G.B. Snow, and G.A.M.S. van Dongen. 1995. The human E48 antigen, highly homologous to the murine Ly-6 antigen ThB, is a GPI-anchored molecule apparently involved in keratinocyte cell-cell adhesion. *J. Cell Biol.* 129:1677-1689.
- Butz, S., and R. Kemler. 1994. Distinct cadherin-catenin complexes in calcium dependent cell-cell adhesion. *FEBS Lett.* 355:195-200.
- Buxton, R.S., and A.I. Magee. 1992. Structure and interactions of desmosomal and other cadherins. *Semin. Cell Biol.* 3:157-167.
- Buxton, R.S., P. Cowin, W.W. Franke, D.R. Garrod, K.J. Green, I.A. King, P.J. Koch, A.I. Magee, D.A. Rees, J.R. Stanley, and M.S. Steinberg. 1993. Nomenclature of the desmosomal cadherins. *J. Cell Biol.* 121:481-483.
- Buxton, R.S., A.I. Magee, I.A. King, and J. Arneemann. 1994. Desmosomal Genes. In *Molecular Biology of Desmosomes and Hemidesmosomes*. J.E. Collins and D.R. Garrod, editors. R.G. Landes Co., Austin, TX. 1-17.
- Chitavev, 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.
- Citi, S. 1994. *Molecular Mechanisms of Epithelial Cell Junctions: from Development to Disease*. R.G. Landes Co., Austin, TX. 1-308.
- Collins, J.E., and D.R. Garrod. 1994. *Molecular Biology of Desmosomes and Hemidesmosomes*. R.G. Landes Co., Austin, TX. 1-131.
- Cowin, P. 1994. Plakoglobin. In *Molecular Biology of Desmosomes and Hemidesmosomes*. J.E. Collins and D.R. Garrod, editors. R.G. Landes Co., Austin, TX. 53-68.
- Cowin, P., and B. Burke. 1996. Cytoskeleton-membrane interactions. *Curr. Opin. Cell Biol.* 8:56-65.
- 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, TX. 141-155.
- Cowin, P., H.-P. Kapprell, and W.W. Franke. 1985. The complement of desmosomal plaque proteins in different cell types. *J. Cell Biol.* 101:1442-1454.
- Cowin, P., H.-P. Kapprell, W.W. Franke, J. Tamkun, and R.O. Hynes. 1986. Plakoglobin: a protein common to different kinds of intercellular adhering junctions. *Cell*. 46:1063-1073.
- Duden, R., and W.W. Franke. 1988. Organization of desmosomal plaque proteins in cells growing at low calcium concentrations. *J. Cell Biol.* 107:1049-1063.
- Evans, G.I., G.K. Lewis, G. Ramsey, and J.M. Bishop. 1985. Isolation of monoclonal antibodies specific for human c-myc proto-oncogene product. *Mol. Cell Biol.* 5:3610-3616.
- Farquhar, M.G., and G.E. Palade. 1963. Junctional complexes in various epithelia. *J. Cell Biol.* 17:375-412.
- Fuchs, E., and K. Weber. 1994. Intermediate filaments: structure, dynamics, function and disease. *Ann. Rev. Biochem.* 63:345-382.
- Fujiwara, S., K. Kohno, A. Iwamatsu, I. Naito, and H. Shinkai. 1996. Identification of a 450-kDa human epidermal autoantigen as a new member of the plectin family. *J. Invest. Dermatol.* 106:1125-1130.
- Garrod, D.R. 1993. Desmosomes and hemidesmosomes. *Curr. Opin. Cell Biol.* 5:30-40.
- Garrod, D.R., and J.E. Collins. 1994. Desmosomes in differentiation and development. In *Molecular Biology of Desmosomes and Hemidesmosomes*. J.E. Collins and D.R. Garrod, editors. R.G. Landes Co., Austin, TX. 19-33.
- Geiger, B., and D. Ginsberg. 1991. The cytoplasmic domain of adherens-type junctions. *Cell Motil. Cytoskel.* 20:1-6.
- 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.* 193:134-143.
- Green, K.J., T.S. Stappenbeck, D.A.D. Parry, and M.L.A. Virata. 1992a. Structure of desmoplakin and its association with intermediate filaments. *J. Dermatol. (Tokyo)*. 19:765-769.
- Green, K.J., M.L.A. Virata, G.W. Elgart, J.R. Stanley, and D.A.D. Parry. 1992b. Comparative structural analysis of desmoplakin, bullous pemphigoid antigen and plectin: members of a new gene family involved in organization of intermediate filaments. *Int. J. Biol. Macromol.* 14:145-153.
- Guo, L., L. Degenstein, J. Dowling, Q.-C. Yu, R. Wollmann, B. Perman, and E. Fuchs. 1995. Gene targeting of BPAG1: abnormalities in mechanical strength and cell migration in stratified epithelia and neurologic degeneration. *Cell*. 81:233-243.
- Hatzfeld, M., G.J. Kristjansson, U. Plessmann, and K. Weber. 1994. Band 6 protein, a major constituent of desmosomes from stratified epithelia, is a novel member of the armadillo multigene family. *J. Cell Sci.* 107:2259-2270.
- Heid, H.W., A. Schmidt, R. Zimbelmann, S. Schafer, S. Winter-Simanowski, S. Stumpp, M. Keith, U. Figge, M. Schinolzer, and W.W. Franke. 1994. Cell type-specific desmosomal plaque proteins of the plakoglobin family: plakophilin 1 (band 6 protein). *Differentiation*. 58:113-131.
- Horiguchi, Y., F. Furukawa, M. Fumita, and S. Imamura. 1994. Ultrastructural localization of E-cadherin cell adhesion molecule on the cytoplasmic membrane of keratinocytes in vivo and in vitro. *J. Histochem. Cytochem.* 42:1333-1340.
- Johnson, K.R., J.E. Lewis, D. Li, J. Wahl, A.P. Soler, K.A. Knudsen, and M.J.

- Whelock. 1993. P- and E-cadherin are in separate complexes in cells expressing both cadherins. *Exp. Cell Res.* 207:252-260.
- Jones, J.C.R. 1988. Characterization of a 125K glycoprotein associated with bovine epithelial desmosomes. *J. Cell Sci.* 89:207-216.
- Jones, J.C.R., and R.D. Goldman. 1985. Intermediate filaments and the initiation of desmosome assembly. *J. Cell Biol.* 101:506-517.
- Jones, J.C.R., and K.J. Green. 1991. Intermediate filament-plasma membrane interactions. *Curr. Opin. Cell Biol.* 3:126-131.
- Jou, T.S., D.B. Stewart, J. Stappert, W.J. Nelson, and J.A. Marrs. 1995. Genetic and biochemical dissection of protein linkages in the cadherin-catenin complex. *Proc. Natl. Acad. Sci. USA.* 92:5067-5071.
- Kapprell, H.-P., P. Cowin, W.W. Franke, and H. Poning. 1985. Biochemical characterization of desmosomal proteins isolated from bovine muzzle epidermis: amino acid and carbohydrate composition. *Eur. J. Cell Biol.* 36:217-229.
- Kapprell, H.-P., P. Cowin, and W.W. Franke. 1987. Biochemical characterization of the soluble form of the junctional plaque protein, plakoglobin, from different cell types. *Eur. J. Biochem.* 166:505-517.
- Kelly, D.E. 1966. Fine structure of desmosomes, hemidesmosomes and an aperiodic globular layer in developing newt epidermis. *J. Cell Biol.* 28:51-72.
- Klymkowsky, M.W., R.H. Miller, and E.B. Lane. 1983. Morphology, behavior, and interaction of cultured epithelial cells after the antibody-induced disruption of keratin filament organization. *J. Cell Biol.* 96:494-509.
- Klymkowsky, M.W., D.R. Shook, and L.A. Maynell. 1992. Evidence that the deep keratin filament system of the *Xenopus* embryo acts to ensure normal gastrulation. *Proc. Natl. Acad. Sci. USA.* 89:8736-8740.
- Knudsen, K.A., and M.J. Wheelock. 1992. Plakoglobin, or an 83-kD homologue distinct from beta-catenin interacts with E-cadherin and N-cadherin. *J. Cell Biol.* 118:671-679.
- Koch, P.J., and W.W. Franke. 1994. Desmosomal cadherins: another growing multigene family of adhesion molecules. *Curr. Opin. Cell Biol.* 6:682-687.
- Korman, N.J., R.W. Eyre, V. Klaus-Kovtun, and J.R. Stanley. 1989. Demonstration of an adhering-junction molecule (plakoglobin) in the autoantigens of pemphigus foliaceus and pemphigus vulgaris. *N. Engl. J. Med.* 321:631-635.
- 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.L. 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.
- Luna, E.J., and A.L. Hitt. 1992. Cytoskeleton-plasma membrane interactions. *Science (Wash. DC).* 258:955-964.
- Magee, A.I., and R.S. Buxton. 1991. Transmembrane molecular assemblies regulated by the greater cadherin family. *Curr. Opin. Cell Biol.* 3:854-861.
- 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.
- Mattey, D.L., and D.R. Garrod. 1986. Calcium-induced desmosome formation in cultured kidney epithelial cells. *J. Cell Sci.* 85:95-111.
- McLean, W.H.I., and E.B. Lane. 1995. Intermediate filaments in disease. *Curr. Opin. Cell Biol.* 7:118-125.
- Miller, K., D. Mattey, H. Measures, C. Hopkins, and D. Garrod. 1987. Localisation of the protein and glycoprotein components of bovine nasal epithelial desmosomes by immunoelectron microscopy. *EMBO (Eur. Mol. Biol. Organ.) J.* 6:885-889.
- Moll, R., W.W. Franke, D.L. Schiller, B. Geiger, and R. Krepler. 1982. The catalog of human cytokeratins: patterns of expression in normal epithelia, tumors and cultured cells. *Cell.* 31:11-24.
- Mueller, H., and W.W. Franke. 1983. Biochemical and immunological characterization of desmoplakins I and II, the major polypeptides of the desmosomal plaque. *J. Mol. Biol.* 163:647-671.
- Nathke, I.S., L. Hinck, J.R. Swedlow, J. Papkoff, and W.J. Nelson. 1994. Defining interactions and distributions of cadherin and catenin complexes in polarized epithelial cells. *J. Cell Biol.* 125:1341-1352.
- O'Keefe, E.J., H.P. Erickson, and V. Bennett. 1989. Desmoplakin I and desmoplakin II: Purification and characterization. *J. Biol. Chem.* 264:8310-8318.
- Ouyang, P., and S.P. Sugrue. 1992. Identification of an epithelial protein related to the desmosome and intermediate filament network. *J. Cell Biol.* 118:1477-1488.
- Peifer, M., P.D. McCrea, K.J. Green, E. Wieschaus, and B.M. Gumbiner. 1992. The vertebrate adhesive junction proteins beta-catenin and plakoglobin and the *Drosophila* segment polarity gene armadillo form a multigene family with similar properties. *J. Cell Biol.* 118:681-691.
- Plott, R.T., M. Amagai, M.C. Udey, and J.R. Stanley. 1994. Pemphigus vulgaris antigen lacks biochemical properties characteristic of classical cadherins. *J. Invest. Dermatol.* 103:168-172.
- Roop, D. 1995. Defects in the barrier. *Science (Wash. DC).* 267:474-475.
- Rubinfeld, B., B. Souza, I. Albert, S. Munemitsu, and P. Polakis. 1995. The APC protein and E-cadherin form similar but independent complexes with alpha-catenin, beta-catenin, and plakoglobin. *J. Biol. Chem.* 270:5549-5555.
- Sacco, P.A., T.M. McGranahan, M.J. Wheelock, and K.R. Johnson. 1995. Identification of plakoglobin domains required for association with N-cadherin and alpha-catenin. *J. Biol. Chem.* 270:20201-20206.
- Schmelz, M., and W.W. Franke. 1993. Complexus adherentes, a new group of desmoplakin-containing junctions in endothelial cells: The syndesmosis connecting retothelial cells of lymph nodes. *Eur. J. Cell Biol.* 61:274-289.
- Schmidt, A., H.W. Heid, S. Schafer, U.A. Nuber, R. Zimbelmann, and W.W. Franke. 1994. Desmosomes and cytoskeletal architecture in epithelial differentiation: cell type-specific plaque components and intermediate filament anchorage. *Eur. J. Cell Biol.* 65:229-245.
- Skallii, O., J.C.R. Jones, R. Gagescu, and R.D. Goldman. 1994. IFAP 300 is common to desmosomes and hemidesmosomes and is a possible linker of intermediate filaments to these junctions. *J. Cell Biol.* 125:159-170.
- Skerrow, C.J., and A.G. Matoltsy. 1974. Chemical characterization of isolated epidermal desmosomes. *J. Cell Biol.* 63:524-530.
- Staehein, L.A. 1974. Structure and function of intercellular junctions. *Int. Rev. Cytol.* 39:191-283.
- Stappenbeck, T.S., and K.J. Green. 1992. The desmoplakin carboxyl terminus coaligns with and specifically disrupts intermediate filament networks when expressed in cultured cells. *J. Cell Biol.* 116:1197-1209.
- Stappenbeck, T.S., E.A. Bornslaeger, C.M. Corcoran, H.H. Luu, M.L.A. Virata, and K.J. Green. 1993. Functional analysis of desmoplakin domains: specification of the interaction with keratin versus vimentin intermediate filament networks. *J. Cell Biol.* 123:691-705.
- Stappenbeck, T.S., J.A. Lamb, C.M. Corcoran, and K.J. Green. 1994. Phosphorylation of the desmoplakin COOH terminus negatively regulates its interaction with keratin intermediate filament networks. *J. Biol. Chem.* 269:29351-29354.
- Steinberg, M.S., H. Shida, G.J. Giudice, M. Shida, N.H. Patel, and O.W. Blaschuk. 1987. On the molecular organization, diversity and functions of desmosomal proteins. In *Junctional Complexes of Epithelial Cells*. G. Bock and S. Clark, editors. John Wiley & Sons, Chichester, England. 3-19.
- Steinert, P.M. 1993. Structure, function and dynamics of keratin intermediate filaments. *J. Invest. Dermatol.* 100:729-734.
- Troyanovsky, S.M., R.B. Troyanovsky, L.G. Eshkind, R.E. Leube, and W.W. Franke. 1994. Identification of amino acid sequence motifs in desmocollin, a desmosomal glycoprotein, that are required for plakoglobin binding and plaque formation. *Proc. Natl. Acad. Sci. USA.* 91:10790-10794.
- Tsukita, S., S. Tsukita, A. Nagafuchi, and S. Yonemura. 1992. Molecular linkage between cadherins and actin filaments in cell-cell adherens junctions. *Curr. Opin. Cell Biol.* 4:834-839.
- Vassar, R., P.A. Coulombe, L. Degenstein, K. Albers, and E. Fuchs. 1991. Mutant keratin expression in transgenic mice causes marked abnormalities resembling a human genetic skin disease. *Cell.* 64:365-380.
- Virata, M.L.A., R.M. Wagner, D.A.D. Parry, and K.J. Green. 1992. Molecular structure of the human desmoplakin I and II amino terminus. *Proc. Natl. Acad. Sci. USA.* 89:544-548.
- Wahl, J.K., P.A. Sacco, T.M. McGranahan-Sadler, L.M. Sauppe, M.J. Wheelock, and K.R. Johnson. 1996. Plakoglobin domains that define its association with the desmosomal cadherins and the classical cadherins: identification of unique and shared domains. *J. Cell Sci.* 109:1043-1054.
- Wiche, G. 1989. Plectin: general overview and appraisal of its potential role as a subunit protein of the cytomatrix. *Crit. Rev. Biochem. Mol. Biol.* 24:41-67.
- Witcher, L.L., 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.