Identification of the Plakoglobin-binding Domain in Desmoglein and Its Role in Plaque Assembly and Intermediate Filament Anchorage

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Abstract. The carboxyterminal cytoplasmic portions (tails) of desmosomal cadherins of both the desmoglein (Dsg) and desmocollin type are integral components of the desmosomal plaque and are involved in desmosome assembly and the anchorage of intermediate-sized filaments. When additional Dsg tails were introduced by cDNA transfection into cultured human epithelial cells, in the form of chimeras with the aminoterminal membrane insertion domain of rat connexin32 (Co32), the resulting stably transfected cells showed a dominant-negative defect specific for desmosomal junctions: despite the continual presence of all desmosomal proteins, the endogenous desmosomes disappeared and the formation of Co32–Dsg chimeric gap junctions was inhibited. Using cell transfection in combination with immunoprecipitation techniques, we have examined a series of deletion mutants of the Dsg1 tail in Co32–Dsg chimeras. We show that upon removal of the last 262 amino acids the truncated Dsg tail still effects the binding of plakoglobin but not of detectable amounts of any catenin and induces the dominant-negative phenotype. However, further truncation or excision of the next 41 amino acids, which correspond to the highly conserved carboxyterminus of the C-domain in other cadherins, abolishes plakoglobin binding and allows desmosomes to reform. Therefore, we conclude that this short segment provides a plakoglobin-binding site and is important for plaque assembly and the specific anchorage of either actin filaments in adherens junctions or IFs in desmosomes.

INTERCELLULAR adhering junctions (9) are important structures contributing to stable and positionally ordered cell–cell attachment as well as to the anchorage and specific intracellular arrangement of cytoskeletal filaments and contain calcium-dependent transmembrane cell adhesion molecules, which are members of the cadherin family of proteins. While intercellular contact formation and adhesion is effected by the aminoterminal and N-glycosylated part of the cadherin molecule, the association of cytoskeletal filaments involves the carboxyterminal intracellular portion and is mediated by certain submembranous dense plaques of 10–35 nm thickness. Despite their structural similarities and the fact that all junctions of this group contain a common major plaque protein, plakoglobin, their biochemical composition is markedly different. In epithelial cells, for example, two major types of adhering junctions can be generally distinguished in relation to composition and the specific type of filament attached (4, 6, 8, 16, 20, 47, 52): (*a*) Actin microfilaments anchor at adherens junctions which occur in diverse sizes, shapes, and positions (e.g., *zonula adhaerens, fascia adhaerens*, and *punctum adhaerens*), and contain E- or N-cadherin whose cytoplasmic portion is associated with certain cytoplasmic proteins such as α- and β-catenin, vinculin, α-actinin, and radixin, all of which contribute to plaque formation.

(*b*) Intermediate-sized filaments (IFs)[1] are anchored at desmosomes (*maculae adhaerentes*) of variable sizes (mostly isodiametric and with diameters in the 0.1–5.0 μm range), the plaque of which contains, in addition to the intracellular portions of the desmosome-specific cadherins, i.e., desmocollin(s) (Dsc) and desmoglein(s) (Dsg), a special set of cytoplasmic proteins such as plakoglobin, desmoplakin, and some other cell type-specific proteins (for examples see 16, 19, 37, 44, 47). Moreover, the desmosomal cadherins represent multigene families whose members can be expressed differently in relation to epithelial differentiation pathways (24, 26), and at least three different genes of each of the Dsg

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1. *Abbreviations used in this paper*: aa, amino acid; Co32, connexin 32; Dsc, desmocollin; Dsg, desmoglein; IF, intermediate-sized filament.

(Dsg1–3) and the Dsc (Dsc1–3) proteins have been distinguished in human tissues (for review and nomenclature see 5, 14, 45).

While in the last few years considerable progress has been made in the identification of the major molecular components of the adhering junctions we are still far from understanding the molecular principles and regulatory mechanisms involved in junction formation and in the anchorage of the specific kind of cytoskeletal filaments to the plaque. Immunoprecipitation experiments and transfection studies using cDNAs with deletions have shown that the cytoplasmic portion (tail) of E-cadherin is necessary for the formation and function of the *zonula adhaerens* (34, 35) and is intimately and stably associated with β-catenin and/or, somewhat less stably, with its close relative plakoglobin (for amino acid [aa] sequence relationship see 3, 10, 12, 30–32, 41, 42) as well as with the vinculin-related protein α-catenin (18, 22, 33–36, 38–40, 52, 54).

Even less is known about the interaction of molecules of desmosomal cadherins with desmosomal plaque proteins and IFs. In cell transfection experiments using cDNA constructs encoding chimeric proteins, we have recently shown that the cytoplasmic carboxyterminal segment of desmocollin Dscla is sufficient for the assembly of a specific plaque that contains desmoplakin and plakoglobin and is competent in IF anchorage (51, see also 13). On the other hand, in epithelial cells transfected with cDNA constructs encoding chimeras combining the connexin32 (Co32) transmembrane portion with a Dsg1 tail we noticed a stable dominant-negative effect, resulting in the inhibition of formation of chimeric junctions as well as of all endogenous desmosomes (51).

To find the mechanism(s) responsible for the unexpected, inhibitory and dispersive dominant-negative effect of Dsg1 tails on desmosome assembly and thus on intracellular IF arrangement and to identify the Dsg tail domain(s) involved and potential molecules binding to it, we have extended these experiments by transfections of constructs in which the tail was partially deleted. Here we report the identification of a short Dsg segment involved in plakoglobin binding which is important for desmosome formation.

Materials and Methods

Plasmid Construction

The construction of a plasmid clone encoding a chimeric polypeptide consisting of the four transmembrane domains of rat liver Co32 and the cytoplasmic segment of bovine Dsg 1 has been described (51). Carboxyterminal deletion mutants were produced from the construct in the Bluescript vector (plasmid clone BlCoDg) with the help of conveniently located unique restriction endonuclease sites and subsequent integration of the new gene fragments (flanked 5′ by HindIII and 3′ by the blunt-ended restriction endonuclease site used for truncation) between the HindIII and blunt-ended NarI sites of plasmid derivative Blx which contains the synthetic oligonucleotide (5′-AAGCTTGGAGGCGCCTGACTAGCTAGGATCC-3′; the three stop codons in all three reading frames are underlined) in the polylinker of the Bluescript vector. The following restriction endonucleases were used for truncation: BalI for construction of BlCoDg(209), NdeI for BlCoDg(134).

Some mutants were constructed with the help of polymerase chain reaction (PCR). For construct BlCoDg(168) primers Dg-O-1 (5′-GGAGGCGCCCCTGGCGGCGGG-3′) and Dg-O-2 (5′TTTGGATCCTACAACAGCCCACAGAGCC-3′) were used in PCR from BlCoDg. The NarI/BamHI PCR fragment was subcloned into the NarI/BamHI-digested vector BlCoDg. In this case the TGA sequence localized further downstream within the multiple cloning site of Bluescript was used as a stop codon. Construct BlCoDgd(168–210) was prepared by ligation of the BalI/XbaI fragment encoding the Dsg1 tail (starting from position 210) into the BamHI/XbaI restriction endonuclease sites of BlCoDg(168). For BlCoDgd(32–75) a PCR fragment was amplified using primers Dg-O-1 and Dg-O-5 (5′-TATAGATCTTGCCCTTCCACTGCCCACGAG-3′) and BlCoDg and the resulting fragment was subcloned with the help of NarI and BglII into BlCoDg.

The HindIII/XbaI inserts of all Bluescript clones were further subcloned into the eukaryotic expression vector pHβAP$_r$-1-neo, containing the neomycin resistance gene (cf. 51). In addition, the coding sequence of rat connexin43 (Co43; cf. 1) was amplified from rat heart cDNA using primers 43-O-1 (5′-AAAGTCGACGTGAAAGAGAGGTGCCCA-3′) and 43-O-2 (5′-AAATCTAGATTAAATCTCCAGGTCATC-3′) and cloned into the SalI/XbaI sites of pHβAP$_r$-1-neo. The correct construction of all recombinant plasmids was checked by nucleotide sequencing.

Cell Culture, DNA Transfections, and Cell Coupling Assays

Human vulvar carcinoma A-431 cell lines stably expressing CoDg (B2 and B5) were described previously (51) and in addition new cell lines were selected from A-431 cells transfected as described (cf. 51). Geneticin was added 72 h after transfection at a final concentration of 1 mg/ ml and at least four independent stable cell lines were established for each construct. In transient transfection experiments cells were fixed 72 h after transfection.

Transfected and untransfected cells were assayed for intercellular coupling by following the spreading of dye (Lucifer Yellow) microinjected into individual cells essentially as described (28 and references cited therein).

Immunofluorescence Microscopy

Cells grown on glass coverslips were fixed and permeabilized either with methanol/acetone or with 3% formaldehyde/0.1% saponin as described (cf. 29). For immunostaining the following primary antibodies were used: (*a*) rabbit serum raised against a synthetic peptide corresponding to the cytoplasmic loop of rat liver Co32 (28); (*b*) rabbit serum against the cytoplasmic carboxyterminal peptide of human Co43 (28); (*c*) murine mAb Dg3.10 against bovine Dsg which reacts with a carboxyterminal epitope of bovine and human Dsg (23, 46); (*d*) a mixture of murine mAbs DpI and DpII&2-2.15, 2.17, and 2.19 against desmoplakin (7); (*e*) guinea pig antibodies against bovine desmoplakin (kindly provided by Dr. H. Heid, German Cancer Research Center, Heidelberg, FRG); (*f*) murine mAbs against plakoglobin as the previously described mAb PG 5.1 (8) and mAb 11E4 (generously provided by Dr. Margaret Wheelock, University of Toledo, OH); (*g*) murine mAb lu-5 against cytokeratins (11); (*h*) rabbit antibodies against E-cadherin (kindly provided by Dr. R. Kemler, Max-Planck-Institute, Freiburg i. Br., FRG); and (*i*) murine mAb bVIN-1 against human vinculin (Sigma Chem. Co., St. Louis, MO). The first antibodies were detected by Texas-red conjugated anti-mouse or anti-guinea pig antibodies or by FITC-conjugated anti-rabbit antibodies (Dianova, Hamburg, FRG) and visualized by epifluorescence microscopy.

For staining of actin-containing structures formaldehyde/saponin fixation was used and cells were treated for 20 min with FITC-phalloidin (Sigma Chem. Co.) at a concentration of 10 μg/ml in PBS, washed 5 min in PBS and embedded in elvanol.

Immunoprecipitation and Immunoblotting

Cells were metabolically labeled with [^{35}S]methionine mostly overnight (150 μCi/10-cm Petri dish in medium with reduced methionine), washed in PBS and briefly incubated in hypotonic buffer (10 mM Tris–HCl, pH 7.4, 2 mM DTT, and 20 μM 4-amindinophenyl-methansulfonyl-fluoride [APMSF] with or without 2 mM EDTA and 2 mM EGTA). The suspension was homogenized, centrifuged at 1000 *g* for 5 min, and the supernatant treated with twice concentrated immunoprecipitation lysis buffer (100 mM Tris–HCl, pH 7.5, 2% NP40, 300 mM NaCl, and 20 μM APMSF and 4 mM EDTA), with or without 1% deoxycholate. After centrifugation (20,000 *g*, 10 min) the supernatants were incubated with the appropriate antibodies (overnight at 4°C) and then for 1 h at room temperature with 15 mg protein A–sepharose (Pharmacia P-L Biochemicals Inc., Milwaukee, WI) equilibrated in immunoprecipitation buffer. Beads were then washed five times with PBS, one time with PBS/1% Triton X-100 and the immune complexes were subjected to SDS-PAGE followed either by autoradiographic detection of labeled polypeptides or immunoblot reaction using the alkaline phosphatase system as described (51).

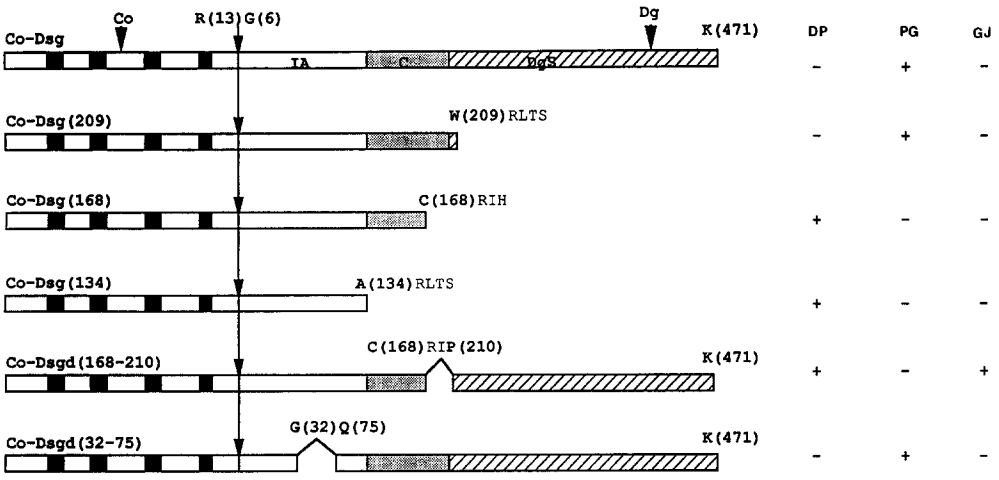

Figure 1. Schematic representation of some of the Co32–Dsg chimeric polypeptides used in the present study and phenotypic characteristics of the human A-431 cells transfected with the corresponding cDNAs. As their aminoterminal part all constructs contained a segment of rat liver Co32 (left of *vertical arrow*) comprising all four transmembrane domains (*black*) and extending to the arginine R(13) located 13 aa positions after the fourth transmembrane region. This residue was directly adjacent to a glycine residue G(6) present in the cytoplasmic tail portions of different Dsg. The complete Dsg tail from G(6) to K(471) was present in construct Co–Dsg, including the intracellular anchor domain (*IA*, *white*), the C-domain (*C*, *stippled*) and the large Dsg-specific extension (*DgS*, *hatched*) whereas various parts of the tail were deleted in the other constructs. aa next to the deleted parts (internal deletions are indicated by *brackets*) are in bold face (giving the relative position from the Dsg transmembrane domain), aa introduced during cloning are also indicated (normal print). The position of the epitopes and major antibody binding sites used for immunological detection are shown by *arrowheads* (*Co* for rabbit antibodies to Co32; *Dg* for murine mAb Dg3.10; 23). Several stably transfected A-431 cell clones synthesizing the specific chimeric polypeptides were analyzed by immunofluorescence microscopy for the presence of desmosomes using desmoplakin antibodies (*DP*), for the colocalization of the Co–Dsg chimeras with plakoglobin (*PG*), and for the capacity of the chimeric proteins to form gap junctions (*GJ*). These results are summarized on the right.

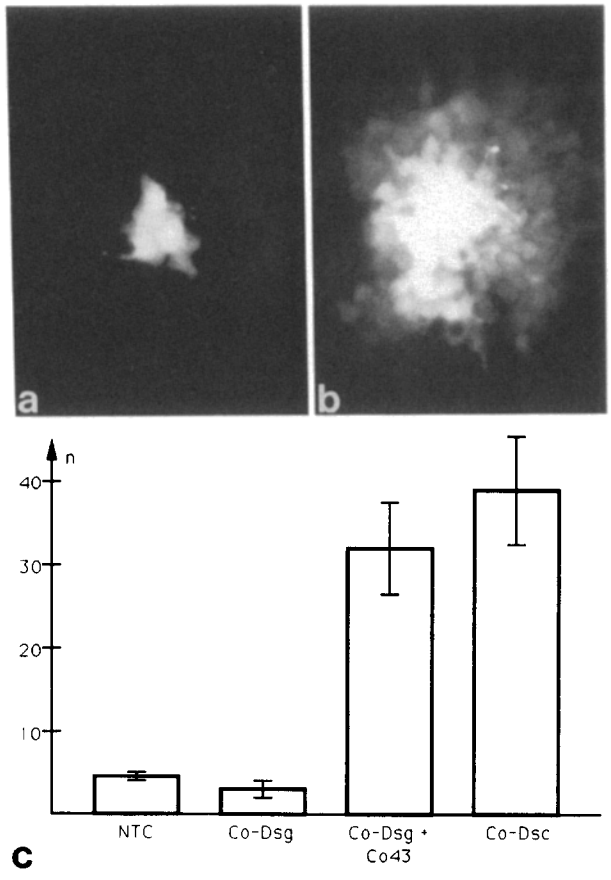

Figure 2. Assays for intercellular coupling and transfer of dye molecules (Lucifer Yellow) microinjected into individual cells and visualized by fluorescence microscopy (*a* and *b*) were evaluated 10 min later by counting the number of positive cells in the neighborhood of the injected cell (at least 10 injections per cell line examined). (*a*) Co–Dsg transfected cells of line B5. (*b*) Cells transfected with cDNAs for both Co–Dsg and Co43. (*c*) Histogram showing the quantitative evaluation (n = number of positive, i.e., coupled cells) for non-transfected A-431 cells (*NTC*), Co-Dsg-transfected cells of line B5, cells double-transfected (B5 cells transfected with cDNA encoding Co43), and Co-Dsc-transfected cells (see 51).

Results

Our observation that the introduction of additional desmoglein (Dsg1) tails in the form of Co–Dsg chimeras resulted, in different cultured epithelial cells, in the disappearance of all desmosomes (51) has led to the working hypothesis that this effect may be due to the depletion of a critical endogenous cytoplasmic molecule that binds to Dsg tails (for N-cadherin see also 21). Therefore, we designed experiments in which the chimera-integrated Dsg1 tails were systematically deleted and mutated (Fig. 1). By studying the synthesis and distribution of desmosomal proteins in A-431 cells transfected with cDNA constructs carrying such deletions and by immunoprecipitation of Dsg complexes we wanted to identify possible partner molecules binding in vivo to certain Dsg segments. In addition, we assayed for cell–cell transfer through the connexons of gap junctions by microinjection of Lucifer Yellow dye and found that Co–Dsg transfected cells were as poorly coupled as normal, i.e., non-transfected A-431 cells whereas cells transfected with desmocollin tail-containing constructs such as Co–Dsc (cf. 51) show markedly increased dye transfer (Fig. 2). All results described in the following have been obtained from stably transfected cell clones.

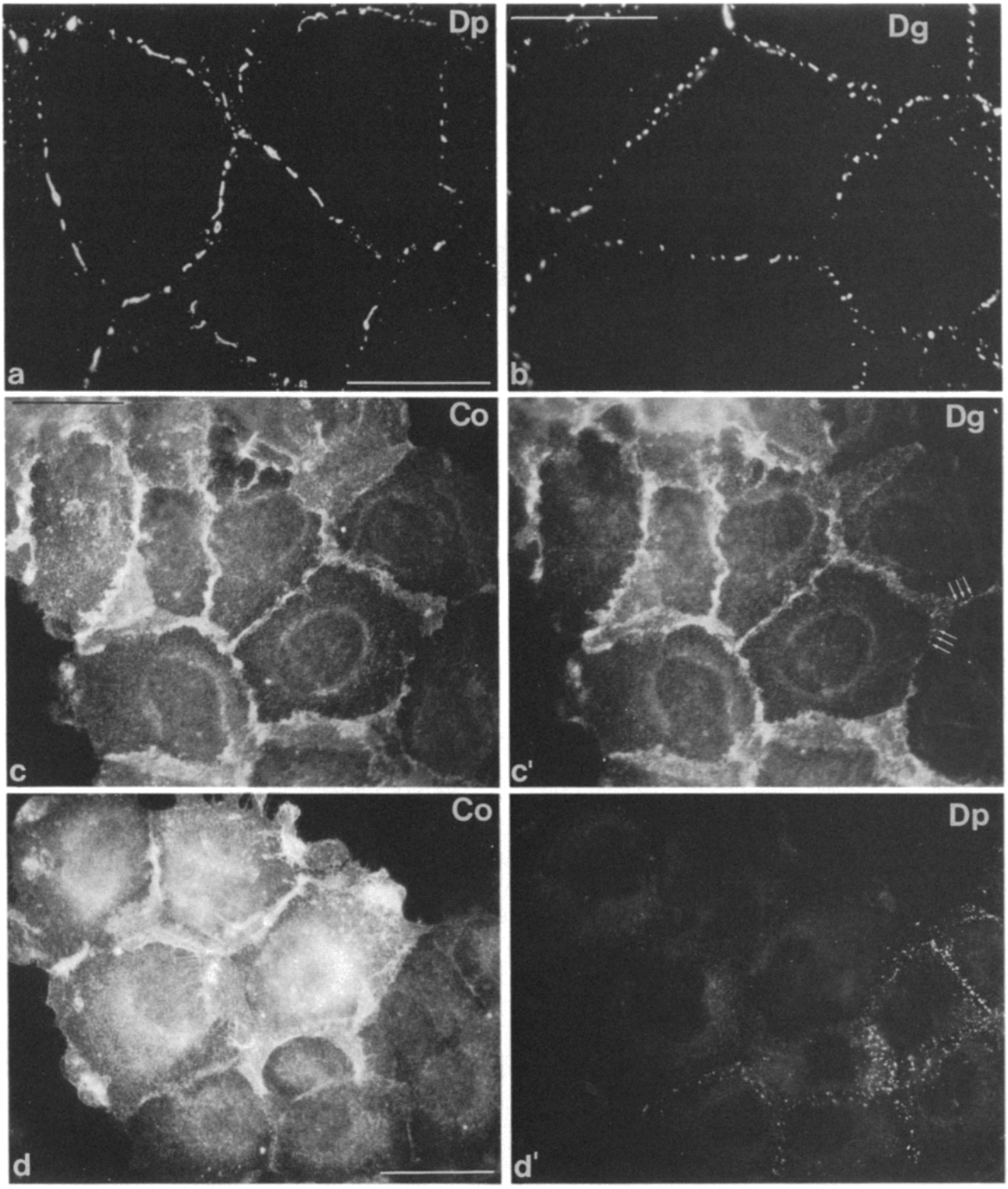

Figure 3. Immunofluorescence microscopy showing the distribution of desmosomal proteins in untransfected human A-431 cells (*a* and *b*) in comparison to A-431 cells transfected with a DNA clone producing the chimeric protein Co–Dsg(209) (*c–d'*). Untransfected cells were reacted with murine mAbs to desmoplakin (*Dp*, *a*) or Dsg (*Dg*, *b*) showing the typical punctate distribution of desmosomes. Transfected A-431 cells shown here were double-stained with rabbit antibodies to Co32 (*Co*, *c* and *d*) and monoclonal antibodies to either desmoglein (*Dg*, *c'*) or desmoplakin (*Dp*, *d'*). Note the absence of dotted arrays of desmosomal staining in most transfected cells, with the exception of a small area with some very small desmosome-like dots in a cluster of cells showing drastically reduced immunofluorescence reaction of the transgene product (*arrows* in *c'* and lower right part of *d* and *d'*). Note also the codistribution of chimera Co–Dsg(209) with the endogenous Dsg2 of A-431 cells, most of which appears to be at the cell surface. The desmoplakin reaction in *d'* is diffusely spread over the cytoplasm in most cells but the fluorescence intensity shown here has been reduced to optimize the appearance of the brilliantly fluorescent recovered desmosomes in the lower right. Bars, 50 μm.

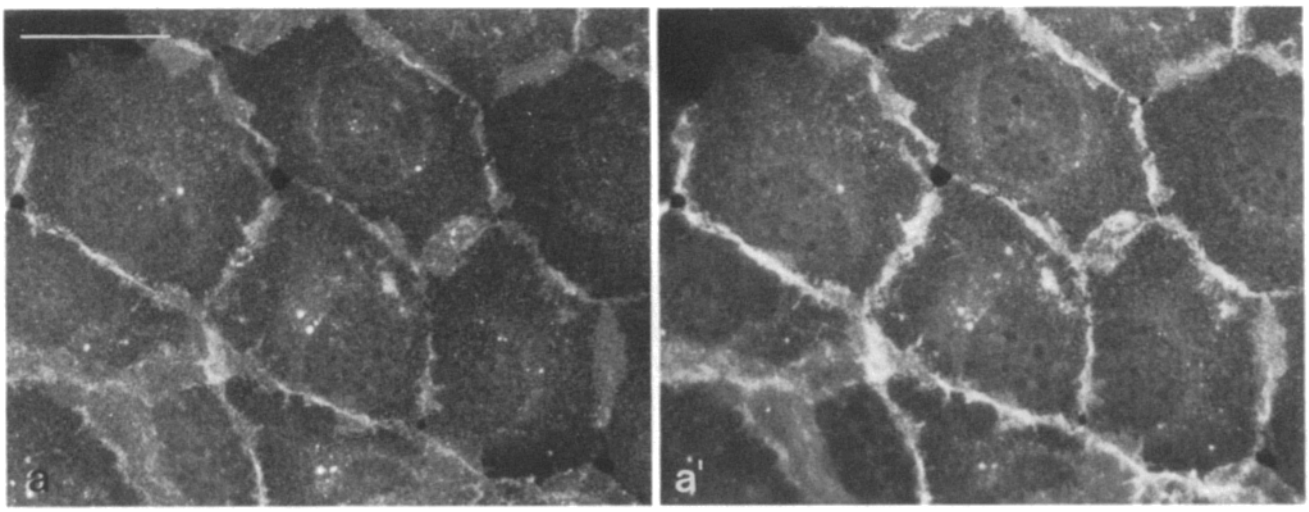

Figure 4. Double label immunofluorescence microscopy of A-431 cells transfected to produce protein Co–Dsg(209) using rabbit antibodies against Co32 (*a*) and monoclonal mouse antibodies against plakoglobin (*a'*) demonstrating the frequent colocalization of both antigens at the cell surface and in intracellular vesicle-like structures. Bar, 50 μm.

In Co–Dsg- and Co–Dsg(209)-expressing Cells Desmosomal Proteins Are Continually Synthesized but Not Assembled into Desmosomes

Recently, we reported a cDNA clone encoding a chimeric protein containing the four transmembrane domains of rat liver Co32 and the entire cytoplasmic tail region of bovine Dsg1. Expression of this clone in the notoriously desmosome-rich human epithelial cells of line A-431 led to two spectacular effects: (*a*) the inability of the synthesized chimeric proteins to assemble into chimeric junctions as, for example, observed for similar constructs with Dscla (51), and (*b*) the disappearance of all the numerous endogenous desmosomes (51).

To study the molecular basis of these effects and the contribution of individual Dsg subdomains we transfected cells with Co–Dsg deletion mutants lacking the codons for the last 262 aa, including the epitope of mAb Dg3.10 (23). Expression of this cloned construct Co–Dsg(209), which encodes a new and much shorter chimeric protein (Fig. 1), also resulted in the disappearance of punctate desmoplakin immunostaining at cell–cell borders and of all desmosomal structures, leaving only a weak and homogenous cytoplasmic fluorescence (data not shown; compare also Fig. 3, *a* and *b* with *d'*). Similar to Co–Dsg, Co–Dsg(209) codistributed with plakoglobin at the cell surface (Fig. 4). The endogenous Dsg, which in this cell line is mostly, if not exclusively Dsg2 (26, 45), was now selectively detected with mAb Dg3.10 and again appeared rather evenly dispersed over the cell surface membrane, particularly along cell–cell contacts (Fig. 3 *c'*). Double label immunofluorescence microscopy showed that this distribution was similar to that of the chimera encoded by Co–Dsg(209), as detected by antibodies specific for rat Co32 (Fig. 3, *c* and *c'*). In addition, some weakly immunostained, dot-like, probably vesicular cytoplasmic structures were also noted at variable frequency. These results indicate that the last 262 aa, with all the three Dsg-specific carboxy-terminal domains, including the repeating units (13, 23), did not contain any information necessary for the negative-dominant effects of desmosome disruption and inhibition of desmosome assembly.

While cells with intense synthesis of the Co–Dsg chimera were totally negative for punctate desmosomal protein immunostaining, small groups of cells that were only weakly positive for Co–Dsg(209) had recovered and showed finely punctate arrays of Dsg and desmoplakin immunostaining along cell–cell borders (compare Fig. 3, *c* and *c'*, *d* and *d'*). Furthermore, Dsg and desmoplakin colocalized in the same small desmosomal dots (not shown) which, however, here appeared to be smaller than those detected in untransfected A-431 cells (compare Fig. 3, *a* and *b* with *c'* and *d'*). This pattern of heterogeneity suggests that low expression of certain Co–Dsg constructs in individual cell colonies allows the recovery of desmosomal structures.

To examine whether the observed changes of desmosome assembly and of desmoplakin staining were due to alterations in the expression and/or the stability of the proteins involved, we performed comparative immunoblot analyses of total cellular proteins (Fig. 5). These results showed that in steady state culture conditions the total amounts and the sizes of the desmosomal proteins were not considerably affected and that neither down-regulation of expression nor degradation of protein had taken place (Fig. 5, *a* and *b*, show examples for Dsg and desmoplakin). We have previously

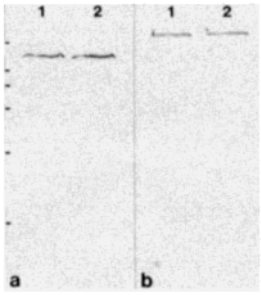

Figure 5. Immunoblot analysis of untransfected A-431 cells (lane *1*) and cells transfected (lane *2*) to express Co–Dsg(209). Equal amounts of protein from total cell lysates were separated by SDS-PAGE, transferred to nitrocellulose, and reacted with monoclonal antibodies to Dsg (*a*) or desmoplakin (*b*). Note the similar intensity of the alkaline phosphatase reaction in the untransfected and the transfected cells. Bars on the left margin denote the relative positions of coelectrophoresed reference proteins (from top to bottom: myosin, 205,000 M_r; β-galactosidase, 116,000 M_r; phosphorylase b, 97,400 M_r; BSA, 67,000 M_r; ovalbumin, 45,000 M_r; carbonic anhydrase, 29,000 M_r).

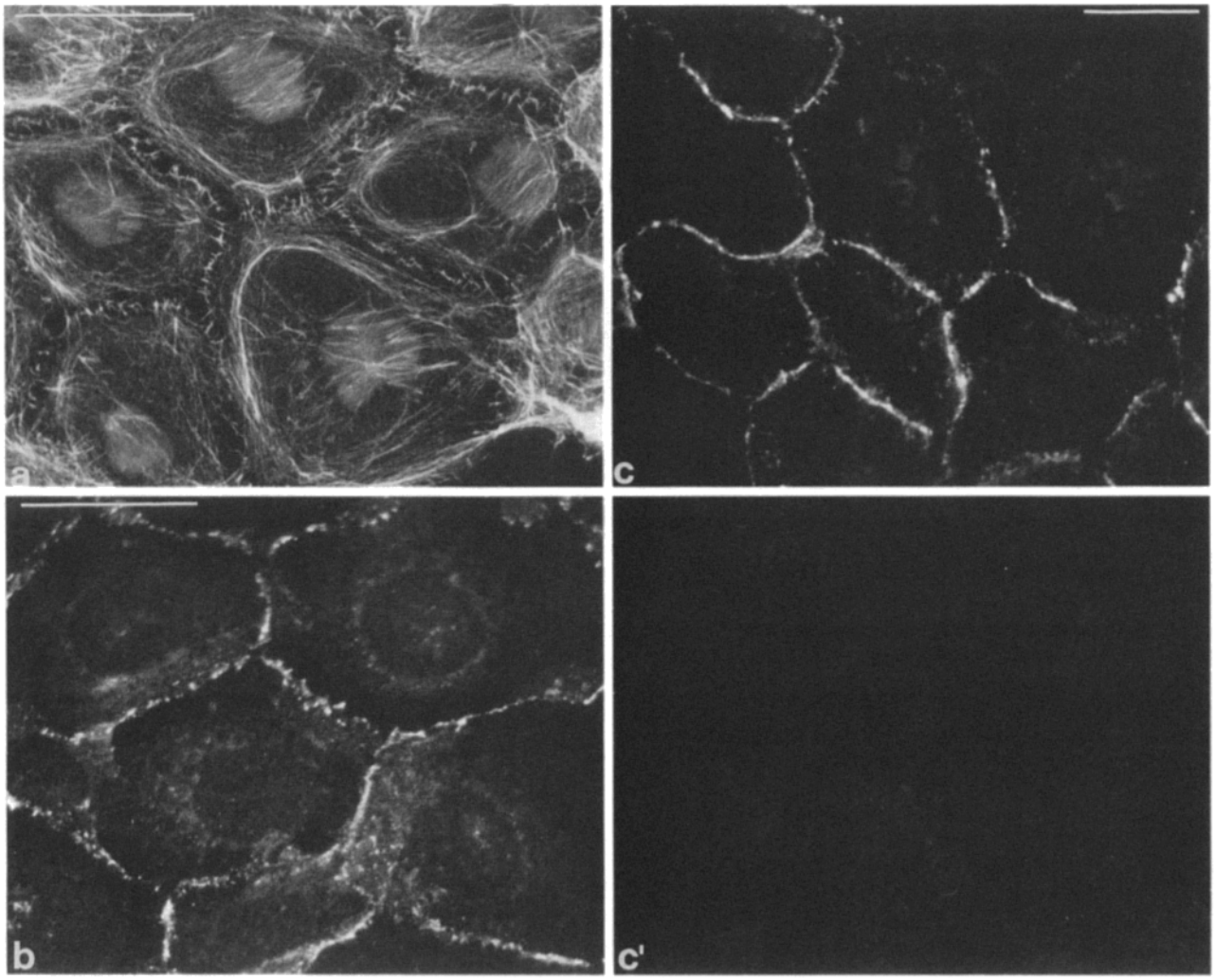

Figure 6. Fluorescence microscopy of A-431 cells (clone B5) transfected to produce chimeric protein Co–Dsg, comparing the distribution of actin-containing filaments (*a*), visualized with FITC-labeled phalloidin, and of E-cadherin (*b* and *c*) with that of desmoplakin as a marker of desmosomes (*c'* shows the same field as *c*) visualized by immunofluorescence using rabbit antibodies (*b* and *c*) or, in double-label immunofluorescence, with a monoclonal murine antibody (*c'*). Two large and representative fields are shown in *b* and *c*, presenting extended membrane staining mostly in small distinct junctions of the *punctum adhaerens* type, in the absence of desmosomes (compare *c* and *c'*). Note also the typical cable arrays of actin filament bundles in *a*, with apparent anchorage sites at cell contact points identifiable by E-cadherin antibodies (as in *b* and *c*). Bars, 50 μm.

shown that in these cells the amounts of the chimeric Co–Dsg protein and the endogenous Dsg were similar (51).

Co-Dsg Chimeras Disrupt Desmosomes Selectively

We then examined whether the expression of the Co–Dsg chimeras and the disappearance of all desmosomes had any effect on the assembly of other cell contact structures, particularly the actin-filament anchoring adhering junctions containing E-cadherin and vinculin. Immunofluorescence microscopy using antibodies to actin, E-cadherin and vinculin showed these proteins in their characteristic localizations, indistinguishable from those seen in nontransfected A-431 cells (Fig. 6). Many of the actin filament cables were intimately associated with plasma membrane attachment structures in regions of cell–cell contact (Fig. 6 *a*) which in turn were strongly positive for E-cadherin (Fig. 6, *b* and *c*) and vinculin (not shown). Double-label immunolocalization revealed the specific effect of transfections with Co–Dsg and Co–Dsg(209) with particular clarity (Fig. 6, *c* and *c'*): Adherens junctions, as identified by the presence of E-cadherin, are present (Fig. 6 *c*), whereas desmosomes are totally absent (Fig. 6 *c'*).

The level of connexin(s) in untransfected A-431 cells and in A-431 cells expressing Co–Dsg (clone B5) was below the level of detection with antibodies against human Co32 and Co43. We therefore transfected B5 cells with a Co43-encoding gene construct. Immunofluorescence microscopy of selected double-transfected cell clones showed the efficient incorporation of both transgene products: as in B5 cells, Co–Dsg was rather evenly distributed over the plasma membrane but Co43 now formed large gap junctions, many of which were negative for Co–Dsg (results not shown), resulting in effective cell-to-cell coupling (Fig. 2). In parallel cotransfection experiments, Co43 colocalized well with the connexin-desmocollin chimera Co–Dsc (see 51).

Although the assembly of connexons is still poorly under-

stood, our data suggest that the Dsg1 tail in Co–Dsg, but not that of Dsc in Co–Dsc chimeras (51), interferes with the ability of the transmembrane part of this connexin to cluster into paracrystalline gap junctions and also prevents the integration of these chimeras into preexisting gap junctions.

The C Domain of the Dsg Tail Is Responsible for Desmosome Disruption

To understand which part of the Dsg tail is important for desmosome disruption we prepared several deletion mutants (Fig. 1). As shown, mutant Co–Dsg(209) lacking the Dsg-specific end domains of 262 aa is still able to disrupt desmosomes. In contrast, neither chimera Co–Dsg(168) which lacks further 41 aa representing the end of the C-domain, nor mutant Co–Dsg(134) lacking the entire C-domain affected desmosome formation, as determined by immunolocalization with desmoplakin and Dsg antibodies in transfected cell lines (Fig. 7). While in some places the Co–Dsg chimeric proteins showed colocalization with plakoglobin we also encountered many plasma membrane regions that were strongly positive for plakoglobin (Fig. 7 *a'*) but negative for Co32 (Fig. 7 *a*).

To test whether this short, 41-aa-long segment of the C-domain is sufficient for desmosome destruction we constructed mutant Co–Dsgd(168–210), with an internal deletion of these aa (Fig. 1). The expression of this chimera did not disrupt desmosomes (Fig. 8). Surprisingly, however, protein Co–Dsgd(168–210) was not only transported to the cell surface but formed gap junction-like clusters (Fig. 8, *a–c*) which, however, were not stained by desmoplakin (Fig. 8 *a'*) and plakoglobin (Fig. 8 *b'*) and did not appear to anchor cytokeratin fibrils (Fig. 8 *c'*). The specificity of this effect was also demonstrated by the introduction of another internal deletion mutant (construct CoDsgd(32–75); cf. Fig. 1) into A-431 cells which induced the same desmosome-negative phenotype as chimera Co–Dsg (not shown).

Plakoglobin Can Bind to the C Domain of Dsg

To determine which polypeptide(s) directly interact with the cytoplasmic Dsgl-tail, in particular with the 41 aa of the C-domain, we performed a series of immunoprecipitation experiments. Using Co32 antibodies, we were, for example, able to precipitate protein Co–Dsg from metabolically labeled A-431 cells of clone B5, as seen by autoradiography (Fig. 9 *a*) and by immunoblot analyses using Dsg and Co32 antibodies (not shown). In these experiments we consistently and specifically observed coimmunoprecipitation of only one additional polypeptide of molecular mass ~83 kD together with Co–Dsg, independent from the specific calcium and desoxycholate addition (Fig. 9 *b*). Immunoblot analysis with mAb PG5.1 showed that this polypeptide was plakoglobin (Fig. 9 *c*). An identical reaction was obtained with mAb 11E4 which recognizes a different plakoglobin epitope (not shown; see also Materials and Methods). Under the same conditions E-cadherin antibodies co-precipitated two major proteins of 90 and 100 kD, i.e., α- and β-catenin, and a miniscule amount of plakoglobin (Fig. 9 *b*), confirming and extending the data of previous authors (e.g., 22, 34, 39; see also Introduction).

Immunoprecipitation experiments in which the different Co32–Dsg1 mutant chimeras were compared (Fig. 9 *c*)

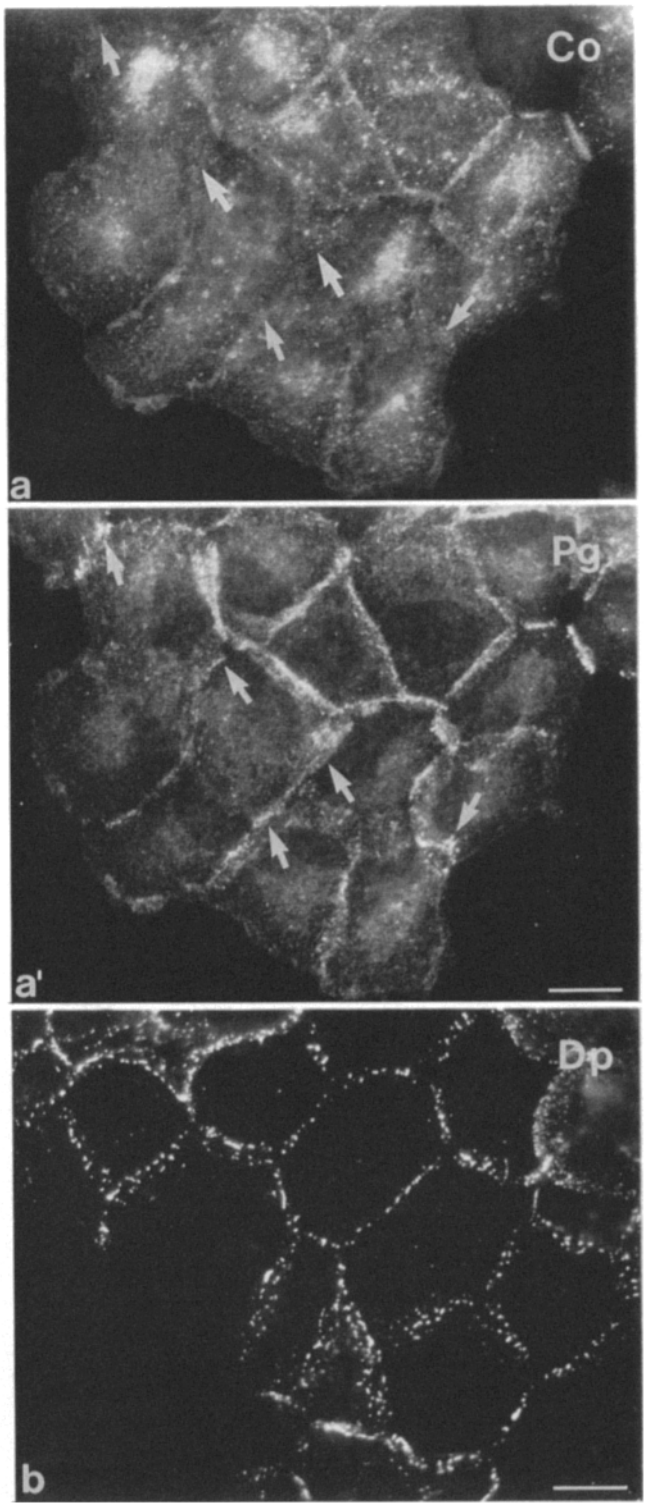

Figure 7. Immunofluorescence micrographs of transfected A-431 cells of subclone 168-a expressing the chimeric tail deletion protein Co–Dsg(168). Co–Dsg(168) as detected by rabbit antibodies to Co32 (*Co*) appears at cell surface sites or in small intracellular aggregates (*a*) which are not stained by monoclonal plakoglobin antibodies (*a'*). Notably, the plasma membrane localization of both proteins differs significantly (*arrows*) although Co–Dsg(168) can be transported to and detected at the cell surface. Immunofluorescence microscopy with monoclonal desmoplakin antibodies (*b*) demonstrates that these cells contain normal-appearing desmosomes. Bars, 50 μm.

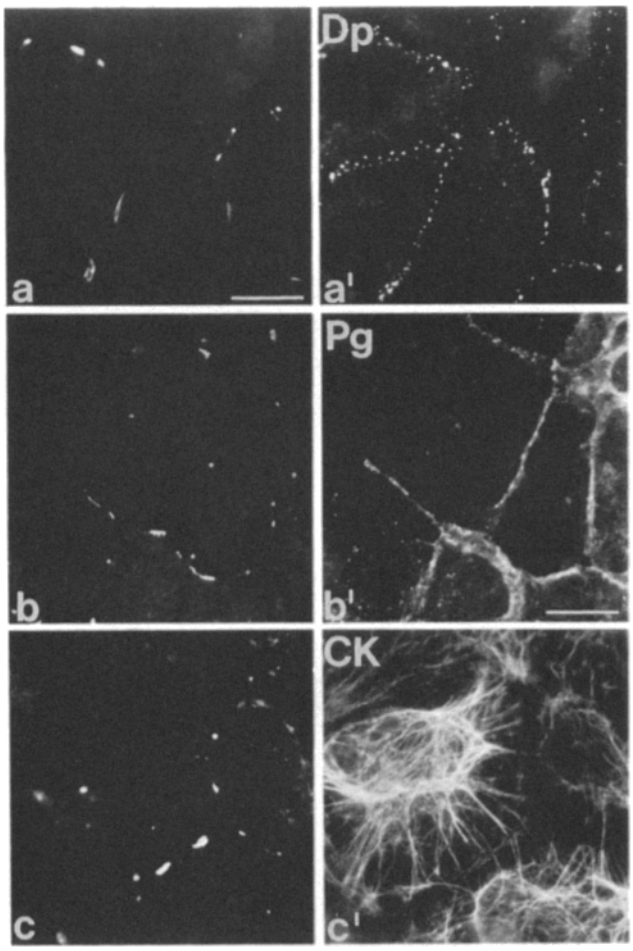

Figure 8. Double label immunofluorescence microscopy of A-431 cells producing the chimeric deletion protein Co-Dsgd(168–210). The left panel (*a–c*) shows the distribution of the mutant deletion protein, reacted with rabbit Co32 antibodies, in comparison with structures immunostained with mAbs to desmoplakin (*a'*, *Dp*), plakoglobin (*b'*, *Pg*) or cytokeratins (*c'*, *CK*). Although Co-Dsgd-(168–210) can form gap junction-like plaque structures these are not codistributed with desmosomeal proteins and do not appear as specific sites of intermediate filament anchoring. Bars, 50 μm.

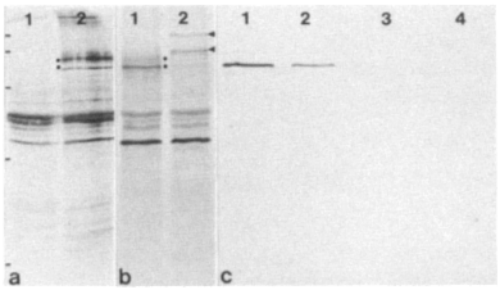

Figure 9. Autoradiograms (*a* and *b*) and immunoblot reaction (*c*) of immunoprecipitates from transfected A-431 cells. Immunoprecipitates were separated by SDS-PAGE (relative positions of marker proteins are mostly as in Fig. 3 and indicated on the left margin: β-galactosidase, phosphorylase b, BSA, ovalbumin, and carbonic anhydrase). (*a*) Immunoprecipitates from Co-Dsg-producing cells (clone B5) obtained with rabbit antibodies against Co43 (lane *1*) or Co32 (lane *2*). Note that a specific Co-Dsg band is only seen in lane *2* and that this also contains plakoglobin (these two polypeptides are indicated by *dots*: Co-Dsg, *top*, plakoglobin, *bottom*). (*b*) Immunoprecipitates from A-431 cells (clone B5) transfected to express chimera Co-Dsg, using rabbit antibodies to rat Co32 (lane *1*) or to E-cadherin (lane *2*). Note coprecipitation (*arrowheads*) of Co-Dsg (*top*) with plakoglobin (*bottom*), in contrast to the coprecipitation of E-cadherin (*top*) with catenin(s) (*bottom*) and a very low amount of plakoglobin. (*c*) Immunoblot reaction of murine monoclonal plakoglobin antibody with proteins in immunoprecipitates obtained with rabbit antibodies against Co32 from A-431 cell lines stably expressing Co-Dsg (lane *1*), Co-Dsgd(209) (lane *2*), Co-Dsgd(168) (lane *3*) and Co-Dsgd(168–210) (lane *4*). Immunoblot reaction, visualized by the alkaline phosphatase detection system, is only seen in lanes *1* and *2*.

demonstrated that Co32 antibodies also precipitated chimera Co-Dsg(209) in association with plakoglobin, whereas plakoglobin was not detected in association with immunoprecipitated chimeric proteins Co-Dsg(168) and Co-Dsgd-(168–210). This was in agreement with our double label immunofluorescence experiments in which plakoglobin colocalized with Co-Dsg and Co-Dsg(209) but not with Co-Dsg(168) or Co-Dsgd(168–210), indicating that the last 41 aa of the C-domain contribute significantly to plakoglobin binding.

Discussion

Desmoglein Dsg1 binds plakoglobin (27, 42, this study). Our detailed mutational analysis now has localized the Dsg1 tail region that is responsible, in transfected cells, for the dominant-negative effect on desmosome formation and stability to a relatively short segment of 41 aa at the end of the intracellular cadherin-specific domain (ICS domain; cf. 23, 45; C-domain in Fig. 1) which is 285 aa away from the carboxyterminus of this protein. This region is homologous to the carboxyterminal domain characteristic of the much shorter desmocollins Dscl-3 type a and the classical cadherins, is most conserved in aa sequence between the diverse cadherins (Fig. 10) (cf. 15, 17, 23–26, 45, 49) and has been shown in E-cadherin to be contained in the region responsible for the binding of plakoglobin and/or the catenins and thus, directly or indirectly, for the specific anchoring of actin microfilaments (3, 18, 20, 22, 30–36, 38–41, 52, 54). The binding of catenins and plakoglobin to other cadherins such as N-cadherin also takes place at the C-domain (cf. 18, 22, 38, see also 21). In a different study, we have further shown that it is this C-domain which in Dsc is needed for the binding of plakoglobin, notably in the central part containing the repeating units (cf. 10, 12, 41), and the assembly of a plaque competent in the specific anchorage of IFs (51, Troyanovsky, S. M., R. B. Troyanovsky, L. G. Eshkind, R. E. Leube, and W. W. Franke, manuscript in preparation). We conclude that the terminal section of the C-domain contains both information common to different cadherins such as that for the binding of plakoglobin and distinguishing information such as that for the exclusion of catenins from desmosomal cadherins and for the specific interaction with other components of the desmosomal plaque.

Considering the high sequence homology in the C-domain of the diverse cadherins, including members of the Dsc and Dsg subfamilies (Fig. 10), it is remarkable to note that this region displays discrete differences in its interaction with

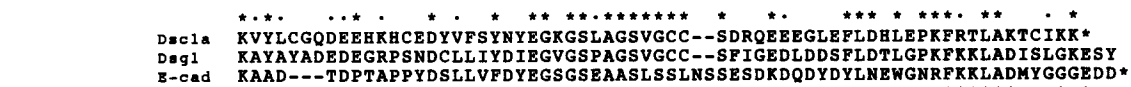

Figure 10. Comparison of the amino acid sequences (one letter code) of the carboxyterminal portion of the tail domains of human desmocollin Dscla (*Dscla*; from reference 50), desmoglein 1 (*Dsgl*; from reference 53) and E-cadherin (*E-cad*; from reference 2) in an optimized alignment to show amino acids identical (*asterisks*) or conservatively exchanged (*dots*) between Dsgl and Dscla (*top*) or Dsgl and E-cadherin (*bottom*).

various members of the plakoglobin/β-catenin/*armadillo* gene product family of proteins: in E-cadherin and some other classical cadherins it can bind β-catenin and/or—although with seemingly lesser stability—plakoglobin (22, 30–32, 42), whereas in desmogleins (this study, 42) and desmocollins (Troyanovsky, S. M., R. B. Troyanovsky, L. G. Eshkind, R. E. Leube, and W. W. Franke, manuscript in preparation) it seems to bind preferentially plakoglobin. A third and even different binding pattern is observed in the APC tumor suppressor gene product of colorectal carcinomas which apparently binds effectively β-catenin (43, 48).

In addition to the C-domain, other regions of the Dsg tail have also a marked influence on the topogenic behavior of the Co–Dsg chimeras. For example, although both C-domain–deficient chimeras Co–Dsg(168) and Co–Dsgd(168–210) cannot bind plakoglobin, the latter protein forms gap junction-like structures in the transfected cells whereas the former does not. A possible explanation might be that the large Dsg-specific domain of 261 aa contributes to the intramembranous lateral assembly of complex, i.e., gap junction-like structures whereas the extensively truncated forms are unable to cluster in this specific way.

The mechanism by which the introduction of Dsg tail domains interferes so dramatically with desmosome assembly and the arrangement of the IF system is still unknown. We can exclude, however, several simple explanations. Clearly, the synthesis of all major desmosomal proteins continues in the cells suffering from the dominant-negative effect, and the resulting proteins are sufficiently stable and correctly inserted into the plasma membrane where they tend to concentrate at cell–cell boundaries but do not aggregate in the form of junctions. Both the normal endogenous desmosomal proteins as well as the chimeric proteins are also not enriched in endocytotically derived vesicles as it has been shown for epithelial cells uncoupled in the presence of low calcium concentrations (for references see 6, 16, 47).

In our previous paper (51) we have mentioned the depletion by competition hypothesis that also appeared attractive to Kintner (21) as an explanation for his results with various deletion forms of N-cadherin in *Xenopus laevis* embryos. Our results showing that the inhibition phenotype correlates with plakoglobin binding are obviously compatible with the hypothesis that the plakoglobin concentration in the cytoplasm and/or at the plasma membrane is critical so that the entrapment of some of it by the extra Dsg tail segments introduced would generally disturb the equilibrium with plakoglobin bound to E-cadherin and the desmosomal cadherins. However, there are still other arguments questioning this explanation and alternative explanations have been discussed elsewhere (51). We are currently analyzing in greater detail the complexes of endogenous Dsg and its mutants that form in the transfected cell and also try to determine the stereochemistry and relative binding affinities of these molecules in vitro.

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Note Added in Proof. After acceptance of this manuscript we learned of an article reporting plakoglobin binding in vitro to the same region of desmoglein that we have identified in living cells in the present paper: 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.

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