

Direct Ca²⁺-dependent Heterophilic Interaction between Desmosomal Cadherins, Desmoglein and Desmocollin, Contributes to Cell–Cell Adhesion

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Abstract. Human fibrosarcoma cells, HT-1080, feature extensive adherens junctions, lack mature desmosomes, and express a single known desmosomal protein, Desmoglein 2 (Dsg2). Transfection of these cells with bovine Desmocollin 1a (Dsc1a) caused dramatic changes in the subcellular distribution of endogenous Dsg2. Both cadherins clustered in the areas of the adherens junctions, whereas only a minor portion of Dsg2 was seen in these areas in the parental cells. Deletion mapping showed that intact extracellular cadherin-like repeats of Dsc1a (Arg¹-Thr¹⁷⁰) are required for the translocation of Dsg2. Deletion of the intracellular C-domain that mediates the interaction of Dsc1a with plakoglobin, or the CSI region that is involved in the binding to desmoplakin, had no effect. Coimmunoprecipitation experiments of cell lysates stably expressing

Dsc1a with anti-Dsc or -Dsg antibodies demonstrate that the desmosomal cadherins, Dsg2 and Dsc1a, are involved in a direct Ca²⁺-dependent interaction. This conclusion was further supported by the results of solid phase binding experiments. These showed that the Dsc1a fragment containing cadherin-like repeats 1 and 2 binds directly to the extracellular portion of Dsg in a Ca²⁺-dependent manner. The contribution of the Dsg/Dsc interaction to cell–cell adhesion was tested by coculturing HT-1080 cells expressing Dsc1a with HT-1080 cells lacking Dsc but expressing myc-tagged plakoglobin (MPg). In the latter cells, MPg and the endogenous Dsg form stable complexes. The observed specific coimmunoprecipitation of MPg by anti-Dsc antibodies in coculture indicates that an intercellular interaction between Dsc1 and Dsg is involved in cell–cell adhesion.

STRUCTURALLY related desmosomes and adherens junctions, collectively termed adhering junctions, are involved in anchoring the cytoskeleton to the plasma membrane, intercellular cell type-specific adhesion, and signaling (Geiger and Ayalon, 1992; Schmidt et al., 1994; Klymkowsky and Parr, 1995; Peifer, 1995; Cowin and Burke, 1996; Gumbiner, 1996). Classic and desmosomal cadherins are featured in both adherens and desmosome junctions. It is widely accepted that classic cadherins mediate homophilic calcium-dependent cell–cell adhesion (Nose et al., 1990; Grunwald, 1993; Shapiro et al., 1995; Briehner et al., 1996; Nagar et al., 1996). An exception to this rule, heterophilic binding of the chicken B-cadherin to LCAM, has been documented (Murphy-Erdosh et al., 1995). On the intracellular face of the plasma membrane, cadherins are integrated into plaques consisting of junctional-specific proteins. These proteins function in the formation of anchoring sites for microfilaments and intermediate filaments (in adherens junctions and desmosomes, respectively) and are critical for adhesion and signaling properties of cadherins (Nagafuchi and Takeichi, 1988;

Ozawa et al., 1989; Green and Jones, 1990; Geiger and Ayalon, 1992; Schmidt et al., 1994).

In contrast with adherens junctions that may contain only one cadherin isoform, desmosomes always include cadherins from two subfamilies, desmogleins (Dsg1–3)¹ and desmocollins (Dsc1–3). Alternative splicing increases Dsc diversity producing long (Dsc a) and short (Dsc b) isoforms that differ in their intracellular domains (Garrod, 1993; Koch and Franke, 1994). Recent experiments with chimeric proteins consisting of the gap junction protein connexin32 and the intracellular regions of desmosome cadherins indicate that Dsg and Dsc have different functions. The CoDsc chimera containing the intracellular portion of Dsc1a nucleated the formation of the intracellular desmosomal plaques. The cytoplasmic domain of Dsg1, in a similar construct, displayed a dominant negative effect on desmosome formation (Troyanovsky et al., 1993, 1994a,b).

Involvement of the desmosomal cadherins in cell–cell adhesion was underscored by cell culture observations that antibodies against the extracellular regions of Dsc interfered with the formation of the epithelial sheet (Cowin et al., 1984). Also, auto anti-Dsg antibodies caused a blistering skin disease (Stanley, 1995). Perturbation in epidermal

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1. *Abbreviations used in this paper:* Dsc, desmocollin; Dsg, desmoglein; MPg, myc-tagged plakoglobin.

cell–cell interactions was found in transgenic animals producing a dominant negative form of Dsg (Allen et al., 1996). Structural similarity between the extracellular repeats of Dsg and Dsc with those of the classic cadherins involved in homophilic adhesion provides additional support for the adhesive functions of the desmosomal cadherins. However, in contrast with classic cadherins, desmosomal cadherins, alone or in combination, failed to support cell–cell adhesion upon expression in nonadhesive fibroblast-like cells (Amagai et al., 1994; Chidgey et al., 1996; Kowalczyk et al., 1996). This suggests that the functional properties of classic and desmosomal cadherins are distinct despite their overall structural homology. Moreover, the molecular mechanism of coassociation of the different desmosomal cadherins in the actual desmosome is not well understood. While direct interactions between desmosomal cadherins were not documented, it seems likely that they are essential for desmosome assembly.

Several observations suggest that efficient desmosome formation, and hence interactions between desmosomal cadherins, may require the function of the classic cadherins (Wheelock and Jensen, 1992; Lewis et al., 1994; Amagai et al., 1995). To investigate this possibility, we expressed bovine Dsc1a in HT-1080 cells that feature extensive adherens junctions and produce endogenous Dsg2. We found that expression of Dsc1a in these cells induces the formation of stable complexes between Dsc and Dsg. In addition, we show a direct Ca^{2+} -dependent interaction between the extracellular regions of two desmosomal cadherins. These observations suggest that heterophilic interactions between desmosomal cadherins are important for targeting these proteins to desmosomes and for cell–cell adhesion.

Materials and Methods

Plasmid Construction

The plasmid BIDc1a encoding the entire sequence of the mature bovine Dsc1a isoform was obtained by subsequent insertions in the Bluescript vector of the XhoI–NotI and XhoI–KpnI inserts cut out from the plasmids BDC 7.5 and BDC 6.1, respectively (Koch et al., 1991). The sequence coding for the leader peptide lacking in BIDc1a was amplified from plasmid pDGK5(B), encoding bovine Dsg1 (Koch et al., 1990). It was inserted upstream to the Dsc1a sequence in BIDc1a between the unique HindIII and NarI sites, resulting in plasmid BILDc1a. The fusion protein encoded by this plasmid contains the entire sequence of the Dsg-derived leader peptide and the entire sequence of the mature Dsc1a. In Dsc1a only the first amino acid residue Arg is replaced with Glu to create a Dsg-specific processing site. The same pDGK5(B) plasmid was used for construction of BIDc(1–170Dg) that contains the coding sequence for the leader peptide and for the Glu¹-Arg¹⁷⁰ sequence of the mature Dsg1 preceding the corresponding sequence of Dsc1a. Deletion mutagenesis for constructing BIDcΔ(697–761) and BIDcΔ(597–609) was done using PCR-mediated site-directed mutagenesis. BIMPg, containing the entire sequence of human plakoglobin tagged on 5' end by 6myc epitope, was constructed using a plasmid HPG Ca 2.1 (Franke et al., 1989) and a plasmid CS26MT provided by Dr. R. Kopan (Washington University, St. Louis, MO). The HindIII–XbaI inserts of all Bluescript subclones were further subcloned into the eukaryotic expression vector pBEHpac18 (Horst et al., 1991) containing the puromycin resistance gene and SV-40 early promoter element.

To express Dsg and Dsc extracellular fragments Dc12M (Arg¹-Asp²¹²), Dg12F (Glu¹-Asp²¹²), and Dg123F (Glu¹-Val³³⁵), in *Escherichia coli*, the QIA-expression system (Qiagen, Chatsworth, CA) was used. The corresponding sequences of the bovine Dsg1 and Dsc1a were amplified, ligated either with single myc or flag (Sigma Chemical Co., St. Louis, MO) sequences, and then inserted in a pQE18 vector. Correct amplification and cloning of all recombinant plasmids was checked by restriction endonu-

lease mapping and nucleotide sequencing. Plasmid CMVSpPg encoding a chimeric protein consisting of the entire synaptophysin and plakoglobin has been described (Chitaev et al., 1996).

Cell Culture, DNA Transfection, and Immunological Methods

The HT-1080 human fibrosarcoma cells were provided by Dr. G. Goldberg (Washington University, St. Louis, MO). The cells were grown in DME (GIBCO BRL, Gaithersburg, MD) supplemented with 10% FCS. Transfection of HT-1080 cells, as well as the selection, growth, immunofluorescence microscopy, and immunoprecipitation, was done as described for A-431 cells (Trojanovsky et al., 1993; Chitaev et al., 1996). The following primary antibodies were used: (a) rabbit polyclonal antibodies against human Dsc2 and Dsg2 (Demlehner et al., 1996); (b) monoclonal murine U114 antibodies against human Dsc3 (Nuber et al., 1996); (c) mAbs Dsg2E-G129 specific for human Dsg2 (Schafer et al., 1996); (d) mAbs 2.10 against bovine Dsc1 (this antibody cross-reacts with human Dsc1); (e) 3.10 specific for human Dsg1 and Dsg2 (Schafer et al., 1996); (f) 5.1 against human plakoglobin and a mixture of antibodies 2.15, 2.17, and 2.19 against desmoplakin (for references describing these antibodies, see Trojanovsky et al., 1994a); (g) rabbit pan-cadherin antibody and mAbs GC-4 against N-cadherin, M2 against FLAG, and 9E10 against myc epitopes (Sigma Chemical Co.); (h) murine mAb against β-catenin (Transduction Laboratories, Lexington, KY); (i) rabbit anti-synaptophysin antibody (DAKO, Hamburg, Germany). For immunoprecipitation 75 μl of supernatants containing 3.10 or 2.10 antibodies was added to each sample. Lysates were precleared by centrifugation at 100,000 g for 1 h before immunoprecipitation.

Solid Phase and Reconstitution Assays

The in vitro solid phase assay was described previously (Chitaev et al., 1996). In brief, Dsg fragments isolated as described previously (Chitaev et al., 1996) were diluted in loading buffer (20 mM Tris HCl, pH 7.8, 1 mM DTT with or without 2 mM EDTA) and immobilized on a 96-well dish and incubated with increasing amounts of Dc12M. Binding was detected by an ELISA assay with myc 9E10 mAb. The solid phase assay was always performed in the absence or presence of 2 mM EDTA added in each solution of the binding assay. This EDTA concentration did not change the affinity of the 9E10 antibody to the myc epitope, as shown by direct ELISA assay.

For the reconstitution assay the Dc12M fragment was mixed with the Dsg fragments, Dg12F, or Dg123F in 1.5 ml PBS in final concentration of 1 μg/ml. For control, Dc12M was not added. Samples were incubated 15 min, and then subsequently treated with 75 μl 9E10 anti-myc antibodies and with 15 μg protein A–Sepharose (Pharmacia, Piscataway, NJ) suspended in PBS. The beads were then washed five times with PBS supplemented with 1% Triton X-100. The immunoprecipitates were analyzed by immunoblotting with different primary antibodies in conjunction with an enhanced chemiluminescence detection system (Boehringer Mannheim Biochemicals, Indianapolis, IN).

Results

Expression of Desmocollin 1a in Human Fibrosarcoma HT-1080 Cells Induces Intracellular Redistribution and Lateral Clustering of Endogenous Desmoglein 2

Although interactions between desmosomal cadherins are likely to be involved in desmosome assembly and in cell–cell adhesion of epithelial tissues, expression of Dsg and/or Dsc in the adhesion-deficient L-fibroblasts did not produce a detectable increase in intercellular adhesion (Amagai et al., 1994; Chidgey et al., 1996; Kowalczyk et al., 1996). Absence of the functional adherens junctions in L-fibroblasts could be one of several reasons contributing to the failure to demonstrate interactions between desmosomal cadherins. From this point of view, human fibrosarcoma HT-1080 cells present an advantage for studying Dsc–Dsg interactions. Supporting previous observations (Sacco et al.,

1995), we have demonstrated (Figs. 1 and 2) that, in contrast with L-fibroblasts, HT-1080 cells exhibit prominent intercellular contacts containing adherens junctions proteins N-cadherin and β -catenin. Surprisingly, we found that HT-1080 cells, despite their fibroblast-like phenotype, synthesize Dsg2 at essentially the same level as epithelial A-431 cells (Fig. 1). By immunofluorescence analysis (see list of the antibodies in Materials and Methods), HT-1080 cells were devoid of other desmosomal proteins such as plakoglobin, desmoplakin, and all known isoforms of Dscs (Fig. 1). Immunofluorescent staining with either monoclonal or polyclonal anti-Dsg antibodies revealed that a major portion of Dsg2 is randomly distributed on the surface of the HT-1080 cells (Fig. 2 *a*). Compared with A-431 cells, only a small fraction of Dsg2 can be detected at cell-cell contact regions where it is colocalized with N-cadherin and β -catenin (Fig. 2 *a'*). Dsg2 was sensitive to the treatment of HT-1080 cells with trypsin (not shown), confirming its cell surface localization.

Since desmosomes contain two distinct cadherins, Dsg and Dsc, we examined whether expression of the full-length bovine Dsc1a can bring about lateral clustering of the endogenous Dsg2 in HT-1080 cells. Double immunofluorescence analysis using polyclonal anti-Dsg2 and monoclonal anti-Dsc1 2.10 antibodies revealed a redistribution of endogenous Dsg2 in HT-1080 cells stably transfected with Dsc1a (HTDc cells). In these cells both desmosomal cadherins Dsg2 and Dsc1a were colocalized in large clusters of cell-cell contact regions (Fig. 2, *b* and *b'*) that were also positive for β -catenin (Fig. 2, *c* and *c'*). Notably, co-distribution of both desmosomal cadherins was found exclusively at cell-cell contacts. For example, Dsc-positive polymorphic vesicles detected in the cytoplasm of the transfected cells were devoid of Dsg (Fig. 2).

To determine the contribution of the intracellular and extracellular domains of Dsc to the assembly of the clusters of desmosomal cadherins, we transfected HT-1080 cells with several mutants of Dsc1a (Fig. 3 *A*). Two deletion mutants were constructed and stably expressed in HT-1080 cells. The first mutant, Dsc Δ (697-761), lacked the entire sequence of the C-domain mediating interaction of Dsc1a with plakoglobin. The second mutant, Dsc Δ (597-609), had an internal deletion of the CSI region involved in binding to desmoplakin (Trojanovsky et al., 1994b). Both deletions had no effect on the capacity of Dsc1a to form lateral clusters that incorporated endogenous Dsg (Fig. 2, *d* and *d'*). Expression of Dsc in HT-1080 cells did not induce synthesis of endogenous desmoplakin or plakoglobin

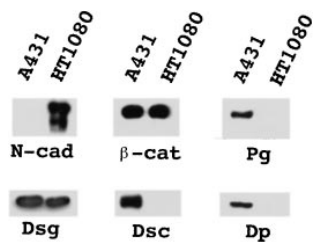


Figure 1. Immunoblot analysis of the junctional proteins synthesized in epithelial A-431 cells and in fibrosarcoma HT-1080 cells. Equal amounts of protein from total lysates of A-431 and HT-1080 cells (lanes A431 and HT1080, respectively) were separated by SDS-PAGE

(8%) and analyzed by immunoblotting with N-cadherin (*N-cad*), β -catenin (β -*cat*), plakoglobin (*Pg*), Dsg2-specific (*Dsg*), rabbit Dsc2 (*Dsc*), and desmoplakin (*Dp*) antibodies.

as determined by either immunofluorescence microscopy or immunoblotting (not shown). Taken together, these observations suggested that known intracellular desmosomal proteins are not required for relocation of Dsg2 in HTDc cells. We then constructed a chimeric protein Dc(1-170Dg) in which the sequence (Arg¹-Thr¹⁷⁰) corresponding to the first, and approximately half of the second, extracellular cadherin-like repeats of Dsc1a was replaced with the homologous sequence of the bovine Dsg1 (Fig. 3 *A*). This chimeric protein was faithfully delivered to the cell surface upon expression in HT-1080 cells; however, it failed to trigger the clustering of endogenous Dsg (Fig. 2, *e* and *e'*). Thus the extracellular region of Dsc1a, but not its intracellular domain, is required to effect lateral clustering of Dsg2 in HT-1080 cells.

Desmosomal Cadherins, Dsg and Dsc, Are Involved in Ca²⁺-dependent Heterophilic Interactions

The ability of the Dsc1a to interact with Dsg2 in HTDc cells was tested in coimmunoprecipitation experiments (Fig. 3 *B*). The total NP-40 lysates of the control HT-1080 cells, and cells stably expressing Dsc1a or its mutants, were immunoprecipitated with anti-Dsc antibodies. The presence of Dsg in the immunoprecipitates was monitored by immunoblot analysis. These experiments revealed that Dsc1a was able to coprecipitate Dsg2 from the HTDc cell lysate (Fig. 3 *B*). Substitution of the homologous sequence from bovine Dsg1 for the extracellular region of Dsc (the Dsc[1-170Dg] mutant) completely abolished Dsg/Dsc interactions in the coimmunoprecipitation assay, whereas deletions of the intracellular domain of Dsc, Dsc Δ (697-761) and Dsc Δ (597-609), had no effect. Therefore, the first two cadherin-like domains of the extracellular portion of Dsc1 are required for Dsg binding. N-cadherin and β -catenin were not detected in immunoprecipitates obtained with anti-Dsc or -Dsg antibodies (not shown), indicating the absence of a strong interaction between desmosomal and classic cadherins.

To address the question of whether Dsg2 directly interacts with Dsc1a or these interactions are mediated by an additional protein(s), the control and HTDc cells were metabolically labeled before immunoprecipitation. A prominent 120-kD band reacting with anti-Dsc antibodies was present in the immunoprecipitates obtained from HTDc cells using mAb 2.10 (Fig. 3 *C*). Only Dsg2 consistently coimmunoprecipitated with Dsc1a as a protein with a molecular mass of 160 kD that reacted with both anti-Dsg 3.10 and G129 antibodies (Fig. 3 *C*). An association between Dsg and Dsc was also detected in similar immunoprecipitation experiments using anti-Dsg antibodies (Fig. 3). The absence of the other specific bands in addition to Dsg or Dsc in these immunoprecipitates suggests a direct interaction between these two proteins in the HTDc cells. A disproportionately small amount of coprecipitating protein in these experiments could be caused, in part, by low solubility of the Dsg/Dsc complexes compared with free forms of both cadherins. Indeed, a significant amount of both desmosomal cadherins was found in the pellets after NP-40 extraction (not shown).

It is well documented that interaction of classic cadherins is Ca²⁺ dependent (Ozawa et al., 1990; Geiger and

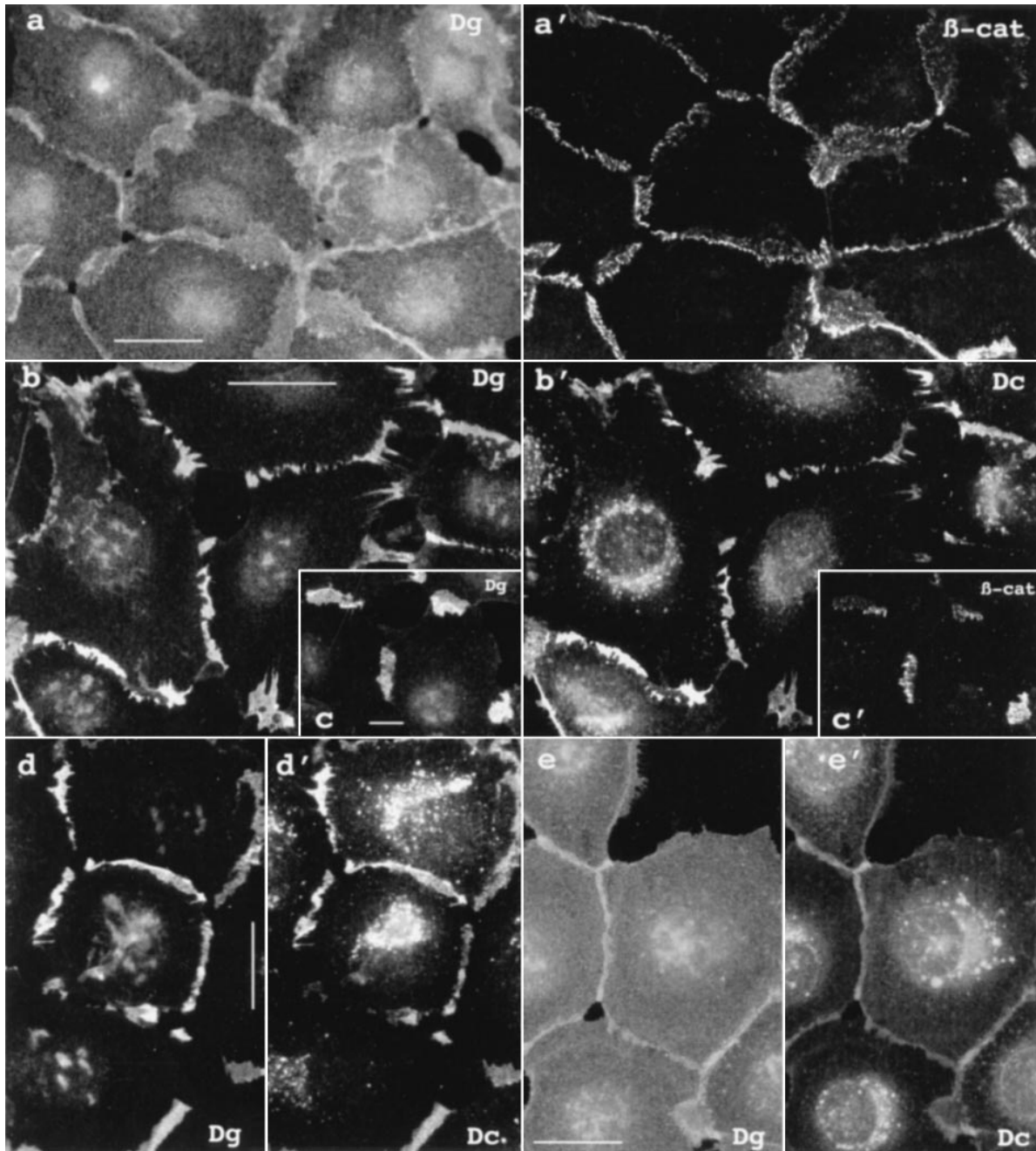


Figure 2. Redistribution of endogenous Dsg2 in HT-1080 cells expressing bovine Dsc1a or its mutants. Double-label immunofluorescence microscopy of control HT-1080 cells (*a* and *a'*) and HT-1080 cells producing Dsc1a (subclone HTDc-7; *b* and *b'* and *c* and *c'*), Dsc Δ (697–761) (subclone HTc-3; *d* and *d'*), and Dsc(1-170Dg) (subclone HTDcg-9; *e* and *e'*), using anti-Dsg serum (*a*–*e*) and mAbs against β -catenin (*a'* and *c'*) or bovine Dsc1a (*b'*, *d'*, and *e'*). Note that only a small portion of Dsg is located at the cell–cell junctions in parental or in HTDcg cells. The majority of Dsg is localized to the junctions in cells expressing Dsc1a or Dsc Δ (697–761). Bars, 25 μ m.

Ayalon, 1992; Gumbiner, 1996; Briehner et al., 1996). To test whether Dsc/Dsg interactions detected in HTDc cells were sensitive to Ca^{2+} , the cells were lysed in the presence of an increasing concentration of EGTA. The data presented at Fig. 4 show that 2 mM EGTA reduced the amount of Dsc/Dsg complexes over twofold. EGTA, however, was unable to completely abolish interactions between the two desmosomal cadherins.

Since immunoprecipitation experiments revealed that the

first two cadherin-like domains of Dsc are required for interactions with Dsg, we constructed and expressed the recombinant protein Dc12M in *E. coli*. This protein consists of the cadherin-like domains 1 and 2 of Dsc followed by the myc epitope and histidine hexamer (Fig. 5 A). To determine whether the Dsg domain(s) is involved in the interaction with Dc12M protein, we constructed the Dsg fragments Dg12F and Dg123F containing its extracellular cadherin-like domains 1 and 2 or 1, 2, and 3, respectively.

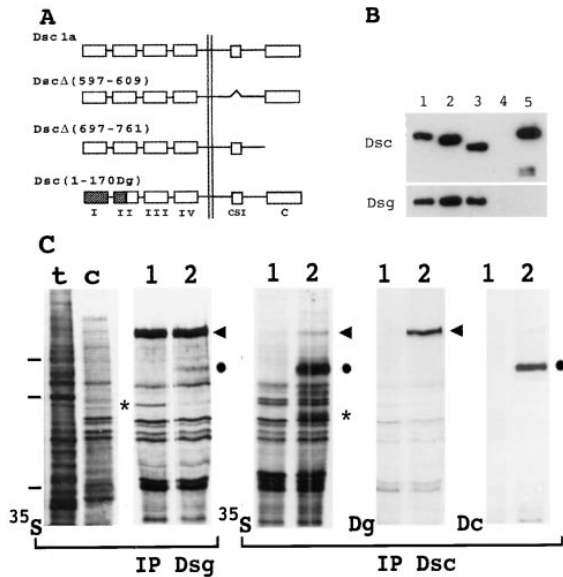


Figure 3. Schematic representation of the Dsc1a gene constructs and coimmunoprecipitation experiments showing Dsc–Dsg interactions. (A) Four extracellular cadherin-like domains (numbered from I to IV), the intracellular conserved sequence motif CSI (I), and the C-domain (C) involved in binding to desmoplakin or plakoglobin, respectively (Trojanovsky et al., 1994b), of bovine Dsc1a are indicated by open boxes. The mutants DscΔ(597–609) and DscΔ(697–761) contain the complete deletion of the CSI or C-domain, respectively. In the mutant Dsc(1–170Dg), the Dsg1-derived sequence (stippled) substitutes for the corresponding sequence of Dsc. The plasma membrane is denoted by the vertical double line. (B) Western blot analysis of immunoprecipitates obtained using anti-Dsc 2.10 antibody from HT-1080 cells transfected to produce Dsc1a (lane 1), DscΔ(597–609) (lane 2), DscΔ(697–761) (lane 3), and Dsc(1–170Dg) (lane 5); control HT-1080 cells (lane 4). Blots were developed either with 2.10 (Dsc) against Dsc1a or with 3.10 (Dsg) against Dsg antibodies. (C) Autoradiograms (³⁵S) of immunoprecipitates obtained with anti-Dsg 3.10 (IP Dsg) or anti-Dsc 2.10 (IP Dsc) antibodies from HT-1080 (lanes 1) or HTDc (lanes 2) cells. Total cell lysate of HTDc cells was loaded in lane t; control immunoprecipitate was obtained by anti-myc antibody in lane c. Immunoprecipitates were separated by SDS-PAGE (8%). Note that anti-Dsg antibody precipitates an equal amount of Dsg (arrowhead) from both cell lines in the IP Dsg experiment; the Dsc band is only seen in lane 2 (dot). Dsc immunoprecipitates in IP Dsc were also analyzed by immunoblotting using Dsg (Dg)- or Dsc1a (Dc)-specific antibodies. Note that bovine Dsc1a and endogenous Dsg are seen only in transfected cells (lane 2). Asterisks on autoradiograms indicate two bands of unknown nature that inconsistently appeared in some of the immunoprecipitates. Bars (left) denote the relative positions of coelectrophoresed reference proteins (from top to bottom: β-galactosidase, 116,000; phosphorylase b, 97,400; BSA, 67,000).

Carboxyl termini of the Dsg-derived polypeptides were tagged by the FLAG epitope and polyhistidine (Fig. 5 A). SDS-PAGE and immunoblot analysis of the recombinant proteins purified by Ni-agarose chromatography showed that the samples contained homogenous polypeptides of the expected molecular mass of 30 kD for Dg12F and Dc12M, and 45 kD for Dc123M (Fig. 5 A).

To analyze direct interactions between Dc12M and Dsg fragments we used a solid phase assay developed previ-

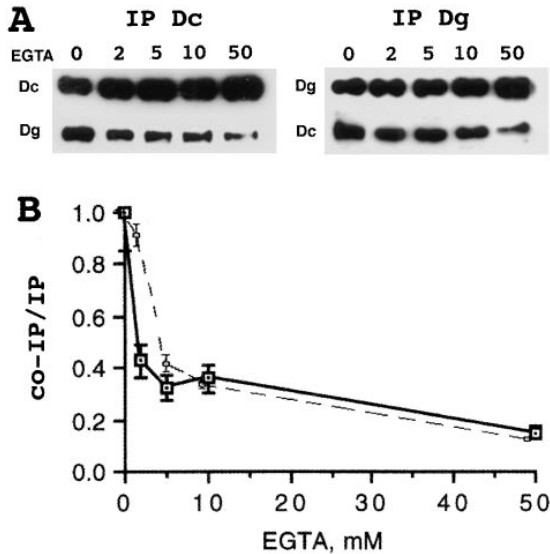


Figure 4. The calcium-dependent coimmunoprecipitation of Dsg and Dsc. (A) HTDc cells were immunoprecipitated using anti-Dsc 2.10 (IP Dc) or anti-Dsg 3.10 (IP Dg) antibodies. Immunoprecipitates were analyzed by immunoblotting with antibodies used for immunoprecipitation (upper line; Dc for IP Dc, or Dg for IP Dg) and with antibodies against the coprecipitated component (bottom line; Dg or Dc). Increasing the EGTA concentration in the lysis buffer raises the amount of the immunoprecipitated component, but decreases the amount of coimmunoprecipitated protein. (B) Bands from the coimmunoprecipitated experiments depicted in A were scanned and the ratios of immunoprecipitated to coimmunoprecipitated bands (Co-IP/IP; open boxes for IP DC and open circles for IP Dg) are shown in the graph. Each point represents an average ratio from four independent experiments. Vertical bars indicate the SD.

ously for examination of plakoglobin–cadherin binding (Chitav et al., 1996). Dsg fragments were immobilized on a 96-well dish and incubated with increasing amounts of Dc12M. Binding was detected by subsequent ELISA assay using anti-myc antibody. In this assay the Dc12M polypeptide interacts with Dg12F very weakly, and only in the presence of calcium ions (Fig. 5 B). Significant binding, however, was observed between Dc12M and Dg123F proteins containing extracellular domains 1–3. Addition of 5 mM EGTA also reduced but did not completely abolish these interactions (Fig. 5 B).

As an alternative approach, the molecular interactions between different Dsg and Dsc fragments were tested in a reconstitution assay. Fig. 5 C shows a representative experiment in which fragments Dc12M were mixed with fragments Dg12F or Dg123F and immunoprecipitated using anti-myc antibody. The results of these experiments are in good agreement with those obtained in the solid phase binding assay, demonstrating the strong interaction between Dc12M with Dg123F and Dg12F fragments.

Heterophilic Binding of Desmosomal Cadherins Contribute to Cell–Cell Adhesion

Endogenous expression of N-cadherin interferes with the measurement by conventional aggregation assays of the adhesive properties of HT-1080 cells that are due to

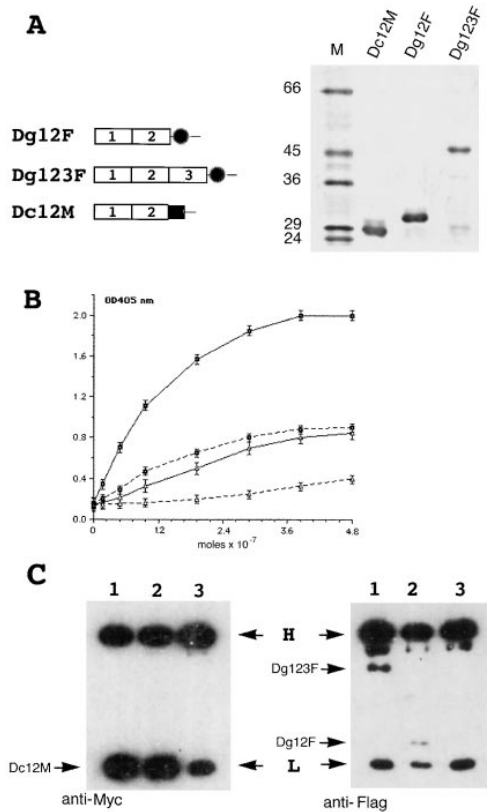


Figure 5. Direct interactions of the extracellular Dsg and Dsc domains in binding assays in vitro. (A) Scheme (left) and SDS-PAGE (right) of recombinant Dsg and Dsc fragments. The fragments Dg12F and Dg123F contain extracellular cadherin-like repeats 1 and 2, or 1, 2, and 3 of bovine Dsg1, respectively, followed by a FLAG epitope (filled circle). Dc12M consists of the first two cadherin-like repeats of the bovine Dsc1a placed in front of the myc epitope (filled box). Each fragment was tagged by polyhistidine (solid line) at the carboxyl terminus. Recombinant proteins after purification using Ni-NTA-agarose were separated by 15% SDS-PAGE. *M*, of the coelectrophoresed size markers in lane *M* is indicated. (B) Solid phase binding assay. For the assay, 100 μ l of a 10 μ g/ml solution of purified Dg12F (open boxes) or Dg123F (open triangles) was applied to each well. Plates were incubated with increasing amounts of Dc12M as indicated. Experiments were performed in the presence of 0.1 mM CaCl_2 (solid line) or 5 mM EDTA (dashed line). Binding was monitored using anti-myc 9E10 mAb. (C) Reconstitution assay. Dg123F (lanes 1 and 3) or Dg12F (lane 2) fragments were incubated for 30 min in the presence (lanes 1 and 2) or absence of Dc12M that was then immunoprecipitated by myc antibody. Coimmunoprecipitation of the Dsg-derived fragments was then monitored by immunoblotting with FLAG antibody (Flag). The parallel gel was stained with myc antibody (Myc). *H* and *L* indicate the positions of the heavy and light IgG chains of the 9E10 antibody used for precipitation. Position of Dc12M comigrating with the light chains; Dg123F and Dg12F are indicated with arrows.

the expression of transfected desmosomal cadherins. To test the contribution of the Dsg/Dsc interaction to cell-cell adhesion, HT-1080 cells expressing Dsc Δ (697-761) were cocultured with HT-1080 cells lacking any form of Dsc but expressing myc-tagged plakoglobin (MPg). In the latter cells, plakoglobin and endogenous Dsg form stable complexes that can be detected by coimmunoprecipitation

(Fig. 6 B). Thus, in this coculture system, specific coimmunoprecipitation of MPg by anti-Dsc antibodies in a Ca^{2+} -dependent fashion will be indicative of intercellular interactions mediated by desmosomal cadherins (Fig. 6 A). The direct complex between Dsc and MPg could not be formed because of the absence of the plakoglobin binding site in Dsc Δ (697-761). A coculture of MPg- and Dsc(1-170Dg)-expressing cells was used as an additional negative control. To verify that the subpopulations of the corresponding cells in both cocultures were present in an approximately equal ratio, aliquots of the cellular lysates were subjected to Western blot analysis with myc- and Dsc-specific antibodies before immunoprecipitation. Experiments shown in Fig. 6 C demonstrate that Dsc Δ (697-761), but not Dsc(1-170Dg), interacts with Dsg/MPg complexes present in the opposing cellular subpopulation of the coculture. In a separate experiment (Fig. 7) the HTDc cells were cocultured with HT-1080 cells stably producing the chimeric protein SyPg (Chitavev et al., 1996). This protein, as we reported previously, binds to classic and desmosomal cadherins. As a result, it is incorporated into cell-cell junctions of the transfected cells. Double immunofluorescence microscopy of these cocultures using polyclonal anti-synaptophysin and monoclonal anti-Dsc antibodies showed that Dsc1a was specifically incorporated into the junctions arising between cells from two subpopulations of Dsc1a-positive and -negative cells.

In addition, to show that interactions between desmosomal cadherins were present at the intracellular interface, we investigated whether the amount of the Dsc/Dsg complex is proportionate to cell density in culture. Intercellular contacts in HT-1080 cells at $\sim 75\%$ confluence are more extensive than in those at low density. Thus, HT-1080 cells expressing Dc Δ (697-761) were plated at a density of 12 and 75% confluence and left overnight to allow intracellular contacts to stabilize. The lysates of low and high density cultures containing the same amount of total protein were immunoprecipitated with an anti-Dsc antibody, and immunoprecipitates were analyzed by immunoblotting (Fig. 6 D). A five- to sixfold reduction in the amount of Dsg in Dsc immunoprecipitates was obtained from cell cultures plated at low as compared with high densities. This shows that Dsg/Dsc interaction in coculture correlates well with the number of intercellular contacts. It is important to note that, while plated at low density, HT-1080 cells always contain some number of cell-cell contacts that cause coimmunoprecipitation of Dsg.

Discussion

A number of studies have shown that expression of Dsg and/or Dsc in adhesion-deficient L-fibroblasts fails to produce detectable levels of intercellular adhesion (Amagai et al., 1994; Chidgey et al., 1996; Kowalczyk et al., 1996). Although intercadherin interactions are very likely to be involved in desmosome assembly and in cell-cell adhesion of the epithelial tissues, no physical association between these two desmosomal cadherins was reported. The major reasons for failure to demonstrate interactions between desmosomal cadherins might have been (a) insolubility of the corresponding complexes in the epithelial cells, and (b) malfunction of Dsg and Dsc in L-fibroblasts in the ab-

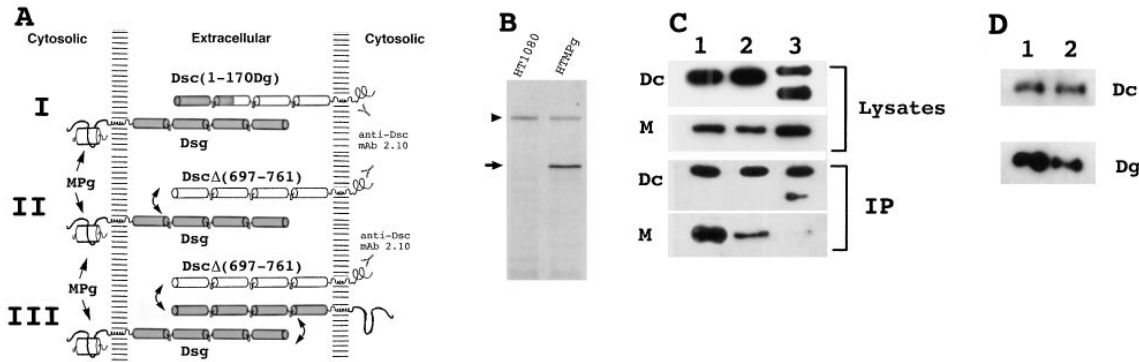


Figure 6. Coimmunoprecipitation of MPg with Dsc Δ (697–761) but not with Dsc(1–170Dg) from cocultures. (A) Schematic representation of the interface between MPg- and Dsc Δ (697–761)-producing cells (II and III), or Dsc(1–170Dg)-producing cells (I). Dsg and Dsc are shown by stippled or open cylinders, respectively. MPg associated with the intracellular region of Dsg in MPg cells is denoted by the single open cylinder. Note that two different models may account for coimmunoprecipitation of MPg with Dsc Δ (697–761). In model II, Dsg and Dsc form a head-to-head complex while, in model III, desmosomal cadherins form side-to-side dimers. (B) Autoradiogram showing strong interactions between endogenous Dsg2 and MPg in MPg-producing cells. Immunoprecipitates were obtained with anti-Dsg 3.10 antibodies from metabolically labeled HT-1080 cells (*HT1080*) or MPg-producing cells (*HTMPg*). Dsg and MPg are indicated by the arrowhead or the arrow. (C) Immunoblot analysis of the total lysates (*Lysates*) and immunoprecipitates (*IP*) obtained with anti-Dsc 2.10 antibodies of the cocultures containing Dsc Δ (697–761) and MPg cells (lanes 1 and 2) or Dsc(1–170Dg) and MPg cells (lane 3). Nitrocellulose blots were developed with anti-Dsc 2.10 (*Dc*) or anti-Myc (*M*) antibodies. (Lane 2) Immunoprecipitates were obtained in the presence of 5 mM EGTA. Note that EGTA or replacement of the Arg¹-Thr¹⁷⁰ Dsc sequence in the Dsc(1–170Dg) mutant abolished the interactions with MPg. Lower band in lane 3 is a degradation product of the Dsc(1–170Dg) chimera. (D) Western blot analysis of immunoprecipitates obtained from dense (lane 1) or sparse (lane 2) HTDc cells using mAb Dsc 2.10. Immunoprecipitated (*Dc*) and coimmunoprecipitated (*Dg*) bands in precipitates were developed by 2.10 or 3.10 antibodies, respectively.

sence of functional adherens junctions and classic cadherins in particular. As an alternative model for study of the interaction between Dsc and Dsg, we chose fibrosarcoma HT-1080 cells. In contrast with L-fibroblasts, they have prominent intercellular contacts containing N-cadherin and β -catenin. HT-1080 cells produce a single desmosomal protein, Dsg2, at the same level as in epithelial A-431 cells. In these cells, however, only a minor portion of this cadherin is localized to cell–cell junctions.

Here we have shown that expression of the full-length Dsc1a in HT-1080 cells results in a redistribution of endogenous Dsg2. Both desmosomal proteins, Dsg2 and Dsc1a, were efficiently incorporated into the cell–cell contacts of HTDc cells, where they become associated with adherens junction proteins, such as N-cadherin and β -catenin. Coimmunoprecipitation experiments revealed that in these cells Dsc1a is able to interact directly with Dsg2, but not with N-cadherin. Notably, formation of Dsc/Dsg complexes was found in the absence of major desmosomal proteins, plakoglobin and desmoplakin, that were undetected in parental HT-1080 cells and in HTDc cells. In addition, deletions of plakoglobin or desmoplakin binding sites in Dsc1a had no effect on its ability to interact with Dsg2, demonstrating that known cytoplasmic interactions of Dsc1a are not required for Dsg/Dsc association. In contrast, replacement of the extracellular domain of Dsc1a with the corresponding sequence of Dsg completely abolished both Dsg redistribution and Dsg/Dsc complex formation in coimmunoprecipitation. Furthermore, association between Dsg and Dsc was calcium dependent, which is characteristic of interactions involving extracellular domains of cadherin. In vitro binding experiments provided further support for a direct interaction between the extra-

cellular segments of two desmosomal cadherins. Using two different binding assays, we have found that Dc12M, a Dsc fragment containing extracellular repeats 1 and 2, binds directly to the extracellular region of Dsg. Chelation of Ca²⁺ ions decreased but did not completely abolish this binding. Our data are not sufficient to conclude whether Dsg/Dsc heterodimers are formed through head-to-head or side-to-side interactions (see hypothetical models of Dsg–Dsc complexes in Fig. 6). Recently, Briehner et al. (1996) showed that lateral dimers of the extracellular region of the C-cadherin in vitro are relatively stable after the removal of Ca²⁺ ions. Similarly, incomplete inactivation of the Dsg–Dsc interaction even in the presence of a high concentration of EGTA suggests that the cadherin dimers have lateral alignment. However, in the solid phase assay, the Dc12M fragment binds more strongly to the Dsg fragment containing extracellular domain 3. This observation may be interpreted to mean that Dsg–Dsc forms antiparallel head-to-head complexes in which the extracellular domains 1 and/or 2 of Dsc bound to domain 3 of Dsg. Additional experiments are required to determine the exact structural features of Dsg/Dsc dimers.

Another important feature of Dsc/Dsg complexes is revealed by the fact that MPg coimmunoprecipitated with anti-Dsc antibodies from the coculture of Dsc Δ (697–761)- and MPg-expressing HT-1080 cells. This unequivocally demonstrated that interacting Dsc and Dsg are derived from opposing cells. The number of Dsc/Dsg complexes directly correlates with the propagation of cell–cell contacts in HTDc coculture. Furthermore, clusters incorporating Dsc and Dsg are also assembled along the contacts with Dsc-negative cells. These observations are consistent with the idea that these complexes are formed only on the in-

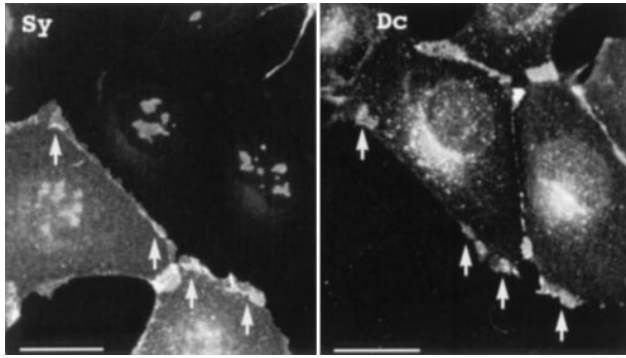


Figure 7. Double-label immunofluorescence microscopy of HTDc cells producing bovine Dsc1a and of HT-1080 cells producing the chimeric protein SyPg in coculture. (Sy) The distribution of the SyPg in Dsc-negative cells visualized by immunostaining with rabbit anti-synaptophysin antibody. This is compared with the localization of the Dsc1a in HTDc cells detected by mAb 2.10 (Dc). Both proteins, although present in opposing cells, form clusters located at the same areas of cell-cell contacts (arrows). Bars, 25 μ m.

interface between two neighboring cells. Thus, heterophilic interactions between desmosomal cadherins are involved in intercellular adhesion of epithelial cells, and corresponding complexes can be functional elements in desmosome assembly. In support of this assumption we found that the bovine Dsc1a as well as its mutants Dsc Δ (697–761) and Dsc Δ (597–609) were efficiently incorporated into human desmosomes upon expression in epithelial A-431 cells (Chitaev, N.A., unpublished results). In contrast, the mutant Dsc(1–170Dg), unable to interact with Dsg in HT-1080 cells, was also unable to form desmosomes in A-431 cells. The question remains, however, how Dsg/Dsc complexes assemble in a mature desmosome. The function of the intracellular desmosomal plaque proteins, such as plakoglobin and desmoplakin, may be necessary for segregation of the Dsg/Dsc complexes from adherens junctions and for further desmosome assembly (Hinck et al., 1994; Allen et al., 1996; Bornslaeger et al., 1996; Chitaev et al., 1996; Demlehner et al., 1996; Ruiz et al., 1996; Troyanovsky et al., 1996). The subsequent expression of these desmosomal proteins in HTDc cells is likely to provide an excellent system for examining the molecular mechanisms involved in this process.

The low level of Dsg/Dsc interactions in sparse cultures of HTDc cells is consistent with the data of Kowalczyk et al. (1996) showing the absence of detectable intercadherin interactions when both Dsg and Dsc are coexpressed in mouse fibroblasts lacking adherens junctions. The requirement of adherens junctions for intercadherin interactions was suggested by observations that the malfunction of adherens junctions, induced either by E-cadherin antibodies or by dominant negative mutants of the classic cadherins, delays desmosome assembly in keratinocytes after raising the calcium concentration (Wheelock and Jensen, 1992; Lewis et al., 1994; Amagai et al., 1995). In HT-1080 cells, Dsc/Dsg clusters were found in areas where N-cadherin- β -catenin complexes are abundant. Therefore, it is reasonable to hypothesize the existence of cross talk between ad-

herens junctions and desmosomes, which allows Dsg/Dsc interactions only after the establishment of the cell type-specific contacts mediated by classic cadherins.

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