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Identification of amino acid sequence motifs in desmocollin, a desmosomal glycoprotein, that are required for plakoglobin binding and plaque formation

(desmosome/cadherin/desmoplakin/adhering junctions/cytoskeleton)

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ABSTRACT **By transfecting epithelial cells with gene constructs encoding chimeric proteins of the transmembrane part of the gap junction protein connexin 32 in combination with various segments of the cytoplasmic part of the desmosomal cadherin desmocollin 1a, we have determined that a relatively short sequence element is necessary for the formation of desmosome-like plaques and for the specific anchorage of bundles of intermediate-sized filaments (IFs). Deletion of as little as the carboxyl-terminal 37 aa resulted in a lack of IF anchorage and binding of the plaque protein plakoglobin, as shown by immunolocalization and immunoprecipitation experiments. In addition, we show that the sequence requirements for the recruitment of desmoplakin, another desmosomal plaque protein, differ and that a short (10 aa) segment of the desmocollin 1a tail, located close to the plasma membrane, is also required for the binding of plakoglobin, as well as of desmoplakin, and also for IF anchorage. The importance of the carboxyl-terminal domain, homologous in diverse types of cadherins, is emphasized, as it must harbor, in a mutually exclusive pattern, the information for assembly of the IF-anchoring desmosomal plaque in desmocollins and for formation of the α-/β-catenin- and vinculin-containing, actin filament-anchoring plaque in E- and N-cadherin.**

Intercellular junctions of the adhering category are symmetric structures providing direct molecular contacts between adjacent cells of many different tissues. The various morphological forms of adhering junctions all represent clusters of transmembrane cell-adhesion glycoproteins of the cadherin superfamily whose extracellular parts make Ca^{2+}-dependent contacts and whose cytoplasmic portions associate with certain cytoplasmic proteins, assembling a plaque at which bundles of cytoskeletal filaments can attach. Two major types of filament-anchoring junctions have been distinguished: (*i*) The diverse forms of adherens junctions (*zonulae adherentes, fasciae adherentes*, and *puncta adherentia*) contain certain "classical" cadherins such as E- and N-cadherin, which are associated with a characteristic ensemble of cytoplasmic proteins, including plakoglobin, α- and β-catenin, vinculin, α-actinin, and radicin and anchor actin microfilaments (1–5).

(*ii*) Desmosomes (*maculae adherentes*) are membrane domains enriched in special cadherins ("desmosomal cadherins")—i.e., one or several representatives of the desmoglein (Dsg; for nomenclature see ref. 6) and the desmocollin (Dsc) subfamilies, the cytoplasmic portions of which nucleate the assembly of a plaque that contains plakoglobin, desmoplakin(s), and certain accessory proteins and anchors intermediate filament (IF) bundles (6–14).

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It is clear from various experiments that the carboxyl-terminal cytoplasmic portions (tails) of the cadherins are crucial in both intramembranous clustering and plaque formation and thereby also in the interaction with the specific cytoskeletal filament system. For classical (e.g., E-, P-, or N-) cadherins, it has been shown by deletion experiments that a 72-aa carboxyl-terminal segment is required to assemble α- and β-catenin, as well as plakoglobin, and to provide anchorage of actin microfilaments (15–19). In epithelial cells stably transfected with gene constructs encoding chimeric proteins with tails of desmosomal cadherins (10) it has been found that the carboxyl-terminal domain of the larger splice form of Dsc—i.e., Dsc1a, but not that of the shorter splice form Dsc1b (for amino acid sequences see refs. 20–25)—can induce the assembly of a desmosome-type plaque that contains plakoglobin and desmoplakin and anchors IFs (26).

To identify amino acid sequence motifs directing desmosomal plaque assembly and IF anchorage we have transfected cells with gene constructs coding for chimeric proteins that combine the transmembrane part of the gap junction protein connexin 32 (Cx32) with a series of terminal and internal deletion mutants of the Dsc tail. Here we show that a remarkably short carboxyl-terminal segment is needed to bind plakoglobin and to effect the formation of desmosomal plaque structures and IF anchorage.

MATERIALS AND METHODS

Construction of Plasmid Clones. Clone BlCoDcI encoding a chimeric polypeptide consisting of the four transmembrane domains of rat liver Cx32 and the cytoplasmic segment of bovine Dsc1a has been described (26). For construction of the deletion mutant BlCoDcΔ(138), BlCoDcI was first cleaved with *Cfr*10I and blunt-ended. The insert was excised with *Hin*dIII and then cloned into the blunt-ended *Nar* I and *Hin*dIII sites of plasmid Blx, which contains three stop codons in different reading frames by insertion of the synthetic oligonucleotide 5′-AAG CTT GGA GGC GCC TGA CTA GCT AGG ATC C-3′ (stop codons are underlined) in the polylinker of the Bluescript vector. BlCoDcΔ(55) was obtained after ligation of the ≈800-bp *Hin*dIII/*Bal* I (blunt-ended) fragment of BlCoDcI into the *Hin*dIII/*Bam*HI (blunt-ended) sites of Bluescript. In this case, an in-frame TGA sequence was next to the inserted DNA.

Other mutants were constructed with the help of PCR. For construct BlCoDcΔ(109) PCR fragments were amplified from

Abbreviations: Cx32, connexin 32; Dsc, desmocollin; Dsg, desmoglein; IF, intermediate filament; C-domain, 65-aa carboxyl-terminal domain of the longer splice variant *a* of all three known members of the Dsc family.

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BlCoDcI sequence with primers DcI-O-510 (see ref. 26) and DcI-O-542 (5′-AAA GAG CTC TCT AGA GCC GCG GTT GGG TGA AGT TGT G-3′), treated with the restriction endonucleases *Nar* I and *Xba* I, and exchanged for the 500-bp *Nar* I/*Xba* I fragment of BlCoDcI. For plasmid BlCoDcIΔ(38-72) two sets of primers were used: DcI-O-510 and DcI-O-543 (5′-TTT ACT AGT TGT GGG GAG TCT AAT ATT-3′) and DcI-O-544 (5′-TTT ACT AGT ACT TTG GAT GCC AAC AAA-3′) and DcI-O-509 (26). The fragments that were amplified from BlCoDcI were treated with *Nar* I/*Spe* I or *Spe* I/*Bam*HI, respectively, and ligated together into the *Nar* I/*Bam*HI sites of BlCoDcI.

For construction of BlCoDcΔ(12-23), a fragment coding for the Dscla tail from Asp-11 with a deletion from Ala-13 to Thr-22 was amplified with amplimers DcI-O-155 (5′-CAA AGA TCT AGA AGG GCC CGG AGA AGA AGT GAT GGA T-3′) and DcI-O-511 (5′-ATT CTA GAA AAA GGC ACA TTT TAT TT-3′) from BlCoDcI, cut with *Bgl* II/*Xba* I and ligated into the *Nar* I/*Xba* I-treated plasmid BlCoDcI in the presence of the annealed complementary oligonucleotides 5′-CGC CAA AAC AGT AAA GAA GTG TTT TCC AGA A-3′ and 5′-GAT CTT CTG GAA AAC ACT TCT TTA CTG TTT TGG-3′ that code for Lys-2 to Asp-11 of Dscla, introducing a Val-12 → Leu mutation.

BlCoDcΔ(12-23) was used for the generation of mutants by introduction of complementary oligonucleotides between the newly introduced *Bgl* II/*Apa* I sites. For control, the wild-type-like sequence 5′-AGAT CTA GCC CAG CAA AAT TTA ATT GTA TCA AAT ACT GAA GGG CCC-3′ (cloning sites are underlined; mutated nucleotides in boldface print result in conservative Val → Leu mutation in *Bgl* II site) was restored (BlL12). For generation of mutants of this region, oligonucleotides were used in which codon TTA was substituted by AAA (mutant BlK17), codon TCA was substituted by GAA (mutant BlE20), codon ACT was substituted by GAA (mutant BlE22), and codon GAA was substituted by AAA (mutant BlK23).

The *Hin*dIII/*Xba* I inserts of all Bluescript clones were further subcloned into the eukaryotic expression vector pHβAP$_r$-1-neo, containing the neomycin resistance gene (compare ref. 26). The correct construction of all recombinant plasmids was checked by nucleotide sequencing.

Methods used for transfecting human epithelial A-431 cells and for the selection and growth of stably CoDcI- and CoDcII-transfected cell clones (sublines D2 and D4) have been described (26).

Immunoprecipitation and Immunoblotting. Cells were lysed and supernatant fractions were subjected to immunoprecipitation essentially as described (27), using rabbit Cx32 antibodies (28). Immune complexes were subjected to SDS/PAGE, followed by immunoblot detection using monoclonal antibodies to plakoglobin [PG5.1 (ref. 29)] or Dsg [Dg3.10 (refs. 30 and 31)]. Immunoblots were analyzed as described (26) with horseradish peroxidase-conjugated secondary antibodies and an enhanced chemiluminescence system (Amersham).

Immunofluorescence and Electron Microscopy. Immunofluorescence microscopy on methanol/acetone-fixed cells and electron microscopy were as described (26), using the following primary antibodies: Cx32 antibodies from rabbit (28), murine monoclonal antibodies against plakoglobin [PG5.1 (ref. 29)], desmoplakin [DPI and II2.15, -2.17, and -2.19 (ref. 32)], Dsg (Dg3.10), and cytokeratins [lu-5 (ref. 33)].

RESULTS

The Plakoglobin-Binding Domain in Dscla. The 65-aa carboxyl-terminal domain of the longer splice variant *a* of all three known members of the Dsc family (C-domain; refs. 6, 25, and 26) is encoded by a single exon (13, 20, 21, 26) and displays high amino acid sequence homology between dif-

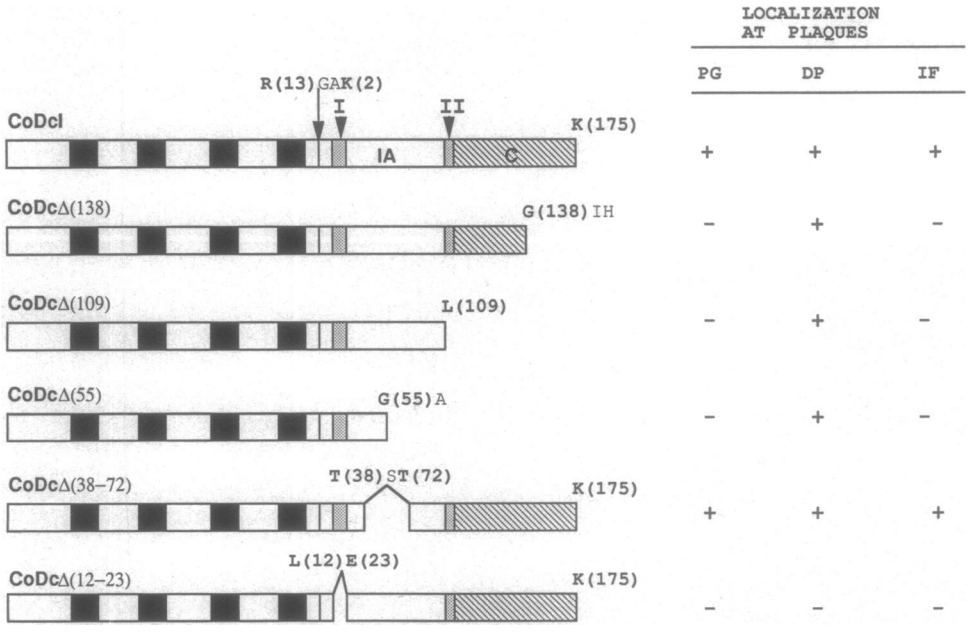


FIG. 1. Schematic representation of several rat liver Cx32/bovine Dscla chimeras (*Left*) and the corresponding phenotypes of chimeric gap junctions in stably transfected A-431 cells (*Right*). The mutants contain the amino-terminal part of Cx32 with its four transmembrane domains (black boxes) and loops, as well as a short portion of the Cx32 cytoplasmic tail region up to Arg-13 [R(13)], in combinations with various Dscla tail segments, starting at Lys-2 [K(2)]. The arrow denotes the junction between elements of the two proteins. The two subdomains of the Dscla intracellular anchoring (IA) domain with the two conserved sequence motifs CSI (I) and CSII (II) and the cadherin-typical C-domain are specifically labeled. Amino acid positions at the various deletion and fusion sites and at the carboxyl termini are in boldface type; amino acid residues introduced by cloning appear in light print. The amino acid numbers (in parentheses) give the distance from the presumed end of the last Cx32 transmembrane domain. Table at right presents scores for the presence (+) or absence (−) of plakoglobin (PG), desmoplakin (DP), and IFs at the chimeric gap junctions formed, as determined by double-label immunofluorescence microscopy.

ferent desmosomal and nondesmosomal cadherins—for example, there is ≈65% aa identity among Dsc1a, Dsc2a, and Dsc3a in both bovine and human protein, and the last 34 aa are particularly well conserved (≈90% identity). To examine the binding and morphogenic capability of this domain, deletion mutants of CoDcI, a chimera of rat Cx32 with a Dsc1a tail, were used for transfection (Fig. 1).

Similar to CoDcI carrying the intact tail of Dsc1a, mutants CoDcΔ(138), missing the last 37 aa of the wild-type molecule, and CoDcΔ(109), missing the entire C-domain, also assembled desmoplakin-positive gap junction-like structures in the transfected cells (Figs. 2 *a–c* and 3 *a* and *b*). In contrast to CoDcI chimera, however, both mutant chimeric junctions did not show significant colocalization with plakoglobin (Figs. 2 *d* and *e* and 3 *c* and *d*) and were not seen with attached IF bundles (Figs. 2 *f* and *g* and 3 *e* and *f*).

In immunoprecipitation with Cx32 antibodies from lysates of stably transfected cells, chimera CoDg containing the entire cytoplasmic domain of Dsg1 (26) coprecipitated plakoglobin (Fig. 4, lane a; compare with ref. 27), as did chimera CoDcI (Fig. 4, lane c). In contrast, chimera CoDcII representing the alternative splice form *b* and containing the entire Dsc1b tail (26), as well as several C-domain deletion mutants such as CoDcΔ(138) (Fig. 4, lanes b and d) and CoDcΔ(109), did not coprecipitate detectable amounts of plakoglobin. In further control experiments, we immunoblotted the immunoprecipitated proteins with Dsg-specific antibodies (for details see ref. 26) to exclude that possible Dsg–Dsc complexes might have contributed to the observed plakoglobin binding of chimera CoDcI. Furthermore, no desmoplakin immunoreactivity was detected in such immunoprecipitates. However, because only a small fraction of total CoDcI was solubilized under our extraction conditions, we cannot exclude that the immunoisolates contained only a subset of CoDc molecules—e.g., those located in the cytoplasm and reported (26) to be negative for desmoplakin.

Determinations of Dsc1a Sites Required for Desmoplakin Accumulation. Surprisingly, chimeras CoDcΔ(138) and CoDcΔ(109), which did not form a plaque revealing plakoglobin, could accumulate desmoplakin (Fig. 3*b*), indicating that desmoplakin binding does not depend on detectable amounts of plakoglobin. This result was confirmed by our analyses (Fig. 1) of further truncation, such as in mutant CoDcΔ(55). In particular, one short segment located close to the membrane—i.e., in the IA region (for terminology see refs. 23 and 31)—markedly affected desmoplakin binding. This IA region is highly variable, except for two conserved sequence motifs that are identical in all three bovine and human Dsc genes known (13, 25, 26): segment CSI (AQQNLIVSNTE) and segment CSII (TQPRLGE; see Fig. 1). When we transfected cells with a series of internal deletion mutants (Fig. 1), CoDcΔ(38-72) could still accumulate desmoplakin,

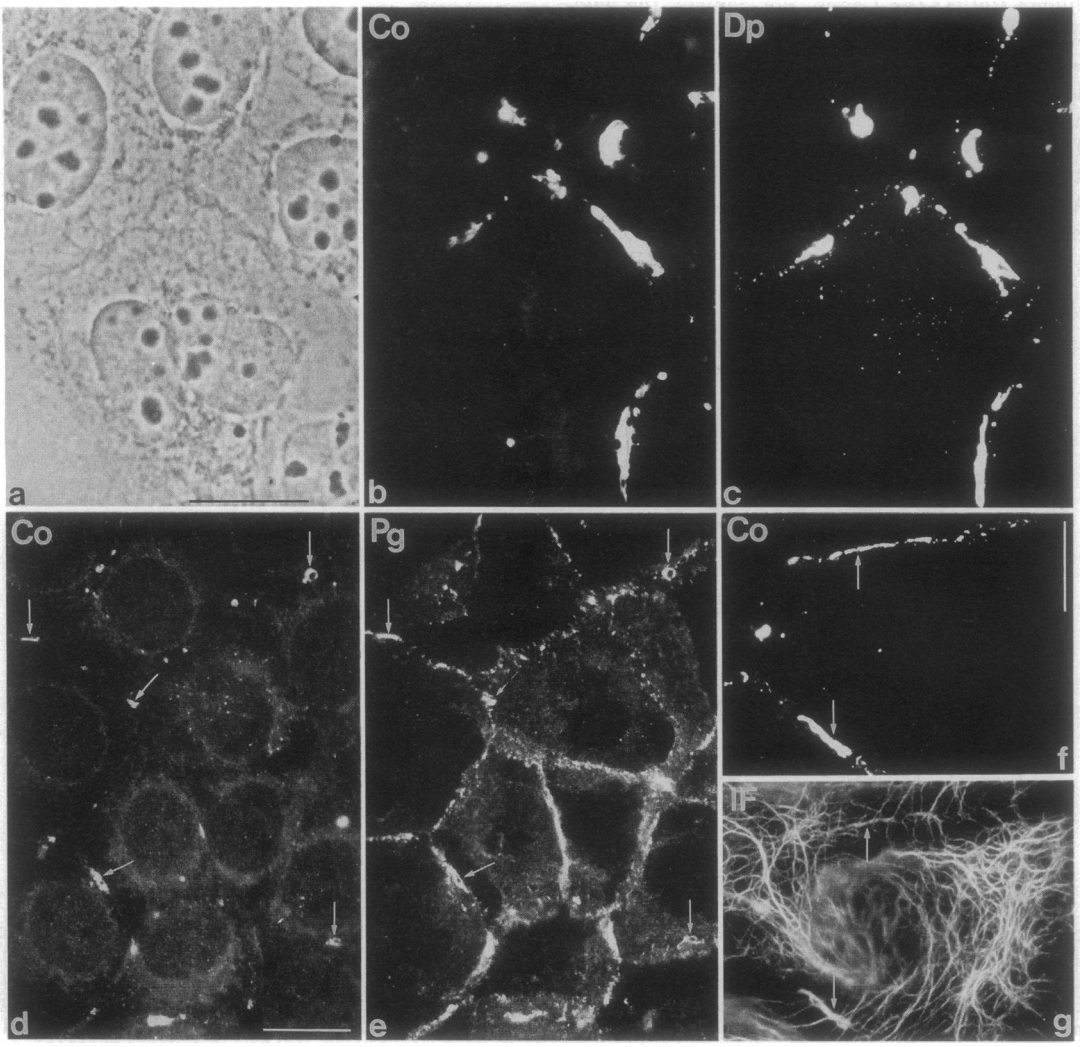


FIG. 2. Double-label immunofluorescence microscopy of A-431 cells stably transfected to express chimera CoDcI containing the entire Dsc1a tail. Note colocalization of Cx32 (Co) with desmoplakin (Dp) (*b* and *c*; corresponding phase-contrast picture is *a*), plakoglobin (Pg) (*d* and *e*), and bundles of cytokeratin IFs (*f* and *g*). Arrows in *d–g* denote some selected sites as examples. [Bar = 25 μm (*a–e*) and 40 μm (*f*, *g*).]

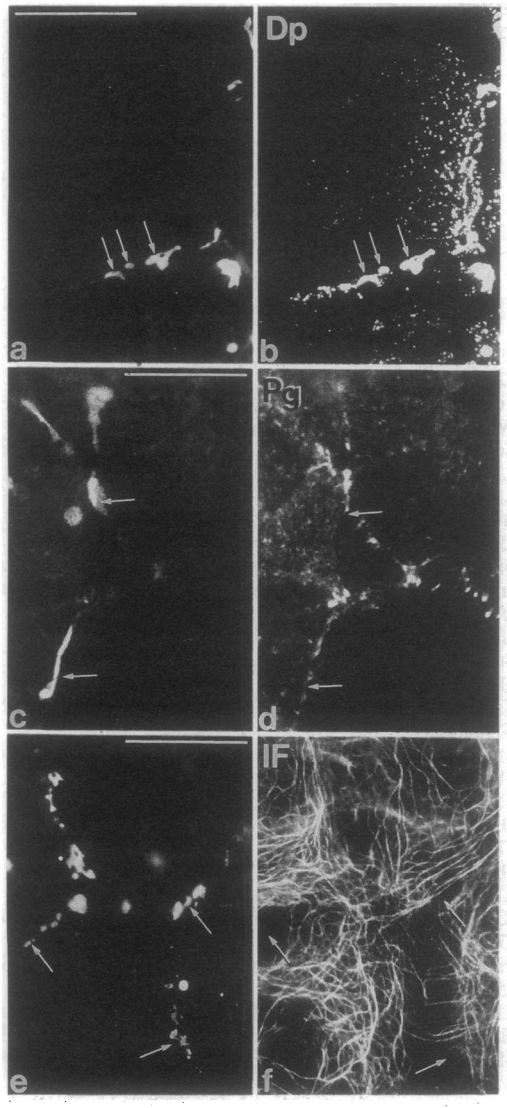


FIG. 3. Double-label immunofluorescence microscopy of A-431 cells stably expressing chimera CoDcΔ(138). Comparison of Cx32 label with that of desmoplakin (*a* and *b*), plakoglobin (*c* and *d*), and cytokeratin IFs (*e* and *f*) shows that in this mutant only desmoplakin detectably accumulates at the chimeric gap junction (e.g., denoted by arrows in *a* and *b*), in contrast to plakoglobin and IFs (some exemplary sites are denoted by arrows in *c–f*). (Bar = 30 μm.)

indicating that, at least in Dscla, the amino acid sequence distance between membrane and C-domain need not be maintained to induce plaque formation and IF anchorage. Deletion of the CSI segment in construct CoDcΔ(12-23), however, resulted in a lack of detectable desmoplakin in the Cx32 chimeric junctions formed (Figs. 1 and 5 *a* and *b*). Interestingly, plakoglobin was also absent from the chimeric structures formed (Fig. 5 *c* and *d*). We also excluded the hypothesis that this lack of binding was due to proteolytic loss of the C-domain in the mutated protein because the intact chimeric molecule was still detected with Cx32 antibodies in immunoblot experiments of total proteins.

The CSI segment contains two potential phosphorylation sites (Ser-20, Thr-22) preceding the negatively charged Glu-22 and a hydrophobic pocket (Leu-17–Ile-18–Val-19). Point mutations of Ser-20 or Thr-22 to glutamate (mutants CoDcE20 and CoDcE22) did not show any significant effect on plakoglobin and desmoplakin binding, whereas mutations of either Leu-17 or Glu-23 to lysine (mutants CoDcK17 and CoDcK23) caused a loss of desmoplakin association, beside

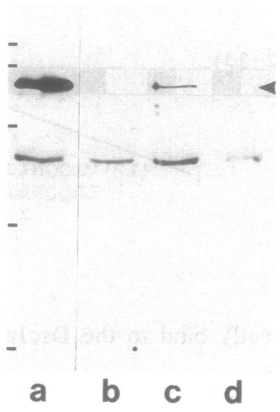


FIG. 4. Immunoblot analysis using plakoglobin antibodies on SDS/PAGE-separated proteins of immunoprecipitates formed by Cx32 antibodies from lysates of cDNA-transfected A-431 cell lines expressing either the Dsg chimera CoDg (lane a) or the Dsc mutant chimeras CoDcΔ(138) (lane b), CoDcI (lane c), and CoDcII (lane d). Arrowhead at right denotes plakoglobin; the lower band corresponds to immunoglobulin heavy chain. Molecular size markers are indicated by bars at left [from top down: phosphorylase a (97.4 kDa), bovine serum albumin (67 kDa), ovalbumin (45 kDa), and carbonic anhydrase (29 kDa)]. Note that plakoglobin is only detected in immunoprecipitates of *a* and *c*.

a somewhat reduced immunostaining for plakoglobin (Fig. 6). These latter mutants also were unable to attach IFs.

DISCUSSION

We have identified a short carboxyl-terminal segment of 37 aa in a Dsc, Dscla, that is crucial for plakoglobin binding and is also required, in the environment of the cortical cytoplasm of an epithelial cell (cf. ref. 26), for plaque formation and IF attachment. From the mutations analyzed, however, we cannot yet exclude additional contributions of other sequences in the Dsc molecule, such as segment CSI (see above and Figs. 1 and 6). In addition, *in vitro* binding experiments using purified Dsc tail polypeptides and plakoglobin, both produced in *Escherichia coli* by recombinant DNA technology, in solid-phase microwell assays have shown that pla-

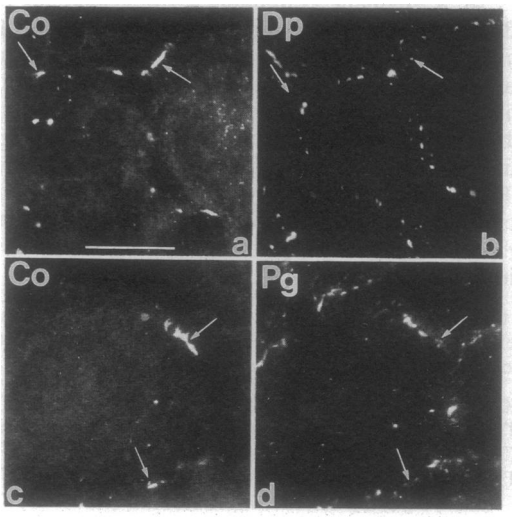
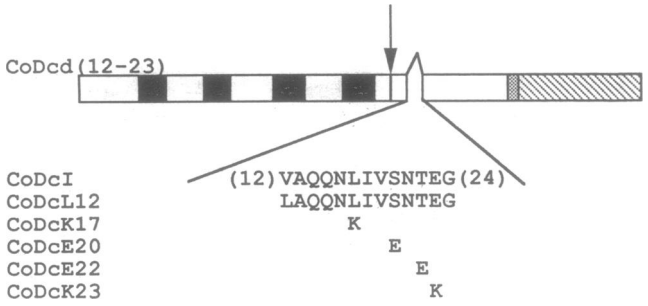


FIG. 5. Double-label immunofluorescence microscopy of A-431 cells producing chimera CoDcΔ(12-23), showing absence of colocalization of Cx32 (Co) with desmoplakin (Dp) (*a* and *b*) and plakoglobin (Pg) (*c* and *d*). (Bar = 25 μm; same magnification in *a–d*).



LOCALIZATION AT PLAQUES	
DP	PG
-	-
+	+
+	+
-	(+)
+	+
+	+
-	(+)

FIG. 6. Schematic from fine analysis of the results of segment-CSI (see Fig. 1) point mutations on association of desmoplakin (DP) and plakoglobin (PG) (assays and symbols as for Fig. 1). Note selective lack of desmoplakin binding in two point mutations (see text). (+), Somewhat reduced reactivity.

koglobin can directly bind to the Dscla tail (unpublished results).

In E- and N-cadherin this C-domain has been shown to be responsible for the binding of plakoglobin and/or β-catenin, together with α-catenin, vinculin, and other proteins, and for the formation of *zonula adherens*-type junctional plaque capable of anchoring actin filaments (e.g., refs. 15–19 and 34). Recently, we have shown that the dominant-negative effect of Dsg (Dsg1) tails in similar chimeric construct transfections on desmosomal plaque formation and IF anchorage resides in the C-domain of Dsg (26, 27). Thus, in spite of the high amino acid sequence homology of this domain in all these cadherins (Dsc, Dsg, and E- and N-cadherin) and in spite of its common interaction with plakoglobin and/or its "sister molecule" β-catenin, this region also must harbor the information to distinguish in its binding specificity between the two different plaque protein ensembles that anchor either actin filaments or IFs.

We have also shown that a short juxtamembranous region of the Dscla tail is involved in the recruitment of desmoplakin that can occur independently from plakoglobin binding [see, e.g., mutant CoDcΔ(55), Fig. 1]. The conserved CSI segment is a plausible candidate for this interaction with desmoplakin because even point mutations in this segment affected desmoplakin localization at the chimeric junctions (Fig. 6); we do not know whether this binding is direct or involves other proteins. The CSI segment also contributes to plakoglobin binding, probably through a conformational influence: deletions or mutations in this region may considerably change conformation, which then no longer allows binding.

The importance of the short (12 aa), juxtamembranous CSI segment on desmoplakin accumulation reminds one of the effects of a segment containing cytoplasmic aa 6–27 of N-cadherin on cell adhesion during embryogenesis of *Xenopus laevis* (34). However, we have to postulate that there are also amino acid sequence elements in Dsc molecules that negatively affect desmoplakin binding, as also indicated by our observation that the shorter Dsc splice variant *b*, which contains an intact CSI region, does not bind desmoplakin (26). Finally, our results show that desmoplakin binding to Dsc tails alone is insufficient to secure IF anchorage, although desmoplakin has been reported to bind to IF under certain circumstances (14).

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