Two-hybrid Analysis Reveals Fundamental Differences in Direct Interactions between Desmoplakin and Cell Type-specific Intermediate Filaments*

(Received for publication, April 9, 1997)

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Desmosomes are cell junctions that act as sites of strong intercellular adhesion and also serve to anchor the intermediate filament (IF) cytoskeleton to the plasma membrane of a variety of cell types. Previous studies demonstrated that the COOH terminus of the desmosomal plaque protein desmoplakin (DP), is required for the association of DP with IF networks in cultured cells and that this domain interacts directly with type II epidermal keratin polypeptides in vitro. However, these studies left open the question of how desmosomes might anchor other IF types known to associate with these junctions. In this report we used yeast two-hybrid and in vitro dot blot assays to further examine the requirements for direct interactions between desmoplakin and various IF types.

Our results confirm the ability of the DP COOH terminus (DPCT) to interact with at least two regions of the head domain of the type II epidermal keratin K1 and also demonstrate that DPCT can interact with the type III IF family members, vimentin and desmin, as well as simple epithelial keratins. Unlike the situation for type II epidermal keratins, the interaction between DPCT and simple epithelial keratins appears to depend on heterodimerization of the type I and II keratin polypeptides, since both are required to detect an interaction. Furthermore, although the interaction between DPCT and K1 requires the keratin head domain, deletion of this domain from the simple epithelial keratins does not compromise interaction with DPCT. The interaction between DPCT and type III or simple epithelial keratins also appeared to be less robust than that between DPCT and K1. In the case of K8/K18, however, the interaction as assessed by yeast two-hybrid assays increased 9-fold when a serine located in a protein kinase A consensus phosphorylation site 23 residues from the end of DP was altered to a glycine. Taken together, these data indicate that DP interacts directly with different IF types in specific ways.

Desmosomes are intercellular junctions that act as cell surface attachment sites for intermediate filaments (IF) (1–8). By anchoring IF at sites of strong intercellular adhesion, desmosomes create a transcellular cytoskeletal network that is thought to confer mechanical integrity to the tissues where they reside. It is not surprising, therefore, that these junctions are particularly prominent in tissues experiencing mechanical stress such as epidermis, where they interact with IF networks comprising K5/K14 and K1/K10 keratin pairs. Desmosomes are also found in a wide variety of other tissues, including simple epithelia expressing the K8/K18 keratin pair, myocardial cells expressing desmin, and the arachnoid and pia of meninges and dendritic reticulum cells of lymph nodes, both of which express vimentin (6, 9).

For a number of years, the most abundant desmosomal plaque proteins, the desmoplakin, have been leading candidates for linking IF to the desmosomal plaque (10–12). Recent studies provide strong support for the hypothesis that these proteins are required for IF attachment to the plaque (13). Desmoplakins I and II are products of a single gene, with predicted molecular masses of 332 and 259 kDa, respectively. They are members of an emerging protein family that currently includes plectin, bullous pemphigoid antigen 1, envoplakin, and perhaps IFAP300 (14–20). Family members share blocks of sequence homology and a common structural organization first described for desmoplakin (14). Desmoplakin I is thought to form an extended homodimer with a central α-helical coiled-coil rod domain about 130 nm in length flanked by globular head and tail domains (14, 21). Desmoplakin II, which is thought to arise from alternative splicing, contains both end domains but lacks approximately two-thirds of the central rod domain (22). Transient transfections of cDNAs encoding polypeptides containing the COOH-terminal domain of DP suggested that this domain is able to associate with keratin IF networks in a number of cultured cell lines, including COS-7 and HeLa, and vimentin IF networks in mesenchymal cells (23, 24). More recently, a bacterially expressed recombinant form of DPCT was demonstrated to interact directly with type II epidermal keratins in vitro, and this association was dependent on the presence of sequences in the N-terminal head domain of K5 (25). However, interactions of DPCT with either simple epithelial keratins (K8/K18) or vimentin were not observed in this latter study, in contrast to the transfection studies discussed above (23). The question, therefore, is whether DP interactions with nonepidermal IF, which in fact are found in many in-

* This work was supported by National Institutes of Health (NIH) Grant RO1 AR35973 and American Cancer Society Grant CB104 (to W. I.), NIH Grant RO1 AR43380, March of Dimes Birth Defects Foundation Grant 1-FY96-0146, and a Faculty Research Award from the Laboratory of Skin Biology, NIAMS, National Institutes of Health, Bethesda, Maryland 20892

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stances in nature, are fundamentally different from those between DP and epidermal IF proteins. Specifically, do DP and nonepidermal IF interact directly, or are other intermediary molecules involved?

In the present study, we have employed the yeast two-hybrid system (26) to directly compare the ability of DPCT to bind to type II epidermal keratins, nonepidermal keratins, and the type III IF polypeptides vimentin and desmin. Furthermore, we have dissected in detail the mechanisms underlying these associations. Consistent with the in vitro binding data reported by Kouklis et al. (25), our results suggest that DPCT interacts with the type II epidermal keratin, K1, and that this association depends on the presence of the K1 NH2 terminus. We also show for the first time that DPCT can interact directly with nonepidermal keratins and type III IF polypeptides. Our data demonstrate that, unlike type II epidermal keratins, which can interact directly with DPCT in the absence of a type I partner, the simple epithelial keratins require the presence of both partner polypeptides to interact with DP. The interaction between K5/K18 and DPCT, but not that between K1 and DP, is enhanced by altering a single serine 23 residues from the carboxyl terminus to a glycine. These data strongly suggest that DP can interact directly with all IF types examined in this study. Importantly, however, the interactions between DP and different IF types are apparently facilitated and regulated by fundamentally different mechanisms at the molecular level.

MATERIALS AND METHODS

Yeast Strains and Media—Saccharomyces cerevisiae strain PCY2 (MATa Δgal4Δgal80 URA3–GAL1-LacZ lys2–801 amber hi3–Δ200 trpl–36Δ leu2–2Δ2Δ101Δamu1 his3–Δ200) was used for all assays. Yeast cultures were grown at 30 °C in either YEPD medium (1% yeast extract, 2% peptone, 0.004% adenine sulfate, and 2% glucose) or glucose minimal medium except when they were used for the in vitro binding assay. In the latter case, yeast was cultured in galactose minimal medium. All plasmid constructions were transformed into Escherichia coli strain XL-1 Blue (Stratagene, La Jolla, CA), and bacteria were grown in LB medium.

Construction of plasmids encoding full-length and truncated versions of the intermediate filament proteins, vimentin and keratins K5 and K18, in pPC62 and pPC86 has been described (30). Polymerase chain reaction (PCR) was performed to generate the coding sequences and a stop codon. The PCR products were then subcloned into pBlueScript II, KS (Stratagene, La Jolla, CA). All constructs were sequenced across the junctions between vector and insert to confirm that the correct sequence had been obtained. In most cases, Western blots were also performed on homogenates of positive colonies after transformation to ensure that the expected proteins were expressed.

Two-hybrid plasmids encoding keratin K1 were obtained by PCR amplification using plasmid clone B227 (K1 cDNA in PGM7 (31)) as template. The 5′ primer, K1-p-1 (5′-GATAAGTCGACAGGAGGTGGTTTGCA-3′) was used to insert a SacI restriction site 5′ to the K1 start site. The 3′ primer, SP6 primer (Promega, Madison, WI), brought a SacI restriction site from the PGM7 vector polynucleotide to the 3′ end of the K1 cDNA. The SacI/SacI fragment was then subcloned into pPC86 to obtain p68-K1. Taking advantage of the absence of SacII restriction sites in the K1 cDNA, a SacI/SacII restriction fragment encoding K1 and a stop site at the 3′ end was excised from p68-K1 and inserted into pPC62 to obtain p62-K1.

DNA encoding the entire carboxyl-terminal domain of desmoplakin I, DPCT (nucleotides 6168–8945), GenBank™ accession number M77830), was amplified from plasmid clone p148 (DPAN, nucleotides 3819–8945 (24)) by PCR with the sense primer DP-p-3 (5′-CATGTTGT-TGACCATATGGGCTCTAGTACATAGGAGTGGTG-3′) and the antisense primer DP-p-4 (5′-GGCCACTTACGACCGGCTCTAGTGTGAAGACTTGAC-3′). The PCR products were then subcloned into the XbaI/SacI site of pPC62 to yield p62-DPCT. Similarly, by using the sense primer DP-p-5 (5′-GATTACGAGCC- CCTTACGACCGGCTCTAGTGTGAAGACTTGAC-3′) and the antisense primer DP-p-6 (5′-GAGGT-GACGCTTACGACCGGCTCTAGTGTGAAGACTTGAC-3′), a PCR fragment was amplified from plasmid p148 and inserted into the EcoRI/SacI site of pPC62 to yield p86-DPCT.

The starting points for two-hybrid constructs for DPCT.Gly23C, in which a serine located 23 amino acids from the carboxyl terminus is altered to a glycine (see Ref. 32 for explanation), were pBlueDPCT and pPC86. pBlueDPCT contained DPCT cloned into a modified Bluescript KS vector in which the polynucleotide's unique SacI site was deleted. p138 encoded the rod and carboxyl-terminal domains of DP with a Ser → Gly alteration at position 23 from the carboxyl terminus (32). A SacI/EcoRI fragment from pBlueDPCT was replaced with the corresponding SacI/EcoRI fragment from p138 containing the Ser → Gly alteration. The resulting plasmid, pBlueDPCT.Gly23C, was then restriction-digested at XbaI/SacII and inserted into the XbaI/SacII site of pPC62 to obtain p62-DPCT.Gly23C. To obtain p86-DPCT.Gly23C, the same XbaI/SacII fragment was blunted by using the Klenow large fragment and ligated into pPC86.

Additional constructs of DPCT and of the rod domain of DP were generated using PCR. Primers used for these constructions, containing the appropriate restriction sites and/or stop codons, are listed in Table I.

Yeast Transformation and β-Galactosidase Assay—Transformation of yeast was performed according to the procedure of Hilt et al. (33) and Meng et al. (30). In some experiments, co-transformations involving three plasmid constructions were carried out. In such cases, the amount of the plasmid encoding Gal4 DNA-binding (DB) domain fusion protein was kept constant, while the concentrations of the other two plasmids encoding Gal4 transcription activation (TA) domain fusion proteins

### TABLE I

| Constructions          | Nucleotides | Primers used in PCR |
|------------------------|-------------|---------------------|
| p62-DPCTΔC             | 6168–8089   | 5′-CTAGTTCTGACGATGGTGGCCTCAG-3′   |
| p86-DPCTΔC             | 6168–8089   | 5′-GAACACCGGGCTAGAGGCTGCTAA-3′   |
| p62-DPCTΔ80            | 6168–7507   | p62-DPCT was digested with NcoI/SacII, blunted, and ligated. |
| p86-DPCTΔ80            | 6168–7507   | p62-DPCT was digested with NcoI/SacII, blunted, and ligated. |
| p62-DP rod             | 3819–6167   | 5′-GGCACCAGGTAGCCTAGGCTGCTAA-3′   |
| p86-DP rod             | 3819–6167   | 5′-GGCACCAGGTAGCCTAGGCTGCTAA-3′   |
| p62-DP rodΔ101         | 3819–5858   | p62-DP rod was digested with XbaI/SacI, blunted, and ligated. |
| p86-DP rodΔ101         | 3819–5858   | p62-DP rod was digested with XbaI/SacI, blunted, and ligated. |
were varied. β-Galactosidase activity was estimated qualitatively by incubation with 5-bromo-4-chloro-3-indolyl β-D-galactoside, and quantified fluorometrically as described by Meng et al. (30).

Preparation of Cell Extracts and Immunoblotting—Protein extracts of "S. cerevisiae" for immunoblotting were prepared as described (30). Protein content in cell lysates was determined by the method of Bradford (34). Immunodetection on Western blots was performed essentially according to the method of Towbin et al. (35). The monoclonal antibodies, Gal4DNA-BDmAB, which is directed against the DNA-binding domain of yeast Gal4 protein, and Gal4ADmAB, which binds the major activation domain of yeast Gal4 protein (CLONTECH Laboratories, Palo Alto, CA) were used as the primary antibody at dilutions recommended by the manufacturer. Alkaline phosphatase-conjugated, affinity-purified, goat anti-mouse IgG (Kirkegaard & Perry Laboratories, Gaithersburg, MD) was used as the secondary antibody at a dilution of 1:2000.

Dot Blot Assay—In vitro protein binding assays were carried out using a modification of the method described by Kouklis et al. (25). Human K1/K10 keratin intermediate filaments (KIF) were extracted, prepared, and assembled as described previously (36). Bovine tongue DP was purified as described by O'Keefe et al. (21). Nitrocellulose filters were loaded with 20 pmol/slot of K1/K10 KIF in KIF assembly buffer (10 mM Tris-HCl (pH 7.6), 1 mM EDTA, 1 mM dithiothreitol). Filters were washed three times with blocking solution containing phosphate-buffered saline plus 5% bovine serum albumin, 1 mM MgCl₂, 0.1% Tween 20 and were then overlaid with 65 μl of desmoplakin at 25 μg/ml (5 pmol) plus peptide in the following amounts: 0, 5 pmol (molar ratio of 1:1), 15 pmol (3:1), or 25 pmol (5:1). Filters were then incubated for 2 h at 4 °C. After one wash with phosphate-buffered saline plus 0.1% Tween 20 and two additional rinses with phosphate-buffered saline plus 0.1% Tween 20 containing 1 mM KCl, filters were processed as for immunoblotting. A primary DP polyclonal antibody raised in rabbit (a generous gift from Dr. Robert Goldman, Northwestern Medical School) was used at 1:100, followed by a secondary antibody conjugated to horseradish peroxidase (Cappel Laboratories, Durham, NC) at 1:5000 or 1:8000 depending on the batch. Filters were washed with phosphate-buffered saline/Tween three times and developed using the Amersham enhanced chemiluminescence kit. Three exposures of 5, 15, and 60 s were taken for each filter. X-ray films were scanned and digitized using NIH Image 1.6. The film image was gridded to delineate a 9 × 9-mm square enclosing each spot, and a density value was assigned to each square. Values were compared with a standard curve generated using purified DP protein at concentrations ranging from 0.1 to 5 nmol to ensure that values were determined on spots within the linear range for the film. The average densities were then expressed as a percentage of the control.

RESULTS

Characterization of Control Plasmids: Dimerization of DP—Controls for the vectors and yeast host used in this study have been presented in a recent report (30). In short, these controls demonstrate that in the absence of cDNA encoding the proteins of interest, the two vectors, pPC62 and pPC86, do not reconstitute Gal4 activity when introduced by transformation into PCY2 cells either alone or together. Co-transformations in which the cDNA insert was omitted from one of the two vectors also produced negative results.

In addition to these controls, several studies have shown that the two-hybrid system is both specific and sensitive in detecting interactions involving IF proteins (30, 37–39). In all cases, transformations of yeast cells with IF protein-encoding Gal4 constructs faithfully reported interactions that were expected to occur and were negative for interactions known not to occur as tested by other approaches. Several additional controls pertained directly to the present study. Sequence analysis of DP predicted that it should form a coiled-coil dimer via its α-helical rod domain (14). Consistent with this prediction, transformation using a pair of DP rod-Gal4 DB and DP rod-Gal4 TA fusion constructs generated β-galactosidase activity, whereas a single DP rod-Gal4 DB construct did not (Fig. 1). Interestingly, β-galactosidase activity fell off dramatically when the rod was truncated by 101 residues at its carboxyl end, suggesting that a site essential for dimerization is present within this stretch of the rod. Furthermore, transformation with a pair of DPCT constructs did not produce measurable β-galactosidase activity, suggesting that this domain does not form homodimers.

DPCT Interacts Directly with Epidermal Type II Keratins—Recent studies employing in vitro binding assays demonstrated a direct interaction between type II epidermal keratins and DPCT (25). Here we show that a direct interaction between DPCT and the type II epidermal keratin, K1, is also readily detected using the two-hybrid system. Co-formation of Gal4 fusion constructs expressing DPCT and K1 into PCY2
cells produced a high level of β-galactosidase activity both in qualitative 5-bromo-4-chloro-3-indoyl β-D-galactose staining of filter lifts (not shown) and in quantitative fluorescence assays (Fig. 2). This interaction apparently does not require the presence of the in vivo type I partner of K1, keratin K10. However, in agreement with the observation that headless K5 could not interact with DPCT (25), removal of the K1 head domain also abolished this interaction completely (Fig. 2).

Multiple Sequences in the K1 Head Domain Contribute to Its Interaction with DPCT—We have adapted an in vitro overlay assay using dot blotting to further explore interactions between K1 and DP in the absence or presence of a series of competing peptides. An interaction between purified native DP and K1 was confirmed (Fig. 3, line 2). However, in the presence of peptide containing contiguous sequences from residues 59–79 of K1, there was significant inhibition of reaction by competition (line 3). Peptides that contained sequences including a GSRS motif immediately upstream of the KSIS motif appeared to compete more effectively (compare lines 4 and 5). A peptide containing a conserved substitution in this region also competed efficiently (line 6), but a peptide in which the lysine residue had been substituted for an uncharged residue (as in the disease nonepidermolytic palmoplantar keratoderma) did not compete (line 7). Similarly, K1 sequences from elsewhere in the molecule (lines 13–15) also did not compete. These data confirm the previous observation that sequences in the vicinity of the KSIS region are critical for binding between type II epidermal keratin polypeptides and DP (25). As discussed in more detail below, peptides containing sequences from the tail region of DP also competed effectively (lines 10–12).

Intriguingly, two peptides from the H1 subdomain about 80 residues downstream of the KSIS motif also effectively competed for binding to DP (lines 16 and 20), but mutations of the sequence shown in line 16 that mimicked ones seen in keratin diseases did not (lines 17 and 18), thus indicating the H1 subdomain is also important for binding of K1 to DP. To examine further the role of this region in the interaction with DPCT, we constructed a yeast two-hybrid vector deleting all but the H1 domain of the K1 head (K1H1). As shown in Fig. 4, this construct brought reporter activity up to 20% of that seen with full-length K1. Together, the peptide competition and two-hybrid results strongly suggest that the H1 region of K1 contributes to its interaction with DP.

**FIG. 3. Dot blot assay.** K1/K10 polypeptides were polymerized and dotted onto filters as described under “Materials and Methods” and incubated with native purified DP in the absence or presence of varying molar ratios (shown at the top) of peptides derived from various regions of K1, DP, or related proteins (shown on the left). Bound DP was detected as described with a polyclonal DP antibody, and dot blots were quantified, yielding the numerical results shown on the right, to assess the ability of each peptide to compete for DP binding to KIF. Data are averages of 3–5 replicates. The dot blot shown here represents one of three x-ray film exposures of a typical experiment; thus, not all apparent dot intensities match the computed numbers. Amino acid residues in boldface type denote mutations seen in known keratin diseases.
as in the intestinal brush border, binding of DPCT to simple epithelial keratins (K8, K18, and K19) was not observed by Kouklis et al. (25). This observation raised the question of whether the association between DP and simple epithelial keratin networks, previously observed in transient transfection experiments (23, 24), was direct or required other accessory proteins. Here we examined this question using two-hybrid analysis. To this end, we prepared constructs encoding full-length versions of the epithelial keratins, K8 and K18, fused to either the TA or DB domains of Gal4, and used them in cotransformations with DPCT-Gal4 fusion constructs.

Initial transformations in which either a K8 or K18 fusion construct was expressed in combination with a DPCT fusion construct showed that neither keratin alone interacts with DPCT (Fig. 5). Thus, the type II epithelial keratin K18 is distinctly different from the type II epidermal keratin K1, which alone interacts with DPCT in overlay assays (25) and by two-hybrid analysis (Fig. 2).

Next we carried out a series of transformations to test whether the presence of both a type I and a type II simple epithelial keratin (presumably in the form of a heterodimer) was necessary for interaction with DPCT. This was accomplished by including in the transformation a K8-TA construct, a K18-TA construct, and a DPCT-DB construct. In a previous study, we had determined that K8 and K18 dimerized strongly in two-hybrid transformations (30). In the transformation described here, however, dimerization of K8 with K18 alone could not activate transcription of the reporter gene, because both keratins were expressed as fusion proteins with the TA domain of Gal4. With the additional presence of the DPCT-DB construct, however, a significant increase in β-galactosidase activity was detected (Fig. 5), strongly suggesting that the DNA binding domain of Gal4 was provided by the DPCT-DB construct as a result of keratin-DP interaction. This, in turn, implies that dimerization of K8 and K18 made possible an interaction between these keratins and DPCT. Of note is that the final level of reporter gene activity was only about 10% of that produced by the interaction between keratin K1 and DPCT. Nonetheless, this level of activity was reproducible by interchanging cDNA inserts and vectors. Western blot analysis of yeast homogenates of positive clones (Fig. 6) further confirmed the presence of appropriately expressed fusion proteins.

We also detected a second important difference between the interaction of DPCT with epidermal keratins and that with simple epithelial keratins. While the site of interaction in the epidermal keratin K1 appears to be in the head domain (25) and its removal abolishes interaction with DPCT, in the case of K8 and K18, head domain removal did not affect their interaction with DPCT significantly (Fig. 5).

**Sequences in DPCT Important for Interactions with Keratin IF—** Previously, it was demonstrated that deletion of the last 68 residues of DPCT abrogated alignment with keratin, but not vimentin, networks in cultured cells (23). To further assess the
role of this region in direct interactions with IF polypeptides, constructs were made in which either the C subdomain (14) or the COOH-terminal 80 residues of DPCT (which included 12 residues of the C subdomain) were deleted. When tested in the two-hybrid assay for interactions with K1 and K8/K18, we found that in each case the reporter gene activity fell to baseline levels (Fig. 7).

The importance of this region was also confirmed by the ability of a peptide corresponding to DP amino acids 2801–2825 (GenBank™ accession number M77830) to inhibit binding of purified DP to KIF in the dot blot assay (Fig. 3, line 12). This peptide contains 20 residues that were previously shown to be critical for interaction of DP with keratin networks in cultured cells. Interestingly, two overlapping peptides upstream of this stretch of amino acids were equally effective at inhibiting binding to K1 (Fig. 3, lines 10 and 11). Peptides modeled after equivalent regions in envoplakin and plectin (lines 8 and 9) also competed but were less effective.

Stappenbeck et al. (32) observed that phosphorylation of a serine 23 residues from the COOH terminus of DP inhibits the interaction of DP with simple epithelial keratin networks in several cell types. To determine if this modulation took place at the level of the keratin dimer, we carried out transformations using a DPCT variant in which the serine residue in question was converted to a glycine, DPCT.GlyC23 (Fig. 8). As with the wild type DPCT, this variant did not interact with either K8 or K18 alone, but it interacted with both keratins to produce a level of reporter gene activity that was nearly 9-fold higher than that obtained using the wild type DPCT construct (Fig. 8). This level approaches that of DPCT-epidermal keratin interaction (see Fig. 2). Interestingly, this amino acid alteration did not significantly affect DPCT's interaction with K1 (compare Figs. 8 and 2), with reporter gene activity levels remaining high in both cases. Finally, the robust interaction between the glycine variant of DPCT and K8/K18 did not appear to depend on either the K8 or K18 head domain; K8ΔH and K18ΔH, headless versions of K8 and K18, produced similar activities upon transformation.

**DPCT Interacts with Type III IF Proteins**—There are many known instances in which non-keratin IF associate with desmosomes. Notable among these are intercalated discs in cardiac muscle, where the IF contain desmin, and the arachnoid and pia of meninges and dendritic reticulum, in which cells have IF containing vimentin (4, 6). It is therefore puzzling that DPCT did not appear to interact with type III IF proteins in the overlay assays of Kouklis et al. (25). One possibility was that the interaction between type III IF proteins and DP may not be as strong and therefore may have escaped detection. We explored this possibility using the two-hybrid assay.

As shown in Fig. 9, the type III IF proteins, vimentin and desmin, do interact with DPCT but at a level about 50% of that of epidermal keratin K1. Of interest is that several modifications of the COOH end of DPCT augmented the interaction significantly. These were the removal of the C subdomain and the terminal 68 residues, removal of the 80 COOH-most residues, and the variant, DPCT.Gly23C. All of these modified
forms of DPCT enhanced interaction with vimentin by about 50% (Fig. 9).

DISCUSSION

The results presented here provide strong confirmation of previous transfection and in vitro biochemical studies demonstrating that DPCT interacts directly with IF (23–25). Also consistent with the previously reported formation of desmosplakin I homodimers (21), the DP rod domain dimerizes as assessed by two-hybrid analysis. Most importantly, however, these studies have extended our current understanding of DP/IF interactions by revealing that DP interacts with different types of IF by fundamentally different mechanisms.

DPCT Interacts Directly with all Types of IFs Tested—An interaction between DP and a type II epidermal keratin (K1 in this study) was observed using the two-hybrid approach, confirming previous biochemical studies that employed in vitro overlay and filter binding assays (25). We were also able to detect direct interactions between DPCT and type III IF proteins, vimentin and desmin, as well as simple epithelial keratins, K8/K18. These results are consistent with the transient transfection data of Stappenbeck and Green (24) and Stappenbeck et al. (23), demonstrating that DPCT aligns with IF networks comprising these IF types.

The results of the present study provide possible explanations for the lack of observed association between DPCT and either simple epithelial keratins or type III IF proteins in previous in vitro biochemical analyses (25). First, our results suggest that in the case of simple epithelial keratins, the type I and type II partner proteins must both be present for an interaction with DPCT to take place. The Kouklis study did not test this possibility, since the experiments were carried out on SDS-polyacrylamide gel electrophoresis-separated proteins.

A second possible explanation may lie in the relative strength of interaction between DP and IF proteins of different types. Transformations with type III IF proteins and DPCT (Fig. 9) yielded $\beta$-galactosidase activities approximately half of that obtained for transformations with the type II epidermal keratin, K1 (Fig. 2). Although, strictly speaking, reporter gene activities in two-hybrid assays do not vary linearly with strengths of interaction, they generally can be used to compare strengths among interactions of a similar type, such as those involving variants of a class of proteins (41). Taken in this context, interactions between DPCT and type III IF proteins may be significantly weaker than those between DPCT and epidermal keratins and could have escaped detection in in vitro binding assays. Indeed, robust alignment of DP with vimentin IF networks in transient transfection assays requires the DP rod domain (23, 24), suggesting that the interaction between full-length DP and vimentin IF in vivo may be strengthened considerably by the formation of DP coiled-coil dimers. In this light, it is perhaps not surprising that a vimentin-DPCT interaction was not detected in the Kouklis study.

Different IF Types Interact with DP via Distinct Sequences in Different IF Domains—The yeast two-hybrid data presented here suggest that the mechanisms by which type II epidermal keratins and simple epithelial keratins or type III IF proteins interact with DPCT are fundamentally different. That is, type II epidermal keratins interact with DPCT via sequences in the NH$_2$-terminal head domain of a single keratin polypeptide chain, whereas simple epithelial keratins (K8/K18) interact via sequences in the rod domain. In the case of K8/K18, the central rod regions are sufficient to interact with DPCT but must be co-expressed, suggesting that the interaction interface requires the tertiary structure of the $\alpha$-helical coiled-coil. Type III proteins may use a similar mechanism, since short regions of the

FIG. 8. Quantitative two-hybrid analysis of interactions demonstrating that a serine — glycine alteration 23 amino acids from the very COOH terminus of DP greatly enhances interactions between DPCT and K8/K18. Gly23C refers to this glycine variant of DP. The interaction between the serine variant and K8/K18 is included at the far left as a reference. The same convention for $x$ and $y$ axes as described in Fig. 1 is used here.

FIG. 9. Quantitative two-hybrid analysis demonstrating interactions between the type III IF proteins vimentin and desmin and DPCT and variants thereof. Note that these interactions yield reporter gene activities about 40–80% of that produced by interaction between DPCT and the epidermal keratin K1.
vimentin rod domain, but not the NH₂-terminal head domain, interact with DPCT (data not shown).

Previous work defined the importance of the K5 head domain in the interaction with DPCT (25). The peptide sequence essential for this interaction contains the KSIS motif found in all epidermal type II keratins, but this motif is in fact absent in the simple epithelial K8, vimentin, and type I keratins. Here we confirm using both in vitro dot blot assays and the yeast two-hybrid assay that like K5, the type II epidermal keratin K1 interacts with DPCT via its NH₂-terminal head domain. We show further that a peptide containing the comparable KSIS sequence effectively competes for K1's interaction with purified native DP (Fig. 3). Thus, this region appears to have general importance for the interaction of type II keratins expressed in stratified squamous epithelia with DP. Furthermore, using both peptide inhibition (Fig. 3) and two-hybrid analyses (Fig. 4), we found that the H1 region, which is downstream of the KSIS peptide but still part of the NH₂-terminal domain, also contributes to the interaction of K1 with DP (Fig. 3, line 16). Thus, multiple sequences in the NH₂ terminus appear to contribute to this interaction.

**Yeast Two-hybrid Results Support the Existence of Regulatory Sequences within the Last 80 Residues of DPCT**—The last 80 residues of DPCT are required for interactions with all keratins assessed in the yeast two-hybrid assay. These data are consistent with previous observations that deletion of the last 68 residues of DP abrogated the ability of DP to align with simple epithelial or epidermal keratins but not vimentin IF networks in cultured cells (23). This study also showed that 20 residues (amino acids 2804–2823) at the beginning of this region are required for this association. The importance of these 20 residues in the interaction between K1 and DP was confirmed here using peptide inhibition analysis, in which a peptide containing this region as well as two peptides just upstream effectively inhibited interaction between K1 and native full-length DP (Fig. 3, lines 10–12). These data are consistent with the observation that the 68 terminal amino acids, although required, are not sufficient for alignment with keratin networks in cultured cells (23).

The most COOH-terminal 80 residues of DP were not required for its interaction with vimentin (Fig. 9). In fact, deletion of these residues actually enhanced this interaction as assessed by quantitative fluorescence assays. This observation is interesting in light of a recent observation by Nikolic et al. (42). These workers reported that the interaction between vimentin and the COOH terminus of the highly related protein, plectin, was dependent on plectin repeat 5, a region equivalent to the B subdomain of DP. This subdomain is retained in our constructs, DPAC and DPΔ80. In fact, the DP subdomain B by itself also interacts with vimentin in two hybrid assays, albeit at lower levels (data not shown).

We previously showed that substitution of a glycine for a serine 23 amino acids from the end of the DP COOH terminus enhances the interaction of DP with simple epithelial keratin networks as assessed by transient transfection assays (32). Consistent with these previous findings, the glycine variant of DPCT exhibited a 9-fold increase in its interaction with K8/K18 in the yeast two-hybrid assay (Fig. 8). It has not been formally demonstrated that SerC23 is phosphorylated in yeast, as was demonstrated for DP expressed in cultured mammalian cells. Nevertheless, phosphorylation of SerC23 is a likely explanation for the observed differences in the behavior of the serine and glycine DPCT variants in the yeast two-hybrid assays. An increase in fluorescence over that observed for wild type DPCT was also observed in transformations with the DPCT.GlyC23 or DPCTΔ80 (which removes the serine residue) and vimentin (Fig. 9), suggesting that this interaction may also be regulated by phosphorylation of SerC23. However, the interaction between DPCT.GlyC23 and K1 was not detectably different as compared with that between DPCT.SerC23 and K1 (compare Fig. 9 with Fig. 2), highlighting another fundamental distinction in how different keratins and DP interact.

Although our data suggest that DP can interact directly with all IF types studied so far, it is nevertheless likely that other desmosome-associated proteins are also involved in anchoring IF to the cell surface in vivo. Plakophilin/band 6 has been shown to interact in vitro in overlay assays with IF polypeptides (43, 44). In addition, the desmoplakin family members IFAP300 and plectin, which also interact directly with IF (18, 45), may enhance the interaction of DP with keratins. Additional family members such as a 450-kDa human epidermal autoantigen (46) and envoplakin (16) also may prove in the future to play a similar role. Consistent with this idea, Ruhrberg et al. (16) recently demonstrated, using high resolution immunogold electron microscopy, that DP and envoplakin are closely associated in situ. In the special case of the assembly of a cornified cell envelope in terminally differentiating squamous epithelia, envoplakin, DP, and type II keratins are closely linked (47). Moreover, the lysine residue of the KSIS motif is essential for transglutaminase cross-linking (40, 47, 48). Finally, other proteins closely associated with IF at the IF-desmosome interface such as the recently cloned pinin molecule, although not yet shown to associate with DP or IF, are also good candidates for enhancing this interaction (49).

The importance of further defining the complex interactions that facilitate linkage of IF to the desmosome in a tissue- and differentiation-specific manner is highlighted by the existence of inherited diseases for which a molecular mechanism has not yet been defined. Already there has been a suggestion that a nonepidermolysis form of the human skin disorder, palmoplantar keratoderma, may arise due to a mutation in keratin K1 that may play a role in its interaction with DPCT (25, 40). Although mutations that give rise to human disease have been identified in the desmoplakin family members plectin (50–52) and bullous pemphigoid antigen 1 (53, 54), none yet have been identified in DP. One attractive hypothesis is that mutations in the DP tail that affect interactions with cell type-specific filament systems may give rise to inherited tissue-specific human diseases.

**Acknowledgments**—We thank all those who generously provided vectors, cDNAs and other reagents including Drs. Pierre Chevray, Daniel Nathans, Robert Evans, Bishr Omary, Joe Rothnagel, Dennis Roop, and Robert Goldman.

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