The Desmoplakin Carboxyl Terminus Coaligns with and Specifically Disrupts Intermediate Filament Networks When Expressed in Cultured Cells

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Abstract. Specific interactions between desmplakins I and II (DP I and II) and other desmosomally cytoskeletal molecules have been difficult to determine in part because of the complexity and insolubility of the desmosome and its constituents. We have used a molecular genetic approach to investigate the role that DP I and II may play in the association of the desmosomal plaque with cytoplasmic intermediate filaments (IF). A series of mammalian expression vectors encoding specific predicted domains of DP I were transiently expressed in cultured cells that form (COS-7) and do not form (NIH-3T3) desmosomes. Sequence encoding a small antigenic peptide was added to the 3' end of each mutant DP cDNA to facilitate immunolocalization of mutant DP protein. Light and electron microscopic observations revealed that DP polypeptides including the 90-kD carboxy-terminal globular domain of DP I specifically colocalized with and ultimately resulted in the complete disruption of IF in both cell lines. This effect was specific for IF as microtubule and microfilament networks were unaltered. This effect was also specific for the carboxyl terminus of DP, as the expression of the 95-kD rod domain of DP I did not visibly alter IF networks. Immunogold localization of COS-7 cells transfected with constructs including the carboxyl terminus of DP demonstrated an accumulation of mutant protein in perinuclear aggregates within which IF subunits were sequestered. These results suggest a role for the DP carboxyl terminus in the attachment of IF to the desmosome in either a direct or indirect manner.

Desmosomes are intercellular junctions that function in cell-cell adhesion and act as specific cell surface attachment sites for intermediate filaments (IF) (for reviews see Steinberg et al., 1987; Garrod et al., 1990; Green and Jones, 1990; Schwarz et al., 1990). The supracellular network of cytoplasmic IF and desmosomes is found in a number of tissues such as epithelia, myocardium, and arachnoid where desmosomes interact with keratin, desmin, and vimentin IF, respectively (Schwarz et al., 1990). While the exact function of IF has yet to be fully understood (Steinert and Roop, 1988; Klymkowsky et al., 1989), the ultrastructure of the IF-desmosome network suggests that in addition to maintaining the integrity and proper relationship among cells, it may impart increased tensile strength and mechanical resistance to whole tissues (Arrn and Staehelin, 1981). Recent work that supports this idea demonstrated that transgenic mice with disrupted IF in the basal layer of stratified epithelia displayed abnormalities in epidermal architecture and blistered easily (Vasser et al., 1991). Although cytoplasmic IF have been extensively investigated, the exact nature and importance of the link to desmosomes is unknown.

Ultrastructurally, desmosomes appear as disc-shaped regions, 0.5–2.0 μm in diameter and ~100-nm thick, symmetrically arranged about two adjacent cell membranes. Between the plasma membranes is a 20–30-nm space which contains the extracellular domains of the transmembrane glycoproteins of the desmosome (or desmoglea). The cytoplasmic portion of the desmosome consists of an electron-dense, trilaminar plaque of variable thickness (10–40 nm), which underlies both cell membranes (Schwarz et al., 1990). IF are thought to interact with the desmosome by looping through the innermost plaque region (Kelly, 1966).

The highly related molecules, desmplakins I and II (DP I and II), are the most abundant constituents of the cytoplasmic plaque region of the desmosome (Mueller and Franke, 1983; Kapprell et al., 1985). Based on an analysis of the predicted amino acid sequence (Green et al., 1990), DP I is predicted to form a homodimer comprising a central α-helical coiled-coil dimer ~130-nm long, flanked by two globular ends corresponding to the amino and carboxy termini of the molecule. This prediction is consistent with the biochemical evidence that purified and crosslinked DP I exists as a dimer in vitro. Furthermore, rotary shadowed images of purified DP I (O'Keefe et al., 1989) appeared dumbbell shaped with a central "rod" of the length predicted by Green.
et al. (1990). The rod domain of DP I is characteristic of many α-fibrous proteins including IF in that it is predominantly composed of a series of heptad repeats (Conway and Parry, 1990). DP II is thought to be derived from an alternatively spliced mRNA of DP I resulting in a greatly shortened rod domain (Green et al., 1990).

One particularly interesting feature of the 851 amino acid carboxyl terminus of DP I and II is a series of three regions that contain 4.6 copies of a 38-residue repeat (Green et al., 1990). The periodicity of the acidic and basic residues of these repeats matches that of the IB rod domain of IF thus indicating a basis for potential ionic interaction between the two molecules. The possibility that DP links IF to the desmosome has been previously suggested based on a number of observations (Green and Jones, 1990). Furthermore, the 38-residue repeat has also been found in the carboxyl terminus of plectin, which is a known IF-associated protein (Wichel et al., 1991), and the 230-kD bullish pemphigoid antigen (Green et al., 1990; Tanaka et al., 1991), which has been localized to the plaque region of hemidesmosomes. Hemidesmosomes act as attachment sites for IF at the dermal-epidermal junction (Green and Jones, 1990), so it is intriguing that this junction contains a plaque component similar to DP in desmosomes.

One difficulty with the proposed DP-mediated linkage of IF to the desmosome is that previous attempts using biochemical techniques have been unable to demonstrate an interaction between DP and IF or any other molecule (O'Keefe et al., 1989). There are several possible explanations for these results. First, if DP does directly bind IF, it is possible that the denaturing conditions used to extract DP from desmosomes during purification affected the extrinsic binding capacity of DP. Another possibility is that DP may interact directly with IF, but only in the presence of accessory proteins that would stabilize the interaction. A third possibility is that one or more linking proteins mediate an indirect association of DP and IF. The possibilities that accessory or linking proteins may be involved must be considered as the desmosomal plaque is complex and a number of other desmosomal molecules have been proposed as IF linkers (Tsukita and Tsukita, 1985; Kapprell et al., 1988; Cartaud et al., 1990; Pasdare et al., 1991). To avoid difficulties inherent with in vitro binding experiments, we have chosen a molecular genetic approach to test the possible interaction between the carboxyl terminus of DP and IF in cells that would contain any putative cofactors or linking proteins. Using the previously characterized partial cDNA of DP I, the carboxyl terminus with and without the rod domain of DP I was expressed in tissue culture cells. By tracking the pattern of expression of mutant protein by immunofluorescence and immunogold EM, we found that the carboxyl terminus of DP specifically coalesced with and eventually resulted in the disruption of IF networks.

**Materials and Methods**

**Cell Culture**

COS-7 African Green monkey kidney cells that constitutively express SV-40 large T antigen, thus replicating plasmids with an SV-40 origin of replication to a high number (Gluzman, 1981) and NIH-3T3 mouse fibroblasts were cultured in DMEM plus 10% FCS and 100 U/ml penicillin and 100 μg/ml streptomycin.
Results

Expression of Mutant DP Domains in COS-7 Cells

cDNA sequences precisely corresponding to the predicted carboxy-terminal globular domain (DPCT), the central rod domain (PDRD), and the carboxyl terminus plus the rod (amino-terminal deletion designated DP.AN) (Green et al., 1990) were cloned into the eukaryotic expression vector, pRC4B (Evans and Scarpulla, 1988). This vector uses the rat cytochrome c promoter and intron enhancer sequences to drive the expression of a cDNA insert. In addition, it encodes an SV-40 origin of replication, which increases the copy number of the plasmid in cells such as COS-7 that express large T antigen (Gluzman, 1981), thus further enhancing expression. Domain-specific constructs of DP cDNA were generated by a combination of PCR mutagenesis and routine subcloning techniques. DNA sequence analysis was used to verify structure of the completed constructs (for details see Materials and Methods). Peptide tags encoding either a 5-amino acid fragment of substance P (Albers and Fuchs, 1987, 1989) or an 11-amino acid epitope of c-myec (Munro and Pelham, 1987) were used to follow the expression of mutant DP in immunolocalization experiments. Peptide tags were used in part because species-specific DP antibodies were not readily available. Two different tags were used to ensure that observations concerning the effects of each domain were not affected by a specific peptide tag. For simplicity, the constructs in Fig. 1, pRC4DPCT, pRC4DP.ROD, and pRC4DP.AN, will be referred to herein as pDPCT, pDP.ROD, and pDP.AN, respectively.

These constructs were transiently transfected into COS-7 cells by calcium phosphate precipitation and cell extracts were processed 48 h after glycerol shock for analysis by SDS-PAGE. A rabbit polyclonal antibody directed against a fusion protein of the carboxyl terminus of DP used for immunoblotting (Angst et al., 1990) reacted only with the 90 and 185-kD proteins expressed by pDPCT and pDP.AN, respectively, as well as the endogenous 240 and 210-kD DP I and II (Boehringer Mannheim Corp., Indianapolis, IN); a 1:10 dilution of a mouse monoclonal, DPI-2.17 (Cowin et al., 1985), which has been epitope mapped to the DP I-specific region of the rod (Nilles, L., and K. Green, unpublished results), reacted with only the 95
and 185-kD proteins expressed by pDP.ROD and pDP.ΔN, respectively, as well as the endogenous DP I. The 9E10.2 mAb directed against an 11-amino acid residue fragment of c-myc reacted on immunoblots only with protein expressed by pDP.CT and pDP.ΔN tagged with this epitope (Evans et al., 1985). Antibodies specific for a fragment of substance P reacted only with proteins tagged with this epitope (not shown).

From the immunoblots, the relative amounts of each DP domain expressed appeared to be approximately equal and in each case appeared to be many times higher than endogenous DP (Fig. 2). To make a quantitative estimate on a population basis, transfected cells were metabolically labeled and extracts were immunoprecipitated using a polyclonal antibody to DP I that was reactive to all three mutants (not shown). Taking into account transfection efficiency, the relative amount of protein expressed by each construct was approximately equivalent and was 15–20-fold higher than endogenous DP I as determined by densitometric scanning of autoradiograms.

Immunolocalization of protein expressed from each construct 48 h after glycerol shock demonstrated interesting and
pressed by pDP ROD in untransfected cells (see Fig. 5a).

in the cytoplasm (Fig. 3, c and d). These cytoplasmic spots appeared to be similar to those formed by the protein expressed by pDP ROD in transfected cells (see Fig. 5a).

**Effect of Mutant DP on Cytoskeletal Systems of COS-7 Cells**

A series of double-label immunofluorescence experiments were performed to determine if endogenous cytoskeletal systems were affected by the overexpression of these mutant proteins. We were particularly interested in possible effects on IF networks. We observed that COS-7 cells express both type III vimentin and type I and II keratins, although the expression of keratin filaments was decreased in sparse cultures typically used for transfection. This phenomenon has been previously observed with cultured epithelial cells (Rheinwald and O’Connell, 1985). We also observed that COS-7 cells were capable of synthesizing desmosomal components, which is consistent with the epithelial nature of these cells. However, under the culture conditions used for transient transfection, desmosomes were formed only occasionally. Therefore we focused our analysis on the effects of each DP domain on IF.

Double label indirect immunofluorescence of COS-7 cells transfected with either pDPCT or pDP.DN revealed a striking reorganization of both keratin and vimentin networks into tight, often perinuclear, aggregates. Fig. 4, b and d demonstrates this for pDPCT and similar results were obtained with pDP AN (not shown). In both cases, the disrupted IF colocalized with the perinuclear aggregate of mutant DP. At this level of expression, >90% of transfected cells had completely disrupted IF, as compared to untransfected COS-7 cells in which 10-15% of the cells were typically observed to have disorganized IF networks. This pattern in untransfected cells is possibly a result of the transient reorganization of IF shown to occur in certain cells during mitosis (Aubin et al., 1980). The disruption was specific for IF as neither the microtubule nor the microfilament systems were affected (Fig. 4, e-h). Intriguingly, the perinuclear aggregate colocalized with the microtubule organizing center (Fig. 4, e and f). IF disruption was also specific for constructs containing the carboxyl terminus of DP, as cells that expressed the rod domain of DP I alone did not have any apparent disruption of vimentin (Fig. 5, a and b) or keratin (Fig. 5, c and d) networks. As in untransfected cells, 10-15% of cells transfected with pDP ROD were observed to have disorganized vimentin networks that did not colocalize with the cytoplasmic aggregates of protein expressed from this vector.

The nature of the carboxy-terminal-induced IF disruption was investigated by examining earlier times after transfection, using the same amount of the expression vector DNA. Mutant DP was first detected by immunofluorescence 9 h after glycerol shock. Protein expressed from both pDPCT and pDP.DN displayed a striking colocalization with intact IF (Fig. 6, a-e-d), while the protein expressed from pDP ROD was diffuse in the cytoplasm (not shown). Occasional local differences in the DPCT and vimentin patterns were observed, presumably because of the additional colocalization of mutant protein with the keratin network. These data were obtained using the 9E10.2 antibody to the c-myc tag (similar results were obtained with DP antibody), thus confirming that the colocalization was with exogenous mutant DP. At times 9–24 h after glycerol shock, many transfected cells contained bundles of IF that colocalized with mutant DP expressed from either pDPCT or pDP.DN. These IF appeared to be in the process of disruption as by 24 h most were completely disrupted in transfected cells. The result of the time course experiment indicated that the IF disruption was not because of the formation of a mutant protein aggregate which then secondarily caused IF to collapse. Similar colocalization was also observed when the amount of plasmid used in the transfection was decreased by two-thirds and cells were assayed at 24 h after glycerol shock. The colocalization and the disruption of IF were observed with DP carboxyl terminus-containing constructs that were tagless or tagged with either substance P or c-myc.

COS-7 cells transfected with pDPCT and pDP.DN were also assayed at 72–144 h after glycerol shock. At these later time points, colonies of transfected COS-7 cells with disrupted IF were observed. By 144 h, the percentage of transfected cells as determined by immunofluorescence was greatly decreased suggesting that the continuous overexpression of these constructs was ultimately toxic to cells. Immunofluorescence of the remaining viable cells expressing mutant DP typically demonstrated either the presence of short IF around the perinuclear aggregate or colocalization of mutant DP with completely reformed IF networks. It is possible that these cells were in the process of recovery and expressed very little, if any, mutant protein.

**Examination of Transfected COS-7 Cells by EM**

The aggregates of mutant protein and disrupted IF in COS-7 cells transfected with pDPCT and pDP.DN were analyzed in more detail at the ultrastructural level by both conventional and immunogold EM. The perinuclear aggregates observed at the light microscope level in cells transfected with pDPCT appeared to comprise an electron-dense accumulation of smaller aggregates in the cytoplasm of the cell that were neither located within vesicles nor associated with the nuclear membrane (Fig. 7, a and b). Immunogold labeling of transfected cells demonstrated that the aggregates contained both mutant DP and vimentin throughout the entire structure (Fig. 7, c and d), implying that the IF network did not simply collapse around a preformed aggregate of mutant DP. As a control, a mAb to β-tubulin specifically labeled the cytoplasm (Fig. 7, e). The aggregates of DPCT were electron dense with no apparent filamentous substructure, aggregates of DP.DN were composed of a filamentous meshwork. The fine filaments of this meshwork were shorter and of smaller diameter than the 8-10-nm cytoplasmic IF found in neighboring nontransfected cells. The second type of aggregate in
Figure 5. Indirect double label immunofluorescence of COS-7 cells transfected with pDP.ROD tagged with substance P 48 h after glycerol shock. a and b were stained with DPI-2.17 and a vimentin polyclonal antibody, respectively. c and d were stained with a rabbit polyclonal antibody directed against DP and RGE 53, directed against K18. Bar, 10 μm.

Expression of Mutant DP Domains in NIH-3T3 Cells Resulted In a Similar Pattern of Immunolocalization

It is possible that the apparent association of the carboxyl terminus of DP with IF in COS-7 cells was because of the presence of other desmosomal proteins present in these cells that acted as cofactors or linking proteins. To test this, mutant DP constructs were transiently transfected into NIH-3T3 mouse fibroblasts that do not form desmosomes. In each construct, the patterns of expression observed by double-label indirect immunofluorescence were similar to those seen in COS-7 cells. Immunofluorescence analysis of NIH-3T3 cells transfected with either pDPCT or pDP.ΔN 48 h after glycerol shock, revealed similar patterns of expression observed at various time points in COS-7 cells. At 48 h after glycerol shock, mutant protein colocalized with intact vimentin networks in certain cells (Fig. 9, a and b) and disrupted vimentin networks in others (Fig. 9, c and d). This variable phenotype was likely to be a result of different levels of expression...
Figure 6. Indirect double label immunofluorescence of COS-7 cells transfected with pDPCT (a and b) or pDPΔN (c and d) 9 h after glycerol shock. a and c were stained with 9E10.2, to identify c-myc tagged mutant DP. b and d were stained with a vimentin polyclonal antibody. At this time point, both mutant DPs colocalize with the vimentin IF system in transfected cells. Bar, 10 μm.

Discussion

We have presented evidence that truncated DP comprising the predicted carboxyl terminus specifically colocalizes with IF in COS-7 and NIH-3T3 cells. When expressed at high levels, IF networks in these cells are ultimately disrupted. Several lines of evidence suggest that this effect is specific both for the carboxyl terminus of DP and for the IF system. First, the rod domain of DP expressed in either cell line neither colocalized with nor disrupted IF. The possibility that differing levels of expression were responsible for the apparent specificity was ruled out by quantifying levels of mutant DP expression in transfected cells. Second, microtubule and microfilament systems were not obviously affected by the overexpression of any domain of DP. Finally, other investigators have overexpressed the entire keratin 18 cDNA in COS-1 cells using a vector with a strong promoter and an SV-40 origin of replication (Blouin et al., 1990). While K18 incorporated into the endogenous filament network, the “excess” K18 accumulated in a juxtanuclear site similar to the aggregates produced in cells overexpressing DPCT. However, in cells overexpressing K18, the keratin network remained undisturbed and the vimentin network was only partially reorganized. Therefore overexpression of an insoluble cytoskeletal molecule is not sufficient to disrupt IF even when the molecule can interact normally with endogenous IF at lower levels of expression.

In addition to the disruption of IF observed in cells that overexpressed DPCT and DPΔN, we also observed coalignment without disruption in cells that were most likely expressing lower amounts of these mutant proteins. This evidence argues for the specificity of the interaction. That the effects described above are specific for the IF system is also reflected in the nature of the disruption and the ultrastructure of the resulting aggregates. Electron microscopical analysis revealed that at high levels of expression, IF did not just collapse into whorls around the nucleus, as occurs in response to heat shock (Shyy et al., 1989) or pharmacological agents such as colchicine (for review see Klymkowsky et al., 1989). Instead, IF in transfected cells appeared to disassemble and
subsequently, IF subunits became reorganized into electron dense (DPCT) or fine filamentous meshworks (DPAN) also containing mutant protein. The apparent disassembly we observed is more in line with observations by Klymkowsky (Klymkowsky, 1981; Klymkowsky et al., 1983), in which microinjection of antibodies directed against intermediate filaments resulted in a disappearance of the IF system. IF disruption has also been observed as a result of expressing of dominant negative mutations of keratins (Albers and Fuchs, 1987, 1989; Kulesh et al., 1989; Lu and Lane, 1990; Trevor, 1990) and the 19-kD product of the EIB oncogene (White and Cipriani, 1989, 1990). However, in these latter cases, an ultrastructural analysis of the affected IF networks was not performed. Therefore, we cannot speculate on the potential similarities with the disrupted filaments we observed.

As described above both DPCT and DPAN were capable of forming aggregates with vimentin. However, as demonstrated by conventional and immunogold EM, the vimentin-containing aggregates were ultrastructurally quite different. Those formed with DPCT were electron dense and not noticeably filamentous while those formed with DPAN comprised a fine filamentous meshwork. In addition, non-vimentin-containing aggregates of DPAN or DP.ROD did not consist of fine filaments. It follows that to form filamentous aggregates, the rod domain and carboxy-terminal domains of DP as well as vimentin were all necessary. It is possible that the rod domain of DPAN allowed the formation of dimers and/or oligomers that interact with vimentin to create higher order structures whose ultrastructure differed so dramatically from the dense aggregates formed by DPCT and vimentin. It may be noteworthy that the DPAN-vimentin-containing aggregates ultrastructurally resemble the meshwork of 4–5-nm fine filaments that comprise the innermost desmosomal plaque (Pirbazari and Kelly, 1985). It is possible that by overexpression of this portion of DP which can associate with itself and vimentin, a large plaque-like structure is assembled in the cytoplasm. Expression of full-length DP including the amino terminus may facilitate the localization of this meshwork to the cell cortex.

Figure 7. EM of COS-7 cells transfected with pDPCT 48 h after glycerol shock. Cells were fixed either for conventional EM (a and b) or for immunogold EM (c–e). Clusters of extremely electron-dense aggregates in cells transfected with this construct, at low (a) and high (b) magnification. Postembedding immunogold labeling of these aggregates was done with the mouse mAbs: 9E10.2, directed against the c-myc peptide tag (c); V9, directed against vimentin (d); and E7, directed against β tubulin (e). The gold particles conjugated to anti-mouse secondary antibodies were 15 nm in diameter. Due to the density of these aggregates, the sections were not counterstained. Therefore, the aggregates appear to be less electron dense. The edge of labeled aggregates was shown in the figures to demonstrate the specificity for either the aggregate (c and d) or for the cytoplasm (e). Note that under the conditions of LR White embedment without counterstaining microtubules cannot be visualized even though their antigenicity is retained (e). N, nucleus. Bars: (a) 1 μm; (b–e) 0.25 μm.
Figure 8. EM of COS-7 cells transfected with pDPΔN 48 h after glycerol shock. Cells were fixed either for conventional EM (a and b) or for immunogold EM (c–f). Large filamentous aggregates were observed near the nucleus (N), as shown in a, and many smaller, electron-dense spots were seen in the cytoplasm of cells transfected with this construct (as shown in b). Postembedding immunogold labeling of transfected cells was carried out with the mouse mAbs: 9E10.2, directed against the c-myc peptide tag (c and e) and, V9, directed against vimentin (d and f). c and d are high magnifications of the filamentous perinuclear aggregate that were vimentin positive. e and f are high magnifications of the cytoplasmic electron-dense spots that were vimentin negative. These sections were counterstained so that the fine filamentous meshwork was visible. Bars: (a) 5 μm; (b) 1 μm; and (c–f) 0.25 μm.
The colocalization of the carboxyl terminus of DP along IF also indicates that this domain of DP may be involved in linking IF to the desmosome. The nature of this linkage still remains to be determined. In vitro binding experiments have so far been unable to demonstrate a direct interaction between DP and IF or any other molecule. One possible explanation for these results, is that the conformation of DP was altered as a result of the denaturing conditions used to solubilize DP during purification, and that this prevented the direct binding of DP to IF. However, it is possible that one or more cellular cofactors or linking proteins may be required to stabilize and/or mediate the association of DP to IF. This would explain the colocalization of DP and IF observed in tissue culture cells when no DP-IF interaction has been reported in vitro. However, the colocalization of the carboxyl terminus of DP with vimentin observed in transfected NIH-3T3 cells makes it unlikely that these factors are other proteins restricted to desmosomes.

Other molecules localized to desmosomes have been demonstrated to bind IF in vitro. However, one of these, DP IV, is only present in desmosomes of stratified epithelia (Kapprell et al., 1988) while others, including desmocalmin (Tsukita and Tsukita, 1985), a 140-kD lamin B related protein (Cartaud et al., 1990), and plectin (Foisner et al., 1991) are present in much smaller amounts than DP in the desmosome. DP IV, desmocalmin, and the 140-kD lamin B related protein are likely to be restricted to desmosomes and therefore, in light of the 3T3 results, are unlikely to be absolutely required for a DP-IF association. This does not exclude the possibility that they play some sort of stabilizing role in the plaque of the desmosome, thus explaining previous in vitro binding data. Certain desmosomal molecules, such as plectin, are not restricted to desmosomes and are also located in mesenchymal tissues. Therefore we cannot rule out that the DP-IF interaction observed in fibroblasts is not stabilized or mediated by such a protein.

The colocalization of DPCT along IF may have implications for the normal process of desmosome assembly. Although it is not clear if intact IF networks are needed for desmosome formation, previous investigators have suggested that DP is translocated to the cell surface in association with IF during the formation of desmosomes (Jones and Goldman, 1985; Pasdar and Nelson, 1988). In these experiments desmosome assembly was induced in cultured epithelial cells by switch-
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