New insights into the molecular basis of desmoplakin- and desmin-related cardiomyopathies

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Summary

Desmosomes are intercellular adhesive complexes that anchor the intermediate filament cytoskeleton to the cell membrane in epithelia and cardiac muscle cells. The desmosomal component desmoplakin plays a key role in tethering various intermediate filament networks through its C-terminal plakin repeat domain. To gain better insight into the cytoskeletal organization of cardiomyocytes, we investigated the association of desmoplakin with desmin by cell transfection, yeast two-hybrid, and/or in vitro binding assays. The results indicate that the association of desmoplakin with desmin depends on sequences within the linker region and C-terminal extremity of desmoplakin, where the B and C subdomains contribute to efficient binding; a potentially phosphorylatable serine residue in the C-terminal extremity of desmoplakin affects its association with desmin; the interaction of desmoplakin with non-filamentous desmin requires sequences contained within the desmin C-terminal rod portion and tail domain in yeast, whereas in in vitro binding studies the desmin tail is dispensable for association; and mutations in either the C-terminus of desmoplakin or the desmin tail linked to inherited cardiomyopathy seem to impair desmoplakin-desmin interaction. These studies increase our understanding of desmoplakin-intermediate filament interactions, which are important for maintenance of cytoarchitecture in cardiomyocytes, and give new insights into the molecular basis of desmoplakin- and desmin-related human diseases.

Key words: Desmoplakin, Desmin, Vimentin, Intermediate filaments, Cardiomyocyte, Cardiomyopathy

Introduction

Desmosomes are multi-protein complexes that play a key role in promoting cell-cell adhesion and in tethering the intermediate filament (IF) system to the cell membrane. These structures are abundant in tissue exposed to mechanical stress, such as the epidermis and heart. They contribute to maintaining the integrity of embryonic tissues during development (reviewed in Getsios et al., 2004). The adhesive core of desmosomes comprises transmembrane glycoproteins of the cadherin family, the desmogleins and the desmocollins. Two members of the armadillo family of proteins, plakoglobin and plakophilins, as well as desmoplakin (DP) link the IF cytoskeleton to the adhesive core of desmosomes by means of linear and lateral interactions between these various molecules (Getsios et al., 2004).

DP, the most abundant desmosomal plaque component, is a member of the plakin family, which also includes plectin, BP230, periplakin and epiplakin (reviewed in Jefferson et al., 2004). These proteins serve as cytolinkers and/or scaffolding proteins, connecting IF to other cytoskeletal networks and/or distinct sites at the plasma membrane. Plakins are predicted to form homodimers with a central coiled-coil domain flanked by globular end domains (Green et al., 1992a; Green et al., 1992b; Ruhrberg and Watt, 1997). The N-terminal region of DP encompasses a so-called plakin domain harboring α-helical bundles (Green et al., 1990) and mediates its localization to desmosomes by binding to plakoglobin and plakophilins (Stappenbeck and Green, 1992; Stappenbeck et al., 1993; Kouklis et al., 1994; Cowin and Burke, 1996; Kowalczyk et al., 1997; Smith and Fuchs, 1998). The C-terminus of DP contains a plakin repeat domain (PRD) consisting of three homologous subdomains, denoted A, B and C, that are interrupted by intervening sequences of varying length (Fig. 1). These subdomains consist of 4.5 copies of a 38-amino acid sequence repeat motif (Green et al., 1990; Choi et al., 2002). The DP tail is able to associate with various IF proteins, such as epidermal and simple epithelial keratins and vimentin (Stappenbeck and Green, 1992; Stappenbeck et al., 1993; Kouklis et al., 1994; Kowalczyk et al., 1997; Meng et al., 1997; Fontao et al., 2003). Recent studies have identified sequences that allow DP to bind to distinct IFs. Specifically, the C subdomain within the C-terminal extremity of DP binds to K5/K14 and K8/K18, while its linker subdomain is able to associate with K8/K18 and vimentin (Fontao et al., 2003). Furthermore, DP-IF interactions

are regulated by phosphorylation of Ser2849 within the DP C-terminus (Stappenbeck at al., 1994; Fontao et al., 2003; Godsel et al., 2005). Crystallographic studies have shown that the B and C subdomains exhibit a globular structure with a conserved groove, the features of which seem to be suitable for an interaction with vimentin (Choi et al., 2002), but the importance of which has yet to be empirically tested. The importance of understanding the mechanisms governing DP-IF associations is highlighted by recent studies indicating that proper attachment of IFs to the desmosomal plaque via DP is required for maintaining cell-cell adhesive strength (Huen et al., 2002).

The importance of DP in vivo is supported by the observation that DP-null mutant mice die at embryonic day 6.5 owing to defects of the extraembryonic endoderm. Furthermore, chimeric morulae expressing DP in extraembryonic tissues do not survive beyond day E9.5 as a result of defects in the developing epidermis, neuroepithelium and heart with perturbation of desmosome assembly and severing of the IF-cell membrane attachment (Gallicano et al., 1998; Gallicano et al., 2001). Finally, pathogenic mutations in the DP gene cause cardiomyopathy and palmo-plantar keratoderma (Armstrong et al., 1999; Norgett et al., 2000).

IF, which are present in all vertebrate cells and tissues, are classified into five families (Herrmann and Aebi, 2004). All IF polypeptides are built from a central rod domain, consisting of α-helical segments, flanked by globular non α-helical N-terminal head and C-terminal tail domains. The assembly of IF into long 10-12 nm caliber polymers, is initiated by the central rod of IF proteins. The process progresses by formation of dimers and tetramers, which further associate following three principal assembly modes. While the N-terminal head domains seem to control IF assembly and stabilization, their tails are thought to regulate lateral packing and stabilization of higher order filament structure (Herrmann and Aebi, 2004).

Desmin, an IF protein type III, is one of the earliest expressed muscle-specific proteins. It is detected in early phase of skeletal (Sejersen and Lendahl, 1993) and cardiac (Kachinsky et al., 1995) muscle differentiation together with vimentin and nestin, the expression of which decline during development (Carlsson et al., 2000). Desmin-null mutant mice show disorganization of myofibril architecture in mechanically stressed striated muscles, including heart (Li et al., 1996; Milner et al., 1996; Wang et al., 2001). Furthermore, desmin gene mutations cause certain forms of muscular dystrophies, with or without cardiomyopathies. Although certain mutations compromise filament assembly (Park et al., 2000; Dalakas et al., 2000; Li and Dalakas, 2001; Bär et al., 2005) at various stages, in other cases the implicated pathogenic mechanisms are unclear (Dalakas et al., 2000; Bär et al., 2004).

Ultrastructural studies have shown that desmin filaments attach in a lateral fashion to the DP-containing region of the desmosomal plaques of the heart, which suggests that special domains exist in desmin that are involved in binding (Kartenbeck et al., 1983). Recent immunoelectron microscopy studies have revealed that, at intercalated discs, desmosomes and fasciae adherentes form a highly integrated system, for which the term of area composita has been proposed (Franke et al., 2006). At the molecular level, although DP was shown to bind to desmin in yeast two-hybrid assays (Meng et al., 1997), little information is available regarding the association of DP and desmin and whether the latter is regulated as in the case of vimentin (Fontao et al., 2003). Characterization of these interactions is key to deciphering the mechanisms by which the junctional region of the intercalated discs mediates the attachment of IF bundles, generating a stress-resistant scaffolding able to integrate the mechanical forces generated during heart contraction. Recent discovery of mutations in humans (reviewed by Sen-Chowdhry et al., 2005) and engineered gene disruption of desmosomal genes (Bierkamp et al., 1996; Ruiz et al., 1996; Grossmann et al., 2004) have unequivocally confirmed the functional interdependence of desmosomal proteins and their importance for the integrity of the cytoarchitecture of cardiac muscle cells.

Therefore, the aim of our study was to investigate the interaction between DP and desmin by defining functionally important sequences implicated in the binding of these molecules with each other by comparative analysis using the yeast two-hybrid system, cell transfection studies and biochemical assays. Furthermore, we sought to gain insights into the impact of pathogenic mutations in DP and desmin on these interactions.

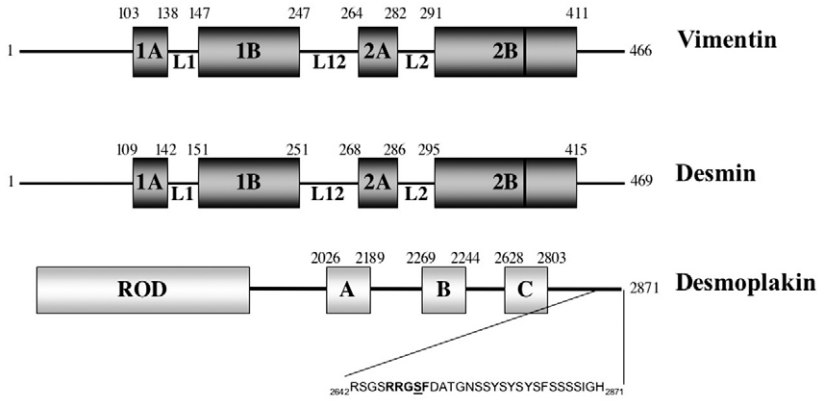

Fig. 1. Human wild type desmin, vimentin and the C-terminal half of DP showing the predicted subdomains with the corresponding amino acid positions. The location of a potential protein kinase A consensus site within the C-terminal extremity of DP is indicated in bold, while the position of Ser2849 is underlined.

Results

DP and desmin are found codistributed in the intercalated disc regions of cardiac myocytes

Previous studies have identified sequences within DP that are important for its interaction with vimentin and keratins (Stappenbeck and Green, 1992; Stappenbeck et al., 1993; Meng et al., 1997; Choi et al., 2002; Fontao et al., 2003), but the interaction of DP with desmin has not yet been characterized. Hence, we first investigated the localization of DP and desmin in longitudinal sections of monkey cardiac muscle by double immunofluorescence microscopy. As expected (Kartenbeck et al., 1983), these two proteins were both found at the level of intercalated discs. Desmin also showed the typical cross-striated labeling in a distinct sarcomeric pattern (Fig. 2).

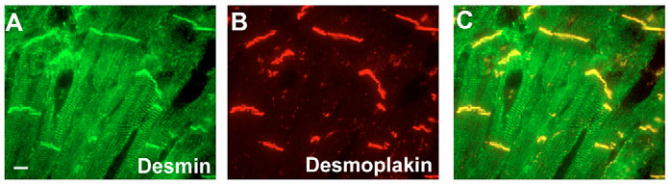

Fig. 2. Expression and localization of DP and desmin in monkey cardiomyocytes was detected by immunofluorescence microscopy (A-C). Desmin was stained with a rabbit antiserum (A) and DP with a monoclonal mouse antibody (B). The two proteins codistribute at intercalated discs, whereas desmin is also found along the cardiac muscle striations (C). Bar, 5 μm.

We then examined the distribution of ectopically expressed DP in a keratinocyte cell line stably expressing desmin, in which desmin forms a keratin-independent cytoskeleton (Magin et al., 2000). To ensure the best codistribution potential of DP, we used a DP mutant carrying the S2849G mutation within the C-terminal extremity, which abrogates a potential phosphorylation site critical for the association of DP with various IF proteins (Stappenbeck et al., 1994; Meng et al., 1997; Fontao et al., 2003; Godsel et al., 2005). Immunofluorescence microscopy studies of transfected cells expressing a DP construct encompassing the B and C subdomains and the C-terminal extremity, green fluorescent protein (GFP)-tagged at its N-terminus, demonstrated that recombinant DP-BC^{S2849G} decorated both the epidermal keratin and the desmin network (Fig. 3). However, the association with desmin appeared to be less favored than that with cytokeratins, suggesting differential binding affinities of DP for these IF networks.

To investigate the targeting of the DP tail to specific subcellular regions in cultured striated muscle cells, the GFP-DP-BC^{S2849G} construct was transiently expressed in primary neonatal rat cardiac myocytes that upon the time point of transfection variably contain assembled myofibrils with mature Z discs (van der Ven et al., 2000). Confocal laser IF microscopy studies showed a precise colocalization of DP-BC^{S2849G} with desmin (Fig. 3): the recombinant DP-BC^{S2849G} decorated the intercalated disc regions as well as the filamentous desmin network, that in some transfected cells has partially rearranged into a transverse orientation (Fig. 3).

Distinct sequences within the DP tail are required for its coalignment with desmin in co-transfected IF-free SW13 cells

To further define the regions of the DP tail required for its coalignment with desmin, we performed co-transfection studies with a series of DP constructs, including the wild-type DP tail, DP-BC, Myc-tagged at their N-terminus, and full-length desmin or vimentin hemagglutinin (HA)-tagged at their C-terminus using IF-free SW13 cells. In transfected SW13 cells, desmin only formed a rudimentary network with short filamentous structures and perinuclear aggregates, as described (Schweitzer et al., 2001; Bär et al., 2006). When co-expressed, both DP-BC and DP-BC^{S2849G} colocalized with desmin (Fig. 4A-D; Table 1) suggesting that, in IF-free SW13 cells, in analogy to other cell lines (Stappenbeck et al., 1994), the presence of S2849 did not have a major impact on the ability of the DP tail to codistribute with desmin. Deletion of the last 51 amino-acid residues from DP had no detectable effect on the codistribution (Table 1). The DP mutant containing the B subdomain and the linker region, DP-BL$^{2194-2566}$, also codistributed with desmin (Fig. 4E, F; Table 1). By contrast, the DP mutant protein containing the C subdomain and the C-

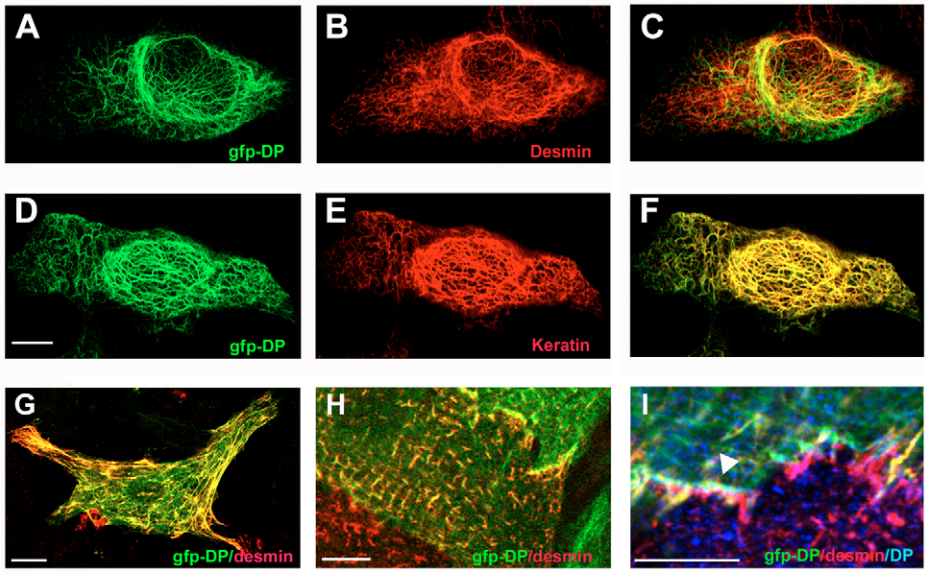

Fig. 3. Ability of DP to become codistributed with both the desmin network and keratin intermediate filaments in a transfected human keratinocyte cell line, that stably expresses desmin (A-F) as well as with the desmin network in transfected primary rat cardiomyocytes (G-I). The cells were transiently transfected with cDNA encoding DP-BC^{S2849G}, GFP-tagged at its N-terminus (A-I). Transfected cells were double-stained with an anti-GFP antiserum (A,D) and either a monoclonal antibody directed against desmin (B) or a monoclonal anti-keratin 14 antibody (E), and subsequently examined by laser confocal microscopy. Overlays are shown in C and F. Primary rat cardiomyocytes were transiently transfected with cDNA encoding GFP-tagged DP-BC^{S2849G} (G-I). The GFP is localized in the fluorescein channel. Cells were also stained with a monoclonal anti-desmin antibody (overlays in G and F) or with both the rabbit NW161 anti-DP antiserum and a monoclonal anti-desmin antibody (overlays in I). DP-BC^{S2849G} is found colocalized with desmin filaments that are either still longitudinal (overlay in G) or already rearranged into a cross-striated pattern (overlay in H). At intercalated discs, DP-BC^{S2849G} colocalizes with endogenous DP and desmin (white color in I, arrows). Notably, in cells in which the GFP-DP-BC^{S2849G} construct strongly stained the region of intercalated discs, the staining pattern of endogenous desmin extending from the junctional region into the cytoplasm appeared disorganized (arrowhead) and reduced (I) suggesting that the DP-tail acted in a dominant negative fashion by competing with the endogenous DP for sites of IF attachment at intercalated discs. Bars, 10 μm.

terminal extremity, DP-C^{S2849G}, did not colocalize with desmin but was present as cytoplasmic aggregates in transfected SW13 cells (Fig. 4G,H; Table 1). The occasional presence of clumped perinuclear material with both DP-C and desmin was most likely related to the overexpression of the recombinant proteins due to the strong CMV promotor and the inability of this cell line to maintain extended filament arrays (Bär et al., 2006), leading eventually to the formation of aggresomes. The latter have been shown to consist of misfolded proteins encircled by collapsed IF proteins (reviewed in Kopito, 2000).

Recombinant proteins encompassing either the linker region, DP-L, or the B subdomain alone, DP-B, were not detected using a polyclonal 9E10.2 antibody (Table 1), possibly owing to a toxic effect, an increased susceptibility to proteolysis of the transgene product in transfected cells, or an impaired accessibility of the antibody to the single Myc tag as suggested (Stappenbeck et al., 1993). Finally, when compared with desmin, the various DP constructs showed the same coalignment potential when co-expressed with an exogenous vimentin network (Table 1). These results suggest that a region encompassing the B subdomain with the linker region of DP contains sequences sufficient to drive the codistribution of DP with either the desmin or the vimentin network.

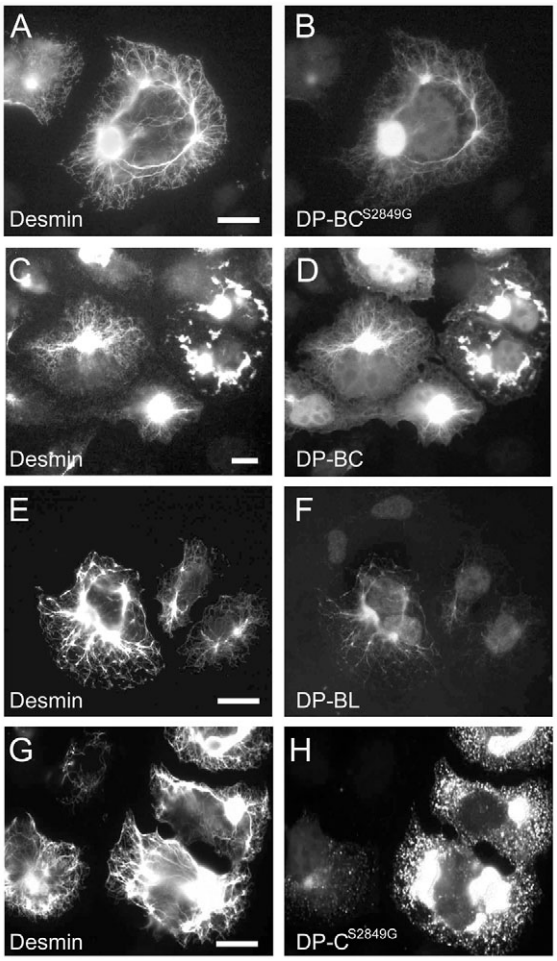

Fig. 4. Codistribution potential of the DP tail with the intermediate filament desmin network. Representatives of double immunofluorescence analysis of IF-free SW13 cells transiently transfected with cDNAs encoding desmin (A,C,E,G) tagged at the C-terminus with an HA epitope and deletion mutants DP-BC^{S2849G} (B), DP-BC (D), DP-BL (F), DP-C^{S2849G} (H) and tagged at their N-terminus with a Myc epitope. When co-expressed, both DP-BC^{S2849G} and DP-BC colocalized with desmin. In some transfected cells, disruption and collapse of the desmin network was observed (see e.g. D and E). The DP mutant containing the B subdomain and the linker region, DP-BL$^{2194-2566}$, also codistributed with desmin, while the DP mutant protein containing the C subdomain and the C-terminal extremity, DP-C^{S2849G}, did not colocalize with desmin but was present as cytoplasmic aggregates in transfected SW13 cells. Bars, 15 μm.

The DP tail associates with desmin in in vitro binding assays

Since by using a nonionic detergent buffer containing 1% Triton X-100 we were unable to solubilize DP and desmin from cultured primary rat cardiac myocytes to perform co-immunoprecipitation studies, we then tested the ability of the DP tail to associate with desmin filaments in in vitro binding assays and compared it with that of vimentin as positive control. Desmin or vimentin filaments were immobilized on nitrocellulose membranes and overlaid with in vitro transcribed and translated recombinant DP proteins that were used as fluid-phase ligands (Fig. 5). Recombinant DP-BC^{S2849G} interacted with desmin, although with an apparent lower affinity compared with vimentin. As depicted in Fig. 5, the binding efficiency of DP-BC^{S2849G} to both desmin and vimentin varied accordingly to the amount of immobilized filaments. Although binding of DP to desmin abruptly decreased when the amount of desmin was below 0.5 μg, the association of DP with vimentin was maintained for up to 0.05 μg immobilized vimentin. Furthermore, DP-BCΔ51 and DP-BL exhibited greatly reduced binding for both desmin and vimentin compared with DP-BC^{S2849G}. Finally, the recombinant proteins encompassing the linker region alone, DP-L, or the C subdomain with either the entire C-terminal extremity, DP-C^{S2849G}, or lacking the C-terminal 51-amino acids, DP-CΔ51, did not detectably interact with either desmin or vimentin. These results, which are in agreement with those obtained in cell transfection studies, indicate that DP-BL contains sequences sufficient for the interaction of DP with both desmin and, as previously reported, vimentin. However, the presence of both B and C repeats, as well as of the C-terminal extremity of DP, are required for efficient binding. Since bacterially expressed proteins are typically not phosphorylated, the presence of Ser2849 in DP is not expected to have a negative impact on the association under the condition of the overlay assays. In keeping with this idea, the ability of wild-type DP-BC recombinant to interact with desmin and vimentin (not shown) was not significantly different from that of DP-BC^{S2849G} (Fig. 8).

Identification of sequences within the DP tail interacting with desmin in yeast two-hybrid assays

To further map the association between DP and desmin, we performed yeast two-hybrid assays using a series of mutants of DP expressed as GAL4 DNA-BD fusion proteins (Fig. 6). Some of the truncated proteins carried the amino acid substitution S2849G within their C-terminal extremity (Stappenbeck et al., 1994; Meng et al., 1997; Fontao et al., 2003). DP-BC^{S2849G}, but not DP-BC, bound to desmin in this assay (Fig. 6). The constructs DP-BCΔ51, DP-BL, and DP-L

Table 1. Summary of data obtained in cell co-transfection experiments in IF-free SW13 cells, in biochemical and yeast two-hybrid studies

	Tranfection studies		Biochemical assays		Yeast two-hybrid analysis	
DP constructs	Desmin	Vimentin	Desmin	Vimentin	Desmin	Vimentin
DP-BC	n.o.	n.o.	+	+	–	–
DP-BC^{S2849G}	+	+	+	+	+	+
DP-BCΔ51	+	+	±	±	±	+
DP-BL	+	+	±	±	±	+
DP-B	n.o.	n.o.	n.d.	n.d.	–	–
DP-L	n.o.	n.o.	–	–	±	±
DP-C^{S2849G}	–	–	–	–	–	–

Cell transfection, in vitro binding and yeast two-hybrid data are based on assays showed in Figs 4, 6 and 7, respectively. + and – indicate positive or negative phenotypes, while ± indicates that binding activity was reduced in either biochemical or yeast two-hybrid assays; n.o. indicates that tagged DP was not observed in transfection studies; n.d., not done.

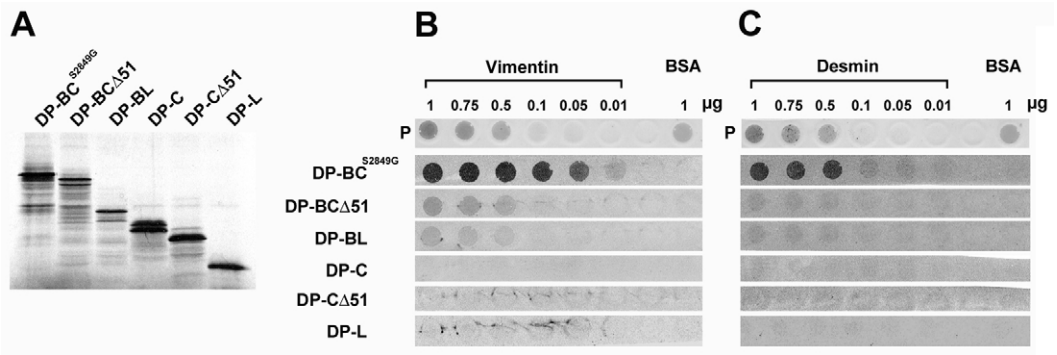

Fig. 5. Dot-blot overlay assays of purified recombinant polymerized intermediate filament vimentin (B) and desmin (C) with radiolabeled fragments of the DP tail. ^{35}S-radiolabeled c-myc-tagged recombinant forms of DP, DP-BC^{S2849G}, DP-BCΔ51, DP-BL, DP-C^{S2849G}, DP-CΔ51 and DP-L were generated by coupled in vitro transcription/translation and analyzed by SDS-PAGE and autoradiography (A). The occasional detection of additional radiolabeled protein bands most likely reflect the presence of partially transcribed and translated products (A). Different amounts of polymerized vimentin (B) and desmin (C) were immobilized on a nitrocellulose membrane by dot blotting and incubated with radiolabeled DP-BC^{S2849G}, DP-BCΔ51, DP-BL, DP-C^{S2849G}, DP-CΔ51, DP-L. Protein loading was verified by amino-black staining of the spotted proteins (P). The binding efficiency of DP-BC^{S2849G} to both desmin and vimentin varied according to the amount of immobilized filaments. While binding of DP to desmin abruptly decreased when the amount of desmin was below 0.5 μg, the association of DP with vimentin was maintained for up to 0.05 μg immobilized vimentin. Furthermore, DP-BCΔ51 and DP-BL exhibited greatly reduced binding for both desmin and vimentin compared with DP-BC^{S2849G}. Finally, DP-L, DP-C^{S2849G}, and DP-CΔ51 did not detectably interact with either desmin or vimentin.

(encompassing residues 2442-2630 of the linker region) did weakly bind to desmin only when tested as GAL4-AD fusion proteins against desmin fused to the GAL4-BD, as inferred from the activation of the reporter gene ADE2 only (Hudson et al., 2004). By contrast, DP-C^{S2849G}, DP-CΔ51 as well as DP-Ct^{S2849G} (consisting of the 79-residue-long C-terminal extremity of DP) never showed binding activity to desmin.

The ability of the various DP constructs to bind to vimentin seemed to be more robust when compared to that of desmin, since DP-BCΔ51, DP-BL and DP-L fused to GAL4 DNA-BD were all able to associate with vimentin (Fig. 6) (Fontao et al., 2003).

Since the C-terminal extremity in DP-BC seems to influence its binding to vimentin (Fontao et al., 2003), we next tested a chimeric protein consisting of the B and C subdomains of

	GAL4 DNA-BD fusion	GAL4 AD fusion	
	DP constructs	Desmin	Vimentin†
DP-BC	2194 B C 2871	-	+#
DP-BC^{S2849G}	2194 B C 2871	+#	+
DP-BCΔ51	2194 B C 2820	+*	+#
DP-BL	2194 B 2566	+*	+
DP-B	2194 B 2444	-	-
DP-L	2442 2630	+*	+#
DP-C^{S2849G}	2606 C 2871	- *	-
DP-CΔ51	2606 C 2820	-	-
BP230-BC	2077 B C 2649	-	-
BP230-BC-DPCt^{S2849G}	B C	+#	+#
DP-Ct^{S2849G}		-	-

Fig. 6. Yeast two-hybrid analysis of interactions between various subdomains of the C-terminal tail of DP fused to GAL4-BD and vimentin or desmin proteins fused to GAL4-AD. + and –, indicate growth or no growth respectively on selective media, tested as described under Materials and Methods. # indicates no growth on medium lacking adenine. * indicates that the interaction was tested between DP fused to GAL4-AD and desmin mutants fused to GAL4-BD.

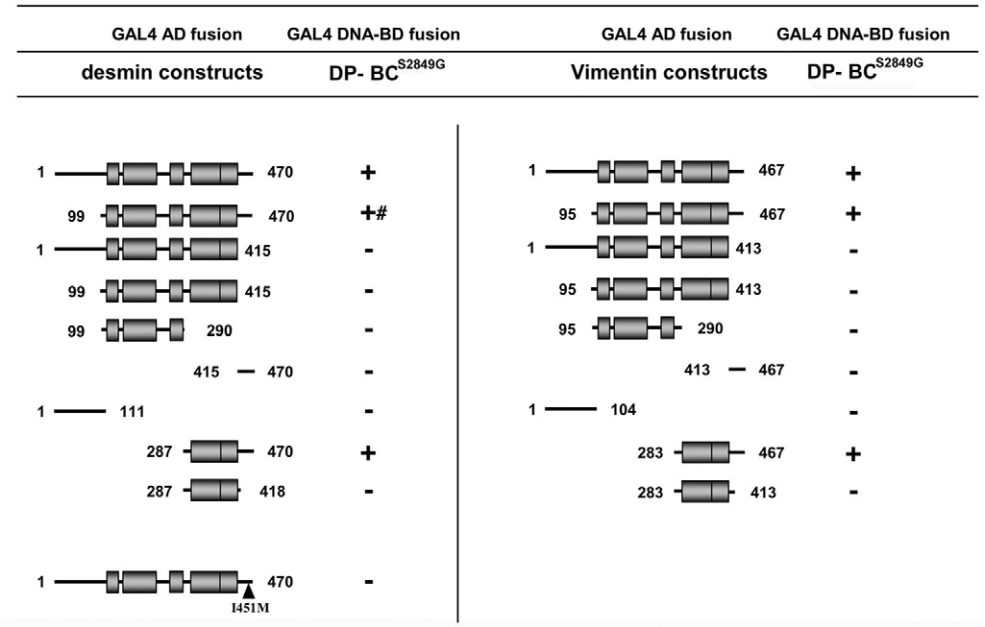

Fig. 7. Yeast two-hybrid analysis of interactions between various constructions of desmin and vimentin proteins fused to GAL4-AD and the DP-BC^{S2849G} fused to GAL4-BD. + and –, indicate growth or no growth, respectively, on selective media, tested as described under Materials and Methods. # indicates no growth on medium lacking adenine.

BP230 fused at its C-terminal extremity to the last C-terminal 51-amino acid stretch of DP with the S2849G substitution, BP230 (BC)-DP Ct^{S2849G}. The C-terminal region of BP230, another plakin family member, contains a B and C subdomain and a linker region exhibiting high homology with those of DP, but does not associate with vimentin in yeast (Fontao et al., 2003). The results indicate that the last 51-amino acid stretch within the C-terminal extremity of DP contains sequences not only affecting the association with vimentin (Fontao et al., 2003), but also to desmin, since the chimeric protein BP230 (BC)-DP Ct^{S2849G}, but not BP230-BC, was able to bind desmin (Fig. 6).

These findings support and extend results from the transfection and in vitro approaches, demonstrating that: (1) although the linker region and C-terminal extremity are critically involved in binding, the presence of the B and C subdomains is also required to ensure a robust interaction of DP with desmin, and (2) in analogy to what is observed with other IF proteins (Stappenbeck et al., 1994; Meng et al., 1997; Fontao et al., 2003), the amino acid substitution S2849G within the C-terminal extremity of DP favors its binding to desmin.

Identification of sequences within desmin and vimentin binding to DP by yeast two-hybrid assays: importance of both their C-terminal rod and tail regions

To characterize sequences within desmin or vimentin important for their association with DP, we generated a series of desmin and vimentin deletion constructs (Fig. 7). We first verified that the constructs containing a portion of the rod domain of desmin or vimentin could make homodimers in yeast (not shown). Whereas deletion of their head domain had no effect on binding to DP-BC^{S2849G}, truncation of the tail domain from either desmin or vimentin completely abolished the interaction. Furthermore, DP-BC^{S2849G} was not able to associate with the heads or tails of either desmin or vimentin with or without their entire rod domain (Fig. 7). To better map the involved interaction sites, we generated constructs encoding the C-terminal portion encompassing the linker sequence L2 and the 2B segment of these IF proteins (Fig. 7). When these constructs were tested against DP-BC^{S2849G}, desmin$^{287-470}$ and vimentin$^{283-467}$ showed binding activity in yeast, while desmin$^{287-418}$ and vimentin$^{283-413}$ did not (Fig. 7). These findings indicate that, in yeast assays, the interaction of DP-BC^{S2849G} with either desmin or vimentin depends on their tail domain, since their deletion abrogates binding; and sequences within the C-terminal region of the rod of both desmin and vimentin contribute to the interaction.

DP interacts with tailless desmin in in vitro binding assays

To verify the participation of the tail of desmin to its binding to DP, we tested the ability of DP-BC^{S2849G} to associate with tailless desmin in pull-down assays. Glutathione S-transferase (GST) fusion proteins encoding the B and C subdomains and the C-terminal extremity of DP and BP230 as control were tested for their ability to associate with in vitro transcribed and translated recombinant desmin proteins used as fluid-phase ligands. In apparent contrast to the findings obtained in yeast, DP-BC^{S2849G}, but not BP230-BC, formed a complex with both wild-type and tailless desmin (Fig. 8). We also carried out comparative overlay assays using wild-type and tailless desmin filaments, that were immobilized on nitrocellulose membranes and overlaid with in vitro transcribed and translated recombinant forms of DP-BC or DP-BC^{S2849G}. There was no detectable difference in binding activities (Fig. 8). Together, these observations indicate that under the conditions of our in vitro binding assays the tail of desmin is dispensable for DP binding.

Deletion of the desmin tail has a negative impact on the colocalization potential of DP with the desmin network in transfected cells

To further investigate in vivo the impact of the desmin tail on the ability of DP to become coaligned with the desmin network, we performed transfection studies using cDNAs for tailless desmin and DP-BC^{S2849G}. In SW13 cells expressing

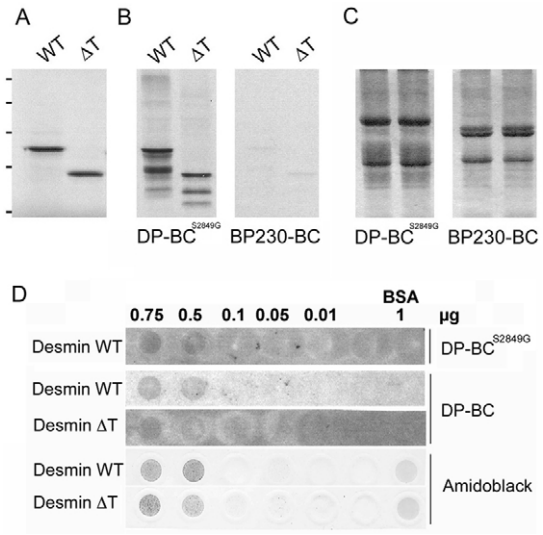

Fig. 8. Interaction of DP with wild-type desmin and tailless desmin in GST pull-down experiments. (A) ^{35}S-radiolabeled recombinant forms of wild-type desmin and tailless desmin were generated by coupled in vitro transcription/translation and analyzed by SDS-PAGE and autoradiography. Their M_r was 54K and 48K, respectively, as predicted on the basis of their cDNA. Ladder: molecular mass markers, M_r from top to bottom: 175K, 83K, 62K, 47.5K and 32.5K. (B) GST-fusion proteins of DP-BC^{S2849G} and of BP230-BC were immobilized on glutathione agarose beads and incubated with either ^{35}S-labeled wild-type desmin or tailless desmin. After washing, samples were resolved by SDS-PAGE and ^{35}S-labeled bound proteins were visualized by autoradiography (C). Note that wild-type and tailless desmin showed a tendency to proteolytic degradation under the conditions of the GST pull-down experiments. (B) Coomassie blue staining of the gel depicted in panel B to verify equimolar loading of GST fusion proteins. (D) Different amounts of polymerized wild-type and tailless desmin were immobilized on a nitrocellulose membrane by dot blotting and incubated with radiolabeled DP-BC. Protein loading was verified by amino-black staining of the spotted proteins. The binding efficiency of DP-BC to both wild-type and tailless desmin was not significantly different and abruptly decreased when the amount of desmin was below 0.5 μg. Note that binding of radiolabeled DP-BC^{S2849G} to wild-type desmin was comparable with that of DP-BC.

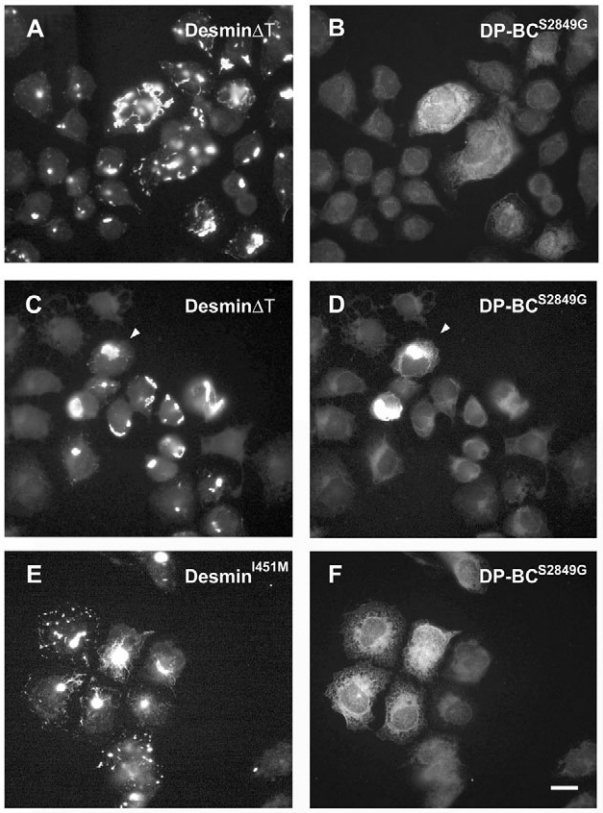

Fig. 9. The potential of the DP tail to become codistributed with the desmin network is affected by sequences contained within the rod and tail domains of desmin. Intermediate-filament-free SW13 cells were transiently co-transfected with the cDNAs encoding DP-BC^{S2849G}, Myc-tagged at the N-terminus (B,D,F) and tailless desmin (DesminΔT) (A,C), or desmin I451M (E). Although in most transfected cells, DP and tailless desmin were found together in spot-like and large cytoplasmic aggregates (C,D), in ~5% of the transfected cells the recombinant DP-BC^{S2849G} was found diffusely distributed in the cytoplasm without obvious colocalization with tailless desmin (A,B). In a few cells expressing both desmin^{I451M} and DP-BC^{S2849G}, DP-BC^{S2849G} did not become coaligned with desmin^{I451M}, but retained a cytoplasmic distribution (E,F). Bar, 20 μm.

tailless desmin, only short thin filamentous structures and cytoplasmic aggregates were observed (Fig. 9A,C) (Bär et al., 2006). In the majority of co-transfected cells, DP and tailless desmin were found together in spot-like or large cytoplasmic aggregates (Fig. 9C,D). However, in a percentage of transfected cells, in which transgene expression was not high (up to 10 out of 100 analyzed cells), as judged by immunofluorescence microscopy, the recombinant DP-BC^{S2849G} was diffusely distributed in the cytoplasm and did not colocalize with desmin (Fig. 9A,B), suggesting that the codistribution potential of DP with tailless desmin in these cells was impaired. In this context, it is possible that the observed perinuclear cytoplasmic aggregates containing both DP-BC^{S2849G} and tailless desmin reflects aggresome formation due to protein overexpression rather than true codistribution. In fact, a profound redistribution of the IF network is observed during the formation of aggresomes. The latter are finally interspersed and surrounded by IF (Kopito, 2000). Together, these findings provide evidence indicating that deletion of the desmin tail exerts a negative impact on the colocalization potential of DP with the desmin network.

Effects of disease-causing mutations on DP-desmin interaction

A recessive mutation in the DP gene leading to a truncated DP protein of 2541 residues lacking the C subdomain and C-terminal extremity has been shown to cause cardiac defects (Norgett et al., 2000). When a similar truncated protein encompassing residues 2194 to 2566 of DP was tested (see DP-BL) in both overlay and yeast two-hybrid assays, the truncated protein exhibited a reduced ability to interact with desmin.

Since our findings suggested an implication of the desmin tail in its binding to DP, we then investigated the effect of a dominant desmin mutation associated with cardiomyopathy. The mutation consisted of a substitution of an Ile to a Met at position 451 within the desmin tail, desmin^{I451M} (Li et al.,

1999; Dalakas et al., 2003). In yeast two-hybrid assays, the mutated molecule, which was able to self-dimerize (not shown) showed no binding activity with DP-BC^{S2849G} (Fig. 7). We next carried out co-transfection studies using IF-free SW13 cells (Fig. 9). When single transfected cells expressing desmin^{I451M} were examined, the majority of cells exhibited, in addition to small spot-like aggregates, thin and short filamentous structures, which suggests that the mutated desmin^{I451M} molecule retained some ability to assemble into higher ordered structures (Fig. 9E) (Dalakas et al., 2003). In the majority of co-transfected cells, DP-BC^{S2849G} was found together with desmin^{I451M} in cytoplasmic aggregates. However, in cells in which transgene expression was not high (5-10% of the cells), as judged by immunofluorescence microscopy, DP-BC^{S2849G} exhibited a diffuse cytoplasmic distribution with no obvious colocalization with desmin^{I451M} similarly to that observed with tailless desmin (see above) (Fig. 9E,F). This observation provides further support to the idea that the desmin tail contributes to its interaction with DP. Together, mutations in either DP or the desmin tail may impair their association.

Discussion

Binding of the desmin IF system to the intercalated disc regions in cardiac myocytes is thought to critically participate in the establishment of a stress-resistant scaffold that integrates the mechanical forces generated during heart contraction. Here we have dissected the interaction of DP, a junctional plaque protein of intercalated discs (Franke et al., 2006), with desmin. Our findings show that DP is able to associate directly with desmin as assessed by biochemical and yeast two-hybrid assays. In extension to previous studies (Meng et al., 1997; Fontao et al., 2003), we show that sequences contained in the linker region and the C-terminus of DP are not only critical for binding to vimentin and cytokeratins (Fontao et al., 2003), but also to desmin. However, the presence of both B and C repeats and flanking sequences is essential for efficient DP binding to desmin. Furthermore, the interaction of DP with non-filamentous desmin and vimentin in yeast assays is dependent on sequences contained within the C-terminal portion of the rod and the tail domain, whereas in in vitro binding studies DP is also able to associate with polymerized tailless desmin. This observation suggests that the assembly state of desmin affects its binding to DP. Finally, we obtained evidence that disease-causing mutations in either the DP or desmin gene may have an as yet unrecognized impact on the ability of DP and desmin to associate with each other.

Identification of sequences within the DP tail required for its association with desmin

Here we demonstrate that regions of DP important for vimentin binding (Stappenbeck et al., 1993; Fontao et al., 2003) (this study) also contain recognition sites contributing to the interaction with desmin. First, in transfection studies, we found that the DP tail is not only able to coalign with the desmin network in non-myogenic cell types, but is also targeted to the subcellular region of cardiac myocytes, where the endogenous desmin network is found. Furthermore, combined yeast and ligand-binding studies indicate that the linker region and the C-terminus of DP are critically implicated in the association with desmin, but that the B and C plakin repeat domains are required to ensure robust binding. This situation is thus consistent with the results of in vitro binding assays showing that a recombinant DP protein encompassing both the B and C subdomains associates better with vimentin than the isolated B or C subdomain (Choi et al., 2002). In this context, it should be noted that although the regions within the DP tail implicated in binding to vimentin and desmin were similar, the association of DP with vimentin appeared to be invariably stronger than that with desmin. Overall the presented results provide further support to the theory that the linker region between the B and C subdomain and C-terminal extremity of DP encompass sequences critical for binding to both desmin and vimentin, while the B and C subdomains participate to ensure a robust and efficient association (Fontao et al., 2003) (Fig. 10).

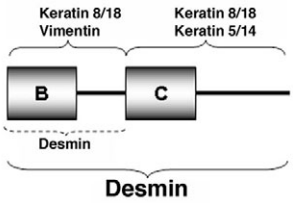

Fig. 10. Binding sites for various IF proteins on the tail of DP. The linker region between the B and C plakin-repeats and the C-terminal extremity contain sequences critical for IF binding and ensure binding specificity. The B and C plakin-repeat domains participate in the association by providing additional binding sites and/or affecting the proper folding of the linker region and C-terminal extremity. While the linker region and the B plakin repeat of DP contain the minimal sequences required for its association with desmin, the C plakin repeat and C-terminal extremity contribute to efficient binding.

The presence of Ser at position 2849 within the C-terminal extremity of DP affects the association with desmin

Phosphorylation of the tail of DP and plectin seems to critically modulate their interaction with various IFs (Fontao et al., 2003; Godsel et al., 2005). The 68-residue long COOH extremity of DP contains smaller repeating units of G-S-R-X, the last of which is modified to G-S-R-R-G-S and may serve as a target sequence for protein kinases. Evidence has been provided indicating that the C-terminal extremity of DP is indeed subject to phosphorylation in vivo in distinct cell lines (Stappenbeck et al., 1994; Godsel et al., 2005) and in the PJ-49A yeast strain utilized here (Fontao et al., 2003). Our results in yeast reveal that the DP-BC mutant carrying the substitution S2849G, but none of the recombinant proteins containing the wild-type COOH extremity of DP, showed binding activity to desmin and vimentin. Recent studies have demonstrated that a DP molecule containing the S2849G substitution shows an enhanced association with IFs in living cells. This phosphorylation-deficient mutant was found at a five-times greater ratio in the Triton X-100 insoluble fraction when compared with the wild-type construct (Godsel et al., 2005). Together, these findings further support the idea that phosphorylation of the DP tail represents a general means by which the association of DP with various IF types is differentially modulated. In this context, evidence has been recently provided indicating that the interaction between cadherin-catenin complexes with the actin cytoskeleton is not

static and stable, but rather dynamic, a means which would enable the cells to undergo constant morphogenetic changes (Yamada et al., 2005). Therefore, further studies are needed to test whether the linkage of desmosomal molecule-containing cell junctions with the IF system is also more dynamic than currently appreciated and to precise the role of phosphorylation events in local regulation of this connection.

An increased importance of the tail domain of desmin for the interaction with DP in yeast, but not in ligand-binding assays

Evidence exists indicating that the rod domain of different IF proteins can mediate their association with distinct cytolinkers, such as DP, BP230, plectin and plakophilins (Foisner et al., 1988; Hofmann et al., 2000; Fontao et al., 2003). Specifically, two studies suggested that the rod domain of vimentin mediates the interaction with DP (Meng et al., 1997; Choi et al., 2002). Crystallographic studies of Choi et al. (Choi et al., 2002) showed that the B and C subdomains of DP exhibit a conserved basic groove, a feature that would potentially allow an interaction with the rod of vimentin. Although our overall findings do not exclude a critical role of the rod domain of desmin and vimentin for binding to DP, they provide evidence that there are additional important sequences in their tail domain that contribute to the interaction. In fact, deletion of their tail domain abrogated their binding to DP in yeast. Furthermore, the colocalization potential of DP with tailless desmin was occasionally impaired in transfected cells. The presence of a missense mutation in the desmin tail linked to cardiomyopathy (see below) had similar consequences. Hence, our findings suggest that the tails of desmin and vimentin participate in the establishment of cytoskeletal architecture by favoring their connection with DP and thus membrane sites.

Intriguingly, yeast two-hybrid assays appeared to be more stringent than in vitro binding assays and transfection studies, in which DP was still able to associate and become coaligned with tailless desmin, respectively. It is possible that the binding sites on the C-terminal rod region are sufficient to ensure the association of DP under the experimental conditions of the overlay or transfection studies, in which DP is tested against a polymerized and reconstituted IF network. By contrast, in yeast, desmin and vimentin are only expected to form either homodimers or tetramers (Meng et al., 1997). Therefore, the assembly state of desmin and vimentin may have a critical impact on the conformation and/or the number of available recognition sites important for their interaction with DP. In this regard, the situation is reminiscent of previous studies indicating that the nonfilamentous tetrameric form of desmin has substantially different binding abilities for calponin from that of polymeric filamentous desmin (Mabuchi et al., 1997).

Recent yeast two-hybrid assays from our laboratory (our unpublished data) indicate that the N-terminal half of the desmin rod mediates its association with plectin, another desmin-binding protein of the plakin family that is also expressed in cardiomyocytes (Reipert et al., 1999). These preliminary observations and current findings support the idea that desmin binds to DP and plectin by using a different set of sequences located in the C-terminal and N-terminal portion of the rod domain, respectively. This may provide a means by which desmin participates in multiple and non-competitive molecular interactions with various cytolinkers to strengthen IF connections. This seems essential in vivo, since the presence of plectin at intercalated disc regions is not sufficient to fully compensate defects of DP function in heart of either humans or mice (Gallicano et al., 2001).

DP and desmin mutations can impair the DP-desmin interaction: evidence for the molecular basis of DP- and desmin-related cardiomyopathies

DP gene mutations have been linked to cardiomyopathies (Armstrong et al., 1999; Norgett at al., 2000; Alcalai et al., 2003). Notably, in myocardial sections from one patient with a mutation leading to a truncated DP protein lacking the C subdomain and C-terminal extremity, desmin did not localize to intercalated discs, which suggests an impairment in the DP-desmin interaction is the underlying causative mechanism (Kaplan et al., 2004). Our findings provide strong support for this idea, since a recombinant DP protein (DP-BL) similar to the above mentioned inherited DP tail truncation (Norgett et al., 2000) showed reduced binding activities to desmin. Strikingly, we found that a recessively inherited missense mutation in the B subdomain (Alcalai et al., 2003) also impairs DP-desmin association, since a DP-BC construct carrying the G2375R substitution was unable to interact with desmin in yeast (not shown). The substitution of this Gly, which adopts a backbone conformation with formation of a sharp turn at the end of the third plakin repeat (Choi et al., 2002), by an Arg is expected to profoundly affect the structure of the B subdomain and thus of the DP tail. This observation further underlines the participation of the plakin repeats to IF-binding.

Desmin mutations may also lead to cardiomyopathy (Goldfarb et al., 1998; Li et al., 1999; Dalakas et al., 2003). These mutations, most of which are located within the 2B segment of the rod, interfere with the IF assembly process at distinct stages, although some mutants form normal-looking IFs (Bär et al., 2005; Bär et al., 2006). In this context, a desmin mutant with a missense mutation in the tail, desmin^{I451M}, exhibited only a partial impairment of IF network formation, which raises the possibility that certain mutations, besides impairing IF assembly, contribute to disease by other mechanisms (Li et al., 1999; Dalakas et al., 2000). Our results supports this idea. First, when tested in yeast, desmin^{I451M} was no longer able to interact with DP, whereas another desmin mutant with a L385P substitution within the 2B segment did (not shown). Furthermore, in transfection studies, the potential of DP to coalign with desmin^{I451M} appeared reduced. Hence, mutations in the desmin tail may have an as yet unrecognized impact on DP-desmin interaction, impairing IF-membrane attachments. The latter have been found to regulate intercellular adhesive strength (Huen et al., 2002). In line with this idea, it should be mentioned that, in desmin-null mutant mice, changes at intercalated discs have been observed, where intercellular gaps form between opposing cardiomyocytes (Thornell et al., 1997).

In conclusion, our study demonstrates for the first time that, in analogy to what was found with vimentin, binding of DP to desmin depends on sequences within the linker region and C-terminal extremity of DP with its B and C subdomains contributing to efficient binding. Furthermore, the C-terminal rod region and tail of desmin contain recognition sites implicated in the interaction with DP. Finally, inherited

mutations in these proteins may critically impair their association. These studies further increase our understanding of DP-IF interactions important for maintenance of cytoarchitecture in cardiac cells and give new insights into the molecular basis of desmosomal proteins and desmin-related human cardiomyopathies.

Materials and Methods

cDNA constructs

Plasmid inserts were generated by restriction digestion or PCR using the proofreading Pfu DNA polymerase (Promega, Madison, WI) and gene-specific sense and antisense primers containing restriction site tags. Primer design was based on human DP, vimentin and desmin sequences (GenBank acc. no. m77830, bc030573 and nm-001927, respectively). Mutagenesis was carried out using the Quick Change site-directed mutagenesis kit (Stratagene, La Jolla, CA). The various DP deletion mutants and IF proteins were cloned into either the yeast GAL4 DNA-BD vector pAS2-1 or GAL4-AD vector pACT2 (Clontech, Palo Alto, CA), into the eukaryotic expression vector pEGFP-C3 (Clontech), pcDNA3-myc vector (Fontao et al., 2003), the prokaryotic expression vector pET15b (Novagen, Madison, WI) and pGEX-2T (Amersham Pharmacia Biotech, Piscataway, NJ). The chimeric construct BP(BC)-DP(Ct) consisting of residues 2077 to 2649 of BP230 fused to residues 2821 to 2871 of DP with or without the S2849G mutation has been previously described (Fontao et al., 2003). Sequences were confirmed by nucleotide sequencing.

Cell culture, transfection and immunofluorescence microscopy studies

The SW13 clone 2l human adrenal carcinoma cell line has been previously described (Sarria et al., 1990). Cells were cultured in DMEM and Ham's F12 media, supplemented with 5% FBS, 100 U/ml glutamine, 100 U/ml penicillin and 100 U/ml streptomycin. Cells were grown at 37°C in a humidified 5% CO_2 atmosphere. The human KEB-3D keratinocyte cell line stably expressing desmin was cultured as described (Magin et al., 2000). Cells were grown to 40-60% confluence on glass coverslips in 6-well tissue-culture plates. Transient transfections were performed with 0.8 μg cDNA using 2 μl of Lipofectamine 2000 (Invitrogen) according to the manufacturer's procedure. Neonatal cardiac cells were isolated from one to two-day-old Wistar rats ventricles by digestion with trypsin-EDTA and type 2 collagenase as described (Springhorn and Clayhorn, 1989). Once the sequential digestions were terminated, the cells were pooled in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Groningen, Netherlands) supplemented with 10% FBS (GibcoBRL, Switzerland), penicillin (100 units/ml) and streptomycin (10 μg/ml) and seeded in 150 cm^2 flasks to allow selective adhesion of cardiac fibroblasts (Sadoshima and Izumo, 1993). Thereafter, cardiomyocytes were decanted from the flasks. Electroporation was performed with cells using Nucleofector™ (Amaxa, Cologne, Germany) according to the manufacturer's protocol. Briefly, 1 μg of pEGFP-C3 (Clontech) or GFP-DP-BC per 10^6 cardiomyocytes were resuspended in a mixture of 100 μl Nucleofector solution and electroporated in the Nucleofector electroporator with the cardiomyocyte specific program (G09). Thereafter, cardiomyocytes were plated on fibronectin-gelatin-coated 12-mm glass slides. After 4 days of culture, transfected rat cardiomyocytes were rinsed twice in PBS, fixed with 2% paraformaldehyde for 15 minutes, rinsed twice in PBS and permeabilized with 0.1% Triton X-100 in PBS for 5 minutes at room temperature. For the other immunofluorescence microscopy studies, cell grown on glass coverslips were fixed with 1% paraformaldehyde in MTSB (0.1 M Pipes, 1 mM EGTA, 4% PEG 4000, pH 6.9) for 10 minutes at 37°C and permeabilized with 0.1% Triton X-100 in PBS for 5 minutes at room temperature. After rinsing in PBS and blocking with 1% BSA in PBS for 30 minutes at 37°C, the cells were incubated with primary antibodies for 30 minutes at 37°C and then washed twice with PBS. Cells were subsequently incubated with secondary antibodies for 30 minutes at 37°C, washed twice, mounted in DAKO medium and viewed under a Zeiss inverted microscope Axiovert 200 (Zeiss, Oberkochen, Germany), under a Zeiss LSM410 confocal inverted laser scanning microscope (Zeiss) for the desmin-transfected keratinocyte cell line and under a LSM 510 Meta confocal scanner mounted on an upright Axioskop 2FS microscope for the transfected cardiomyocytes. Tissue sections of monkey heart were purchased (Inova Diagnostics Inc., San Diego, CA).

Antibodies

The following immunoreagents were used: mouse monoclonal antibody (mAb) directed against the HA epitope tag (12CA5), mAb 9E10 against the Myc epitope tag (Boehringer Mannheim Corporation, CA), mAb GFP-2 against GFP (Santa Cruz Biotechnology, Santa Cruz, CA), mAb D33 against desmin (Dako, Hamburg, Germany), mAb anti desmin (LabVision, CA), the rabbit NW161 anti-desmoplakin antiserum (Bornslaeger et al., 1996), mAb RCK107 directed against K14 (Monosan, Uden, The Netherlands), and anti-vimentin mAb clone V9 (Immunotech, Marseille, France); rabbit SC-805 anti-serum against hemagglutinin (HA) epitope tag, rabbit anti-GFP antiserum and rabbit H76 anti-desmin antiserum (Santa Cruz Biotechnology, Santa Cruz, CA), GP53 guinea pig anti-vimentin antiserum (Progen Biotechnik GMBH, Heidelberg, Germany). Secondary antibodies were purchased from Molecular Probes (Eugene, OR), Alexa Fluor 488-conjugated goat anti-rabbit IgG, Alexa Fluor 488 goat anti-guinea pig, Alexa Fluor 568 goat anti-rabbit, Alexa Fluor 488 FITC goat anti-mouse, TRITC-conjugated donkey anti-mouse IgG and Cy5-conjugated AffiniPure Donkey anti-Mouse IgG (Jackson ImmunoResearch Laboratories, PA).

Yeast two-hybrid assays

Yeast two-hybrid assays were performed as previously described (Fontao et al., 2003; Gontier et al., 2005). The vectors used were the yeast GAL4-AD and GAL4-BD expression vectors pACT2 and pAS2.1, respectively (Clontech).

Metabolic labeling of c-myc and HA-tagged proteins

^{35}S-methionine-labeled recombinant forms of DP and desmin were generated by coupled in vitro transcription/translation of pcDNA3-myc constructs using the Quick TNT coupled reticulocyte lysate system (Promega). Non-incorporated amino acids were removed from the in vitro translation mixture (50 μl) using Ultrafree 0.5-Biomax 5K (Millipore) filters. The translation mixture was diluted into 2 ml binding buffer (20 mM HEPES, 10 mM PIPES, 0.2 mM $CaCl_2$, 2 mM $MgCl_2$, 50 mM KCl, pH 7.2) supplemented with 0.1% (wt/vol) of BSA.

Expression and purification of recombinant proteins

cDNA fragments encoding the B and C subdomains of DP and BP230 were subcloned in frame with the C-terminus sequence of GST into pGEX-2T (Amersham Pharmacia Biotech). *Escherichia coli* BL21(DE3) (Novagen) was transformed with these constructs or the vector pGEX-2T without insert. Expression of GST or GST-fusion proteins were induced by adding 0.5 mM IPTG in the broth medium for 3 hours. Bacteria were collected by centrifugation, resuspended in phosphate-buffered saline (PBS) supplemented with 1% Triton X-100 and 5 mM EDTA and lyzed by sonication. Purification of GST-fusion proteins was performed as previously described (Geerts et al., 1999). Protein concentration was determined with the protein assay reagent from Bio-Rad (Bio-Rad, Hercules) using bovine serum albumin (BSA) as a standard.

Overlay assays

IF proteins (human vimentin, a gift of H. Herrmann, Heidelberg, Germany, and human desmin, Progen) were first equilibrated by dialysis against a buffer consisting of 6 M urea, 10 mM Tris-HCl, 10 mM β-mercaptoethanol, pH 8.0 for 1 hour at 4°C. Additional dialyses were performed with 3 M urea, 10 mM Tris-HCl, 10 mM β-mercaptoethanol, pH 8.0 for 4 hours at 4°C, then with 5 mM Tris-HCl, 10 mM β-mercaptoethanol, pH 8.0 for overnight at 4°C and finally with 10 mM Tris-HCl, 2 mM β-mercaptoethanol, 5 mM EDTA, pH 8.0 for 3 hours at 4°C. The proteins were polymerized by addition of 1/10 (vol/vol) of 0.2 M Tris/HCl, 1.6 M NaCl, pH 7.0 for 1 hour at room temperature. The quality of the filaments was verified by electron microscopy after negative staining with uranyl acetate. One to 0.01 μg of the polymerisation mixture was spotted onto a nitrocellulose membrane using a Dot-Blot apparatus (Schleider and Schuell, Keene, NH). Membranes were subsequently washed in binding buffer and incubated overnight at 4°C in blocking buffer [binding buffer supplemented with 2% (wt/vol) of heat-treated BSA]. Nitrocellulose strips were then incubated overnight at 4°C with ^{35}S-methionine-labeled proteins prepared as described above. After subsequent washes with binding buffer supplemented with 0.1% BSA and with binding buffer, the nitrocellulose strips were air-dried and bound proteins were visualised by autoradiography.

GST pull-down assays

To reduce non-specific binding to the GST moiety in subsequent steps, ^{35}S-methionine-labeled recombinant forms of desmin, prepared as described above, were diluted in binding buffer supplemented with 1% heat inactivated BSA and preabsorbed for 1 hour at room temperature to 100 μg of GST immobilized to glutathione beads. The preabsorbed mixture was then incubated for 1 hour at room temperature with 10 μg of GST-DP-BC or GST-BP230-BC immobilized on glutathione. After four washes in binding buffer, beads were resuspended in SDS-sample buffer, heated five minutes at 100°C and loaded on a 10% polyacrylamide gel. Bound proteins were visualized by autoradiography.

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