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A unique and specific interaction between αT-catenin and plakophilin-2 in the area composita, the mixed-type junctional structure of cardiac intercalated discs

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Summary

Alpha-catenins play key functional roles in cadherin-catenin cell-cell adhesion complexes. We previously reported on αT-catenin, a novel member of the α-catenin protein family. αT-catenin is expressed predominantly in cardiomyocytes, where it colocalizes with αE-catenin at the intercalated discs. Whether αT- and αE-catenin have specific or synergistic functions remains unknown. In this study we used the yeast two-hybrid approach to identify specific functions of αT-catenin. An interaction between αT-catenin and plakophilins was observed and subsequently confirmed by co-immunoprecipitation and colocalization. Interaction with the amino-terminal part of plakophilins appeared to be specific for the central 'adhesion-modulation' domain of αT-catenin. In addition, we showed, by immuno-electron microscopy, that desmosomal proteins in the heart localize not only to the desmosomes in the intercalated discs but also at adhering junctions with hybrid composition. We found that in the latter junctions, endogenous plakophilin-2 colocalizes with αT-catenin. By providing an extra link between the cadherin-catenin complex and intermediate filaments, the binding of αT-catenin to plakophilin-2 is proposed to be a means of modulating and strengthening cell-cell adhesion between cardiac muscle cells. This could explain the devastating effect of plakophilin-2 mutations on cell junction stability in intercalated discs, which lead to cardiac muscle malfunction.

Key words: Catenin, Plakophilin, Intercalated disc, Heart, Cell junction, Desmosome

Introduction

The structural integrity of the heart is maintained by the end-to-end connections between cardiomyocytes. These specialized cell-cell contacts, called intercalated discs, ensure mechanical and electrochemical coupling of the contractile cells. The intercalated disc was originally described as consisting of three main junctional complexes: adherens junctions of the fascia adhaerens type, desmosomes (maculae adhaerentes) and gap junctions (Forbes and Sperelakis, 1985). It has been proposed that adherens junctions provide strong cell-cell adhesion mediated by linkage of the cadherin-catenin complex to the actin cytoskeleton (Gumbiner, 2000), whereas desmosomes provide additional structural support through the interaction of desmosomal cadherins with intermediate filaments (Huber, 2003). Adjacent cells communicate through gap junctions (Giepmans, 2004), aggregated channels that connect the cells and permit the exchange of small regulatory proteins and ions. To mediate the normal cell-cell interaction between cardiomyocytes, which is indispensable for cardiac functionality, these three different junctional complexes must be properly organized in the intercalated discs. Knockout experiments revealed that absence of adherens junctions (Linask et al., 1997; Olson et al., 2002; Kostetskii et al., 2005; Sheikh et al., 2006) or desmosomes (Bierkamp et al., 1996; Ruiz et al., 1996; Grossmann et al., 2004) has devastating consequences for heart development and viability. Moreover, mutations in structural proteins that are involved in the organization of the intercalated disc often lead to cardiomyopathy and heart failure (Maeda et al., 1997; McKoy et al., 2000; Norgett et al., 2000; Ferreira-Cornwell et al., 2002; Gerull et al., 2004; Prakasa and Calkins, 2005; Awad et al., 2006a; Awad et al., 2006b; Heuser et al., 2006; Pilichou et al., 2006; Uzumcu et al., 2006; Yang et al., 2006). It is interesting that these intercalated discs were recently described as containing mainly a novel, mixed-type junctional structure instead of classical adherens junctions (Borrmann et al., 2006; Franke et al., 2006). This so-called area composita is composed of two junction types: some are desmosome-like, and the majority is similar to fasciae adhaerentes but consisting of several desmosomal proteins in addition to proteins typical of epithelial adherens junctions.

Alpha-catenins are key molecules of the classical cadherin-mediated cell-cell adhesion complex. For years it has been thought that alpha-catenins provide the indispensable link to

the actin cytoskeleton (Kobielak and Fuchs, 2004). In the classical model for adherens junctions, α-catenins anchor the cadherin-catenin complex to the actin cytoskeleton by binding to the armadillo proteins β-catenin and/or plakoglobin on one side, and by interacting directly or indirectly with actin filaments on the other side. However, Drees et al. (Drees et al., 2005) and Yamada et al. (Yamada et al., 2005) suggested, on the basis of compelling in vitro data, that this model might not be correct. They postulated that α-catenins stabilize the cell-cell contacts by functioning as a molecular switch that regulates actin dynamics at the site of the adherens junction.

Three α-catenins are known: the ubiquitously expressed αE-catenin (Herrenknecht et al., 1991; Nagafuchi et al., 1991), neural αN-catenin (Hirano et al., 1992), and αT-catenin, which we earlier identified as a member of the α-catenin family with a restricted expression pattern (Janssens et al., 2001). These three α-catenins are highly similar but differ in expression pattern. Our previous study showed that αT-catenin is especially abundant in heart tissue, where it is co-expressed with αE-catenin at intercalated discs, in the peritubular myoid cells of testis, and in smaller amounts in skeletal muscle and brain. In vitro, αN- and αT-catenin can substitute for the adhesive functions of αE-catenin, but their restricted expression patterns indicate additional isoform-specific or synergistic functions. This hypothesis was recently confirmed by the inability of endogenous αT-catenin expression to rescue heart-specific αE-catenin knockout in mice (Sheikh et al., 2006). Ablation of αE-catenin expression leads to defects in cardiomyocyte structural integrity; this results in unique forms of cardiomyopathy and susceptibility of the heart wall to rupture after cardiac stress.

It is well established that all other types of cell-cell junctions depend hierarchically on adherens junctions (Gumbiner et al., 1988; Taniguchi et al., 2005), although it is becoming increasingly clear that desmosomes are probably equally important in establishing efficient cell-cell adhesion (Hatsell and Cowin, 2001; Vasioukhin et al., 2001). Desmosomes consist of transmembrane desmosomal cadherins (desmocollins and desmogleins), which are clustered, reinforced and tethered to the intermediate filament cytoskeleton by cytoplasmic plaque proteins, such as desmoplakins, plakoglobin and plakophilins (Ishii and Green, 2001). Plakoglobin is the only protein reported to be present in both classical adherens junctions and desmosomes (Zhurinsky et al., 2000). Plakophilins are members of the armadillo protein family. They are composed of a basic N-terminal head domain, an armadillo repeat region of nine imperfect repeats, and a short C-terminal tail (Bonné et al., 1999; Choi and Weis, 2005). Plakophilins can interact with multiple components of the complex, such as desmogleins, desmocollins, desmoplakins, plakoglobin and keratin intermediate filaments (Bonné et al., 2003; Chen et al., 2002). Thus, plakophilins are thought to zip up desmosomal cadherins and tighten the desmosomal plaque. Whereas expression of plakophilin-1 (PKP1) and -3 (PKP3) is mostly restricted to subtypes of epithelial tissues (Bonné et al., 1999; Moll et al., 1997), plakophilin-2 (PKP2) displays a broader expression pattern and can also be found in non-epithelial cells, e.g. cardiomyocytes and lymph node follicle cells (Mertens et al., 1996).

In this paper, we propose a unique function for αT-catenin that sheds light on a possible role in muscle cells. We show that αT-catenin interacts specifically with desmosomal plakophilins, whereas αE-catenin and αN-catenin do not. We confirmed this interaction by co-immunoprecipitation and showed that it is mediated by the αT-catenin adhesion-modulation-domain (AMD). In addition, we demonstrated by immuno-electron microscopy (immuno-EM) that fascia adhaerens-like adhering junctions in the intercalated disc are composed of the cadherin-catenin complex, but that desmosomal proteins also localize to these specialized adhering junctions, which we name 'hybrid adhering junctions'. These data indicate that binding of αT-catenin to plakophilins can strengthen cell-cell adhesion in contractile cells by the formation of a unique type of junction that combines elements of classical desmosomes and adherens junctions. This specific function for αT-catenin may explain why two very similar α-catenins (αE- and αT-catenin) are coexpressed in the same junction.

Table 1. Yeast two-hybrid interaction assays: αT-catenin specifically interacts with plakophilin-1, -2 and -3

Number	Bait plasmid	Prey plasmid	Interaction
A			
1	pGBT9-α-catulin	pGADT7-hPKP3	–
2	pGBT9-αE-catenin	pGADT7-hPKP3	–
3	pGBT9-αN-catenin	pGADT7-hPKP3	–
4	pGBT9-αT-catenin	pGADT7-hPKP3	+
5	pGBKT7-αT-catenin	pGADT7-hPKP3	+
6	pGBKT7-hPKP3	pGADT7-αT-catenin	+
7	pGBKT7-hPKP3	pGAD424-αE-catenin	–
8	pGBKT7-hPKP3	pGAD424-αN-catenin	–
B			
9	peGBKT7-hαT-catenin	pGAD424-β-catenin(N-term)	+
10	peGBKT7-hαT-catenin	pGADT7-hPKP1	+
11	pAS2-hPKP2	pGADT7-αT-catenin	+
12	pAS2-hPKP2	pGAD424-αE-catenin	–
C			
13	pGBKT7-p0071 head ΔC2	pGADT7-αT-catenin	–
14	pGBKT7-p0071 head ΔN1	pGADT7-αT-catenin	–
15	pdGBKT7-hδ-catenin short	pGADT7-αT-catenin	–
16	peGBKT7-hαT-catenin	pdGADT7-hδ-catenin short	–
17	pGBKT7-p120ctn-3AC	pGADT7-αT-catenin	–
18	pGBKT7-mARVCF CM	pGADT7-αT-catenin	–

Results

αT-catenin interacts specifically with plakophilins

When screening for αT-catenin-specific interactions in the yeast two-hybrid system, we unexpectedly found an interaction with PKP3. We therefore tested other α-catenin bait constructs by cotransforming with the PKP3 prey. The interaction turned out to be specific for αT-catenin (Table 1A, rows 1-5), which was confirmed by exchange of bait and prey inserts (Table 1A, rows 6-8). Not only PKP3 but also PKP1, PKP2 and of course β-catenin interacted with αT-catenin in this assay (Table 1B, rows 9-12). Other armadillo proteins with considerable sequence similarity to plakophilins could not bind αT-catenin. No interaction was seen with p0071 (also known as plakophilin-4), delta-catenin, p120ctn, or ARVCF (Table 1C, rows 13-18).

To identify the αT-catenin and plakophilin domains responsible for this mutual interaction, we generated a series of deletion mutants and a chimeric construct in which the N-terminal halves of αT-catenin and αE-catenin were swapped. Using different C-terminally truncated constructs of αT-catenin,

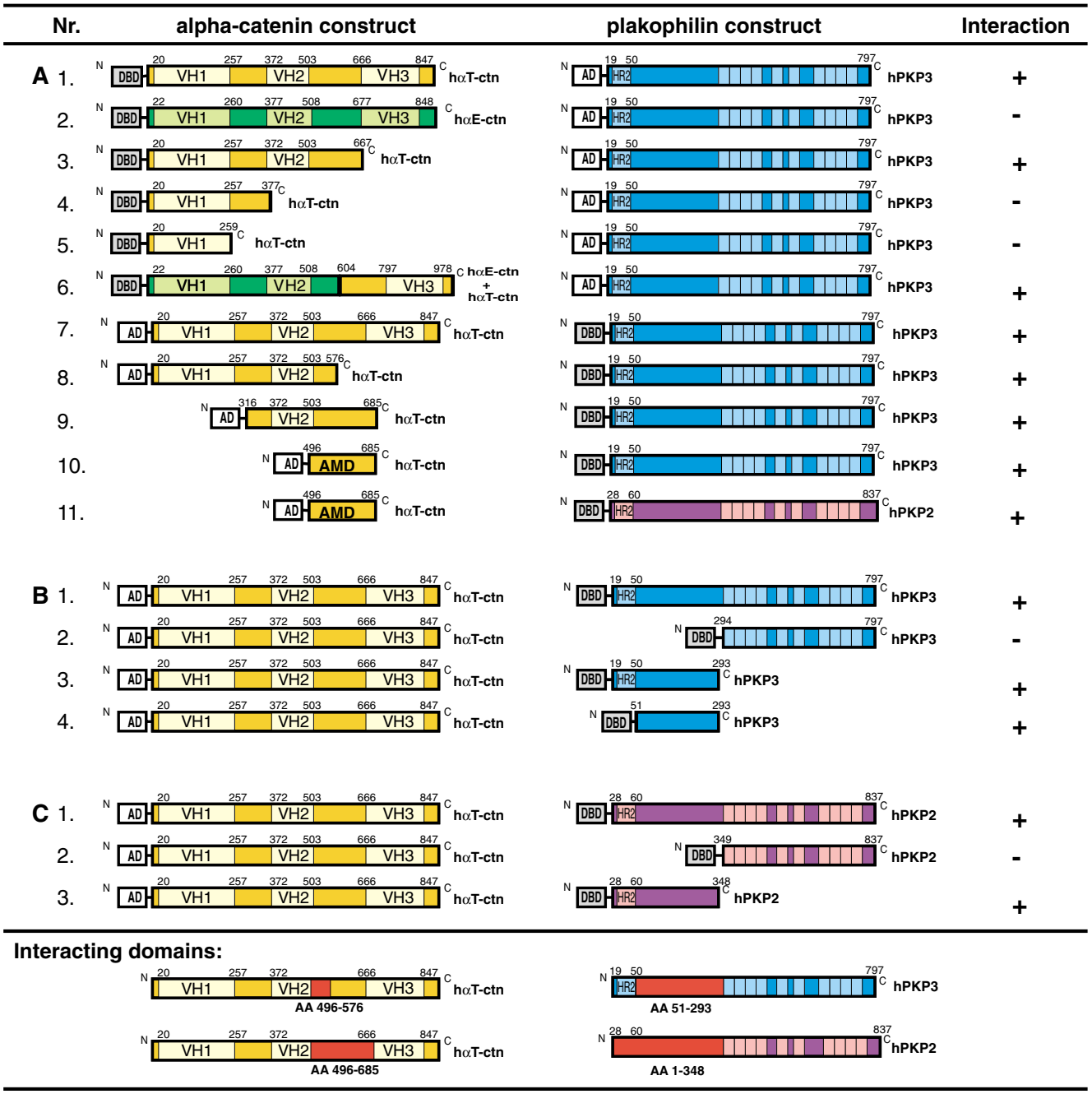

Fig. 1. Delineation of the domains mediating mutual interactions between αT-catenin and plakophilins (PKP3 and PKP2). Yeast two-hybrid analysis was performed on various deletion mutants of (A) αT-catenin (yellow), (B) PKP3 (blue) and (C) PKP2 (purple). We also used a chimeric construct in which the amino-terminal half of αT-catenin was replaced with that of αE-catenin (green; row A.6). Each open reading frame (αT-catenin, PKP3 and PKP2) was fused to the GAL4-DNA binding domain (DBD, grey boxes) or to the GAL4-activating domain (AD, white boxes). The narrowed down interacting domains are depicted at the bottom in red. Amino acid residue (AA) numbers indicate the positions of the corresponding domains. VH, vinculin homology domain; AMD, adhesion modulation domain; N, amino terminus; C, carboxyl terminus.

we found that the fragment containing AA 2-667 still interacted with PKP3, in contrast to a smaller αT-catenin fragment comprising AA 2-377 or AA 2-259 (Fig. 1A, rows 3-5). The construct in which the C-terminal half of αT-catenin was fused to the N-terminal half of αE-catenin could also interact with PKP3 (Fig. 1A, row 6), and so it became clear that the central domain of αT-catenin (CD: AA 316-685) is essential for this interaction. Indeed, αT-catenin fragments containing either this central domain or the so-called central adhesion-modulation domain (AMD: AA 496-685) (Imamura et al., 1999) can bind PKP3 (Fig. 1A, rows 9-10). Because the AA 1-576 fragment of αT-catenin also bound PKP3 (Fig. 1A, row 8), the PKP3-interaction domain was narrowed down to AA 496-576, within the adhesion modulation domain of αT-catenin.

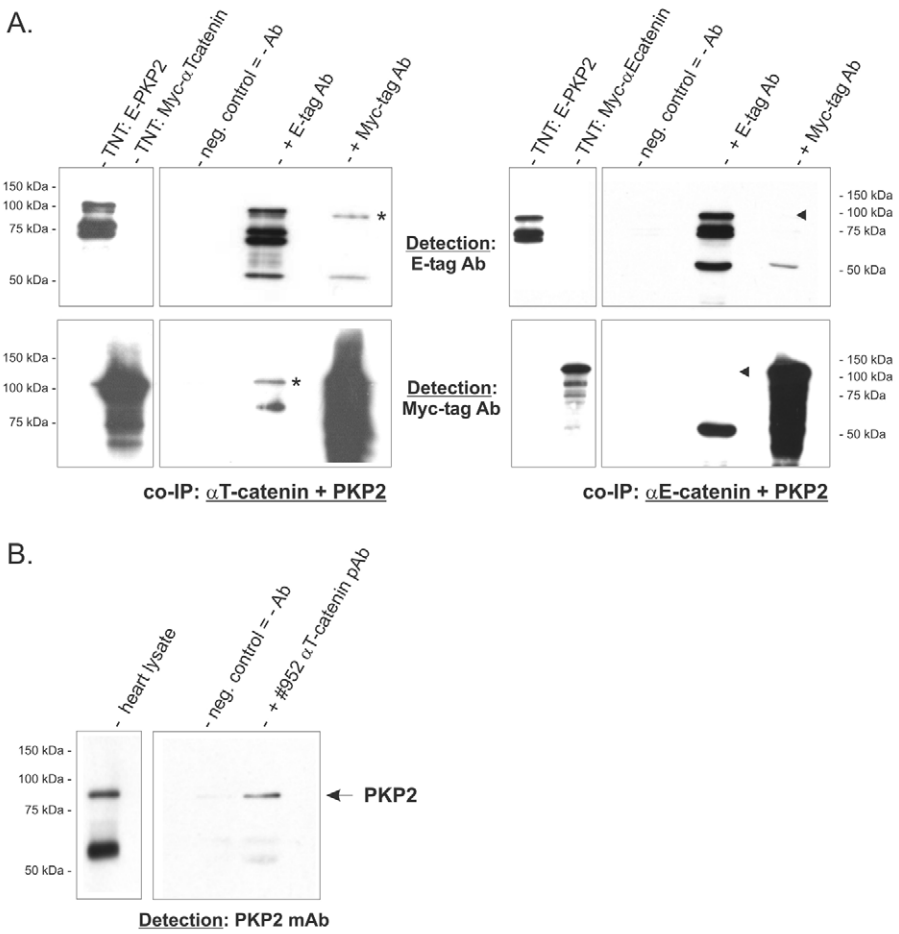

Fig. 2. Co-immunoprecipitation confirms the unique and specific interaction between αT-catenin and PKP2 both in vitro and in vivo. (A) Myc-tagged αT-catenin (114 kDa, left panel), Myc-tagged αE-catenin (111 kDa, right panel) and E-tagged full-length human PKP2 (100 kDa) were produced by coupled transcription-translation in vitro. Proteins immunoprecipitated with anti-Myc or anti-E-tag antibodies were analyzed by western blot. Interaction between αT-catenin and PKP2 is clearly observed (left panel; asterisk), but no interaction is seen between αE-catenin and PKP2 (right panel; arrowhead). (B) Endogenous PKP2 was co-immunoprecipitated with αT-catenin from mouse heart lysate. αT-catenin was specifically immunoprecipitated with polyclonal antibody 952, followed by western blot analysis using a PKP2-specific monoclonal antibody.

For PKP3, we found that the N-terminal domain is necessary for interaction, but the C-terminal *Arm* domain can be deleted, as can HR2, the main N-terminal homology region of plakophilins (Fig. 1B, rows 1-4).

Because αT-catenin is expressed predominantly in cardiac muscle (Janssens et al., 2001), we focused on its interaction with plakophilin-2, the only plakophilin expressed in cardiomyocytes (Mertens et al., 1996). Similar interaction domains for plakophilin-2 were found: a central domain comprising the AMD (AA 496-685) of αT-catenin could bind the amino-terminal head domain of plakophilin-2 in the yeast two-hybrid assay (Fig. 1A, row 11; Fig. 1C, rows 1-3). This direct interaction was confirmed by co-immunoprecipitation (co-IP) of in vitro synthesized Myc-tagged αT-catenin and E-tagged PKP2 (Fig. 2A). By contrast, in vitro-synthesized Myc-tagged αE-catenin was not co-immunoprecipitated with E-tagged PKP2, and vice versa. This confirms our yeast two-hybrid data and points to a specific function of αT-catenin. It is noteworthy that we also confirmed the interaction of αT-catenin with PKP1 and PKP3 by both colocalization and co-IP after cotransfection of either MCF7/AZ or HEK293T cells (data not shown). In addition, we were able to co-immunoprecipitate PKP2 from a heart lysate using an αT-catenin-specific antibody, showing that PKP2 and αT-catenin can be found in the same protein complex in vivo (Fig. 2B).

αT-catenin colocalizes with PKP2 and desmin at hybrid adhering junctions of the intercalated discs of adult heart

A rat monoclonal antibody, 1159_12A4S4, was generated against a peptide derived from the C terminus of murine αT-catenin. The specificity of this antibody for mouse αT-catenin was shown on lysates of HEK293T cells transfected with the respective GFP-tagged α-catenin constructs (Fig. 3). This specific monoclonal antibody was used to analyze the expression of αT-catenin by immunohistochemistry (Fig. 4) and immuno-EM (Fig. 5) on sections of mouse heart. As described before (Borrmann et al., 2006; Franke et al., 2006) a mixed-type junctional structure (area composita) was apparent at the intercalated discs. Strong labeling for αT-catenin was seen at the fascia adhaerens-like junctions, where αT-catenin colocalized with other molecules of the cadherin-catenin complex, such as β-catenin and αE-catenin, but also with desmosomal proteins such as PKP2, desmoglein and desmoplakin. This was apparent from both single-label (Fig. 5A) and double-label (Fig. 5B) experiments. By contrast, no localization of either α- or β-catenin could be seen at desmosome-like junctions of the intercalated discs. These results were quantified and are shown in Fig. 5D. Moreover, double-label immuno-EM for αT-catenin and the intermediate filament protein desmin revealed desmin immunoreactivity in the junctions that contain αT-catenin (Fig. 5C).

We thus showed that interaction with PKP2 is a specific property of αT-catenin, and that recruitment of PKP2 and other desmosomal proteins to a hybrid adhering junction is found specifically in intercalated discs of cardiomyocytes.

Discussion

We had previously identified αT-catenin and shown that it has high sequence similarity to the other two known α-catenins, αE- and αN-catenin (Janssens et al., 2001). Introduction of αT-catenin in a colon carcinoma cell line that is αE-catenin-negative rescued cell-cell adhesion, implying that αT-catenin

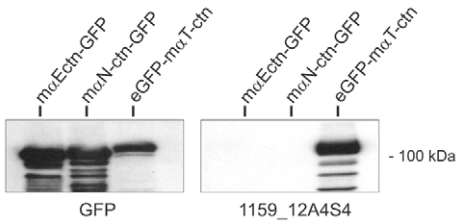

Fig. 3. Rat monoclonal antibody 1159_12A4S4 specifically detects αT-catenin (right panel). Antibody specificity was tested by western blot analysis of HEK-293 cells transfected with constructs expressing GFP-tagged mouse α-catenins, as indicated above the lanes. Anti-GFP antibody was used to detect all fusion proteins as a positive control for transfection efficiency (left panel).

can substitute for the adhesive functions of αE-catenin. However, co-expression of αT-catenin and αE-catenin at the intercalated discs in heart tissue indicated that each α-catenin has a specific or a synergistic function. αT-catenin is abundant in heart muscle cells, which are exposed to strong mechanical stress and consequently need a robust cell-cell adhesion structure. We hypothesized that αT-catenin may serve this purpose by reinforcing the classical cadherin-catenin complex by binding specific interaction partners, which may distinguish it from the other α-catenins.

Using the yeast two-hybrid approach, we have now found that interaction with plakophilins is a specific function of αT-catenin. In this context, PKP2 is the most relevant plakophilin, as it is expressed in cardiomyocytes, whereas PKP1 and PKP3 are restricted mainly to epithelial tissues, in which αT-catenin has not been detected (Bonné et al., 1999; Janssens et al., 2001; Mertens et al., 1996; Moll et al., 1997). In order to identify potential interaction domains, we used a variety of αT-catenin and plakophilin constructs. We showed that a central 81-AA domain of αT-catenin (AA 496-576) mediates interaction with the N-terminal domain of plakophilins. This domain is homologous to the proposed adhesion-modulation domain (Imamura et al., 1999), which has been shown to be essential for αE-catenin's role in strengthening adhesion. The adhesion modulation domain consists of a bundle of four anti-parallel α-helices (Yang et al., 2001) predicted to serve as a platform for hydrophobic interactions. Binding of this domain to plakophilins and thus linking of the cadherin-catenin complex to the intermediate filaments via plakophilins could strengthen

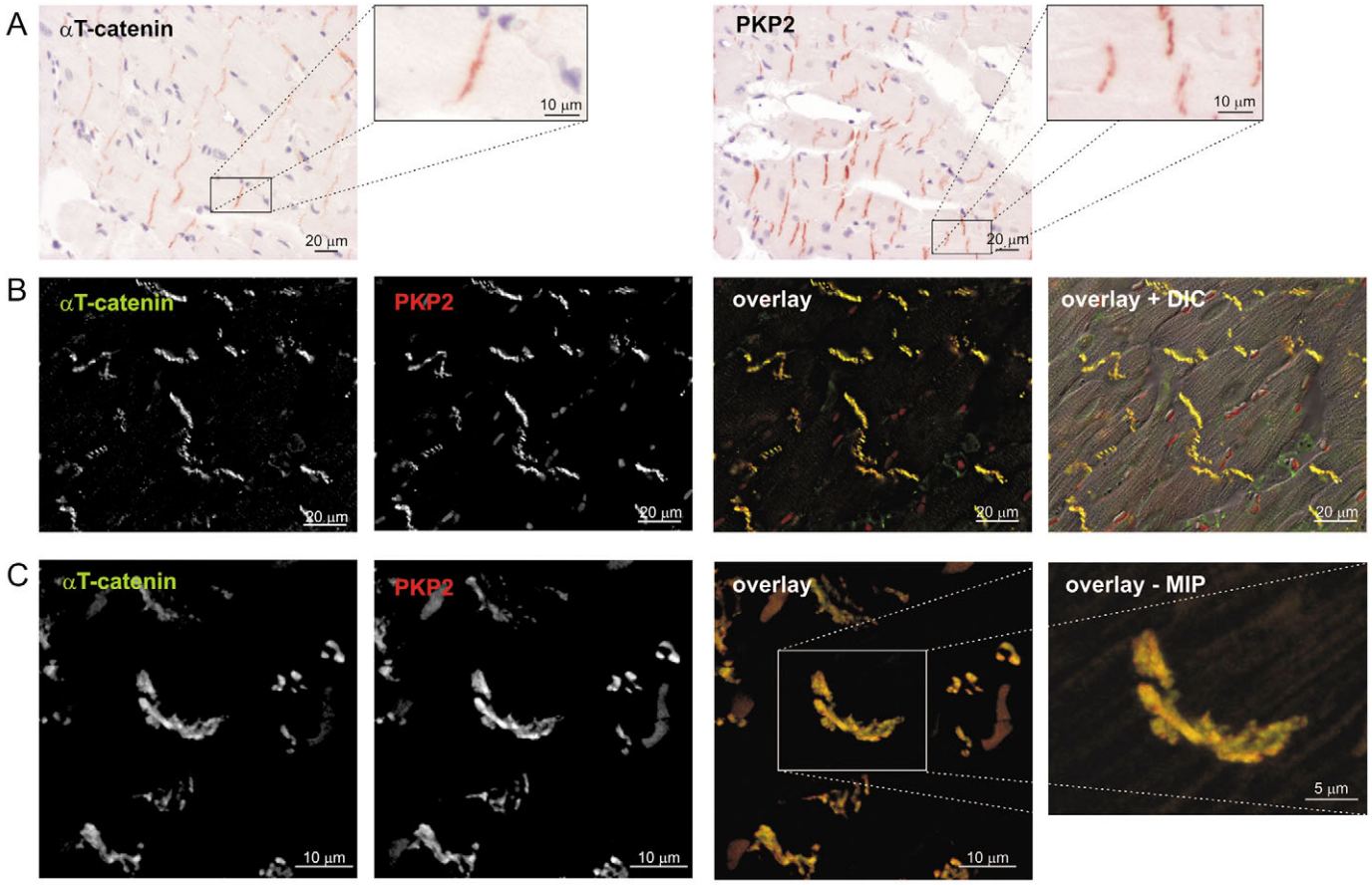

Fig. 4. Immunohistochemical staining identifies colocalization of αT-catenin and PKP2 at the intercalated discs of adult mouse heart. Tissue was fixed in paraformaldehyde, embedded in paraffin, and sections were stained using specific antibodies followed by histochemical detection. (A) Counterstaining with Hematoxylin and Eosin, (B) conventional immunofluorescence and (C) confocal laser scanning microscopy. Overlay images of red and green fluorescence are shown in the third column. In B, the rightmost panel shows double fluorescence combined with differential interference contrast microscopy (DIC). In C, maximum intensity projections (MIPs) of 29 z-sections of 0.25 μm were made (rightmost panel).

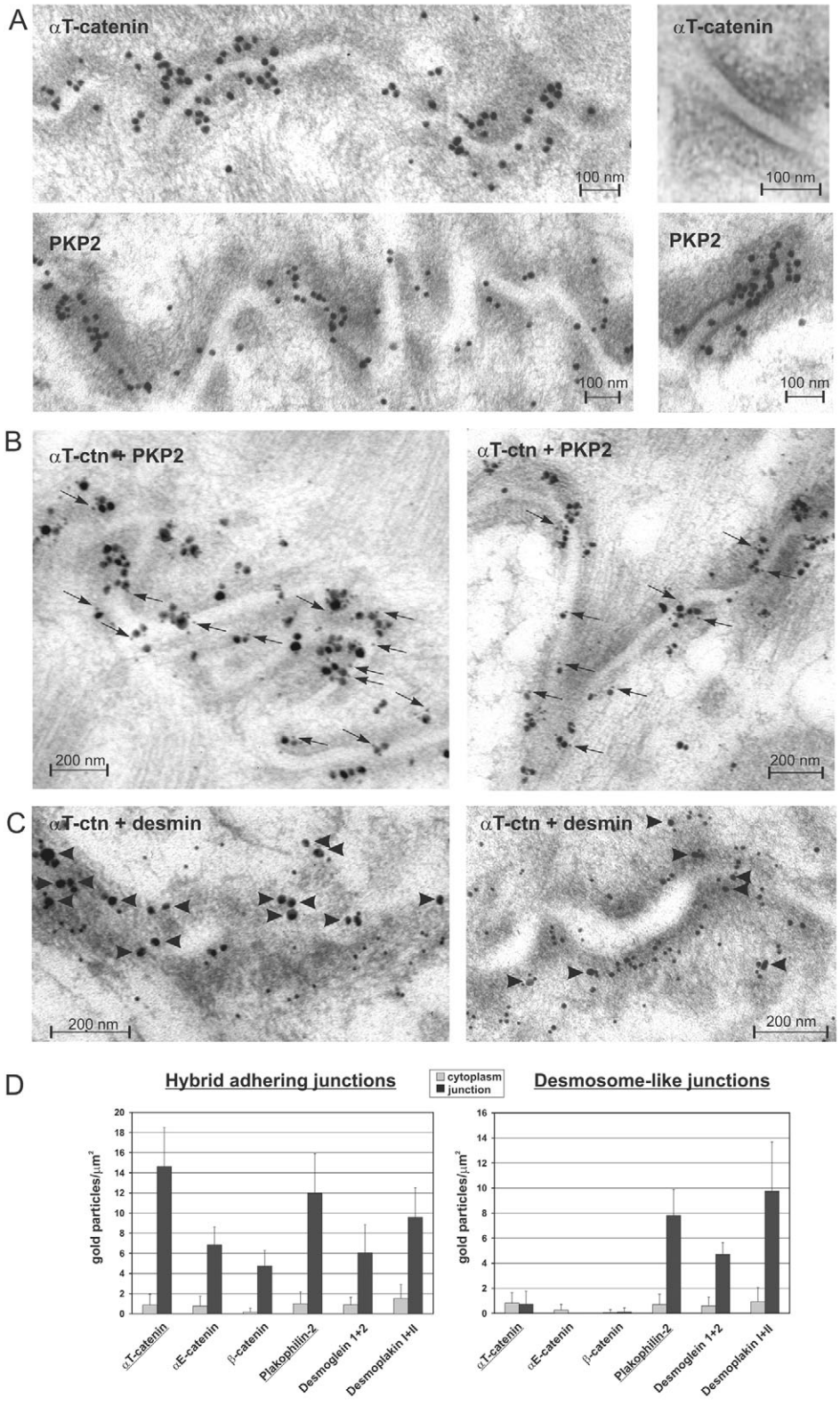

Fig. 5. Immuno-EM identifies colocalization of αT-catenin and PKP2 at adhering junctions of the intercalated discs of cardiomyocytes. (A) Single labeling with silver amplification of αT-catenin and PKP2 at fascia adhaerens-like junctions (left panel) and desmosome-like junctions (right panel). Whereas PKP2 is detected in both junction types, αT-catenin is confined to the fascia adhaerens-like junction type, which we named 'hybrid adhering junctions'. (B) Consecutive double labeling at the mixed-type junctional structure (area composita): detection of αT-catenin by silver amplification (large dots) was followed by PKP2 labeling (10-nm gold particles, small dots, arrows). (C) Detection of intermediate filaments at the area composita by consecutive double labeling: αT-catenin detection by silver amplification (big dots, indicated by arrowheads) was followed by desmin labeling (10-nm gold particles, small dots).
(D) Schematic representation of the number of gold particles counted at the site of hybrid adhering junctions (left, black bars) and desmosome-like junctions (right, black bars) in comparison to the cytoplasmic background label (grey bars) after immunogold labeling of several cadherin/catenin-associated proteins (αT-catenin, αE-catenin and β-catenin) and several desmosomal proteins (PKP2, desmoglein and desmoplakin). The classic cadherin-catenin complex is found only at the hybrid adhering junctions, whereas desmosomal proteins are detected at both the desmosome-like and the hybrid adhering junctions of the intercalated disc.

intercellular adhesion. We then confirmed the interaction between αT-catenin and plakophilin-2 by co-immunoprecipitation.

Additionally, we demonstrated that fascia adhaerens-like adhering junctions between cardiomyocytes are not composed solely of the cadherin-catenin complex. Using immuno-EM, we showed that desmosomal proteins are also localized at these cell-cell contact sites. This has been suggested by Grossmann and colleagues (Grossmann et al., 2004), who found that both desmosomes and fascia adhaerens-like adhering junctions of the

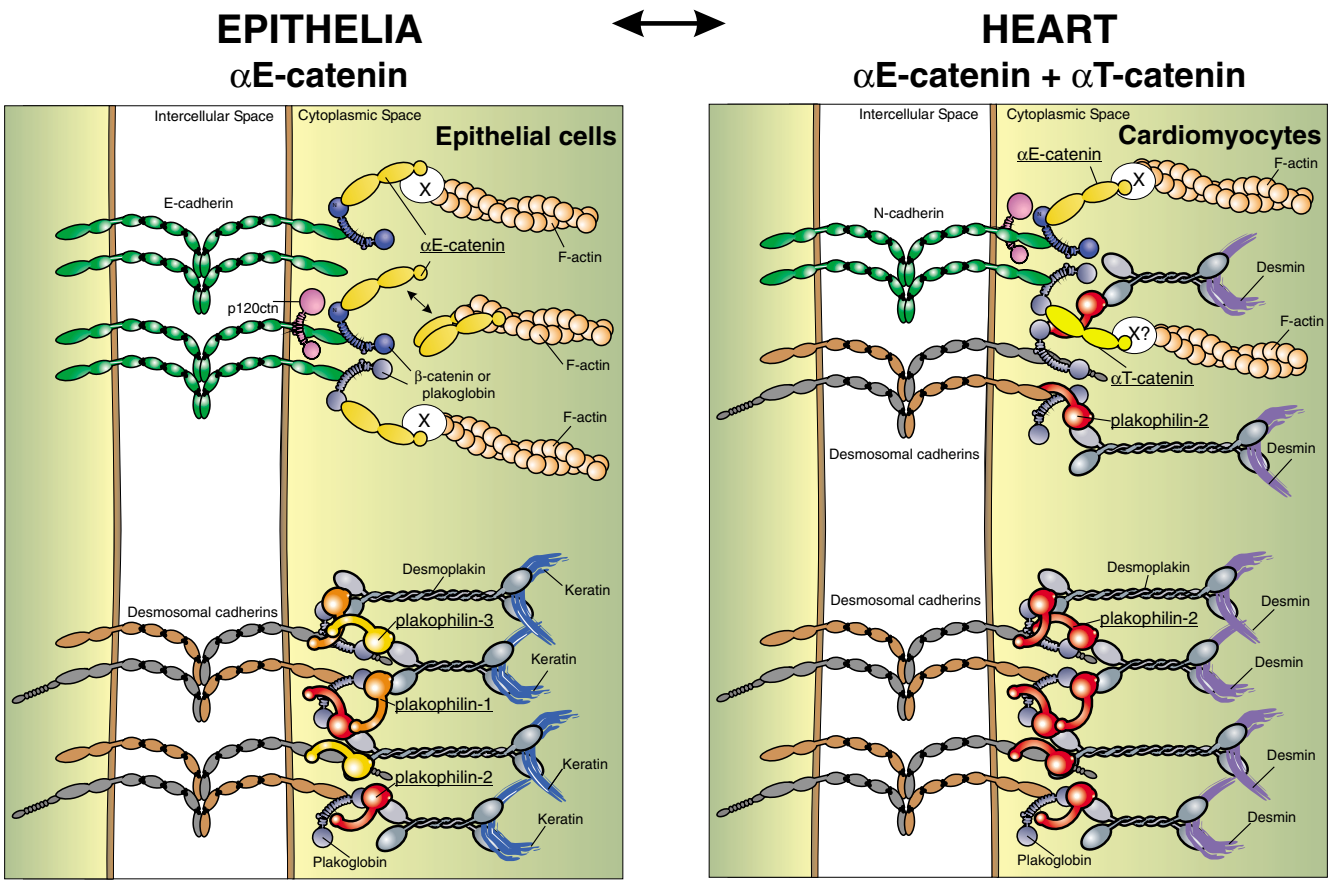

Fig. 6. Models for cadherin-based cell-cell junctions in epithelia (left) and in heart (right panel). In the heart, αT-catenin recruits desmosomal proteins to hybrid adhering junctions (top drawings), thereby forming, together with desmosomes (see below), an area composita, which is an enforced, mixed-type junctional structure attached to both the actin cytoskeleton and the intermediate filaments. By contrast, epithelial tissues do not express αT-catenin, and therefore their adherens junctions are not attached to the intermediate filaments. The composition of typical desmosomes (drawings at the bottom) is largely similar in the two tissue types. Although desmosomes are shown here as containing simultaneously all three types of plakophilins as well as heterophilically binding desmosomal cadherins, we are not aware of evidence for such high complexity in individual desmosomes.

intercalated discs are disturbed in the hearts of PKP2-deficient mouse embryos, which exhibit lethal alterations in heart morphogenesis and stability at mid-gestation. More recently, Franke and colleagues (Borrmann et al., 2006; Franke et al., 2006) have reported on the occurrence of these unusually complex adhering junctions at the intercalated discs. Our data indicate that binding of αT-catenin to PKP2 could be a means of strengthening cell-cell adhesion in contractile cells, by the formation of an exclusive type of hybrid junction that combines elements of classic desmosomes and adherens junctions, and in this way providing an extra link from the cadherin-catenin complex to intermediate filaments (Fig. 6). The tissue-restricted expression of αT-catenin and the specificity of its interaction with PKP2 explain why this exclusive junction is not seen in other cell types and why two very similar α-catenins (αE- and αT-catenin) are co-expressed at the intercalated discs.

One could expect that PKP2 or αT-catenin mutations that prevent the interaction of these proteins would also result in a less stable intercalated disc, leading to vulnerability of cardiac muscle to mechanical stress. Indeed, PKP2 mutations are common in arrhythmogenic right ventricular cardiomyopathy (ARVC) (Gerull et al., 2004; Prakasa and Calkins, 2005), probably the second most common cause of unexpected sudden deaths among young people and athletes. However, most of these PKP2 mutations would affect not only the αT-catenin-plakophilin interaction at the hybrid adhering junctions of the heart, but also the formation of functional desmosomes at the intercalated disc. It is worthwhile mentioning that the human αT-catenin gene, *CTNNA3*, is mapped to chromosome 10q21.3 (Janssens et al., 2003). As this region contains a gene for autosomal dominant familial dilated cardiomyopathy, and as high levels of αT-catenin occur in healthy heart tissue, *CTNNA3* was proposed as a potential cause of this common heart disease. However, until now no αT-catenin mutations have been reported in patients with heart failure (Janssens et al., 2003).

The interaction between αT-catenin and plakophilins provides strong evidence for a physical link in the heart between the cadherin-catenin complex, linked to the actin cytoskeleton, and desmosomal proteins, associated with the intermediate filaments. Previously, an interaction was reported between endogenous β-catenin and overexpressed plakophilin-2 in epithelial cells (Chen et al., 2002). The physiological

relevance of this observation is unclear, as epithelial cells do no form the hybrid adhering junctions typical of intercalated discs in the heart. The novel concept of hybrid adhering junctions may help to explain some outstanding questions about the crosstalk between the proteins of adherens junctions and those of desmosomes, not only in heart but also in other tissues. It remains to be determined whether plakophilins and other desmosomal proteins are also recruited to adherens junctions in other cell types that express αT-catenin, e.g. testis, brain and skeletal muscle.

In summary, we describe here a novel αT-catenin function that sheds light on a possible role for this particular α-catenin isoform in cardiac muscle, in which it is abundantly expressed. Although αT-catenin is co-expressed with αE-catenin at intercalated discs of cardiomyocytes, we propose that it has the specific function of binding to PKP2, which αE-catenin cannot do. Through this interaction αT-catenin may be involved in recruiting desmosomal proteins to hybrid adhering junctions, and thereby forming a mixed-type, reinforced junction (area composita) at the intercalated disc that is attached to both intermediate filaments and the actin cytoskeleton (Fig. 6). Further untangling of this web of intermolecular connections may yield precious information concerning the establishment of stable cell-cell contacts in these specialized cardiomyocytes, which could serve as a paradigm for other cell systems. In this respect, we look forward to studying the αT-catenin-null mouse.

Materials and Methods

Construction of expression plasmids

Full-length αE-catenin cDNA was excised from pDR2αE-ctn (Janssens et al., 2001) as two fragments, *Eco*47III-*Sph*I and *Sph*I-*Sal*I, which were then ligated in *Sma*I-*Sal*I-digested pGBT9 (Clontech, Palo Alto, CA, USA). This yielded pGBT9-αE-catenin, in which full-length αE-catenin [nucleotides (nt) 69-3433; amino acid residues (AA) 1-906] is fused in frame with the GAL4 DNA binding domain. Similarly, ligation to pGAD424 (Clontech) yielded pGAD424-αE-catenin, in which full-length αE-catenin is fused in frame with the GAL4 activation domain. Full-length αE-catenin cDNA was amplified for Gateway cloning (Invitrogen, Carlsbad, CA. USA) with gene-specific primers containing the AttB sites: MCBU#2422 (5′-GGGACAAGTTTGTACAAAAAAGCAGGCTTGACTGCTGTCCATGCAGGCA-ACATAA-3′) and MCBU#2423 (5′-GGGGACCACTTTGTACAAGAAAGCT-GGGTGTTCACCACCTGGACTGGTTCAGTCT-3′). The amplified fragment was precipitated with polyethylene glycol (PEG) and inserted in pDONR207 (Invitrogen) by the BP recombination reaction, yielding pENTR207-hαE-catenin (nt 72-2930; AA 2-906). LR reaction between this entry clone and destination vector pdCS2MT produced expression plasmid pdCS2Myc-αEcatenin, in which the N terminus of αE-catenin (nt 72-2930; AA 2-906) is fused to a Myc tag.

The cDNA for human αN-catenin was kindly provided as pPN-hANCTN by C. Petit (Claverie et al., 1993). Nearly full-length αN-catenin was amplified from pPN-hANCTN with primers MCBU#137 (5′-ACCCCCCGGGGGCAACTTCACCT-ATCATTC-3′ containing a *Xma*I site) and MCBU#138 (5′-GCCGCCGC-CTTCCTTTTCATTTCCGCTCTT-3′). The PCR fragment was digested with *Xma*I and *Ban*I and ligated together with a *Ban*I-*Hin*dIII fragment of pPN-hANCTN in *Xma*I-*Hin*dIII-digested pBluescriptIIKS+ (Stratagene, La Jolla, CA, USA), yielding pBlue-αN-ctn(9-2838). pGBT9-αN-catenin and pGAD424-αN-catenin were generated by cloning an *Xma*I-*Sal*I restriction fragment of pBlue-αN-ctn, comprising nt 9-2838 (AA 1-953) of the αN-catenin cDNA, into pGBT9 and pGAD424 (both Clontech), respectively.

M. Takeichi (Kobe, Japan) kindly provided the α-catenin expression plasmids pCA-αEcat-EGFP and pCA-αNcat-EGFP (Abe et al., 2004).

The 5′ region of the α-catulin cDNA was amplified by PCR with primers MCBU#711 (5′-AGGGGGCAGTGGCTGAAGAAAGAAGATATC-3′) and MCBU#725 (5′-TATTAGATATCGCCTCTCCCGGACCCGCC-3′). The PCR product was cut with *Eco*RV and *Mun*I, and ligated in a three-point reaction with a *Mun*I-*Sal*I fragment of pGEMT-α-ctl(1-2264) (Janssens et al., 1999) into the *Bam*HI(blunted)-*Sal*I-digested two-hybrid vector pGBT9 (Clontech). The resultant construct, pGBT9-α-catulin, comprised nt 50-2264 of the cDNA, encoding AA2-735.

To clone full-length αT-catenin cDNA in pGBT9 (Clontech) in fusion with the GAL4 binding domain, four consecutive constructs were made. A PCR product of 1134 bp was synthesized with primers MCBU#1607 (5′-AGAATTCTC-AGCTGAAACACCAATCAC-3′) and MCBU#1609 (5′-AGGATCCTGCGAA-GGTCTCTTGTCT-3′) using a pGEMTeasy-αT-ctn-RACE2 clone as template (Janssens et al., 2001). The product was digested with *Eco*RI and *Bam*HI and ligated to the *Eco*RI and *Bam*HI sites of pGBT9, yielding pGBT9-αT-ctn(179-953; AA2-259). This construct was opened with *Pst*I, and a 1111-bp fragment from clone pGEMT-αT-ctn-RACE2 was inserted to obtain pGBT9-αT-ctn(179-1306; AA2-377). From this construct, the 1082-bp *Eco*RI-*Ssp*I insert was isolated and ligated together with fragment *Ssp*I-*Sal*I from pGEMTeasy-αT-ctn-RACE1 in pGBT9 restricted with *Eco*RI-*Sal*I. This yielded pGBT9-αT-ctn(179-2176; AA2-667).

To obtain an overlapping 3′ clone, a PCR product of 890 bp was synthesized with primers MCBU#1610 (5′-GGATGATAATCAATTTGTGGACATCTC-3′) and MCBU#1608 (5′-GGGATCCGTAGATTTGTCTTCCTCTAA-3′). For this PCR, template cDNA was synthesized from RNA prepared with the RNeasy kit (Qiagen, Chatsworth, CA, USA) from the PC-3 prostate cancer cell line. The PCR product was cut with *Bgl*II and *Sal*I, and inserted in the *Bgl*II-*Sal*I restricted pGBT9-αT-ctn(179-2176: AA2-667) to obtain pGBT9-αT-ctn(179-2860; AA2-895). The *Eco*RI-*Sac*I insert of pGBT9-αT-ctn(179-2860: AA2-895) and the *Sac*I-*Not*I fragment of the original EST clone pT3T7-EST728263 (containing the full 3′-UTR) were together ligated in *Eco*RI-*Not*I-digested pGBKT7 (Clontech), yielding pGBKT7-αT-ctn(179-3024; AA2-895). The insert of this construct was excised with *Not*I, blunted, cut with *Eco*RI, and inserted in *Bam*HI(blunt)-*Eco*RI restricted pGADT7 (Clontech), yielding pGADT7-αT-ctn(179-3024; AA2-895). A C-terminal truncation of this construct was made by ligating an *Eco*RI-*Sca*I fragment of this plasmid into pGADT7 restricted with *Bam*HI(blunt) and *Eco*RI, yielding pGADT7-αT-ctn(179-1904; AA2-576).

To fuse the N-terminal half of αE-catenin to the C-terminal half of αT-catenin, we made use of the *Nco*I sites in the corresponding cDNAs. Thus, a *Sal*I-*Nco*I fragment of pGBT9-αE-ctn(69-3433; AA1-906) was ligated to an *Nco*I-*Sal*I fragment of pGBT9-αT-ctn(179-2860; AA2-895) to yield pGBT9-αE(69-1882; AA1-604)αT(1609-2860; AA478-895)-ctn. Matchmaker-3 two-hybrid vectors (Clontech) were converted for Gateway cloning (Invitrogen) by ligating the rfB cassette in the blunted *Eco*RI-*Bam*HI sites of pGADT7 and in the blunted *Eco*RI-*Not*I sites of pGBKT7, yielding destination vectors pdGADT7 and pdGBKT7. Full-length human αT-catenin cDNA was amplified for Gateway cloning (Invitrogen) with gene-specific primers containing the AttB sites: MCBU#2424 (5′-GGGGA-CAAGTTTGTACAAAAAAGCAGGCTTGTCAGCTGAAACACCAATCACATT-G-3′) and MCBU#2425 (5′-GGGGACCACTTTGTACAAGAAAGCTGGGTG-TGGTTAGGCAGGATTTTGTCATATAG-3′). The amplified fragments were precipitated with PEG and inserted in pDONR207 (Invitrogen) by the BP recombination reaction, yielding pENTR207-hαTctn(179-2887). This entry clone was transferred to pdGBKT7 and pdGADT7 by Gateway LR cloning (Invitrogen), yielding peGBKT7-hαTctn(179-2887; AA2-895) and peGADT7-hαTctn(179-2887; AA2-895).

Fragments of α-catenins were amplified for Gateway cloning (Invitrogen) with gene-specific primers containing only 12 bp of the AttB sites. The full AttB sites were added by performing a second PCR reaction with primers MCBU#2923 (5′-GGGGACAAGTTTGTACAAAAAAGCAGGCT-3′) and MCBU#2924 (5′-GGG-GACCACTTTGTACAAGAAAGCTGGGT-3′). PCR products were precipitated with PEG and inserted in pDONR207 (Invitrogen) by the BP recombination reaction. A fragment containing the adhesion modulation domain (AMD, AA 509-643) of αT-catenin was amplified with primers MCBU#2897 (5′-AAAAAGC-AGGCTCAGAATTCGATGCTGTAGATGACATTACA-3′) and MCBU#2896 (5′-AGAAAGCTGGGTCGGATCCTTCAGCATCAAGCTTACTCTT-3′). The Gateway BP reaction produced the entry clone pENTR207-hαTctn/AMD(1664-2236; AA496-685), encoding αT-catenin AA 496-685.

The larger central domain of αT-catenin (CD, AA 316-685) was amplified with primers MCBU #2895 (5′-AAAAAGCAGGCTCTGAATTCGATTCTTCCTGTA-CGCGGGAC-3′) and MCBU #2896 (mentioned above). The BP reaction produced the entry clone pENTR207-hαTctn/CD(1122-2236; AA316-685). By performing an LR reaction between these entry clones and destination vector pdGADT7, expression clones peGADT7-hαT-ctn/AMD(1664-2236; AA496-685) and peGADT7-hαT-ctn/CD(1122-2236; AA316-685) were obtained. The LR Gateway reaction between the entry clone pENTR2B mαT-ctn FL(120-2979; AA1-895) (Busby et al., 2004) and destination vector pdEGFP (B. Janssens, Study of two novel members of the human alpha-catenin family. PhD thesis, Ghent University, 2002) produced a plasmid for eukaryotic overexpression of mouse αT-catenin with its N terminus fused to eGFP; this plasmid was designated pdEGFP-mαTctn FL(120-2979; AA1-895). As previously described, plasmid pdCS3-mαTctn FL(120-2979; AA1-895) was obtained in a similar way (Busby et al., 2004).

Full-length β-catenin cDNA was kindly provided by J. Behrens (Erlangen, Germany) as plasmid pBAT-βCAT. The N-terminal fragment (bp 239-717), corresponding to β-ctn AA 14-172, was obtained as an *Nco*I-*Pst*I restriction fragment. The *Nco*I cut end was filled in with *Pfu* polymerase, and the fragment was cloned in the *Sma*I-*Pst*I opened pGAD424, yielding pGAD424-β-catenin(N-term), containing nt 239-717 of the β-catenin cDNA, encoding AA 14-172.

Cloning of pGBKT7-hp120ctn-3AC, pGBKT7-hPKP3 (encoding AA 1-797 of human PKP3), pGBKT7-hPKP3arm (encoding AA 294-797) and pGBKT7-hPKP3headΔHR2 (encoding AA 51–293) has been described (Bonné et al., 2003).

PKP3 was amplified with primers MCBU #1846 (5′-ATACGAATTCCAGGA-CGGTAACTTCCTG-3′) and MCBU#1970 (5′-ATACGAATTCAGGACCCAG-GAAGTCCTCCT-3′), cut with *Eco*RI, and cloned into pGADT7 (Clontech) to obtain pGADT7-hPKP3 (encoding AA 1-797). pGAD424-hPKP-1 (Hatzfeld et al., 2000) was cut with *Eco*RI and *Sal*I and cloned into the *Eco*RI/*Xho*I sites of pGADT7, yielding pGADT7-hPKP1 (encoding AA 1-727 of human PKP1), and also into the *Eco*RI/*Sal*I sites of pEGFP-C2 (Clontech), yielding pEGFP-hPKP1.

Bait plasmids pAS2-hPKP2 (encoding AA 1-837 of human PKP2), pAS2-hPKP2a head domain (encoding AA 1-348) and pAS2-hPKP2 ARM domain (encoding AA 349-837) (Chen et al., 2002) were kindly provided by K. Green (Chicago, USA). Full-length hPKP2 was cut out with *Eco*RI/*Sal*I and cloned in the *Eco*RI-*Xho*I restricted GATEWAY vector, pENTR3C, yielding plasmid pENTR3C hPKP2. An LR reaction between this entry clone and destination vector pdCS2E-tag yielded expression clone pdCS2E-PKP2, in which the N terminus of PKP2 (AA 1-837) is fused to the E-tag. M. Hatzfeld (Halle, Germany) kindly provided us with the Y2H plasmids pGBKT7-p0071 head ΔC2 (encoding AA 1-208 of p0071), pGBKT7-p0071 head ΔN1 (encoding AA 209-509) (Hatzfeld and Nachtsheim, 1996), and pGBKT7-mARVCF-CM (encoding AA 229-962).

Delta-catenin cDNA was excised (*Mlu*I-*Not*I) from pClneoMyc delta-catenin-8 (Ide et al., 1999), kindly provided by Y. Hata and Y. Takai (Tokyo, Japan), and ligated in *Xmn*I-*Not*I restricted pENTR2B, yielding plasmid pehδCTNshort, encoding AA 1-792. LR reactions between this entry vector and destination vectors pdGADT7 and pdGBKT7 produced pdGADT7hδCTNshort and pdGBKT7hδCTNshort, respectively.

Yeast two-hybrid assay

The Matchmaker 2 and 3 systems (Clontech) were used with the Y190 and AH109 yeast strains for yeast two-hybrid interaction tests. Cotransformants were obtained by standard methods (Gietz and Woods, 1995) and plated on minimal synthetic drop-out medium (SD) lacking leucine and tryptophan. After 3 days, colonies were picked and grown overnight in SD without leucine and tryptophan. Replica plates selecting for prey-bait interactions in transformed Y190 yeasts were made in SD medium lacking leucine, tryptophan and histidine, but containing 0.07 M potassium phosphate, 40 mM 3-amino-triazol to suppress leaky His expression, and 80 mg/ml X-β-GAL (Duchefa, Haarlem, The Netherlands). Replica plates selecting for prey-bait interactions in transformed AH109 yeasts were made in SD medium lacking leucine, tryptophan, histidine and alanine, but containing 20 mg/ml X-α-GAL (Duchefa). None of the two-hybrid constructs used showed auto-activation in our system (data not shown).

Co-immunoprecipitation

In order to obtain Myc-tagged αT-catenin, Myc-tagged αE-catenin, and E-tagged PKP2, coupled in vitro transcription/translation was performed using the TnT-Coupled Reticulocyte Lysate System (Promega) according to the manufacturer's instructions. For co-immunoprecipitation, 50 μl of the proteins synthesized in vitro were supplemented with 300 μl binding buffer consisting of PBS containing 1% Nonidet P-40 and a protease inhibitor cocktail (Complete; Roche Diagnostics, Mannheim, Germany). They were incubated overnight with 4 μg of either anti-Myc monoclonal antibody clone 9E10 or anti-E-tag monoclonal antibody (GE Healthcare, Chalfont St Giles, UK), after which 50 μl of 50% protein-G Sepharose (Amersham, Uppsala, Sweden) were added. After incubation for 3 hours, the Sepharose beads were washed three times with binding buffer.

For co-immunoprecipitation of the endogenous proteins, mouse heart lysate was prepared in RIPA buffer (150 mM NaCl, 5 mM EDTA, 20 mM Hepes pH 7.5, 1% Nonidet P-40, 0.2% desoxycholate, 0.1% SDS and a protease inhibitor cocktail). The lysate was centrifuged and a sample containing 800 μg protein was incubated overnight with the αT-catenin-specific polyclonal antibody 952, after which 50 μl of 50% protein-G Sepharose beads (Amersham) was added. After incubation for 3 hours, the beads were washed four times with binding buffer (140 mM NaCl, 5 mM EDTA, 20 mM Hepes pH 7.5, 1% Nonidet P-40, and a protease inhibitor cocktail). The washed Sepharose beads were combined with 6× sample loading buffer (0.35 M Tris-HCl pH 6.8, 10.3% SDS, 36% glycerol, 5% β-mercaptoethanol and 0.012% Bromophenol Blue), and boiled for 5 minutes. Samples were analyzed by SDS-PAGE and western blotting. Proteins were detected with primary anti-Myc polyclonal antibody (1:1000; MBL International, Woburn, MA, USA), anti-E-tag monoclonal antibody (1:1000; GE Healthcare), or plakophilin-2-specific monoclonal antibody (1:200; Progen), followed by horseradish peroxidase-coupled anti-mouse or anti-rabbit secondary antibody (1:3000; Amersham), and visualized with the ECL detection system (Amersham).

Generation of rat monoclonal antibodies

To generate monoclonal antibodies, peptide 1159 (MLAPKEDRLNANKNL-C), corresponding to the C terminus of mouse αT-catenin, was coupled to keyhole-limpet hemocyanin (Sigma), combined with Titermax (Sigma) as adjuvant, and injected into three rats. Boosts were given at intervals of at least 2 weeks. Sera were tested on peptide-ELISA until a titer of 1:5000 without loss of reactivity was obtained. Fusing spleen cells with Sp20Ag14 myeloma cells generated hybridomas. Supernatants of hybridoma cell lines positive for peptide-ELISA were tested by western blotting and immunofluorescence for recognition of αT-catenin fused N-terminally to Myc in cells transfected with plasmid pdCS3-mαTctn FL(120-2979). After subcloning, one hybridoma cell line, 1159_12A4S4, was further cultured for antibody production. Supernatant was purified and concentrated using a protein-A Sepharose column.

Immunohistochemistry

Paraffin sections of paraformaldehyde-fixed mouse heart tissues were deparaffinized with HistoclearII (National Diagnostics). Antigen was retrieved in EDTA-buffered Antigen Retrieval Solution, pH 9 (Prosan) in a microwave oven. To block endogenous peroxidase activity, sections were incubated with 3% hydrogen peroxidase for 5 minutes. After washing with PBS, they were incubated for 1 hour with primary antibodies: mouse monoclonal anti-PKP2 antibody (1:10; Progen), or rat monoclonal 1159_12A4S4 against αT-catenin (1:25), both diluted in PBS containing 0.4% gelatin. Secondary antibodies used were biotin-labeled goat anti-mouse IgG (LAB2 kit; Dako Cytomations, Glostrup, Denmark) or goat anti-rat IgG (1:300; Dako Cytomations); they were subsequently linked to streptavidin conjugated to horseradish peroxidase. Detection was carried out by incubation for 10 minutes with the chromogenic peroxidase substrate 3-amino-9-ethylcarbazole (AEC, LSAB2-kit, Dako Cytomations). After counterstaining with Hematoxylin, sections were mounted with Aquatex (Merck, Whitehouse Station, NJ, USA). Samples were examined with an Olympus BX51 microscope and images were recorded with a Coolsnap camera (Photometrics, Tucson, AZ, USA) using RSImage software (Roper Scientific, Trenton, NJ, USA).

Secondary antibodies used for colocalization were goat anti-mouse IgG labeled with Alexa Fluor 594 (1:500; Invitrogen) and goat anti-rat IgG labeled with Alexa Fluor 488 (1:500; Invitrogen). Sections were mounted with Vectashield. Fluorescence and DIC images were obtained using a BX61 microscope equipped with epifluorescent optics (60× NA 1.42 oil-immersion objective) and a Fluoview CCD camera. Green and red fluorescence signals were separated using a standard GFP and Dsred filter cube (Olympus Belgium, Belgium). Images were overlaid using the CellM software. 3D images were taken using an SP5 AOBS confocal microscope (Leica Microsystems, Germany). Simultaneous two-channel recording was performed with a 63× NA 1.4 oil-immersion objective using a pinhole size of 1 Airy unit and excitation wavelengths of 488 and 543 nm. The fluorescence emission signals were monitored with two spectral photomultiplier tubes (PMTs; bandwidth: 515-525 and 590-610). In order to make maximum intensity projections (MIPs), 29 z-sections of 0.25 μm were made. Colocalization analysis was done using the Leica application suite software. MIPs and 3D projections were made using the Leica Deblur software.

Immuno-EM

Mouse heart tissue was excised, briefly immersed in hexadecane, and frozen immediately in a high-pressure freezer (EM Pact, Leica Microsystems, Vienna, Austria). Freeze-substitution was carried out in a Leica EM AFS. Heart tissue was substituted in dry acetone over 4 days as follows: –90°C for 26 hours, 2°C per hour increase for 15 hours, –60°C for 8 hours, 2°C per hour increase for 15 hours, and –30°C for 8 hours. Samples were then slowly warmed up to 4°C. They were infiltrated stepwise over 3 days at 4°C in LR-White, hard grade (London Resin, Basingstoke, UK), and embedded in capsules. Polymerization was performed by UV illumination for 24 hours at 4°C followed by 16 hours at 60°C. Ultrathin sections of gold interference color were cut using an ultramicrotome (Ultracut E, Reichert-Jung) and collected on formvar-coated copper slot grids. All immunolabeling steps were performed in a humid chamber at room temperature. Grids were floated upside down on 25-μl aliquots of blocking solution (5% BSA, 1% FSG in PBS) for 15 minutes, and then washed for 5 minutes (1% BSA in PBS). They were incubated in primary antibodies diluted in 1% BSA in PBS for 60 minutes and washed four times for 5 minutes (0.1% BSA in PBS). Where appropriate, the grids were labeled with unconjugated bridging antibodies (DakoCytomation) in 1% BSA in PBS, and washed five times for 5 minutes with 0.1% BSA in PBS. Finally, grids were incubated for 25 minutes with 10-nm colloidal gold-protein A conjugates (PAG_{10nm}, Department of Cell Biology, Utrecht University, Utrecht, The Netherlands), and silver amplification (Aurion, The Netherlands) was done for 60 minutes. Sequential colocalization was performed as described previously (Bienz et al., 1986): incubation with the αT-catenin rat monoclonal antibody followed by a bridging antibody and PAG_{10nm}. After silver amplification for 120 minutes, the grids were incubated with either the anti-PKP2 mouse monoclonal Ab or the anti-desmin rabbit polyclonal antibody, followed by a bridging antibody and PAG_{10nm}. Sections were post-stained with uranyl acetate in an LKB ultrastainer for 30 minutes at 40°C, and with lead stain for 5 minutes at 20°C. Control experiments consisted of treating sections either with PAG_{10nm} alone or with bridging antibodies and PAG_{10nm}. Grids were viewed with a JEOL 1010 TEM at 80 kV. In order to quantify the number of gold particles per square micrometer, the TEM negatives were digitalized and the areas of fascia adhaerens-like (hybrid adhering) junctions and desmosome-like junctions were analyzed using the ImageJ analysis program, Scion Image (developed at the US National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image). The data were derived from at least triplicate labeling experiments, and in total at least

10 micrographs of both junction types were used, corresponding to 10 μm^2 cytoplasmic surface for each of the primary antibodies.

The following primary antibodies were used for immuno-EM: rat monoclonal mouse αT-catenin-specific antibody 1159_12A4S4 (diluted 1:10), rabbit polyclonal desmin (1:500; Abcam), αE-catenin- (1:500; Sigma) and β-catenin-specific antibody (1:500; Sigma), mouse monoclonal PKP2-specific (1:10; Progen), desmoglein1+2-specific antibody (1:50; Progen), and desmoplakin I+II-specific antibody (1:10; Roche Diagnostics).

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