αT-Catenin: a novel tissue-specific β-catenin-binding protein mediating strong cell-cell adhesion

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SUMMARY

Cadherins are major cell-cell adhesion proteins whose cytoplasmic domains bind to catenin proteins. Strong intercellular adhesion depends on linkage of the cadherin/catenin complex to the actin cytoskeleton via α-catenin. To date, it is not clear how different cell types achieve the variable strength of cell-cell adhesion clearly needed in a multicellular organism. Here, we report the cloning and molecular characterization of αT(testis)-catenin, a novel human cDNA encoding a protein with homology to both human αE(epithelial)-catenin and αN(neural)-catenin. Although originally discovered in testis, αT-catenin is expressed in other tissues, the highest levels being observed in heart. Immunohistochemical analysis showed human αT-catenin localization at intercalated discs of cardiomyocytes and in peritubular myoid cells of testis. In cells transfected with αT-catenin cDNA, interaction with β-catenin was demonstrated by co-immunoprecipitation. Transfection of α-catenin-deficient colon carcinoma cells recruited E-cadherin and β-catenin to cell-cell contacts and functional cadherin-mediated cell-cell adhesion was restored in this way. Moreover, compaction of these cells was at least as prominent as in the case of cells expressing endogenous αE-catenin. We propose that αT-catenin is necessary for the formation of stretch-resistant cell-cell adhesion complexes, in particular, muscle cells.

Key words: Catenin, Cadherin, Muscle, Heart, Testis

INTRODUCTION

The αE-catenin protein, a component of the epithelial cadherin/catenin adhesion complex, is a well-known invasion suppressor. To reach full functionality of the cadherin/catenin cell-cell adhesion complex, it is necessary to link the complex to the actin cytoskeleton. α-Catenins provide this link by binding to either β-catenin or plakoglobin through their N-terminal domain, and by binding actin directly, through their C-terminus, or indirectly, for example by binding to the actin-binding molecule α-actinin (Rudiger, 1998). It has been shown that loss of αE-catenin affects cell-cell adhesion and promotes tumorigenicity (Ewing et al., 1995). In several invasive cells, αE-catenin defects are observed, and introduction of exogenous αE- or αN-catenin can restore cell-cell aggregation and counteract invasiveness (Hirano et al., 1992; van Hengel et al., 1997; Watabe et al., 1994).

In contrast to the growing family of cadherins (Nollet et al., 2000; Yagi and Takeichi, 2000), the family of α-catenins is so far quite small. The αE-catenin protein is ubiquitously expressed, mainly in epithelial tissues. αN-catenin shows about 75% identity to αE-catenin, but its expression is restricted to neural tissues (Hirano et al., 1992). By analogy with αE-catenin, it can also bind to β-catenin or plakoglobin and is supposed to bind α-actinin and actin. Although vinculin shows much less identity (22%) to αE- or αN-catenin, it shares some properties with α-catenins. Vinculin is mainly found in focal adhesions, where it forms the link between the extracellular matrix-binding integrins and the actin cytoskeleton by binding to both the integrin-binding molecule talin and to F-actin (Burridge and Mangeat, 1984). Vinculin is sometimes found in cell-cell contacts as well, and it may even be able to take over the function of αE-catenin by binding directly to β-catenin (Hazan et al., 1997). However, vinculin has been reported to bind to a central region of αE-catenin and to be essential for apical junctional organization (Watabe-Uchida et al., 1998). Moreover, vinculin contains a unique central proline-rich hinge domain, which is absent in the α-catenins and which allows the vinculin tail to bind to the head, thus masking some binding sites (Johnson and Craig, 1995). For the recently reported α-catulin (Janssens et al., 1999), the identity to both α-catenins and vinculin is about 25% at the amino acid level, but no functional evidence for adhesive properties of α-catulin has yet been found. In addition to their structural role, it is becoming clear that α-catenins and vinculin also have a regulatory function in the coordination of assembly and disassembly of adherens junctions (Rudiger, 1998).

Here, we report on a novel α-catenin with about 55% identity and 70% similarity to other α-catenins. We demonstrate the tissue-specific expression of this αT-catenin and its localization to cell-cell junctions of cardiomyocytes. Moreover, testicular peritubular myoid cells are shown to

express αT-catenin. Further, we prove that αT-catenin can functionally restore cell-cell adhesion in colon cancer cells lacking α-catenins. Together, these data indicate that this novel αT-catenin is involved in the formation of specific cell-cell contacts in particular types of muscle cells.

MATERIALS AND METHODS

Cell cultures

Most of the cell lines used were purchased from the American Cell Type Culture Collection (ATCC, Rockville, MD). HCT-8/E8, HCT-8/E11R1 and HCT-8/R1 cell lines were obtained by subcloning of the human ileocecal adenocarcinoma cell line HCT-8 (CCL-224), where E stands for epithelioid and R for round-cell variants lacking αE-catenin (van Hengel et al., 1997). PC-3 (CRL-1435) is a human prostate carcinoma cell line and HEK-293 (CRL-1573) is a human embryonic kidney fibroblast cell line.

Cloning of human αT-catenin cDNA

A human αT-catenin-specific expressed sequence tag (EST) clone was identified by BLAST analysis (Altschul et al., 1990) and requested from the IMAGE consortium UK-HGMP Resource Center (Hinxton, UK). Expression of the corresponding transcript was confirmed by RT-PCR on PC-3 mRNA template using primers MCB967 (5′-TGAGGCAGAAAAAGAAAAGA-3′) and MCB968 (5′-AGTGTGGTTAGGCAGGATT-3′). To complete the cDNA sequence we performed two consecutive 5′ Marathon™ RACE experiments on a human testis Marathon cDNA preparation (Clontech, Palo Alto, CA). For the first 5′ RACE, the gene-specific primer was MCB1027 (5′-AATCTGCCGAGCAAGGACATCCA-3′) and the nested primer was MCB1028 (5′-TCAGGCAGTTGAGTCATCTTAGC-3′). RACE-PCR was performed on a Perkin Elmer 2400 thermal cycler (Perkin Elmer, Foster City, CA) following the supplied protocol (touchdown PCR). Obtained RACE fragments were purified from agarose gel using QIAquick™ columns (Qiagen, Chatsworth, CA) and then cloned in the pGEMT® vector (Promega, Madison, WI). The resulting plasmid was called pGEMT-haTctn-RACE1. As the cloned cDNA appeared to be incomplete, a second 5′-RACE experiment was performed with gene-specific primer MCB1254 (5′-ACCCGTGACGATGTGAGCA-ACTC-3′) and nested primer MCB1255 (5′-GAGCTGTCTGCGAA-GGTCTCTTG-3′). The obtained fragment was cloned in the pGEMT®-easy vector (Promega), and called pGEMTeasy-haTctn-RACE2.

All DNA sequences were determined by the dideoxy chain termination method (Sanger et al., 1977), using fluorescent dye terminators in a 377ABI automated DNA sequencer (Applied Biosystems, Foster City, CA). Sequences were assembled and compared by use of the software packages DNAstar (DNASTAR, Madison, WI) and Staden gap4 (Bonfield et al., 1995). Protein alignments and amino acid similarities were calculated using the CLUSTAL W program (Higgins and Sharp, 1989; Thompson et al., 1994) and GCG software. Alignments were shaded using the WWW-BOXSHADE server (http://ulrec3.unil.ch/softward/BOX_form.html).

Expression analysis by RT-PCR

Expression analysis using the human Rapid-Scan panel (OriGene Technologies, Rockville, MD) was performed on 100-times diluted template, followed by nested PCR (1/10 of the end volume of the first reaction was used). Primers MCB967 (5′-TGAGGCAGAAAAAG-AAAAGA-3′) and MCB968 (5′-AGTGTGGTTAGGCAGGATT-3′) were used for the first PCR, yielding a product of 743 bp. For nested PCR, primers MCB967 (5′-TGAGGCAGAAAAAGAAAAGA-3′) and MCB1010 (5′-GCTGAGCCTCGTCTGAC-3′) were combined, yielding a smaller product of 630 bp. The end-point determination method used does not allow a reliable determination of expression levels to be deduced from the amount of PCR product visualized on gel. Therefore, visual presence of a signal (even weak) was scored as positive, and complete absence was scored as negative. As a control, an αE-catenin-specific product of 747 bp was amplified with primers MCB53 (5′-CTTCGGGCCTCTGGAATTTA-3′) and MCB73 (5′-CGACATCAGGGTGCTGTAGG-3′). Amplified products were checked for specificity by sequence analysis, showing that the double bands observed for heart and testis after nested RT-PCR indeed correspond to the larger primary product and the smaller nested product predicted.

For RT-PCR analysis of mouse tissues, RNA was prepared from different tissues with the RNAeasy method (Qiagen) and cDNA was prepared using a commercial kit (Life Technologies, Paisley, UK). For mouse αT-catenin, primers MCB2461 (5′-CCCCAATGTTTT-ATGT-TAT-3′) and MCB2463 (5′-GGGGAGAACTCATCGTAT-3′) were designed on the sequence of an EST clone (GenBank accession no. AW556211), resulting in amplification of a 442-bp product. For mouse αE-catenin (GenBank accession no. NM_009818), a 733-bp product was amplified with primers MCB2636 (5′-GAAGGCCC-CTGAGAAGAA-3′) and MCB2637 (5′-CCCGAATAAAGCAAC-TCCAT-3′). For mouse αN-catenin (GenBank accession no. NM_009819) an 858-bp product was amplified with primers MCB2479 (5′-GCCCTGATTGAGTTTGATAA-3′) and MCB2480 (5′-CCCAGCTTCATAGTTCTCC-3′). As a control, a 452-bp fragment of mouse GAPDH was amplified with primers MCB2219 (5′-ACCACAGTCCATGCCATCAC-3′) and MCB2220 (5′-TCCA-CCACCCTGTTGCTGTA-3′).

Eukaryotic expression plasmids

After completion of plasmid constructs as described below, all clones were checked by DNA sequencing. For all PCRs, Pfu polymerase (Stratagene, La Jolla, CA) with proofreading activity was used. A PCR product of 1134 bp was synthesized with primers MCB1607 (5′-AGAATTCTCAGCTGAAACACCAATCAC-3′) and MCB1609 (5′-AGGATCCTGCGAAGGTCTCTTGTCT-3′) using the pGEMTeasy-haTctn-RACE2 clone as a template. This product, restricted with *Eco*RI and *Pst*I, was combined with a *Pst*I-*Ssp*I fragment of pGEMT-haTctn-RACE2, an *Ssp*I-*Bgl*II fragment from pGEMTeasy-haTctn-RACE1, and a *Bgl*II-*Sal*I fragment of a 3′ overlapping PCR product generated with primers MCB1610 (5′-GGATGATAATCAATTTGT-GGACATCTC-3′) and MCB1608 (5′-GGGATCCGTAGATTTG-TCTTCCTCTAA-3′) on RNA from the PC-3 prostate cancer cell line. The full-length human αT-catenin cDNA was inserted in the *Eco*RI-*Sal*I-digested pEGFPC2 vector (Clontech), in order to obtain an in-frame N-terminal fusion with the GFP protein. The resulting plasmid was called pEGFPC2-haTctn(179-2860). An *Eco*RI-*Bst*EII fragment from clone pGEMT-RACE2 was combined with a *Bst*EII-*Not*I fragment of pEGFPC2-haTctnCTN(179-2860) in the *Eco*RI-*Not*I-digested vector pEF6MycHisA (Invitrogen, San Diego, CA), yielding construct pEF6MH-haTctn(1-2860) encoding a C-terminal fusion between human αT-catenin and the Myc and His epitopes.

Screening of a human fetal kidney 5′ stretch cDNA library in vector λDR2 (Clontech) with an αE-catenin-specific probe resulted in the isolation of the pDR2αECTN plasmid, from which an *Eco*47III-*Sal*I fragment corresponded to the full-length αE-catenin cDNA (GenBank accession no. D14705). The latter fragment was purified and ligated in the *Eco*RI-*Sal*I-digested pEGFPC2 vector (Clontech), yielding plasmid pEGFPC2-haEctn(69-3454).

A full-length cDNA fragment of human αN-catenin (GenBank accession No. M94151) was prepared from plasmid pPN-hANCTN (Claverie et al., 1993), kindly provided by C. Petit (Institut Pasteur, Paris). This fragment was then cloned in the *Eco*RI-*Sal*I-digested pEGFPC2 vector (Clontech), yielding plasmid pEGFPC2-haNctn(39-2885).

Transfection experiments

For vaccinia virus-mediated transient overexpression, α-catenins were

cloned in the pE/L-GFP vector (Frischknecht et al., 1999). Cells were transfected with Lipofectin (Life Technologies) and simultaneously co-infected with vaccinia virus strain ΔA36R, which does not make actin tails (Parkinson and Smith, 1994). At 4 to 30 hours after transfection, high levels of expression under control of the vaccinia virus early/late promoter (E/L) (Chakrabarti et al., 1997) were obtained of the cloned cDNA, N-terminally fused to GFP. To this end, human αT-catenin cDNA was amplified with Taq+Precision polymerase (Stratagene) using primers containing a 5′ *Not*I site and a 3′ *Eco*RI site (MCB2386, 5′-GGGGGCGGCCGCGGAGGGTCA-GCTGAAACACCAATCACATTG-3′ and MCB2387, 5′-CCCCG-AATTCGCCGTGTGGTTAGGCAGGATTTTGTCATATAG-3′) and cloned into the *Not*I-*Eco*RI sites of the pE/L-GFP vector, yielding plasmid pE/L-GFP-haTctn(179-2913).

HEK-293 cells were transiently transfected by the calcium phosphate method. For stable transfection of HCT-8/R1 carcinoma cells, 4×10^6 cells were electroporated (Easyject; Eurogentec, Seraing, Belgium) with 10 μg of plasmid pEF6MH-haTctn(1-2860). Cells were plated and cultured in the presence of 6 μg/ml blasticidin (Invitrogen) to select for stable transfectants. Colonies of blasticidin-resistant cells were isolated and tested by immunofluorescence and western blotting for expression of αT-catenin. One positive clone was isolated and called HCT-8/R1/T31. As a negative control we transfected HCT-8/R1 cells with the empty pEF6MH vector, resulting in stable clones called HCT-8/R1/1743. As reported previously (van Hengel et al., 1997), related HCT-8/E11R1 cells were stably transfected with αN-catenin cDNA and a positive clone was designated HRpCαN2.

Antibodies

For the generation of polyclonal antibodies, peptide #952 with sequence C-KIHPLQVMSEFRGRQIY, corresponding to the C-terminus of the human αT-catenin protein, was coupled to keyhole-limpet hemocyanin (Sigma, St Louis, MO) and injected into each of three rabbits using Titermax (Sigma) as adjuvant. Boosts were given with intervals of minimum two weeks. Sera were tested by ELISA on the peptide used for injection, using nonrelevant peptide as a negative control. For western blotting, a dilution of 1:1000 was used and recognition of αT-catenin was inhibited by incubation of the polyclonal antibody in blocking solution containing 0.5 to 2.5% antigenic peptide for 1 hour prior to use.

Monoclonal antibodies were generated by coupling N-terminal peptide #892, with sequence C-VANKSDLQKTYQKL, to keyhole-limpet hemocyanin, followed by injection into C57Bl/6 mice. Boosts were given with intervals of 2 weeks, and sera were tested by peptide-ELISA until a titer of 1:10,000 without loss of reactivity was obtained after 6 weeks. Hybridomas were generated by fusion of spleen cells with Sp20_Ag14 myeloma cells. Supernatants of hybridoma cell lines, positive in peptide-ELISA, were tested by western blotting and immunofluorescence for recognition of the αT-catenin protein, fused at its N-terminus to GFP, in lysates of various cells transfected with plasmid pEGFPC2-haTctn(179-2860). One hybridoma cell line, designated 892_24D2S after subcloning, was deposited with the Belgian Coordinated Collections of Microorganisms (BCCM^{TM}) (no. LMBP 5537CB) and used for further analysis.

Other primary antibodies used were polyclonal αE-catenin- and β-catenin-specific antibodies (Sigma), monoclonal αE-catenin- and β-catenin-specific antibodies (Transduction, Lexington, KY), anti-E-cadherin monoclonal antibodies HECD-1 (Takara Biochemicals, Otsu, Japan) and MB2 (Bracke et al., 1993), anti-N-cadherin monoclonal antibody (Zymed, San Francisco, CA), monoclonal anti-desmin antibody clone 33 (Biogenex, San Ramon, CA), anti-desmoglein clone 10G11 (Progen, Heidelberg, Germany), anti-ZO-1 (Zymed) and anti-Myc-tag clone 9E10 (Oncogene, Cambridge, MA).

SDS-PAGE and western blot analysis

Protein lysates from various mouse tissues were prepared by isolating the tissues from normal BALB/c mice and mixing them in Laemmli buffer (Laemmli, 1970). Debris was removed by centrifugation and protein concentration was measured by the BioRad DC kit (BioRad, Richmond, CA). Lysates from subconfluent cultures of cell lines were also prepared in Laemmli buffer, followed by sonication and centrifugation. Of each protein lysate, 40 μg was diluted with 6× sample buffer (0.35 M Tris-HCl, pH 6.8, 10.28% SDS, 36% glycerol, 5% β-mercaptoethanol, 0.012% bromophenol blue), boiled for 5 minutes and subjected to separation on 10% polyacrylamide gels. Proteins were transferred onto Immobilon-P membranes (Millipore, Bredford, MA) and blocked with 5% nonfat dry milk, 0.1% Tween-20 in Tris-buffered saline buffer (100 mM Tris-HCl, pH 7.4, 1.4 M NaCl) prior to incubation with the primary antibody. Detection was carried out by phosphatase-coupled secondary antibodies (Sigma) and nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) as a substrate.

For co-immunoprecipitation, lysates of transfected HEK-293 cells were prepared in PBS containing 1% Nonidet P-40 and a protease inhibitor cocktail (Boehringer). Lysates of mouse tissues were prepared in extraction buffer containing 25 mM Tris/HCl (pH 7.5), 1 mM EDTA, 1 mM EGTA, 100 mM NaCl, 1% Triton X-100, 0.5% Nonidet P-40 and the protease inhibitor cocktail. Lysate (800 μg) was incubated overnight with 4 μg of the respective antibody, after which 100 μl of 50% protein-G Sepharose (Amersham Pharmacia Biotech, Rainham, UK) was added to monoclonal antibodies, whereas protein-A Sepharose (Amersham Pharmacia Biotech) was added to polyclonal antibodies. After 2 hours of incubation, the Sepharose beads were washed three times with PBS containing 0.1% Nonidet P-40, followed by boiling for 5 minutes in Laemmli buffer. Samples were split in two parts before being subjected to SDS-PAGE and western blotting. On these western blots, protein was detected by the ECL detection system using secondary antibodies coupled to horseradish peroxidase (Amersham Pharmacia Biotech).

Immunodetection in cryosections of human tissues

For immunohistochemistry, frozen sections of human heart and testis tissues were air dried and fixed in aceton at 4°C for 10 minutes. The sections were washed in PBS and preincubated with 10% goat serum for 10 minutes. They were then incubated for 30 minutes with crude monoclonal 892_24D2S hybridoma supernatant, which was diluted in PBS with 1% BSA, or with clone 33 anti-desmin antibodies. The secondary antibody used was biotin-labeled goat-anti-mouse Ig (Dako, Carpinteria, CA), which was subsequently linked to streptavidin-biotin complex coupled to horseradish peroxidase. Detection was carried out by a 5-minute incubation with the chromogenic peroxidase substrate diaminobenzidine (Biogenex). Cell nuclei were counterstained with hematoxylin, after which the slides were dehydrated in alcohol, cleared in toluol and mounted.

For double immunofluorescent staining, frozen sections were air dried, fixed in acetone at 4°C for 10 minutes, washed in PBS and preincubated with 10% goat serum for 10 minutes. The slides were then incubated for 45 minutes with mixtures of primary antibodies diluted in PBS: either 1:5 monoclonal antibody 892_24D2S plus 1:500 polyclonal anti-αE-catenin, or 1:500 polyclonal antibody #952 plus 1:500 monoclonal anti-N-cadherin. The secondary goat anti-mouse IgG and goat anti-rabbit IgG antibodies used were labeled with FITC or TRITC (Santa Cruz Biotechnology, Santa Cruz, CA), or with Alexa 488 or Alexa 594 (Molecular Probes, Eugene, OR).

Immunocytochemistry of cell cultures

Cells were grown on glass coverslips until confluency, rinsed briefly with PBS and fixed with either ice-cold methanol for 1 minute, or with 3% paraformaldehyde (Merck, Darmstadt, Germany) for 10 minutes at room temperature, followed by permeabilization in 0.2% Triton X-100 (Sigma) for 2 minutes. Cells were then incubated for 30 minutes with primary antibody diluted in blocking solution (20 mM Tris/HCl pH 7.5, 154 mM NaCl, 2 mM EDTA, 2 mM $MgCl_2$, with 1% BSA

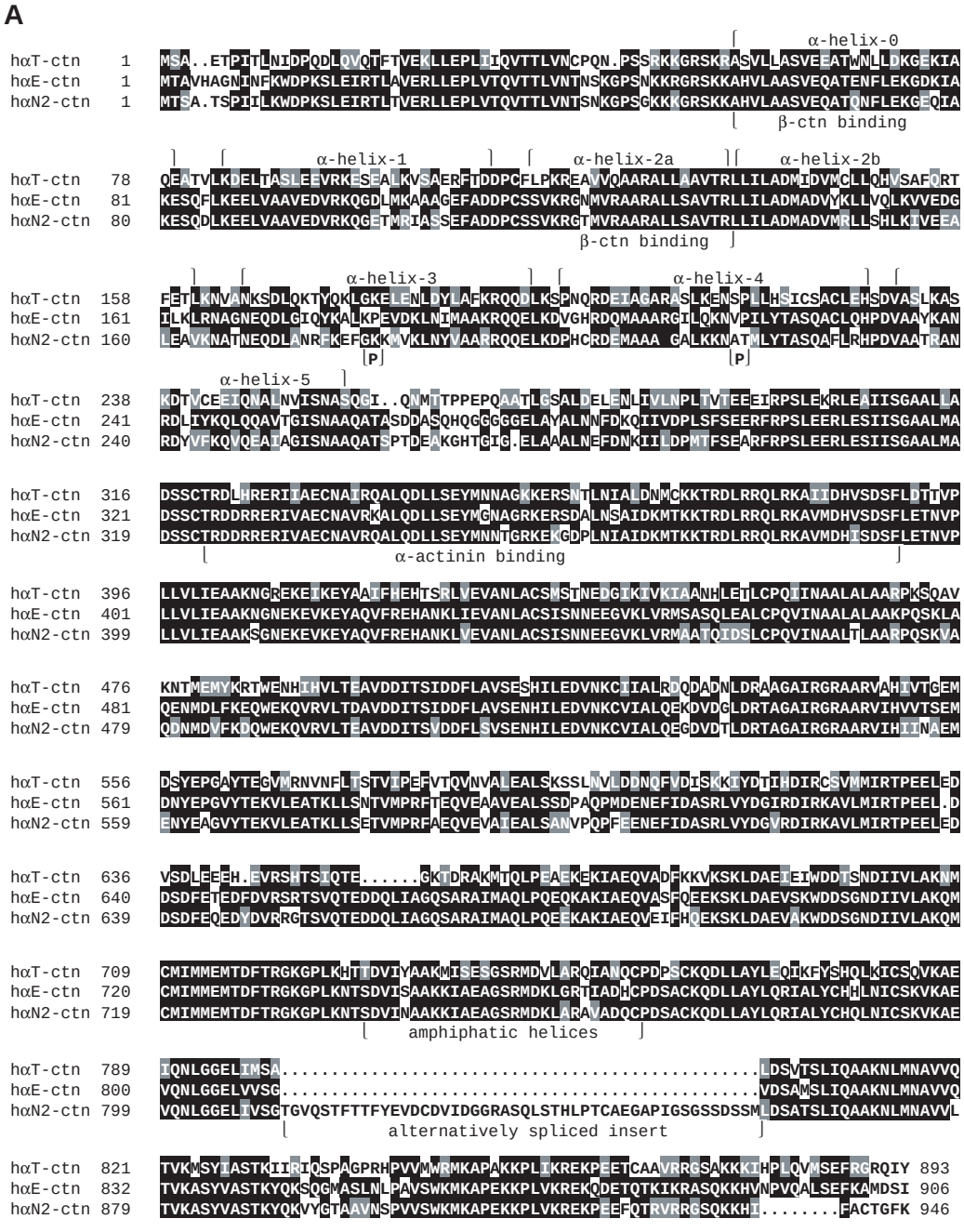

Fig. 1. Sequence comparison between the novel αT-catenin and other members of the human α-catenin/vinculin family. (A) Alignment of the protein sequences by the CLUSTAL W method (Higgins and Sharp, 1989). The location of N-terminal α-helices as determined for αE-catenin (Pokutta and Weis, 2000) is shown on top of the sequences. Only one of two characteristic proline residues, inducing kinks in the α-helices of αE-catenin (marked with P below the sequences) is conserved in αT-catenin. Other annotated domains of αE-catenin are: β-catenin-binding domains (Pokutta and Weiss, 2000; Huber et al., 1997), the α-actinin-binding domain (Nieset et al., 1997), amphiphatic helices possibly responsible for actin binding (Rudiger, 1998). Also indicated is the position of an alternatively spliced insert generally found for αN-catenin (Claverie et al., 1993). The sequences aligned here are available from GenBank under accession nos. AF091606 (αT-catenin), D14705 (αE-catenin) and M94151 (αN2-catenin). (B) Schematic protein alignments including amino acid identities (%) between the three main vinculin homology domains (black boxes) (Herrenknecht et al., 1991). As reported before (Janssens et al., 1999), α-catulin and vinculin are clearly more distant members of the α-catenin/vinculin family. The total size of the depicted proteins is indicated at the right (aa, amino acid residues), whereas residues flanking various domains are indicated by their codon numbers. N, amino-terminus; C, carboxy-terminus.

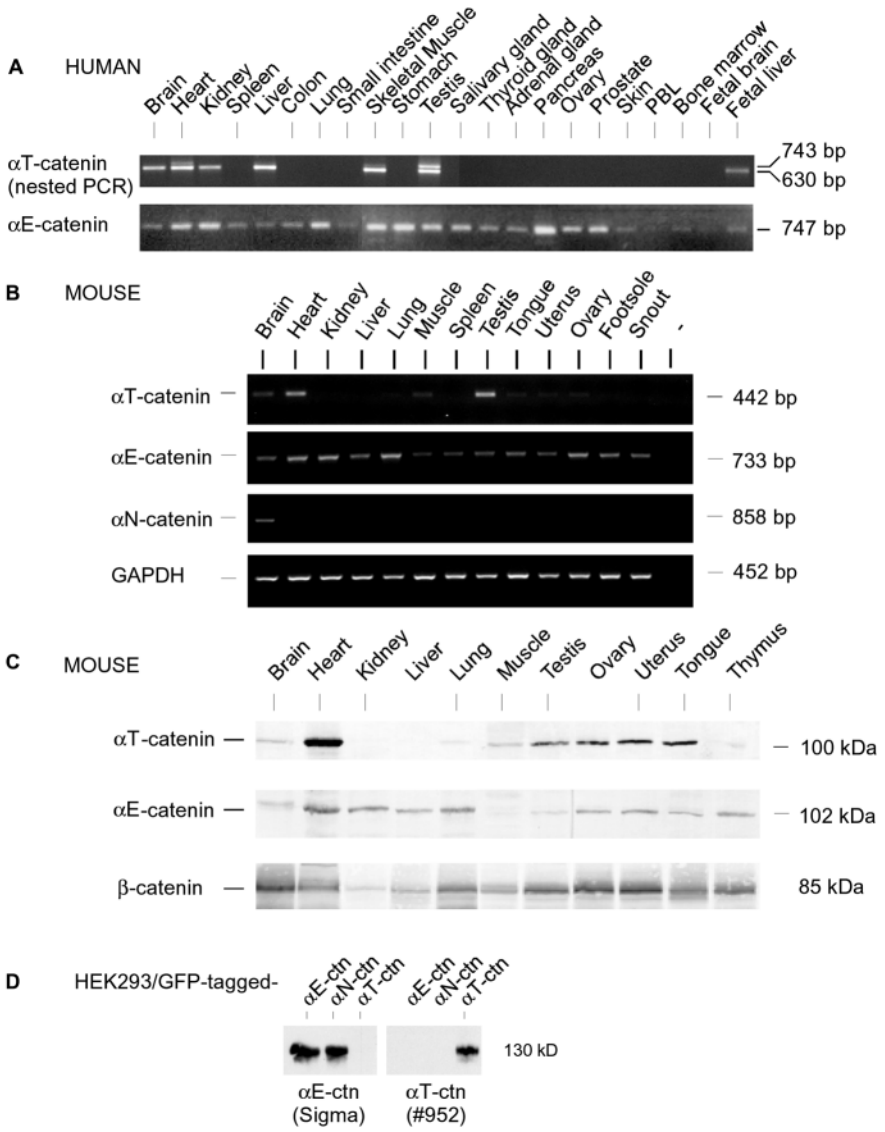

Fig. 2. Tissue-specific expression patterns of αT-catenin. (A) Rapid-scan RT-PCR expression analysis of human αT-catenin and αE-catenin mRNAs. The specific 743-bp product of the first reaction was visible in heart, testis and skeletal muscle (not shown). After nested PCR, this first product of 743 bp is still visible, whereas the nested PCR product of 630 bp is detectable in the same three samples and a few more (brain, kidney, liver, fetal liver). PCR with αE-catenin-specific primers (yielding a 747-bp product) reveals expression in most tissues. PBL, peripheral blood lymphocytes. (B) RT-PCR analysis of αE-catenin, αT-catenin and αN-catenin mRNAs in mouse organs. GAPDH mRNA analysis served as a positive control. (C) Western blot analysis of αT-catenin, αE-catenin and β-catenin protein expression in various mouse organs. For detection of αT-catenin, polyclonal serum #952 was applied. In brain tissue, the 104-kDa band revealed by anti-αE-catenin most likely corresponds to crossreacting αN2-catenin protein. (D) Antibody specificity was tested by western blot analysis of HEK-293 cells transfected with GFP-tagged α-catenins: a commercial αE-catenin antibody (Sigma) crossreacts with αN-catenin but not with αT-catenin, whereas serum #952 is highly specific for αT-catenin.

and 1% goat serum), washed in PBS, and incubated for 30 minutes with secondary antibodies diluted in blocking solution. Secondary anti-mouse IgG or anti-rabbit IgG antibodies were coupled to either Alexa 594 or Alexa 488 (Molecular Probes). Finally, cells were treated for 10 seconds with a 4′-6-diamidine-2-phenylindole-dihydrochloride solution (DAPI; Roche Diagnostics, Mannheim, Germany) to mark nuclear DNA, followed by mounting in Vectashield (Vector Laboratories, Burlingame, CA) to prevent photobleaching. Samples were examined with a Zeiss Axiophot microscope and images were recorded with a high-performance charge-coupled digital camera (Cohu, San Diego, CA) and NIH image software (version 1.62), or with a MicroMAX camera (Princeton, Trenton, NJ) and MetaMorph software (Image Universal Corporation, New York, NY).

Aggregation assays

Cell-cell adhesion was numerically evaluated in a fast aggregation assay as described before (Bracke et al., 1993). In brief, cultures were dissociated into single-cell suspensions under E-cadherin-saving conditions using collagenase. They were incubated under Gyrotory shaking (New Brunswick Scientific, New Brunswick, NJ) at 80 rpm for 30 minutes in an isotonic buffer containing either 1 mM EGTA or 1.25 mM Ca^{2+}. E-cadherin could be functionally blocked by treatment with MB2 anti-cadherin monoclonal antibody, starting 30 minutes before aggregation at 4°C and continued throughout aggregation at 37°C. The volume distribution (expressed as % of the total cell volume) in function of the particle diameter was measured by an LS200 particle size analyzer (Coulter Electronics Ltd, Luton, UK), at the start of the incubation at 37°C (t0) and after 30 minutes (t30). The Kolmogoronov-Smirnov test was applied to cumulative fast aggregation curves to analyze statistical differences.

Slow aggregation was performed as described (Boterberg et al., 2000). Briefly, single-cell suspensions were seeded onto a semi-solid agar medium. After 24 hours, aggregate formation was evaluated subjectively by phase-contrast microscopy.

For the compaction and decompaction assays, cells were seeded at a density of 6×10^5 cells/6 ml of RPMI supplemented with 10% fetal bovine serum. The suspended cells were incubated on a Gyrotory shaker (New Brunswick, NJ) at 80 rpm for 3 days. Aggregate formation was evaluated, after which the multicellular spheroids were dissociated by passing 30 times through Pasteur pipettes with a diameter of 1.5 mm (Takeda et al., 1995). Compaction and decompaction were evaluated qualitatively by phase-contrast microscopy and quantitatively by measuring the volume distribution (% of total cell volume) in function of the particle diameter using an LS200 particle size analyzer (Coulter).

RESULTS

Isolation of a novel α-catenin cDNA

By performing BLAST analyses (Altschul et al., 1990) with αE-catenin sequences as a query, two human EST sequences

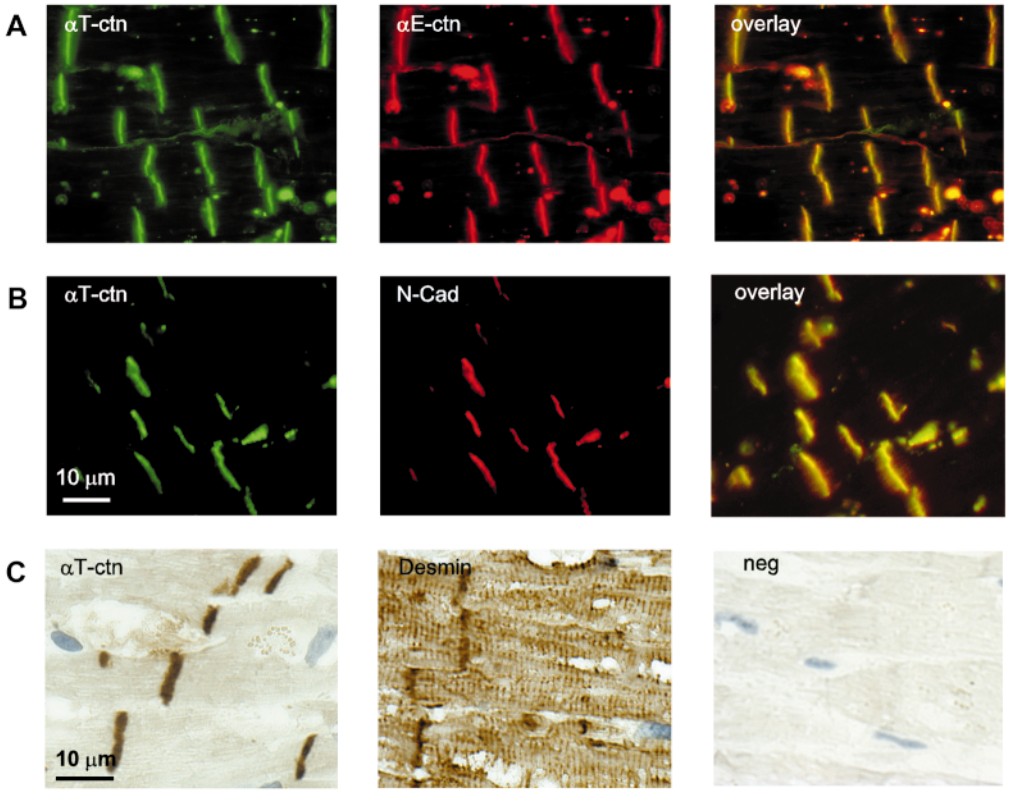

Fig. 3. Immunolocalization of αT-catenin in cryosections of human heart. (A) Double immunofluorescent staining of αT-catenin (monoclonal antibody 892_24D2S) and αE-catenin (polyclonal antibody) shows colocalization of the α-catenin proteins at intercalated discs of cardiomyocytes. (B) Double immunofluorescent staining of αT-catenin (polyclonal antibody #952) and N-cadherin (monoclonal antibody) shows colocalization at intercalated discs of cardiomyocytes. (C) Immunohistochemical staining for αT-catenin (monoclonal antibody 892_24D2S) or desmin (monoclonal antibody 33) shows that αT-catenin is localized at the intercalated discs of cardiomyocytes, while desmin is present also at Z-discs. In the negative control (neg), only secondary antibody was used.

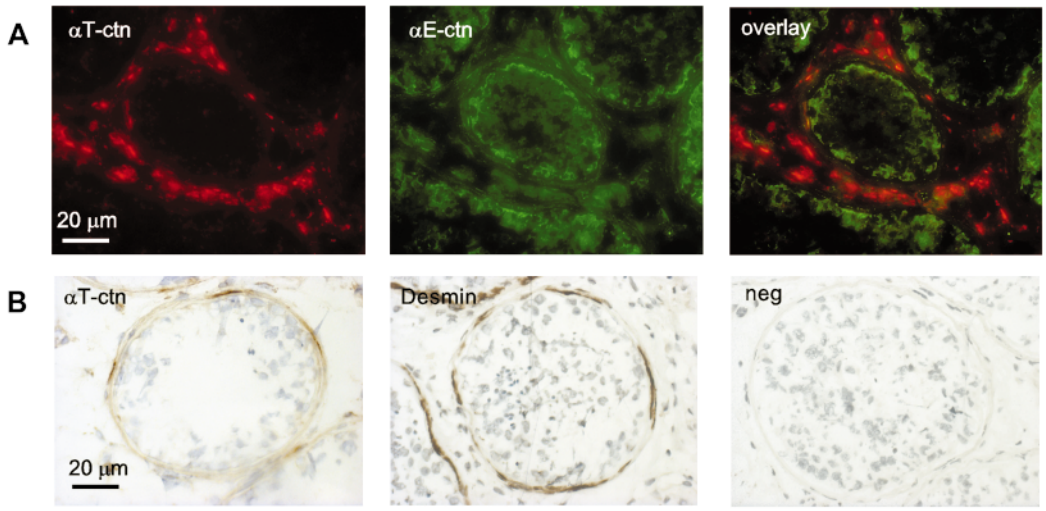

Fig. 4. Immunolocalization of αT-catenin in cryosections of human testis. (A) Double immunofluorescent staining of αT-catenin (monoclonal antibody 892_24D2S) and αE-catenin (polyclonal antibody) shows differential localization of these two related proteins. The αT-catenin is present in peritubular cells, clearly separated from αE-catenin, which is present in cells within the seminiferous tubules. (B) Immunohistochemical staining of consecutive sections for αT-catenin (monoclonal antibody 892_24D2S) and desmin (monoclonal antibody 33) demonstrates that αT-catenin is localized in desmin-expressing peritubular myoid cells. In the negative control (neg), only secondary antibody was used.

(GenBank accession nos. AA393647 and AA400832), both originating from IMAGE clone 728263, were found to be similar but not identical to αE- or αN-catenin. By RT-PCR, we confirmed faint expression of this novel transcript in the αE-catenin-negative prostate carcinoma cell line PC-3 (data not shown). Two consecutive 5′ RACE experiments using human testis mRNA as a template provided us with a full-length cDNA sequence (GenBank accession no. AF091606). The 3024 bp sequence contains a Kozak-consensus start codon (Kozak, 1991) at position 176, preceded by an in-frame stop codon at position 137. The stop codon terminating the ORF is located at position 2861, and a putative poly-adenylation signal (ATAAAA) is seen in the 3′ untranslated region 38 bp before the end of the sequence. The ORF encodes a protein of 895 amino acid residues, with a predicted molecular weight of 100 kDa and an overall identity to αE-catenin (102 kDa) and αN-catenin (104 kDa) of about 58% and 56%, respectively (Table 1; Fig. 1). Homology with either vinculin or α-catulin (Janssens et al., 1999) was significantly lower. Thus, we considered this novel molecule a true α-catenin family

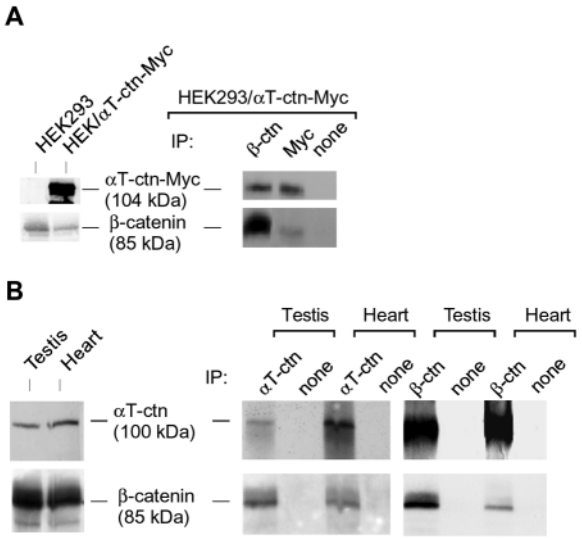

Fig. 5. Confirmation of αT-catenin/β-catenin interactions by co-immunoprecipitation (IP). (A) IP from HEK-293 cells transfected with a plasmid encoding Myc-tagged αT-catenin. In the western blots (left), which serve as controls for efficient transfection, αT-catenin was detected by monoclonal antibody 892_24D2S and β-catenin by a polyclonal antibody. The IP results (right) were obtained either with monoclonal anti-β-catenin antibody or with monoclonal anti-Myc antibody. SDS-PAGE was followed by western blotting. A mixture of both antibodies was then used to probe this blot. In mock transfected cells, only β-catenin was detected as expected (data not shown). (B) IP from mouse tissues, performed with polyclonal antibody #952, specific for αT-catenin, and a polyclonal antibody specific for β-catenin (Sigma). After western blotting of total lysates (left) and coimmunoprecipitates (right), αT-catenin and β-catenin were detected by use of the same antibodies.

member, and designated it αT-catenin because its transcript was discovered in a testis-derived cDNA library.

αT-catenin is highly expressed in heart and testis

A human cDNA Rapid Scan panel was screened by PCR for αT-catenin expression. A first PCR reaction revealed expression in mainly heart and testis (data not shown), whereas a second nested PCR also amplified low amounts in some additional tissues such as brain, kidney, liver (adult and fetal) and skeletal muscle (Fig. 2A). Compared with the ubiquitously expressed αE-catenin, the novel αT-catenin shows a very restricted expression pattern (Fig. 2A). These findings were confirmed by RT-PCR analysis of several mouse tissues (Fig. 2B). The brain-specific expression of mouse αN-catenin mRNA is in line with the literature (Hirano et al., 1992).

Table 1. Percentage of identities (and similarities) of members of the α-catenin/vinculin family*

	Vinculin	α-Catulin	αT-Catenin	αE-Catenin	αN-Catenin
Vinculin	100	18.6 (23.7)	17.3 (30.3)	22.5 (31.0)	22.3 (30.0)
α-Catulin		100	19.7 (32.1)	24.8 (33.9)	25.6 (32.8)
αT-Catenin			100	56.1 (73.7)	58.5 (69.6)
αE-Catenin				100	76.5 (83.1)
αN-Catenin					100

*MegAlign sequence comparison and distance calculation with GCG software (http://www.BEN.ac.be). Aligned sequences are available from GenBank under accession nos. M33308 (vinculin), U97067 (α-catulin), AF091606 (αT-catenin), D14705 (αE-catenin) and M94151 (αN2-catenin).

Polyclonal and monoclonal antibodies were generated against peptides derived from, respectively, the C- and the N-terminus of human αT-catenin. Upon analysis of several mouse tissue lysates by western blotting, we observed a specific cross-species reaction of polyclonal serum #952 with mouse αT-catenin (Fig. 2C). The highest expression of mouse αT-catenin protein was found in heart, but testis, ovary, uterus and tongue also scored positive. Low amounts were seen in brain, lung, skeletal muscle and thymus. The same mouse multiple tissue blot was incubated with antibodies specific for αE- and β-catenin. Both proteins were expressed ubiquitously. Compared with αT-catenin, αE-catenin protein levels seemed to be lower in heart and testis, but higher in kidney, liver, lung and thymus (Fig. 2C). The specificity of the α-catenin antibodies used was shown on lysates of HEK-293 cells transfected with the respective GFP-tagged α-catenin constructs (Fig. 2D). A commerical 'αE-catenin' antibody crossreacted with αN-catenin but not with αT-catenin, whereas polyclonal antibody #952 turned out to be specific for αT-catenin. The latter interaction with αT-catenin on western blot could be abolished by preincubation of antibody #952 with the immunizing peptide.

We then investigated the localization of αT-catenin protein in frozen tissue sections of human heart and testis. In human cardiomyocytes, αT-catenin protein was clearly located at cell-cell contacts (i.e. intercalated discs; Fig. 3). In double labelling experiments, it co-localized with αE-catenin (Fig. 3A) as well

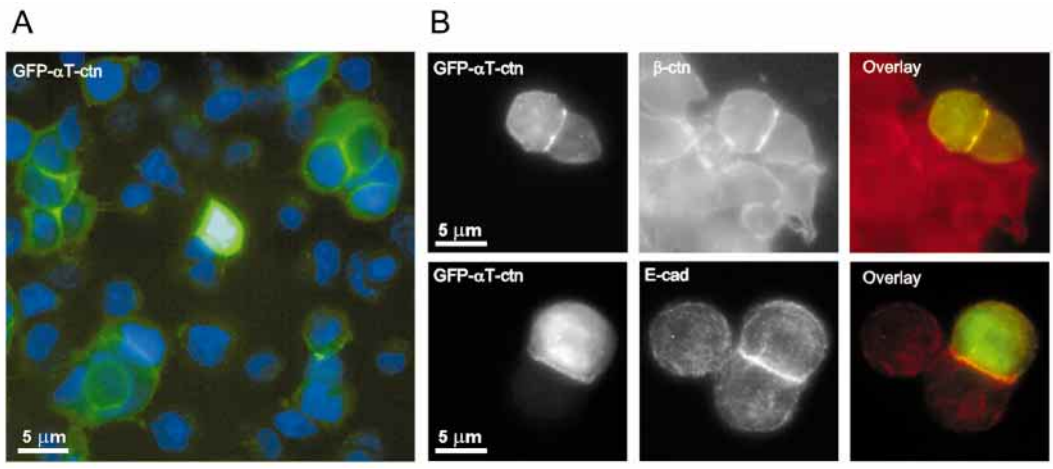

Fig. 6. Transient overexpression of αT-catenin in α-catenin-negative HCT-8/R1 colon carcinoma cells restores cadherin/catenin-mediated cell-cell adhesion. At 10 hours after transfection with pE/L-GFP-haTctn plasmid and simultaneous infection with ΔA36R vaccinia virus, opposing cells expressing GFP-αT-catenin show increased fluorescence at their common cell-cell contacts (A). This results in recruitment of β-catenin and E-cadherin to the same intercellular contact sites (B).

as N-cadherin (Fig. 3B). The muscle marker desmin could be detected at both intercalated discs and sarcomeric Z-lines, whereas αT-catenin expression was confined to intercalated discs (Fig. 3C). In human testis, αT-catenin protein was detected mainly in spindle-shaped cells surrounding testicular tubuli (Fig. 4). Interestingly, αT-catenin did not co-localize here with αE-catenin, as the latter showed an abundant intratubular expression pattern (Fig. 4A). The αT-catenin-expressing cells in testis correspond to desmin-positive cells (Fig. 4B), and therefore could be identified as peritubular myoid cells. These stainings suggest that αT-catenin expression is confined to specific muscle cell types, although detailed immunohistological studies of additional tissues are warranted in this respect.

αT-catenin interacts with β-catenin and functionally restores cell aggregation in α-catenin-negative cancer cells

The localization of αT-catenin at particular cell-cell contacts suggests an interaction between αT-catenin and the cadherin/catenin complexes. Interaction between αT-catenin and β-catenin was confirmed by coimmunoprecipitation either from lysates of HEK-293 cells overexpressing Myc-tagged αT-catenin, or from mouse heart and testis tissue extracts (Fig. 5).

To assess whether αT-catenin binding to β-catenin has functional implications for the formation of cell-cell contacts, we carried out rescue experiments by overexpression of αT-catenin in round HCT-8/R1 cells lacking α-catenins (Vermeulen et al., 1995; Vermeulen et al., 1999). Vaccinia virus-mediated expression was used to obtain high transient transfection efficiencies (30-70%). Cell-cell adhesion was found to be restored if neighboring cells expressed the ectopic protein that became enriched at cell-cell contacts, whereas solitary expressing cells remained round with diffuse expression of the ectopic protein (Fig. 6A). Moreover, when GFP-tagged αT-catenin was overexpressed in neighboring cells, its enrichment in cell-cell contacts recruited both β-catenin and E-cadherin to these sites (Fig. 6B). However, when αT-catenin was overexpressed for longer time periods, it tended to form cytoplasmic rod-like aggregates (data not shown).

To quantify the restoration of cell-cell adhesion by αT-catenin expression in HCT-8/R1 cells, these cells were transfected with a plasmid encoding Myc-tagged αT-catenin. Using polyclonal antibody #952, one transfectant was found to express ectopic αT-catenin and called HCT-8/R1/T31. Immunofluorescent analysis of HCT-8/R1/T31 cells with anti-Myc antibodies showed that the αT-catenin-Myc protein was localized at restored cell-cell contacts (Fig. 7). Components of the adherens junctions (E-cadherin, β-catenin and plakoglobin) were recruited to such αT-catenin-positive sites, but desmosomes (desmoglein-2) and tight junctions (ZO-1, occludin) also showed reassembly (Fig. 7).

By using the fast aggregation assay, we showed that HCT-8/R1/T31 cells are strongly aggregating, in contrast to nonaggregating parental HCT-8/R1 cells (Fig. 8). HCT-8/R1/T31 cells aggregated to an extent similar to that of αE-catenin-positive HCT-8/E8 cells and HRpCαN2 cells transfected with αN-catenin cDNA. These differences were significant (Table 2), showing that αT-catenin can functionally restore aggregation.

In a slow aggregation assay, αT-catenin-transfected HCT-8/R1/T31 cells were compacting to the same extent as αE-

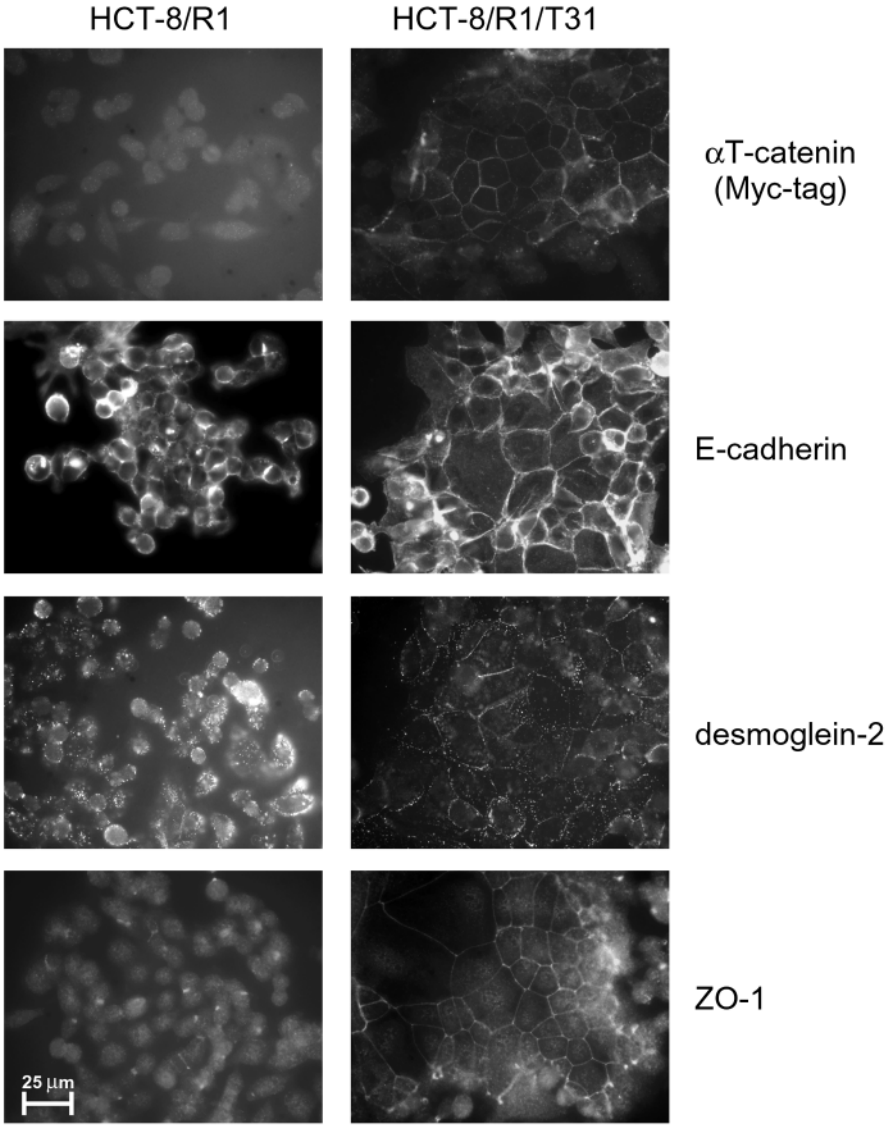

Fig. 7. Relocalization of multiple cell-cell adhesion components in stably transfected colon cancer cells, expressing Myc-tagged αT-catenin. The α-catenin-negative parental HCT-8/R1 cells (left panels) were compared to the cloned transfectant HCT-8/R1/T31 (right panels). Cells were stained for the Myc tag (exogenous αT-catenin), for E-cadherin, desmoglein-2 or ZO-1 antigens.

catenin-positive HCT-8/E8 cells (Fig. 9B). In a quantitative compaction/decompaction assay (Fig. 9C-E) we showed that multicellular spheroids of HCT-8/R1/T31 cells, like these of HCT-8/E8 cells, cannot be readily dissociated by repeated pipetting.

DISCUSSION

On the basis of sequence database analysis we discovered a novel member of the α-catenin family. Starting from a single EST clone derived from testis mRNA, a novel full-length cDNA was cloned, which we called αT-catenin. The encoded novel protein has about the same predicted size as other α-catenins (100 kDa) and shows an overall amino acid identity of 57%. When the three main vinculin homology domains previously proposed (Herrenknecht et al., 1991) were separately aligned, the highest sequence conservation was found in the C-terminal domain (Fig. 1B; 71.5% identity). It is noteworthy that the sequence conservation between the three presently known α-catenins is particularly elevated in some functional domains, such as binding sites for β-catenin, α-actinin and actin. Hence, αT-catenin is probably able to function as a typical α-catenin. In line with this observation, we demonstrated binding of αT-catenin to β-catenin by coimmunoprecipitation.

The 3D structure of an N-terminal fragment of the αE-catenin protein was resolved recently and shown to consist of antiparallel α-helices, which together form a four-helix bundle either by homodimerization or by binding to β-catenin (Pokutta and Weis, 2000). A typical feature of this αE-catenin structure is a tandem kink of 30°, generated by two proline residues in helices α3 and α4, respectively (marked by [P] in Fig. 1A). It is noteworthy that such proline residues are completely absent from the αN-catenin protein sequence, while only one is present in the αT-catenin sequence. The domain for binding to β-catenin is located in the very N-terminal part of α-catenins. Although it was reported that a small fragment, corresponding to α-helix-2a (Fig. 1A), can bind to β-catenin (Huber et al., 1997), it was more recently proposed on the basis of the 3D structure of the αE-catenin fragment, that α-helix-0 is also needed for binding to β-catenin (Pokutta and Weis, 2000). The amino acid sequences of both α-helix-0 and α-helix-2a seem to be well conserved across the three α-catenin types, along with their ability to bind to β-catenin.

As shown by RT-PCR and immunoblotting analysis, αT-catenin shows a restricted tissue-specific expression pattern, with remarkably abundant mRNA levels in heart and testis. Protein levels were particularly high in heart. In addition, specific transcripts were detected in brain and skeletal muscle of both man and mouse, and in tongue, uterus and ovary of mouse; this was confirmed at the protein level. Some discrepancy was observed between mRNA expression levels in kidney and liver of man versus mouse. This could be due partly to species differences. A wide variety of cell lines was checked by RT-PCR and western blotting, but all lacked detectable αT-catenin (data not shown). Overall, our expression analysis showed clearly that αT-catenin is more tissue-specific than αE-catenin or β-catenin, although less tissue-restricted than αN-catenin. The expression of αT-catenin in heart is particularly striking. In this context, it is remarkable that metavinculin, a member of the α-catenin/vinculin family, is

Table 2. Kolmogoronov-Smirnov test for statistical differences between fast aggregation curves

Curve 1	Curve 2	KS 1*	KS 2*
t0 HCT-8/R1	t30 HCT-8/R1	531	236
t30 HCT-8/R1	t30 HCT-8/R1 + MB2	718	270
t0 HCT-8/E8	t30 HCT-8/E8	3976	2958
t30 HCT-8/E8	t30 HCT-8/E8 + MB2	3511	3541
t0 HRpCαN2	t30 HRpCαN2	1610	2040
t30 HRpCαN2	t30 HRpCαN2 + MB2	1840	1720
t0 HCT-8/R1/T31	t30 HCT-8/R1/T31	3560	3580
t30 HCT-8/R1/T31	t30 HCT-8/R1/T31 + MB2	4020	3911

*Kolmogoronov-Smirnov values (KS) higher than 1000 correspond to a significant difference between curves (p=0.001); KS 1 and KS 2 are derived from two independent experiments.

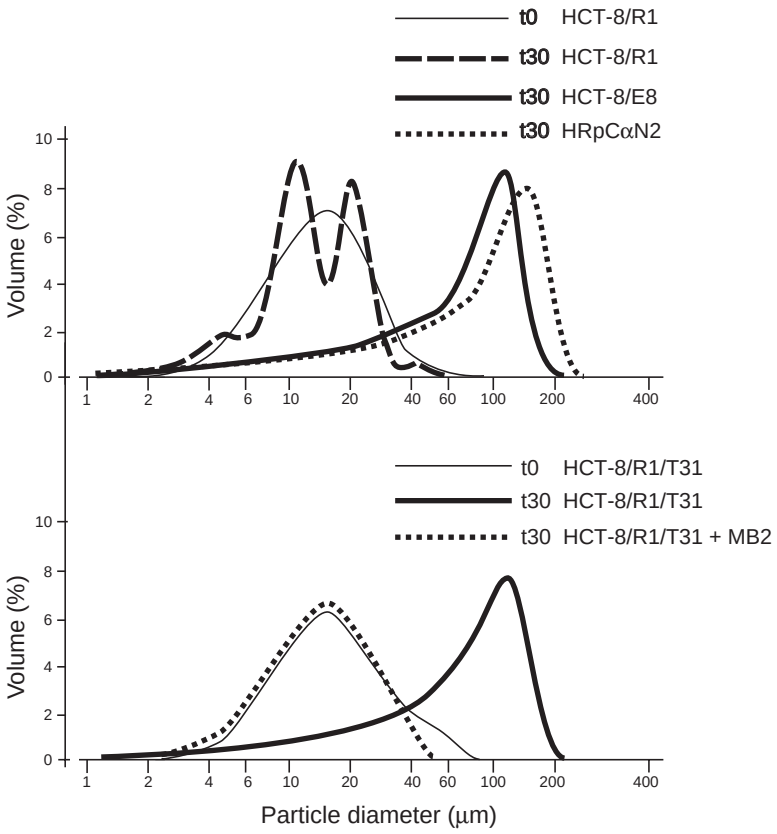

Fig. 8. Fast aggregation of α-catenin-negative HCT-8/R1 colon cancer cells is restored upon stable transfection with αT-catenin cDNA. After preparation of single-cell suspensions, cell aggregation was measured by determination of the volume distribution (%) in function of the particle diameter (μm) at the starting point (t0) and after 30 minutes (t30). HCT-8/R1, HCT-8/E11R1 and HCT-8/E8 cells were all obtained by subcloning HCT-8 cells, but only HCT-8/E8 cells express endogenous αE-catenin. HRpCαN2 is a cloned transfectant of HCT-8/E11R1 cells expressing exogenous αN-catenin (van Hengel et al., 1997); HCT-8/R1/T31 is a cloned transfectant of HCT-8/R1 cells expressing exogenous αT-catenin. MB2 is a monoclonal E-cadherin blocking antibody.

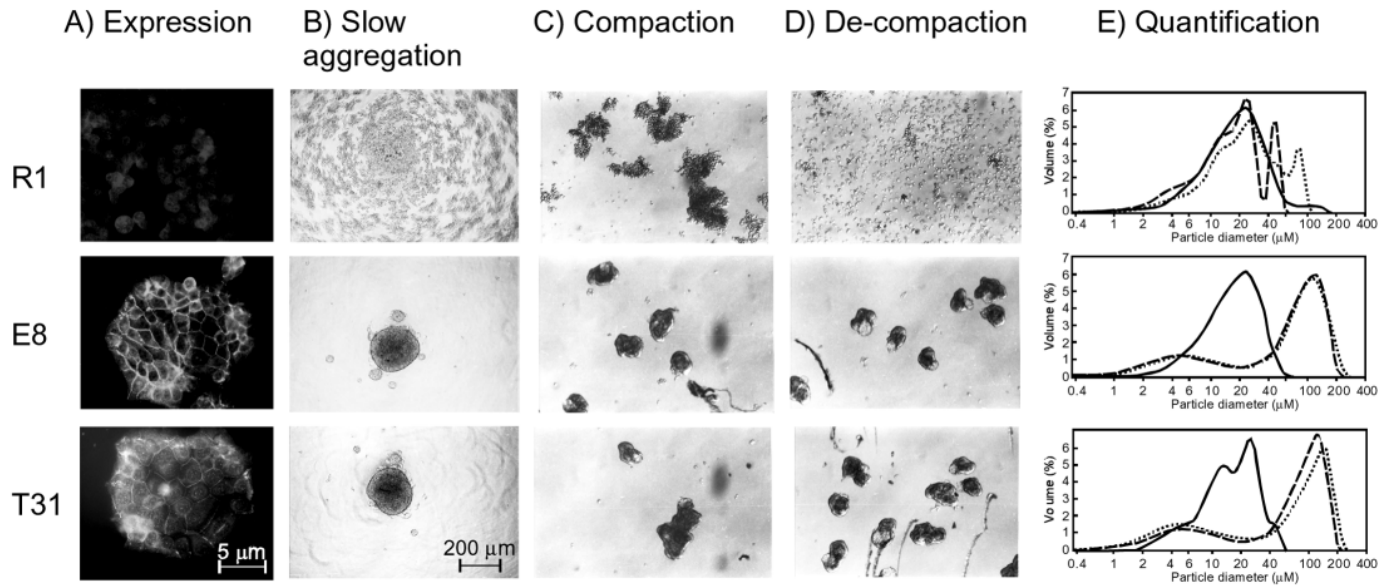

Fig. 9. Slow aggregation, compaction and decompaction assays on various related HCT-8 colon cancer cells. (A) Representative immunofluorescent staining for α-catenins in the cell lines used: no expression in untransfected HCT-8/R1 cells (R1), strong expression of αE-catenin in untransfected HCT-8/E8 cells (E8), and moderate expression of exogenous αT-catenin in transfectant HCT-8/R1/T31 (T31). (B) After slow aggregation of single-cell suspensions for 24 hours on semi-solid agar, images of representative cultures were taken. No aggregation is seen in cultures of HCT-8/R1 cells. Cells expressing exogenous αT-catenin form compacted large aggregates, similar to those of cells expressing endogenous αE-catenin. (C-D) Compaction-decompaction assays. (C) After Gyrotory shaking of suspended cell cultures for 3 days, cell aggregates were similar to the results obtained in (B). (D) Small spheroids of HCT-8/R1 cells were dissociated by repeated pipetting, whereas no decompaction could be seen of larger spheroids of HCT-8/E8 and HCT-8/R1/T31 cells. (E) Quantification of compaction/decompaction by volume distribution in function of the particle diameter: unbroken line, before compaction; dashed line, compaction; dotted line, decompaction after pipetting.

specifically expressed in cardiac and smooth muscle (Belkin et al., 1988; Gimona et al., 1987).

The polyclonal and monoclonal antibodies generated by us against αT-catenin peptides turned out to be specific without crossreaction with αE-catenin or αN-catenin. Likewise, all α-catenin-specific antibodies tested hardly crossreacted with αT-catenin protein in western blot analyses. This means that in all previously reported analyses of adhesion complexes, including the specialized intercalated discs between individual heart muscle cells, αT-catenin protein was probably not detected. The availability of αT-catenin-specific antibodies without crossreactivity will allow previous findings to be re-evaluated. In intercalated discs N-cadherin is the most important junction molecule (Volk and Geiger, 1984; Volk and Geiger, 1986). N-cadherin is known to be linked at its cytoplasmic site to β-catenin or plakoglobin (Hertig et al., 1996), but much less is known about the particular link between this complex and the actin cytoskeleton, more particularly the heart-specific myofibrils. It has been suggested that this link is very important, as plakoglobin null-mutant embryos die from embryonic day 10.5 onwards, owing to an impairment in the architecture of myocardiac intercalated discs (Bierkamp et al., 1996; Ruiz et al., 1996). We detected colocalization of αE- and αT-catenins in intercalated discs.

In human testis cryosections, we detected αT-catenin protein in peritubular myoid cells. Interestingly, αE-catenin is not co-localizing here with αT-catenin as it is present in intratubular cells. This differential localization points towards a specific function for αT-catenin. It was shown for peritubular myoid cells in the mouse that they express P-cadherin and β-catenin at their cell membranes from day 15 after birth (Lin and DePhilip, 1996), when actin filaments are fully organized in a characteristic orthogonal pattern (Vogl et al., 1995). In maturating peritubular cells of the rat, αE- and β-catenin were detected but not plakoglobin (Byers et al., 1994). We may propose a role for αT-catenin in the formation of a complex between P-cadherin and F-actin, thus stabilizing the cell-cell contacts between testicular peritubular myoid cells.

To show functionality of αT-catenin in cell-cell adhesion complexes, expression of ectopic αT-catenin in HCT-8/R1 cells lacking α-catenins was found to functionally restore adhesive properties. Vaccinia virus-mediated transient overexpression of αT-catenin in these cells clearly resulted in recruitment of β-catenin to cell-cell contact sites. Stable transfection with a plasmid encoding Myc-tagged αT-catenin resulted in restoration of the cadherin-based adhesion complex, but also of structures resembling tight junctions and desmosomes. We showed restoration of strong cell-cell adhesiveness in both fast and slow aggregation assays. Thus, we showed that αT-catenin, like αE- and αN-catenin, functions in cadherin-mediated cell-cell adhesion by providing the necessary link between β-catenin and the actin cytoskeleton.

The fact that αT-catenin co-immunoprecipitated with β-catenin from testis and heart tissues points towards a specific function in adhesion complexes of these tissues. The relevance of co-expression of αT- and αE-catenin in cardiomyocytes remains to be elucidated. It is conceivable that stronger anchoring of adhesion complexes to the actin cytoskeleton is needed in heart muscle tissues, where αT-catenin is mainly

expressed. αT-catenin may reinforce the cell-cell adhesion junctions by forming molecular complexes similar to but tighter than those containing αE-catenin. Furthermore, it is possible that αT-catenin distinguishes itself from other α-catenins by binding to specific interaction partners.

In summary, we report an interesting novel member of the α-catenin family. Although αT-catenin can be detected in the same tissues as αE-catenin, enriched levels, especially in cardiomyocytes and testicular peritubular myoid cells, point at a crucial role in tissue-specific cell-cell adhesion junctions. Introduction of ectopic αT-catenin in carcinoma cells with defective αE-catenin expression restores functional cadherin-catenin complexes. Further analysis of αT-catenin may contribute to understanding how particular tissues achieve different ways of cell-cell adhesion by use of specific α-catenins. It will be challenging to discover αT-catenin-specific binding partners and functions that are not shared by the other α-catenins.

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