

Assembly of the cadherin-catenin complex in vitro with recombinant proteins

Hermann Aberle, Stefan Butz, Jörg Stappert*, Helge Weissig†, Rolf Kemler‡ and Heinz Hoschuetzky

Max-Planck-Institut für Immunbiologie, D-79108 Freiburg, Germany

*Present address: Stanford University, Department of Molecular and Cellular Physiology, School of Medicine, Stanford, CA 94305-5426, USA

†Present address: La Jolla Cancer Research Foundation, 10901 North Torrey Pines Road, La Jolla, CA 92037, USA

‡Author for correspondence

SUMMARY

The cytoplasmic domain of classical cadherins is tightly associated with three proteins termed α -, β - and γ -catenin. These accessory proteins are of central importance for the adhesive properties of this class of cell adhesion molecules. In order to examine the molecular architecture of the cadherin-catenin complex in more detail we have expressed the catenins and the cytoplasmic domain of E-cadherin as fusion proteins in *Escherichia coli*, and analyzed the interaction of purified recombinant cadherin and catenins in combinatorial protein-protein interaction experiments. The cytoplasmic domain of E-cadherin cannot directly associate with α -catenin but interacts with high affinity with β -catenin, whereas the binding of γ -catenin (plakoglo-

bin) to E-cadherin is less efficient. α - and β -catenin assemble into a 1:1 heterodimeric complex. The analysis of various truncated β -catenins revealed that an α -catenin binding site in β -catenin is localized between amino acid positions 120 and 151. The central role of β -catenin for the assembly of the heterotrimeric E-cadherin/ α -catenin/ β -catenin complex in mixing experiments with all components was demonstrated. The reconstitution in vitro of the cadherin-catenin complex should allow the study of the interaction with signalling molecules and with the actin-based cytoskeleton.

Key words: cadherin, catenin, reconstitution, interaction

INTRODUCTION

Catenins are peripheral cytoplasmic proteins, which were first identified in association with the epithelial cell adhesion molecule E-cadherin (uvomorulin) in immunoprecipitation experiments with anti-E-cadherin antibodies (Ozawa et al., 1989; Nagafuchi and Takeichi, 1989). Analysis of various truncated E-cadherin polypeptides expressed in mouse L cells led to the definition of a 72 amino acid region within the cytoplasmic domain of E-cadherin that mediates the interaction with catenins (Ozawa et al., 1990). Additional deletion and point mutations within this 72 amino acid domain delimited the catenin-binding site to a 30 amino acid peptide (E-cadherin, amino acid positions 677-706) (Stappert and Kemler, 1994).

Besides E-cadherin, other classical cadherins like mouse N- and P-cadherin, *Xenopus* N-cadherin and chicken L-CAM were also found to be associated with catenins (for review, see Kemler, 1992). At present the major biological function of catenins is thought to be a molecular link between cadherins and the actin-based cytoskeleton (Stappert and Kemler, 1993). Their association with cadherins seems to be essential for the manifestation of full cell-cell adhesion activity. Catenins are also part of a higher order sub-membranous protein network by which cadherins are connected to other integral membrane proteins (McNeill et al., 1990). Recently it was reported that catenins can also be found associated with tumor suppressor gene products (Rubinfeld et al., 1993; Su et al., 1993) and that catenins are substrates for tyrosine kinases (Matsuyoshi et al.,

1992; Hamaguchi et al., 1993; Behrens et al., 1993; Shibamoto et al., 1994; Hoschuetzky et al., in press). These experimental observations led to the hypothesis that catenins might represent important proteins in the regulation of the extracellular adhesive properties of cadherins and in their interaction with the cytoskeleton (Kemler, 1993).

On the basis of their different molecular masses, catenins have been designated α -catenin (102 kDa), β -catenin (88 kDa), and γ -catenin (80 kDa). A combination of biochemical analyses - including pulse-chase and cross-linking experiments and DNase I binding studies - indicated that β -catenin interacts more directly with the cytoplasmic domain of E-cadherin than does α -catenin (Ozawa and Kemler, 1992). In contrast, α -catenin seems to mediate the interaction with actin filaments (Ozawa et al., 1990). Primary structure analysis of α - and β -catenin revealed a close resemblance to already known proteins also involved in cell-cell and cell-matrix interactions. α -Catenin shares sequence similarity with vinculin, a peripheral cytoplasmic protein localized in focal contacts and in adherens junctions (Geiger et al., 1980; Burridge et al., 1988). α -Catenin has been cloned in mouse (Herrenknecht et al., 1991; Nagafuchi et al., 1991), human (Claverie et al., 1993; Oda et al., 1993a), chicken (Hirano et al., 1992) and *Drosophila* ($D\alpha$ -catenin, Oda et al., 1993b). Sequence analysis revealed the existence of at least two isoforms of α -catenin (αE and αN). β -Catenin has been cloned in *Xenopus* (McCrea et al., 1991), mouse (Butz et al., 1992) and invertebrates (Rosenthal, 1993). The inferred primary amino acid sequences revealed similar-

ity to the product of the *Drosophila* segment polarity gene *armadillo* (Riggleman et al., 1990; Peifer and Wieschaus, 1990) and to plakoglobin, a component of desmosomes and adherens plaques (Cowin et al., 1986; Franke et al., 1989). Peptide pattern analysis (Ozawa et al., 1989) and immunochemical data (Peifer et al., 1992) suggested that γ -catenin might be identical or closely related to plakoglobin, and this was further substantiated, since plakoglobin is a component of the cadherin-catenin complex (Knudsen and Wheelock, 1992; Piepenhagen and Nelson, 1993).

To analyze the molecular interactions between catenins and the cytoplasmic domain of E-cadherin in more detail we have expressed the corresponding proteins in *Escherichia coli* and subjected the purified recombinant proteins to in vitro association experiments in order to reconstitute the cadherin-catenin complex.

MATERIALS AND METHODS

Chemicals and reagents

Restriction endonucleases and other molecular biological reagents were purchased from Boehringer. *Taq* polymerase, ECL detection kit and X-ray films were from Amersham, and peroxidase and alkaline phosphatase-labeled secondary antibodies from Dianova. (Hamburg, Germany). Oligonucleotides were synthesized on an Applied Biosystems model 394A synthesizer with β -cyanochemistry. Peptides were synthesized on an Applied Biosystems model 431A, using Fmoc chemistry.

Antibodies

Polyclonal antibodies against synthetic peptides corresponding to α -catenin (peptide M12K, amino acids 891-901), to β -catenin (peptide M14M, amino acids 1-14; peptide P14L, amino acids 768-781) and to plakoglobin (peptide D15A, amino acids 731-745) were raised in New Zealand White rabbits as described (Herrenknecht et al., 1991; Butz et al., 1992). Briefly, the peptides were conjugated to keyhole limpet hemocyanin (KLH) via glutaraldehyde. The peptide D15A was synthesized on a polystyrene-polyoxyethylene graft copolymer carrying a branched hepta-lysine and used directly for immunization (Butz et al., 1994) and for affinity-purification of the respective antibodies.

A polyclonal antibody specific for the cytoplasmic domain of murine E-cadherin was generated against the peptide L30D - corresponding to amino acids 677-706 - conjugated with glutaraldehyde to KLH.

Peptide-specific antibodies were isolated from pooled sera by affinity chromatography on the corresponding peptide affinity columns (peptides were conjugated to activated Sepharose 4B). Antibodies were eluted with 100 mM glycine-HCl, pH 2.5, neutralized with 1 M Tris-HCl, pH 8, and stabilized with 1 mg/ml BSA and 0.02% (w/v) sodium azide.

The monoclonal antibody, mab-GST-19, specific for the pGEX2T (Pharmacia) encoded glutathione S-transferase (GST), has been described (Flaswinkel and Reth, 1994).

Construction of expression plasmids

Recombinant DNA work was done according to standard procedures (Sambrook et al., 1989). Colonies expressing recombinant proteins were identified by colony immunoscreening, restriction analysis and immunoblotting.

MBP expression construct of the cytoplasmic domain of E-cadherin (ECT)

The expression plasmid pGEXUC1, coding for the cytoplasmic

domain of murine E-cadherin (ECT, amino acids 580-728) fused to the C terminus of the GST of pGEX2T (Pharmacia), has been described (Marrs et al., 1993). pGEXUC1 was digested with *EcoRI* and the 5' protruding ends were filled in with Klenow polymerase. After *BamHI* digestion ECT was subcloned into the *BamHI/SmaI* sites of pQE40 (Diagen), excised from pQE40ECT with *BamHI/SalI* and ligated into the corresponding restriction sites of the pMAL-C2 vector (Biolabs).

α -Catenin expression construct

The α -catenin cDNA (Herrenknecht et al., 1991) was amplified by overlapping-extension PCR. With a first set of primers a new *BspHI* site was added to the 5'-end of α -catenin (p α -cat5', nt 73-92 of cDNA: 5'-GTGGAATTCTCATGACTGCCGTCCACGCAGG), while the internal *BspHI* site in α -catenin was deleted by silent mutation (p Δ BspHIAS, nt 2251-2229: 5'-GGTCATCTCCATCATTATCATGCAC). With a second set of primers the overlapping region necessary for extension was synthesized (p Δ BspHIS, nt 2229-2253: 5'-GTGCATGATAATGATGGAGATGACC), and a unique *BglIII* site was introduced to the 3' end of α -catenin (p α -cat3', nt 2782-2765: 5'-ATAAAGCTTCAAGATCTGTCCATGGCTTTGAACTCGC). By combining the two PCR fragments, full-length α -catenin was amplified, using the primer pair p α -cat5' and p α -cat3'. The amplified sequence was digested with *BspHI* and *BglIII*, and cloned in frame into the *NcoI/BglIII* site of the His6-tag expression vector pQE60 (Diagen). In the final step most of the PCR-amplified α -catenin sequence was replaced by authentic α -catenin cDNA using the unique restriction sites *NarI* and *EcoNI*. The PCR-amplified 5' and 3' regions of the recombinant α -catenin were verified by sequence analysis.

GST expression constructs of β -catenin

Two *EcoRI* fragments covering the entire β -catenin coding region (Butz et al., 1992) were subcloned into the pSKII vector. The two plasmids were termed pSK8E2 (N-terminal coding 1.3 kb fragment, amino acids 1-422, 5'-3' orientation) and pSK8E1 (C-terminal coding 2.1 kb fragment, amino acids 422-781, 3'-5' orientation). In order to express full-length β -catenin, as well as various β -catenin domains, these two vectors were used as PCR templates. Synthetic PCR primers contained sequences coding for the N or C terminus of the desired polypeptides flanked by additional sequences for restriction sites, initiation or termination codons. The PCR products were blunt-end ligated and subcloned into the *EcoRV* site of pSKII. The amplified sequences were verified by sequencing. For the generation of expression cassettes, inserts in opposite orientations were excised with *BamHI* at an insert-specific unique restriction site. The two isolated fragments were ligated into the *BamHI* site of pSKII. By this procedure each expression sequence was flanked by *BamHI/SmaI/PstI/EcoRI* restriction sites.

To generate a β -catenin full-length expression construct the cDNA sequence coding for the β -catenin N terminus (amino acids 1-119) was amplified with the primer pairs MKNDEI (5'-CATATGGC-TACTCAAGCTGACC) and MKNTERM (5'-CTATTAGGGAT-GAGCAGCGTCAAAC) on the pSK8E2 template. The PCR product was blunt-end ligated and cloned in both orientations into the *EcoRV* site of pSKII. The corresponding vectors were termed pSKNTERM5' and 3'. The entire β -catenin coding sequence (pSK β tot) was assembled by combining the *BamHI/SphI* fragment of pSKNTERM1, the *SphI/EcoRI* fragment of pSK8E2, the *EcoRI/SstI* fragment of pSK8E1 and the *SstI/BamHI* fragment of pSK β EH2(HH)I. pSK β EH2(HH)I was obtained by digesting pSK8E2 with *HindIII* and ligating the resulting C-terminal coding *HindIII* fragment (nt 1362-2460 of cDNA) in the corresponding site of pSKII (3'-5' orientation).

The expression cassette coding for the β -catenin N-terminal sequence (amino acids 1-119) was generated by digesting the two vectors pSKNTERM5' and 3' with *BamHI/HincII*. The gel-purified fragments (coding for amino acids 1-57 and 58-119) were simul-

taneously ligated into *Bam*HI-digested pSKII, and the product was designated pSKBNTERM.

The expression cassette corresponding to the β -catenin C-terminal sequence (amino acids 683-781), the pSKEH2(HH)I template, was amplified using the primer pair MKCTERM (5'-ATGAGGACAGAGCAATGGCT) and T3 (5'-TTAACCCCTCACTAAAG). The PCR product was subcloned into the *Eco*RV site of pSKII (pSKCTERM). The final expression cassette coding for the C-terminal domain of β -catenin was obtained using the unique *Eco*RV site within this fragment. Two pSKCTERM plasmids containing inserts in opposite orientations were *Bam*HI/*Eco*RV digested and the fragments (for amino acids 683-708 and 709-781) simultaneously ligated into *Bam*HI-digested pSKII (designated pSKBCTERM).

For the construction of pSK Δ NTERM a PCR reaction was carried out on the pSK8E2 template with the primer pair Δ NTERM (5'-ATGACTAATGTCCAGCGCTTG) and β P4rev (5'-GGTCTGTCA-GATGAAG). The PCR product was inserted into the *Eco*RV site of pSKII (pSK Δ NTERM). pSK β tot was digested with *Xba*I/*Bgl*II to remove all but the very C-terminal most coding sequences (amino acids 695-781) and ligated with the *Xba*I/*Bgl*II fragment of pSK Δ NTERM (for amino acids 120-302). The resulting cDNA construct (pSK Δ BgIII) is missing a 1177 bp *Bgl*II fragment (coding for amino acids 303-694 of the β -catenin core region). Therefore, pSK Δ BgIII was digested with *Bgl*II and ligated to the 1177 bp *Bgl*II fragment of pSK β tot.

A cDNA construct (pSK Δ CTERM) lacking the C-terminal coding sequences was obtained with a PCR reaction on pSK8E1 with the primer pair β P6 (5'-ATCAGGACACCCAACG) and Δ CTERM (5'-CTATTACAGATCCCCTCAGAAATAAGCTTTTGCTCCTC-GAAGAGGGAAGTGGTCAG). The primer Δ CTERM codes for 13 additional amino acids after the C terminus, comprising a myc epitope. The product was ligated into *Eco*RV-digested pSKII. The final vector pSK Δ CTERM consists of an N-terminal coding *Bam*HI/*Sac*I fragment (for amino acids 1-576) from pSK β tot and a C-terminal coding *Bam*HI/*Sac*I fragment (for amino acids 577-683) from pSK Δ CTERM.

A plasmid (pSKBCORE) coding for the β -catenin core region (amino acids 120-683) was generated from the *Xba*I/*Bgl*II fragment of pSKBNTERM.

pGEX β 124 was generated by digesting pGEX β tot with *Eco*47III/*Not*I, fill-in reaction and blunt-end religation. pGEX β 151 was obtained by digesting pGEX4T1 with *Not*I, fill-in reaction and subsequent digestion with *Bam*HI. The gel-purified vector was ligated to the *Bam*HI/*Bsa*AI fragment of pGEX β tot. pGEX β 183 was obtained by digesting pGEX β tot with *Hind*III/*Not*I, followed by the fill-in reaction and blunt-end religation.

Generation of a GST-plakoglobin fusion construct

The N-terminal sequence of the human plakoglobin cDNA (clone HPG Ca 2.1, Franke et al., 1989) was amplified with the primer pair plako53 5'-ATATGCCGCGGATCCATGGAGGTGATGAACCT-GATGG and plako35 5'-AGCTGTCCCTCGCCTGACAC (nt 396-415). The sense primer introduced a *Bam*HI restriction site directly upstream of the start codon. The PCR product was digested with *Bam*HI/*Sst*I and ligated into the corresponding restriction sites of the cDNA clone HPG Ca 2.1reverse, in which the insert has been inverted. The resulting plasmid construct was digested with *Bam*HI/*Xho*I and cloned into the corresponding restriction sites of the expression vector pGEX4T1 (Pharmacia).

Expression and purification of GST-fusion proteins

GST-fusion proteins were expressed in Epicurian Coli^R XL-1 Blue MRF (Stratagene). The bacteria were grown in LB medium supplemented with 200 μ g/ml ampicillin and 2% (w/v) glucose. Expression of recombinant proteins was induced with 1 mM IPTG for 60 minutes at 30°C at an A_{600} of 0.5. The bacteria were pelleted and resuspended

in 10 volumes of PBS containing 1% (v/v) Triton X-100, 1 mM MgCl₂, 10 μ g/ml DNase I, 10 μ g/ml RNase, 10 μ g/ml leupeptin, 10 μ g/ml PMSF, 10 μ g/ml soybean trypsin inhibitor and 0.1 u/ml α ₂-macroglobulin. The bacteria were lysed by 2 passages through a French pressure cell (18,000 lbf/in²; 1 lbf/in² \approx 6.9 kPa). Cell debris was removed by centrifugation (10 minutes, 14,000 g). GST-fusion proteins were isolated by affinity chromatography on glutathione-agarose (Sigma), eluted with 10 mM glutathione in 100 mM Tris-HCl, pH 8, 10 μ g/ml leupeptin and dialyzed against 50 mM HEPES-NaOH, pH 7.4. Protein solutions were adjusted to 50% glycerol (v/v) and stored at -20°C.

Expression and purification of recombinant α -catenin

α -Catenin carrying a C-terminal histidine tag was expressed in *E. coli* M15 (Quiagen). The bacteria were grown in LB medium supplemented with 100 μ g/ml ampicillin, 20 μ g/ml kanamycin and 2% (w/v) glucose.

Growth of bacteria, induction of the recombinant proteins and cell lysis were done as described above. Fusion proteins were isolated by affinity chromatography on a Ni²⁺-chelate resin (Diagen) equilibrated with lysis buffer. After adsorption of the proteins the resin was extensively washed with lysis buffer followed by 50 mM sodium acetate, pH 6.2. α -Catenin was eluted with 100 mM imidazole-HCl, pH 6.8. Eluted proteins were immediately adjusted to 100 mM HEPES-NaOH, pH 7.4, 10 μ g/ml leupeptin, dialyzed against 50 mM HEPES-NaOH, pH 7.4, and processed as described above.

Expression and purification of MBP-ECT

Growth of bacteria, induction of the recombinant proteins and cell lysis were done as described above for GST-fusion proteins. Cleared lysates were dialyzed against 20 mM Tris-HCl, pH 8 (buffer A). MBP-ECT was isolated by high-resolution anion-exchange chromatography on a TMAE-EMD 650 S column (Merck). Proteins were eluted with a NaCl gradient in buffer A (flow rate 1 ml/min, gradient slope 5 mM/ml). Fractions were analyzed by SDS-PAGE and immunoblotting. MBP-ECT-containing fractions were dialyzed against 50 mM HEPES-NaOH, pH 7.5, and processed as described above.

In vitro reconstitution

All materials used in the in vitro reconstitution experiments was pre-coated with 0.2% Triton X-100. Recombinant proteins were mixed in buffer B (10 mM HEPES-NaOH, pH 7.4, 100 mM KCl, 1 mM MgCl₂, 0.1% (v/v) Triton X-100) and incubated for 30 minutes at room temperature. Precipitated material was removed by centrifugation (5 minutes, 14,000 g) and protein complexes were isolated by affinity purification on glutathione-agarose. After 15 minutes of incubation at 4°C the beads were washed 5 times with double-concentrated buffer B. Specifically bound proteins were eluted with SDS, separated by SDS-PAGE and analyzed by Coomassie Blue staining or immunoblotting.

Blot overlay analysis

Recombinant proteins were separated by SDS-PAGE and electroblotted onto nitrocellulose membranes. Duplicate membranes were stained with Amido black (0.1% (w/v) in 42% ethanol/10% acetic acid) in order to confirm protein transfer. Free membrane binding sites were blocked in TST buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% (v/v) Tween 20). Membranes were incubated with 10 μ g/ml recombinant protein in TST for 60 minutes at room temperature. After extensive washing with TST, the nitrocellulose membranes were incubated with the corresponding catenin-specific antibody, followed by an alkaline phosphatase-conjugated secondary antibody. Control experiments were performed by incubating the membranes with a non-committal protein or by omitting either the incubation with recombinant catenins or the anti-catenin antibodies.

Other methods

SDS-PAGE and immunoblotting were done as described (Ozawa et al., 1989). Gels and blots were standardized with prestained molecular mass markers (Sigma). Immunoblots were developed with peroxidase-labeled secondary antibodies (Dianova) followed by enhanced chemiluminescence (ECL, Amersham) or with alkaline phosphatase-labeled antibodies (Dianova) and the color reaction (BCIP/NBT, Promega). Protein concentrations were determined with the BCA protein assay (Pierce).

RESULTS

Purification and characterization of recombinant proteins

The cytoplasmic domains of E-cadherin (ECT), α - and β -catenin and plakoglobin were expressed as fusion proteins carrying as a tag either glutathione S-transferase (GST), six histidine residues (His6) or maltose binding protein (MBP) necessary for their subsequent utilization in combinatorial protein-protein interaction assays. A summary of the various fusion proteins as well as β -catenin deletion constructs is given in Table 1. The growth conditions of the bacteria as well as the lysis procedure were optimized for both high solubility of fusion proteins and minimal proteolytic degradation. Soluble fusion proteins were purified to apparent homogeneity. The sizes of the recombinant proteins differed from those of the native proteins due to the addition of tag sequences, but each protein was recognized by its respective antibody in immunoblots (Fig. 1) and immunoprecipitation experiments (not shown). From these experiments it is concluded that soluble fusion proteins are similar to native proteins with respect to antibody binding. It should be noted that in reconstitution assays the re-isolation of proteins by the His6-tag led to high background due to the affinity of unrelated proteins and β -catenin to Ni^{2+} -chelating resins. The MBP-tagged cytoplasmic domain of E-cadherin displayed only a very low affinity to the corresponding amylose affinity resin, so this recombinant protein was purified by high-resolution ion-exchange chromatography. Because of the above problems, the GST-tag

was used for repurification of protein complexes in all further reconstitution assays.

Heterodimer formation in solution

Recombinant α -catenin with a His6-tag at the C terminus was mixed in solution with recombinant proteins carrying a GST-tag, followed by subsequent affinity purification with glutathione resins. As can be seen in Fig. 2A, recombinant α -catenin was unable to complex with the cytoplasmic domain of E-cadherin but was associated with both β -catenin and plakoglobin. Due to the GST-tag, the recombinant plakoglobin is larger, exhibiting a similar electrophoretic mobility to recombinant α -catenin (Fig. 2A). The association of α -catenin with plakoglobin or with β -catenin was confirmed with anti- α -catenin antibodies in immunoblots (not shown). Binding of β -catenin and plakoglobin to ECT, the cytoplasmic domain of E-cadherin, was studied using a GST- β -catenin/MBP-ECT and a GST-plakoglobin/MBP-ECT combination (Fig. 2B). Under these experimental conditions only β -catenin bound to ECT, indicating that β -catenin has a higher binding affinity to ECT than does plakoglobin (see below). A possible heterodimerization of β -catenin and plakoglobin in solution could not be investigated, since both fusion proteins contained a GST-tag.

The heterodimerization experiments of the components of the cadherin-catenin complex were carried out under buffer conditions that resembled the ionic strength of the cytoplasm and were similar to the buffer conditions used for the isolation of the cadherin-catenin complex from metabolically labeled eukaryotic cells (Ozawa et al., 1989). However, heterodimers

Table 1. Recombinant cadherin and catenins

Protein	Amino acids	Tag-sequence	Antibody
ECT	580-728	GST (N)	L30D
ECT	580-728	MBP (N)	L30D
α -Catenin	1-906	His6 (C)	M12K (C)
β -Catenin	1-781	GST (N)	M14M (N), P14L (C)
β - Δ CTerm	1-683	GST (N)	M14M (C)
β -N119	1-119	GST (N)	M14M (N)
β -N124	1-124	GST (N)	M14M (N)
β -N151	1-151	GST (N)	M14M (N)
β -N183	1-183	GST (N)	M14M (N)
β -CORE	120-683	GST (N)	myc (C)
β - Δ NTERM	120-683	GST (N)	P14L (C)
β -CTERM	683-781	GST (N)	P14L (C)
Plakoglobin	1-745	GST (N)	D15A (C)

The fusion proteins carry a tag sequence at either the N or the C terminus. Proteins were recognized by their respective antibody as indicated. ECT, cytoplasmic domain of murine E-cadherin; GST, glutathione S-transferase; MBP, maltose binding protein; N, C, tag sequence or epitope at N or C terminus.

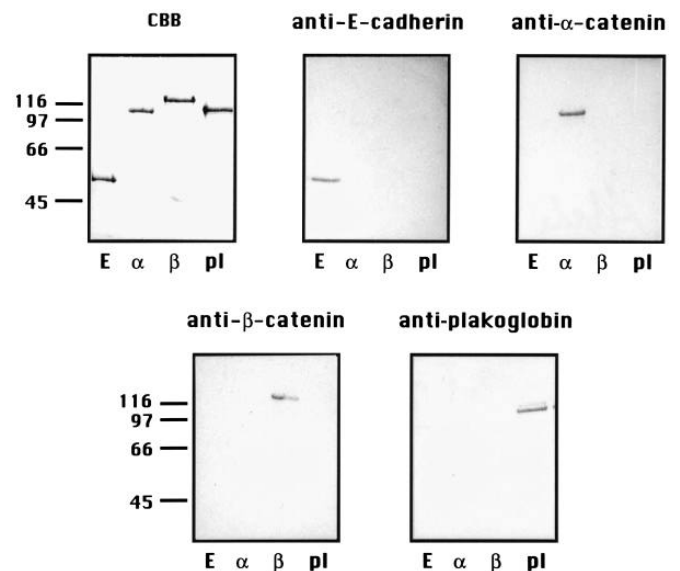


Fig. 1. Characterization of recombinant cadherin and catenins. The cytoplasmic domain of murine E-cadherin (ECT), α - and β -catenin and plakoglobin were expressed in *E. coli* and affinity purified from the soluble fraction of the bacterial lysates as described in Materials and Methods. Fusion proteins were separated by SDS-PAGE and blotted onto nitrocellulose filters. The identity of the Coomassie Blue-stained proteins was verified with antibodies specific for the respective proteins. Positions of molecular mass markers are indicated on the left (in kDa). Note that the apparent molecular masses of the recombinant proteins differ from those of native proteins due to the addition of the tag sequences.

formed equally well at different pH values (pH 6-8.5) or high salt (1 M NaCl) and were stable for days at room temperature.

Heterodimerization of proteins could also be demonstrated by nitrocellulose filter overlay detection. Purified MBP-ECT was recognized by anti-E-cadherin antibodies and interacted with recombinant β -catenin in overlay experiments (Fig. 3). Recombinant proteins separated by SDS-PAGE and blotted onto nitrocellulose filters were incubated with soluble proteins, which were then detected with their respective antibodies. Here again, α -catenin did not interact directly with ECT, but bound equally well to both recombinant β -catenin and plakoglobin (Fig. 4). β -Catenin and plakoglobin each interacted with ECT and recombinant α -catenin (Fig. 4). Remarkably, recombinant β -catenin appeared to bind more efficiently to the cytoplasmic domain of E-cadherin than does recombinant plakoglobin (Fig. 4). This indicates that plakoglobin has a certain affinity for the cytoplasmic domain of E-cadherin, although this interaction could not be detected in the binding experiments in solution described above.

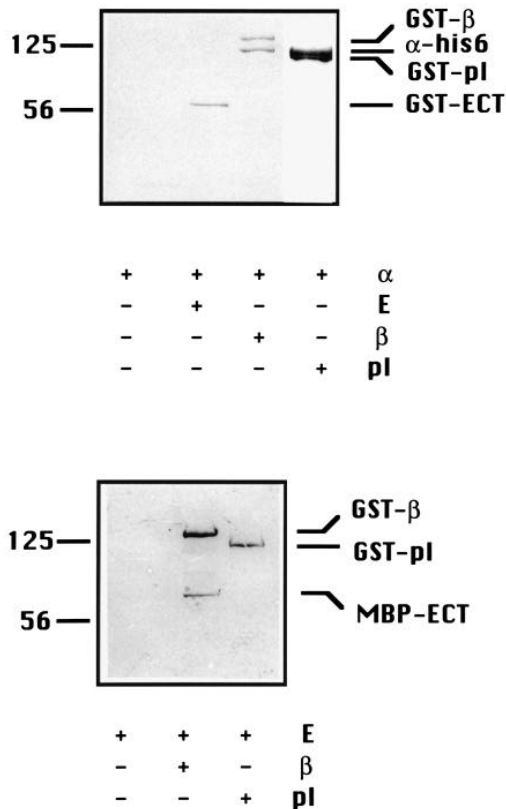


Fig. 2. Formation of heterodimeric protein complexes. (A) Heterodimers with α -catenin: α -catenin-His was incubated with either GST-fusion proteins of β -catenin, plakoglobin (pl) or ECT, the cytoplasmic domain of E-cadherin. Protein complexes were affinity purified with glutathione-agarose. Bound proteins were eluted with SDS, separated by SDS-PAGE, and Coomassie Blue stained. The positions of the recombinant proteins are indicated. (B) Heterodimers with E-cadherin: the cytoplasmic domain of E-cadherin fused to maltose binding protein (MBP) was incubated with GST- β -catenin or GST-plakoglobin. Protein complexes were purified with glutathione-agarose, eluted with SDS, and the proteins separated by SDS-PAGE were Coomassie Blue stained. The positions of the recombinant proteins are indicated.

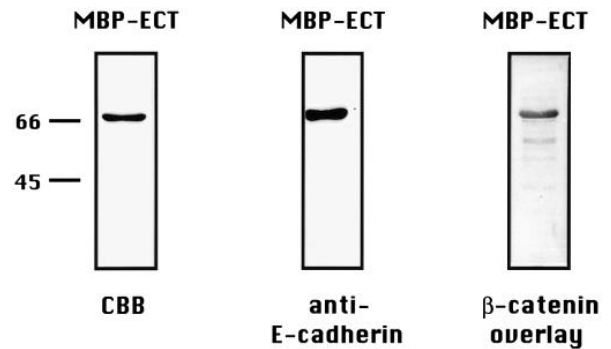


Fig. 3. Binding of recombinant β -catenin to the cytoplasmic domain of E-cadherin in overlay experiments. MBP-ECT, the maltose binding protein fused to the cytoplasmic domain of E-cadherin, was purified as described in Materials and Methods and stained with Coomassie Blue (CBB) or with anti-E-cadherin (anti-L30D). In overlay experiments GST- β -catenin binds to MBP-ECT as revealed with an anti- β -catenin antibody (anti-M14K).

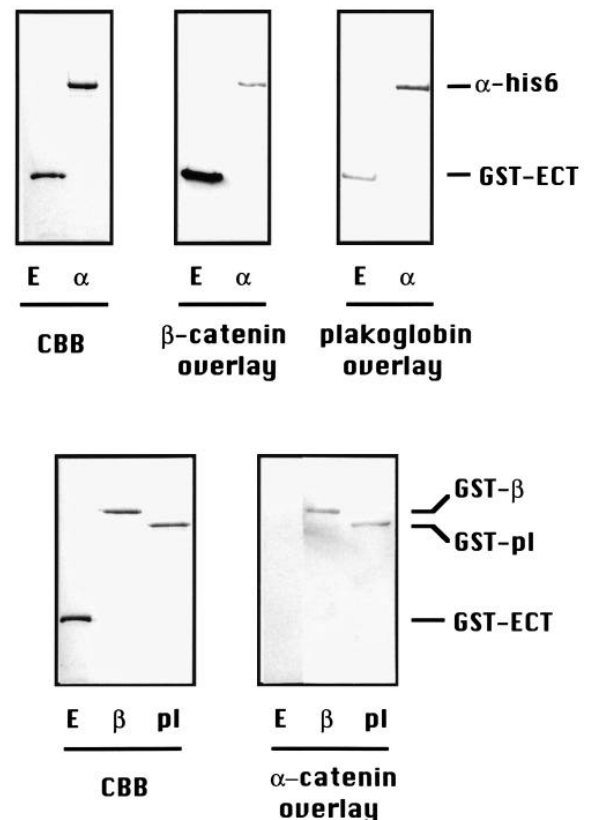


Fig. 4. Blot overlay analysis of the interaction of catenin and cadherins. (A) Overlay with β -catenin and plakoglobin: recombinant E-cadherin and α -catenin were separated by SDS-PAGE and blotted onto nitrocellulose filters. Blots were incubated with recombinant β -catenin or plakoglobin as indicated. Bound β -catenin or plakoglobin were detected with the peptide-specific antibodies P14L and D15A. (B) Overlay with α -catenin: recombinant E-cadherin, β -catenin and plakoglobin were separated by SDS-PAGE and blotted onto nitrocellulose filters. Blots were incubated with recombinant α -catenin. Bound α -catenin was then detected with the peptide-specific antibody M12K.

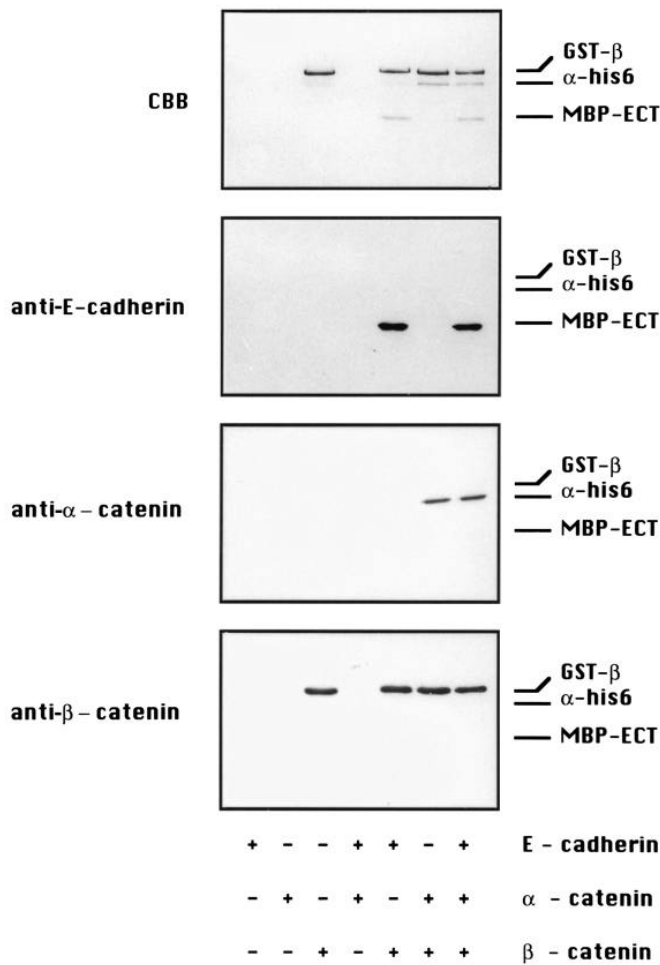


Fig. 5. Recombinant E-cadherin, α -catenin and β -catenin associate to form a heterotrimeric complex. For the *in vitro* reconstitution of cadherin-catenin complexes recombinant E-cadherin linked to maltose binding protein (MBP), α -catenin with a C-terminal His-tag and GST fusion proteins of β -catenin were coincubated in combinations as indicated. Protein complexes were affinity isolated on glutathione-agarose and analyzed by SDS-PAGE and immunoblotting. Gels were stained with Coomassie Blue (CBB), and immunoblots were incubated with the indicated anti-peptide antibodies.

Taken together these experiments demonstrate that recombinant β -catenin and plakoglobin can interact both with recombinant α -catenin and with the cytoplasmic domain of E-cadherin, while α -catenin forms only heterodimers with recombinant β -catenin or plakoglobin.

In vitro assembly of the cadherin-catenin complex

Recombinant E-cadherin (MBP-ECT), GST- β -catenin and α -catenin-His6 were mixed in various combinations and purified by binding to glutathione-agarose. Specifically bound proteins were then eluted under denaturing conditions and separated by SDS-PAGE. Coomassie Blue staining of the gels revealed that the three proteins had the capacity to associate into a heterotrimeric complex, as is also shown by immunoblots with the corresponding anti-peptide antibodies (Fig. 5). These results clearly demonstrate the central role of β -catenin in the

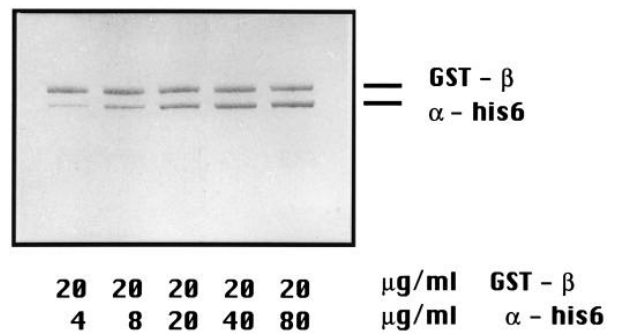
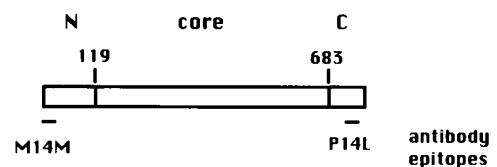


Fig. 6. Recombinant α -catenin and β -catenin associate to form a 1:1 complex. GST- β -catenin was incubated with increasing concentrations of α -catenin-His as indicated. Protein complexes were isolated with glutathione-agarose and analyzed by SDS-PAGE.

assembly of the complex, since only β -catenin was able to interact with both α -catenin and the cytoplasmic domain of E-cadherin if experiments were performed with proteins in solution. They also indicate that none of the fusion partners used interfered with either complex formation or the subsequent purification of protein complexes on glutathione-agarose.

Identification of an α -catenin binding site in β -catenin

The molecular interaction between α - and β -catenin was studied in more detail; a constant amount of recombinant β -



β	—————	AA	1 - 781
β - ΔC	—————	AA	1 - 683
β - N119	—	AA	1 - 119
β - N124	—	AA	1 - 124
β - N151	—	AA	1 - 151
β - N183	—	AA	1 - 183
β - CR	—————	AA	120 - 683
β - ΔN	—————	AA	120 - 781
β - C	—	AA	684 - 781

Fig. 7. Scheme of β -catenin structure and GST fusion constructs of β -catenin used to map the α -catenin binding site. The primary sequence of β -catenin can be subdivided into three domains according to the sequence homology among β -catenin, plakoglobin and armadillo (Reynolds et al., 1992). The central core region (CR) of β -catenin reveals the highest degree of homology among the three proteins. The respective N- and C-terminal domains exhibit less strong homology. GST fusion proteins of β -catenin and of various deletions were constructed as indicated. AA, amino acid.

catenin was mixed with increasing concentrations of α -catenin (Fig. 6). Protein heterodimers were isolated and analyzed by SDS-PAGE. These experiments indicate that the recombinant proteins assembled in a 1:1 ratio.

To characterize further the interaction between recombinant α - and β -catenin, different β -catenin domains were expressed as GST-fusion proteins (see Table 1 and Fig. 7). β -Catenin domains were roughly defined into three regions with high or low sequence homology to the closely related proteins plakoglobin and armadillo. The central region of β -catenin exhibiting the highest degree of homology was designated as β -catenin core region. Fusion proteins were isolated from the soluble fraction of bacterial lysates; binding studies with recombinant α -catenin indicated that α -catenin binds to the N-terminal half of β -catenin, although the most N-terminal domain of β -catenin (amino acid positions 1-119) seemed not to exhibit binding activity (Fig. 8A). These results suggest that α -catenin binds close to the hydrophobic repeats of β -catenin or that the expression of deletion constructs led to structural alterations that interfered with α -catenin binding. To distinguish between these possibilities additional deletion constructs covering β -catenin amino acid positions 1-183 were expressed as GST-fusion proteins (Fig. 7). When soluble recombinant

proteins were mixed with α -catenin and purified by glutathione-agarose, α -catenin interacted with β -catenin polypeptides containing either amino acid positions 1-151 or amino acid positions 1-183 (Fig. 8B). These results clearly demonstrate that an α -catenin-binding site in β -catenin is located between amino acid positions 120 and 151. Sequence comparison revealed that this region is well conserved in the β -catenin/plakoglobin/armadillo protein family from different species. Homology to this sequence is also found in p120 protein, a kinase substrate localized in adherens junctions of epithelial cells, which has been reported to contain hydrophobic repeats homologous to the β -catenin protein family (Reynolds et al., 1992). This suggests that p120 might have α -catenin binding properties.

DISCUSSION

Biochemical analysis on eukaryotic cells directly has already provided important information about the molecular organization of the E-cadherin-catenin complex, but the amount of purified proteins was generally insufficient for a more detailed analysis. In pulse-chase experiments it was shown that β -catenin binds to the E-cadherin precursor polypeptide and that α -catenin seems to be involved in mediating the connection of the cadherin-catenin complex with the actin filament network (Ozawa et al., 1990; Ozawa and Kemler, 1992). However, it was not clear whether α -catenin can bind directly the cytoplasmic domain of E-cadherin. The relative position of γ -catenin in the complex was less well understood, since the amount of this component varied depending on cell types and biochemical methods. It was mainly for this reason that γ -catenin was placed in the periphery of the complex (Kemler, 1992). γ -Catenin might be identical to plakoglobin (Knudsen and Wheelock, 1992) and plakoglobin appears to bind, as does β -catenin, directly to E-cadherin (Näthke et al., 1994; S. Butz and R. Kemler, in press).

Considering the biological importance of catenins for cadherin function, we have expressed the components of the cadherin-catenin complex as bacterial fusion proteins to obtain a sufficient amount of soluble protein for structural and functional analysis. Our results indicate that recombinant proteins exhibit many of the properties of native proteins, which suggests that neither the expression in bacteria nor the purification procedure has had any apparent effect on protein conformation. It should however be noted that we had in preliminary experiments expressed several constructs with different tags and not all recombinant proteins turned out to be suitable for reconstitution experiments, being either largely insoluble or becoming degraded during the purification procedure. For example, recombinant proteins with dihydrofolate reductase (DHFR) as a tag were generally insoluble and recombinant β -catenin with a His6-tag at the N terminus exhibited no binding activity. It was also necessary to optimize the purification procedure for each recombinant protein. Thus it was possible to obtain unambiguous results in the protein binding studies where virtually no contaminations were detected after Coomassie Blue staining, and no non-specific binding to glutathione-agarose was observed in immunoblots. We were able to assemble the E-cadherin-catenin complex with recombinant proteins and show that this assembly depends most directly on

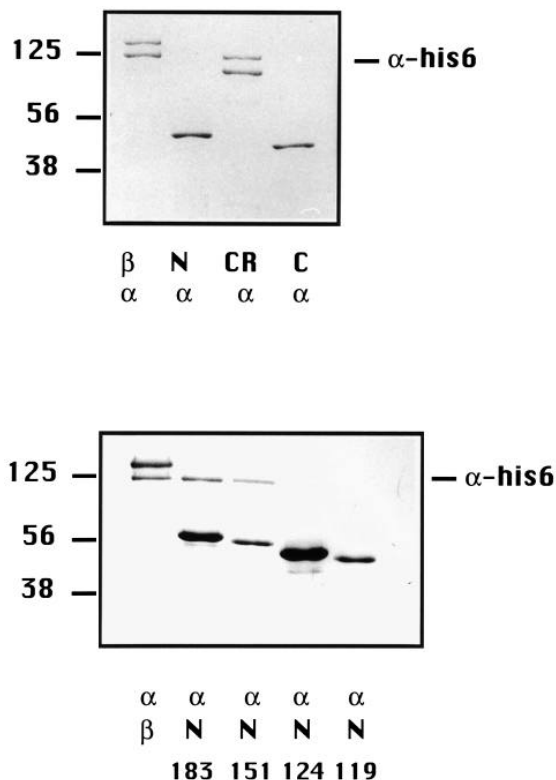


Fig. 8. Mapping of the α -catenin binding site in β -catenin. (A) His-tagged α -catenin was co-incubated with β -catenin, the N-terminal domain (N), the core region (CR) and the C-terminal domain (C) each fused to GST. Protein complexes were affinity purified, analyzed by SDS-PAGE and stained with Coomassie Brilliant Blue. (B) Recombinant α -catenin was co-incubated with different β -catenin peptides covering amino acid positions 1-183 (see Fig. 7) and protein complexes were analyzed as described above. These experiments demonstrate that an α -catenin binding site is localized in β -catenin between amino acid positions 120 and 151.

β -catenin, which can bind to α -catenin and to the cytoplasmic domain of E-cadherin. Under the same experimental conditions plakoglobin could not substitute for β -catenin in the assembly of the E-cadherin-catenin complex although plakoglobin was able to bind to the cytoplasmic domain of E-cadherin in overlays and interacted with α -catenin. Most likely β -catenin exhibits a higher binding affinity to E-cadherin than plakoglobin, as indicated by the difference in binding efficiency of the two proteins in overlay experiments. Binding of β -catenin to E-cadherin is mediated by the conserved core region of the protein (amino acid positions 120-683). β -Catenin and plakoglobin show the highest degree of homology in this region, suggesting that the interaction of plakoglobin with E-cadherin occurs also in this region. The interaction of β -catenin with α -catenin was studied in more detail, indicating that the two proteins interact in a 1:1 stoichiometry. We have also identified an α -catenin binding site in β -catenin (amino acid positions 120-151).

The assembly in vitro of the E-cadherin-catenin complex reported here should allow study of the influence of signalling molecules on the assembly and disassembly of the complex. It has been reported that catenins are substrates for tyrosine phosphorylation, but the consequence of this modification is at present not well understood. Finally, it will be of interest to see whether the reconstitution experiments can be extended to study the interaction of the E-cadherin-catenin complex with actin filaments.

We gratefully thank Dr James Nelson, Stanford University, USA, for the expression plasmid pGEXUC1, coding for the cytoplasmic domain of murine E-cadherin. We thank Johann Wirsching for technical assistance, Lore Lay for preparing the photographs, and Rosemary Schneider for typing the manuscript. We are grateful to Dr Randy Cassada for critically reading the manuscript.

REFERENCES

- Behrens, J., Vakaet, L., Friis, R., Winterhager, E., VanRoy, F., Mareel, M. M. and Birchmeier, W. (1993). Loss of epithelial differentiation and gain of invasiveness correlates with tyrosine phosphorylation of the E-cadherin/ β -catenin complex in cells transformed with a temperature-sensitive v-src gene. *J. Cell Biol.* **120**, 757-766.
- Burridge, K., Fath, K., Kelley, T., Nuckolls, G., Turner, C., (1988). Focal adhesion-transmembrane junctions between the extracellular matrix and the cytoskeleton. *Annu. Rev. Cell Biol.* **4**, 487-525.
- Butz, S. and Kemler, R. (1994). Distinct cadherin catenin complexes in Ca^{2+} -dependent cell-cell adhesion. *FEBS Lett.* (in press).
- Butz, S., Stappert, J., Weissig, H. and Kemler, R. (1992). Plakoglobin and β -catenin: distinct but closely related [letter]. *Science* **257**, 1142-1144.
- Butz, S., Rawer, S., Rapp, W. and Birsner, U. (1994). Immunization and affinity purification of antibodies using resin-immobilized lysine-branched synthetic peptides. *Pept. Res.* **7**, 20-23.
- Clavier, J. M., Hardelin, J.P., Legouis, R., Levilliers, J., Bougueleret, L., Mattei, M. G. and Petit, C. (1993). Characterization and chromosomal assignment of a human cDNA encoding a protein related to the murine 102-kDa cadherin-associated protein (α -catenin). *Genomics* **15**, 13-20.
- Cowin, P., Kapprell, H.-P., Franke, W. W., Tamkun, J. and Hynes, R. O. (1986). Plakoglobin: a protein common to different kinds of intercellular adhering junctions. *Cell* **46**, 1063-1073.
- Flaswinkel, H. and Reth, M. (1994). Dual role of tyrosine activation motif of the Ig- α protein during signal transduction via the B-cell antigen receptor. *EMBO J.* **13**, 83-89.
- Franke, W. W., Goldschmidt, M. D., Zimbelmann, R., Mueller, H. M., Schiller, D. L. and Cowin, P. (1989). Molecular cloning and amino acid sequence of human plakoglobin, the common junctional plaque protein. *Biochemistry* **86**, 4027-4031.
- Geiger, B., Tokuyasu, K. T., Dutton, A. H. and Singer, S. J. (1980). Vinculin, an intracellular protein localized at specialized sites where microfilament bundles terminate at cell membranes. *Proc. Nat. Acad. Sci. USA* **77**, 4127-4131.
- Hamaguchi, M., Matsuyoshi, N., Ohnishi, Y., Gotoh, B., Takeichi, M. and Nagai, Y. (1993). p60^{v-src} causes tyrosine phosphorylation and inactivation of the N-cadherin-catenin cell adhesion system. *EMBO J.* **12**, 307-314.
- Herrenknecht, K., Ozawa, M., Eckerskorn, C., Lottspeich, F., Lenter, M. and Kemler, R. (1991). The uvomorulin-anchorage protein α -catenin is a vinculin homologue. *Proc. Nat. Acad. Sci. USA* **88**, 9156-9160.
- Hirano, S., Kimoto, N., Shimoyama, Y., Hirohashi, S. and Takeichi, M. (1992). Identification of a neural α -catenin as a key regulator of cadherin function and multicellular organization. *Cell* **70**, 293-301.
- Hoschuetzky, H., Aberle, H. and Kemler, R. (1994). β -catenin mediates the interaction of the cadherin-catenin complex with epidermal growth factor receptor. *J. Cell Biol.* (in press).
- Kemler, R. (1992). Classical cadherins. *Semin. Cell Biol.* **3**, 149-155.
- Kemler, R. (1993). From cadherins to catenins: cytoplasmic protein interactions and regulation of cell adhesion. *Trends Genet.* **9**, 317-321.
- Knudsen, K. A. and Wheelock, M. J. (1992). Plakoglobin, or an 83-kD homologue distinct from β -catenin, interacts with E-cadherin and N-cadherin. *J. Cell Biol.* **118**, 671-679.
- Marrs, J. A., Napolitano, E. W., Murphy-Erdosh, C., Mays, R. W., Reichardt, L. F. and Nelson, W. J. (1993). Distinguishing roles of the membrane-cytoskeleton and cadherin mediated cell-cell adhesion in generating different Na^+, K^+ -ATPase distributions in polarized epithelia. *J. Cell Biol.* **123**, 149-164.
- Matsuyoshi, N., Hamaguchi, M., Taniguchi, S., Nagafuchi, A., Tsukita, S. and Takeichi, M. (1992). Cadherin-mediated cell-cell adhesion is perturbed by v-src tyrosine phosphorylation in metastatic fibroblasts. *J. Cell Biol.* **118**, 703-714.
- McCrea, P. D., Turck, C. W. and Gumbiner, B. (1991). A homolog of the *armadillo* protein in *Drosophila* (plakoglobin) associated with E-cadherin. *Science* **254**, 1359-1361.
- McNeill, H., Ozawa, M., Kemler, R. and Nelson W. J. (1990). Novel function of the cell adhesion molecule E-cadherin as an inducer of cell surface polarity. *Cell* **62**, 309-316.
- Nagafuchi, A. and Takeichi, M. (1989). Transmembrane control of cadherin-mediated cell adhesion: a 94 kDa protein functionally associated with a specific region of the cytoplasmic domain of E-cadherin. *Cell Regul.* **1**, 37-44.
- Nagafuchi, A., Takeichi, M. and Tsukita, S. (1991). The 102 kD cadherin-associated protein: similarity to vinculin and posttranscriptional regulation of expression. *Cell* **65**, 849-857.
- Näthke, S. I., Hinck, L., Swedlow, J. R., Papkoff, J. and Nelson, W. J. (1994). Defining interactions and distributions of cadherin and catenin complexes in polarized cells. *J. Cell Biol.* **125**, 1341-1352.
- Oda, T., Kanai, Y., Shimoyama, Y., Nagafuchi, A., Tsukita, S. and Hirohashi, S. (1993a). Cloning of the human α -catenin cDNA and its aberrant mRNA in a human cancer cell line. *Biochem. Biophys. Res. Commun.* **193**, 897-904.
- Oda, H., Uemura, T., Shiomi, K., Nagafuchi, A., Tsukita, S. and Takeichi, M. (1993b). Identification of a *Drosophila* homolog of α -catenin and its association with the *armadillo* protein. *J. Cell Biol.* **121**, 1133-1140.
- Ozawa, M., Baribault, H. and Kemler, R. (1989). The cytoplasmic domain of the cell adhesion molecule uvomorulin associates with three independent proteins structurally related in different species. *EMBO J.* **8**, 1711-1717.
- Ozawa, M., Ringwald, M. and Kemler, R. (1990). Uvomorulin-catenin complex formation is regulated by a specific domain in the cytoplasmic region of the cell adhesion molecule. *Proc. Nat. Acad. Sci. USA* **87**, 4246-4250.
- Ozawa, M. and Kemler, R. (1992). Molecular organization of the uvomorulin-catenin complex. *J. Cell Biol.* **116**, 989-996.
- Peifer, M. and Wieschaus, E. (1990). The segment polarity gene *armadillo* encodes a functionally modular protein that is the *Drosophila* homolog of human plakoglobin. *Cell* **63**, 1167-1178.
- Peifer, M., McCrea, P. D., Green, K. J., Wieschaus, E. and Gumbiner, B. M. (1992). The vertebrate adhesive junction proteins β -catenin and plakoglobin and the *Drosophila* segment polarity gene *armadillo* form a multigene family with similar properties. *J. Cell Biol.* **3**, 681-691.
- Piepenhagen, P. A. and Nelson, W. J. (1993). Defining E-cadherin-associated protein complexes in epithelial cells: plakoglobin, β -catenin and γ -catenin are distinct components. *J. Cell Sci.* **104**, 751-762.
- Reynolds, A. B., Herbert, L., Cleveland, L., Berg, S. T. and Gaut, J. R.

- (1992). p120, a novel substrate of protein tyrosine kinase receptors and of p60^{v-src}, is related to cadherin-binding factors β -catenin, plakoglobin and *armadillo*. *Oncogene* **7**, 2439-2445.
- Riggleman, B., Schedl, P. and Wieschaus, E.** (1990). Spatial expression of the *Drosophila* segment polarity gene *armadillo* is posttranscriptionally regulated by wingless. *Cell* **63**, 549-560.
- Rosenthal, E.** (1993). Identification of homologues to β -catenin/plakoglobin/armadillo in two invertebrates, *Urechis caupo* and *Tripneuste gratilla*. *Biochim. Biophys. Acta* **1173**, 337-341.
- Rubinfeld, B., Souza, B., Albert, I., Müller, O., Chamberlain, S. H., Masiarz, F. R., Munemitsu, S. and Polakis, P.** (1993). Association of the APC gene product with β -catenin. *Science* **262**, 1731-1734.
- Sambrook, J., Fritsch, E. F. and Maniatis, T.** (1989). *Molecular Cloning: A Laboratory Manual*, 2nd edn, New York: Cold Spring Harbor Laboratory Press.
- Shibamoto, S., Hayakawa, M., Takeuchi, K., Hori, T., Oku, N., Miyazawa, K., Kitamura N., Takeichi, M. and Ito, F.** (1994). Tyrosine phosphorylation of β -catenin and plakoglobin enhanced by hepatocyte growth factor and epidermal growth factor in human carcinoma cells. *Cell Adhes. Commun.* **1**, 295-305.
- Stappert, J. and Kemler, R.** (1993). Intracellular associations of adhesion molecules. *Curr. Opin. Neurobiol.* **3**, 60-66.
- Stappert, J. and Kemler R.** (1994). A short core region of E-cadherin is essential for catenin binding and is highly phosphorylated. *Cell Adhes. Commun.* **2**, 319-327.
- Su, L.-K., Vogelstein, B. and Kinzler, K. W.** (1993). Association of the APC tumor suppressor protein with catenins. *Science* **262**, 1734-1737.

(Received 12 July 1994 - Accepted 29 August 1994)