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The amino- and carboxyl-terminal tails of β-catenin reduce its affinity for desmoglein 2

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SUMMARY

Beta-catenin and plakoglobin are members of the armadillo family of proteins and were first identified as components of intercellular adhering junctions. In the adherens junction β-catenin and plakoglobin serve to link classical cadherins to the actin-based cytoskeleton. In the desmosome plakoglobin links the desmosomal cadherins, the desmogleins and the desmocollins, to the intermediate filament cytoskeleton. β-catenin is not a component of the desmosome. Previously we have shown that the central armadillo repeat region of plakoglobin is the site for desmosomal cadherin binding. We hypothesized that the unique amino- and/or carboxyl-terminal ends of β-catenin may regulate its exclusion from the desmosomal plaque. To test this hypothesis we used chimeras between β-catenin and plakoglobin to identify domain(s) that modulate association with desmoglein 2. Chimeric constructs, each capable of associating with classical cadherins, were assayed for association with the desmosomal cadherin desmoglein 2. Addition of either the N- or C-terminal tail of β-catenin to the armadillo repeats of plakoglobin did not interfere with desmoglein 2 association. However, when both β-catenin amino terminus and carboxyl terminus were added to the plakoglobin armadillo repeats, association with desmoglein 2 was diminished. Removal of the first 26 amino acids from this construct restored association. We show evidence for direct protein-protein interactions between the amino- and carboxyl-terminal tails of β-catenin and propose that a sequence in the first 26 amino acids of β-catenin along with its carboxyl-terminal tail decrease its affinity for desmoglein and prevent its inclusion in the desmosome.

Key words: β-Catenin, Desmoglein, Plakoglobin, N-Cadherin

INTRODUCTION

Epithelial cell-cell adhesion is important in maintaining tissue integrity during development as well as in adulthood. Two adhering type junctions are responsible for strong cell-cell adhesion, the adherens junction and the desmosome. Each of these junctions is comprised of a transmembrane cadherin and a complex cytoplasmic plaque that serves to link the cadherin to actin microfilaments or the intermediate filament cytoskeleton. The adherens junction is composed of a classical cadherin (e.g. E-, P- or N-cadherin) linked to β-catenin or plakoglobin (Aberle et al., 1994; Jou et al., 1995). Alpha-catenin then links the cadherin/catenin complex to the actin cytoskeleton through interactions with α-actinin, vinculin, ZO-1, and actin filaments (Imamura et al., 1999; Nieset et al., 1997; Rimm et al., 1995; Watabe-Uchida et al., 1998). Desmosomes contain the desmosomal cadherins, desmogleins and desmocollins, that are linked to the intermediate filament cytoskeleton through interactions with plakoglobin (Korman et al., 1989; Troyanovsky et al., 1994a,b) and desmoplakin (Kowalczyk et al., 1997) as well as other cell type specific plaque proteins, such as the plakophilin family of proteins (Bonne et al., 1999; Heid et al., 1994; Mertens et al., 1996; Schmidt et al., 1997). Thus, plakoglobin is found in both adherens junctions and desmosomes (Cowin et al., 1986) while β-catenin is restricted to the adherens junction.

Beta-catenin and plakoglobin are members of the armadillo family of proteins, and were first identified as proteins that co-immunoprecipitated with cadherins (Butz et al., 1992; Franke et al., 1989; McCrea et al., 1991; Ozawa et al., 1989; Riggleman et al., 1989). Armadillo family members share a common structural motif termed the ‘armadillo repeat domain’ which consists of a variable number of imperfect 40-42 amino acid repeats. Beta-catenin and plakoglobin are comprised of 12 central armadillo repeats with unique amino- and carboxyl-terminal domains. Beta-catenin and plakoglobin share 76% identity in the armadillo repeat domain (Fouquet et al., 1992). In contrast, these two molecules share only 29% identity in the C-terminal tail and 41% identity in the N-terminal tail. The armadillo repeat domain of β-catenin has been crystallized and its structure determined (Huber et al., 1997). This domain forms a superhelix of helices that features a positively charged surface which may be important for protein-protein interactions with the classical cadherins as well as other

partners such as axin/conductin and APC (Behrens et al., 1998; Ikeda et al., 1998; Rubinfeld et al., 1993; Sakanaka et al., 1998).

In desmosomes, plakoglobin is bound to the cytoplasmic domain of the desmogleins and the desmocollins (Korman et al., 1989; Mathur et al., 1994; Troyanovsky et al., 1994a,b; Wahl et al., 1996). The domain of plakoglobin required for this association resides in the armadillo repeats and overlaps the amino-terminal sequences which interact with α-catenin in the adherens junction (Chitaev et al., 1998; Wahl et al., 1996). Plakoglobin mediates the association of the intermediate filament cytoskeleton to the desmosomal plaque by associating with the amino terminus of desmoplakin (Kowalczyk et al., 1997). Thus, plakoglobin plays a central role in linking transmembrane cadherin molecules to two different cytoskeletal systems.

Beta-catenin and plakoglobin are highly homologous and interact with many of the same partners; however, one exception is the desmosomal cadherins. Plakoglobin associates with both classical and desmosomal cadherins while β-catenin associates only with the classical cadherins. The current study was designed to identify the domains of β-catenin which are responsible for discriminating between classical and desmosomal cadherins. We have constructed β-catenin/plakoglobin chimeras, expressed them in A431 and HT1080 cells and used co-immunoprecipitations to assay for association between the chimeras and the cadherins. We have identified domains in the amino- and carboxyl-termini of β-catenin which reduce its affinity for the desmosomal cadherins. Understanding the protein-protein interactions of β-catenin and plakoglobin with classical and desmosomal cadherins may help elucidate the organization and assembly of these junctions.

MATERIALS AND METHODS

Cell culture

A431 human epidermoid carcinoma cells and HT1080 human fibrosarcoma cells were obtained from ATCC (Rockville, MD) and maintained in Dulbecco's modified Eagle's medium (Sigma Chemical Co., St Louis, MO) supplemented with 10% fetal bovine serum (Hyclone Laboratories, Logan, UT).

Detergent extraction of cells

Confluent monolayers were rinsed three times with phosphate buffered saline (PBS) and extracted in TNE extraction buffer (10 mM Tris acetate, pH 8.0, 0.5% NP-40, 1 mM EDTA, 2 mM phenylmethylsulfonyl fluoride). The cells were placed on ice, scraped and triturated vigorously for 10 minutes. Insoluble material was pelleted by centrifugation at 14,000 ***g*** for 15 minutes at 4°C, and the supernatant was stored at −70°C.

Antibodies and immunoprecipitations

Mouse monoclonal antibodies against desmoglein 2 (6D8), P-cadherin (6A9), N-cadherin (13A9) and the amino terminus of plakoglobin (11E4) have been described (Johnson et al., 1993; Lewis et al., 1994; Sacco et al., 1995). The PG 5.1 antibody was a gift from Dr Werner W. Franke (German Cancer Research Center, Heidelburg, Germany). The 9E10.2 hybridoma was a gift from Dr Kathleen J. Green (Northwestern University, Chicago IL). HECD-1 was a gift from Dr Masatoshi Takeichi (Kyoto University, Kyoto, Japan). 6E3 is a mouse monoclonal anti-β-catenin antibody which recognizes an amino-terminal epitope. 5H10 is a mouse monoclonal anti-β-catenin antibody that recognizes an epitope in the carboxyl-terminal domain. Both 6E3 and 5H10 were generated using bacterially expressed fusion proteins as antigens (Johnson et al., 1993).

All polypropylene tubes were rinsed with 0.1% NP-40 and dried prior to use in immunoprecipitations. A 300-500 μl aliquot of TNE extract was added to 300 μl of hybridoma conditioned medium and gently mixed at 4°C for 30 minutes. 50 μl packed anti-mouse IgG affinity gel (ICN Biochemical Co., Costa Mesa, CA) was added and mixing continued for 30 minutes. Immune complexes were washed five times with TBST (10 mM Tris-HCl, pH 7.5, 150 M NaCl, 0.05% Tween-20). After the final wash the packed beads were resuspended in 2× Laemmli sample buffer, boiled for 5 minutes, and the relevant proteins resolved by SDS-PAGE.

Electrophoresis and immunoblotting

Polyacrylamide gel electrophoresis in the presence of SDS (SDS-PAGE) was done according to the procedure of Laemmli (1970). Electrophoresis materials were from Fisher Scientific (Pittsburgh, PA). High molecular mass markers were from Sigma Chemical Co. SDS-PAGE resolved proteins were transferred to nitrocellulose for immunoblots. Nitrocellulose sheets were blocked using 5% non-fat dry milk for 45 minutes, rinsed 15 minutes and two times 5 minutes in TBST. Primary antibodies were added (1:100) and incubation continued for one hour. Primary antibodies were removed by one 15 minute and two 5 minute washes in TBST. Horseradish peroxidase conjugated anti-mouse IgG (Jackson Immunochemicals, West Grove, PA) was added at a 1:10,000 dilution and incubated 1 hour. Nitrocellulose sheets were washed once for 15 minutes and four times 5 minutes in TBST. Supersignal Chemiluminescent kit (Pierce Chemical Co., Rockford IL) was used for detection of immunoreactive bands. Alternatively, bands were detected colorimetrically using alkaline phosphatase conjugated secondary antibodies (Jackson Immunochemicals) and nitroblue tetrazolium and 4-bromo-3-chloro-2-indol phosphate (Sigma Chemical Co).

Molecular constructions

All constructs were derived from a human plakoglobin cDNA kindly provided by Dr W. W. Franke and a chicken β-catenin cDNA isolated in our laboratory (Johnson et al., 1993). All constructions were produced by standard methods. All PCR generated fragments and ligation junctions were sequenced to verify sequence and reading frame. Details of the following constructs are available upon request.

Plakoglobin repeats

PCR was used to place a start codon immediately upstream from AA 123 of human plakoglobin. Our repeat domain construct terminates at AA 687. **β-cat repeats**: PCR was used to place a start codon upstream of AA 132 of chicken β-catenin. The repeat domain construct extends to AA 693 of β-catenin. **βN/pgR/**: PCR was used to generate a cDNA corresponding to AA 1-175 of chicken β-catenin, which was then joined to a fragment encoding AA 167-687 of human plakoglobin. **/pgR/βC**: A cDNA coding for AA 695-781 of chicken β-catenin was joined to a fragment encoding AA 123-687 of human plakoglobin. **βN/pgR/βC:** A fragment encoding AA 1-131 of chicken β-catenin was ligated to a fragment encoding AA 123-687 of human plakoglobin that had in turn been ligated to a fragment encoding AA 695-781 of chicken β-catenin. **βN/pgR/pgC**: A fragment encoding AA 1-131 of chicken β-catenin was ligated to a PCR generated fragment encoding AA 123-745 of human plakoglobin. **pgN/pgR/βC**: PCR was used to generate a cDNA fragment encoding amino acids 1-687 of human plakoglobin which was ligated to a PCR generated cDNA fragment encoding amino acids 695-781 of chicken β-catenin. **–26AAβN/pgR/βC:** *Exo*III digestions were used to generate β-catenin with an amino-terminal truncation. A restriction fragment from the 5′ end of the βN/pgR/βC construction was replaced by the corresponding fragment from the truncated β-catenin to generate

–26AAβN/pgR/βC. –26AA βN/PgR/βC was generated with and without an amino-terminal 2×-myc tag. **βN/PgR/βC-60AA:** A fragment encoding AA 1-131 of chicken β-catenin was ligated to a fragment encoding AA 123-687 of human plakoglobin that had been ligated to a PCR fragment encoding AA 695-720 of chicken β-catenin.

Each fragment was subcloned into a modified form of pSPUTK to provide the 5′ UTR of β-globin (Falcone and Andrews, 1991). Where indicated, a 2×-myc tag (MSEQKLISEEDLSMSEQKLISEEDLS) was added at the amino terminus. Constructs were moved into pLKneo or pLKpac for expression in mammalian cells (Hirt et al., 1992; Sacco et al., 1995; Wahl et al., 1996). The pLKneo expression vector was obtained from Dr Nicholas Fasel (University of Lausanne). pLK-pac was made by exchanging the neo gene (G418 resistance gene) with the pac (puromycin resistance) gene (Islam et al., 1996).

Transfections

Transfections were done by the calcium phosphate method using a mammalian transfection kit from Stratagene. G418 (Gibco BRL) and/or puromycin at 1 mg/ml or 2 μg/ml, respectively, were added to DMEM for maintaining transfectants. Dexamethasone (10^{-7} M) was used for inducing transfected gene expression.

Yeast two-hybrid analysis

The yeast strain EGY48 and the plasmids pJG4-5 and pSH18-34 were obtained from the laboratory of Dr Roger Brent, and the plasmid pGILDA, a derivative of pEG202, was obtained from OriGene Technologies, Inc. (Rockville, MD). In contrast to pEG202, production of LexA fusion proteins in pGILDA is galactose-inducible. The plasmid pJG4-5Δ was derived from pJG4-5 by removing the coding region between the *Eco*RV and *Hpa*I restriction sites of the B42 activation domain (Ma and Ptashne, 1987). This procedure removed 65 codons from the 79 amino acid B42 activation domain while retaining the nuclear localization signal and the influenza virus epitope tag. Expression of fusion proteins in pJG4-5Δ is galactose inducible.

The C terminus of β-catenin (AA 695-781) was fused to the LexA DNA binding domain (LexA-βC). We found that constitutive production of LexA-βC was toxic to EGY48 and thus used pGILDA so that production of LexA-βC would be galactose-inducible. The N-terminal fragments of β-catenin were inserted into pJG4-5Δ rather than pJG4-5 so that the N-terminal fragments would not be fused to the strong B42 activation domain. Restriction fragments or PCR products encoding the relevant portions of chicken β-catenin were inserted into the vectors. Details of the constructions are available upon request. The α-catenin and α-actinin constructs have been described previously (Nieset et al., 1997).

The expression constructs were transformed into EGY48 and the yeast were plated on selective media as previously described (Nieset et al., 1997). Colonies were picked and grown overnight in medium containing glucose and leucine and lacking uracil, histidine and tryptophan to select for plasmids. Aliquots of the cultures were diluted into galactose/raffinose medium with the same amino acid composition as above and grown for 18 hours prior to assay for β-galactosidase activity. Beta-galactosidase was assayed as previously described (Nieset et al., 1997) and activities expressed as Miller units.

RESULTS

Endogenous β-catenin does not associate with desmoglein 2

We chose the cervical carcinoma cell-line A431 for these studies because it expresses the classical cadherins, E- and P-cadherin, as well as α-catenin, β-catenin and plakoglobin (Johnson et al., 1993; Wahl et al., 1996). In addition, these cells express desmoplakin, desmogleins and desmocollins and form numerous desmosomes (Palka and Green, 1997; Wahl et al., 1996). Although desmosomal components are known to be relatively insoluble in non-ionic detergents, we were able to extract sufficient desmoglein 2 from A431 cells to conduct these experiments. To verify that endogenous β-catenin did not associate with desmosomal cadherins in A431 cells we immunoprecipitated extracts of A431 cells with antibodies specific for either the amino- or carboxyl terminus of β-catenin and assayed by immunoblot for the presence of desmoglein 2, E-cadherin and P-cadherin. Each β-catenin antibody co-immunoprecipitated E-cadherin and P-cadherin as expected, but did not co-immunoprecipitate desmoglein 2 (Fig. 1, lanes 2 and 3). In contrast, an antibody specific for the amino terminus of plakoglobin co-immunoprecipitated E-cadherin, P-cadherin and desmoglein 2, as previously shown (Fig. 1, lane 1; Wahl et al., 1996).

β-Catenin or plakoglobin armadillo repeats are sufficient for association with desmoglein 2

We previously reported that the minimum domain of plakoglobin that was necessary for association with desmoglein 2 was the armadillo repeat region (Wahl et al., 1996). Since the armadillo repeat regions of plakoglobin and β-catenin share 76% identity (Fouquet et al., 1992) we hypothesized that the armadillo repeat region of β-catenin alone may also be able to associate with desmoglein 2. We used a construct that was myc-tagged at the amino terminus to facilitate selective immunoprecipitations. Surprisingly, when we expressed the β-catenin armadillo repeat region in A431 cells it co-immunoprecipitated with E-cadherin but not with desmoglein 2 (Fig. 2, lanes 1 and 2). This is in contrast with the armadillo repeat region of plakoglobin, which associated with both E-cadherin and desmoglein 2 in A431 cells (Wahl et al., 1996). There are two alternative explanations for this result: (1) the β-catenin repeat region alone is sufficient to distinguish between classical and desmosomal cadherins; or (2) the β-catenin repeat region does not associate with desmoglein 2 with a high enough affinity to compete with the endogenous plakoglobin. To distinguish between these two possibilities, we used HT1080 cells that express N-cadherin and β-catenin but undetectable levels of plakoglobin (Sacco et al., 1995). HT1080 cells express desmoglein 2 but do not express any other known desmosomal components and do not form desmosomes (Chitaev and Troyanovsky, 1997). Stable transfectants were selected and immunoprecipitations were done with anti-myc antibodies. The β-catenin armadillo repeat domain interacted with both N-cadherin and desmoglein 2 (Fig. 2, lanes 3 and 4). These results indicate that the armadillo repeat region of β-catenin alone cannot distinguish between the classical cadherins and the desmosomal cadherins and suggests that the specificity resides within the amino- and/or carboxyl-terminal domains of β-catenin. A comparison of the results from A431 cells vs HT1080 cells suggests that the armadillo repeats of β-catenin have a lower affinity for desmoglein 2 than do the armadillo repeats of plakoglobin.

The β-catenin amino terminus together with its carboxyl terminus prevents association with desmoglein 2

In order to analyze the domains of β-catenin that restrict its

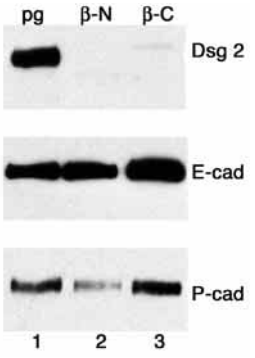

Fig. 1. β-catenin does not co-immunoprecipitate with desmosomal cadherins. NP-40 extract from A431 cells was immunoprecipitated with antibodies against plakoglobin (11E4, lane 1) β-catenin amino terminus (6E3, lane 2) or β-catenin carboxyl terminus (5H10, lane 3). Immunoprecipitates were resolved by SDS-PAGE and immunoblotted with antibodies against desmoglein 2 (6D8), E-cadherin (HECD-1) or P-cadherin (6A9).

binding to the classical cadherins, we constructed chimeric molecules containing various domains of β-catenin and plakoglobin and generated stable A431 cell lines expressing each construct (Fig. 3A). We knew from previous experiments that the plakoglobin armadillo repeat domain, alone, is capable of readily associating with desmoglein 2 in A431 cells (Sacco et al., 1995; Wahl et al., 1996). Thus, we chose the armadillo repeat domain of plakoglobin and added different combinations of β-catenin or plakoglobin amino- and carboxyl-terminal tails. Our hypothesis was that addition of some portion of β-catenin to the armadillo repeat region of plakoglobin would prevent the chimera from associating with desmoglein 2. Fig. 3A shows a schematic of the β-catenin/plakoglobin chimeras constructed for this study. These chimeras were transfected into A431 cells and stable cell-lines were selected. Fig. 3B, lanes 1-7 is an immunoblot using monoclonal antibody PG5.1 that recognizes AA 669-681 of plakoglobin (Wahl et al., 1996). These results demonstrate that each chimera was expressed in A431 cells at a level sufficient for co-immunoprecipitation assays. Endogenous plakoglobin is the most prominent band in each lane, and asterisks point out the chimeric proteins. One chimera, pgN/pgR/βC (lane 5), co-migrated with endogenous plakoglobin, so we immunoblotted this extract with antibodies against β-catenin to verify its expression (lane 5a). Endogenous β-catenin is larger than the chimera and migrated more slowly on SDS-PAGE. To show that generating chimeras between β-catenin and plakoglobin did not produce serious problems in secondary structure each chimera was shown to co-immunoprecipitate with P-cadherin from A431 cells (Fig. 3C).

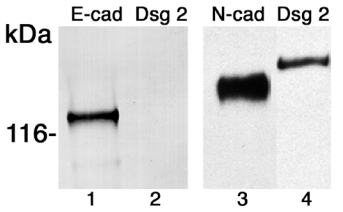

Fig. 2. The armadillo repeat domain of β-catenin co-immunoprecipitates with desmoglein 2 from HT1080 cells but not from A431 cells. A431 cells and HT1080 cells were transfected with the β-catenin armadillo repeat domain fused to an amino-terminal 2× myc tag. The anti-myc antibody, 9E10.2, was used to immunoprecipitate the tagged molecule from lysates of A431 cells (lanes 1 and 2) or from lysates of HT1080 cells (lanes 3 and 4). The immunoprecipitation reactions were resolved by SDS-PAGE and immunoblotted using antibodies against E-cadherin (HECD-1, lane 1), N-cadherin (13A9, lane 3) or desmoglein 2 (6D8, lanes 2 and 4).

To assay for association between the transfected chimeric proteins and desmoglein 2, immunoprecipitations were done using the appropriate antibody against either the amino- or carboxyl terminus of β-catenin and immunoblotting for desmoglein 2. Since the endogenous β-catenin did not associate with desmoglein 2, any desmoglein 2 in the immunoprecipitation reaction must be associated with the chimera. When β-catenin amino- or carboxyl-terminal sequences alone were joined to the plakoglobin armadillo repeats (βN/pgR/ or /pgR/βC) the chimeras were capable of associating with desmoglein 2 (Fig. 3D, lanes 1 and 2). The βN/pgR/ construct contains the β-catenin amino-terminal domain plus β-catenin armadillo repeat 1 followed by plakoglobin armadillo repeats 2-12. Chitaev et al. (1998) previously showed that amino acids in plakoglobin armadillo repeats 1-3 are essential for association with desmogleins. The fact that desmoglein 2 co-immunoprecipitated with the βN/pgR/ chimera indicates that sequences in the first armadillo repeat of β-catenin can substitute for plakoglobin armadillo repeat 1 in binding to desmoglein 2. When both the β-catenin amino- and carboxyl-termini were present (βN/pgR/βC), the association of the chimeric molecule with desmoglein 2 was dramatically reduced (Fig. 3D, lane 3). However, a chimera composed of the plakoglobin amino terminus and armadillo repeats fused with the β-catenin carboxyl terminus (pgN/pgR/βC) readily associated with desmoglein 2 (Fig. 3D, lane 5). These data indicate that the amino- and carboxyl-terminal tails of β-catenin must both be present to interfere with desmoglein 2 interactions. To further test this hypothesis, we examined a chimera composed of the β-catenin amino terminus fused to the plakoglobin armadillo repeats and carboxyl terminus (βN/pgR/pgC) for its ability to associate with desmoglein 2. Surprisingly, this chimera also showed reduced association with desmoglein 2 (Fig. 3D, lane 4). These results led us to conclude that the amino terminus of β-catenin along with its carboxyl terminus prevents β-catenin from associating with desmosomal cadherins. The amino terminus provides the specificity since the carboxyl terminus of either β-catenin or plakoglobin will suffice to interfere with association of chimeric molecules with desmoglein 2. Since the βN/pgR/ chimera does associate with desmoglein 2, the amino terminus of β-catenin alone is not sufficient to modulate association with desmoglein 2. Thus, we propose a model whereby the amino terminus of β-catenin interacts with its carboxyl-terminal tail to disrupt desmoglein 2 interactions. If this is the case, the carboxyl-terminal tail of plakoglobin must also be able to interact with the amino terminus of β-catenin.

Amino acids 1-26 of β-catenin disrupt association with desmoglein 2 in A431 cells

To more precisely determine the β-catenin sequences responsible for interfering with desmoglein 2 interactions we deleted sequences from the βN/pgR/βC construct. Deletion of the first 26 amino acids from the β-catenin amino terminus increased the ability of this chimera to associate with desmoglein 2 (Fig. 3D, lane 6). Replacement of the deleted amino acids with a similar number of amino acids in the form of 2 copies of a myc epitope did not decrease association with desmoglein 2, suggesting that specific β-catenin amino acids are involved in modulating its interactions with desmoglein 2 (Fig. 3D, lane 7). When carboxyl-terminal deletions were

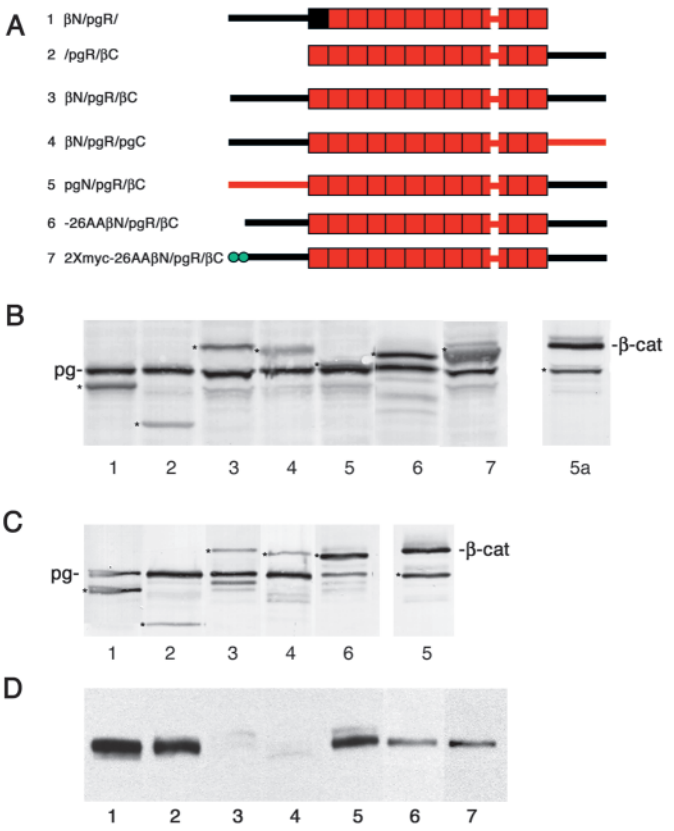

Fig. 3. Chimeras containing β-catenin amino-terminal sequences do not co-immunoprecipitate with desmoglein 2. (A) Chimeras comprised of the plakoglobin armadillo repeat domain and various combinations of β-catenin amino- and/or carboxyl-termini were constructed. Black lines and boxes represent β-catenin while red lines and boxes represent plakoglobin. Green circles represent the myc tag. (B) Chimeric molecules were transfected into A431 cells, extracts were resolved by SDS-PAGE and the expression level of each chimera (pointed out by an asterisk) was verified by immunoblotting with antibodies against plakoglobin (PG5.1, lanes 1-7). The most prominent band in each lane is endogenous full-length plakoglobin. The pgN/pgR/βC chimera (lane 5) co-migrated with endogenous plakoglobin, so we also immunoblotted this extract with antibodies against the carboxyl terminus of β-catenin (5H10, lane 5a). (C) Each chimera was immunoprecipitated with antibodies against P-cadherin and immunoblotted with antibodies against plakoglobin (PG5.1, lanes 1-4 and 6) or against β-catenin (5H10, lane 5). P-cadherin associated with both the endogenous protein and the transfected chimera; the chimera is pointed out with an asterisk. (D) Each chimera was immunoprecipitated with antibodies against β-catenin, 6E3 (lanes 1 and 4) or 5H10 (lanes 2, 3, 5, 6 7). Immunoprecipitation reactions were immunoblotted with anti-desmoglein 2 (6D8).

performed on βN/pgR/βC, removing 20, 40 or 60 amino acids, the truncated chimeras still did not associate with desmoglein 2 (data not shown). However, removal of the entire β-catenin carboxyl-terminal tail (βN/pgR/) restored the ability of this chimera to in associate with desmoglein 2 (Fig. 3D, lane 1). Thus, we conclude that the most amino-terminal 26 amino acids in β-catenin in conjunction with amino acids in the carboxyl-terminal tail prevent its association with desmoglein 2.

Chimeric molecules cannot compete with endogenous plakoglobin for binding desmoglein 2

A431 cells express desmosomal components and form well defined desmosomes (Palka and Green, 1997). Thus in the experiments described above, it was necessary for our chimeric molecules to compete with the endogenous plakoglobin for binding to desmoglein 2. Fig. 2 clearly demonstrates that the isolated armadillo repeat region of β-catenin cannot successfully compete with endogenous plakoglobin for association with desmoglein in A431 cells even though it is capable of associating with desmoglein in the plakoglobin-negative HT1080 cells. To eliminate the problem of competition and directly address the ability of the chimeras to interact with desmosomal cadherins, we turned to the HT1080 cells. Desmoglein 2 did not co-immunoprecipitate with endogenous β-catenin. This indicated that β-catenin was incapable of interacting with desmoglein 2 in HT1080 cells, and not that it simply was unable to compete with plakoglobin for this interaction (Fig. 4A). We transfected HT1080 cells with those chimeric molecules which were unable to associate with desmoglein 2 in A431 cells (i.e. βN/pgR/βC, βN/pgR/pgC, and βN/pgR/βC-60AA) and obtained stable cell lines expressing significant levels of the transgene as indicated by immunoblotting with anti-plakoglobin antibodies (PG5.1, Fig. 4B lanes 1-3, respectively). When these three chimeras were immunoprecipitated from extracts of HT1080 cells with antibodies against the β-catenin amino terminus, desmoglein 2 did co-immunoprecipitate (Fig. 4C), suggesting that these chimeras are capable of associating with desmoglein 2, but cannot efficiently compete with endogenous plakoglobin for this interaction.

To examine this competition more closely we co-transfected full-length plakoglobin and βN/pgR/βC into HT1080 cells. In these cells, plakoglobin and the chimera were expressed at similar levels (Fig. 5A). As expected, anti-plakoglobin antibodies efficiently co-immunoprecipitated desmoglein 2 (Fig. 5B, lane 1). In contrast, anti-β-catenin antibodies co-immunoprecipitated desmoglein 2 at reduced levels when compared to plakoglobin (Fig. 5B, lane 2). These data suggest that, even in HT1080 cells, full-length plakoglobin competes more efficiently than the chimera for binding to desmoglein 2.

Direct evidence for protein-protein interactions between the N- and C-termini of β-catenin

We wanted to determine if there was a direct interaction between the N- and C-terminal ends of β-catenin. To determine if two proteins interact with one another using a typical yeast two-hybrid system, one test protein is fused to a DNA binding domain (such as LexA in pGILDA) and the other test protein is fused to a transcriptional activation domain (such as B42 in pJG4-5). If the two proteins interact, a functioning transcriptional activator is formed leading to synthesis of one or more reporters. This appoach is useful only if at least one of the test proteins does not itself activate transcription.

Since LexA fusions of either the N- or C terminus of β-catenin activate transcription (Hecht et al., 1999), we modified the system so that we could test if the N terminus of β-catenin could modulate the strong transcriptional activity of the C terminus. We prepared a plasmid we call pJG4-5Δ, in which we deleted most of the B42 activation domain from pJG4-5.

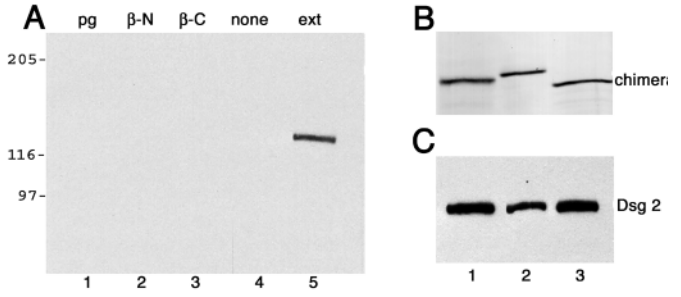

Fig. 4. Chimeras that do not co-immunoprecipitate with desmoglein 2 from A431 cells do co-immunoprecipitate with desmoglein 2 from HT1080 cells. (A) Extracts from untransfected HT1080 cells were immunoprecipitated with antibodies against plakoglobin (11E4, lane 1), β-catenin amino terminus (6E3, lane 2), β-catenin carboxyl terminus (5H10, lane 3) or no antibody (lane 4) and resolved by SDS-PAGE. NP-40 cell extract was run in lane 5. The gel was transferred to nitrocellulose and immunoblotted with an antibody specific for desmoglein 2 (6D8). (B) Extracts of HT1080 cells transfected with βN/pgR/βC (lane 1), βN/pgR/pgC (lane 2) and βN/pgR/βC-60AA (lane 3) were resolved by SDS-PAGE and immunoblotted with antibodies against plakoglobin (PG5.1). (C) Extracts of the above transfectants were immunoprecipitated with antibodies against β-catenin, resolved by SDS-PAGE and immunoblotted with antibodies against anti-desmoglein 2 (6D8).

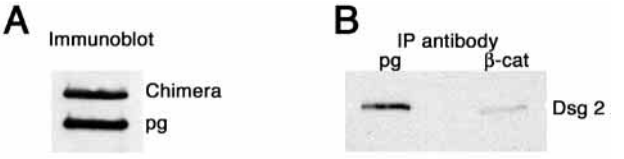

Fig. 5. Plakoglobin competes with the chimeras for binding to Desmoglein 2. HT1080 cells were co-transfected with βN/pgR/βC and plakoglobin. (A) Cell extracts were resolved by SDS-PAGE and immunoblotted with antibodies against plakoglobin (PG5.1). (B) Extracts were immunoprecipitated with antibodies against plakoglobin (11E4, lane 1) or β-catenin (5H10, lane 2), resolved by SDS-PAGE and immunoblotted with antibodies against desmoglein 2 (6D8).

Thus, N-terminal fragments of β-catenin expressed from pJG4-5Δ would not be fused to an additional transcriptional activator.

To verify that pJG4-5Δ lacks transcriptional activity, we tested fragments of α-catenin (amino acids 325-394) and α-actinin (amino acids 479-529) that we previously showed interact with one another (Nieset et al., 1997). Alpha-catenin was inserted into pEG202, and α-actinin was inserted into either pJG4-5 or pJG4-5Δ. Table 1 shows that virtually all the galactose-inducible β-galactosidase activity is missing when α-actinin is cloned into pJG4-5Δ indicating that we have indeed removed the transcriptional activation function from pJG4-5.

The C terminus of β-catenin was cloned into pGILDA (pGILDA-βC) and the N-terminal fragments were cloned into pJG4-5Δ (pJG4-5ΔβN). Compared to pJG4-5Δ, co-transformation of pJG4-5ΔβN with pGILDA-βC resulted in significant repression of β-galactosidase activity suggesting that the N terminus of β-catenin interacted with its C terminus (Table 2). An additional N-terminal fragment of β-catenin was tested showing that the first 26 amino acids of β-catenin can also interact with the C terminus. Thus, the data obtained with the modified yeast-two hybrid system was consistent with the co-immunoprecipitation data presented above.

DISCUSSION

The two primary structures that mediate adhesive interactions between epithelial cells are the adherens junction and the desmosome. These two structures are similar to one another in that they are composed of transmembrane proteins that are members of the cadherin family and are linked to the cytoskeleton through interactions with plaque proteins. However, the adherens junction and the desmosome differ from one another in very important ways. The transmembrane components of the adherens junction are classical cadherins that generally interact with one another in a homotypic manner. However, to organize functional desmosomes, cells must

Table 1. Characterization of pJG4-5Δ

DBD plasmid	AD plasmid	β-Gal activitye on glucose	β-Gal activity on galactose	Number of replicas
pEG202-α-cat	pJG4-5-α-catenin	6.1±0.4	150±16	3
pEG202-α-cat	pJG4-5Δ-α-catenin	6.8±1.5	10.9±4.9	3

Alpha-catenin was inserted into pEG202 and α-actinin was inserted into either pJG4-5 or pJG4-5Δ. Galactose-inducible β-galactosidase activity is substantially decreased when α-actinin is cloned into pJG4-5Δ indicating that we deleted the transcriptional activation domain. DBD: DNA binding domain. AD: activation domain. Beta-galactosidase activity is expressed in Miller units.

Table 2. Interaction between the amino and carboxal ends of β-catenin

DBD plasmid	Repression plasmid	β-Gal activity	Number of replicas
pGILDA-βC (aa 695-781)	pJG4-5Δ	3465±512	8
		3652±327	8
pGILDA-β-C (aa 695-781)	pJG4-5Δ–β-N (aa 1-131)	1473±168	8
		1794±247	8
pGILDA-β-C (aa 695-781)	pJG4-5Δ–β-N (aa 1-26)	1950±455	8

Amino acids 695 to 781 of the C terminus of β-catenin was cloned into pGILDA (pGILDA-βC). N-terminal fragments (amino acids 1-131 or 1-26) were cloned into pJG4-5Δ (pJG4-5ΔβN). Each experiment was replicated 8 times and the standard deviation is indicated. Beta-galactosidase activity is expressed in Miller units.

express both desmoglein and desmocollin, which appear to interact with one another in a heterotypic manner (Chitaev and Troyanovsky, 1997). Another important difference between these two adhesive structures is that the adherens junction is linked to the actin cytoskeleton while the desmosome is linked to the intermediate filament cytoskeleton.

The vast majority of the plaque proteins of the desmosome and the adherens junction are distinct from one another. However, the direct link to the cadherin in both cases is a member of the armadillo family of proteins. In the adherens junction the classical cadherin is linked directly to either β-catenin or plakoglobin. In their structural role in the adherens junction these two proteins appear to be interchangeable. The desmosomal cadherins are linked directly to plakoglobin but not to β-catenin. Since plakoglobin and β-catenin are highly homologous in the central armadillo repeat region which is the domain that mediates interactions with the cadherins, it is intriguing that both armadillo family members interact with the classical cadherins while only plakoglobin interacts with the desmosomal cadherins.

In addition to their structural role in junctions, β-catenin and plakoglobin have been implicated in cellular signaling events that also require interactions with a variety of cellular proteins. Normally, β-catenin stability is controlled by the phosphorylation due to GSK3β, which targets the protein for proteolysis by the ubiquitin pathway. Upon activation of the wnt pathway, GSK 3β is inhibited and cytosolic β-catenin levels increase. Increased cytosolic β-catenin is then available to enter the nucleus and associate with the TCF family of transcription factors and activate β-catenin/TCF responsive genes. The stability of plakoglobin is regulated in a manner similar to that of β-catenin, and plakoglobin can also bind TCF family members (Behrens, 1999; Behrens et al., 1996; Rubinfeld et al., 1995; Simcha et al., 1998). Thus, β-catenin and plakoglobin are highly homologous and interact with many of the same partners; however, one exception is the way in which these two proteins interact with members of the cadherin family of proteins. Plakoglobin associates with classical and desmosomal cadherins while β-catenin associates only with the classical cadherins. The current study was designed to identify the domains of β-catenin which are responsible for discriminating between classical and desmosomal cadherins.

When β-catenin amino- and carboxyl-terminal tails were fused to the plakoglobin armadillo repeats (βN/pgR/βC), the chimera did not associate with desmoglein 2 in A431 cells, although it still bound P-cadherin, E-cadherin and α-catenin. In contrast, the chimera containing the amino terminus of plakoglobin and the carboxyl terminus of β-catenin (pgN/pgR/βC) was capable of associating with desmoglein 2. These data suggest that the amino-terminal domain of β-catenin contains sequences that regulate its association with desmoglein 2. To address this possibility, we constructed amino-terminal deletions of the βN/pgR/βC chimera and showed that the first 26 amino acids are responsible for decreasing the affinity of the chimera for desmoglein 2.

Exactly how the first 26 amino acids of β-catenin decrease its affinity for desmoglein 2 is not clear. However, we speculate that intramolecular interactions between the amino- and carboxyl-terminal tails of β-catenin are responsible for this activity. The chimera that contains the entire amino terminus of β-catenin fused to the plakoglobin armadillo repeats but lacking a carboxyl-terminal tail (βN/pgR/) readily associated with desmoglein 2. This is consistent with our notion that there is an interaction between the amino- and carboxyl-termini of β-catenin that modulates the affinity of β-catenin for desmoglein 2 and may regulate the interaction of β-catenin with other partners. Interestingly, the chimera that is composed of the amino terminus of β-catenin fused to the plakoglobin armadillo repeats and the plakoglobin carboxyl-terminal tail (βN/pgR/pgC) also did not associate with desmoglein 2 in A431 cells. This led us to speculate that β-catenin and plakoglobin are functionally more similar in the carboxyl-terminal tail region than a mere comparison of the amino acid sequences would suggest. In vitro binding assays using recombinant fusion proteins containing the amino- and carboxyl-terminal domains were negative. Thus, an interaction between the amino- and carboxyl-termini of β-catenin may be too weak to detect using an in vitro assay or may only occur intramolecularly in the context of a full-length molecule. Cox et al. (1999) recently showed that the C terminus of Drosophila armadillo can interact, in the yeast two hybrid system, with the armadillo repeats, which is consistent with this idea. The transactivation function of β-catenin and plakoglobin prevented us from using the typical yeast two-hybrid assay to look for direct interactions between the amino- and carboxyl-termini, since both the amino- and carboxyl-termini activated transcription when fused to a DNA binding domain. However, we were able to use a modified yeast two-hybrid assay to present evidence for a direct interaction between the amino- and carboxyl-terminal tails of β-catenin.

Previously, Chitaev et al. (1998) performed alanine scanning mutagenesis of plakoglobin and identified residues that were crucial for association with desmoglein in an in vitro binding assay. These authors identified residues in armadillo repeats 1 and 3 as being critical for desmoglein 1 association in vitro. In our βN/pgR/ construct, armadillo repeat 1 of plakoglobin has been replaced with the armadillo repeat 1 of β-catenin. Our binding data show that this chimera interacts with desmoglein 2 suggesting that repeat 1 of plakoglobin can be substituted with β-catenin sequences and still mediate association with desmoglein 2. This further substantiates the importance of the amino-terminal region of β-catenin rather than the armadillo repeats alone in specifying with which cadherins a particular armadillo family member will associate.

It is quite clear that full-length endogenous β-catenin does not associate with desmosomal cadherins under normal conditions. However, β-catenin has been reported to associate with desmoglein in epidermal cells from plakoglobin–/– mice, in keratinocytes derived from these embryos (Bierkamp et al., 1999) and in A431 cells overexpressing desmoglein 1 (Norvell and Green, 1998). One possible explanation for this aberrant interaction in the plakoglobin–/– mice is simply competition. That is, β-catenin has the ability to weakly interact with desmoglein. However, when plakoglobin is present in the cell, β-catenin cannot compete for interactions with desmoglein. To address the possibility of competition, we transfected the chimeras that did not associate with desmoglein 2 in A431 cells into HT1080 cells that do not express detectable levels of endogenous plakoglobin. Immunoprecipitations from HT1080 transfectants showed that these chimeras were able to associate with desmoglein 2. When we co-transfected plakoglobin and βN/pgR/βC into HT1080 cells and again assayed for

association with desmoglein 2 a diminished level of the chimera was present in the co-immunoprecipitation assay, suggesting that full length plakoglobin is capable of competing off the chimera. However, in contrast to the situation with the plakoglobin–/– mice, we did not see an association between endogenous full-length β-catenin and desmoglein 2 in the HT1080 cells.

A431 cells do not express desmoglein 1 (Schafer et al., 1994). Using A431 cells overexpressing desmoglein 1, or a chimera containing the extracellular domain of E-cadherin and the cytoplasmic domain of desmoglein 1, low levels of endogenous β-catenin were shown to associate with the desmoglein 1 cytoplasmic tail (Norvell and Green, 1998). We propose that introduction of more binding sites for endogenous plakoglobin depleted the endogenous plakoglobin and excess desmoglein 1 cytoplasmic tail could then be bound by some free β-catenin. β-Catenin has only been shown to associate with desmosomal cadherins under non-physiological conditions. Under normal circumstances plakoglobin appears to be the predominant, if not exclusive cytoplasmic binding partner for desmogleins.

In conclusion, the current study demonstrates that amino acids 1-26 of β-catenin function to reduce its association with desmoglein 2 thus restricting β-catenin to the adherens junction. In addition, our data suggest that the amino- and carboxyl-terminal tails of β-catenin interact with one another, perhaps modulating the activity of any potential interaction sites on β-catenin for desmoglein 2.

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