Identification of the Domain of \( \alpha \)-Catenin Involved in Its Association with \( \beta \)-Catenin and Plakoglobin (\( \gamma \)-Catenin)*

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Hiroya Obama and Masayuki Ozawa‡

From the Department of Biochemistry, Faculty of Medicine, Kagoshima University, 8-35-1 Sakuragaoka, Kagoshima 890, Japan

\( \alpha \)-Catenin is a 102-kDa protein exhibiting homology to vinculin, and it forms complexes with cadherins or the tumor-suppressor gene product adenomatous polyposis coli through binding to \( \beta \)-catenin or plakoglobin (\( \gamma \)-catenin). The incorporation of \( \alpha \)-catenin into the cadherin-catenin complexes is a prerequisite for expression of the cell-adhesive activity of cadherins. Using an in vitro assay system involving bacterially expressed proteins, we localized a region in \( \alpha \)-catenin required for molecular interaction with \( \beta \)-catenin and plakoglobin. Analysis of various truncated \( \alpha \)-catenin molecules revealed that amino-terminal residues 48–163 are able to bind to \( \beta \)-catenin and plakoglobin. Consistent with the observation that \( \beta \)-catenin and plakoglobin bind to the same region of \( \alpha \)-catenin, \( \beta \)-catenin competed with the binding of plakoglobin to \( \alpha \)-catenin and vice versa. Under the conditions used, \( \beta \)-catenin bound to \( \alpha \)-catenin with higher affinity than did plakoglobin. Scatchard analysis indicated that the affinity of the interaction between \( \alpha \)-catenin and \( \beta \)-catenin or that between \( \alpha \)-catenin and plakoglobin was moderately strong (\( K_d = 3.8 \times 10^{-8} \) and \( 7.7 \times 10^{-8} \), respectively). When transfected into L cells expressing E-cadherin, the amino-terminal region of \( \alpha \)-catenin (from residue 1 to 226) formed complexes with \( \beta \)-catenin supporting the in vitro binding experiment results.

Cadhers are a major group of calcium-dependent cell-cell adhesion molecules that bind through a homophilic mechanism and that are localized to specialized intercellular junctions called adherens junctions (1, 2). The cadherins are transmembrane proteins possessing an extracellular calcium-binding segment and an intracellular domain that is highly conserved (~90% identity) among most members of the family. Deletion of the conserved intracellular segment results in cadherin inactivation even if the extracellular binding domain seems to remain intact (3). The cytoplasmic domain of cadherins interacts with three molecules termed catenins (\( \alpha \), \( \beta \), and \( \gamma \)), and the resultant complexes seem to associate with cortical actin filaments (4). This interaction between cadherins and catenins is essential for cadherin-mediated adhesion and the association of the complexes with the cytoskeleton (5–7).

Two of the catenins have been cloned. \( \alpha \)-Catenin is homologous to vinculin, a cytoskeleton-associated protein (8, 9). \( \beta \)-Catenin is homologous to plakoglobin (a protein found in both adherens junctions and desmosomes) and Armadillo (a segment polarity gene product in Drosophila melanogaster (10–13)). Immunological data suggest that \( \gamma \)-catenin is identical to plakoglobin (14, 15).

Recent in vitro and in vivo experiments have shown that \( \beta \)-catenin and plakoglobin bind directly to the cytoplasmic domain of E-cadherin, while \( \alpha \)-catenin binds directly to \( \beta \)-catenin or plakoglobin (16–20). The amino-terminal parts of \( \beta \)-catenin and plakoglobin have been shown to comprise their \( \alpha \)-catenin-binding sites, and the central core region, which is composed of 13 copies of the so-called Armadillo repeat, is involved in the association with cadherins. The latter is also involved in the complex formation with the adenomatous polyposis coli tumor-suppressor protein and in the case of plakoglobin, with desmosigines (desmosomal catenins). The region of \( \alpha \)-catenin responsible for \( \beta \)-catenin and plakoglobin binding, however, had not been identified. In this study, we report experiments that revealed the region in human \( \alpha \)-catenin responsible for the binding of \( \beta \)-catenin and plakoglobin.

MATERIALS AND METHODS

Expression of \( \alpha \)-Catenin as a Fusion Protein with the Maltose-binding Protein—A full-length cDNA clone for human \( \alpha \)-catenin has been described (20). To express \( \alpha \)-catenin as a fusion protein with the maltose-binding protein (MBP) in Escherichia coli cells, cDNA encoding the protein was cloned into an MBP fusion vector (pMALc, New England Biolabs Inc.). cDNA fragments encoding various regions of \( \alpha \)-catenin were generated by using convenient restriction enzyme sites within the cDNA clones or by means of the polymerase chain reaction. The combinations of restriction enzymes used were: BamHI and SalI, BamHI and HindIII, MluI and SalI, BamHI and StuI, BamHI and SpelI, BamHI and XhoI, BamHI and ApaI, XhoI and SpelI, ApaI and SpelI, and XhoI and SpelI. For the polymerase chain reaction, three sense primers (5’GAAGATCT-TTAAATAGAAGAGGAGG, 5’GAAGATCTAAAATTTGCGAAGGAG, and 5’GAAGATCTGAGTTCCAGATAG) and two antisense primers (5’GAAAGCTTCAAGATACCATCTTC and 5’GAAAGCTTGAATACCATCTTC) were used. The reaction mixture was subjected to 30 cycles of denaturation (93 °C, 1 min), annealing (45 °C, 1 min), and extension (72 °C, 1 min). The cDNA fragments were subcloned in frame into the vector, and the plasmid DNAs were introduced into JM109 cells. MBP fusion proteins were purified by affinity chromatography on columns of amylose resin (New England Biolabs Inc.) as described previously (20).

Expression of \( \beta \)-Catenin and Plakoglobin as Fusion Proteins with Glutathione S-Transferase or MBP—The cDNA clones for human plakoglobin and \( \beta \)-catenin have been described (20, 21). The full-length \( \beta \)-catenin cDNA with a BamHI 5’–3’ SalI orientation in Bluescript II KS(+) vector was excised by digestion with BamHI and SalI and then cloned into the BamHI/SalI sites of the glutathione S-transferase (GST) fusion protein (pGEX-4T3, Pharmacia Biotech Inc.) vectors. The GST fusion protein vectors containing cDNA for the entire coding region of plakoglobin have been described (20).

Nitrocellulose Blot Overlay Assay—The binding of GST fusion proteins to MBP fusion proteins was visualized as follows. Purified MBP

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‡ To whom correspondence should be addressed. Tel.: 81-99-275-8358; Fax: 81-99-264-5618.

1 The abbreviations used are: MBP, maltose-binding protein; GST, glutathione S-transferase; HA, hemagglutinin; PAGE, polyacrylamide gel electrophoresis.
fusion proteins (50 ng) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then electroblotted onto nitrocellulose filters as described previously (20). The filters were incubated in phosphate-buffered saline containing 5% nonfat dried milk for 30 min and with GST fusion proteins (100 µg/ml) for 2 h. The filters were then washed with phosphate-buffered saline containing 0.05% Tween 20. The MBP fusion proteins bound to proteins on the filters were detected by incubation with affinity-purified anti-GST antibodies followed by horseradish peroxidase-labeled F(ab')2 fragments of goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc.) and 4-chloro-1-naphthol as a substrate. For competitive binding assay of β-catenin and plakoglobin, affinity-purified antibodies against β-catenin or plakoglobin were used to detect the respective fusion protein bound to α-catenin. To quantify the binding, the peroxidase-labeled antibody was replaced by the 125I-labeled F(ab')2 fragment of goat anti-rabbit IgG (7.5 µCi/µg, DuPont NEN), and bound radioactivity was determined using a scintillation counter.

Antibodies—Rabbit antibodies against GST were prepared and purified as described previously (20). A monoclonal antibody against GST was purchased from Santa Cruz Biotechnology. Monospecific antibodies against the carboxyl-terminal part of plakoglobin or the full-length β-catenin have been described previously (20, 21). A monoclonal antibody against GST, directed against hemagglutinin (HA) was kindly provided by Dr. A. Yoshimura (Kurume University, Fukuoka, Japan).

Scatchard Analysis of GST/β-catenin or GST/Plakoglobin Binding to α-Catenin—The α-catenin fusion proteins (10 µg) containing residues 1–906 (the entire protein, MBP/α-CN-(1–906) or residues 48–163 (MBP/α-CN-(48–163)) were electroblotted onto nitrocellulose filters after SDS-PAGE. Each filter was incubated with varying concentrations (0–20 µg/ml) of GST/β-catenin or GST/plakoglobin, and the bound GST/β-catenin or GST/plakoglobin was detected by incubation with monoclonal anti-GST antibodies followed by the horseradish peroxidase-labeled antibody (125I-labeled F(ab')2 fragment of goat anti-rabbit IgG) (7.5 µCi/µg, DuPont NEN), and bound radioactivity was determined using a scintillation counter.

Localization of the β-Catenin-binding Site on α-Catenin—To localize the sites for β-catenin and plakoglobin binding in α-catenin, different regions of the α-catenin molecule (Fig. 1) were expressed as maltose-binding protein (MBP) fusion proteins in E. coli binding protein (MBP) fusion proteins in amylose resin gels and then run on 8% polyacrylamide gels. The proteins were stained with either Coomasie Brilliant Blue (A) or incubated with anti-MBP antibodies (C), GST/β-catenin (B and D), or GST/plakoglobin (E) after transfer to a nitrocellulose membrane. The binding of GST/β-catenin to the MBP/α-catenin fusion proteins was visualized as described under “Materials and Methods.” The band indicated by the arrowhead in B may represent a bacterial stress protein (DnaK) (33) co-purified with the MBP/α-catenin fusion proteins and recognized by the antibodies used in the experiments. The degree of contamination of the protein varies depending on the fusion protein.

RESULTS

Localization of the β-Catenin-binding Site on α-Catenin Using α-Catenin Fusion Proteins—To localize the sites for β-catenin and plakoglobin binding in α-catenin, different regions of the α-catenin molecule (Fig. 1) were expressed as maltose-binding protein (MBP) fusion proteins in E. coli and then purified to homogeneity by affinity chromatography on amylose resin. Each fusion protein migrated on an SDS-PAGE gel as a 4-chloro-1-naphthol as a substrate. For competitive binding assay of β-catenin and plakoglobin, affinity-purified antibodies against β-catenin or plakoglobin were used to detect the respective fusion protein bound to α-catenin. To quantify the binding, the peroxidase-labeled antibody was replaced by the 125I-labeled F(ab')2 fragment of goat anti-rabbit IgG (7.5 µCi/µg, DuPont NEN), and bound radioactivity was determined using a scintillation counter.

transfected cells were analyzed as described below. To isolate stable transfectants, Madin-Darby canine kidney cells (1 × 10⁶) were transfected with pC-αCN1–226 as described above except that the voltage was set at 600 V. Stable transfectants were selected and cloned as described before (4). The cells were lysed in 10 mM Tris-HCl buffer, pH 7.5, containing 0.5% Nonidet P-40 and 1 mM phenylmethanesulfonyl fluoride. The truncated α-catenin with the HA tag was collected with the anti-HA monoclonal antibody 12CA5, which had been preabsorbed to protein A-Sepharose CL-4B. The immune complex was washed with a washing buffer (10 mM Tris-HCl, pH 7.5, 0.5% Nonidet P-40, 0.5 M NaCl, 1 mM phenylmethanesulfonyl fluoride).

As shown in Fig. 2B, GST/β-catenin bound to the α-catenin fusion proteins containing residues 1–906 (the entire protein, MBP/α-CN-(1–906) and residues 1–449 (the amino-terminal half of the protein, MBP/α-CN-(1–449), but it did not bind to a...
protein spanning residues 326–906 (the carboxyl-terminal two-thirds of the protein, MBP/α-CN-326–906). The binding appeared to be specific for these portions of the MBP fusion proteins as no binding was observed between GST/β-catenin and MBP alone. Furthermore, GST itself had no ability to bind to MBP/α-CN-(1–906) or MBP/α-CN-(1–449) (data not shown), indicating that the β-catenin portion of GST/β-catenin is responsible for the binding between GST/β-catenin and the MBP fusion proteins. The binding of GST/β-catenin to the MBP fusion proteins was abolished if GST/β-catenin was boiled for 5 min immediately before the blot overlaying was performed (data not shown). The results indicate that β-catenin binding to α-catenin is limited to the amino-terminal 449 residues of the α-catenin molecule.

To further define the β-catenin-binding site within the amino-terminal 449 residues of the α-catenin molecule, we expressed a series of α-catenin fusion proteins with deletions from the amino terminus or from the carboxyl terminus of MBP/α-CN-(1–449). These proteins were then examined as to their ability to bind to β-catenin. Although the α-catenin fusion proteins containing residues 48–163 exhibited such an ability, those with deletions in residues 48–163 did not (Fig. 2D). These results suggest that the β-catenin-binding site in α-catenin is localized within residues 48–163 (as summarized and shown in Fig. 1).

Localization of the Plakoglobin-Binding Site in α-Catenin—

The high degree of sequence identity (82%) between the α-catenin-binding site of β-catenin and that of plakoglobin (20) suggested that the binding site of α-catenin for β-catenin and plakoglobin is the same. To determine whether this is the case or not, we analyzed the binding of the plakoglobin fusion protein with GST (GST/plakoglobin) to the α-catenin fusion proteins as above. As in the case of GST/β-catenin binding, GST/plakoglobin bound to the α-catenin fusion proteins containing residues 48–163 but not to the α-catenin fusion proteins without these residues (Fig. 2E). These results suggest that the plakoglobin-binding site in α-catenin is also localized within residues 48–163.

To confirm that β-catenin and plakoglobin bind to the same region of α-catenin, a competition experiment was carried out. When GST/plakoglobin was included on the incubation of GST/β-catenin with an α-catenin fusion protein, the binding of GST/β-catenin to the α-catenin fusion protein decreased with increasing amounts of GST/plakoglobin and vice versa (Fig. 3). The binding of GST/plakoglobin, however, seems to be more sensitive to the presence of the competitor. The presence of an approximately 12-fold molar excess of GST/plakoglobin reduced the binding of GST/β-catenin, but the presence of an approximately 4-fold molar excess of GST/plakoglobin did not (Fig. 3A). The binding of GST/plakoglobin to an α-catenin fusion protein was significantly reduced by the presence of a 4-fold molar excess of GST/β-catenin and almost completely inhibited by the presence of a 12-fold molar excess of GST/β-catenin (Fig. 3B). These results may suggest that β-catenin has a higher affinity to α-catenin than plakoglobin.

To better characterize the interaction of β-catenin or plakoglobin with α-catenin, Scatchard analysis was carried out (Fig. 4). Increasing concentrations of GST/β-catenin or GST/plakoglobin were incubated with MBP/α-catenin (residues 1–906) fixed on nitrocellulose membranes followed by quantification of the amounts of the bound GST/β-catenin or GST/plakoglobin. The results demonstrated that GST/β-catenin bound to α-catenin with a dissociation constant (Kd) of 3.8 × 10⁻⁸ M and that GST/plakoglobin bound to α-catenin with a Kd of 7.7 × 10⁻⁸ M. When the truncated α-catenin with residues 48–163 was subjected to the same analysis, Kd values of 1.5 × 10⁻⁸ M and 5.6 × 10⁻⁸ M were obtained for GST/β-catenin and GST/plakoglobin, respectively.

Residues 1–220 of α-Catenin Are Sufficient for β-Catenin Binding in Vivo—To prove that the results obtained in in vitro binding experiments reflect the activity of α-catenin in vivo, we expressed the amino-terminal region of α-catenin in living cells. We constructed an expression vector containing a cDNA encoding residues 1–226 of α-catenin and a sequence of an epitope of HA. The presence of the HA tag at the carboxyl terminus of the truncated α-catenin enabled us to collect the expressed protein by immunoprecipitation. An L cell line ex-
pressing E-cadherin (and thus also expressing β-catenin) was transiently transfected with an expression vector, and the expressed α-catenin was collected with the anti-HA monoclonal antibodies to determine whether or not it could bind to β-catenin. After SDS-PAGE and transfer to a nitrocellulose membrane, the collected materials were probed with antibodies to β-catenin. As shown in Fig. 5, β-catenin was precipitated by anti-HA antibodies from the cells transfected with the expression vector but not from the cells transfected with a control vector. Therefore, the truncated α-catenin containing amino-terminal residues 1–226 was able to bind to β-catenin.

To estimate the amount of β-catenin bound to the mutant α-catenin in cells, Madin-Darby canine kidney cells were transfected with the same vector, and stable transfectants were isolated. The amounts of β-catenin co-precipitated with the truncated α-catenin were compared with those of β-catenin in the total cell lysates. Quantitative analysis revealed that about 5% of β-catenin in the lysates was associated with the mutant α-catenin (data not shown).

**DISCUSSION**

Using an *in vitro* binding system, we analyzed the molecular interaction of α-catenin with β-catenin and plakoglobin and localized the binding site for the latter two molecules in α-catenin. Furthermore, by expressing the amino-terminal region of α-catenin in living cells, we obtained evidence that the region can bind to β-catenin *in vivo*. During the preparation of this manuscript, we noticed a report that the amino-terminal 606 amino acids of α-catenin bind to β-catenin in a yeast two-hybrid system (23). Our results indicating that amino-terminal residues 48–163 of α-catenin bind to β-catenin are consistent with this observation and also further extend it. Furthermore, we showed that plakoglobin and β-catenin bind to the same region in α-catenin. Scatchard analysis revealed that β-catenin binds to α-catenin with higher affinity ($K_d = 3.8 \times 10^{-8}$ M) than plakoglobin ($K_d = 7.7 \times 10^{-8}$ M).

cDNA cloning revealed that α-catenin is a vinculin-related protein. Vinculin is a cytoskeletal protein associated with both cell-cell and cell-extracellular matrix adhesions-type junctions (24, 25). The homology between vinculin and α-catenin is restricted to three major regions in the amino-terminal, central, and carboxyl-terminal parts of the two proteins. Vinculin has been shown to bind to F-actin (26) and talin (27). Thus, the latter protein can bind directly to the cytoplasmic domain of β1 integrins (28, 29), which are members of the integrin superfamily of transmembrane heterodimeric glycoproteins. The actin-binding site of vinculin has been localized to the carboxy-terminal region (26), whereas the talin-binding site is in the amino-terminal region of residues 1–258 (30). Therefore, we found similarities between α-catenin and vinculin not only in the primary sequence but also in the position of the region used for hierarchical molecular interactions in cell adhesion molecule complexes. The amino-terminal region of both proteins is involved in the interaction with a molecule (β-catenin in the case of α-catenin and talin in the case of vinculin) that binds directly to the cell adhesion molecules (cadherins and integrins, respectively). The 116 amino acid residues identified in human α-catenin as the β-catenin- and plakoglobin-binding site in this study, however, show only a 19.8% identity to the sequence of human vinculin. Therefore, it is not surprising that these two proteins interact with distinct proteins despite their overall structural similarity.

The alternative splicing of an mRNA primary transcript is a widespread means of generating structurally and functionally distinct protein isoforms and contributes to tissue-specific and developmentally regulated patterns of gene expression. The presence of splice variants of α-catenin and αn-catenin (a neural form of α-catenin) has been reported (31, 32), although their biological activities have not been determined. The variant forms included a 24- or 48-amino acid insertion in their carboxy-terminal regions, respectively. The present study suggests that these variant forms have similar varieties, if not identical, abilities to bind to β-catenin and plakoglobin.

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