Journal of Cell Science 110, 1013-1022 (1997)
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JCS8132

Characterization of the interactions of αcatenin with αactinin and βcatenin/plakoglobin

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SUMMARY

Cadherins are calcium-dependent, cell surface glycoproteins involved in cell-cell adhesion. To function in cell-cell adhesion, the transmembrane cadherin molecule must be associated with the cytoskeleton via cytoplasmic proteins known as catenins. Three catenins, αcatenin, βcatenin and γcatenin (also known as plakoglobin), have been identified. βcatenin or plakoglobin is associated directly with the cadherin; αcatenin binds to βcatenin/plakoglobin and serves to link the cadherin/catenin complex to the actin cytoskeleton. The domains on the cadherin and βcatenin/plakoglobin that are responsible for protein-protein interactions have been mapped. However, little is known about the molecular interactions between αcatenin and βcatenin/plakoglobin or about the interactions between αcatenin and the cytoskeleton. In this study we have used the yeast two-hybrid system to map the domains on αcatenin that allow it to associate with βcatenin/plakoglobin and with αactinin. We also identify a region on αactinin that is responsible for its interaction with αcatenin. The yeast two-hybrid data were confirmed with biochemical studies.

Key words: Cadherin, Cytoskeleton, αCatenin, αActinin, βCatenin, Plakoglobin

INTRODUCTION

Adherens junctions are intercellular structures particularly prominent in epithelia and the myocardium that function in cell-to-cell adhesion and appear at regions of close cell-cell apposition as two parallel intracellular plaques into which actin filaments insert (Geiger et al., 1990). The classical cadherins are the transmembrane components of these junctions and play a critical role in calcium dependent cell-cell interactions, important both in developmental processes and in maintenance of normal tissue architecture. These cadherins contain a highly conserved cytoplasmic domain that indirectly associates with the actin cytoskeleton via proteins termed catenins.

The catenins were identified by their ability to co-immunoprecipitate with the cadherins and were named αcatenin, βcatenin and γcatenin according to their mobility on SDS-PAGE (Nagafuchi and Takeichi, 1988; Ozawa et al., 1989; Wheelock and Knudsen, 1991; McCrea et al., 1991; McCrea and Gumbiner, 1991). βCatenin is a 95 kDa protein that shares about 65% identity with γcatenin (McCrea et al., 1991; Fouquet et al., 1992), an 82 kDa protein also known as plakoglobin (Knudsen and Wheelock, 1992; Peifer et al., 1992). βCatenin and plakoglobin associate directly with the cadherin and can substitute for one another in the cadherin-catenin complex (Butz and Kemler, 1994; Hinck et al., 1994; Näthke et al., 1994; Sacco et al., 1995). αCatenin is a 102 kDa protein that shares some homology with vinculin (Nagafuchi et al., 1991; Herrenknecht et al., 1991) and is associated with the cadherin indirectly through its interaction with βcatenin or plakoglobin. Thus, the adherens junction is composed of transmembrane cadherin molecules each of which is associated directly with either βcatenin or plakoglobin which in turn is associated directly with αcatenin. αCatenin mediates the interaction between the cadherin-catenin complex and the actin cytoskeleton through its associations with αactinin (Knudsen et al., 1995) and actin filaments (Rimm et al., 1995).

The domains on the cadherin molecule that interact with βcatenin or plakoglobin (Nagafuchi and Takeichi, 1988; Ozawa et al., 1990; Stappert and Kemler, 1994; Jou et al., 1995), the domains on βcatenin and plakoglobin that interact with cadherin (Hülsken et al., 1994; Sacco et al., 1995; Wahl et al., 1996), and the domains on βcatenin and plakoglobin that interact with αcatenin (Aberle et al., 1994; Hülsken et al., 1994; Jou et al., 1995; Rubinfeld et al., 1995; Sacco et al., 1995; Ozawa et al., 1995; Wahl et al., 1996; Aberle et al., 1996) have been described. In this study we describe the domains on αcatenin and αactinin that interact with each other to link the cadherin/catenin complex to the cytoskeleton. In addition we specify a domain on αcatenin that, by binding either βcatenin or plakoglobin, allows it to associate with cadherins.

MATERIALS AND METHODS

Molecular constructions

The full-length cDNA clone for human non-muscle αactinin was a gift from Dr D. J. Kwiatkowski, Harvard Medical School (Youssoufian et al., 1990). The cDNA clones for chicken α(E)-catenin and chicken βcatenin have been described (Johnson et al., 1993). A full-length human plakoglobin clone (Franke et al., 1989) was a gift from Dr W. W. Franke, German Cancer Research Center, Heidelberg. DNA-binding domain (DBD) and activation domain (AD) fusions for yeast two-hybrid analysis were produced by standard methods, including shuttling restriction fragments into intermediate vectors or blunt ending to allow the in-frame insertion into yeast plasmids. Exonuclease III deletions of a selected portion of αcatenin were constructed as described (Johnson et al., 1993). All ligation junctions were sequenced to verify the construction and the reading frame. Details of the constructions are available upon request. The *Escherichia coli* strain JM109 (Yanisch-Perron et al., 1985) was used as a host.

An E-cadherin/αcatenin chimera was constructed for expression in cultured cells. A fragment encoding amino acids (aa) 294-506 of α catenin was subcloned into pCHA (Pati, 1992). The insert was joined to a fragment of human E-cadherin cDNA (K. Johnson, unpublished) encoding aa 1-792. This final construction was inserted into the eukaryotic expression vector pLKneo (Hirt et al., 1992). Amino acids 1-737 of E-cadherin were inserted into pLKneo and used as a negative control.

Recombinant fusion proteins between maltose-binding protein and the protein of interest (MBP fusions) were expressed in *E. coli* JM109 using the pMal-c2 expression system essentially as described by the manufacturer (New England Biolabs, Beverly, MA). Soluble extracts of induced bacterial cultures were prepared essentially as described by the manufacturer.

Two-hybrid analysis

The yeast strain EGY48 (*MAT**a** trp1 ura3 his3 leu2:: p3LexA$_{op}$*-LEU2) and the plasmids utilized in the two-hybrid system (pEG202, pJG4-5, pSH18-34, pSH17-4, pRFHM1 and pJK101) were obtained from the laboratory of Dr Roger Brent, Massachusetts General Hospital, Boston, MA (Golemis and Brent, 1992; Zervos et al., 1993; Gyuris et al., 1993). Yeast growth and transformation as well as two-hybrid selection and assays were performed essentially as described (Ausubel et al., 1996). EGY48 containing the lacZ reporter plasmid (pSH18-34) was transformed with a derivative of pEG202 where the LexA DNA-binding domain (DBD) was fused to a portion of α catenin. None of the LexA/αcatenin fusions used in this study activated transcription of either reporter in the absence of an activation domain, and each was shown to repress transcription of the lacZ reporter in pJK101 indicating that the DBD fusion was able to enter the nucleus and bind LexA operators. Expression of each LexA/α catenin fusion protein in EGY48 was verified by immunoblotting. The yeast were then transformed with a pJG4-5 derivative where the B42 activation domain (AD) was fused to a fragment of either βcatenin, plakoglobin or αactinin. Transformants were grown in liquid galactose-containing dropout medium supplemented with leucine to induce expression of the AD-fusion protein before plating onto galactose plates lacking leucine. A minimum of 8 yeast colonies from at least two independent transformations which grew on these plates were tested further. The expression of the AD-fusion protein in EGY48 was verified by immunoblotting. The galactose-dependence of the leucine prototrophy was tested as described by Ausubel et al. (1996) and the galactose-inducible βgalactosidase activity was examined using a filter assay (Ausubel et al., 1996). For an interaction to be scored as positive, the yeast were required to grow significantly better on galactose than glucose plates lacking leucine, and exhibit significantly greater βgalactosidase activity in the presence of galactose than glucose in the filter assay. For selected interactions, units of βgalactosidase activity were determined for 4 independent clones in both galactose- and glucose-containing medium using a standard liquid assay (Ausubel et al., 1996) and the average value was reported.

Antibodies and other reagents

Rabbit polyclonal antiserum against E-cadherin, rat monoclonal antibody E9 against human E-cadherin and mouse monoclonal antibodies 1G5 against αcatenin, 12F7 and 5H10 against βcatenin and 4F11 against plakoglobin have been described (Wheelock et al., 1987; Johnson et al., 1993; Wahl et al., 1996). Anti-LexA rabbit polyclonal antiserum was a gift from Dr Barak Cohen (Massachusetts General Hospital, Boston, MA). Mouse monoclonal antibody 12CA5 recognizing an influenza virus hemaglutinin epitope was purchased from Berkeley Antibody Company (Berkeley, CA). A mouse monoclonal antibody against maltose-binding protein was generated as described (Johnson et al., 1993). Mouse monoclonal antibodies against αactinin, BM75.2 and mAb1682, were purchased from Sigma Chemical Company (St Louis, MO) and Chemicon International Inc. (Temecula, CA), respectively. Secondary antibodies were purchased from Jackson Immunoresearch Laboratories (West Grove, PA).

Electrophoresis and immunoblotting

Polyacrylamide slab gel electrophoresis in the presence of SDS (SDS-PAGE) was performed according to the procedure of Laemmli (1970) with materials from Bio-Rad (Richmond, CA). Molecular mass markers were from Sigma. SDS-PAGE-resolved proteins were transferred to nitrocellulose and immunoblotted as described (Knudsen and Wheelock, 1992).

Cell culture and immunofluorescence

A431 cells were obtained from the American Type Culture Collection (Rockville, MD) and maintained in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum (Hyclone, Logan, UT). The A431D cell line is a derivative of A431 that has ceased to express cadherins (see Fig. 3) due to chronic treatment with dexamethasone. Cells were transfected using the calcium phosphate method with reagents from Stratagene (La Jolla, CA) and stable transfectants were selected in G418 (GibcoBRL, Gaithersburg, MD). The transfected cells were fixed with ice-cold methanol and stained for double immunofluorescence with antibodies against E-cadherin and α actinin followed by appropriate secondary antibodies conjugated to either fluorescein or rhodamine.

E-cadherin 'patching'

To 'patch' E-cadherin in transfected cells, polyclonal anti-E-cadherin was diluted 1:50 in culture medium and added to living cells for 2 hours at 37°C. The cells were then washed thoroughly with phosphate buffered saline and processed for immunofluorescence microscopy.

Immunoprecipitation

A 1 ml sample of cell extract was mixed with 100 μl of monoclonal antibody supernatant at 4°C as described (Sacco et al., 1995). After 30 minutes, 100 μl of packed anti-mouse IgG-Sepharose (Organon Teknika-Cappel, Durham, NC) was added, and mixing was continued for 30 minutes. The Sepharose-bound immune complexes were washed 5 times with 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% Tween-20. The pellets were solubilized and resolved by SDS-PAGE.

In vitro binding assays

Monolayers of colon carcinoma SW707 cells (gift of Dr Menhard Herlyn, Wistar Institute, Philadelphia, PA) were washed with phosphate buffered saline at room temperature and scraped from the flask on ice using 5 ml/225-cm^2 flask 10 mM Tris-HCl, pH 8.0, 1 mM EDTA (TE) containing 2 mM phenylmethylsulfonyl fluoride. The cells were transferred to a Dounce homogenizer and homogenized on ice. The resulting suspension was centrifuged at 15,000 ***g*** for 10 minutes at 4°C and the supernatant stored at -80°C. Before use in the

binding assay, Nonidet P-40 (BDH, Poole, UK) was added to 0.05% (v/v), and the salt concentration was adjusted to 150 mM with NaCl. Microfuge tubes used in the assays were coated with 0.1% (v/v) Triton X-100 to reduce non-specific binding. A 40 μl sample of amylose resin (New England Biolabs) was mixed for 10 minutes at 4°C with bacterial extract containing the recombinant MBP fusion protein. The resin with the bound MBP fusion protein was pelleted and washed once with 10 mM Tris, 0.5% (v/v) NP-40, 1 mM EDTA (TNE). A 100 μl sample of purified bovine serum albumin (BSA, 10 mg/ml) was added to block non-specific binding and the samples mixed for 10 minutes at 4°C, after which the resin was pelleted and the supernatant aspirated. A 100 μl portion of SW707 extract was added and the samples were incubated for 10 minutes at 4°C. The amylose resin-bound complexes were pelleted and washed 5 times with TNE. The pellets were boiled in Laemmli sample buffer (Laemmli, 1970) and resolved by SDS-PAGE.

RESULTS

Determination of a direct interaction between α catenin and αactinin

αCatenin has been shown to link the cadherin/catenin complex to the cytoskeleton (Ozawa et al., 1990; Nagafuchi et al., 1994; Watabe et al., 1994). We and others have previously shown that αactinin co-localizes with αcatenin at cell-cell borders in cells that form adherens junctions (Tokuyasu et al., 1981; Knudsen et al., 1995). In addition, we presented data suggesting a direct interaction between αcatenin and αactinin (Knudsen et al., 1995). To further characterize this interaction and determine whether or not it occurs by direct binding between the two proteins, we employed yeast two-hybrid analysis and confirmed our results with transfection experiments in cultured cells.

Using the two-hybrid system we tested for protein-protein interactions between αcatenin and αactinin. When we tested two fragments of αcatenin with three fragments of αactinin we found that aa 6-506 of αcatenin interacted with aa 479-892 of αactinin (see Table 1). To narrow down the site on αcatenin that interacts with αactinin, several amino- and carboxyl-terminal deletions were tested. Results obtained with the most informative deletions are summarized in Fig. 1. A construct encoding only aa 325-394 of αcatenin interacted with aa 479-529 of αactinin while a construct encoding aa 325-377 did not; thus aa 325-394 of αcatenin were sufficient for the interaction with αactinin.

Table 1. Yeast two-hybrid interactions between αcatenin and αactinin

αCatenin DBD fusion Amino acids:	αActinin AD fusion Amino acids: 1-88	89-478	479-892
6-506	–	–	+
507-905	–	–	–

Two DBD/αcatenin fusions (*Pst*I to *Cla*I, aa 6-506; *Cla*I to end, 507-905) were tested for interaction with three AD/αactinin fusions (start to *Bgl*II, aa 1-88; *Bgl*II to *Bgl*II, 89 to 478; *Bgl*II to end; 479 to 892). Only one pair (+) showed galactose inducible growth on plates lacking leucine and also galactose inducible βgalactose activity in the filter assay.

To confirm that the αactinin-interaction domain on α catenin identified by the two hybrid studies also interacted with native full-length αactinin we examined the interaction in vivo using A431D cells. A431D cells are a derivative of A431 cells that do not express a classical cadherin but do express αactinin (Lewis et al., 1997); they also express catenins which are present in the cytosol. Comparisons of the localizations of αactinin and E-cadherin in A431 vs A431D cells is shown in Fig. 2A-D. αActinin is abundant in the A431D cells but does not localize to cell-cell borders in the absence of E-cadherin as it does in the A431 cells. A431D cells were transfected with a chimeric protein consisting of human E-cadherin lacking the βcatenin/plakoglobin binding site, joined directly to aa 294-506 of αcatenin, which includes the αactinin binding site identified in the yeast two-hybrid assay. The chimeric fusion protein localized to sites of cell-cell contact (Fig. 2F). In addition, it was able to recruit αactinin (Fig. 2E) to the plasma membrane. Arrows in Fig. 2E and F point out regions where the fusion protein and αactinin co-localize. For a control, we transfected A431D cells with a truncated E-cadherin construct that included the extracellular domain and the transmembrane domain, but did not include the cytoplasmic

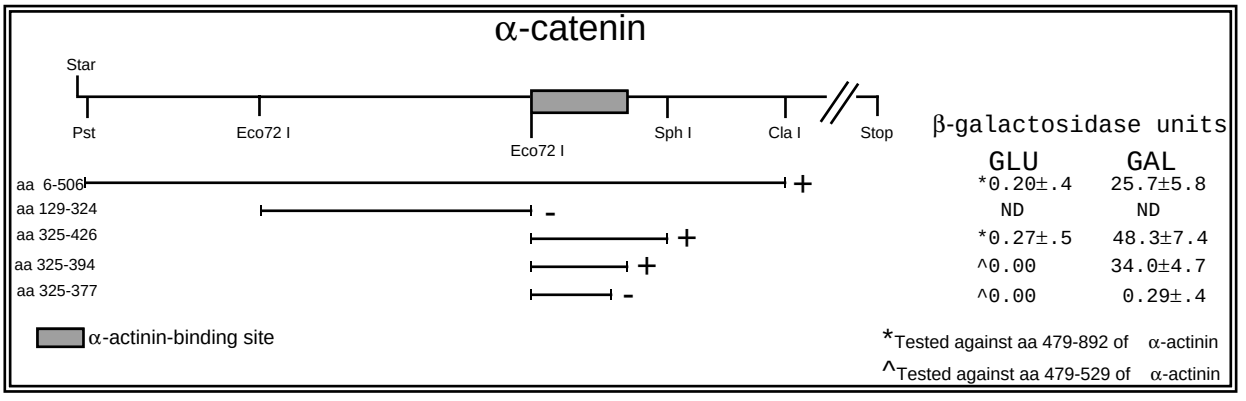


Fig. 1. Identification of the αactinin-binding site in αcatenin. The indicated restriction fragments of αcatenin were used to create DBD fusions and tested against an AD/αactinin fusion with aa 479-892 or 479-529. The *Eco*72I to *Sph*I fragment of αcatenin encodes aa 325-426. Subsequently, exonuclease III deletions narrowed the region to aa 325-394. + indicates galactose inducible growth on plates lacking leucine as well as galactose inducible βgalactose activity in the filter assay. Units of βgalactose activity (± standard deviation) in liquid cultures grown in medium containing glucose (glu) or galactose (gal) are given in the table inset. ND, not done.

domain and thus was not able to bind the catenins. This control construct was not able to recruit αactinin to regions of cell-cell contact. Fig. 2G and H show the localization of αactinin and truncated E-cadherin, respectively, in the control cells. Arrows point out regions of cell-cell contact. In Fig. 3, extracts of the transfected cells were immunoblotted with antibodies against E-cadherin. The chimeric E-cadherin/αcatenin molecule migrated at approximately 125 kDa (lane 1), the E-cadherin extracellular domain in the control cells migrated at approximately 90 kDa (lane 2), and full length E-cadherin migrated at 120 kDa (lane 4). The A431D cell extract did not express any E-cadherin (lane 3). The light band at 110 kDa in lane 2 is probably the unprocessed precursor of the chimeric protein.

Since αactinin is very abundant in A431D cells we felt that co-localization was not sufficient to convince us of an in vivo interaction between the transfected fusion protein and the endogenous αactinin. Therefore we performed an antibody patching experiment to further substantiate the interaction. A431D cells transfected with either the chimeric E-cadherin/α catenin molecule or the truncated E-cadherin construct were treated with polyclonal antibodies directed against the extra-

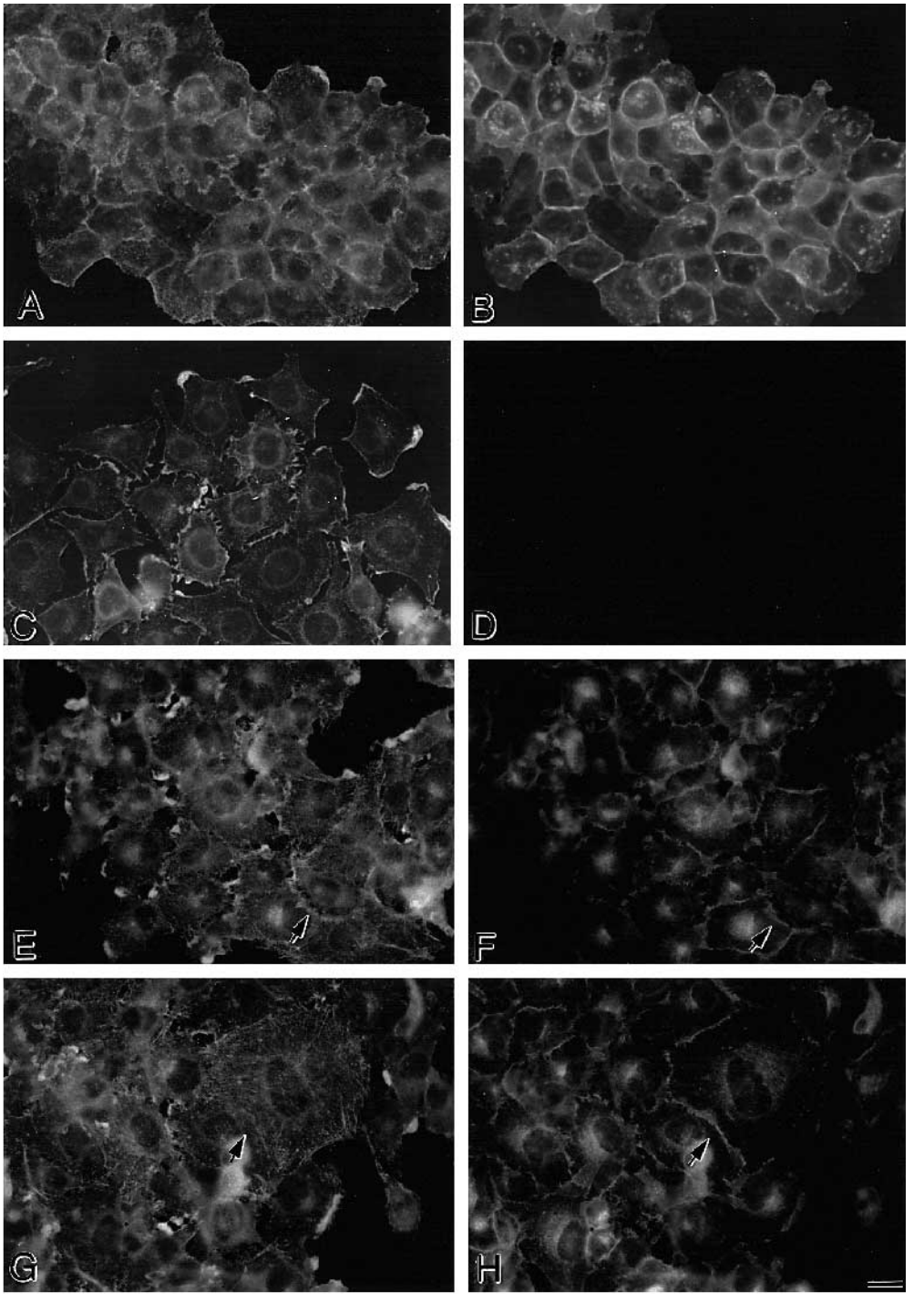


Fig. 2. Co-localization of E-cadherin and αactinin in transfected cells. A431 cells and A431D cells were processed for immunofluorescence with mouse antibodies against αactinin and rabbit antibodies against E-cadherin. (A and B) The localization of αactinin and E-cadherin, respectively, in A431 cells. Note the co-localization of these two molecules at cell-cell borders. (C and D) The localization of αactinin and E-cadherin, respectively, in A431D cells which have limited cell-cell contact due to a loss of E-cadherin. A chimera between E-cadherin (aa 1-792) and α catenin (aa 294-506) was transfected into A431D cells. (F) The localization of the chimera using rabbit anti-E-cadherin and (E) the co-localization of αactinin using monoclonal antibody mAb1682. As a control a truncated E-cadherin molecule that contained the extracellular and transmembrane domains (aa 1-737) was transfected into A431D cells. (H) The localization of E-cadherin and (G) the localization of αactinin. Arrows in E-H point out cell borders. Bar, 30 μm.

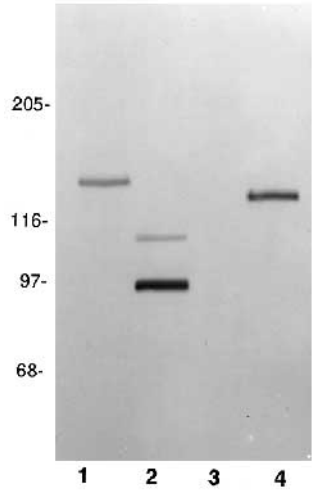


Fig. 3. Expression of transfected proteins. Extracts of A431 cells (lane 4), A431D cells (lane 3), A431D cells transfected with the chimeric E-cadherin/αcatenin molecule (lane 1), and A431 cells transfected with the truncated E-cadherin molecule (lane 2) were resolved by SDS-PAGE and immunoblotted with monoclonal antibody E-9 against E-cadherin. Molecular mass markers are indicated. The light band at 110 kDa in lane 2 is most likely the unprocessed precursor of the chimeric protein.

cellular domain of E-cadherin. When living cells expressing the chimeric E-cadherin/αcatenin construct were treated with antibody, the chimeric molecule was redistributed to patches on the top surfaces of the cells (see Fig. 4B). αActinin co-localized in the patches along with the chimeric molecule (Fig. 4A). Fig. 4E and F show a magnified portion of Fig. 4A and B, respectively, to more clearly point out the co-localization of the chimeric E-cadherin/αcatenin protein and αactinin (note the arrows). When the control cells expressing the extracellular and transmembrane domains of E-cadherin without the cytoplasmic domain were treated with polyclonal antibodies against E-cadherin, the transfected protein was not able to efficiently patch (Fig. 4D). This was most likely due to the fact that the truncated cadherin was not connected to the actin cytoskeleton. However, a few regions of cadherin redistribution could be seen, and these regions did not contain αactinin (Fig. 4C). When these figures were enlarged (Fig. 4G and H), it was clear that αactinin did not redistribute with the truncated cadherin molecule (arrows). To more directly demonstrate an association between αactinin and the cadherin/αcatenin fusion protein we prepared NP-40 extracts of the cells, immunoprecipitated the fusion protein with anti-E-cadherin antibodies, resolved the immunoprecipitation reaction on SDS-PAGE and immunoblotted the reaction products with antibodies against α actinin. Fig. 5 shows that αactinin co-immunoprecipitated with the E-cadherin/αcatenin fusion protein (lane 1) but did not co-immunoprecipitate with the truncated E-cadherin (lane 2). Thus, the results with the A431D cells support the results of the yeast two-hybrid system and indicate a direct interaction between the αactinin binding domain of αcatenin and full-length αactinin.

Identification of a domain on αactinin that interacts with αcatenin

We sought to identify a domain on αactinin that would interact with αcatenin in two-hybrid experiments. Several αactinin constructions in pJG4-5 were tested for binding to a DBD fusion containing aa 6-506 of αcatenin and selected results are presented in Fig. 6. These experiments identified aa 479-529 of αactinin as sufficient to interact with αcatenin. Additional experiments showed that aa 479-529 of αactinin interacted with aa 325-394 of αcatenin (i.e. the αactinin binding site).

Rimm et al. (1995) have reported that both an amino (aa 1-228) and a carboxyl (aa 461-907) terminal fragment of human α(E)-catenin bind actin filaments in vitro. Neither of these fragments of α(E)-catenin contained the αactinin interaction domain identified in our two-hybrid experiments. We tested for interactions between αcatenin and βactin using two-hybrid assays and found no consistent evidence for an interaction (data not shown). However, these results are consistent with the data presented by Rimm et al. (1995) and indicate that filamentous, rather than monomeric, actin is required for αcatenin binding.

Identification of a domain on αcatenin that interacts with βcatenin and plakoglobin

Previous studies from our laboratory and others have identified the domain on plakoglobin and βcatenin that interacts with α catenin as residing in the amino-terminal region near the first armadillo repeat (Aberle et al., 1994; Hülsken et al., 1994; Ozawa et al., 1995; Sacco et al., 1995; Wahl et al., 1996; Aberle et al., 1996). Previous results from Jou et al. (1995) indicated the amino-terminal 606 aa of αcatenin would associate with β catenin. To more carefully determine which domain(s) on α catenin was involved, we employed the yeast two-hybrid system and in vitro binding assays.

Restriction fragments encoding amino acids (aa) 13-551 and 13-196 of βcatenin and aa 1-578 and 1-232 of plakoglobin were inserted into pJG4-5. Each of the βcatenin and plakoglobin AD fusion proteins included the domain that has been shown to interact with αcatenin (Hülsken et al., 1994; Aberle et al., 1994; Sacco et al., 1995). Fig. 7 presents representative results with plakoglobin. Based on amino- and carboxyl-terminal deletions, we identified the region from aa 97-148 of αcatenin as necessary for interaction with plakoglobin. When the same αcatenin constructs were tested against AD/βcatenin fusions that included the αcatenin interaction site (aa 13-551 or 13-196; Hülsken et al. 1994; Jou et al., 1995), we obtained identical results (data not shown). This indicates that the site of interaction between αcatenin and βcatenin is similar to that between αcatenin and plakoglobin.

An in vitro protein-protein binding assay was used to confirm the yeast-two hybrid data. Fragments of αcatenin encoding aa 6-148, 6-135, 97-282 or 129-324 were expressed in bacteria as MBP fusion proteins. Each fusion protein was tested for its ability to interact with βcatenin in extracts from SW707 cells by mixing the cell extract with the fusion protein immobilized on amylose resin. SW707 cells were chosen for these experiments because they express high levels of soluble βcatenin that is not complexed with αcatenin (M. J. Wheelock, unpublished). Fig. 8 presents the analysis of each precipitate immunoblotted for both βcatenin and the MBP/αcatenin fusion proteins. The nitrocellulose paper was cut horizontally between the 97 kDa and 68 kDa markers; the top half was immunoblotted with a monoclonal antibody against βcatenin, whereas the bottom half was blotted with a monoclonal antibody that rec-

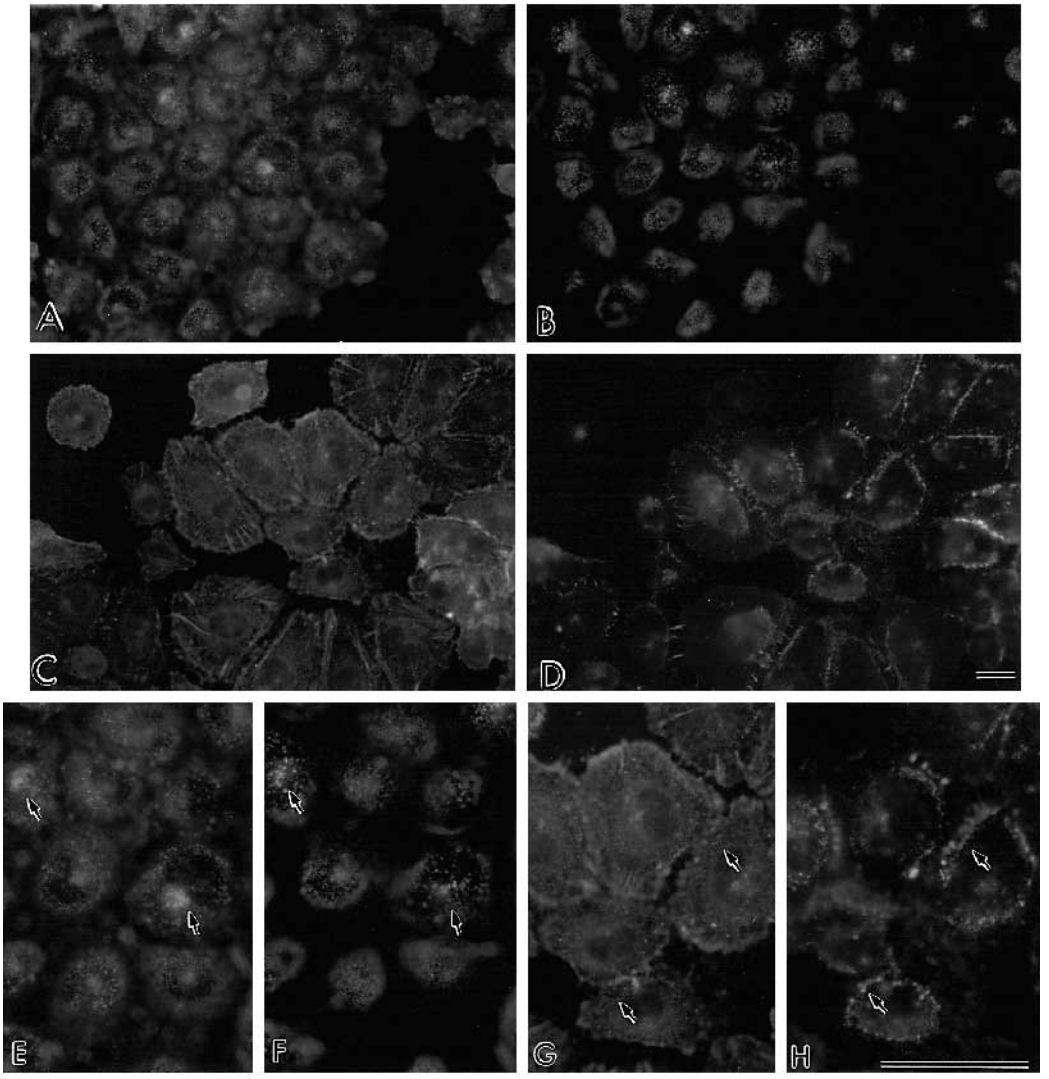


Fig. 4. Co-patching of E-cadherin and αactinin in transfected cells. The transfected cells described in Fig. 2 were incubated with polyclonal antiserum against the extracellular domain of E-cadherin to 'patch' the cadherin molecule. (A and B) The localization of E-cadherin (B) and αactinin (A) after the antibody treatment in the cells transfected with the chimeric E-cadherin/α catenin molecule. (E and F) An enlargement of a selected area of A and B. Prominent areas where E-cadherin (F) and αactinin (E) are co-localized are indicated by arrows. (C and D) The localization of E-cadherin (D) and αactinin (C) after patching in the cells transfected with the truncated E-cadherin molecule. The truncated E-cadherin does not patch efficiently, but regions that are rich in E-cadherin do not show co-localization of αactinin. When regions of C and D were enlarged (G and H), the lack of co-localization was evident and is indicated by arrows. Bar, 30 μm.

ognized the fusion protein. We found that βcatenin bound to the MBP fusion protein that included aa 6-148 of αcatenin (lane 2) and to the one that included aa 97-282 (lane 4) but did not bind to the MBP fusion protein that included aa 6-135 (lane 1) nor to the one that included aa 129-324 (lane 3). These results are consistent with the yeast two-hybrid data.

DISCUSSION

The proteins that make up the junctional complexes of cells have been implicated in a number of cellular events in addition to their more obvious structural role. An important aspect to understanding how these complex structures function lies in understanding how they are assembled. Previous studies from several laboratories have mapped the domains on the cadherins that interact with βcatenin and plakoglobin and the domains on βcatenin and plakoglobin that interact with the cadherin and α catenin (reviewed by Wheelock et al., 1996). In this study we have examined the interactions of αcatenin with αactinin, β catenin and plakoglobin. Our results are summarized as a model depicted in Fig. 9.

Previously we showed that αactinin co-localizes with and

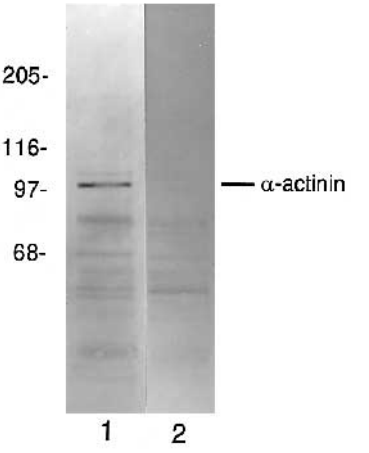


Fig. 5. Co-immunoprecipitation of E-cadherin and αactinin in transfected cells. Extracts of A431D cells transfected with the chimeric E-cadherin/αcatenin molecule (lane 1), or A431 cells transfected with the truncated E-cadherin molecule (lane 2) were immunoprecipitated with antibodies against E-cadherin. The immunoprecipitation reactions were resolved by SDS-PAGE and immunoblotted with monoclonal antibody BM75.2 against αactinin. Molecular mass markers are indicated.

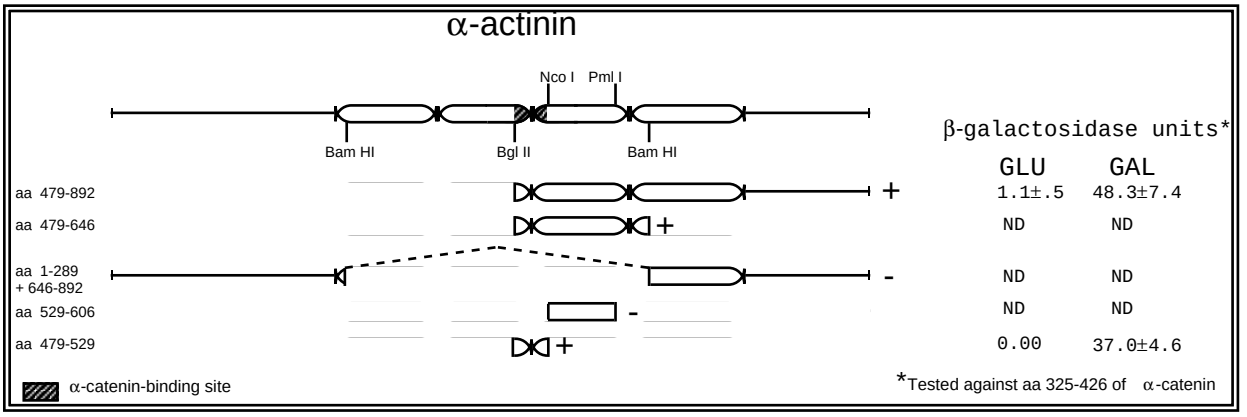


Fig. 6. Identification of the αcatenin-binding site in αactinin. AD/αactinin fusions were created using the indicated restriction fragments and internal deletions. Each was tested against a DBD/αcatenin fusion containing aa 325-426 of αcatenin. The smallest fragment of αactinin to remain positive was *Bgl*II to *Nco*I (aa 479-529). ND, not done.

interacts with the cadherin/catenin complex in cells and suggested that this occurred via a direct interaction between α actinin and αcatenin (Knudsen et al., 1995). In studies reported here we used the yeast two-hybrid system to demonstrate a direct interaction between the two proteins and to identify the region of each molecule involved. Our results indicate that aa 325-394 of αcatenin and aa 479-529 of αactinin are sufficient for interaction of the two proteins. When a portion of αcatenin that included the αactinin binding site was fused to a carboxyl-terminally truncated E-cadherin, the chimeric molecule was able to recruit αactinin to the membrane of transfected cells. These observations are consistent with our finding that αactinin does not associate with αcatenin in cells unless αcatenin is associated with βcatenin and cadherin (Knudsen et al., 1995). We postulate that conformational changes induced by protein-protein interactions in vivo expose the active binding sites of α catenin and αactinin. This model is analogous to the binding of F-actin to vinculin in which the F-actin binding site on vinculin is masked by self-association (Johnson and Craig, 1995a). These authors proposed that the association of vinculin with junctional proteins reveals the F-actin binding site. Such regulatory mechanisms may have important roles in controlling the assembly of the adherens junction and its attachment to the cytoskeleton. This may be especially important during embryogenesis and wound healing when old cell-cell contacts are broken and new ones are formed. It has also been reported that acidic phospholipids bind near the carboxyl terminus of vinculin exposing the actin-binding site (Johnson and Craig, 1995b; Weekes et al., 1996). Since αcatenin and vinculin share regions of homology, it will be interesting to determine if any of the regulatory mechanisms involving vinculin have counterparts in αcatenin.

αActinin is known to form anti-parallel dimers (Flood et al., 1995; Wallraff et al., 1986); our data showing an interaction between small regions of αcatenin and αactinin suggest that one molecule of αcatenin may interact with one member of the αactinin dimer. Thus, it is possible that the interaction of two molecules of αcatenin, each present in a distinct cadherin/catenin complex, with two αactinin molecules present in a dimer may contribute to the formation and stabil-ization of adherens junctions. The adherens junction may then be further stabilized by vinculin binding to αactinin and actin.

Our data indicate that sequences at one spectrin repeat boundary of αactinin are involved in its interaction with α catenin. The proteins of the spectrin family are elongated with varying numbers of spectrin repeats; αactinin has four such repeats. The crystal structure of repeat 14 of *Drosophila* α spectrin has been determined; it consists of a three-helix bundle (helices A, B and C from amino- to carboxyl terminus) with

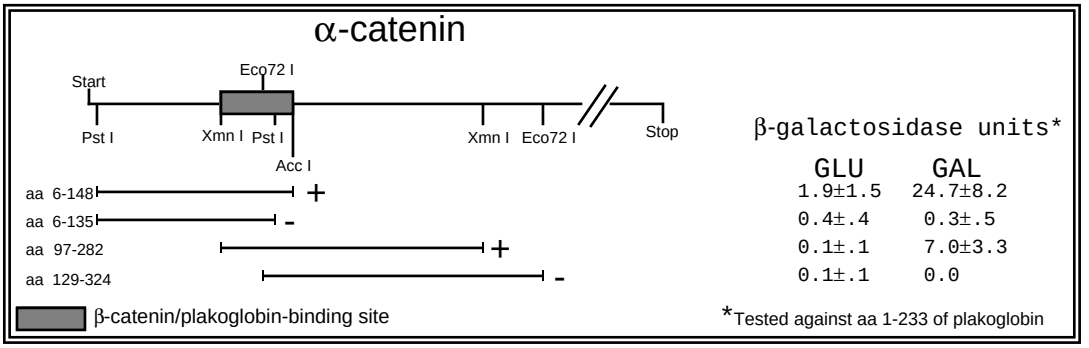


Fig. 7. Identification of the βcatenin/plakoglobin-binding site in αcatenin. Selected restriction fragments were used to create DBD/αcatenin fusions. These were tested against two AD/plakoglobin fusions and two AD/βcatenin fusions with identical results. Based upon the amino- and carboxyl-terminal deletions, aa 97 to 148 are necessary for interaction with plakoglobin or βcatenin.

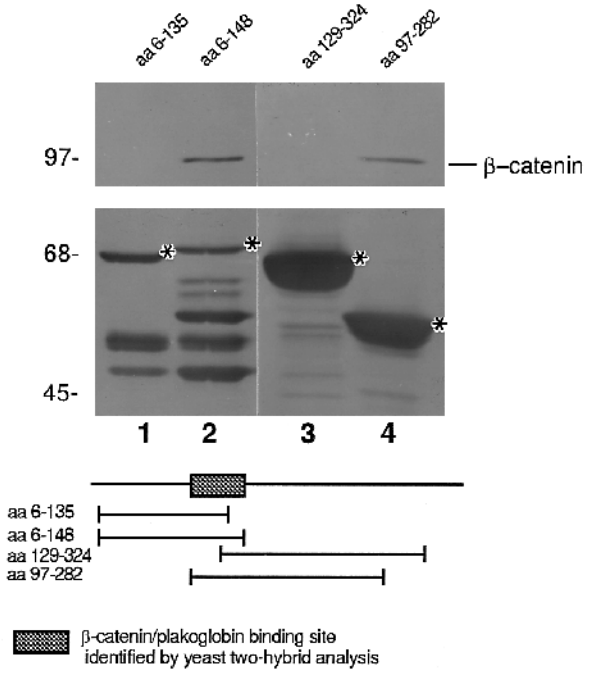
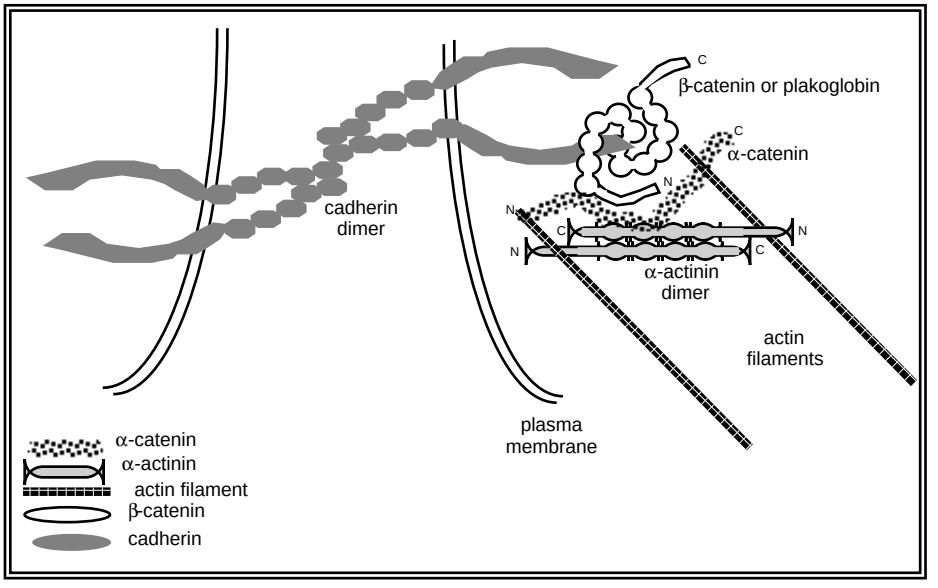


Fig. 8. In vitro analysis of protein-protein interactions between αcatenin and βcatenin. Amylose resin was incubated with extracts containing MBP/αcatenin fusions of aa 6-135, or 6-148, 129-324, or 97-282, washed, and then incubated with a soluble extract of SW707 cells. The proteins bound to the resin were separated by SDS-PAGE, transferred to nitrocellulose and incubated with antibodies specific for βcatenin (top portion) or the MBP/αcatenin fusion protein (bottom portion). βCatenin bound to fusion proteins including aa 6-148 (lane 2; 69 kDa) and 97-282 (lane 4; 55 kDa) but not to fusion proteins including aa 6-135 (lane 1; 68 kDa) or 129-324 (lane 3; 66 kDa). Asterisks indicate the fusion proteins in the bottom part of the figure. Bands that migrate below these bands are breakdown products that are recognized by the anti-maltose-binding protein antibody. A diagram of the constructs and the βcatenin/plakoglobin-binding site on αcatenin are shown below the gel. Molecular mass markers (kDa) are indicated on the left.

flexible segments between the αhelices (Yan et al., 1993). Hydrophobic interactions were predicted to stabilize interactions between adjacent repeats. However, the repeats in α actinin are longer than those of spectrin (114-125 aa compared to 106 aa) implying that additional short sequence elements exist at the amino-termini of the αactinin repeats (Gilmore et al., 1994). When spectrin repeats are precisely expressed in *E. coli*, they are protease resistant, suggesting they fold to form compact domains. However, recombinant proteins containing incomplete repeats are not expected to form stable folded molecules (Winograd et al., 1991; Gilmore et al., 1994; Kahana et al., 1994). Based upon the crystal structure, the portion of α actinin identified in our study (aa 479-529) includes 2/3 of helix C of repeat 2 (including Gly487), the extra segment present in αactinin repeats, and 3/4 of helix A of repeat 3 (including Pro520 and Gly527). Since this fragment of αactinin does not include a whole repeat, spans a repeat boundary and includes several potential helix destabilizing residues, it is unlikely that the helices fold into native conformations. Another construct that showed positive interaction with α catenin in the two-hybrid assay included all of repeats 3 and 4 plus flanking sequences (aa 479-892, see Fig. 6). From other studies (Winograd et al., 1991; Gilmore et al., 1994; Kahana et al., 1994; Menhart et al., 1996), we would predict that the helices of repeat 3 and 4 in this longer recombinant protein folded properly. Taken together, these data raise the possibility that the helices of repeats 2 and 3 may not be involved in direct interaction with αcatenin but that the additional sequence elements at the amino terminus of repeat 3 may play a role in the interaction.

The interaction between vinculin and αactinin has been characterized. Using blot overlay procedures, an αactinin binding site on residues 1-107 of vinculin has been identified (Kroemker et al., 1994). Although the amino-termini of vinculin and αcatenin are weakly similar, the αactinin binding site on αcatenin does not map to its amino terminus. In addition, blot overlay techniques have identified a vinculin

Fig. 9. Model of the interactions between the cadherins, catenins and the cytoskeleton. This model is based on the data presented in this manuscript along with data presented by others. The cadherin is presented as a dimer based on the work of Shapiro et al. (1995) and Overduin et al. (1995). Cadherin extracellular interactions are based on the work of Nagar et al. (1996). The interactions between cadherin and β catenin/plakoglobin are based on our work (this manuscript; Sacco et al., 1995; Wahl et al., 1996) as well as that of others (Nagafuchi and Takeichi, 1988; Ozawa et al., 1990; Stappert and Kemler, 1994; Aberle et al., 1994, 1996; Hülsken et al., 1994; Rubinfeld et al., 1995; Jou et al., 1995). The interactions between αcatenin and αactinin/actin filaments are based on the data presented in this paper and that presented by Knudsen et al. (1995) and Rimm et al. (1995).



binding site at the carboxyl terminus of spectrin repeat 4 of α actinin (McGregor et al., 1994). We did not find that this region of αactinin was necessary for its interaction with αcatenin. The lack of correspondence between these binding sites shows that questions remain concerning the significance of the homology between αcatenin and vinculin.

Two different types of αcatenin have been described; α(E)-catenin is present in a wide variety of cells and α(N)-catenin is found in neural tissues (Hirano et al., 1992). The β catenin/plakoglobin binding site identified in chicken α(E)-catenin with the two-hybrid assay is 88% identical to the corresponding region of chicken α(N)-catenin while the αactinin binding site is 89% identical. Thus, it is likely that α(N)-catenin interacts with βcatenin/plakoglobin and αactinin in a manner similar to that of α(E)-catenin. Each of the different αcatenins is alternatively spliced near its carboxyl terminus (Rimm et al., 1994; Claverie et al., 1993). The regions of α catenin involved in binding αactinin and βcatenin/plakoglobin do not include the alternative splice site. In addition, the site we have identified on αactinin is not involved in alternative splicing (Arimura et al., 1988; Waites et al., 1992) suggesting that the interactions between these proteins are not limited to particular isoforms.

In addition to the interactions between αcatenin and α actinin, it is likely that other interactions help to anchor the cadherin/catenin complex to the actin cytoskeleton. Rimm et al. (1995) found two domains, aa 1-228 and 461-907, of human α(E)-catenin (neither of which contain the αactinin binding site) that bind actin filaments in vitro. Nagafuchi et al. (1994) constructed chimeras between E-cadherin and the amino (aa 1-508) or carboxyl (aa 509-906) terminal portion of mouse α(E)-catenin. These authors found that the carboxyl-terminal chimera, which may include the more carboxyl-terminal actin binding site, provided full adhesive activity in their assays. In contrast, the amino-terminal chimera, which included actin, α actinin, and βcatenin/plakoglobin binding sites, interacted with the cytoskeleton in an in vitro assay but did not produce full adhesive activity by the cells. At first glance one might conclude from the data of Nagafuchi et al. (1994) that the carboxyl-terminal actin binding site is more important for interaction with the cytoskeleton. However, the adhesion mediated by the E-cadherin/carboxyl αcatenin fusion protein appeared to be *more* stable than adhesion mediated by a normal cadherin/βcatenin/αcatenin complex. It is possible that the α actinin binding domain provides a more flexible (and thus more regulated) interaction with the cytoskeleton. It also is possible that the αactinin binding domain in the E-cadherin/amino α catenin fusion protein was not active. This possibility is supported by the observation that, although the fusion protein contained the site for binding βcatenin, it failed to recruit β catenin to the membrane in cells (Nagafuchi et al., 1994). These authors did not examine αactinin localization. More studies will be needed to unravel the biological significance of the myriad protein-protein interactions in the adherens junction and the contribution of the αcatenin/αactinin interaction to cadherin-mediated cell-cell adhesion.

The authors thank Drs W. W. Franke, D. J. Kwiatkowski, R. Brent, B. Cohen, and M. Herlyn for reagents. We acknowledge the excellent technical assistance of James K. Wahl III and Tammy Sadler both from the University of Toledo. This work was supported by NIH GM51188 and by grants from the Ohio Chapters of The American Cancer Society and The American Heart Association and by the Ohio Board of Regents to M.J.W. and K.R.J.

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(Received 6 November 1996 - Accepted 6 February 1997)