The Cadherin Cytoplasmic Domain Is Unstructured in the Absence of β-Catenin

A POSSIBLE MECHANISM FOR REGULATING CADHERIN TURNOVER

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Cadherins are single pass transmembrane proteins that mediate Ca\(^{2+}\)-dependent homophilic cell-cell adhesion by linking the cytoskeletons of adjacent cells. In adherens junctions, the cytoplasmic domain of cadherins bind to β-catenin, which in turn binds to the actin-associated protein α-catenin. The physical properties of the E-cadherin cytoplasmic domain and its interactions with β-catenin have been investigated. Proteolytic sensitivity, tryptophan fluorescence, circular dichroism, and \(^1\)H NMR measurements indicate that murine E-cadherin cytoplasmic domain is unstructured. Upon binding to β-catenin, the domain becomes resistant to proteolysis, suggesting that it structures upon binding. Cadherin-β-catenin complex stability is modestly dependent on ionic strength, indicating that, contrary to previous proposals, the interaction is not dominated by electrostatics. Comparison of 18 cadherin sequences indicates that their cytoplasmic domains are unlikely to be structured in isolation. This analysis also reveals the presence of PEST sequences, motifs associated with ubiquitin/proteosome degradation, that overlap the previously identified β-catenin-binding site. It is proposed that binding of cadherins to β-catenin prevents recognition of degradation signals that are exposed in the unstructured cadherin cytoplasmic domain, favoring a cell surface population of catenin-bound cadherins capable of participating in cell adhesion.

The formation and maintenance of solid tissues depends upon specific and regulated intercellular adhesion (1). Cadherins are single pass transmembrane adhesion proteins that link the cytoskeletons of adjacent cells in two kinds of intercellular junctions: the adherens junction and the desmosome. These structures play a critical role in tissue development, including cell segregation, condensation, polarization, and differentiation. Cadherin-mediated linkage of cytoskeletal networks imparts resistance to mechanical stress and enables concerted motions required by morphogenic processes. Defects in cadherin-mediated adhesion are associated with several characteristics of malignant transformation, such as dedifferentiation, high mobility, and invasive growth (2, 3).

Adherens junctions are sites of cell-cell contact that link the actin cytoskeletons of adjacent cells (4). Cadherin extracellular domains on opposing membranes mediate specific Ca\(^{2+}\)-dependent, homotypic interactions. The cytoplasmic domains bind to β-catenin, which in turn binds to the actin-associated protein α-catenin (4, 5). An analogous adhesion system exists in Drosophila, with armadillo and DE-cadherin the orthologues of β-catenin and E-cadherin, respectively (6). Deletion mutagenesis studies have mapped the regions of E-cadherin and β-catenin required for association. The C-terminal 72 residues of E-cadherin are necessary and sufficient for β-catenin binding (7), and a 30-amin acid stretch within this region has been proposed to be the “core” β-catenin binding sequence (8).

The primary structure of β-catenin consists of an N-terminal region of 140 amino acids, followed by a 524-residue domain that contains 12 repeats of 42 amino acids known as armadillo (arm) repeats (9) and a 119-residue C-terminal tail. The arm repeat domain is required for association with cadherins (10, 11). The three-dimensional structure of the arm repeat domain showed that each arm repeat comprises three helices, with the repeats packing to form a superhelix of helices (12). The superhelix features a shallow groove with a positively charged surface potential. The core β-catenin-binding region of E-cadherin, which has a calculated pI of 3.3, was proposed to bind within the positively charged groove presented by the β-catenin armadillo domain (12).

Several mechanisms appear to modulate cadherin-based adhesion. Cadherins associate with β-catenin shortly after biosynthesis, while still in the endoplasmic reticulum, and the two proteins move together to the cell surface, where they associate with α-catenin (13). Failure of cadherins to associate with β-catenin leads to retention in the endoplasmic reticulum and degradation of cadherin (14). Adherens junction formation is also affected by phosphorylation (15, 16). For example, phosphorylation of serines in the the cadherin cytoplasmic tail by casein kinase II and glycogen synthase kinase-3 kinases increases the affinity of cadherin for β-catenin (16). Moreover, adherens junctions are enriched in protein-tyrosine kinases and phosphatases, some of which bind the cadherin-catenin complex directly (17–20). Tyrosine kinases target several adherens junction components that could modulate junctional stability, including β-catenin (21) and the arm repeat protein p120ctn (22–24), which binds the cadherin cytoplasmic domain at a site distinct from β-catenin (25, 26).

β-Catenin also plays a central role in the Wnt/Wg growth
factor signaling pathway that controls cell fate determination during embryogenesis (reviewed in Ref. 27). In this role β-catenin acts as a transcriptional coactivator when bound to members of the lymphoid enhancer factor/T-cell factor (LeF/Tcf) transcription factor family (28, 29). Wnt signaling activates transcription by blocking or slowing the normally rapid turnover of β-catenin, thereby elevating cytosolic levels of β-catenin and promoting formation of an active β-catenin-transcription factor complex (27). The cytosolic concentration of β-catenin is normally maintained below the signaling threshold by a multicomponent complex that targets β-catenin for ubiquitination and proteasomal degradation. This protein complex contains APC, the product of the adenomatous polyposis coli gene, the serine/threonine kinase GSK3β, and Axin. The β-catenin binding sequences in LEF-1 and APC are largely electronegative, and by polymerase chain reaction from a sequence encoding the DE-cadherin cytoplasmic domain was amplified and the resulting overhangs were filled with DNA polymerase I large depending on which ligand is bound, 3 h after induction, and the cell paste was stored at 70 °C. The β-catenin fusion proteins were batch affinity purified from lysates with glutathione-agarose beads (Sigma). GST–β-catenin fusion protein was obtained by eluting the protein with a buffer containing 50 mM reduced glutathione. E- and DE-cadherin cytoplasmic domains and β-catenin were obtained by cleaving the glutathione-agarose-bound fusion proteins with bovine thrombin (Sigma). Thrombin can cleave β-catenin internally at Arg59 or Arg60. Thus, digestion of the GST-β-catenin fusion yields full-length β-catenin and two other major species. These fragments, which differ only at their N termini, copurify and are collectively named β76. Anion exchange and size exclusion chromatography were used to purify all protein products to near homogeneity.

Cadherin–β-Catenin Stoichiometry Experiments—Mixtures of rEcat or rDEc and various β-catenin constructs were incubated for more than 1 h at 4 °C and injected onto an Amersham Pharmacia Biotech HR 10/30 Superdex 200 size exclusion column equilibrated with 50 mM Tris-HCl, pH 7.8, 200 mM NaCl, 20 mM EDTA, and 1 mM DTT.

Endogenous versus Recombinant E-Cadherin–β-Catenin Binding Studies—MDCK cells (type II J) were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. Rabbit polyclonal antibody for the E-cadherin cytoplasmic domain was described previously (33). Mouse monoclonal antibodies for E-cadherin and β-catenin were purchased from Transduction Laboratories. Polyclonal anti-E-cadherin and preimmune sera were covalently coupled to Sepharose. In each case, a ratio of 10 μl of serum to 75 ml of a 50% slurry of protein A-Sepharose was incubated for 12 h at 4 °C. The Sepharose was washed with 0.2 M NaCl, pH 9.0, and the antibodies were cross-linked to the protein A with dimethylimelimitide (Pierce).

Cell extracts were prepared by washing 2 × 107 plated MDCK cells with ice-cold Tris-saline (20 mM Tris-HCl, pH 7.5, 154 mM NaCl) and scraping the cells in 2 ml of DTEB (0.5% Nonidet P-40, 50 mM Tris-HCl, pH 8.0, 100 mM NaCl) supplemented with protease and phosphatase inhibitors (0.1 mM Na3VO4, 50 mM NaF, 1 mM Pefabloc (Roche Molecular Biochemicals), and 10 mg/ml each of leupeptin, aprotinin, peptatin-A, chymostatin, and antipain). Insoluble material was removed with a 10-min incubation on ice before centrifugation at 20,800 × g for 15 min. The resulting supernatant was “precleared” by incubating it with immobilized preimmune serum for 45 min at 4 °C, centrifuging it for an additional 5 min at 20,800 × g, and taking the new supernatant.

Precleared extract supernatant was incubated for 2 h at 4 °C with Sepharose-immobilized polyclonal E-cadherin antibody. Postincubation Sepharose was washed once with DTEB and twice with NaCl-supplemented DTEB. All washes were for 10 min at 4 °C and 10 different NaCl concentrations were used. Sepharose beads were resuspended in DTEB, centrifuged, and washed with 1 ml of DTEB and boiled for 5 min in SDS sample buffer. After separation on 6.5% SDS-PAGE gels, immunoprecipitated proteins were transferred to Immobilon-P polyvinylidene fluoride membrane (Millipore Corp.) and detected with murine monoclonal primary antibodies specific for E-cadherin and β-catenin and 0.1 μCi/ml 125I-labeled goat anti-mouse secondary antibody (ICN Pharmaceuticals, Irvine, CA). Immunoblots were exposed to x-ray film (X-Omat AR, Eastman Kodak Co.) and quantified using a Molecular Dynamics Storm 820 PhosphorImager system.

To compare the behavior of recombinant β-catenin-E-cadherin complex with that of endogenous complex, 1:1 stoichiometric mixtures of the recombinant proteins were incubated on ice for 16 h and mock cleaved as described above. Immunoprecipitations and washes were carried out as described above. Immunoprecipitates were separated on 12% SDS-PAGE gels and processed for immunoblotting and quantitation as described above.

Tryptophan Fluorescence and Fluorescence Anisotropy—Tryptophan fluorescence emission and anisotropy experiments utilized an SLM 8000 C spectrophluorometer, featuring a T-format optical pathway and double and single grating excitation and emission monochromators, respectively. Data were taken at room temperature from continuously stirred samples. A 290 nm (8 nm bandpass) excitation wavelength was used for all experiments. Fluorescence emission spectra were recorded from 300 to 420 nm using a 4-nm emission bandpass and a 2.0-nm wavelength increment with a 1-s integration period. Tryptophan (12 mM), β76 (1 mM), and E- and DE-cadherin cytoplasmic tail (12 mM) samples were prepared using 10 μg protein/ml and were excited with Tris-HCl or HEPES, pH 7.0–8.5, 20 mM NaCl with 1 mM DTT or 10 μM merocyaanine present for β76 and DE-cadherin. E-cadherin emission spectra were also measured under denaturing conditions (100 mM Tris-HCl, pH 7.0, 6 mM guanidine HCl). Two-channel anisotropy data were collected using the “single point polarization” option and Schott WG-335 long pass filters instead of emission monochromators. Data

1 The abbreviations used are: rEcat, recombinant cytoplasmic domain of E-cadherin; rDEc, recombinant cytoplasmic domain of DE-cadherin; β76, thrombospondin fragment of β-catenin; β59, armadillo repeat region of β-catenin; GST, glutathione S-transferase; DT, diithiothreitol; BSA, bovine serum albumin; CHES, 2-(cyclohexylamino)ethanesulfonic acid; MDCK, Madin-Darby canine kidney; Al, aliphatic index.
were collected under native conditions (10 mM Tris-HCl, pH 8.5, 20 mM NaCl, 1 mM DTT) for E-cadherin (12 nm) and DE-cadherin (6, 3, or 1.5 nm) cytoplasmic tails, β76 (2 nm), β-catenin (3.5 nm), bovine serum albumin (BSA, 3 nm), and tryptophan (12 or 14 nm). Data were also collected under denaturing conditions (100 mM Tris-HCl, pH 7, 6 M guanidine HCl, and 1 mM DTT) for E- and DE- (6 nm) cadherin cytoplasmic tails, β76, BSA, and tryptophan.

Circular Dichroism—Circular dichroism data were measured using an Aviv 60DS spectropolarimeter equipped with a Peltier temperature control unit (Hewlett-Packard 89100A). The spectropolarimeter was calibrated with (+)-10-camphorsulfonic acid and a 1-mm path length was used for all experiments. Spectra of E- and DE-cadherin cytoplasmic domains were measured in 10 mM phosphate, pH 7, at 0 °C. These spectra were measured in two parts, with 25 and 10 nm samples being used for 280–205- and 220–186-nm wavelength ranges, respectively. The two halves of each spectrum were scaled using the known sample concentrations.

NMR Measurements—NMR spectra were acquired using a General Electric GN-Omega instrument operating at 500 MHz and a 680 μM sample of E-cadherin cytoplasmic domain in 5 mM Tris-HCl, pH 8, 10 mM NaCl, 0.35 mM trimethyl silyl propionate, and 50% D2O. Presaturation was used to suppress the H2O peak, and trimethyl silyl propionate was employed as the chemical shift standard. The sample was shimmmed briefly before acquisition of one-dimensional 1H NMR spectra of 256 scans each (4096 real points; spectral width, 7000 Hz) at 50, 25, and 3 °C. NMR data were processed using Felix, version 2.30, from Biosym Technologies (San Diego, CA). The free induction decay was processed conservatively by Fourier transformation without premultiplication. Following phasing, the base lines were corrected by fitting to a zero order polynomial function.

Poly-l-glutamate versus E-Cadherin Binding Competition—GST-β-catenin fusion protein and glutathione-agarose were mixed for 45 min at room temperature in a binding buffer comprising 200 mM Tris-HCl, pH 8.5, 2 mM DTT, and either 100, 200, or 400 mM NaCl. Poly-l-glutamate (P1818 or P4636, with average molecular masses of roughly 1,000 and 11,000 Da, Sigma) was then added, and the mixtures were incubated an additional 45 min. E-cadherin was added, and after an additional 45 min incubation, the agarose beads were spun down, washed three times with the appropriate binding buffer, and boiled in reducing SDS-PAGE sample buffer. The sample supernatants were analyzed by SDS-PAGE. In each case, after the addition of E-cadherin, the incubation mixture was 0.25 μM in GST-β-catenin, 3 μM in E-cadherin cytoplasmic domain, and 0, 3, 9, or 27 μM in poly-l-glutamate. Different E-cadherin and poly-l-glutamate stocks were made using the appropriate binding buffers.

Limited Proteolysis of Cadherin-β-Catenin Complexes—Approximately 25 μM recombinant E-cadherin cytoplasmic domain (rECyt) alone or mixed in 1:1 stoichiometric amounts with either full-length β-catenin or the armadillo repeat region (β59) (12) was subjected to limited proteolysis with subtilisin. A control mixture containing 25 μM rECyt and 30 μM bovine serum albumin was also digested. Mixtures were incubated at 4 °C for 1 h and digested for 20 min at room temperature with subtilisin concentrations ranging from 0.08 to 1.3 μg/ml. Digestion were carried out in a buffered solution comprising 100 mM CHES, pH 9.2, 2 mM CaCl2, and 5 mM DTT. This pH is suboptimal for subtilisin but is required for β59 solubility. In separate experiments, rECyt-β-catenin complex was digested with endoproteinase Glu-C using concentrations ranging from 0.4 to 27 μg/ml; these reactions were carried out in 50–100 mM Tris-HCl, pH 8.5, 2 mM CaCl2, and 5 mM DTT. rDE cyt-β-catenin complex was also digested with subtilisin as described for rECyt. Subtilisin digestions were stopped by adding phenylmethylsulfonyl fluoride to a final concentration of 8 mM, and endoproteinase Glu-C digestions were stopped by boiling.

Sequence Analysis—Cytoskeletal tail sequences from 18 different vertebrate subtypes of classical/type I and atypical/type II cadherins were analyzed. These sequences are mostly of human origin and represent 6 type I cadherins (E-cadherin, N-cadherin, P-cadherin, R-cadherin, M-cadherin, and Xenopus laevis EP-cadherin; GenBank™ accession numbers Z13009, M34064, X63629, L34059, D83542, and U70477, respectively) and 12 type II cadherins (cadherin-5, cadherin-6, cadherin-7, cadherin-8, cadherin-9, cadherin-10, cadherin-11, cadherin-12, cadherin-18, cadherin-19, cadherin-20, and Rattus norvegicus PB-cadherin; GenBank™ accession numbers X79981, D31784, J007611, L34060, AB035302, AF039747, L40456, L40457, U93925, J007607, AF217258, and D38348, respectively) as described in a recent phylogenetic analysis of the cadherin superfamily (34). The PEST-FIND program (35) was used with a sequence window of 10 as implemented at EMNet Austria. Aliphatic and instability indices were calculated using the ProtParam tool at the ExPaSy proteomics server. An “aspartic acid index,” which compares the normalized change in total aspartic acid content relative to total glutamic acid content, is defined as 1.207 (ASP/GLU) where 1.207 is the ratio of the compositional percentage for Glu (numerator) and Asp as found in SWISS-PROT Release 38.0 and %ASP and %GLU represent the compositional percentages found in a given cadherin cytoplasmic tail.

RESULTS

Recombinant Cadherin Cytoplasmic Domains Are Not Folded in Isolation—Fluorescence, circular dichroism, and proton NMR were used to characterize the folded state of recombinant E-cadherin and DE-cadherin cytoplasmic domains (rE cyt and rDE cyt respectively). E cyt contains a single tryptophan that is located near the C terminus of the protein. DE cyt has two tryptophans that are near the N and C termini, the latter in a position distinct from the tryptophan in E cyt. In a folded protein, the tryptophan indole ring is frequently buried within a hydrophobic core or is otherwise shielded from solvent, resulting in a blue shift of the fluorescence maximum relative to free tryptophan (36). The tryptophan emission maximum for β76, a thrombin-generated fragment of β-catenin (see “Experimental Procedures”), is significantly blue-shifted relative to tryptophan alone, whereas the maxima for E cyt and DE cyt are not (Table I). Thus, the single E cyt tryptophan and two DE cyt tryptophans appear to be solvent exposed.

Folded and denatured proteins commonly display significantly different fluorescence anisotropy values because of the loss of rotational freedom that occurs when a tryptophan indole ring is buried in a hydrophobic core. β-Catenin and BSA yield anisotropy values of −0.085 when folded and −0.035 when denatured with 6 M guanidine HCl (Table I). In contrast, recombinant E- and DE-cadherin cytoplasmic domains have anisotropy values comparable with those observed with dena-
tured proteins, and these values do not change significantly when the cadherins are subjected to denaturants (Table I).

The fluorescence anisotropy data suggest that the E cyto and DEcyto domains are unfolded under native conditions. However, regions of a folded protein that lack structure, such as large loops or unstructured N and C termini, might give similar results. CD spectroscopy was therefore used to probe for the presence of a regular protein secondary structure. The spectra measured for rEcyto and rDEcyto at 0 °C are essentially identical and feature a single minimum in mean residue ellipticity at ~202 nm (Fig. 1). This spectrum indicates a lack of secondary structure, which would be expected of an unstructured polypeptide (37, 38).

To eliminate the possibility that the cytoplasmic tail has a defined conformation without regular secondary structure, one-dimensional 1H NMR spectra were measured at 3, 25, and 50 °C (Fig. 2). At 50 °C, peaks with the chemical shift values of random coil peptides (39) are observed at 0.95 ppm (δ and γ protons of Ile, Leu, and Val), 2.10 ppm (ε protons of Met), and 6.81 ppm (Tyr ring protons). Resolved peak doublets at 7.47 and 7.58 ppm are consistent with exposed C7 and C4 Trp protons, as one would find in an unfolded protein. A broad, very low shoulder between 8 and 9 ppm suggests that amide protons are unprotected and in rapid exchange with solvent protons and deuterons. These data indicate that rEcyto is unfolded at 50 °C. Spectra recorded at 25 and 3 °C are similar to that of the 50 °C spectrum (Fig. 2). However, amide proton peaks appear at 25 °C and become more prominent at 3 °C, with a single small alkyl peak becoming resolved at 0.70 ppm. The appearance of amide proton peaks may result from secondary structure formation, or from a lower rate of solvent exchange resulting from the decreased temperature. Because the amide resonances lack the chemical shift dispersion that accompanies secondary structure formation, the latter explanation is more likely. Thus, rEcyto appears to be largely unstructured at 50, 25, and 3 °C.

Recombinant E-Cadherin Cytoplasmic Domain and Endogenous E-Cadherin Have Similar β-Catenin Binding Properties—The β-catenin binding properties of rEcyto were compared with those of E-cadherin isolated from eukaryotic cells. Full-length β-catenin, as well as fragments lacking the first ~90 amino acids (β76; see "Experimental Procedures") or comprising the arm repeats (β59) (12) were used in these experiments. Size exclusion chromatography (Table II) and native gel electrophoresis (data not shown) were used to separate mixtures comprising 2:1, 1.5:1, 1:1, 1:1.5, and 1:2 molar ratios of β76 and rEcyto, appears to be largely unstructured at 50, 25, and 3 °C.

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To compare directly the stability of recombinant and endogenous E-cadherin-β-catenin complexes, we examined the sensitivity of each complex to salt washes, a common method of testing protein-protein interactions in cell extracts. Endogenous E-cadherin-β-catenin complex was immunoprecipitated from MDCK cell extracts with polyclonal anti-E-cadherin antibodies attached to Sepharose beads. Recombinant β-catenin-Ecyto complexes were prepared in parallel and immunoprecipitated using the same buffer conditions. After washing the immunoprecipitates, the beads were incubated with buffers containing increasing concentrations of NaCl. The amount of complex resistant to dissociation was assayed by Western blots of the post-wash bead-associated proteins with cadherin and β-catenin-specific antibodies. The stability of the recombinant complex in NaCl was found to be similar to that observed with the endogenous proteins (Fig. 3). Although the two curves agree within experimental error, the recombinant complex was consistently slightly less stable than the endogenous complex.

It is possible that the increased stability of the endogenous complex is the result of post-translational modifications, such as phosphorylation (16).

Electrostatic Complementarity Is Not a Dominant Factor in E-Cadherin-β-Catenin Complex Stability—The positively charged groove presented by β-catenin and the overall negative charge of the β-catenin-binding regions of E-cadherin, LEF-1, and APC suggests that electrostatic complementarity has a major role in the interaction of these proteins with β-catenin. As a simple test of this hypothesis, we examined the salt dependence of the interaction. As described above (Fig. 3), increasing NaCl concentrations reduce the stability of the E-cadherin-β-catenin complex. However, roughly 50% of the cadherin-β-catenin complex remains in NaCl concentrations as high as 2.5 M, suggesting that electrostatics make only a modest contribution to complex stability. To further gauge the contribution of electrostatics in complex formation, we tested whether the poly-L-glutamate polyanion P1818 (1000 Da; average length, 10 residues) could act as competitive inhibitor of rEcyto-β-catenin complex formation. P1818 is a weak competi-

Fig. 3. The stability of recombinant and endogenous E-cadherin-β-catenin complexes as a function of NaCl concentration. Recombinant (Rec and RECOMB) Ecyto-β-catenin and endogenous E-cadherin-β-catenin complexes from MDCK cell extracts were immunoprecipitated using an immobilized anti-E-cadherin antibody. Samples were washed with buffers containing different NaCl concentrations and boiled, and the proteins were separated by SDS-PAGE. β-catenin and E-cadherin were detected by Western blotting with 125I-labeled antibody and quantitated using a phosphorimaging system. Each plotted data point is the mean β-catenin-E-cadherin ratio from four experiments. The S.D. for each point is displayed as a bar. Cad, cadherin; β-cat, β-catenin.


**Cadherin Cytoplasmic Domain**

**FIG. 4.** E-cadherin-β-catenin complex formation in the presence of poly-L-glutamate. GST-β-catenin fusion protein was bound to glutathione agarose and incubated with an equimolar amount of E<sub>cyto</sub> in the presence of various amounts of poly-L-glutamate. Postincubation beads were washed with buffer, and the bound proteins were analyzed by SDS-PAGE. The experiment was carried out using three different incubation buffers that differed only in NaCl concentration. The molar excess of poly-L-glutamate and the concentration of NaCl in the incubation buffer are shown above the lanes. The positions of molecular mass standards are marked with arrows on the left side of the figure and labeled in kilodaltons. The positions of GST-β-catenin and E<sub>cyto</sub> bands are labeled on the right.

... of the cadherin-β-catenin complex, it is not a dominant factor in the stability of the complex.

β-Catenin Binding Protects E<sub>cyto</sub> from Proteolysis—The cadherin cytoplasmic domain is unstructured in isolation, but it could become structured or fold upon binding β-catenin. Compact globular domains typically exhibit some resistance to proteolytic degradation, so limited proteolysis is one means of ascertaining structural stability. The relatively nonspecific protease subtilisin and the acid-specific protease endoproteinase Glu-C (V8 protease) both readily digest R<sub>cyto</sub>. However, in the presence of β-catenin, what appears to be full-length R<sub>cyto</sub> remains at subtilisin (Fig. 5) and endoproteinase Glu-C (data not shown) concentrations that completely degrade R<sub>cyto</sub> alone (Fig. 5, lanes 5 and 7 or lanes 12 and 14). Similar protection results were obtained with the DE<sub>cyto</sub>-β-catenin complex and subtilisin (data not shown). β59, the armadillo repeat domain of β-catenin (12), is thought to contain the entire cadherin-binding site. This fragment also protects R<sub>cyto</sub> from degradation but does so less effectively than full-length β-catenin (Fig. 5, lanes 7 and 9 or lanes 14 and 16). BSA+R<sub>cyto</sub> mixtures were used to test whether the protection afforded by full-length β-catenin was simply due to the addition of an alternative substrate for subtilisin. The amount of BSA used in the digests was chosen to provide the same number of peptide bonds as full-length β-catenin. Adding BSA protects R<sub>cyto</sub>, somewhat (Fig. 5, lanes 5, 10, and 11), but the protection is substantially weaker than that afforded by β-catenin and slightly weaker than that provided by β59 (Fig. 5, lanes 9 and 11 or lanes 16 and 18).

**Instability Is Reflected in the Sequences of the Cadherin Cytoplasmic Tails**—A protein that is normally unstructured in solution might be expected to have a higher than average number of charged/polar residues and a lower than average number of hydrophobic residues. Analysis of 18 type I and type II cadherins shows that they contain on average 41% more charged residues (Arg, Asp, Glu, and Lys) and 29% fewer aliphatic residues (Ile, Leu, Met, Phe, and Val) relative to the average amino acid composition of the proteins in the SWISS-PROT data base (Release 38). Given that the average calculated PI for the cytoplasmic tails is 4.4, it is not surprising that most of the increase in charge is from a higher than usual number of aspartic and glutamic acid residues. In most cases the compositional increase in Asp far outweighs that of Glu, as reflected in the “Asp index,” which we define as the normalized change in total aspartic acid content relative to total glutamic acid content (Fig. 6). It is interesting to speculate that this bias is related to the lack of tertiary structure seen in the domain; the side chain of Asp is less hydrophobic than that of Glu, making it less likely to form favorable packing interactions with other residues. The aliphatic index (AI), a measure of hydrophobicity and thermostability (41), was also calculated from these sequences. This is a compositional index based upon the sum of the mole percentages of Ala, Val, Ile, and Leu weighted by the relative side chain volumes. Proteins from thermophilic bacteria have been found to have a significantly higher AI value (mean of 92.6, S.D. of 10.6) than proteins from mesophilic organisms (mean of 78.8, S.D. of 14.5). Thus, a higher AI value is correlated with higher thermostability. The sequences from the cadherin cytoplasmic tails have AI values (mean of 64.4, S.D. of 4.2) significantly below that found for mesophilic proteins.

Cadherins appear to be targeted for degradation when not bound to β-catenin (14). Because the PEST sequence motif is correlated with rapid protein turnover in vivo (35), we used the PEST-FIND program to identify potential PEST sequences within the cadherin cytoplasmic domains. PEST sequences contain Pro, Glu or Asp, and Ser or Thr and are flanked by but do not contain basic residues (His, Arg, and Lys). PEST sequences with PEST-FIND scores greater than +5 are considered the best candidates for being degradation signals, but many proteins containing sequences with lower PEST scores are known to be degraded (35). Of the 18 cadherin cytoplasmic domains analyzed, 15 have positive scoring PEST sequences that contain or overlap the serine-rich, minimal β-catenin-binding region in the C-terminal half of the domain; 12 of these sequences score greater than +3, and 4 have values greater than +5 (Fig. 6). This minimal β-catenin-binding region of cadherin also has two features frequently found in synthetic signals that target proteins to the ubiquitin-proteosome system in *Saccharomyces cerevisiae*: a high content of serine and threonine residues and a frequently recurring sequence motif, (bulky hydrophobic)-(S or T)-(S or T)-(bulky hydrophobic) (42). The sequence motif Leu-Ser-Ser-Leu is very highly conserved within the cadherin cytoplasmic tail and is located within the minimal β-catenin-binding region (Fig. 6). It should also be noted that five of the six type I (E-, N-, R-, P-, and EP-cadherins) and two of the twelve type II (cadherin-8 and cadherin-20) cadherins had positive scoring PEST sequences in the membrane-proximal, N-terminal half of the cytoplasmic domain. All of these membrane-proximal type I cadherin PEST sequences scored greater than +3.5. This region harbors the p120<sup>ctn</sup>-binding site (25, 26).

We calculated the instability index (II), a measure that is based upon a correlation between the stability of a protein in vivo and the frequency of certain dipeptides in its sequence (43), for each of the 18 sequences analyzed above. Index values greater than 40 are indicative of metabolic instability, which is defined as an in vivo half-life of less than 5 h. The average cadherin cytoplasmic tail II value was 56.6 (Fig. 6), and only the cadherin-8 cytoplasmic tail was predicted to be stable.

**DISCUSSION**

The cytoplasmic tail is the most highly conserved domain among type I cadherins (34). With a length of ~150 residues, it...
Easily enough to be an independently folded structural unit. We expressed the E- and DE-cadherin cytoplasmic do-
 mains in E. coli and purified them to homogeneity. Tryptophan
fluorescence, circular dichroism, and one-dimensional proton
NMR studies all lead to the same surprising conclusion: rEcyto
and rDEcyto are unfolded in solution. The recombinant domains
appear to have the same biochemical properties as those of
endogenous cadherins. They form 1:1 stoichiometric complexes
with β-catenin, and the stability of the rE cyto-
β-catenin complex as a function of salt concentration is comparable with that
of endogenous E-cadherin-
β-catenin complex. In addition,
rEcyto and rDE cyto can be concentrated to 40 and 20 mg/ml,
respectively, without the aggregation that would occur with a
"misfolded" protein. Thus, it is unlikely that the recombinant
proteins are simply misfolded in bacteria, and it is highly
probable that endogenous cadherin cytoplasmic domains are
also unfolded.

β-Catenin appears to protect full-length rEcyto from degra-
dation under conditions that completely degrade rEcyto alone.

The β-catenin-binding site of E-cadherin is thought to lie
within the C-terminal 72 residues of the 150-amino acid cyto-
plasmic tail, with the minimal binding site encompassing just
30 residues (8). Given the relatively short binding sequence
and the unfolded state of Ecyto, proteolytic protection by seques-
tration of the entire domain seems unlikely. The simplest ex-
planation is that Ecyto adopts a defined conformation upon
binding to β-catenin. We interpret the observed difference in
protection provided by full-length β-catenin and β59 as evi-
dence that regions of β-catenin outside the arm repeat domain
interact with E-cadherin. A structuring of cadherin upon bind-
ing to β-catenin may facilitate interactions between cadherins
and other junctional components such as p120 ctn.

The acidic nature of the β-catenin-binding regions of cad-
herins, APC, and LF/E/Te transcription factors led us to pro-
pose that these otherwise unrelated proteins bind as extended
polypeptides in the electrostatically positive groove present
in the arm repeat region of β-catenin (12). Indirect support for the
groove binding model comes from the observation that nuclear
localization signal peptides bind in an extended conformation
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Recent site-directed mutagenesis data have shown that APC and LEF-1 bind within the groove of β-catenin (30). As cadherins compete with APC and LEF-1 for binding to β-catenin (11, 30), it is likely that cadherin also interacts with this region. Although the groove appears to be the binding site for β-catenin ligands, our results indicate that electrostatic complementarity is not a major contributor to the stability of the cadherin-β-catenin complex. This is consistent with experimental and theoretical studies showing that the energetically favorable interactions between complementary charges do not always compensate for the unfavorable desolvation of the participating charged groups (45, 46). Although charged and polar interactions may not have a dominant role in complex stability, they are generally important for specificity, because the cost of desolvating polar groups upon burial in an interface must be offset by electrostatically complementary interactions. In addition, long range electrostatic interactions may be used to enhance rates of association, because they can provide attractive forces even during molecular rotation and realignment (47).

Electrostatic complementarity between β-catenin and Eγ₉₋₁₀ may simply be a consequence of maintaining the cadherin cytoplasmic domain in an extended, unstructured state that can readily bind to β-catenin. When unfolded, a typical globular protein has poor solubility properties and is prone to aggregation. Thus, it is not surprising that the cadherin cytoplasmic domains have compositions skewed toward charged amino acids at the expense of aliphatic residues. Such a composition is likely to favor an extended conformation, because charge-charge repulsion will reduce the likelihood of self-association. β-Catenin may present an electrostatically positive surface to complement the acidic character required to maintain cadherin as an unstructured protein.

The lack of structure seen in the uncomplexed cadherin tail may be related to the turnover of cadherins. E-cadherin has a relatively short half-life (<5 h) in MDCK cells (48). β-Catenin associates with E-cadherin shortly after cadherin synthesis (13), and mutants deficient for β-catenin binding are retained within the endoplasmic reticulum and are rapidly degraded (14). We have shown here that the majority of cadherin cytoplasmic domains contain PEST sequence motifs. The PEST sequences may function as signals for the degradation of “cate-
nin-free” or uncomplexed cadherins.

Some of the cadherin tail sequences have low PEST scores (Fig. 6), but other low scoring PEST sequences are known to be proteolytic degradation signals (35). Moreover, it has been shown that phosphorylation may activate a latent or low-scoring PEST sequence (35, 49–53). A well studied example is the degradation of IkB proteins, inhibitors of the transcription factor NF-κB (54). The presence and phosphorylation of PEST sequences in IkBs is required for a rapid and constitutive turnover of free IkBs that may facilitate sustained NF-κB activity (55–58). A well studied example is the degradation of IkB proteins, inhibitors of the transcription factor NF-κB (54). The presence and phosphorylation of PEST sequences in IkBs is required for a rapid and constitutive turnover of free IkBs that may facilitate sustained NF-κB activity (55–58).

Interestingly, a subclass of constitutively active Ras superfamily members binds to and possibly sequesters the PEST regions of free IkB proteins, regulating their turnover (59). The E-cadherin PEST sequence is phosphorylated in a serine-rich region that is highly conserved among type I cadherins and is also present in type II cadherins (Fig. 6) (8), suggesting that phosphorylation can activate these degradation signals as well.

The PEST and β-catenin-binding regions of cadherins overlap extensively, and phosphorylation of the serines in this region has been shown to increase the affinity of E-cadherin for β-catenin (16). It is not known when or where in the cell cadherins are phosphorylated. Binding of cadherins to β-catenin may prevent phosphorylation by sequestering the PEST serines from kinases. Those cadherin molecules that have al-
ready been phosphorylated will have increased affinity for β-catenin. Upon β-catenin binding, these activated PEST sequences would again be sequestered, this time from recognition by the degradation machinery. Either or both of these scenarios would lead to selective degradation of those cadherin molecules that fail to bind to β-catenin.

Given that the cytoplasmic domains of cadherins are unstruc-
tured in the absence of β-catenin, they are likely to be good substrates for kinases as well as the cellular protein degradation machinery. Functional adhesion requires cadherins to be linked to the cytoskeleton through β- and α-catenins, so free cadherin molecules at the cell surface might act as competitive inhibitors of adhesion. Cytosolic levels of β-catenin are tightly controlled to prevent inappropriate activation of Wnt-responsive genes. Increasing β-catenin levels by induction of the Wnt pathway increases the formation of cad-
herin-catenin complex and cell-cell adhesion in some cell lines (60, 61). Likewise, the increased level of total β-catenin follow-
ing overexpression of cadherin in other cell lines likely reflects stabilization of β-catenin molecules that would otherwise be turned over by the Wnt pathway (25, 62–64). These observa-
tions indicate that cadherin and β-catenin turnover are cou-
pled. Cadherins that are not associated with β-catenin may be targeted for degradation by sequences in their unstructured cytoplasmic domains, reducing the population of catenin-free cadherins at the cell surface.

Acknowledgments—We thank Y. Wang and S. Fridman for technical assistance, J. Ames and K. Ng for advice on fluorescence experiments, T. Schwarz for kindly providing the Drosophila head cDNA library, and K. Spink and S. Pokutta for discussions and comments on the manuscript.

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