β-Catenin is a structural component of adherens junctions, where it binds to the cytoplasmic domain of cadherin cell adhesion molecules. β-Catenin is also a transcriptional coactivator in the Wnt signaling pathway, where it binds to Tcf/Lef family transcription factors. In the absence of a Wnt signal, nonjunctional β-catenin is present in a multiprotein complex containing the proteins axin and adenomatous polyposis coli (APC), both of which bind directly to β-catenin. The thermodynamics of β-catenin binding to E-cadherin, Lef-1, APC, axin, and the transcriptional inhibitor ICAT have been determined by isothermal titration calorimetry. Most of the interactions showed large, unfavorable entropy changes, consistent with these ligands being natively unstructured in the absence of β-catenin. Phosphorylation of serine residues present in a sequence motif common to cadherins and APC increased the affinity for β-catenin 300–700-fold, and surface plasmon resonance measurements revealed that phosphorylation of E-cadherin both enhanced its on rate and decreased its off rate. The effects of the N- and C-terminal “tails” that flank the β-catenin armadillo repeat domain on ligand binding have also been investigated using constructs lacking one or both tails. Contrary to earlier studies that employed less direct binding assays, the tails did not affect the affinity of β-catenin for tight ligands such as E-cadherin, Lef-1, and phosphorylated APC. However, the β-catenin C-terminal tail was found to decrease the affinity for the weaker ligands APC and axin, suggesting that this region may have a regulatory role in β-catenin degradation.

The protein β-catenin serves several roles in the development and maintenance of multicellular organisms. It is a structural component of cell-cell contacts and is also a transcriptional coactivator in the Wnt signaling pathway that controls cell fate determination. The multiple functions of this protein depend upon its interactions with several distinct protein ligands. In adherens junctions, β-catenin interacts with the cytoplasmic domain of classical cadherins and with α-catenin, which functionally connects the cadherin-catenin complex to the actin cytoskeleton. The levels of nonjunctional β-catenin are determined by the presence or absence of Wnt growth factor stimulation. In the absence of a Wnt, cytosolic β-catenin is targeted for degradation by a multiprotein complex containing axin and the adenomatous polyposis coli protein (APC). The axin-APC complex binds to β-catenin and also recruits casein kinase 1 (CK1) and glycogen synthase kinase-3β (GSK-3β). These kinases phosphorylate β-catenin, flagging it for destruction by the ubiquitin-proteasome pathway. In the presence of a Wnt signal, phosphorylation of β-catenin is inhibited. The resulting stabilized pool of β-catenin translocates to the nucleus, where it binds to the Tcf/Lef family transcription factors. The β-catenin-Tcf complex recruits general transcription factors, resulting in a complex that activates Wnt target genes.

The primary structure of β-catenin consists of an N-terminal region of ~150 amino acids, followed by a central armadillo (arm) repeat domain of ~520 residues and a C-terminal “tail” of about 100 amino acids. Many β-catenin ligands, including cadherins, Tcfs, APC, axin, and the transcriptional inhibitor ICAT, bind to the arm repeat domain. This region consists of 12 arm repeats, each of which is composed of three α-helices, which pack together to form a highly elongated structure with a positively charged groove (1). Crystal structures of the β-catenin arm repeat domain bound to the E-cadherin cytoplasmic domain (2), several Tcfs (3–5), APC (6–8), and ICAT (9, 10) have revealed that an extended peptide present in these ligands binds to the portion of the groove formed by repeats 5–9 (Fig. 1). In addition to this common binding feature, each ligand forms unique interactions with other portions of the β-catenin arm domain. For example, sequences in E-cadherin N- and C-terminal to the extended groove-binding peptide interact with all of the other arm repeats, and ICAT has an N-terminal helical domain that interacts with arm repeats 11 and 12. Tcfs contain an amphipathic helix C-terminal to the groove-binding peptide that interacts with repeats 3–5. This region is also exploited when APC 20-mers or cadherin are phosphorylated; phosphorylation of serine residues in the sequence SLSSL shared by these proteins results in ordering of this region and formation of interactions similar to those made by the Tcf helix. In the case of APC, binding of the phosphorylated sequence results in additional contacts made by the 18 amino acids C-terminal to the SLSSL sequence (7, 8). Axin is unique among the known arm-domain binding ligands because it does not interact with the repeat 5–9 groove; instead, it binds as a 17-residue α-helix to the groove formed by repeats 3 and 4 (11).

Several studies of the interaction between β-catenin and Tcf-4, E-cadherin, and APC have identified key residues or “hot spots” in the ligands responsible for the majority of the binding energy. The extended peptide common to most β-catenin ligands can be described by the consensus DX\[^{\theta}\]DX\[^{2-7}\]E, where \(\theta\) is an aliphatic hydrophobic amino acid, and \(\phi\) is an aromatic side chain (9). Mutation and binding studies with Tcf-4 peptides indicate that the consensus Asp and the aromatic side chain confer much of the binding energy in the groove (12, 13). Tcf-4 residues Leu\(^{41}\) and Leu\(^{46}\), which are present on one side of an
The helices of the arm domain of β-catenin are drawn as cylinders and colored gray, except for H3 helices that are shown in blue. Each arm repeat is numbered from 1 to 12 as shown below each repeat. All β-catenin-ligand complex structures were superimposed using the β-catenin coordinates, and the relative position of each ligand is shown separately. The N and C termini of each protein are labeled as N and C, respectively. Dashed lines indicate missing residues in crystal structures. The helices of the ligands are shown as cylinders. The helical domain of ICAT is colored in yellow. The region that becomes ordered upon phosphorylation of Eγcyt and APC-R3, is colored in red, and the other regions are shown in green.

Most of the β-catenin ligands discussed herein are natively unstructured proteins, i.e. they do not adopt a single ordered structure in the absence of β-catenin or other partners. Nuclear magnetic resonance, circular dichroism, and fluorescence anisotropy measurements have shown that the entire cadherin cytoplasmic domain is unstructured in the absence of β-catenin (14), and circular dichroism measurements of Tcf-(1–65) and Tcf-(1–130) likewise show a lack of secondary structure (15). The regions of APC and axin that bind to β-catenin are short peptides that are unlikely to form stable structures in solution, and in the case of APC-R3 the lack of electron density for the last 25 residues in the unphosphorylated structure provides direct crystallographic evidence for a lack of structure when not bound to β-catenin (8). The lack of structure appears to have several functional implications. For example, β-catenin colocalizes with cadherin as soon as the latter appears in the endoplasmic reticulum; the two proteins move as a unit to the cell surface (16), and disruption of this interaction results in proteosomal degradation of cadherin due to exposure of a PEST sequence present in cadherin (14, 17). In the case of cadherins and APC, the lack of structure of certain regions when not bound to β-catenin probably enables modification by kinases and phosphatases that can modulate binding affinity (2). In the cell, it is likely that large portions of these proteins are bound to β-catenin or other partners. For example, in the absence of β-catenin Tcf/Lefs serve as transcriptional repressors through their association with TLE/Groucho family proteins (18–22).

In contrast to the wealth of structural knowledge of the β-catenin arm domain and its interactions with ligands, little is known about the N- and C-terminal regions. The N- and C-tails of β-catenin appear to have important roles in preventing β-catenin from binding to desmosomal cadherins, which share significant similarity with classical cadherins found in adherens junctions (23, 24). Several reports suggest that these tails modulate the E-cadherin-β-catenin interaction by binding directly to the arm domain, thereby competing with the ligand (25, 26). The C-tail appears to interact directly with the arm domain, an interaction that has been mapped to the last 22 amino acids of the protein (26). The N-tail also appears to interact with the arm domain, but only in the presence of the C-tail (26). Although E-cadherin and Tcf5 have overlapping interactions with the arm domain, Tcf binding was found to be independent of the C-tail (25, 27).

Although crystal structures have defined the atomic basis of ligand interactions with β-catenin, they do not provide any information about thermodynamics. Understanding the strengths of these interactions is important for dissecting the mechanism of adhesion (28, 29), the APC-axin destruction complex (8), and how β-catenin can displace corepressors from Tcf5 as part of transcriptional activation (30). Moreover, dysregulation of β-catenin destruction and consequent inappropriate transcriptional activation by β-catenin underlie a number of cancers (31), so there is intense interest in developing inhibitors that can selectively block its interactions with Tcf5 while permitting its interactions with components of the destruction complex and cell adhesion molecules (32). However, the interactions of β-catenin with many of its ligands have been assessed by qualitative or only semi-quantitative means.

**EXPERIMENTAL PROCEDURES**

**Construct Design and Overexpression in Escherichia coli—**Four different murine β-catenin constructs, encoding the full-length protein (β-cat-full:1–781), the N-terminal + arm domain (β-cat-arm-Nt:1–671), the arm + C-terminal domain (β-cat-arm-Ct:134–781), and the arm domain alone (β-cat-arm:134–671) were used in these studies (Fig. 2). All were expressed as glutathione S-transferase (GST) fusion proteins. The β-cat-arm construct contains a thrombin cleavage site after the GST (1); the other constructs have an N-terminal GST followed by a tobacco etch virus (TEV) protease recognition site using a modified pGEX-KG vector, pGEX-TEV. DNA fragments encoding β-cat-arm-Nt and β-cat-arm-Ct were obtained by PCR and inserted into pGEX-TEV. The full cytoplasmic domain of murine E-cadherin (Eγcyt;577–728) (14) was used for ITC experiments and pulldown assays. Full-length ICAT and the helical domain of ICAT (residues 1–61; ICAT-h) were used for ITC experiments and were expressed as a GST-fused form and an N-terminal His6-tagged form, respectively, as described (9). Two different murine Lef-1 constructs, Lef-1-(1–65) and Lef-1-(1–131), encoding residues from 1 to 65 and from 1 to 131, respectively, were expressed as...
N-terminal His-tagged forms as described (30). Phosphorylated and nonphosphorylated 20-amino acid repeat 3 of APC (APC-R3:1484–1528) and the β-catenin binding domain of human axin fragment (residues 436–498) were used for ITC experiments and were expressed as GST fusion proteins as described previously (8). Each protein was overexpressed in E. coli BL21 cells. Cells were grown at 37 °C until the A600 of cells reached 0.6–0.8 and were then induced with 0.5 mM isopropyl thiogalactoside. Cells were harvested by centrifugation after additional growth of cells for 3–4 h at 30 °C, and the cell paste was stored at −80 °C.

**Vector Construction for Expression of Biotinylated Ecat**—A new vector, pAN-N1, that adds a biotin ligase consensus site to the N terminus of a protein was constructed from the vector pAN-1 (Avidity, LLC). A consensus cleavage site for the TEV protease was placed between the biotin ligase consensus and the new multiple cloning site (MCS) so that the affinity tag could be removed. Part of the biotinylation consensus, the TEV site, and the MCS was generated by overlap-extension PCR using standard methods and the following three oligonucleotides: 5′-AATTTCGACGCGAAGATTGAAGTCGACGACCGTGATTATCTTTACAG-3′; 5′-CATGAAAACCTGTATTTCCAAGGAATCTGGAAGTCCAGGCCTGACGGC-3′; and 5′-TATAAGGCTCTAAGGATTTCCGGAATTCGACGACCGTGATTATCTTTACAG-3′. The PCR product and the pAN-1 vector were digested with BstBI and HindIII, and the biotinylation/TEV/MCS insert was ligated into pAN-1, generating pAN-N1. The PCR insert, in conjunction with a portion of the biotin consensus tag from pAN-1, encoded the following N-terminal fusion sequence: MSGLNIDFEAKWIEYLFQSGARGLQ-GPPTAEF. The biotin ligase consensus site consists of residues 3–17 (35, 36). Cloning into the BamHI site leaves a single serine between the C terminus of the TEV consensus site and the sequence of interest.

pAN-N1 and a construct described previously containing the mouse E-cadherin cytoplasmic tail (Ecat) (37) were digested with BamHI and EcoRI. The digest product, encoding residues 580–728 of mature E-cadherin, was ligated into pAN-N1 to yield pAN-Ecat. E. coli strain BL21 containing an IPTG-inducible biotin-ligase gene (bira) in vector pACYC-184 (Avidity, LLC) was transformed with the IPTG-inducible pAN-Ecat. This system did not reliably produce sufficient quantities of biotinylated Ecat possibly because induced biotin ligase expression competed with Ecat fusion protein synthesis. In an attempt to improve the yield of biotinylated Ecat fusion protein, the biotinylation/TEV/MCS region of pAN-N1 was moved to vector pET28a (Novagen), which utilizes a stronger bacteriophage T7 expression system. The pAN-N1 biotinylation/TEV/MCS region was PCR-amplified using the following oligonucleotides: A, 5′-ATACATGCGGGCCCTGAACGACATCTTC-3′, and B, 5′-ATACATGCGGGCCCTGAACGACATCTTC-3′. The PCR product and the pAN-1 vector were digested with NcoI and HindIII, and the biotinylation/TEV/MCS insert was ligated into pAN-1, generating pAN-N1. The PCR insert, in conjunction with a portion of the biotin consensus tag from pAN-1, encoded the following N-terminal fusion sequence: MSGLNIDFEAKWIEYLFQSGARGLQ-GPPTAEF. The biotin ligase consensus site consists of residues 3–17 (33, 34). Biotin-protein ligase covalently couples biotin to residue Lys12. Residue Glu17 functions as the C-terminal residue of the biotin ligase consensus and the N-terminal glutamate of the TEV protease consensus site (ENLYFQG) (35, 36). Cloning into the BamHI site leaves a single serine between the C terminus of the TEV consensus site and the sequence of interest.

**Purification of the Recombinant Proteins**—For ITC experiments, β-cat-full, β-cat-arm, β-cat-arm-Nt, β-cat-arm-Ct, Ecat, ICAT, axin, and APC-R3 were purified from their GST fusion proteins, and Lef-1-(1–65), Lef-1-(1–131), and ICAT-h were purified from the N-terminal His-tagged forms. Protease inhibitor mixture (Complete™, Roche Applied Science) was added into thawed cell paste, and the cells were lysed by French press. After centrifugation of lysed cells at 40,000 rpm for 30 min, cleared lysates were removed and incubated with glutathione-agarose beads for GST fusion proteins or Ni2+-NTA-agarose beads for His-tagged protein for 1 h at 4 °C. For GST fusion proteins, glutathione-agarose beads were washed first with phosphate-buffered saline containing 1 mM NaCl, 0.005% Tween 20, and 5 mM dithiothreitol (DTT) and then with cleavage buffer consisting of 25 mM Tris-Cl, pH 8.5, 100 mM NaCl, and 2 mM DTT. Cleavage of the GST tag was performed by the addition of TEV protease (β-cat-full, β-cat-arm-Nt, β-cat-arm-Ct, APC-R3, and axin) or thrombin (β-cat-arm, Ecat, and ICAT) and the cleavage reaction was incubated at 4 °C for overnight (TEV protease) or for 2 h (thrombin). The cleaved protein was collected from flow-through and loaded onto a Mono Q column (Amersham Biosciences) equilibrated with buffer E (25 mM ethanalamine, pH 9.5, 2 mM DTT, and 0.5 mM EDTA) containing 50 mM NaCl for β-cat-arm and buffer A (25 mM Tris-Cl, pH 8.5, 2 mM DTT, and 0.5 mM EDTA) containing 50 mM NaCl for others and was eluted with a linear gradient of NaCl. Mono Q-purified protein was loaded onto a Superdex 200 gel filtration column (Amersham Biosciences) equilibrated with buffer T1 (25 mM Tris-Cl, pH 8.0, 2 mM DTT, and 0.15 mM KCl), buffer T2 (25 mM Tris-Cl, pH 8.8, 2 mM DTT, and 0.1 mM NaCl), or buffer H (25 mM HEPESSodium, pH 7.5, 75 mM NaCl and 0.5 mM EDTA) for ITC experiments.

For purification of Lef-1-(1–65), Lef-1-(1–131), and ICAT-h with the N-terminal His tag, Ni2+-NTA-agarose beads were washed with 10 mM imidazole, after 1 h of incubation. Each His6-tagged protein was eluted using 300 mM imidazole, and its His6 tag was removed by TEV protease. After incubation of the cleavage reaction mixture at 4 °C overnight, imidazole was dialyzed out to reload the cleavage reaction onto Ni2+-NTA-agarose beads. Cleaved protein was collected from flow-through and was further purified using gel filtration chromatography, equilibrated with buffer T2 or H. Each purified protein was spin-concentrated, and the final concentrations were determined by UV absorption at 280 nm.

Phosphorylated Ecat was prepared as described using purified Ecat and commercially available casein kinase II (New England Biolabs) (2), and the protein was repurified by Mono Q and gel filtration chromatography. APC-R3 and phosphoAPC-R3 were obtained as described previously (8).

**Purification and Phosphorylation of Biotinylated Ecat**—The biotinylated Ecat, used for Ecat-β-catenin BLAcore experiments was purified from a culture of E. coli BL21 containing vector pAN-Ecat (above) and the bira gene in vector pACYC-184 (Avidity, LLC). Cells were grown in Superbroth containing 150 μg/ml ampicillin and 20 μg/ml chloramphenicol, with biotin added to 50 μM at mid-log growth phase. Protein expression from both vectors was induced with 200 μM IPTG during late log phase, and the cells were harvested after an additional 3 h of growth. Cell pellets were resuspended in minimal volumes of resuspension buffer (phosphate-buffered saline with 1 mM NaCl, 0.05% (v/v) Tween 20, and 0.1% (v/v) β-mercaptoethanol) and frozen. Phenylmethylsulfonyl fluoride, aprotinin, and pepstatin A (Sigma) were added to thawed cell paste to concentrations of 2 mM and 4 μg/ml, respectively. Approximately 200 Kunitz units of DNase I (Sigma) were added per ml of cell paste, and the cells were lysed with a French press (SLM-Aminco). Protease inhibitors were added again, including EDTA, which had a final concentration of 2 mM. Lysate was clarified by centrifugation, and the supernatant was incubated for 45 min at 4 °C with UltraLink Immobilized Monomeric Avidin matrix (Pierce) prepared as suggested by the manufacturer. A small column was poured with the matrix/lysate slurry
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and washed with 10 column volumes of wash buffer (50 mM Tris-HCl, pH 8.0, 75 mM NaCl). Biotinylated E\textsubscript{cyto} was eluted with elution buffer (50 mM Tris-HCl, pH 8.75, 150 mM NaCl) and further purified by size exclusion chromatography utilizing a Superdex 200 Prep-grade 26/60 column (Amersham Biosciences) equilibrated with Superdex buffer (25 mM Tris-HCl, pH 8.5, 150 mM NaCl). E\textsubscript{cyto} peak fractions from the Superdex column were pooled, diluted 2-fold with Mono Q buffer A (20 mM Tris-HCl, pH 8.5), and bound to an anion exchange column (Mono Q HR 16/10, Amersham Biosciences). Biotinylated E\textsubscript{cyto} was eluted with a gradient from 10 to 30% Mono Q buffer B (20 mM Tris-HCl, pH 8.5, 2 mM NaCl).

Phosphorylated biotin-E\textsubscript{cyto} (biotin-pE\textsubscript{cyto}) was generated using biotin-E\textsubscript{cyto} purified from a culture of E. coli BL21 containing vector pET28ah-bE\textsubscript{cyto} (above) and the birA gene in vector pACYC-184 (Avidity, LLC). Cells were grown in Luria broth containing 25 µg/ml kanamycin and 15 µg/ml chloramphenicol. Biotin and IPTG were added simultaneously at mid-log phase to concentrations of 50 and 600 µM, respectively, and the culture was harvested 2 h after induction. Cell pellets were resuspended in resuspension buffer (above) and lysed with a French press, and the lysate was clarified by centrifugation. Phenylmethylsulfonyl fluoride, EDTA, aprotinin, and pepstatin A (Sigma) were added to the lysate supernatant to concentrations of 2 and 2 mM and 4 and 2 µg/ml, respectively. Ammonium sulfate was added to ~15% of saturation at 4°C, the solution gently agitated for 1 h at 4°C, and any precipitate pelleted by centrifugation. Precipitated protein was resuspended in buffer urea (50 mM Tris-HCl, pH 8.5, 150 mM NaCl, 6 M urea) and purified without urea by gel filtration on a Superdex 200 Prep-grade 26/60 column equilibrated with 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 2 mM DTT. Additional purification utilized a Mono Q anion exchange column equilibrated with Mono Q buffer A (above). Biotin-E\textsubscript{cyto} was eluted as a single well defined peak during a 10–30% gradient of Mono Q buffer B (above). Pulldown experiments with avidin beads demonstrated that a significant fraction of the resulting E\textsubscript{cyto} was biotinylated (data not shown).

Biotin-E\textsubscript{cyto} was phosphorylated in a 2-ml reaction comprising 0.8 mg/ml biotin-E\textsubscript{cyto}, 1× casein kinase II reaction buffer (New England Biolabs), 80 mM Tris-HCl, pH 7.5, 9 mM pH-adjusted ATP, pH 7.3, 10 mM MgCl\textsubscript{2}, and 25,000 units of casein kinase II (New England Biolabs). After a 6-h incubation at 30°C, the reaction was applied to an anion exchange column (Mono Q HR 16/10, Amersham Biosciences) equilibrated with buffer A (50 mM imidazole, pH 7.2, 4 M urea) and eluted with a gradient from 10 to 20% buffer B (50 mM imidazole, pH 7.2, 4 M urea, 2 M NaCl). Mono Q fractions containing the largest single peak of highly phosphorylated pE\textsubscript{cyto} were pooled and concentrated. This peak had very modest shoulders and consisted of biotin-E\textsubscript{cyto} molecules with 3–5 added phosphate groups, as assessed by mass spectroscopy. The final buffer for the biotin-pE\textsubscript{cyto} stock was 25 mM Tris-HCl, pH 8.0, 150 mM NaCl.

Isothermal Titration Calorimetry—ITC measurements were performed at 30°C using a VP-ITC calorimeter (Microcal, Inc.). All purified ligands and β-catenin were concentrated to 200–350 and 8–25 µM, respectively, in H, T1, or T2 buffer. Each titration experiment, except for phosphoE\textsubscript{cyto}, was initiated by a 3- or 4-µl injection, followed by 25–40 7- or 8-µl injections. Blank titrations, which were carried out by injecting ligand into H, T1, or T2 buffer depending on the particular experiment, were subtracted from each data set. The association constant $K_a$, the enthalpy change $\Delta H$, and the stoichiometry $n$ were obtained from fitting the data (38) using the Origin software package (Microcal, Inc.). The dissociation constant $K_d$, the free energy change $\Delta G$, and the entropy change $\Delta S$ were obtained from the basic thermodynamic relationships $K_d = K_a^{-1}$, $\Delta G = -RT\ln K_a$, and $\Delta G = \Delta H - T\Delta S$. For the titration experiment with the very tight ligand phosphoE\textsubscript{cyto}, the displacement method was performed using nonphosphorylated E\textsubscript{cyto} as a competitor (39). In this method, a less strongly bound ligand is used as a competitive inhibitor, and its binding parameters, determined by a separate conventional ITC experiment, are used for calculation of thermodynamic parameters of more strongly bound ligand. Before injecting phosphoE\textsubscript{cyto}, β-catenin in the ITC cell was fully saturated with E\textsubscript{cyto}. Then 8 µl of 300 µM phosphoE\textsubscript{cyto} was injected 20 times after a first 4-µl injection. The raw ITC data showed a relatively small signal because the measured change in heat was because of the contributions of the heat of binding of the strong ligand phosphoE\textsubscript{cyto} and the heat of dissociation of E\textsubscript{cyto} from the complex with β-catenin, which is of opposite sign. Data analysis were performed by nonlinear regression fitting to the displacement model, which was kindly provided by Dr. B. Sigurskjold and installed in Origin version 5.0. The ITC data for the APC-R3 and phosphorylated APC-R3 interactions have been published previously (8) but are included here for comparison with the other ligands.

Surface Plasmon Resonance Data Collection—Data were measured on a Biacore 3000 surface plasmon resonance (SPR) detection system (Biacore AB). Biotin-E\textsubscript{cyto} (BE\textsubscript{cyto}) and phosphorylated biotin-E\textsubscript{cyto} (PBE\textsubscript{cyto}) molecules were captured on streptavidin-coated sensor chips (Sensor Chip SA, Biacore AB). A single covalent biotin at a defined N-terminal location in the E\textsubscript{cyto} proteins produces an oriented capture system, with the E\textsubscript{cyto} domains presented in the orientation one would expect to find in vivo. In each chip, the first of the four flow cells (FC1) did not have any bound E\textsubscript{cyto}, and was used as a control. Flow cells 2–4 (FC2–FC4) had increasing amounts of captured BE\textsubscript{cyto} or PBE\textsubscript{cyto}. Single BE\textsubscript{cyto} and PBE\textsubscript{cyto} chips were used for all of the experiments. The results presented here are derived from FC2, because this had the lowest coupling levels and therefore the lowest levels of mass transport, steric hindrance, and aggregation effects. All experiments were carried out at a flow rate of 100 µl/min to minimize mass transport effects. Each SPR sensorgram consisted of a “blank” buffer injection followed by a 140-s injection of full-length β-catenin with a 370-s dissociation period. The E\textsubscript{cyto} chip surface had to be regenerated between sensorgrams with two 12-s injections of 10 mM HCl because the SPR signal was not returning to baseline in a timely manner.

Each SPR binding experiment tested seven β-catenin concentrations, with three replicates per concentration. Binding to BE\textsubscript{cyto} utilized β-catenin concentrations of 400, 200, 100, 50, 25, 12.5, and 6.25 nM. Binding to phosphoBE\textsubscript{cyto} utilized a different set of serial dilutions that included β-catenin concentrations of 12.5, 6.25, 3.12, 1.6, 0.8, 0.4, and 0.2 nM. The replicates were measured in a partially randomized order with the same “randomized” order used for each experiment. SPR experiments (7 concentrations and 21 replicates) were carried out using a wide range of KCl concentrations for both BE\textsubscript{cyto} and phosphoBE\textsubscript{cyto}. Each binding buffer consisted of 25 mM Tris-HCl, pH 8.0, 2 mM dithiothreitol, 0.005% P2O surfactant and KCl at 150, 300, 600, or 900 mM. The Biacore 3000 was cleaned between experiments using the manufacturer’s desorbance protocol with the E\textsubscript{cyto}-streptavidin chip undocked. Each experiment was preceded by a surface performance test that utilized six cycles of β-catenin injection and HCl regeneration steps. The surface performance test provided evidence that a stable base line had been reached after the desorbance procedure.

The BIAevaluation 3.0 software package (Biacore AB) was used for data processing and fitting. The FC1 reference sensorgrams were subtracted from the appropriate data sensorgrams. The blank buffer injection portion of each sensorgram was then subtracted from the subsequent β-catenin injection (data) portion of the sensorgram.
Sensorgrams were then aligned along the x (time) and y (response unit) axes. It became clear that each injection/regeneration cycle produced a slight reduction in the binding capacity of the chip. This reduction was significant given the large number of sensorgrams collected during each experiment. An average loss of signal per cycle was determined for each flow cell within an experiment, and a correction was applied to each sensorgram. The correction was generally on the order of 0.7%/cycle. Sensorgrams with obvious pathologies were discarded, and the fully adjusted replicate curves for each β-catenin concentration were then averaged. The seven averaged curves from each experiment were then simultaneously and globally fit.

RESULTS AND DISCUSSION

Comparative Binding of Full-length β-Catenin to Different Ligands—The thermodynamics of full-length β-catenin binding to nine different ligands were measured by ITC at pH 7.5 (Figs. 2 and 3). The complete 151-amino acid cytoplasmic domain of E-cadherin (Ecyto) was used in this study and is the same construct that was cocrystallized with the β-catenin arm domain (2). Two constructs of Lef-1 were studied, spanning residues 1–65 or 1–131. The shorter Lef-1 construct corresponds to the conserved β-catenin-binding motif present in most Tcf isoforms, and this region of Xenopus Tcf-3 (3) and human Tcf-4 (4, 5) was cocrystallized with the arm domain. The longer Lef-1 construct was recently identified as a proteolytically protected region when full-length Lef-1 was bound to β-catenin; it contains sequences outside of the structurally characterized β-catenin binding region that are also conserved among Tcfs (30).

APC binds to β-catenin through short consensus peptide repeats of 15 or 20 amino acids, and phosphorylation of a sequence motif unique to the 20-mers by CK1 and GSK-3β enhances the interaction with β-catenin. The APC 20-mer repeat 3 (APC-R3), both unmodified and homogeneously phosphorylated by CK1 and GSK-3β, was cocrystallized with β-catenin, and ITC data for this interaction were reported (8).

The axin construct, residues 436–498, corresponds to the same construct used in the cocrystal structure with β-catenin (11). Full-length ICAT contains a helical domain that binds to arm repeats 11 and 12 and a C-terminal extended region that binds in the groove similarly to cadherin, Tcfs, and APC (9, 10). The helical domain, comprising the first 61 amino acids, can be made as a soluble
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TABLE 1

ITC measurements of ligand binding to full-length β-catenin

All experiments were performed at 30 °C. Titration experiments of Ecyto and phosphoryl Ecyto were carried out in T1 buffer, which is the same used in the BIAcore experiments, in order to compare their results. The other ligands were titrated using H buffer.

| Ligand          | $K_D$ (nM) | $\Delta H$ (kcal/mol) | $\Delta S$ (kcal/mol) | $\Delta G$ (kcal/mol) |
|-----------------|------------|-----------------------|-----------------------|-----------------------|
| H buffer        |            |                       |                       |                       |
| Ecyto           | $36 \pm 4 \times 10^{-9}$ | $-35.9 \pm 0.4$ | $-25.6$ | $-10.3$ |
| Leif-1 (1–65)   | $49 \pm 3 \times 10^{-9}$ | $-14.7 \pm 0.1$ | $-6.0$ | $-10.1$ |
| Leif-1 (1–131)  | $20 \pm 6 \times 10^{-9}$ | $-16.7 \pm 0.2$ | $-6.0$ | $-10.7$ |
| ICAT            | $3.1 \pm 1 \times 10^{-9}$ | $-19.8 \pm 0.1$ | $-8.0$ | $-11.8$ |
| ICAT-h          | $12.0 \pm 1 \times 10^{-9}$ | $-12.5 \pm 0.1$ | $-1.5$ | $-11.0$ |
| APC-R3          | $3.1 \pm 0 \times 10^{-10}$ | $-4.5 \pm 0.1$ | $3.1$ | $-7.6$ |
| PhosphoAPC-R3   | $10 \pm 1 \times 10^{-10}$ | $-31.2 \pm 0.2$ | $-20.1$ | $-11.1$ |
| Axin            | $1.3 \pm 0 \times 10^{-10}$ | $-6.9 \pm 0.2$ | $1.3$ | $-8.2$ |
| T1 buffer       |            |                       |                       |                       |
| Ecyto           | $46 \pm 4 \times 10^{-9}$ | $-39.2 \pm 0.2$ | $-29.0$ | $-10.2$ |
| PhosphoEcyto    | $52 \pm 5 \times 10^{-12}$ | $-53.5 \pm 1.6$ | $-39.2$ | $-14.3$ |

*H buffer consists of the following: 25 mM HEPES, pH 7.5, buffer containing 75 mM NaCl and 0.5 mM EDTA.
*T1 buffer consists of the following: 25 mM Tris-Cl, pH 8.0, buffer containing 150 mM KCl and 2 mM DTT.

protein that binds to β-catenin (9) and is designated ICAT-h. The binding isotherms were fitted to a single binding site, I:1 model, as observed in the crystal structures of these ligand complexes with β-catenin.

The affinities of β-catenin ligands vary over a wide range (Table 1) and can be classified into three groups. APC-R3 and axin belong to the low affinity group with dissociation constants in the single micromolar range, whereas Ecyto, Leif-1 (1–61), Leif-1 (1–131), phosphorylated APC, ICAT, and ICAT-h have $K_D$ values in the range of tens of nanomolar. PhosphoEcyto is an exceptionally avid ligand, with a $K_D$ of 52 pm. There is a rough correlation between the size of the ligand-β-catenin interface and the free energy of binding, and between the enthalpy of binding and the number of contacts formed (Table 2; Fig. 4A). However, the enthalpic and entropic contributions vary substantially in an example of enthalpy-entropy compensation. For example, nonphosphorylated cadherin binds with about the same affinity as Leif-1 and yet shows much larger changes in both enthalpy and entropy.

The wide variation in affinities of β-catenin for its ligands presumably reflects different functional requirements. For example, the strong interaction with E-cadherin is probably important for ensuring delivery of cadherin to the cell surface, as disruption of the interaction results in cadherin destruction (14, 16, 17). Likewise, the high affinity of β-catenin for Tcf/Lefs is probably required to displace TLE/Groucho family corepressors from Tcf/Lef to initiate transcriptional activation (30). On the other hand, the weak affinity for axin and nonphosphorylated APC is likely tied to turnover of the β-catenin destruction complex (for discussion see Ref. 8). It should be noted that although several of these ligands bind to the same site on β-catenin and can compete in vitro, there is no evidence that they do so under normal physiological circumstances, possibly as a consequence of discrete subcellular localization. Therefore, the relative affinities of these ligands and their resultant ability to compete for β-catenin may not be related to any requirement for direct competition among them in vivo.

Most of the reactions studied, apart from APC-R3 and axin, display unfavorable negative entropy and favorable negative enthalpy changes. The unfavorable entropies of most of the interactions correlate with the ligand being unstructured in the absence of β-catenin; presumably there is a large configurational entropy penalty associated with forming the ordered, bound structure. The magnitude of the unfavorable entropy change correlates well with the number of ligand residues that are immobilized in the interface (Table 2; Fig. 4B). Most interestingly, the ICAT helical domain has a small unfavorable binding entropy, despite the fact that it, unlike the other ligands, is structured in isolation (9). The reactions with APC-R3 and axin have favorable positive entropy changes, but their affinities for β-catenin are relatively weak, with $K_D$ values of 3.1 and 1.3 μM, respectively. The positive $\Delta S$ values for these ligands may reflect the relatively small number of ordered residues in these ligands, such that the loss in configurational entropy upon binding does not dominate the favorable entropy of the hydrophobic effect, displacement of bound water from solvated polar groups, and other factors.

Comparison with Previous Studies—Thermodynamic analyses of β-catenin binding to Tcf-4 (12, 15), APC 20-mers (40), and a number of peptides from β-catenin ligands (13) have been reported. Each of these studies used buffer conditions and/or temperatures somewhat different from those used here and from one another, but in general the agreement of these studies is good. The effect of buffers, in particular pH, on the strength of the interactions is discussed below.) The conditions of the present study and those used by Knapp and co-workers (12, 15) for Tcf-4 are similar, and their results, as well those of Gail et al. (13), indicate that Tcf-4 binds with 3- or 4-fold higher affinity than Leif-1. There are no structural data for the Leif-1 interaction, and given the similarity of the Leif-1 and Tcf-4 sequences, the basis of their affinity difference is unclear.

Thermodynamics of E-cadherin Binding—The E-cadherin cytoplasmic domain interacts with the entire β-catenin arm repeat domain (2) (Fig. 1). The 151-amino acid domain is unstructured in isolation (14), but most of the last 100 residues become structured upon binding to β-catenin (2). The interaction involves five distinct regions of the cadherin primary structure. Region 2, residues 639–666, contains a helix that binds to arm repeats 11 and 12. This is followed by region 3 (residues 667–684), which is the conserved peptide that binds to arm repeats 5–9. Region 4, residues 685–694, binds to β-catenin only when Ser 686 and Ser 692 are phosphorylated. Cadherin region 5 (residues 698–723) consists of two helices that bind to the first two β-catenin arm repeats.

FIGURE 3. Experimental calorimetric data for ligand binding to full-length β-catenin. All isothermal calorimetric experiments were performed at 30 °C. All ligands, except for Ecyto and phosphoryl Ecyto, were injected into β-catenin full-H buffer; for Ecyto and phosphoryl Ecyto, T1 buffer was used. In each figure, the top panel shows the base-line-subtracted heat signal obtained by a series of injections of each ligand into the ITC cell containing β-catenin full; the blank injection of ligand into the ITC cell containing only buffer is also shown and has been vertically offset for clarity. The bottom panels show the binding curves calculated using the best fit parameters obtained by a nonlinear least squares fit. For experiments performed in replicate, a representative example is shown.
Thermodynamics of β-Catenin-Ligand Interactions

TABLE 2
Buried surface area and number of contacts

| Ligand          | Protein Data Bank ID code | Total no. residues | Total buried area | Interface buried area | Interface no. contacts | No. contacts |
|-----------------|----------------------------|--------------------|-------------------|-----------------------|------------------------|--------------|
| E-cadherin      | I17X                       | 151                | 31                | 3193                  | 22                     | 202          |
| PhosphoE-cadherin | I17W                      | 151                | 39                | 5042                  | 34                     | 280          |
| Axin            | I17X                       | 51                 | 13                | 2397                  | 28                     | 180          |
| hTcf-4          | I17W                       | 38                 | 26                | 2418                  | 24                     | 211          |
| ICAT-h          | I1M1E                      | 49                 | 19                | 2585                  | 20                     | 156          |
| hTcf-4          | I1M1E                      | 81                 | 25                | 2937                  | 28                     | 180          |
| ICAT            | I1M1E                      | 61                 | 11                | 1201                  | 6                      | 45           |
| Axin            | I1M1E                      | 47                 | 13                | 1795                  | 14                     | 130          |
| PhosphoAPC-R3   | ITH1                       | 47                 | 23                | 3674                  | 27                     | 225          |
| Axin            | 1IQZ7                      | 70                 | 9                 | 1346                  | 5                      | 63           |

The 40 nM dissociation constant of E-cadherin (E-cadherin) derives from highly favorable negative enthalpy and unfavorable negative entropy changes. The entropy change reflects the large number of cadherin residues that become ordered upon binding to β-catenin; the entropy change is 20–95 cal mol⁻¹ K⁻¹ more negative than those of the other ligands (Table 1). However, this entropy penalty is compensated by the favorable enthalpy changes 5–31 kcal mol⁻¹ larger than the other ligands.

Phosphorylation of E-cadherin results in larger favorable enthalpy and unfavorable entropy changes, but the enthalpy change dominates to give an affinity increase of almost 3 orders of magnitude (Table 1). This interaction was too strong to measure in a direct titration experiment but could be obtained by the displacement method in which β-catenin was saturated with nonphosphorylated E-cadherin, whose binding constant had been determined (see “Experimental Procedures”) (39). The dominance of the more favorable enthalpy change (ΔΔH = −14 kcal mol⁻¹) over the more modest entropy change (ΔΔTS = −10 kcal mol⁻¹) can be rationalized by the crystal structures of the phosphorylated and unphosphorylated cadherins (2). Phosphorylation leads to the specific binding of E-cadherin residues 685–694 that are otherwise disordered, and these residues form a number of polar and nonpolar contacts. Several of these polar interactions are buried, which would be expected to contribute favorably to the binding enthalpy. Although an additional 10 residues become ordered upon phosphorylation, the sequences on either side of the phosphorylated region adopt a single, bound structure regardless of the phosphorylation state of E-cadherin. Thus, the phosphorylated region is already constrained by interactions on either side, so the structuring upon binding of this region probably involves relatively little extra loss of conformational entropy.

The effect of phosphorylation on the interaction of β-catenin was further investigated by surface plasmon resonance spectroscopy. The E-cadherin cytoplasmic domain was expressed with an N-terminal recognition sequence for the E. coli BirA gene product, such that the construct could be enzymatically biotinylated at a single site outside of the cadherin sequence. The biotinylated protein was immobilized on a streptavidin surface on a BiACore chip. Full-length β-catenin flowed over the chip, and the change in binding as a function of time was measured. Fitting the data with simple 1:1 binding models (Table 3) gave KD values of 18 nM and 81 pM for nonphosphorylated and phosphorylated cadherin, respectively, which are very close to the values obtained by ITC (Fig. 5, A and B). The good agreement in the KD values indicates that although the 1:1 models do not give particularly good fits to the data (probably reflecting more complicated kinetic processes associated with the structuring of cadherin upon binding to β-catenin), they are nonetheless reasonable approximations. The fitted rate constants show that phosphorylation increases the on rate 13-fold and decreases the off rate 18-fold (Table 3).

Better fits to the nonphosphorylated data were obtained using a model in which there are two distinct molecular species of E-cadherin on the chip (“heterogeneous ligand” model in Table 3; Fig. 5C). The improved fit of the two-species model may simply be due to increasing
the number of parameters, but other models using similar numbers of parameters did not fit the data as well (data not shown). The two-species model can be rationalized by the fact that Pro672 is in the cis conformation, which allows binding of regions 2 and 3 to H9252-catenin (2), whereas in the unbound, unstructured state Pro672 is presumably in equilibration between cis and trans isomers. Region 3, the groove-binding peptide, could bind if this proline is trans, but the preceding region 2 (a helix that binds to arm repeats 11 and 12) would not, giving a lower affinity. Indeed, phosphorylation of H9252-catenin Tyr654 disrupts binding of region 2 and is associated with a 10-fold diminution in binding affinity, similar to the difference in affinities of the two species modeled in the BIAcore fitting.

Lef-1 Binding—ITC experiments were performed using two different constructs of Lef-1, Lef-1-(1–65) and Lef-1-(1–131). Their dissociation constants were determined as 49 and 20 nM, respectively. The 2.5-fold increase in affinity, although small, is statistically significant and implies that contacts with β-catenin are formed by Lef-1-(66–131) beyond those of the canonical β-catenin binding domain spanning residues 1–65 seen in the crystal structures of the homologous XTcf-3 and hTcf-4 sequences. The additional contacts are consistent with the protection from proteolysis of this region when full-length Lef-1 is bound to β-catenin (30). The thermodynamic parameters for the interaction of Lef-1 with full-length β-catenin differ somewhat from data published previously for Tcf-4-(1–53) binding to the arm domain. Fluorescence measurements of Tcf4-(1–53) binding to the arm domain and the full domain of β-catenin give similar KD values (13), so the differences in these studies likely reflect real differences between Lef-1 and Tcf-4. Although the sequences of the Lef-1 and Tcf-4 β-catenin-binding regions are very similar, including the same residues at the hot spots that contribute most of the binding energy, minor sequence differences presumably give rise to their different thermodynamic parameters.

Compared with the other β-catenin ligands, the entropy penalty for Lef-1 binding is smaller than would be expected from the number of residues observed in the interface of XTcf-3 (3) and human Tcf-4 (4, 5) structures. This may be due to the fact that β-catenin Lys312 can form alternative interaction with acidic residues at the C-terminal end of the conserved groove-binding sequence of the ligand, as shown in the two crystal structures of human Tcf-4 bound to β-catenin (4, 5). This would be expected to contribute favorably to the binding entropy, and thereby partially offset the penalty of structuring the Tcf/Lef sequence.

ICAT Binding—ICAT was identified as an inhibitor of the interaction of β-catenin with Tcf (41), and it binds competitively with Tcf4, cad-

**TABLE 3**

| Ligand       | Fitting model | \( k_a \) | \( k_d \) | \( k_a \) | \( k_d \) | \( R_{max} \) | \( R_{max} \) | \( \chi^2 \) |
|--------------|---------------|-----------|-----------|-----------|-----------|--------------|--------------|--------|
| E\(_{cyto}\) | 1:1           | \( 3.56 \times 10^5 \) | \( 6.49 \times 10^{-3} \) | NA        | NA        | 45.6         | NA           | 0.614  |
| E\(_{cyto}\) | Heteroligand  | \( 4.39 \times 10^5 \) | \( 5.15 \times 10^{-3} \) | \( 1.11 \times 10^2 \) | \( 1.41 \times 10^{-2} \) | 34           | 16.7         | 0.326  |
| PhosphoE\(_{cyto}\) | 1:1 | \( 4.49 \times 10^6 \) | \( 3.67 \times 10^{-4} \) | NA        | NA        | 99.8         | NA           | 2.11   |

**FIGURE 5.** BIAcore experiments of unphosphorylated and phosphorylated E\(_{cyto}\). Sensorgrams of full-length β-catenin binding to biotinylated E\(_{cyto}\), immobilized on streptavidin chips. Background subtracted data are shown in black, and the fit curves shown in red. A, biotinylated E\(_{cyto}\) data fit with a simple 1:1 binding model. B, biotinylated phosphoE\(_{cyto}\) data fit with a simple 1:1 binding model. C, biotinylated E\(_{cyto}\) data fit with a model in which two distinct species of E\(_{cyto}\) are present on the chip.
**Thermodynamics of β-Catenin-Ligand Interactions**

| β-Catenin constructs | $K_D$ | $\Delta H$ | $\Delta S$ | $\Delta G$ | No. exp. |
|----------------------|-------|------------|------------|------------|---------|
|                      | $\mu$ | kcal/mol   | kcal/mol   | kcal/mol   |         |
| T2 buffer$^a$        |       |             |            |            |         |
| β-cat-full            | $83 \times 10^{-9}$ | $-39.5 \pm 0.2$ | $-29.7 \pm 0.5$ | $-9.8 \pm 0.1$ | 2       |
| β-cat-arm             | $93 \times 10^{-9}$ | $-39.7 \pm 0.5$ | $-30.0 \pm 3.0$ | $-9.7 \pm 0.1$ | 3       |
| β-cat-arm-Nt          | $113 \times 10^{-9}$ | $-43.3 \pm 0.4$ | $-33.7$ | $-9.6$ | 1       |
| β-cat-arm-Ct          | $60 \times 10^{-9}$ | $-34.8 \pm 0.2$ | $-24.8$ | $-10.0$ | 1       |
| T1 buffer$^b$        |       |             |            |            |         |
| β-cat-full            | $46 \times 10^{-9}$ | $-39.2 \pm 0.2$ | $-29.0$ | $-10.2$ | 1       |
| β-cat-arm             | $74 \times 10^{-9}$ | $-41.1 \pm 0.3$ | $-31.2$ | $-9.9$ | 1       |
| β-cat-arm-Nt          | $58 \times 10^{-9}$ | $-36.5 \pm 0.6$ | $-26.5$ | $-10.0$ | 1       |

$^a$ T2 buffer consists of the following: 25 mM Tris-Cl, pH 8.8, buffer containing 100 mM NaCl and 2 mM DTT.

$^b$ T1 buffer consists of the following: 25 mM Tris-Cl, pH 8.0, buffer containing 150 mM KCl and 2 mM DTT.

herins, and the general transcription factor CBP/p300 (27). ICAT binds to β-catenin with a $K_D$ of 3 nM, stronger than Lef-1 or E-cadherin and comparable with Tcf4. The helical domain of ICAT also binds to β-catenin with high affinity ($K_D = 12$ nM), which is consistent with the notion that this region serves as a high affinity anchor that allows the extended C-terminal tail to compete effectively with other ligands bound in the groove of β-catenin (9, 10). The ICAT helical domain is unique among β-catenin ligands in that it is structured in the absence of β-catenin, which implies that it has no loss of conformational entropy upon binding. This is reflected in the small, albeit slightly unfavorable, $\Delta S$ value (Table 1) and likely explains its high affinity even though it buries a relatively small amount of surface area relative to other β-catenin ligands (Table 2). The interaction with the ICAT helical domain occurs largely through formation of helix-helix packing interactions, in particular the hydrophobic interactions of ICAT residues Tyr$^{15}$, Val$^{22}$, Leu$^{26}$, and Met$^{27}$ with hydrophobic residues of β-catenin repeat 12. The extended region of ICAT alone is not sufficient for binding to β-catenin (9, 10), even though it buries 1700 Å$^2$ of surface upon binding and forms similar contacts with the arm repeat 5–9 groove seen as those present in the E-cadherin, Tcf, and APC complexes. This appears to be a general feature of these β-catenin ligands; and even though they share a highly conserved, critical set of interactions in the β-catenin arm repeat 5–9 groove, high affinity depends upon flanking regions that form interactions unique to each ligand.

**Axin and APC Binding**—Only 17 of the 70 amino acids present in the β-catenin binding domain of axin used in the crystallographic analysis were visible in β-catenin/axin structure (11), and 14 of 47 residues are ordered in β-catenin-APC-R3 complex structure (8). The small number of ordered residues associated with these interactions is consistent with a relatively small loss of translational, rotational, and conformational entropy associated with complex formation. This loss of entropy is overcome by entropically favorable hydrophobic interaction between β-catenin and APC-R3 or axin, resulting in the overall positive entropy changes. In the β-catenin-axin structure, 5 hydrophobic residues of axin, Ile$^{272}$, Leu$^{273}$, Val$^{277}$, Val$^{480}$, and Met$^{481}$, form hydrophobic contact β-catenin arm repeats 3 and 4. In the case of APC-R3, Leu$^{1488}$, Leu$^{1489}$, Phe$^{1491}$, Ala$^{1492}$, and Pro$^{1497}$ interact with β-catenin arm repeats 5–8. In addition to hydrophobic interactions, there are hydrophilic and hydrogen bond interactions that contribute to the favorable enthalpy change. Asp$^{376}$ from axin and Asp$^{3486}$ and Glu$^{1494}$ from APC-R3 form salt bridges with β-catenin Lys$^{292}$, Lys$^{435}$, and Lys$^{312}$, respectively. However, the surface areas buried in the interface with β-catenin are much smaller for axin and APC than those of the other ligands, which correlates with their relatively small binding enthalpies (Tables 1 and 2).

Phosphorylation of six serine residues of APC-R3 dramatically increases its affinity to a $K_D$ of 10 nM, which corresponds to 300 times tighter binding than the nonphosphorylated form (7, 8). Phosphorylation results in the ordering of an additional 25 amino acids relative to the nonphosphorylated R3 structure, which likely accounts for the change to an unfavorable $\Delta S$. The unfavorable entropy change is compensated by a much larger, favorable enthalpy change because of formation of many new contacts with β-catenin (Tables 1 and 2) (8).

**Effects of the β-Catenin N-terminal and C-terminal Tails**—The C-terminal tail of β-catenin has been reported to diminish the affinity of β-catenin for E-cadherin by binding directly to the arm repeats and directly competing with E-cadherin (25, 26). These experiments were performed by incubating GST fusion proteins of β-catenin constructs lacking one or both tails with E-cadherin or the purified tail, affinity-purifying the resulting complexes on glutathione-agarose beads, and detecting the bound proteins by Western blotting with an antibody to cadherin or the tail. By using similar methods, it was reported that the C-tail inhibits binding of cadherin but not Tcf-4 to β-catenin (27). Direct binding to the tail by the arm repeats was also detected by blot overlay assay, in which β-catenin is run on SDS-PAGE, transferred to nitrocellulose, incubated with the tail domain, and Western-blotted with antibody to the tail. The arm repeats were reported to bind to a peptide comprising the last 22 amino acids of β-catenin covalently coupled to Sepharose beads.

Because pulldown and blot overlay assays do not give absolute affinities, ITC was used to compare the affinities of four different constructs of β-catenin, with or without one or both tails (β-cat-full, β-cat-arm, β-cat-arm-Nt, and β-cat-arm-Ct), for Ecyto, Lef-1-(1–65), the ICAT helical domain (ICAT-h), APC-R3, and axin (Tables 4–8). The poor solubility and tendency of the β-catenin arm domain to aggregate at pH 7.5 or 8.0 required that these comparative experiments be carried out at pH 8.8. Comparison of the affinities of full-length β-catenin at these pH values with those at more neutral pH shows little or no effect on binding. The affinities of some of the ligands are independent of pH in this range, whereas others, in particular Ecyto and APC-R3, show slightly weaker affinities corresponding to very small energy differences at the higher pH. Therefore, comparison of different β-catenin constructs binding to the same ligand at pH 8.8 provides a meaningful comparison set for assessing the effect of the β-catenin tails.

The data show that the presence or absence of either or both β-catenin tail regions has no significant effect on binding to the strong ligands Ecyto, phosphoAPC, Lef-1, and ICAT-h. Most interestingly, the presence of the C-tail appears to weaken the affinity of axin slightly, at most 7-fold (compare β-cat-arm to β-cat-arm-Ct), and has a somewhat stronger effect on APC-R3, changing the affinity by up to 20-fold. The
E-cadherin for full-length blotting; data not shown). Moreover, comparison of full-length and down assays and staining with Coomassie Blue (instead of Western

**TABLE 5**

| β-Catenin constructs | $K_D$ | $\Delta H$ | $T \Delta S$ | $\Delta G$ | No. exp. |
|----------------------|-------|------------|--------------|------------|---------|
| β-cat-full           | $5.0 \times 10^{-9}$ | $-16.0 \pm 0.1$ | $-9.8 \pm 1.1$ | $-10.0 \pm 0.1$ | 1       |
| β-cat-arm            | $1.1 \times 10^{-9}$ | $-21.0 \pm 0.2$ | $-12.0 \pm 0.3$ | $-14.0 \pm 0.4$ | 1       |
| β-cat-arm-Nt         | $2.4 \times 10^{-9}$ | $-25.0 \pm 0.1$ | $-15.0 \pm 0.3$ | $-18.0 \pm 0.4$ | 1       |
| β-cat-arm-Ct         | $4.7 \times 10^{-9}$ | $-31.0 \pm 0.2$ | $-22.0 \pm 0.3$ | $-25.0 \pm 0.4$ | 1       |

**TABLE 6**

| β-Catenin constructs | $K_D$ | $\Delta H$ | $T \Delta S$ | $\Delta G$ | No. exp. |
|----------------------|-------|------------|--------------|------------|---------|
| β-cat-full           | $1.1 \times 10^{-9}$ | $-27.0 \pm 0.1$ | $-19.0 \pm 0.3$ | $-29.0 \pm 0.4$ | 1       |
| β-cat-arm            | $1.0 \times 10^{-9}$ | $-30.0 \pm 0.2$ | $-21.0 \pm 0.3$ | $-31.0 \pm 0.4$ | 1       |
| β-cat-arm-Nt         | $4.8 \times 10^{-9}$ | $-28.0 \pm 0.1$ | $-21.0 \pm 0.3$ | $-31.0 \pm 0.4$ | 1       |
| β-cat-arm-Ct         | $1.5 \times 10^{-9}$ | $-36.0 \pm 0.2$ | $-26.0 \pm 0.3$ | $-36.0 \pm 0.4$ | 1       |

**TABLE 7**

| APC-R3 constructs | $K_D$ | $\Delta H$ | $T \Delta S$ | $\Delta G$ | No. exp. |
|------------------|-------|------------|--------------|------------|---------|
| APC-R3 (T2 buffer) | $1.1 \times 10^{-9}$ | $-16.0 \pm 0.1$ | $-11.0 \pm 0.2$ | $-17.0 \pm 0.3$ | 1       |
| APC-R3 (H buffer) | $1.0 \times 10^{-9}$ | $-16.0 \pm 0.1$ | $-11.0 \pm 0.2$ | $-17.0 \pm 0.3$ | 1       |

**TABLE 8**

| β-Catenin constructs | $K_D$ | $\Delta H$ | $T \Delta S$ | $\Delta G$ | No. exp. |
|----------------------|-------|------------|--------------|------------|---------|
| β-cat-full           | $1.6 \times 10^{-9}$ | $-10.0 \pm 0.2$ | $-6.0 \pm 0.3$ | $-15.0 \pm 0.4$ | 1       |
| β-cat-arm            | $0.5 \times 10^{-9}$ | $-14.0 \pm 0.2$ | $-8.0 \pm 0.3$ | $-23.0 \pm 0.4$ | 1       |
| β-cat-arm-Nt         | $0.6 \times 10^{-9}$ | $-12.0 \pm 0.2$ | $-6.0 \pm 0.3$ | $-21.0 \pm 0.3$ | 1       |
| β-cat-arm-Ct         | $2.2 \times 10^{-9}$ | $-15.0 \pm 0.3$ | $-9.0 \pm 0.4$ | $-23.0 \pm 0.5$ | 1       |

ITC data indicate that the C-tail does not block or interfere with $E_{cyst}$ binding to β-catenin. These results contradict an earlier study showing that the arm domain alone (β-cat-arm) and the C-tail deletion mutant (β-cat-arm-Nt) bind to $E_{cyst}$, with higher affinities than wild type (β-cat-full) or the N-tail deletion mutant (β-cat-arm-Ct). Also, the mutant lacking the N-tail was reported to bind very little $E_{cyst}$ (26), but ITC data clearly show that this mutant (β-cat-arm-Ct) binds to $E_{cyst}$ with a $K_D$ of 60 nM, which is similar or even lower than those of β-cat-arm-Nt and β-cat-arm. The reason for this discrepancy is not apparent, but differences in experimental conditions or the method of detection may be issues. We note, however, that we have found similar binding affinities of $E_{cyst}$ for the four different constructs of β-catenin using GST pull-down assays and staining with Coomassie Blue (instead of Western blotting; data not shown). Moreover, comparison of full-length and β-cat-arm-Nt at pH 8.0 reveals no effect of the C-tail on binding. Finally, comparison of all four β-catenin constructs at pH 8.8 shows no effect of the C-tail, and there is only a very small (2-fold) difference in affinity of E-cadherin for full-length β-catenin at pH 8.0 versus 8.8.

We also tested whether the C-tail can bind to the β-catenin arm domain in trans, as reported earlier. Using Coomassie-stained gels in GST pulldown assays, we could not detect binding of the tail to the arm domain even when a 170-fold molar excess of the C-tail (1.4 mM) was added to 8 mM GST-β-cat-arm that had been immobilized on glutathione-agarose (data not shown). To more closely replicate earlier experiments, these pulldown experiments were also analyzed by Western blotting using a monoclonal antibody to the C-tail of β-catenin. Although C-tail was detected, the intensity of each bound band was similar to that of the background binding control of the C-tail to glutathione-agarose beads (data not shown). Castañó et al. (26) suggested that the C-terminal 22 amino acids of β-catenin specifically bind to the arm domain with similar interactions with those observed in $E_{cyst}$-β-cat-arm structure, based on the sequence alignments of C-tail (760–781) and $E_{cyst}$ (655–677). This proposal aligns key hydrophobic and acidic residues present on the face of a helix in E-cadherin (region 2; see Ref. 2), and the arm repeats bound to this peptide immobilized on agarose. However, this alignment places the C-tail sequence Pro-Pro-Gly...
sequence in the middle of the α-helix in the E_caten structure, which is extremely unlikely. We speculate that the interaction observed is because of the basic nature of the arm repeats, which might bind to the acidic peptide; the negative control with unmodified agarose beads might not show this interaction.

E_caten, Lef-1, and APC-R3 form virtually identical interactions with the arm repeat 5–9 groove, yet only APC-R3 appears to be affected by the presence of the C-tail. These results indicate that the proposed mechanism in which the C-tail binds back to the arm domain and acts as a competitive inhibitor cannot be correct. If there is an equilibrium between an "open" form of β-catenin in which the arm domain is accessible to a ligand and a "closed" form in which the tail binds in the repeat 5–9 groove, then the fraction of accessible open form will be the same for all ligands under a given experimental condition. In this case, the equilibrium constants for all of the ligands that interact with this site would have to be diminished by the same factor. However, E_caten and Lef-1, both of which bind to the groove at arm repeats 5–9, show no dependence on the tails, whereas APC-R3, which also binds to this region, is affected. Indeed APC-R3 and axin bind to nonoverlapping regions of the arm domain and show effects of the C-tail, but none of the other ligands that shares one or both of these sites (E_caten, Lef-1) shows no region, is affected. Indeed APC-R3 and axin bind to nonoverlapping regions of the arm domain and show effects of the C-tail, but none of the other ligands that shares one or both of these sites (E_caten, Lef-1) shows no

Overall, the thermodynamics of ligand binding to β-catenin clearly show that the β-catenin tails can affect the affinity of the interaction of the destruction complex ligands axin and APC with the arm domain, which may imply a role in regulating β-catenin turnover. However, the results rule out models in which the tails bind to the arm domain and thereby act as competitive inhibitors. Further studies will be required to understand the molecular basis of this effect.

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