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Biochemical and structural characterization of β -catenin interactions with non-phosphorylated and CK2-phosphorylated Lef-1

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Abstract

In the Wnt/ β -catenin signaling pathway, β -catenin activates target genes through its interactions with the TCF/Lef family of transcription factors. The crystal structures of complexes between the β -catenin armadillo domain and the Lef-1 N-terminal domain show that the overall conformation and many of the interactions are similar to other published structures of TCFs bound to β -catenin. However, a second salt bridge in other TCF- β -catenin structures is absent in the structure of β -catenin-Lef-1 complex, indicating that this feature is not obligatory for β -catenin binding. Casein Kinase II (CK2) has been shown to act as a positive regulator of Wnt signaling, and that Lef-1 is a substrate of CK2. *In vitro* phosphorylation of purified Lef-1 was used to examine the effect of CK2 on the interaction of Lef-1 with β -catenin. Mass spectrometry data show that CK2 phosphorylation of Lef-1 N-terminal domain results in a single phosphorylation site at Ser 40. Isothermal titration calorimetry revealed that β -catenin binds to non-phosphorylated or CK2 phosphorylated Lef-1 with the same affinity, which is consistent with the absence of phosphoSer40 interactions in the crystal structure of phosphorylated Lef-1 N-terminal domain bound to β -catenin. These data indicate that the effect of CK2 on the Wnt/ β -catenin pathway does not appear to be at the level of the Lef-1- β -catenin interaction.

Keywords

Wnt signaling; TCF; crystal structure; Lef; CK2; phosphorylation; binding affinity

Introduction

The Wnt/ β -catenin signaling pathway has essential roles in specification of cell fate, stem cell differentiation, and oncogenesis^{1, 2}. Binding of a Wnt protein to cell surface co-receptors results in the stabilization of cytosolic β -catenin, a transcriptional coactivator. The stabilized β -catenin enters the nucleus and binds the Tcf/Lef family of DNA binding proteins, which includes TCF1, LEF-1, TCF3, and TCF4³. The β -catenin/Tcf complex is thought to recruit general transcription factors that ultimately result in the activation of target genes. β -Catenin binds to a conserved N-terminal region present in Tcf family

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members. In the absence of Wnt/ β -catenin signaling, TCF/Lef is bound to Groucho/TLE-family co-repressors, which keeps Wnt target genes turned off^{3; 4}.

The primary structure of β -catenin features an N-terminal “tail” predicted to have no intrinsic three-dimensional structure, a central armadillo (arm) repeat domain and an unstructured C-terminal acidic tail that is essential for transcriptional activation. The arm domain, comprised of twelve armadillo repeats which form an elongated superhelix of α helices, mediates binding to TCF/LEF proteins, the β -catenin destruction complex components Axin and Adenomatous Polyposis Coli (APC), and the cytoplasmic domain of classical cadherins. TCFs, APC and cadherins share a characteristic amino acid sequence motif $Dx\theta\theta x\phi x_{2-7}E$, where θ is an aliphatic hydrophobic and ϕ is an aromatic residue, which crystal structures have shown bind to the β -catenin superhelical groove that spans arm repeats 5–10 (see Refs. ^{5; 6} and references therein). The hydrophobic residues participate in non-polar contacts, and the conserved acids form salt bridges with positively charged residues of β -catenin. All Tcf/Lef family members feature a cluster of negatively charged amino acids in the sequence between 23–29 (human Tcf-4 numbering), which includes the conserved glutamate.

Casein Kinase II (CK2), a ubiquitous and highly conserved serine/threonine kinase, functions as a positive regulator of Wnt signaling, and appears to be upregulated in many cancers^{7; 8}. CK2 enhances β -catenin mediated transcription activation by Lef-1. Chromatin immunoprecipitation experiments show that CK2 and β -catenin cycle on and off DNA out of phase with the co-repressor TLE1⁹, consistent with the competitive binding of β -catenin and TLE1 for Lef-1¹⁰. CK2 phosphorylates the Lef-1 N-terminal domain, which appears to increase the affinity of Lef-1 for β -catenin and so would favor activating complexes^{9; 11}.

In order to compare the interactions of Tcf family members with β -catenin, we have determined crystal structures of non-phosphorylated and CK2-phosphorylated Lef-1 bound to the armadillo domain of β -catenin and Lef-1. Previous isothermal titration calorimetry (ITC) experiments have suggested that the Tcf-4 and Lef-1 affinities for β -catenin are somewhat different^{5; 12}. Here, we present ITC measurements of Lef-1 and Tcf4 binding to β -catenin under identical conditions, which show that Lef-1 binds slightly less strongly than Tcf4 to β -catenin. The small affinity difference correlates with the absence of a salt bridge in Lef-1 that is present in all other β -catenin ligands. We also find that CK2 phosphorylation of Lef-1 produces no change in affinity or in the structure of the complex, indicating that the effects of CK2 on β -catenin-mediated transcription are not at the level of the Lef-1- β -catenin interaction.

Results

Overall structure of the β -catenin–Lef-1 complex

The complex of the proteolytically-defined murine β -catenin arm repeat domain, residues 134–671¹³, referred to as β -cat-arm, and the N-terminal β -catenin binding domain (β BD) of murine Lef-1, residues 1–63, was purified and crystallized. The structure of the complex was determined by molecular replacement and refined at 2.9 Å (Table 1; Fig. 1). There is one β -cat-arm–Lef-1(1–63) complex in the asymmetric unit, and the two proteins form a 1:1 complex. Residues 157–664 of β -catenin are visible, except for a break between residues 550–561. Residues 11–25 and 48–60 of Lef-1(1–63) are observed; the remaining residues are not visible and are presumably disordered.

The structure of the β -catenin arm domain in the complex is very similar to previously published structures of this domain alone and bound to other ligands. Likewise, the bound conformation of Lef-1 is similar to that of other TCF family members (see below). Lef-1

runs anti-parallel to the long axis of β -catenin, and lies along the positively charged groove of that spans arm repeats 2–10 (Fig. 1). Residues 11–25 of Lef-1 bind as an extended peptide to arm repeats 5–10, and residues 48–60 form an amphipathic α helix that binds to repeats 2–5 (Fig. 2).

The extended N-terminal region of Lef-1 binds to β -catenin using most of the conserved sequence motif Dx00x ϕ x₂–7E. There are relatively few direct contacts between Lef-1 residues 11–18 and β -catenin, which include a salt bridge between Asp12 and Arg515, and the backbone carbonyl oxygen of Gly11 forms a hydrogen bond with Arg582 (Fig. 2c). The first residue of the conserved motif, Asp19, forms salt bridges with Lys435 and Arg474 and non-polar contacts with Cys429 and His470 of β -catenin (Fig. 2d). In addition, β -catenin Asn430 forms hydrogen bonds with the backbone in this region. The side chain of Lef-1 Met21 packs against β -catenin residues Asp390, Thr393, Asn426 and Cys429 (Fig. 2d). The side chain of Ile22 packs against Asn426, which also forms hydrogen bonds with the Lef-1 backbone. Phe24 packs primarily against Arg386, and also Gly422 (Fig. 2d). The conserved Asp19, Met21, Ile22 and Phe24 superimpose very well with those of the known Tcf– β -catenin crystal structures, suggesting they are major determinants of binding specificity (see below). A striking difference with other Tcfs, however, is the absence of any salt bridge involving the glutamate of the Dx00x ϕ x₂–7E motif, Glu27, which is disordered.

Effect of CK2 phosphorylation of Lef-1 on binding to β -catenin

CK2 targets the consensus substrate sequence motif S/TxxD/E¹⁴. A previous study of human Lef-1 reported that Ser42 and Ser61, equivalent to murine Lef-1 Ser40 and Ser59, are phosphorylated by CK2⁹. Phosphorylated murine Lef-1(1–63), designated pLef-1(1–63), was prepared by treating the purified recombinant protein with CK2. MALDI-TOF mass spectrometry of this material gave two mass peaks with a difference of 80 Daltons, which corresponds to the mass of a phosphate group, indicating mono-phosphorylation of Lef-1(1–63) (Fig. 3a). To map the phosphorylation site, pLef-1(1–63) was digested with trypsin and the fragments analyzed by LCQ mass spectrometry. The peptide containing Ser 40 (residues 35–51) was found in two mass peaks of 2014.18 and 2094.16 Da, whereas the peptide containing Ser 59 (residues 53–65) was present in a single mass peak 1346.47 Da, which is the unphosphorylated mass of the peptide. Furthermore, MS/MS fragmentation patterns of the digested fragments confirm that Ser 40 is phosphorylated (data not shown). Therefore, only Ser 40 of Lef-1(1–63) is phosphorylated by CK2, and Ser 59 is not phosphorylated, which is consistent with the fact that Ser 40, but not Ser59, is a consensus CK2 phosphorylation site.

The difference between the results presented here and the previous study of human Lef-1⁹ is not readily explained. The sequences of human and murine Lef-1 β -catenin binding domain are almost identical, except that human Lef-1 has two extra glycines in the N-terminal poly-Gly region starting at residue 5. We considered that the use of different length Lef-1 fragments gave rise to the discrepancy: the study with human Lef-1 employed a construct in which the β -catenin binding domain 1–75 was fused directly to the C-terminal HMG domain¹⁵, whereas we used murine Lef-1 residues 1–65. To test whether the presence of additional residues C-terminal to the conserved β -catenin binding gave rise to the additional phosphorylation sites, a longer murine Lef-1 fragment, comprising residues 1–131, was purified and phosphorylated with CK2 *in vitro*, and analyzed by mass spectrometry. Again, only Ser40 was phosphorylated (data not shown). A possible explanation for the difference in the two studies is a possible contamination of other kinases in the CK2 preparation, although both groups obtained CK2 from the same supplier (K. Jones, personal communication).

In order to assess the possible effects of CK2 phosphorylation on the Lef-1- β -catenin interaction, the complex of the CK2-phosphorylated Lef-1(1–63) and β -cat-arm was purified and crystallized. Note that only about 70% of the Lef-1(1–63) in this complex was phosphorylated in the complex used in this analysis, but the crystals were obtained under different conditions from those of the non-phosphorylated complex. Also, the non-phosphorylated and pLef-1 complexes crystallize in the same tetragonal space group, but the length of the *c* axis is considerably shorter in the pLef-1(1–63) complex, giving a correspondingly lower solvent content and a higher resolution diffraction limit of 2.4 Å.

The overall structures of non-phosphorylated Lef-1(1–63) and phosphorylated Lef-1(1–63) bound to β -catenin are almost identical, except that residues 26–41 of Lef-1 are visible in the phosphorylated structure, where they form an extended loop and participate in both intra- and intermolecular contacts (Fig. 3b,c). Importantly, the temperature factors of Lef-1 undergo a striking increase at Ser40, and the side chain of this phosphoserine is disordered. pLef-1 Gln31 forms an intramolecular hydrogen bond with Lys34, and Glu33 of pLef-1(1–63) interacts with Arg386 of β -catenin (Fig. 3b). The backbone of Lef-1 at residues Glu33 and Lys34 also contacts β -catenin Trp383.

The order of the Lef-1 26–42 region in the phosphorylated structure can be attributed to crystal packing interactions. The phospho-Lef-1- β -catenin complex crystallizes in the same tetragonal space group, but with a smaller *c* axis, than the non-phosphorylated complex (Table 1). In this smaller lattice, Lef-1 Asp26, Gly28, Pro30, Gln31, Glu33, Phe36, Ala37, and Glu38 interact directly with neighboring β -catenin molecules (Fig. 3c); note that Glu33 forms a salt bridge with Arg647 of a neighbor as well as Arg386 (see above). It seems likely that the differences in crystallization conditions, possibly due to the altered charge on the exposed phosphoSer40, give rise to this change in the lattice. Lattice formation likely selects one of a number of low energy conformations sampled by this loop in solution, which would imply that the salt bridge formed between Glu33 and Arg386 can occur at least transiently. The significance of the latter interaction is unclear, however, as the Lef-1 mutant K32A/E33A/K34A has no effect on β -catenin binding in a pulldown assay¹⁶.

Slightly more of the Lef-1 α -helix is visible in the phosphorylated structure (residues 45–60 vs. 48–60) (Fig. 1), which may be due to the relative order of the preceding stretch of Lef-1. The helix binds principally through hydrophobic residues on one face that pack into hydrophobic pockets on β -catenin (Leu48, Ile51, the aliphatic portion of Lys52, Leu55 and Val56). Asp47 forms a salt bridge with Arg376 (Fig. 2a, b). In both structures, the Ser59 side chain is poorly ordered, even though it faces β -catenin. Modeling shows that if Ser59 was phosphorylated, the phosphoserine side chain could not be accommodated without displacing the helix away from β -catenin, again consistent with the mass spectrometry results that CK2 treatment did not phosphorylate this residue.

To confirm that phosphorylation of Lef-1 Ser40 by CK2 does not affect the interaction with β -catenin, we measured the binding affinity of β -catenin and purified Lef-1(1–63) or pLef-1(1–63) by ITC, using pLef-1(1–63) prepared by treating purified Lef-1(1–63) with CK2 and then purified C18 reverse phase HPLC to separate the phosphorylated and unphosphorylated polypeptides (Fig. 4a,b). The affinities of β -catenin for Lef-1(1–63) and pLef-1(1–63) are 23 and 35 nM, respectively, and the differences in the measured affinities and enthalpies are statistically insignificant (Table 2). Overall, the disorder of Lef-1 residues 26–42 in the non-phosphorylated structure and the absence of significant thermodynamic differences in β -catenin binding by non-phosphorylated and phosphorylated Lef-1 indicate that residues 26–42, including Glu33, do not contribute significantly to the binding energy, irrespective of Ser40 phosphorylation by CK2.

Comparison with other TCFs

The high-resolution structure of the pLef-1(1–63) affords the opportunity to compare the structures of several TCF family members bound to β -catenin. The backbone structures of all the known TCFs and Lef-1 in their β -catenin bound conformations are superimposed in Fig. 5a. TCF/Lef proteins all bind similarly to β -catenin with an extended N-terminal region that binds to arm repeats 5–10 and a C-terminal α -helix that interacts with repeats 2–5. The region in between is typically disordered, with the exception of one TCF4 structure, which contains an additional kinked α helix¹⁷.

The disorder of the first 10 residues of Lef-1 is consistent with the disorder of the equivalent region in human TCF4 structures^{17; 18} and with deletion mutagenesis indicating that this region does not contribute significantly to binding^{12; 18; 19; 20}. Residues 11–18 adopt a conformation different from the equivalent portions of other Tcfs, which may be due to differences in lattice packing, as neighboring molecules of β -catenin interact with some of this region of Lef-1. (Interestingly, pLef-1 Cys16 forms a disulfide bond with β -catenin Cys350 from a lattice neighbor, even though its conformation is the same in the non-phosphorylated structure.) It is also possible that some of the difference arises from the insertion of Pro13 between two acids that are otherwise adjacent in sequence in the other Tcfs (Fig. 5b); Lef-1 Asp12 forms a salt bridge with β -catenin Arg515 (Fig. 2c), whereas the equivalent interaction is absent in other TCF- β -catenin structures.

Lef-1 Asp19 represents the start of the conserved Dx00x0x2_7E β -catenin binding motif, and Asp19, Met21, Ile22, and Phe24 interact with β -catenin similarly to other ligands (Fig. 2d, 5b,c). The extensive interactions of the conserved Asp suggest that it is crucial for binding for β -catenin. Mutation of β -catenin residues that interact with Asp19 (Lys435, His470) and Phe24 (Arg386) interfere strongly with binding to β -catenin^{17; 20; 21}. Mutation of Lef-1 Asp1916 or the equivalent TCF4 Asp16 to Ala^{12; 19} dramatically reduces binding to β -catenin. Curiously, mutation of any one of the conserved hydrophobic residues of the motif to Ala in TCF4 has little effect on binding affinity, although the enthalpic and entropic contributions change¹².

The most striking difference between Lef-1 and other TCFs is the absence of the second salt bridge mediated by the conserved glutamate. The interactions of this glutamate with β -catenin vary significantly among the TCF/Lef proteins as well as other β -catenin ligands. In the TCF4- β -catenin complex structures, TCF4 adopts two different modes of interactions with β -catenin: either Glu24 or Glu29 of TCF4 can form a salt bridge with Lys312 (Fig. 5b,d), and Glu24 of xTCF3 likewise interacts with β -catenin Lys312^{17; 18; 21} (Fig. 5b,d). The glutamate in the conserved motifs of E-cadherin, a 20 amino acid repeat of APC, and the transcriptional inhibitor ICAT also forms a salt bridge with β -catenin Lys312^{6; 22; 23; 24; 25}. In contrast, in the 15 amino acid repeats of APC the conserved glutamate forms a salt bridge with β -catenin Lys345, rather than Lys312²⁶.

In the Lef-1 complexes, neither β -catenin Lys312 nor Lys345 makes a direct contact with Lef-1. Lys312 instead forms a salt bridge with Glu649 of a neighboring β -catenin molecule in the crystal lattices of both non-phosphorylated and phosphorylated complexes. Lef-1 residues 26–41, which include both of the glutamates (27 and 33) that form the second salt bridge in the other TCF structures, are disordered in the non-phosphorylated Lef-1 complex. In the phosphorylated complex, Glu27 is poorly ordered and faces solvent, whereas Glu33 interacts with β -catenin Arg386 (Fig. 3b), an interaction that appears to be stabilized by contact with a neighboring molecule in the lattice (see above). Although all TCF family members have a negatively charged cluster in this region, a proline (Pro 30) is inserted into Lef-1 relative to TCF4 and TCF3 (Fig. 5b), which might restrict the chain from forming the second salt bridge. In any case, the absence of the second salt bridge is consistent with the

observation that triple mutants of Lef-1 which remove either of the acids homologous to those in TCF4 that interact with β -catenin Lys312, K25A/D26A/E27A or K32A/E33A/K34A, have no effect on binding to β -catenin¹⁶. In contrast, mutation of the homologous residues (Asp24 and Asp29) in xTCF3 and TCF4 weakens the interaction^{12; 17; 21}, and the Lys312A mutation causes a reduction in binding to TCF4²⁷, although the effects of these changes are much smaller than those that alter the first salt bridge involving the conserved Asp and Lys435.

Given the difference in the structures of TCF-4 and Lef-1 bound to β -catenin, it was of interest to compare the thermodynamics of these complexes. The binding affinity of β -catenin for TCF4 (residues 1–57) was measured by ITC. TCF4 binds to β -catenin with a K_D of 16 nM, which is only slightly stronger than the Lef-1 interaction (Fig. 4c, Table 2). Other ITC studies have reported somewhat higher affinities for TCF4^{12; 28}. The difference is likely due to the difference in buffer conditions, as seen in ITC measurements of APC 20-mer repeat 3 binding to β -catenin^{5; 23; 25; 29}. The small difference in binding affinities between TCF4 and Lef-1 correlates with both the modest contribution that the conserved glutamate makes to the interaction with β -catenin seen in TCF4, and the absence of the second salt bridge in Lef-1.

Discussion

Crystal structures have defined a consensus peptide motif Dx00x0x₂₋₇E for binding to the groove formed by arm repeats 5–10 of β -catenin. These ligands are intrinsically unstructured sequences that bind as extended peptides^{28; 29; 30}. The hydrogen bonding potential of the peptide backbone amide and carbonyl groups is satisfied by a regular ladder of polar residues, typically asparagine, present in the groove of β -catenin in the same position of successive arm repeats. The side chains of the first four conserved residues of the sequence motif Dx00x0x₂₋₇E, *i.e.*, the aspartate, the two adjacent aliphatic non-polar residues, and the aromatic are seen to mediate very similar interactions with β -catenin (the second aliphatic residue can be replaced by methylene groups of a polar side chain like lysine in some cases). Unlike the fixed position of the first four conserved residues in the motif, the conserved glutamate occurs at a variable spacing from the aromatic residue. The Lef-1 data demonstrate that this glutamate does not necessarily contribute to the binding interaction. It is possible that it contributes to the kinetics of binding by helping to correctly position the peptide in the initial encounter with β -catenin, or that Lef-1 represents a singular example in which changes in the surrounding sequence, such as insertion of prolines, renders it unable to form direct, stable contacts.

Various lines of evidence indicate that CK2 serves as an activator of Wnt signaling through its action on various components of the pathway, but the molecular details are not clear^{8; 9; 11; 31}. CK2 phosphorylates β -catenin at Thr393 in the arm region, which results in increased stability of β -catenin and activation of Wnt signaling^{32; 33}. Modeling shows that an acceptable phosphothreonine conformation can be accommodated on the structures presented here with no steric clash (data not shown). In this conformation, the closest approach to the bound Lef-1 ligand is 4.2 Å between a phosphate oxygen and Sy of Met21; the Thr methyl group is in the same position as shown in Fig. 2d and contacts Cε of Met21. More interestingly, the phosphate group can form a hydrogen bond with β -catenin Asn430, which binds to the backbone of extended peptide β -catenin ligands, including Lef-1 Asp19 (Fig. 2d). The effect of this potential interaction is not easy to predict, but it might stabilize the conformation of Asn430. Note that the side chain of Glu20 points away from the protein and so is not expected to interact with a phosphorylated Thr393.

Although we find that CK2 phosphorylation of Lef-1 does not enhance its interaction with β -catenin, it has been shown that this modification decreases Lef-1 binding to the Gro/TLE1 co-repressor⁹. Since TLE1 binds antagonistically with β -catenin to DNA-bound Lef-1, decreasing affinity for TLE1 would also favor transcriptional activation. Further mechanistic studies will be required to explore this hypothesis.

Methods

Protein expression and purification

Constructs encoding the full-length β -catenin (residues 1–781) and the arm domain (β -cat-arm; residues 134–671) were expressed as glutathione S-transferase (GST) fusion proteins. The β -cat-arm construct contains a thrombin cleavage site after the GST. The full-length β -catenin construct has an N-terminal GST followed by a tobacco etch virus (TEV) protease recognition site using a modified pGEX-KG vector, pGEX-TEV. The β -catenin binding regions of murine Lef-1 and human TCF4, comprising residues 1–65 and 1–57 respectively, were produced with a N-terminal His₆-tag and a cleavage site for TEV protease. Cells were grown in LB medium at 37°C to an A₆₀₀ of 0.6–0.8, induced with 0.5 mM or 1 mM isopropyl- β -D-thiogalactopyranoside for β -catenin and Lef-1, respectively, and grown for another 3–4 hr at 30°C.

β -catenin cell pellets were re-suspended in 20 mM pH 8.0 Tris-Cl, 250 mM NaCl, 10% glycerol, 2 mM β -mercaptoethanol; Lef-1 was re-suspended in the same buffer with pH 7.5 Tris-Cl. Before cell lysis, protease inhibitor mixture (CompleteTM; Roche Applied Science), 2.5 μ g/ml pepstatin, and 0.2 mM PMSF were added. Cells were lysed using an SLM-AMINCO French Press at maximum pressure of 1200 psi, and lysates were spun at 18,000 $\times$ g for 30 minutes at 4°C to remove any insoluble material. Clarified lysates were incubated with glutathione-agarose beads for GST fusion proteins or Ni²⁺-NTA-agarose beads for His₆-tagged protein for 1 hr at 4°C.

For GST fusion proteins, glutathione-agarose beads were washed first with phosphate-buffered saline containing 1 M NaCl, 0.005% Tween 20, and 5 mM dithiothreitol (DTT) and then with cleavage buffer consisting of 25 mM pH 8.5 Tris-Cl, 100 mM NaCl, and 2 mM DTT. Cleavage of the GST tag was performed with the addition of TEV protease (4°C overnight for full-length β -catenin) or thrombin (4°C for 2 h for β -cat-arm). The cleaved protein was collected from the flow-through and loaded onto a Mono Q column (GE Healthcare) equilibrated with buffer A (25 mM ethanolamine pH 9.5, 1 mM DTT, 1 mM EDTA) for β -cat-arm and buffer B (25 mM pH 8.5 Tris-Cl, 2 mM DTT, 0.5 mM EDTA) for full-length β -catenin and was eluted with a linear gradient of NaCl. Mono Q purified full-length β -catenin was loaded onto a Superdex 200 gel filtration column (GE Healthcare) equilibrated with buffer T2 (25 mM pH 8.8 Tris-Cl, 2 mM DTT, and 0.1 M NaCl).

For purification of Lef-1 and TCF4 with the N-terminal His₆-tag, Ni²⁺-NTA resin was washed in buffer containing 20 mM pH 7.5 Tris-Cl, 250 mM NaCl, 2 mM β -mercaptoethanol, 20 mM imidazole, and eluted in the same buffer containing 250 mM imidazole and 150 mM NaCl. For ITC, the eluted protein was further purified using gel filtration chromatography equilibrated with buffer T2. For crystallization, the eluted protein was cleaved with 0.025 mg/ml TEV protease and dialyzed in buffer consisting of 20 mM pH 7.5 Tris-Cl, 100 mM NaCl, 5 mM β -mercaptoethanol at 4°C for 16 hours. The cleaved His₆-tag was removed by incubating cleaved protein with Ni²⁺-NTA-Agarose, and flow-through was collected.

The β -cat-arm–Lef-1(1–63) complex was made by mixing β -cat-arm with excess of cleaved Lef-1(1–63). The protein mixture was concentrated using an Amicon Centricon Plus 20

concentrator (PES membrane, 5000 MWCO) before loaded onto a Superdex 200 gel filtration column equilibrated with buffer A and 75 mM NaCl, and the eluted complex was concentrated to 7.5mg/ml.

Phosphorylated Lef-1(1–63) was prepared by incubating purified Lef-1(1–63) with CK2 (New England Biolabs) overnight at 30°C, using approximately 0.05 units/pmol Lef-1. For crystallization experiments the phosphorylated material was further purified by MonoQ and gel filtration chromatography. For ITC experiments, phosphorylated and non-phosphorylated Lef-1(1–63) were separated by HPLC on a C-18 reverse phase column (Vydac 218TP510) using a 5 – 65% acetonitrile gradient in 0.1% trifluoroacetic acid. The volatile solvents were removed by lyophilization and the peptide resuspended in buffer for ITC.

Crystallization, data collection and processing

Crystals resembling clusters of plates were obtained using the hanging drop vapor diffusion method at 23°C, with a well solution of 4.2% PEG 4000, 100 mM sodium citrate pH 5.0 (β -cat-arm–Lef-1(1–63) complex), and 7% PEG 3000, 100 mM sodium citrate pH 5.0 (β -cat-arm–pLef-1(1–63) complex), using a protein solution of 100 μ M β -cat-arm and Lef-1.

Diffraction data for each complex were measured from a single crystal at the Stanford Synchrotron Radiation Laboratory beamlines 11-1 (β -cat-arm–Lef-1(1–63)) and 9-1 (β -cat-arm–pLef-1(1–63)). The crystals were adapted directly to a buffer containing well solution plus 20% glycerol for β -cat-arm–Lef-1(1–63) crystals and 20% PEG 200 for β -cat-arm–pLef-1(1–63) crystals, and frozen in liquid nitrogen. For the β -cat-arm–Lef-1(1–63) complex, a total of 60° of data were collected in 3 segments, using a 90 second exposure and 1° oscillation frames, with a 370 mm crystal-to-detector distance on MAR325 CCD detector. For the β -cat-arm–pLef-1(1–63) complex, a total of 50° of data were collected in 2 segments, using a 40 second exposure and 1° oscillation frames, with a 350 mm crystal-to-detector distance on an ADSC Q315R CCD detector. Diffraction data were processed and scaled using Mosflm and Scala^{34, 35}. There is one β -catenin–Lef-1(1–63) or β -catenin–pLef-1(1–63) complex per asymmetric unit, with a solvent content of 57% and 51%, respectively.

Phasing, model building and refinement

The structure of the phosphorylated complex was solved first due to its higher resolution, and the refined model of the phosphorylated structure was used to solve the non-phosphorylated structure. Phases for the phosphorylated structure were obtained by molecular replacement with Phaser³⁶, using β -catenin molecule A from the β -catenin–ICAT complex structure⁶ (Protein Data Bank ID 1M1E). The Z-scores for the rotation and translation functions were 10.0 and 20.7, respectively. Rigid-body refinement in CNS³⁷ using data from 99 – 3Å gave R and R_{free} of 48.8% and 49.1%, respectively. For the non-phosphorylated structure, the rotation and translation function Z-scores were 20.3 and 51.8. Rigid-body refinement in CNS using data from 99 – 3Å gave R and R_{free} of 29.4% and 26.8%, respectively. The model of the complex was built in Coot³⁸. Refinement was performed using CNS, Phenix³⁹ and BUSTER⁴⁰. For test sets, 4% of reflections for β -catenin–Lef-1(1–63) complex and 6% of reflections for β -catenin–pLef-1(1–63) complex were removed. Bulk solvent and anisotropic temperature factor corrections were applied throughout the refinement. Final refinement statistics are shown in Table 1.

Isothermal Titration Calorimetry

ITC measurements were performed at 30°C using a VP-ITC calorimeter (Microcal, Inc.). Purified Lef-1(1–63), pLef-1(1–63), TCF4(1–57) and full-length β -catenin were

concentrated to 80 – 100 μM and 8 – 12 μM respectively in T2 buffer. Lef-1 or Tcf4 was injected into a solution of β -catenin. Each titration experiment was initiated by two 1- μl injections, followed by 30–35 8–10 μl injections. Blank titrations were carried out by injecting Lef-1(1–63) into T2 buffer (25 mM pH 8.8 Tris-Cl, 2 mM DTT, and 0.1 M NaCl). The association constant K_A , enthalpy change ΔH , and the stoichiometry n were obtained from fitting the data⁴¹ using the Origin software package (Microcal, Inc.). The dissociation constant K_D , the free energy ΔG , and the entropy change ΔS , were obtained from the basic thermodynamic relationships $K_D = K_A^{-1}$, $\Delta G = -RT\ln K_A$, and $\Delta G = \Delta H - T\Delta S$.

Accession numbers

Coordinates and structure factors have been deposited in the Protein Data Bank, with accession numbers 3OUW (non-phosphorylated Lef-1(1–63) complex) and 3OUX (phosphorylated Lef-1(1–63) complex).

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Abbreviations

CK2	Casein Kinase II
ITC	Isothermal Titration Calorimetry
TCF	T-cell factor
LEF	lymphoid enhancing-binding factor

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Fig. 1. Overall view of the β -cat-arm-Lef-1(1-63) complex

Ribbon representation of the complexes. The β -catenin arm domain is shown in grey. The non-phosphorylated Lef-1(1-63) is shown in blue, and the phosphorylated Lef-1(1-63) in turquoise. The sequence of Lef-1(1-63) is shown at the bottom, and the visible portions of the sequence are indicated for the two structures as a solid line for the extended peptide region and a rectangle for the α helix. Disordered regions are indicated by dashed lines.

Fig. 2. Details of the Lef-1- β -catenin interaction

Lef-1 is shown in turquoise, and β -catenin is shown in grey. Lef-1 residues are marked with red text, and β -catenin residues with black text. Side chains that form specific interactions are shown in stick representation, with red, blue and yellow representing oxygen, nitrogen, and sulfur atoms, respectively. Hydrogen bonds and van der Waals contacts are shown with dashed black lines. In all panels the higher-resolution phosphorylated Lef-1 coordinates are shown, but, with the exception of residues 45–47 at the start of the α helix, the interactions are the same in the phosphorylated and non-phosphorylated structures. (A) and (B), two views of the Lef-1 α helix interactions. (C), interactions of Lef-1 11–18. (D), interactions of Lef-1 19–25, which contains the conserved β -catenin binding motif residues Asp19, Met21, Ile22 and Phe24.

Fig. 3. CK2 phosphorylation of Lef-1

(A) MALDI-TOF mass spectrum of CK2-phosphorylated Lef-1(1–63) shows two peaks with that differ by 80 Da. (B) Interactions of pLef-1 residues 26–41 with β-catenin. The color scheme is the same used in Fig. 2. (C) Interactions of pLef-1 residues 26–41 with crystal lattice neighbors. The left panel gives an overview of this region interacting with its partner molecule in the asymmetric unit (grey) and with two neighboring molecules of β-catenin shown in pink and gold. The central panel shows the interaction of Arg647 from the gold neighbor this Lef-1, and the right panel shows interactions of the pink neighbor.

Fig. 4. ITC measurements of the β -catenin interaction with Lef-1 and Tcf-4

(A) Reverse-phase separation of CK2-phosphorylated from unphosphorylated Lef-1(1–63). (B) ITC measurements of non-phosphorylated Lef-1(1–63) (left) or phosphorylated Lef-1(1–63) (right) binding to full-length β -catenin. The top panel shows the heat signal obtained by a series of injections of the ligand into the ITC cell; the bottom panels show the binding curves calculated using the best fit parameters obtained by a nonlinear least squares fit. Each experiment was repeated multiple times (see Table 2); a representative example is shown. (C) ITC measurement of human Tcf-4(1–57) binding to β -catenin.

Fig. 5. Comparison of β -catenin-bound conformations of mLef-1, hTCF4 and xTCF3

(A) Ribbon diagram showing the overlay of β -catenin and TCF/Lef ligands. The coordinates of β -catenin from the crystal structures bound to Lef-1 (turquoise), pLef-1 (blue), TCF4¹⁷ (yellow; PDB code 1jdh), TCF4¹⁸ (purple; PDB code 1jpw), xTCF3²¹ (orange; PDB code 1g3j), were superimposed, and model of β -catenin was removed to show the relative position of each ligand. (B) Amino acid sequences of N-terminal extended β -catenin binding regions of mouse Lef-1, human TCF4, and *Xenopus* TCF3 are aligned, colored as in (A). The two TCF4 alignments correspond to the alternative ways in which the second salt bridge formed by the conserved glutamic acid is seen in different crystal structures^{17; 18}. A structure-based alignment of the conserved β -catenin binding regions from murine E-cadherin²⁴, human ICAT^{6; 22}, APC 20-mer repeat 3^{23; 25} and APC 15-mer repeat A²⁶ is shown in black, along with the consensus motif. (C) Overlay of β -catenin-bound TCF/Lef showing structural conservation of the first four consensus residues. (D) Overlay of β -catenin-bound TCF/Lef in the vicinity of the glutamate of the consensus motif, showing alternative positions of this residue.

Table 1Crystallographic data for Lef-1- β -cat arm complexes

	Non-phosphorylated	Phosphorylated
<u>Data Collection</u>		
Resolution (Å) (last shell)	103. - 2.9 (3.06-2.90)	99.5 - 2.4 (2.46-2.40)
Space group	I422	I422
Unit cell dimension (Å)		
<i>a</i> , <i>b</i>	116.9	115.6
<i>c</i>	214.2	195.9
R_{merge}^a	0.102 (0.396)	0.107 (0.615)
Average $I/\sigma(I)$	13.0 (2.1)	12.2 (2.2)
Completeness (%)	96.5 (82.1)	99.9 (99.8)
Average multiplicity	4.9 (2.8)	4.6 (4.6)
<u>Refinement and Model Geometry</u>		
Number of reflections	15960	26264
Working set	15330	24734
Test set	630	1530
R_{cryst}^b	0.203	0.199
R_{free}^b	0.256	0.264
Number of model atoms		
Protein	3964	4164
Solvent	–	97
Temperature factors (Å ²)		
Wilson B from data	66.0	43.4
Average B of model atoms	79.4	65.7
Anisotropic B tensor		
$B_{11} = B_{22}$	–16.7	–9.6
B_{33}	33.4	19.1
Bond length rmsd from ideal (Å)	0.008	0.008
Bond angle rmsd from ideal (°)	1.2	1.1
Ramachandran plot ^c		
% in core regions	86.5	92.8
% in allowed regions	12.9	6.4
% in generously allowed regions	0.4	0.8
% disallowed	0.2	0.0

Values in parentheses are for the highest resolution shell. Rmsd, root-mean square deviation.

^a $R_{\text{merge}} = \sum_h \sum_i |I_i(h) - \langle I(h) \rangle| / \sum_h \sum_i I_i(h)$, where $I_i(h)$ is the i^{th} measurement of reflection h , and $\langle I(h) \rangle$ is the weighted mean of all measurements of h .

^b $R = \sum_h |F_{\text{obs}}(h)| - |F_{\text{calc}}(h)| / \sum_h |F_{\text{obs}}(h)|$. R_{cryst} and R_{free} were calculated using the Working and Test reflection sets, respectively.

^cAs defined in Procheck⁴²

Table 2
Thermodynamics of Lef/Tcf- β -catenin interactions determined by ITC

The data shown represent the averaged values from the indicated number of experiments.

Ligand	K _D (M)	ΔH (kcal/mol)	T ΔS (kcal/mol)	ΔG (kcal/mol)	No. exp.
Lef-1(1-63)	$23 (\pm 3) \times 10^{-9}$	-13.1 ± 1.5	-2.7 ± 1.6	-10.6 ± 0.1	4
pLef-1(1-63)	$35 (\pm 12) \times 10^{-9}$	-12.3 ± 1.7	-1.9 ± 1.7	-10.3 ± 0.2	3
Tcf4(1-57)	$16 (\pm 3) \times 10^{-9}$	-12.5 ± 0.1	-1.7 ± 0.2	-10.8 ± 0.1	2