Protein kinase CK2 is a ubiquitous serine/threonine kinase involved in many biological processes. It is over-expressed in many malignancies including rodent and human breast cancer, and is up-regulated in Wnt-transformed mammary epithelial cells, where it can be found in a complex with dishevelled and β-catenin. β-Catenin is a substrate for CK2 and inhibition of CK2 reduces levels of β-catenin and dishevelled. Here we report that inhibition of CK2 using pharmacologic agents or expression of kinase inactive subunits reduces β-catenin-dependent transcription and protein levels in a proteasome-dependent fashion. The major region of phosphorylation of β-catenin by CK2 is the central armadillo repeat domain, where carrier proteins like axin and the adenomatous polyposis coli gene product APC interact with β-catenin. The major CK2 phosphorylation site in this domain is Thr383, a solvent-accessible residue in a key hinge region of the molecule. Mutation of this single amino acid reduces β-catenin phosphorylation, co-transcriptional activity, and stability. Thus, CK2 is a positive regulator of Wnt signaling through phosphorylation of β-catenin at Thr383, leading to proteasome resistance and increased protein and co-transcriptional activity.

Protein kinase CK2 is ubiquitously expressed in both the cytoplasm and nucleus of eukaryotic cells. It is highly conserved through evolution; mammalian CK2 can substitute for each other but are highly homologous to each other but are encoded by different genes (5). Activity is regulated by the β subunit, which confers some of the substrate specificity (6). In S. cerevisiae, deletion of either of the catalytic subunits results in a normal phenotype, but deletion of both leads to growth arrest (7). In mammals, the α′ subunit is required for normal male germ cell development (8).

CK2 is not known to be regulated by second messengers, but its activity is enhanced by polyamines and polylysines (9–11) and inhibited by apigenin (chrysin) (12), 6-dichloro-1-β-D-ribofuranosylbenzimidazole (13), and emodin (14). Biochemically, CK2 is unusual in that it is one of the few kinases that can efficiently utilize either ATP or GTP as the phosphoryl donor (15), a property that is very useful experimentally to identify its activity.

CK2 phosphorylates serine or threonine in acidic domains, with S/TXXD/E being the canonical motif (16–19). CK2 regulates many fundamental cellular processes. Of particular interest with respect to cancer biology, CK2 phosphorylates many transcription factors, proto-oncoproteins, and tumor suppressor proteins including c-Myc (20), Max (21), p53 (22), Mdm-2 (23), c-Jun (24), SV40 large T antigen (25), and others.

CK2 phosphorylation can regulate DNA binding, e.g. for c-Jun (24) or Max (21) or nuclear translocation, e.g. for SV40 large T antigen (25). However, one of the important functions of CK2 phosphorylation appears to be the regulation of protein stability. Access of critical cellular proto-oncoproteins to the proteasome is frequently regulated by phosphorylation. CK2 phosphorylates c-Myc in the C terminus (20) and we have recently found that this stabilizes c-Myc protein levels in cells (26). Phosphorylation of PTEN by CK2 stabilizes it, although this inhibits its lipid phosphatase activity and raises the level of phospho-AKT in cells (27–29). On the other hand, phosphorylation of IκB in its C-terminal PEST domain accelerates degradation, favoring NFκB activation (30–34). Each of these activities can contribute to cell proliferation and transformation.

Studies in Drosophila have implicated CK2 in the Wnt pathway, an important pathway during embryonic development, as the signaling intermediate dishevelled (Dvl) was found to associate with and be a substrate for CK2 (35). In mouse mammary epithelial cells, we found that Wnt signaling increases CK2 levels and activity. β-Catenin complexes with CK2 and Dvl and is itself a substrate of CK2. CK2 inhibition in these cells reduces the steady-state levels of both β-catenin and Dvl (36). In addition to its role in development, the Wnt pathway is increasingly recognized to play a role in human cancers, through mutation of genes encoding β-catenin itself or its regulators, chiefly APC, the adenomatous polyposis coli gene product, or axin. These two proteins act as a bridge between β-catenin and GSK3β, facilitating GSK3β phosphorylation of β-catenin at the N terminus in Ser33, Ser37, and Thr41. These phosphorylations require a prior “priming” phosphorylation at Ser45 by CK1α (37). N-terminal phosphorylation of β-catenin leads to its degradation by the proteasome. Wnt signaling can be regulated by other kinases and phosphatases including casein kinase-1ε (CK1ε) (38–42), protein phosphatase-2A (43–45), and protein phosphatase 2C (46). Mutations of β-catenin,
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APC, or axin found in cancers stabilize β-catenin and favor its nuclear translocation, to serve as a required cofactor for TCF/LEF-dependent transcription (47, 48).

Here we report that inhibition of CK2 activity causes a reduction in LEF-1 transcriptional activity due to accelerated degradation of β-catenin via the proteasome pathway. The major site of phosphorylation by CK2 is Thr\(^{395}\) of β-catenin, a residue in a solvent-exposed region as determined by analysis of the three-dimensional x-ray structure of the central domain of the β-catenin molecule (9). Ala substitution at position 395 produced a mutant β-catenin with reduced stability in Xenopus oocytes and reduced co-transcriptional activity in mammalian cells, confirming the direct action of CK2 on phosphorylation and function of β-catenin.

**MATERIALS AND METHODS**

**Tissue Culture and Reporter Assays**—Cost and Wnt-1-c57mg (36) cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 2% l-glutamine, 1% penicillin/streptomycin (Mediatech Inc. Cclggo, Herndon, VA). For some experiments, Cos7 and Wnt-1-c57mg cells were treated with pharmacologic agents to inhibit kinase activity. Apigenin (chrysin), a flavonoid that is a selective inhibitor of CK2, was used at 80 μM and wortmannin, a selective inhibitor of phosphatidylinositol 3-kinase, was used at 25 μM for 2 h. For other experiments, Wnt-1-c57mg cells were treated with 10 μM MG132 overnight to inhibit the 26 S proteasome complex, and then with apigenin for 2 h. For transient transfection, subconfluent COS7 cells were incubated with DNA (1 μg of DNA per plasmid) and FuGENE-6 liposome reagent (3 μl per 1 μg of DNA) (Roche Diagnostics) that were mixed according to the manufacturer’s instructions. For the reporter assay, 1 × 10\(^5\) COS7 cells were plated into 6-well plates and on the following day, the transfection was performed. Equal amounts of plasmids encoding the luciferase reporter and β-galactosidase were used. Total DNA was balanced with the addition of empty vector. For some experiments, Wnt-1-c57mg cells were treated with 80 μM apigenin. After 24 h, the cells were harvested and assayed for the ability to stimulate transcription of a LEF-1-dependent luciferase reporter and for ectomunglovalgir-β-galactosidase activity. Cells were harvested in 1× reporter lysis buffer by scraping. 10 μl of protein extract was assayed for luciferase activity by incubating with 50 μl of firefly luciferase substrate (Promega) and immediately reading on a luminometer (MGM Instruments) for 10 s. Samples were assayed in duplicate and the luciferase counts were normalized by dividing by the values from the β-galactosidase reporter assay. For the β-galactosidase reporter assay, 10 μl of protein extract was diluted with 40 μl of 1× lysis buffer in a 96-well plate, β-Galactosidase assay was used as a standard, diluted in varying amounts of 1× lysis buffer. 50 μl of 2× assay buffer (Promega) was added to each of the diluted samples. After mixing, the plate was incubated at 37 °C for 30 min or until a faint yellow color developed. The reactions were stopped with 150 μl of 1 M sodium carbonate and absorbance of the samples was immediately read at 415 nm on a plate reader.

**Western Analysis**—Equal amounts of protein determined by BCA protein assay were diluted with 2× sample loading buffer (100 mM Tris-CI, pH 6.8, 200 mM diiothreitol, 4% SDS, 0.2% bromphenol blue, 20% glycerol), boiled, and loaded onto polyacrylamide gels. Electrophoresis was performed in a Bio-Rad Mini Protean II gel system at 110 V for 90 min. After electrophoresis, gels were transferred onto nitrocellulose membranes (Schleicher & Schuell Inc.) using a semi-dry electroblotter (Owl Scientific, Woburn, MA) at 400 mA constant current for 60 min. Western blots were performed by chemiluminescence in Signaling Solution (Pierce). A monoclonal antibody against β-actin (Sigma) was used to confirm equal loading of the Western blot membranes for samples. When using the same membrane for sequential antibody detection, the membrane was briefly stripped at 65 °C for 5 min in stripping buffer (62.5 mM Tris, pH 6.8, 100 mM β-mercaptoethanol, 2% SDS), washed 2–3 times in wash buffer, and blocked again in 5% milk.

In vitro Kinase Assay—Extracts from COS7 cells (10 μg) were assayed for CK2 kinase activity in kinase buffer (100 mM Tris, pH 8.0, 20 mM MgCl\(_2\), 100 mM NaCl, 50 mM KCl, 1 μM Na\(_2\)VO\(_4\), and 1× phosphatase inhibitor mixture (Sigma)). Each sample was assayed with and without CK2-specific synthetic peptide, RREREETREEE, to investigate the activity of the CK2 enzyme present in the lysates. Samples incubated without the substrate peptide were used as a background control and the resulting radioactive counts later subtracted from the radioactive counts of their peptide counterparts. Samples were assayed in duplicates. Upon labeling for 20 min at 37 °C with 5 μCi of γ\(^{32}\)P[\(^{32}\)P]GTP, radioactive counts were placed on P81 filter circles and washed 4 times with 150 mM Na\(_3\)PO\(_4\). P81 filter circles were then analyzed on an automatic β-counter.

Free or bead-bound GST-β-catenin was incubated with recombinant CK2 enzyme (10–50 units) and 5 μCi of γ\(^{32}\)P[\(^{32}\)P]GTP at 37 °C for 20 min in kinase buffer (100 mM Tris, pH 8.0, 20 mM MgCl\(_2\), 100 mM NaCl, 50 mM KCl, 1 μM Na\(_2\)VO\(_4\), and 1× phosphatase inhibitor mixture). After the phosphorylation reaction, bead-bound GST-β-catenin proteins were washed 2–3 times with the kinase buffer to eliminate unincorporated γ\(^{32}\)P[\(^{32}\)P]GTP. For soluble GST-β-catenin protein, the kinase reactions were stopped by adding 2× sample loading buffer and boiling. These samples were then centrifuged and loaded onto a denaturing SDS-polyacrylamide gel for electrophoresis as described below. The gel was transferred onto a nitrocellulose membrane and 32P-labeled proteins were visualized by autoradiography. Efficiency of protein transfer was confirmed by Western blotting with either β-catenin or GST antibodies (see above).

Stoichiometry determinations were performed using 1 pmol of purified recombinant wild-type or mutant GST-β-catenin substrate (see below) or synthetic CK2 peptide. Kinase assays were performed as described above using 50 units of recombinant CK2 (1 unit catalyzes 1 pmol/min) and 50 μM γ\(^{32}\)P[\(^{32}\)P]GTP (1760 dpm/pmol). Incorporated counts were determined using P81 filter circles. Raw counts/min were adjusted according to the counting efficiency and values from reactions containing CK2 enzyme and buffer only were subtracted to control for auto-phosphorylation of CK2. For phosphorylation, moles of phosphate incorporated per mol of substrate were calculated for duplicate samples.

Recombinant β-Catenin-GST Fusion Protein and Truncations—The full-length β-catenin cDNA (from pcI-β-catenin, kindly provided by Dr. B. Vogelstein), encoding a 781-amino acid residue protein, was subcloned into pOEX-2T (Amersham Biosciences) for bacterial expression of a recombinant GST-β-catenin fusion protein. C-terminal deletions were generated by digestion with EcoRI, producing a cDNA encoding an 421-amino acid residue β-catenin protein retaining 7 CK2 consensus phosphorylation sites (β-cateninΔ7) or with Xhol, producing a cDNA encoding an 88-amino acid residue N-terminal fragment retaining 2 CK2 consensus phosphorylation sites (β-cateninΔ2) (Fig. 3A). These β-catenin constructs or the pOEX-2T vector alone were transiently transfected into competent cells (Invitrogen) and grown in LB with 50 μg/ml ampicillin until the absorbance at 595 nm was between 0.6 and 1.0. The culture was then induced with 0.5 mM (final concentration) isopropyl-1-thio-β-D-galactopyranoside for 3–4 h. After induction, cells were pelleted at 5,000 rpm and resuspended in NETN buffer (100 mM NaCl, 1 mM EDTA, 20 mM Tris, pH 8.0, 0.5% Nonidet P-40) supplemented with protease inhibitors. Cells were sonicated for 10 s, three times, to ensure complete disruption. Sonicates were pelleted at 15,000 rpm, and the supernatant was filtered before incubating with glutathione-Sepharose 4B beads (Roche Diagnostics). After 2 h of incubation at 4 °C with rotation, the beads were washed 3 times with NETN buffer, and centrifuged at 500 rpm at 4 °C. Aliquots were eluted in 3 fractions by competition with free glutathione (3 mg/ml) in 0.5× Tris, pH 7.4, buffer. Eluted fractions were electrophoresed on SDS-polyacrylamide gels for protein analysis, in a Bio-Rad mini gel system. Gels were either stained with Coomassie Brilliant Blue or Western blotting was performed as described below to detect recombinant β-catenin tagged protein. GST-GST protein was prepared in the same manner.

Site-directed Mutagenesis—To identify the site of CK2 phosphorylation in β-catenin experimentally, site-directed mutagenesis was performed. Sequence-specific PCR primers containing the desired mutations were designed according to Stratagene’s guidelines. For PCR, 100 μl of plasmid DNA was denatured at 95°C for 30 s and subjected to 18 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 1 min, and extension at 68°C for 16 min (2 s/kb). This protocol generated mutant PCR products containing staggered nicks. The PCR products were then subjected to DpnI digestion to restrict the
CK2 Phosphorylation of Thr^{392} Regulates β-Catenin

Apigenin-induced Degradation of β-Catenin Is Blocked by MG132, a Proteasome Inhibitor—Normally, β-catenin is ubiquitinated and degraded by the proteasome pathway. To determine whether or not β-catenin underphosphorylated by CK2 is degraded by the same pathway, MG132 was used to block proteasome activity (53). Treatment of Wnt-1 expressing C57MG cells with MG132 yielded ubiquitinated forms of β-catenin that appear on a Western blot as a ladder of high molecular weight immunoreactive protein because of the addition of varying numbers of ubiquitin molecules (Fig. 2, lane 4). Whereas treatment of these cells with apigenin led to decreased protein levels of β-catenin (Fig. 2, lanes 2 and 3), co-treatment of cells with MG132 and apigenin completely prevented the degradation of β-catenin (Fig. 2, lanes 5 and 6); the β-catenin expression level was not reduced and the ladder of ubiquitin conjugates appeared (Fig. 2). This suggests that apigenin-induced degradation of β-catenin is mediated by the proteasome machinery.

β-Catenin Is Phosphorylated by CK2 between Amino Acids 89 and 421—To identify the site(s) of CK2 phosphorylation in β-catenin, we began by studying progressively truncated proteins (Fig. 3A). A cDNA encoding glutathione S-transferase attached to the N terminus of β-catenin (GST-β-catenin) was engineered in pGEX-2T, and recombinant GST-β-catenin was used as a substrate for phosphorylation by recombinant CK2. The 118-kDa full-length fusion protein was phosphorylated, whereas the 26-kDa GST protein itself was not (Fig. 3B, lanes 1 and 4). Note that there are phosphoprotein bands of ~42 and 28 kDa that appear in lane 1 along with the GST-β-catenin-phosphorylated band. The mobility of these bands is consistent with that of the α and β subunits of CK2 itself, and most likely represent recombinant CK2 that copurifies with the GST-β-catenin fusion protein and autophosphorylates itself. No bands of the molecular weight of CK2 appear in lane 4, i.e. CK2 does not bind to GST alone. To identify the region within β-catenin that is phosphorylated by CK2, two truncated forms of GST-β-catenin were examined. One, GST-β-cateninΔ1, encompassing approximately half of β-catenin up to amino acid 421, including 7 consensus phosphorylation sites, was phosphorylated by CK2 (Fig. 3B, lane 2), whereas a shorter truncation, GST-β-cateninΔ2, including only the N-terminal 89 amino acids and 2 CK2 consensus phosphorylation sites, was not phos-
CK2 Phosphorylation of Thr^{393} Regulates β-Catenin

Inhibition of CK2 reduces β-catenin/LEF-1 transcription. A, COS7 cells (1 x 10^6 cells/well) were co-transfected with plasmids encoding LEF-1 luciferase, β-galactosidase, and/or β-catenin (1 μg each unless otherwise noted) either in the absence (-Lef) or presence (+Lef) of LEF-1 cDNA. Two different amounts of β-catenin plasmid (0.1 g and 0.5 μg) were used for each set of transfections. 24 h post-transfection, the cells were treated with the inhibitors (INH) apigenin (80 μM) or wortmannin (25 μM) for an additional 2 h, then the cells were harvested and luciferase and β-galactosidase assays were performed. The graph shows luciferase lumimometer counts normalized to β-galactosidase activity to control for transfection efficiency. Two independent experiments gave similar results. B, COS7 cells were co-transfected with LEF-1, LEF-1 luciferase, β-galactosidase, and β-catenin cDNAs. One set was left untreated, one set was treated with 80 μM apigenin, and the third set was co-transfected with CK2α and α' kinase inactive mutants. CK2 kinase activity was measured by the incorporation of [γ-32P]GTP into the CK2-specific substrate peptide RRREEEEE, subtracting background counts obtained without the peptide and normalizing to β-galactosidase activity. C, Western blot analysis of these samples demonstrated that treatment with 80 μM apigenin or overexpression of CK2 kinase inactive mutants decreased β-catenin expression (upper panel); β-actin was used as a loading control (lower panel). D, the same extracts used in B and C were used for LEF-1-dependent luciferase activity. A representative graph of luciferase lumimometer counts normalized to β-galactosidase activity is shown (mean ± S.D. of the duplicate transfections); three other independent experiments had similar results.
sites in β-catenin are the GSK3β consensus phosphorylation sites at Ser39 and Ser47, in fact when those are mutated, GSK3β will still phosphorylate β-catenin (54, 55). Thus, it is likely that the regulation of the interaction of β-catenin with its many partners may be regulated in complex ways by phosphorylation. To identify the major sites of phosphorylation by CK2, a series of site-directed mutants were engineered and confirmed by sequencing. Most of the targeted amino acid residues were in the midportion of the molecule, identified by the truncation mutants as the major region of CK2 phosphorylation. In addition, we mutated Ser29 and weak CK2 sites at Thr289 and Ser403, because of their proximity to the series of four sequential GSK3β sites between Ser29 and Ser33, to determine whether there was some interaction between the GSK3β and CK2 phosphorylation events. We also mutated one weak CK2 site in the C-terminal portion of the molecule at Thr693, as a control. Mutations of these sites alone or in combination demonstrated that mutants in which Thr393 was changed to Ala (T393A) were much reduced in phosphorylation by recombinant CK2 (Fig. 4). Mutants of Ser29 to Ala (S29A) were also less phosphorylated but this mutation was not studied further because the truncations indicated that most phosphorylation occurs between residues 89 and 421.

The importance of phosphorylation at Thr393 was further substantiated by stoichiometric calculations of incorporated phosphate (Table I). By these measurements, CK2 was capable of catalyzing the incorporation of 2.01 mol of phosphate into each mol of wild-type β-catenin, but only 0.92 mol of phosphate per mol of T393A mutant. Synthetic CK2 peptide, RRREEEIVK, which possesses a single site for phosphorylation, was used as a positive control, and gave a value of 0.99 mol of phosphate/mol. Thr393 is of particular interest because it is in the armadillo repeat domain of β-catenin, in a region important for interaction with other proteins including APC, axin, and TCF (3, 56, 57). Examination of the crystal structure of the armadillo repeat region, residues 150–665, of β-catenin with its C-terminal portion of the molecule at Thr693, as a control.

In contrast, CK2 consensus phosphorylation sites at Ser179, Ser246, Ser605, and Ser646 are relatively inaccessible in the model.

T393A β-Catenin Has Reduced Co-transcriptional Activity—

To assess the functional importance of the Thr393 phosphorylation site in β-catenin, the wild-type and mutant cDNAs were transfected into COS7 cells along with the LEP-1 reporter, and co-transcriptional activity was determined. The T393A mutant was consistently less efficient as a co-transcriptional activator than the wild-type β-catenin (Fig. 6A). However, in the presence of the CK2 inhibitor apigenin, the co-transcriptional response of the wild-type β-catenin was reduced, whereas that of the T393A mutant was not (Fig. 6B).

β-Catenin T393A Is Degraded More Rapidly Than Wild-type β-Catenin—In these transfection experiments, we found that the levels of expression of β-catenin T393A were consistently

FIG. 4. Thr393 and Ser29 of β-catenin are phosphorylated by CK2. Ala mutations at specific CK2 consensus phosphorylation sites were engineered. Recombinant GST-β-catenin fusion proteins were synthesized from the mutated sequences and an in vitro kinase assay with recombinant CK2 was performed (upper panels). Relative amounts of fusion protein used for the in vitro kinase assay were determined by Western blotting of the same membrane with GST antibody (lower panels). Mutation of Thr393 significantly reduces phosphorylation. Mutation of Thr289, Ser403, Thr289, Thr289, or Thr693 did not significantly reduce phosphorylation. Note that even with mutation of both T393A and S29A, there is still some residual phosphorylation of β-catenin.

FIG. 3. β-Catenin is phosphorylated by CK2 between amino acids 89 and 421. A, a linear representation of full-length (FL) β-catenin and two truncation mutants. The boxes (□) indicate the positions of Ser or Thr in CK2 consensus phosphorylation sites (S/TD/E) whereas the circles (○) and diamonds (♦) indicate positions of GSK3β (S/TXXS/T*) and CDK (SP) consensus phosphorylation sites, respectively. As noted by multiple symbols, some positions could potentially be phosphorylated by more than one kinase. The CK2 sites are also labeled numerically by amino acid residue. GST full-length β-catenin is 118 kDa and contains nine CK2 sites. GST-β-cateninΔ1 includes residues 1–421 and encodes a protein of 72 kDa containing seven CK2 sites. GST-β-cateninΔ2 includes residues 1–89 and encodes a protein of 36 kDa containing two CK2 sites. B, GST-β-catenin fusion proteins as well as GST alone were phosphorylated using [γ-32P]GTP in vitro with recombinant CK2 (upper panel). In the first lane 1, a phosphoprotein band can be seen at 118 kDa (upper panel), migrating identically to the immunoreactive FL GST-β-catenin band (lower panel). In addition, phosphoprotein bands appear at about 45 and 28 kDa, which are not associated with β-catenin immunoreactive bands. These bands co-migrate with CK2α and CK2δ (not shown) and are presumed because of autophosphorylation of the enzyme. Rapidly migrating immunoreactive bands seen in the Western blot (lower panel) may be degradation fragments of the fusion protein or cross-reacting bands. In lane 2, we see that the poorly expressed GST-β-cateninΔ1 protein is also phosphorylated; the ratio of the intensity of the phosphoprotein band and the immunoreactive band for FL β-catenin and Δ1 were the same, indicating that they are phosphorylated equally well. GST-β-cateninΔ2 is well expressed and not phosphorylated at all (third lane), nor is GST itself phosphorylated (fourth lane). Upper panel is an autoradiogram of the CK2 in vitro kinase (IVK) reactions, whereas the lower panel is a Western blot (WB) for GST.
The recombinant wild-type and T393A mutant GST-\(\beta\)-catenin proteins were phosphorylated in vitro by recombinant CK2 and the moles of phosphate incorporated per mol of substrate per min were calculated. The synthetic CK2 peptide substrate (RRREEETEEE), which has only 1 phosphorylatable site, was used as a control. Numbers represent mean ± S.D. of duplicate determinations.

| Sample          | Moles of phosphate incorporated per mol of substrate |
|-----------------|------------------------------------------------------|
| Wild-type \(\beta\)-catenin | 2.01 ± 0.45                                           |
| T393A \(\beta\)-catenin    | 0.92 ± 0.05                                           |
| RRREEETEEE            | 0.99 ± 0.34                                           |

less than those of the wild-type protein (Fig. 6C). Based on our studies of the effect of CK2 phosphorylation on stability of other proteins, we postulated that this phosphorylation mutant of \(\beta\)-catenin might have reduced stability. However, we could not accurately determine the half-life of transfected \(\beta\)-catenin proteins in cycloheximide-treated mammalian cells (data not shown). To measure \(\beta\)-catenin protein stability more accurately, we microinjected recombinant proteins into Xenopus embryos. Microinjection of purified protein has the advantage of avoiding differences in translation efficiency of transfected plasmid constructs. Furthermore, the Xenopus embryo has been a standard system for studying the function and regulation of \(\beta\)-catenin turnover because all the appropriate accessory proteins and enzymes are present. Equal amounts of recombinant wild-type GST-\(\beta\)-catenin and T393A GST-\(\beta\)-catenin fusion proteins were injected into the animal pole of both blastomeres of the embryos at the two-cell stage. Embryos were harvested periodically after injection and lysates were prepared for analysis of \(\beta\)-catenin protein levels by Western blot. A representative experiment demonstrated that T393A \(\beta\)-catenin was degraded more rapidly than the wild-type \(\beta\)-catenin (Fig. 6D, upper panel). In four independent experiments, the half-life for wild-type GST-\(\beta\)-catenin was 25 ± 7.9 min whereas that of GST-T393A \(\beta\)-catenin was shorter, 10.5 ± 4.1 min (\(p = 0.017\)). These results indicate that the phosphorylation of \(\beta\)-catenin at Thr\(^{393}\) by CK2 positively regulates \(\beta\)-catenin stability.

**DISCUSSION**

The Wnt signaling pathway involves remarkably complex mechanisms for regulating levels of free cytoplasmic \(\beta\)-catenin that is available to translocate to the nucleus and serve as a cofactor for TCF/LEF-mediated transcription. There are more than 16 mammalian Wnt family members that interact with the 11 frizzled family receptors and with LRP and proteoglycan co-receptors. These receptors transmit a signal through the 3 Dvl proteins that leads to the repression of GSK3\beta activity, perhaps through interaction with Frat/GBP (59). Inhibition of GSK3\beta reduces phosphorylation of \(\beta\)-catenin at N-terminal serines, which reduces its proteasomal degradation. However, free \(\beta\)-catenin levels also depend upon interactions with the carrier proteins axin, axin-related proteins, and APC. Multiple kinases and phosphatases regulate the interaction and stability of these proteins, including CK1\(\varepsilon\), protein phosphatase 2A, and protein phosphatase 2C.

Work of Willert and colleagues (35) demonstrated that the ubiquitous, highly conserved, and constitutively active serine/threonine kinase CK2 can bind and phosphorylate Dvl proteins in insect cells. In a previous publication (36), we found that this interaction also occurs in mammalian cells. Surprisingly, we identified \(\beta\)-catenin in this complex as well, and \(\beta\)-catenin turned out to be the preferential substrate of CK2; pharmacologic inhibition of CK2 accelerated the degradation of \(\beta\)-catenin. Here we demonstrate that inhibition of CK2 activity, either pharmacologically or by expression of kinase inactive subunits that can act in a dominant negative fashion not only reduces \(\beta\)-catenin protein levels in the cells, but also reduces TCF/LEF-dependent transcription (total \(\beta\)-catenin protein levels and co-transcriptional activity do not always correlate (60)). Our data demonstrate that CK2 stabilizes \(\beta\)-catenin protein and thereby regulates Wnt signaling, although we cannot exclude the possibility that phosphorylation modifies interaction with TCF/LEF or other partners. Truncation mutants of \(\beta\)-catenin allowed us to narrow down the site of phosphorylation to five predicted consensus sites in the mid-portion of \(\beta\)-catenin, and analysis of the armadillo crystal structure indicated that one of these (Thr\(^{393}\)) was likely to be in a solvent-exposed loop accessible to CK2. Site-directed mutagenesis confirmed that Thr\(^{393}\) is a major site of phosphorylation, and the functional importance of phosphorylation at this site was confirmed in experiments demonstrating that this mutant has reduced stability and reduced co-transcriptional activity.

It was difficult to demonstrate a difference in \(\beta\)-catenin protein stability in mammalian cells, most likely because cytoplasmic \(\beta\)-catenin is a minor pool in these cells. Therefore, we turned to the Xenopus laevis system, a standard system for studying Wnt signaling pathway activities (61–65). This system has been used to demonstrate that phosphorylation in the N terminus of \(\beta\)-catenin by GSK3\beta accelerates degradation upon injection into the animal pole of Xenopus embryos (55, 60). Here, we find that the single site mutant at Thr\(^{393}\) resulted in a more than 50% reduction in half-life compared with the wild-type \(\beta\)-catenin protein.

The major CK2 phosphorylation site at Thr\(^{393}\) lies in a solvent-exposed loop of the armadillo repeat region of the mole-

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3 R. Nusse, www.stanford.edu/~rnusse/wntwindow.html.
CK2 Phosphorylation of Thr\textsuperscript{393} Regulates \(\beta\)-Catenin

Although the 88-amino acid \(\beta\)-catenin\textsubscript{A2} truncation mutant was not efficiently phosphorylated by CK2, we nonetheless examined the CK2 consensus sites near the GSK3\(\beta\) sites at Ser\textsuperscript{53}, Ser\textsuperscript{17}, and Thr\textsuperscript{41} in the context of the full-length molecule. S29A also resulted in a significant reduction of phosphorylation but T59A and S60A double mutation did not. Thr\textsuperscript{102}, Thr\textsuperscript{112}, Ser\textsuperscript{179}, and Thr\textsuperscript{693} were other consensus phosphorylation sites whose mutation did not alter overall phosphorylation in our experiments (Fig. 4). Another group also recently found that mutation of Ser\textsuperscript{25} reduced CK2 phosphorylation of \(\beta\)-catenin (69). In their system, Thr\textsuperscript{102} and Thr\textsuperscript{112} were also reported to be phosphorylated, although these data were not shown. The Ser\textsuperscript{25}, Thr\textsuperscript{102}, and Thr\textsuperscript{112} triple mutant yielded a \(\beta\)-catenin of augmented stability. CK2 phosphorylation enhanced binding to GSK3\(\beta\) and \(\alpha\)-catenin, and this appeared to favor adhesion. This has been reported previously, although increased adhesion was explained by phosphorylation of E-cadherin itself in the prior report (70). We have not examined the effect of CK2 phosphorylation upon adhesive functions, but rather have focused upon the critical role of \(\beta\)-catenin in Wnt signaling, for which CK2 appears to have a positive regulatory role. If CK2 stabilizes cytoplasmic \(\beta\)-catenin and promotes Wnt signaling, it should be capable of promoting duplication of the dorsal axis during development of Xenopus embryos. We have found that this is in fact the case.\textsuperscript{4} Furthermore, tumors that have elevated levels of CK2 should exhibit evidence of Wnt pathway activation, and we have shown that in mammary tumors arising in MMTV-CK2\textsubscript{A2} transgenic mice, \(\beta\)-catenin levels are high (58). Thus, it appears that CK2 phosphorylation regulates both adhesive and signaling functions of \(\beta\)-catenin and its partners in a complex fashion, contributing to the finely tuned regulation of this developmentally important pathway.

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