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Research Article

A second protein kinase CK1-mediated step negatively regulates Wnt signalling by disrupting the lymphocyte enhancer factor-1/β-catenin complex

A. Hämmerlein [‡], J. Weiske and O. Huber *

Institute of Clinical Chemistry and Pathobiochemistry, Charité Campus Benjamin Franklin, Hindenburgdamm 30, 12200 Berlin (Germany), Fax: +49 30 8445 4152, e-mail: otmar.huber@charite.de

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Abstract. Deregulated activation of the canonical Wnt signalling pathway leads to stabilization of β-catenin and is critically involved in carcinogenesis by an inappropriate induction of lymphocyte enhancer factor (LEF-1)/β-catenin-dependent transcription of Wnt target genes. Phosphorylation of the pathway components β-catenin, Dishevelled, Axin and APC (adenomatous polyposis coli) by glycogen synthase kinase-3β, CK1 and CK2 is of central importance in the regulation of the β-catenin destruction complex. Here, we identify CK1 and CK2 as major kinases that directly bind to and phosphorylate LEF-1 inducing distinct, kinase-specific changes in the LEF-1/DNA complex. Moreover, CK1-dependent phosphorylation in contrast to CK2 disrupts the association of β-catenin and LEF-1 but does not impair DNA binding of LEF-1. Sequential phosphorylation assays revealed that for efficient disruption of the LEF-1/β-catenin complex, β-catenin also has to be phosphorylated. Consistent with these observations, CK1-dependent phosphorylation inhibits, whereas CK2 activates LEF-1/β-catenin transcriptional activity in reporter gene assays. These data are in line with a negative regulatory function of CK1 in the Wnt signalling pathway, where CK1 in addition to the β-catenin destruction complex at a second level acts as a negative regulator of the LEF-1/β-catenin transcription complex, thereby protecting cells from development of cancer.

Key words. LEF-1; β-catenin; Wnt signalling; CK1; CK2.

The Wnt signalling pathway regulates multiple processes during embryonic development and in the adult organism. Activation of the canonical Wnt signalling pathway increases the steady-state level of β-catenin and induces LEF-1/β-catenin-dependent transcription of Wnt target genes frequently misregulated in human tumours [1]. In unstimulated cells, β-catenin is rapidly targeted to degradation by the ubiquitin-proteasome system. This requires phosphorylation of β-catenin in a high-molecular-weight protein complex including the adenomatous polyposis coli tumor suppressor protein (APC), Axin/Conductin, glycogen synthase kinase-3β (GSK3β) and CK1 (formerly known as casein kinase I). After stimulation of the pathway, phosphorylation of β-catenin, Axin, and APC is inhibited and β-catenin is released from the destruction complex. However, there is also evidence for a GSK3β-independent mechanism where Wnt signalling regulates β-catenin levels by negative regulation of cellular Axin levels [2]. β-Catenin subsequently translocates to the nucleus where it forms a complex with LEF/TCF transcription factors and activates transcription of Wnt target genes such as c-myc [3], cyclin D1 [4, 5] and others (see http://www.stanford.edu/~rnusse/wntwindow.html) often upregulated during carcinogenesis.

Multiple proteins have been identified that regulate Wnt signalling [1]. They either act at the level of Wnt signal reception on the cell surface, modulate the activity of the β-catenin destruction complex or affect the activity of the

* Corresponding author.
‡ Present address: ABDA – Federal Union of German Associations of Pharmacists, Jägerstr. 49/50, 10117 Berlin (Germany)

LEF-1/TCF-β-catenin transcription complex. A principal role of protein phosphorylation by the serine/threonine protein kinases CK1 and CK2 (formerly known as casein kinase II) in the Wnt signalling pathway is commonly accepted. However, there are opposing results with respect to their activating or inhibiting functions [6, 7]. CK1 comprises a large family of protein kinases, namely CK1α, β, γ1, γ2, γ1 δ and ε. All members share at least 50% amino acid identity within the catalytic domain [8]. The isoforms may have different functions, however in vitro studies using different isoforms have not shown significant differences in the preferred target sequence [9]. The CK2 holoenzyme is a tetrameric protein complex composed of two catalytic α and two non-catalytic β subunits. Both the isolated catalytic subunits and the holoenzyme are constitutively active. The β subunits are assumed to play a role as anchoring or docking elements for protein substrates and effectors [10].

LEF-1/TCF family members act as architectural transcription factors [11] which are unable to activate transcription in the absence of co-factors. The identification of a variety of nuclear binding partners for LEF-1/TCF and β-catenin revealed that the activation of Wnt target gene transcription is complex [12]. LEF-1/TCFs rather function as transcriptional repressors by interaction with co-repressors such as CtBP and TLE/groucho family members. Association with β-catenin overcomes the repressive state and induces context-dependent transcription of target genes [12]. With respect to Wnt signalling, BCL9/Legless-mediated recruitment of Pygopus to the nuclear TCF/β-catenin complex was reported to be required for Wnt/Wg signalling [13, 14]. Binding of other factors such as CBP/p300 [15], Pontin52/Reptin52 [16] and Brg-1 [17] to the TCF/β-catenin transcription complex appears to modulate transcription by remodelling the chromatin structure. The complexity is increased even more by the different capacities of the multiple TCF splice variants to activate target gene transcription [18, 19].

Phosphorylation of LEF-1/TCFs by the MAP kinase related Nemo-like kinase (NLK) inhibits binding of the LEF-1/TCF-β-catenin complex to target DNA and/or alters localization of LEF-1/TCFs [20]. In the search for protein kinases that phosphorylate LEF-1, we confirm that protein kinases CK1 and CK2 directly bind to and use LEF-1 as a substrate. In this study we focus on the nuclear aspects of CK1- and CK2-dependent phosphorylation in the Wnt signalling pathway. CK1-dependent phosphorylation disrupts LEF-1/β-catenin complex formation whereas phosphorylation by CK2 does not impair the LEF-1/β-catenin interaction. These observations clearly show that CK1 acts as a negative regulator of the β-catenin signalling activity at the level of the β-catenin destruction complex and at the level of the LEF-1/β-catenin transcription complex thereby preventing carcinogenesis.

Materials and methods

Cell culture

HEK293 and SW480 cells were cultured as reported previously [16]. The human colon adenocarcinoma cell line HT29 was cultured in RPMI-1640 medium, 10% (v/v) FCS, 100 units/ml penicillin and 100 µg/ml streptomycin.

Reagents, enzymes and antibodies

Recombinant casein kinase 1δ and casein kinase 2 were obtained from New England Biolabs. [^{32}P]γATP and [^{32}P]γGTP were purchased from Amersham Biosciences and NEN. Apigenin was from Sigma, heparin from Biochrom KG and CK1-7 from Medac. Goat polyclonal anti-CK1δ (N-19) antibody was obtained from Santa Cruz Biotechnology, mouse anti-FLAG M2 antibody from Sigma, anti-HA (clone 12CA5) and anti-CK2α (clone 1AD9) antibodies were from Chemicon International, anti-β-catenin antibody from BD Transduction Laboratories and monoclonal anti-LEF-1 (clone 13E5) antibody was from NanoTools.

Plasmid construction and transfection

Expression vectors for human CK1ε and human CK2α'-HA and CK2β-myc were generous gifts from Dr. D. M. Virshup and Dr. D. W. Litchfield, respectively. Expression and purification of GST fusion proteins of mouse LEF-1 and corresponding deletion constructs have been described previously [21]. For eukaryotic expression of LEF-1 with an N-terminal FLAG-tag, murine LEF-1 cDNA was cut out of pGEX-LEF-1 and ligated into the *Bam*HI site of pFLAG-CMV4 (Sigma). An expression vector for CK1ε-myc$_6$ was generated by PCR amplification of CK1ε cDNA and subsequent ligation of the *Bgl*II-digested PCR-product into the *Bam*HI site of pCS2+myc. Cells were transfected with the calcium phosphate method.

Pull-down assays

In vitro association assays with GST-LEF-1 and purified recombinant CK1δ were performed in association buffer [20 mM Tris/HCl pH 8.0, 50 mM KCl, 1 mM $MgCl_2$, 0.01% (v/v) Triton X-100] as reported previously [21]. After annealing of the oligonucleotides 5′-TTG GGC GGG ACA CCC TTT GAA GTA CCG AGC AGC TGC CCA TTG GCG-3′ and 5′-biotin-CGC CAA TGG GCA GCT GCT CGG TAC TTC AAA GGG TGT CCC GCC CAA-3′, the DNA probe was bound to Dynabeads M280 Streptavidin (Dynal Biotech) magnetic beads and 25 µl of the bead suspension was subsequently used for pull-down experiments. Equal amounts of GST-LEF-1 fusion proteins were incubated in 200 µl association buffer [25 mM NaCl, 20 mM Tris pH 8.0, 300 mM sucrose, 2 mM $MgCl_2$, 0.1% (v/v) Triton X-100] for 30 min at 4 °C under constant agitation. Beads were sedimented by magnetic force, washed three times and resuspended in 100 µl

association buffer. Phosphorylation was performed as mentioned below. CK1δ activity was blocked by 100 µM CK1-7 [30 min, room temperature (RT)]. Samples were then incubated with mouse β-catenin-His_6 for 30 min at RT under constant agitation. Protein complexes bound to the beads were analysed by Western blotting.

Kinase assays

In vitro phosphorylation of LEF-1 was performed by incubating GST-LEF-1 fusion protein in CK1 or CK2 buffer according to the manufacturer's instructions in the presence of 0.2 mM ATP, 2 µCi [^{32}P]γATP and 25 U purified CK1δ or CK2. For sequential phosphorylation, 2.4 µg GST-LEF-1 was incubated in 200 µl association buffer with 30 µl GSH beads for 30 min at 4 °C under constant agitation. Beads were sedimented by centrifugation, washed three times and resuspended in 100 µl association buffer. Phosphorylation was performed as mentioned before. In parallel, 2.4 µg β-catenin-His_6 in 100 µl association buffer was either phosphorylated by CK1δ or not. If required, CK1δ activity was inhibited with CK1-7. Proteins were then mixed and incubated at RT under constant agitation for 30 min and protein complexes were analysed as described above.

Immunoprecipitations and immunoblotting

HEK293 cells transiently transfected with 2.5 µg pFLAG-LEF-1 and 1.5 µg pCS2+-CK1ε or 1.75 µg pRc/CMV-HA-CK2α′ + 1.75 µg pRc/CMV-myc-CK2β were lysed in AP buffer [10 mM Hepes/NaOH pH 7.4, 0.1 M KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 0.1% (v/v) Triton X-100] and the insoluble fraction was removed by centrifugation (20,800 g, 4 °C, 10 min). The supernatant was precleared with Protein A-Sepharose beads. Three micrograms anti-FLAG M2 antibody prebound to Protein A-Sepharose was added and immunoprecipitations were performed as described previously [22].

Electrophoretic mobility shift assays

GST-LEF-1 was incubated with CK1δ or CK2, β-catenin-His_6, anti-LEF-1 or anti-β-catenin antibodies in a volume of 5.5 µl for 30 min and subsequently a [^{32}P]-labelled probe containing the LEF-1/TCF binding site 1 of the mouse T-brachyury gene promoter (5′-GCT GCT CGG TAC TTC AAA GGG TGT CCC GCC C-3′; 5′-GGG CGG GAC ACC CTT TGA AGT ACC GAG CAG C-3′) was added resulting in a final volume of 10 µl DNA-binding reaction in EMSA buffer [25 mM Tris, 190 mM glycine, 5.5 mM EDTA] containing 0.2 µg/µl dIdC, and 10 mg/ml BSA. After 30 min, electrophoresis was performed using 4% or 8% non-denaturing polyacrylamide gels in 0.25 × TBE buffer.

Reporter gene assays

Reporter gene assays were performed with the Dual-Luciferase Reporter Assay System (Promega). HEK293 cells (5×10^5) were transiently transfected by the calcium phosphate method: 0.2 µg Siamois-luciferase construct [23] and 0.1 µg pHRL-TK (*Renilla* luciferase) were transfected with different combinations of pFLAG-LEF-1 (0.2 µg) or pCS2+-CK1ε-myc_6 (0.4 µg), pRc/CMV-HA-CK2α′ (0.2 µg), pRc/CMV-myc-CK2β (0.2 µg), pCS2+-β-catenin (1.0 µg). The total amount of transfected DNA was equalized by addition of pcDNA3. Luciferase activity was measured in a Lumat LB9507 luminometer (Berthold Technologies) 48 h after transfection and normalized to *Renilla* luciferase expression.

Results

CK1 and CK2 directly interact with and phosphorylate LEF-1

In an approach to identify kinases that use LEF-1 as a substrate, recombinant GST-LEF-1 fusion protein was incubated with cell lysates of HT29, HEK293 and SW480 cells and associated proteins were pulled down with GSH-agarose beads. The isolated protein complexes were directly incubated with [^{32}P]γATP and subsequently analysed by SDS-PAGE and autoradiography. A highly phosphorylated band corresponding to GST-LEF-1 was detectable independent of the cell type used (not shown). Phospho-amino acid analysis revealed that the kinase(s) associated with GST-LEF-1 are members of the Ser/Thr protein kinase family. Predominantly serine residues and to a minor extent threonine residues were phosphorylated, whereas tyrosine phosphorylation was not detectable (fig. 1A). Similar results were obtained when the proteins associated with GST-LEF-1 were eluted with high-salt buffer prior to the phosphorylation assays. GST as a control did not pull down a kinase activity. Phosphorylation of GST-LEF-1 was inhibited in the presence of the known CK2 inhibitor heparin (fig. 1B). Consistently, GST-LEF-1 was phosphorylated by recombinant purified CK2, and this phosphorylation was inhibited in the presence of the CK2 inhibitor apigenin (fig. 1C). To further confirm that CK2 is associated with GST-LEF-1, the ability of CK2 to use GTP as phosphoryl donor with almost the same effectiveness as ATP was tested [24]. In the presence of [^{32}P]γGTP, GST-LEF-1 was phosphorylated but to a lesser extent than with [^{32}P]γATP (fig. 1D). When the specificity of the kinase assays was tested by using GST-β-catenin and GST-Pontin52 as substrates, GST-β-catenin was phosphorylated by CK2 in the presence of [^{32}P]γATP and [^{32}P]γGTP consistent with recent observations that β-catenin is a substrate for CK2 [25, 26]. In contrast, GST-Pontin52 phosphorylation was not detectable in the presence of [^{32}P]γGTP or [^{32}P]γATP under these assay conditions. These results confirm previously published results that GST-LEF-1 is a substrate of CK2 [18] and, moreover, suggested that in addition to CK2, other ki-

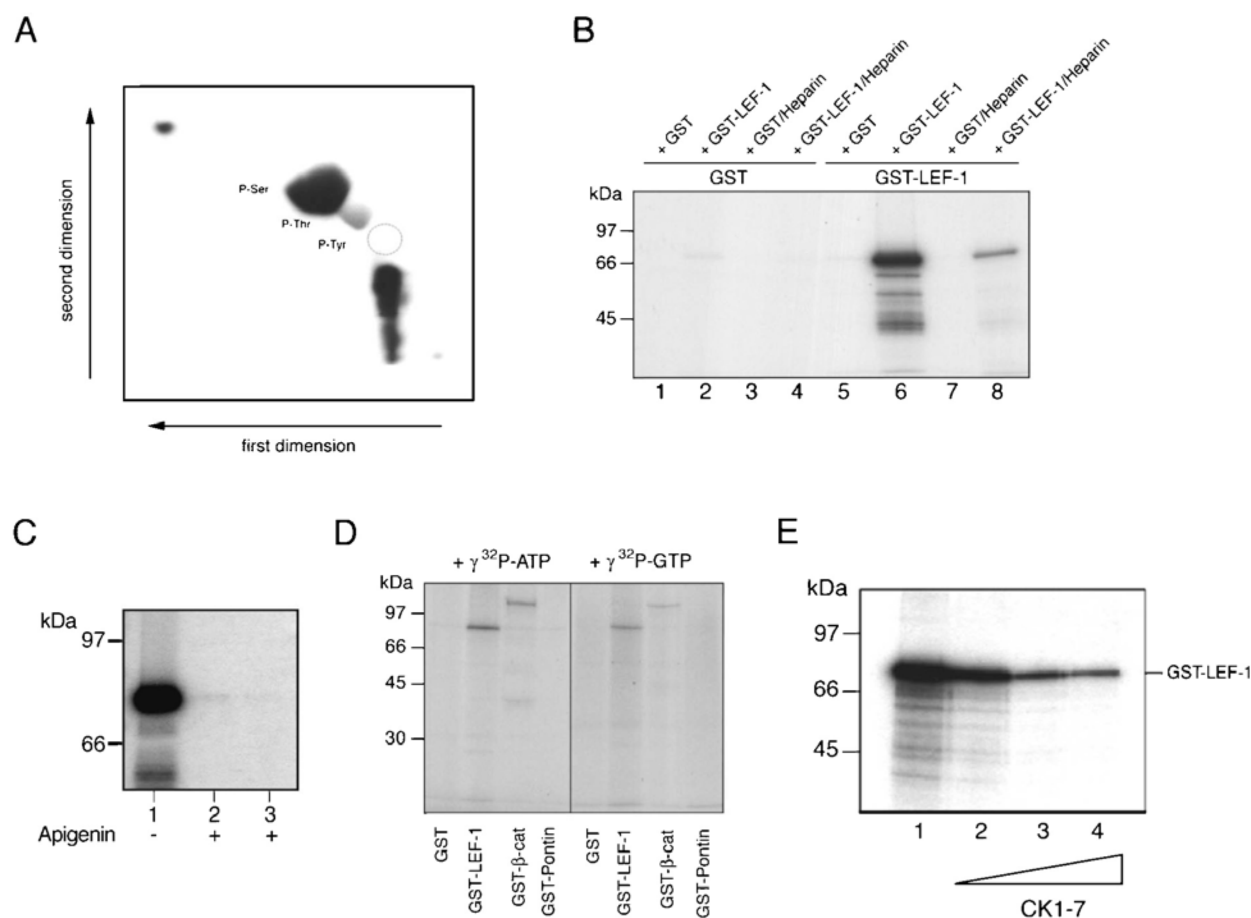
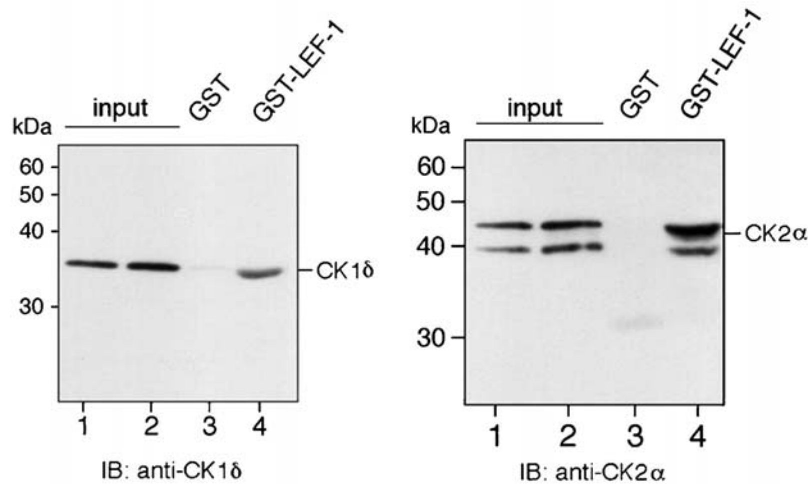
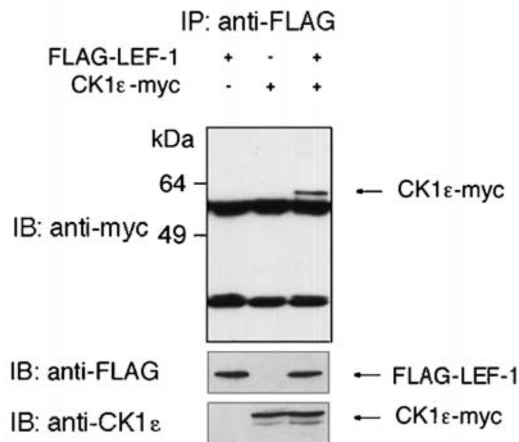


Figure 1. Isolation and characterisation of LEF-1-associated kinases. (*A*) GST-LEF-1 was used to pull down LEF-1-interacting proteins from HT29 cell lysates. Precipitated proteins were eluted in high-salt buffer and tested for kinase activity in phosphorylation assays with GST-LEF-1 as substrate. Phospho-amino acid analysis identified the kinase(s) as Ser/Thr kinase family members. Predominantly serine residues and to a minor extent threonine residues were phosphorylated, whereas tyrosine phosphorylation was not detectable. (*B*) GST-LEF-1 (lanes 5–8) but not GST (lanes 1–4) pulled down kinase activity from HT29 cell lysates. Eluted kinases phosphorylated GST-LEF-1 (lanes 6, 8) but not GST (lane 5, 7). Phosphorylation was inhibited in the presence of the CK2 inhibitor heparin (lane 8). (*C*) Purified recombinant GST-LEF-1 was phosphorylated by recombinant CK2 ($\alpha_2\beta_2$) (lane 1). Phosphorylation was inhibited in the presence of the CK2 inhibitor apigenin (lane 2, 20 µM; lane 3, 40 µM). (*D*) GST-LEF-1 (lanes 2 and 6) and GST-β-catenin (lanes 3 and 7) but not GST-Pontin52 (lanes 4 and 8) were phosphorylated by kinases isolated from HT29 cell lysates in the presence of [^{32}P]γATP and [^{32}P]γGTP. No phosphorylation was detectable on GST (lanes 1, 5) as a control. (*E*) Phosphorylation of GST-LEF-1 with recombinant CK1δ was inhibited by increasing concentrations of the CK1 inhibitor CK1-7 (lanes: 1, control; 2, 0.1 mM; 3, 0.5 mM; 4, 1.0 mM CK1-7).

nase(s) were precipitated with GST-LEF-1. Since *Xenopus* TCF3 was shown to be a substrate of CK1ε [27], CK1ε/δ appeared good candidates for kinases to be pulled down by GST-LEF-1. To test this, the CK1 inhibitor CK1-7 was added to kinase assays with recombinant CK1δ resulting in a dose-dependent reduction in GST-LEF-1 phosphorylation (fig. 1E). Taken together, these results indicate that CK1 and CK2 are the two major kinases associated with GST-LEF-1 under the conditions of our pull-down assays.

These interactions are direct, since purified recombinant CK1δ and CK2 were both able to bind to purified recombinant GST-LEF-1 but not to GST as a control (fig. 2A). Under the conditions of our in vitro association assays, about 10–20% of the kinase input was pulled down. To show that CK1 and CK2 are also associated with LEF-1 in vivo, co-immunoprecipitation experiments were performed with lysates from HEK293 cells transiently transfected with FLAG-tagged LEF-1 and CK1ε-myc$_6$ or CK2α-HA/CK2β-myc. Anti-FLAG M2 antibody co-precipitated CK1ε-myc$_6$ or CK2α-HA from cells co-transfected with either one of the kinases and FLAG-LEF-1 but not when FLAG-LEF-1 or a kinase were transfected alone (fig. 2B, C). The result obtained with CK1δ in the in vitro association assay can be compared with data obtained in the co-immunopreciptiation assay with CK1ε. This was not unexpected since these CK1 family members are highly homologous. Both kinases contain carboxy-terminal extensions and their catalytic domains reveal 98% identity at the amino acid level [8].





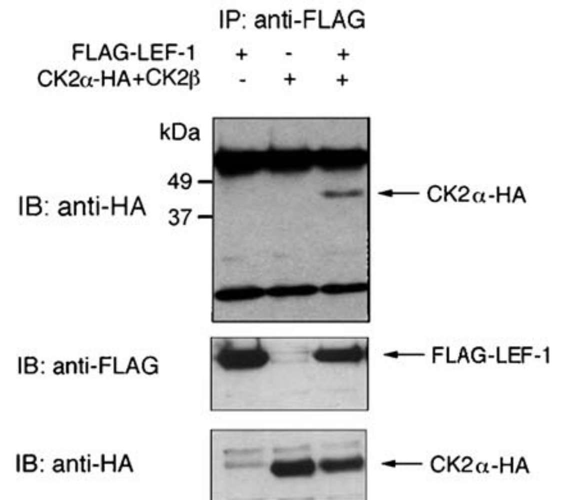


Figure 2. Association of CK1 and CK2 with LEF-1. (*A*) Recombinant CK1δ and CK2 directly interact with LEF-1. Recombinant GST (lane 3) or GST-LEF-1 (lane 4) fusion proteins were incubated with purified recombinant CK1δ (left panel) or CK2 (right panel). Protein complexes were precipitated with GSH-agarose beads and analysed in Western blots with anti-CK1δ or anti-CK2α antibodies. The lower-molecular-weight band detected by the anti-CK2α antibody represents a degradation product of the CK2α subunit. Lanes 1, 2: 10% of the total kinase input; lanes 3, 4: kinases isolated in pull-down assays. (*B*, *C*) CK1ε-myc interacts with FLAG-LEF-1 in cell lysates of transiently transfected HEK293 cells as shown by co-immunoprecipitation experiments, and CK2α-HA/CK2β-myc co-precipitates with FLAG-LEF-1. FLAG-LEF-1 was immunoprecipitated from cell lysates with anti-FLAG M2 antibody and associated CK1ε and CK2α were detected with an anti-myc (9E10) (*B*) and anti-HA (12CA5) (*C*) antibody, respectively. Lower panels show lysate controls for FLAG-LEF-1 and CK1ε-myc or CK2α-HA. The presented gels are representative of at least three independent experiments.

Phosphorylation of LEF-1 induces distinct changes in the LEF-1/DNA complex

DNA binding of LEF-1 in response to phosphorylation was analysed in EMSAs with purified recombinant proteins and a DNA fragment representing the LEF-1/TCF binding site 1 in the mouse T-brachyury promoter [28]. Binding of GST-LEF-1 to the DNA, but not GST as a control, induced a characteristic mobility shift (fig. 3A, lanes 1, 2, 7). Addition of CK1δ or CK2 did not result in a further shift (lane 3, left and right panel). However, addition of CK1δ or CK2 together with ATP, now allowing phosphorylation of LEF-1, induced a distinct but small supershift (lane 4). In the presence of CTP (lane 5) or γS-ATP (lane 6) as non-hydrolysable phosphate donors instead of ATP, the phosphorylation-dependent mobility shift was no longer detectable. Multiple control experiments confirmed that this phosphorylation-dependent retardation was not induced by binding of the kinases to LEF-1, nor does it or represent autophosphorylated kinase (not shown). The affinity of both kinases for LEF-1 appeared to be too low to form a stable complex under these assay conditions.

The mobility shift induced by CK1δ-dependent phosphorylation appeared slightly stronger than the shift detected

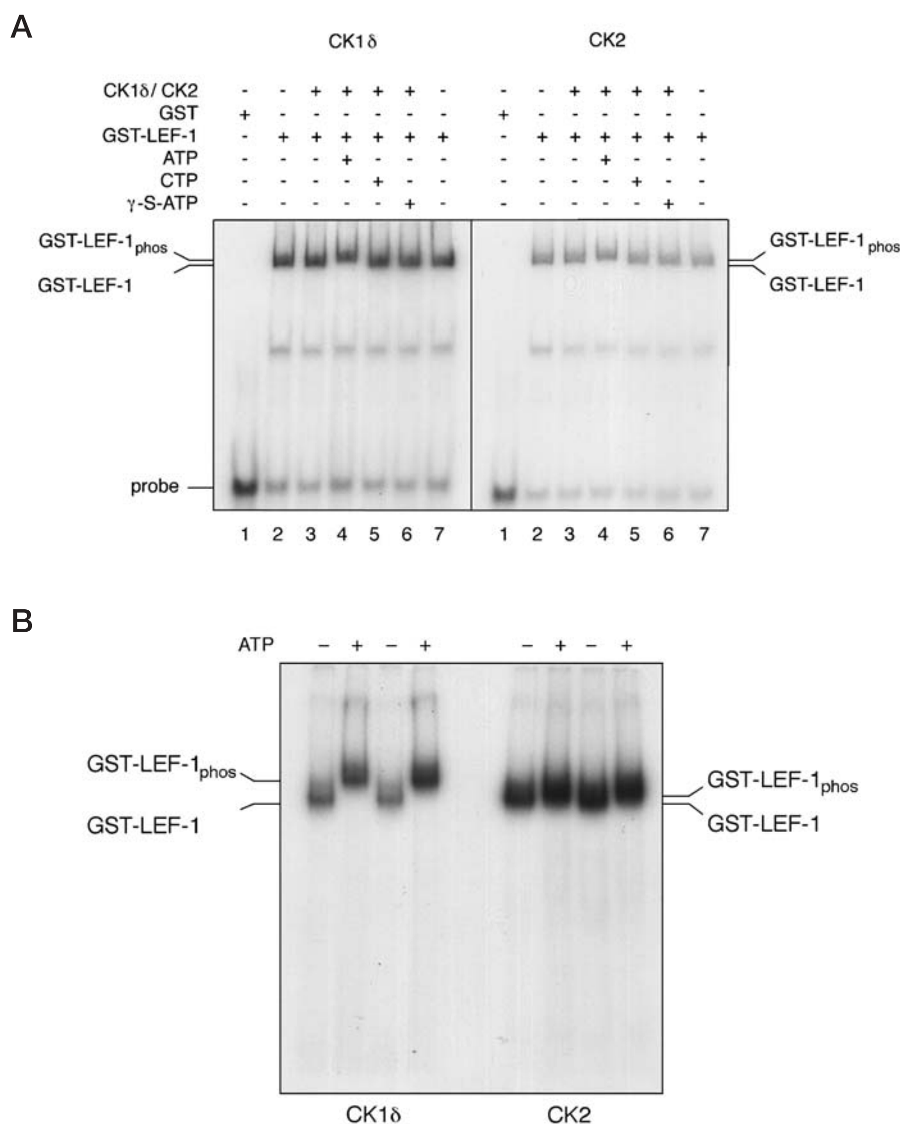


Figure 3. LEF-1 phosphorylation by CK1 and CK2 induces conformational changes in the LEF-1/DNA complex. (*A*) The electrophoretic mobility shift induced by binding of GST-LEF-1 to the DNA probe (lanes 2 and 7) is increased after phosphorylation of GST-LEF-1 by CK1δ or CK2 (lane 4 left and right panel). This supershift was not detectable in the absence of ATP (lane 3) or in the presence of CTP (lane 5) or γS-ATP (lane 6). GST as a control did not bind to the DNA probe (lane 1). (*B*) CK1δ- and CK2-dependent phosphorylation induced distinct changes in the mobilities of the GST-LEF-1/DNA complexes. Protein-DNA complexes were separated on 8% native polyacrylamide gels. Long runs of the gels had to be performed to clearly reveal the distinct differences in retardation and the free probes therefore ran out of the gel. To clearly visualize the differences in mobility, each probe was loaded twice. The presented gels are representative of at least three independent experiments.

in response to CK2-dependent phosphorylation. These distinct differences in retardation were resolved more clearly in long runs of the electrophoretic mobility shift gels (fig. 3B). Indeed, phosphorylation of LEF-1 by CK1δ resulted in a more prominent shift than phosphorylation by CK2. Since further negative charges introduced by phosphorylation are expected to increase the mobility of the LEF-1/DNA-complex under native electrophoresis conditions of the EMSA, the observed mobility shifts appear to represent distinct structural changes in the LEF-1/DNA complex induced by CK1δ- and CK2-dependent phosphorylation. These differences in mobility in response to phosphorylation were confirmed in the presence of a monoclonal anti-LEF-1 antibody (not shown).

CK1-dependent phosphorylation disrupts the LEF-1/β-catenin complex

Addition of recombinant β-catenin-His_6 induced a typical supershift in the EMSA corresponding to the formation of a β-catenin-His_6/GST-LEF-1/DNA complex (Fig. 4A, compare lanes 2 and 8). Binding of anti-LEF-1 antibody resulted in a mobility shift distinct from the shift induced

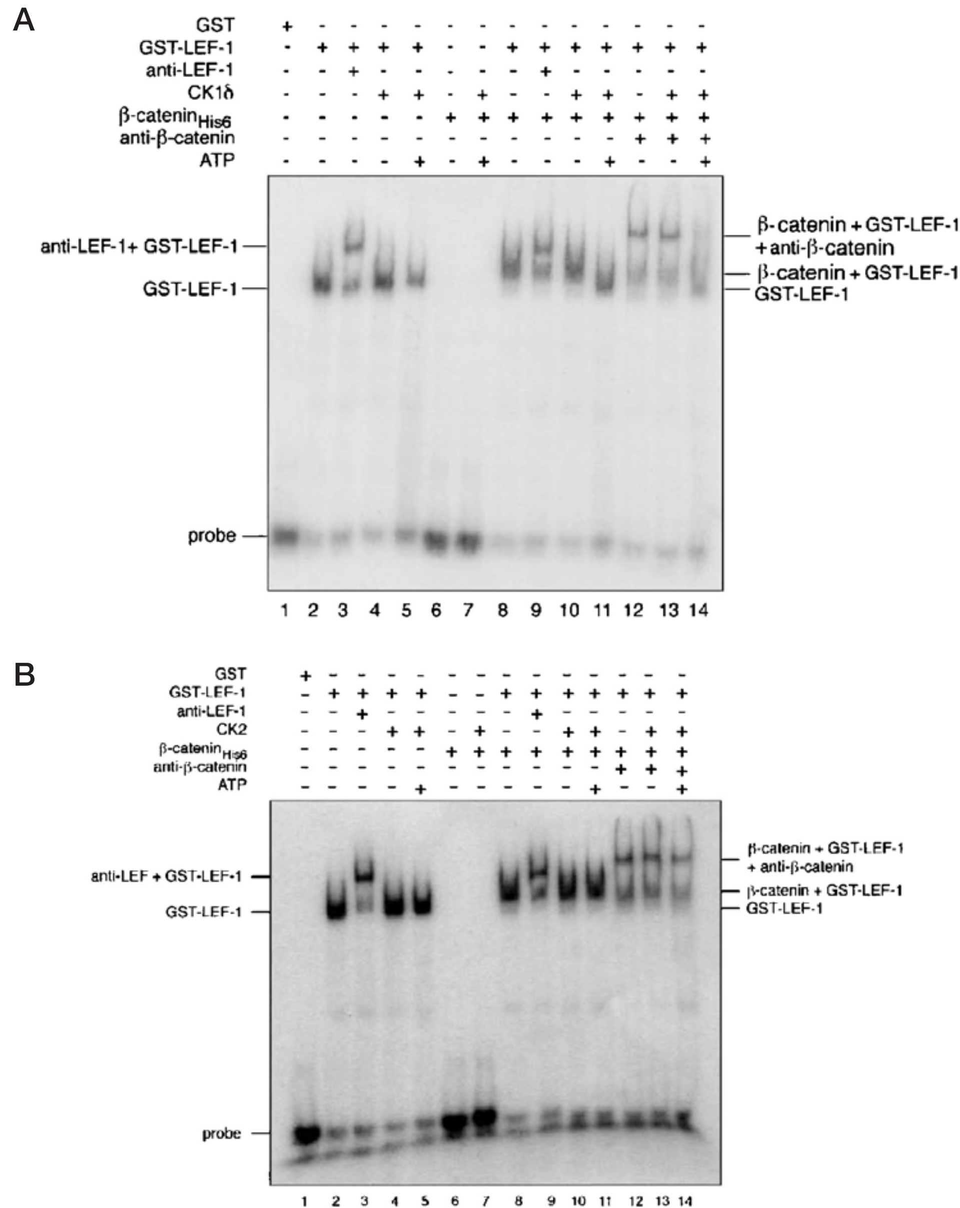


Figure 4. CK1-dependent phosphorylation disrupts the LEF-1/β-catenin complex. (*A*) EMSAs were performed in the presence of different combinations of GST-LEF-1, CK1δ, β-catenin-His$_6$, anti-LEF-1 (clone 13E5) or anti-β-catenin antibody on 4% native polyacrylamide gels. Addition of β-catenin-His$_6$ resulted in a characteristic mobility shift (lane 8), which was further enhanced in the presence of anti-β-catenin antibody (lane 12). In the presence of β-catenin-His$_6$, the anti-LEF-1 antibody induced a mobility shift (lane 9) that was identical to the shift detectable in the absence of β-catenin-His$_6$ (lane 3), indicating that this antibody disrupts the β-catenin-His$_6$/GST-LEF-1 complex. Addition of CK1δ to the LEF-1/β-catenin-His$_6$ complex showed no effect (lane 10). However, in the presence of ATP, the β-catenin-His$_6$/GST-LEF-1 complex was no longer detectable and only the GST-LEF-1 shift was detectable (lane 11). These disruptive effects on the β-catenin-His$_6$/GST-LEF-1 could be confirmed by the addition of anti-β-catenin antibody (lanes 12–14). (*B*) CK2-dependent phosphorylation did not dissociate the GST-LEF-1/β-catenin-His$_6$ complex in an identical experimental set-up as shown in (*A*). (*C*) Addition of calf intestinal phosphatase (CIP) had no effect on the formation of the GST-LEF-1/DNA, the β-catenin-His$_6$/GST-LEF-1/DNA and the anti-β-catenin/β-catenin-His$_6$/GST-LEF-1/DNA complexes in the presence of CK1δ alone (lanes 2–4). However, in the presence of ATP, the CK1δ-induced disruption of the β-catenin-His$_6$/GST-LEF-1 complex was nearly completely abolished by CIP (compare lanes 5 and 6). In these assays, a Cy5-labelled probe was used to specifically analyse the effects of protein phosphorylation/dephosphorylation and to avoid interference with probe dephosphorylation by CIP. Unfortunately, under these conditions, the separation of the GST-LEF-1/DNA complex from the β-catenin-His$_6$/GST-LEF-1/DNA complex was less efficient. Consistent with previous results, CIP had no effect on complex formation in CK2-dependent phosphorylation reactions (not shown). Analysis was performed by fluorescence imaging on a Fuji FLA-3000 Imager. At least three independent experiments were performed.

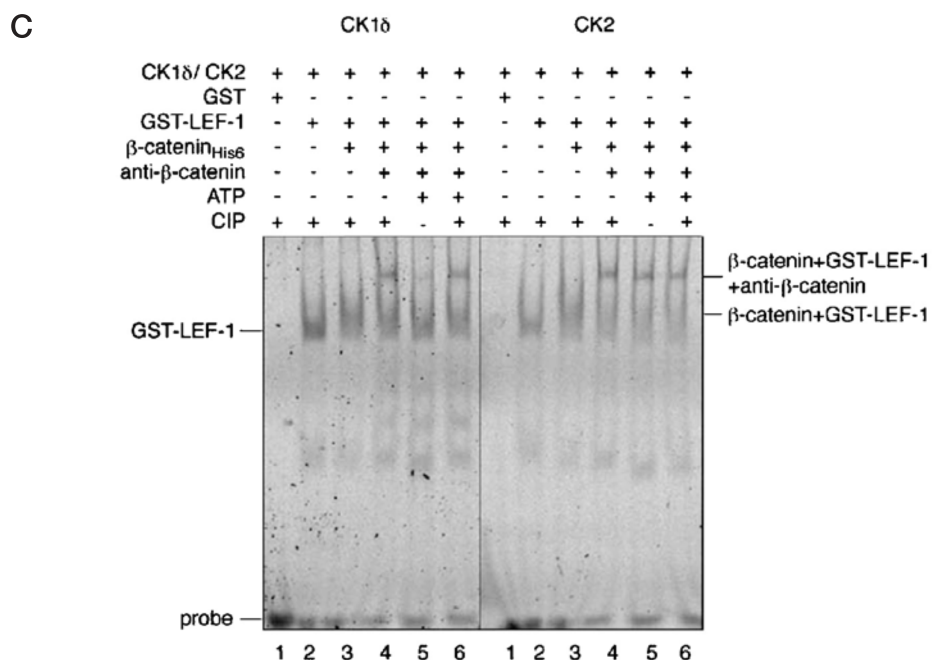


Figure 4 (continued)

by *β*-catenin-His$_6$ (fig. 4A, lane 3). In the presence of anti-LEF-1 antibody, the LEF-1/*β*-catenin interaction appeared to be partially blocked, since in the presence of *β*-catenin-His$_6$, no further shift was detectable (fig. 4A, compare lanes 3 and 9). Addition of CK1*δ*, did not change gel retardation. However, in the presence of ATP and CK1*δ*, the binding of *β*-catenin-His$_6$/GST-LEF-1 was no longer detectable and only the shift induced by binding of phosphorylated GST-LEF-1 to the probe remained detectable (lanes 10/11). Addition of an anti-*β*-catenin antibody induced a further retardation of the DNA probe corresponding to an anti-*β*-catenin/*β*-catenin-His$_6$/GST-LEF-1/DNA complex (lane 12). Again, addition of CK1*δ* alone showed no effect, whereas in the presence of ATP, the anti-*β*-catenin/*β*-catenin-His$_6$/GST-LEF-1/DNA complex was no longer detectable, indicating that phosphorylation by CK1*δ* inhibits the GST-LEF-1/*β*-catenin-His$_6$ interaction (lanes 13, 14). Interestingly, when the same experiment was performed with CK2, no effect on the *β*-catenin-His$_6$/GST-LEF-1/DNA complex upon phosphorylation was observed (Fig. 4B). In control experiments performed in the presence of calf intestinal phosphatase (CIP), the CK1*δ*-induced disruption of the *β*-catenin-His$_6$/GST-LEF-1 complex was nearly completely abolished (fig. 4C).

Since both LEF-1 and *β*-catenin are substrates for CK1, phosphorylation of both proteins may contribute to the release of *β*-catenin from the complex. In pull-down experiments with recombinant GST-LEF-1 and *β*-catenin-His$_6$ proteins, disruption of the preformed complex by addition of recombinant CK1*δ* was inhibited in the presence of the CK1 inhibitor CK1-7 (fig. 5A). Sequential phosphorylations were performed to differentiate between the effects induced by phosphorylation of LEF-1 or *β*-catenin. GST-LEF-1 prebound to GSH-agarose beads was prephosphorylated with CK1*δ* and *β*-catenin was added. Subsequently, complexes were isolated with GSH-agarose beads. This results in a reduction in the amount in *β*-catenin-His$_6$ bound to GST-LEF-1 (fig. 5B, lane 5). When the kinase was blocked with the inhibitor CK1-7 before addition of *β*-catenin-His$_6$, no decrease in *β*-catenin-His$_6$ binding to GST-LEF-1 was detectable (fig. 5B, lane 6). This indicates that phosphorylation of LEF-1 alone is not sufficient to inhibit complex formation. In contrast, when both proteins were prephosphorylated before association, only minor amounts of *β*-catenin-His$_6$ were pulled down (fig. 5B, lane 7). However, when *β*-catenin-His$_6$ was prephosphorylated and CK1*δ* was inhibited with CK1-7 before addition of GST-LEF-1, binding of *β*-catenin-His$_6$ again was not impaired (fig. 5B, lane 8). Thus also phosphorylation of *β*-catenin alone does not impair complex formation. When *β*-catenin-His$_6$ was prephosphorylated and mixed with GST-LEF-1 without removal of CK1*δ*, nearly no binding of *β*-catenin-His$_6$ was detectable (fig. 5B, lane 9) since both *β*-catenin and GST-LEF-1 can be phosphorylated. However, the reduced phosphorylation-induced shift in mobility of GST-LEF-1 suggests that not all sites in GST-LEF-1 are phosphorylated. In control experiments where *β*-catenin-His$_6$ was incubated with CK1*δ* or ATP alone, binding of *β*-catenin-His$_6$ to GST-LEF-1 was not impaired (fig. 5B, lanes 10/11). From these experiments we conclude that both LEF-1 and *β*-catenin have to

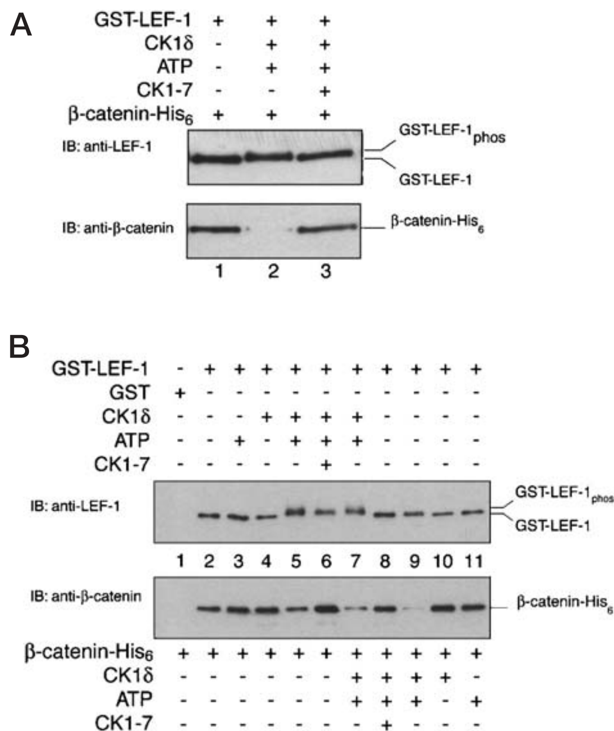


Figure 5. Phosphorylation of both LEF-1 and β-catenin contribute to the disruption of the LEF-1/β-catenin complex. (*A*) A complex of GST-LEF-1 and β-catenin-His$_6$ was preformed (lane 1). Phosphorylation with CK1δ disrupted the complex in the absence of CK1-7 (lane 2), whereas in the presence of CK1-7 (lane 3), the complex remained intact. (*B*) GST-LEF-1/β-catenin-His$_6$ complex formation after sequential phosphorylation with CK1δ. In lanes 2–6, GST-LEF-1 prebound to GSH-agarose beads was incubated as indicated and subsequently β-catenin-His$_6$ was added. In lane 7, GST-LEF-1 and β-catenin-His$_6$ were phosphorylated independently and subsequently mixed for complex formation. In lanes 8–11, β-catenin-His$_6$ was incubated as indicated and subsequently added to GST-LEF-1. Pull-down was performed with GSH-agarose beads and protein complexes were analysed by Western blotting.

be phosphorylated for an efficient disruption/inhibition of complex formation.

CK1 and CK2 modulate the transcriptional activity of the LEF-1/β-catenin complex

To investigate the effects of CK1- and CK2-dependent phosphorylation on LEF-1/β-catenin transcriptional activity in a cellular context, reporter gene assays with a Siamois-luciferase reporter construct were performed in HEK293 cells (fig. 6). Transfection of CK1ε or CK2 alone induced minor changes leading to a small inhibition or activation of the reporter gene activities, respectively. Co-transfection of LEF-1 resulted in a similar minor reduction in the presence of CK1ε, whereas CK2 induced a small increase in the transcriptional activity. However, in the presence of LEF-1 and β-catenin, repressive and activating effects of CK1ε and CK2, respectively, were clearly detectable. The increase in activity induced by CK2 may be explained by conformational changes in response to LEF-1 phosphorylation that may support efficient formation of an activated transcription complex at the Siamois promoter.

Serine 40 in the β-catenin-binding domain of LEF-1 is critical for LEF-1/β-catenin interaction

The amino acid sequence of LEF-1 contains multiple putative CK1 and CK2 phosphorylation sites. In in vitro kinase assays with C-terminally deleted GST-LEF-1 fusion proteins, all constructs including the N-terminal 56 amino acids of LEF-1 were phosphorylated by the recombinant kinases. In addition, CK2 phosphorylates a construct containing only the N-terminal 34 amino acids of LEF-1 (fig. 7A, B). Consistent with the experimental data, analysis of the amino acid sequence revealed a serine residue at position 40 (Ser40) of LEF-1 as a potential target for CK1 phosphorylation. To investigate this in more detail, Ser40 was mutated to Ala generating LEF-1 S40A. In in vitro kinase assays, phosphorylation of GST-LEF-1 S40A by CK1δ was reduced to about 58% compared to GST-LEF-1 (not shown). Since Ser40 is located within the β-catenin-binding domain of LEF-1, we next analysed whether the mutated GST-LEF-1 S40A protein is impaired in binding to β-catenin or binding to DNA. We therefore used a biotinylated DNA probe to pull down DNA-bound GST-LEF-1/β-catenin-His$_6$ complexes with streptavidin magnetic beads. Mutation of LEF-1 did not affect GST-LEF-1 binding to DNA. However, association of β-catenin with mutated GST-LEF-1 S40A was nearly lost. Moreover, when Ser40 was changed to glutamate to generate a situation similar to phosphorylated Ser40, binding of β-catenin was also efficiently blocked (fig. 7C). This result suggests that Ser40 in LEF-1 is critical for the interaction with β-catenin, and phosphorylation of Ser40 contributes to the observed disruption of the β-catenin/LEF-1 complex. However, this does not exclude that phosphorylation of other Ser/Thr residues in LEF-1 may modulate nucleoprotein complexes at Wnt target gene promoters.

Discussion

CK1 and CK2 represent two highly conserved serine/threonine kinases that are ubiquitously expressed in eukaryotic cells and have multiple cytoplasmic and nuclear functions [8, 10]. Here we show that both CK1δ/ε and CK2 directly bind to and phosphorylate LEF-1. Phosphorylation of LEF-1 by CK1 or CK2 may induce a conformational change in the LEF-1 protein that is subsequently transduced to the DNA. This is in line with original findings that LEF-1 acts as an architectural transcription factor [29]. Therefore, CK1 and CK2 appear to be involved in the regulation of the assembly of higher-order nucleo-

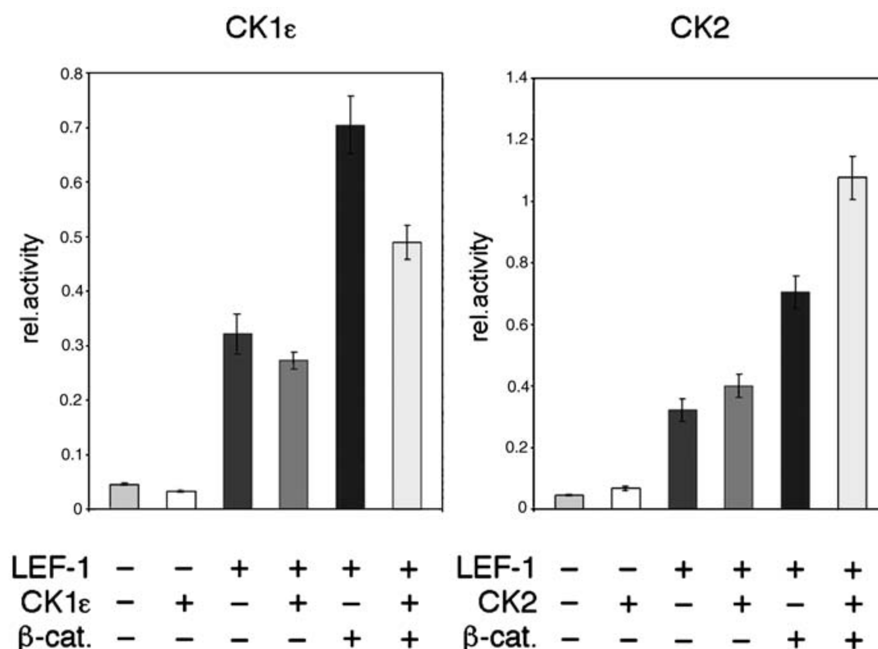


Figure 6. CK1 and CK2 modulate LEF-1/β-catenin transcriptional activity. For dual luciferase reporter gene assays, HEK293 cells were transiently transfected with a Siamois-luciferase reporter plasmid and the pHRL-TK (*Renilla* luciferase) plasmid to normalize transfection efficiency. Expression plasmids for LEF-1, β-catenin, CK1ε and CK2 were co-transfected in various combinations as indicated. Results are mean values of four independent experiments.

protein complexes at the promoters of LEF-1-regulated genes. Consistent with the inhibitory activity of CK1ε in reporter assays, we observed that phosphorylation of LEF-1 by CK1 inhibits formation of the LEF-1/β-catenin complex. Interestingly, CK2 did not alter the LEF-1/β-catenin interaction. In this respect, CK2 activity was recently reported to increase the affinity of LEF-1/TCF transcription factors to DNA [18]. Our experiments did not unequivocally confirm this observation, but this may be due to different assay conditions. Moreover, CK2-dependent phosphorylation was shown to block binding of plakoglobin to TCF4 and this appears to increase the transcriptional activity of the TCF4/β-catenin complex [30].

Although the functions of CK1 and CK2 in the Wnt signalling pathway have been intensively studied, their roles are still ambiguous. Whether CK1 and CK2 regulate all TCF family members in the same way as LEF-1 is not clear. In contrast to our results, phosphorylation of XTCF3 by CK1ε in *Xenopus* oocyte extracts promotes the interaction of XTCF3 with β-catenin [27] and an activating effect for CK1 in reporter gene assays was detected [31, 32]. This discrepancy with our results may either be explained by specific features of the proteins themselves or by the presence of specific factors in the oocyte cytosol. TCFs differ from LEF-1 in their C-terminal extensions, which contain additional putative phosphorylation sites. Moreover, they were recently shown to differ in their potential to activate transcription of specific target genes [33, 34]. CK1- and CK2-dependent phosphorylation may therefore induce promoter-specific effects, which result from the architectural changes induced by LEF-1 phosphorylation. These changes promote or inhibit context-specific assembly of high-molecular-weight nucleoprotein complexes, which regulate target gene transcription. However, the reporter gene activity measured in these assays is the final outcome of CK1 and CK2 activity in the transfected cells and does not differentiate between the effects of CK1 or CK2 activity on the LEF-1/β-catenin transcription complex and/or on the β-catenin destruction complex.

CK1 and CK2 affect the Wnt pathway at the level of the β-catenin destruction complex and in the nucleus at the level of the LEF-1/TCF/β-catenin transcription complex. Many components of these complexes are potential substrates for both kinases. Whether Wnt signalling affects the role and/or target specificity of CK1 and CK2 is not clear. Moreover, there are controversial observations whether the different CK1 isoforms (α and δ/ε) play both positive and negative or different roles. In this respect, the effects of CK1α on the LEF-1/β-catenin complex are currently unknown.

Consistent with our observations, numerous recent studies provide further evidence for a negative role of CK1. β-Catenin has been reported to be phosphorylated by a dual-kinase mechanism where CK1 (α, δ, ε) prephosphorylates β-catenin as a prerequisite for subsequent GSK3β-dependent phosphorylation [35, 36]. Diversin, an ankyrin repeat protein, has been shown to recruit

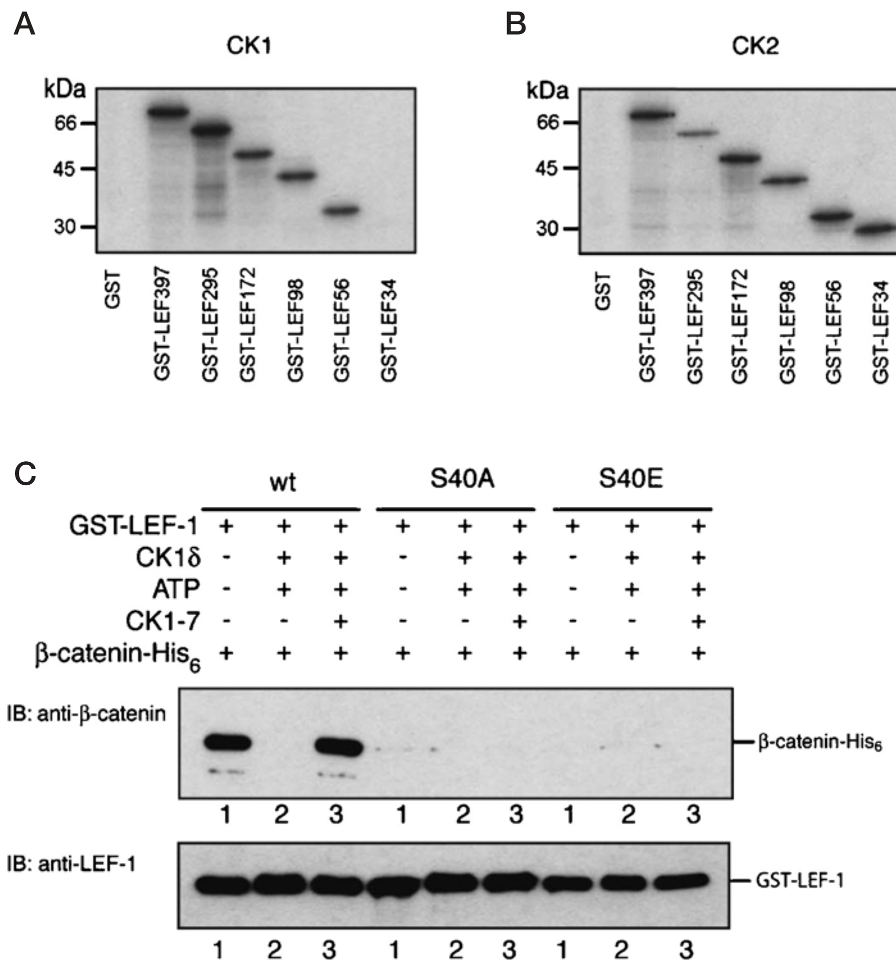


Figure 7. Serine 40 in LEF-1 is critical for the LEF-1/β-catenin interaction. (*A, B*)In vitro phosphorylation was performed with recombinant CK1δ (*A*) or CK2 (*B*) on comparable amounts of GST, GST-LEF-1 and C-terminal-truncated GST-LEF-1 fusion proteins. Numbers indicate the C-terminal amino acids of the constructs. (*C*) Substitution of Ser40 in GST-LEF-1 with alanine or glutamate does not affect binding of GST-LEF-1 to DNA (lower panel) but impairs formation of an LEF-1/β-catenin complex. GST-LEF-1/β-catenin-His_6 complexes bound to the biotinylated DNA probe were pulled down with streptavidin magnetic beads. The presented gels are representatives of at least three independent experiments.

CK1ε to the Axin/Conductin-GSK3β complex and to promote β-catenin degradation [37]. Moreover, Axin was shown to support the CK1ε-dependent phosphorylation of APC which is proposed to be involved in the down-regulation of β-catenin [38]. In vivo experiments in *Drosophila* using RNA interference to inactivate CK1α and ε resulted in elevated levels of Armadillo protein, again consistent with a negative role of CK1 in Wnt/Wg signalling [39]. Further studies, however, now suggest that CK1ε has no negative effect on Wg signalling in *Drosophila* [40].

In contrast, overexpression of CK1 induces axis duplication, stabilization of β-catenin and transcription of target genes in *Xenopus* embryos [31, 41]. This stimulatory effect on Wnt signalling appears not to be isoform specific; however, there are contradictory results in the literature with respect to CK1α [31, 42, 43]. There is evidence that CK1δ/ε act as positive regulators of the canonical Wnt pathway through Dishevelled, resulting in destabilization of the β-catenin destruction complex [41, 44–46]. This appears to be mediated by an enhanced formation of a complex between Dishevelled and Frat-1 [47]. Moreover, Wnt signalling was recently shown to stimulate CK1ε activity [48].

Until recently, knowledge about the role of CK2 was more consistent. In *Drosophila* cells, CK2 associates with and phosphorylates Dishevelled, and overexpression of the Wingless receptor DFz2 stimulated phosphorylation of Dsh [49]. Moreover, Wnt-transfected cells exhibit an increased level and activity of CK2. In these cells, CK2 and β-catenin associate with Dvl proteins, and inhibition of CK2 activity by apigenin accelerates the rate of degradation of β-catenin and Dvl [25] suggesting a positive role for CK2 in Wnt signalling. Phosphorylation of Thr393 in

the Armadillo repeat region appears to be important for the stabilization of β-catenin [50]. Our results, showing an increased activity of the LEF-1/β-catenin transcription complex in cells transfected with CK2, together with the observation that CK2 did not disrupt the LEF-1/β-catenin transcription complex, support a stimulatory function for CK2 in the Wnt signalling pathway. This is consistent with recent results showing that phosphorylation of TCF4 by CK2 did not impair binding to β-catenin [30]. Also in favour of a positive regulatory function of CK2 in Wnt signalling is the recent observation that the C-terminal domain of APC inhibits CK2 activity [51]. This domain is deleted in many colorectal cancer cells and thus may result in a sustained CK2 activity contributing to enhanced β-catenin signalling. However, the role of CK2 in the Wnt pathway is now no longer unequivocally clear since the E2 ubiquitin-conjugating enzyme UBC3B has been shown to be a CK2 substrate which upon phosphorylation interacts with β-TrCP and enhances β-catenin degradation by regulating β-TrCP substrate recognition [52]. This clearly favours a negative regulatory role of CK2 on Wnt signalling. Moreover, CK2 in conjunction with Axin and GSK3β was recently reported to act as a negative regulator of β-catenin cytoplasmic stability [26]. Currently, these apparent discrepancies are difficult to explain. Activation of the pathway by binding of Wnt proteins to the Frizzled/LRP receptor complex may either lead to a relocalization and/or to the activation of different isoforms of the kinases or of substrate proteins. In the unstimulated situation, CK1 and CK2 support degradation of β-catenin and/or destabilization of the LEF-1/β-catenin complex. Wnt stimulation then induces inter- and/or intramolecular changes, e.g. high-affinity binding to new interaction partners or pathway components, which may result in changes of the phosphorylation sites used in the different protein complexes and, as a result, a switch from a negative to a positive regulatory effect in a context-dependent manner. The observed differences may therefore depend on the experimental systems used to analyse this question. Overexpression of Wnt pathway components after transient transfections or RNA injections in embryos may allow the formation of protein complexes that may not represent physiological situations.

In conclusion, our findings provide evidence that CK1 and CK2 are not only involved in Wnt signalling by regulating β-catenin stability at the level of the β-catenin destruction complex but, in addition, modulate the pathway at the level of the LEF-1/β-catenin transcription complex. CK1-dependent phosphorylation of both LEF-1 and β-catenin disrupts the LEF-1/β-catenin transcription complex and, together with its putative stimulatory effect on β-catenin degradation, provides a second step for CK1 in the negative control of Wnt signalling in cells. With respect to the dramatic consequences that are induced by deregulated activation of Wnt target genes resulting in carcinogenesis, a co-ordinated regulation at two levels appears to be an elegant mechanism to tightly control canonical Wnt signalling.

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