The Transcriptional Factor Tcf-4 Contains Different Binding Sites for β-Catenin and Plakoglobin*

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β-Catenin and plakoglobin are two related armadillo proteins necessary for the establishment of adhesion junctions and desmosomes. Moreover, β-catenin can also act as a transcriptional co-activator through its interaction with the members of Tcf/LEF-1 transcriptional factor family. We show here that Tcf-4 can be phosphorylated in vitro by protein kinase CK2 stoichiometrically in amino acids Ser-58–Ser-59–Ser-60. Phosphorylation of these residues does not modify the interaction of Tcf-4 with β-catenin but reduces its association to plakoglobin. The binding sites of Tcf-4 for these two proteins were compared; whereas β-catenin requires the N-terminal first 50 amino acids, plakoglobin interacts mainly with residues 51–80. Tcf-4(51–80) binds plakoglobin in the region of armadillo repeats 1–6. Ternary complexes composed by β-catenin/Tcf-4/plakoglobin could be detected in vitro, demonstrating that simultaneous binding of the two armadillo proteins to Tcf-4 is possible. Experiments performed using a Tcf-4 mutant with decreased interaction to plakoglobin demonstrated that binding to this protein negatively affected the transcriptional activity of Tcf-4. These results indicate that Tcf-4 contains two different sites for binding of β-catenin and plakoglobin, and the interaction of the latter hinders the transcriptional activity of the complex.

β-Catenin and its closely related homologue plakoglobin play a crucial role in cell-cell adhesion. In adherens junctions, β-catenin and plakoglobin independently link the cytosolic domain of cadherins to α-catenin, which in turn directly or indirectly interacts with the actin filaments (for reviews, see Refs. 1–3). In addition, plakoglobin is a component of the desmosomes, where it mediates the association of the desmosomal cadherins, desmoglein and desmocollin, to the intermediate filament cytoskeleton (4, 5). β-Catenin and plakoglobin are members of the armadillo family of proteins, which share a common structural motif, important for protein-protein interactions. Both molecules contain 12 central armadillo repeats (comprised of 42 amino acids each) with a 76% identity (6). The crystal structure of the armadillo domain of β-catenin forms a superhelix that features a positively charged groove that can serve as a binding surface for its many partners (7). In contrast, the N and C termini of β-catenin and plakoglobin are acidic and share only a 29 and 41% identity, respectively. It has been proposed that the terminal domains of catenins could form intramolecular interactions with the armadillo domain that negatively regulate its association with several proteins such as desmoglein (8), E-cadherin, and the TATA-binding protein (9).

Besides its role in cell adhesion, β-catenin also has a signaling activity as a member of the Wnt pathway (10, 11). The signaling role of β-catenin is mediated through its interaction with transcription factors of the T cell factor (Tcf) family. In this complex, Tcf provides the DNA binding domain, whereas β-catenin contributes the transactivation domain, allowing the subsequent activation of specific target genes involved in embryonic development (11, 12). The formation of Tcf-β-catenin complexes depends on the amount of free β-catenin. Thus, β-catenin exhibits a dual localization, at the membrane as part of the adherens junction complexes and at the nucleus, where it is involved in transcriptional activation. Tyrosine phosphorylation may participate in the regulation of these two pools of β-catenin (13). It has been demonstrated that phosphorylation of β-catenin Tyr-654 controls β-catenin-E-cadherin association (14). Additionally, β-catenin levels are efficiently regulated by the proteasome degradation system (15, 16). The targeting for β-catenin degradation involves phosphorylation within its N terminus by glycogen synthase kinase 3β (15, 17) in a complex that includes the tumor suppressor product of adenomatous polyposis coli gene, axin, and β-TRCP/ slimb (12, 18–20), which results in β-catenin ubiquitination and subsequent degradation. The binding of Wnt to its Frizzled receptor results in the inhibition of glycogen synthase kinase 3β activity and subsequent increase in β-catenin levels (21, 22). This is followed by the translocation of β-catenin into the nucleus, where it binds

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1 The abbreviations used are: TBP, TATA-binding protein; Tcf, family of the T cell transcription factors; GST, glutathione S-transferase; mAb, monoclonal antibody; MDCK, Madin-Darby canine kidney cells.
to the N terminus of LEF/Tcf transcription factors, forming a complex that activates target genes (23–26).

It is well accepted that deregulation of β-catenin signaling, which results in its accumulation and improper activation of transcriptional targets, is associated with tumors in a variety of tissues (27–31). Elevated levels of β-catenin/Tcf-4 are found in the majority of human colon tumor cell lines. This is mainly due to stabilization of β-catenin by different mechanisms, which are through either (i) mutations in the adenomatous polyposis coli gene, which result in truncated adenomatous polyposis coli proteins that impede GSK3-β phosphorylation of β-catenin and no longer regulate β-catenin levels (32, 33) or (ii) stabilizing mutations in the N-terminal tail of β-catenin, which also interfere with GSK3-β phosphorylation (29, 31), leading to β-catenin accumulation in the cytoplasm and nucleus (28, 30).

Although the signaling role of β-catenin is well established, it is not clear whether plakoglobin also acts as a specific transcriptional activator of LEF/Tcf-responsive genes. Plakoglobin also binds to the N terminus of Tcf-4 with an affinity similar to that of β-catenin but does not seem to directly activate transcription (34, 35). Moreover, these authors report that in plakoglobin-transfected cells, plakoglobin was unable to form a ternary complex containing the LEF-1 transcription factor and the LEF-1 binding DNA sequence (36). This inhibitory effect of plakoglobin was assigned to a negative regulation of the catenin N- and C-terminal domains.

The ubiquitously expressed serine/threonine protein kinase CK2 has been implicated in the regulation of cell growth and proliferation because it is up-regulated in many human tumors (37, 38). More recently, CK2 has been described as a positive regulator of the Wnt pathway, leading to the accumulation of β-catenin (39). These authors report that β-catenin is a substrate of this kinase. When we tried to reproduce these results, we observed that purified β-catenin is not a direct substrate of CK2 in vitro. In contrast, this kinase efficiently phosphorylates the N-terminal domain of human Tcf-4 (hTcf-4). We show that, although phosphorylation of hTcf-4 affects its binding to plakoglobin, it does not modify its interaction with β-catenin. This result allowed us to identify close but different hTcf-4 binding regions for β-catenin and plakoglobin.

**EXPERIMENTAL PROCEDURES**

*Generation of hTcf-4 Mutants—Point mutants S58A/S59A/S60A, S58E, and S60E were obtained using the QuikChange™ site-mutation kit (Stratagene, La Jolla, CA). A polymerase chain reaction was performed using Pfx polymerase, pCDNA1.1His-Tcf-4 as template and oligonucleotide primers containing each mutation. Sense primers used for generation of S58A/S59A/S60A, S58E, and S60E were, respectively, 5′-GAATTCCGAGGCGGA-3′/H9252 and 5′-GAACTCAGCTCCAGCT-3′/H9252, 5′-AAAGAATT-CTTCCGCCTCCTCG-3′ and 5′-GAACTCAGCTCCGGA-3′. Modified nucleotides respect the hTcf-4 sequence (GenBank™ accession number Y11306) are indicated in bold. The amplified fragment was treated with DpnI, which digests the parental construct. Finally, the nicked plasmid was transformed and sequenced. The expression plasmid pGEX coding the 1–80-amino acid fragment of hTcf-4 mutants was generated from pGEX-6P1-full-length-Tcf-4 mutants by cutting with Smal and XhoI, filling in, and ligating. Deletion mutant 1–30 was generated by cutting pGEX-6P1-Tcf-4 with EcoI.CRI-NotI, filling in, and ligating. Mutants 1–51, 1–110, 10–51, and 51–110 were amplified from entire Tcf-4 cDNA by polymerase chain reaction using oligonucleotides corresponding to nucleotide sequences 1–15, 28–42, 151–165, 153–138, and 331–315. The amplification fragments were inserted into the BamHI-XhoI or EcoRI-XhoI sites of a pGEX-6P1 plasmid. The 51–80 mutant was obtained from pGEX-6P1-Tcf-4(51–110) cutting with Smal and NotI, filling in, and ligating. All mutants were verified by sequencing.*

*Expression of Recombinant Proteins—The complete cDNA for human plakoglobin, kindly provided by A. Ben-Ze’ev (Weisman Institute, Israel) and A. Cano (Universitat Autonoma de Madrid, Spain), was cloned in the EcoRI site of pGEX-6P2. A DNA fragment comprising the 12 armadillo repeats (amino acids 111–672) was amplified from the entire plakoglobin cDNA by polymerase chain reaction using oligonucleotides corresponding to the nucleotide sequences 331–348 and 2016–1999. Plakoglobin armadillo fragments comprising repeats 1–6 (amino acids 111–385) or 7–12 (amino acids 380–672) were amplified by polymerase chain reaction using oligonucleotides corresponding to sequences 331–348 and 1138–1138 or 1138–1155 and 2016–1999, respectively. All the amplification fragments were inserted in the BamHI-SmaI sites of a pGEX-6P1 plasmid. The plakoglobin fragment comprising amino acids 1–113 was generated from pGEX-6P1-plakoglobin by cutting with PstI and EcoRI. Blunt ends were generated with Klenow enzyme, and the 460-base pair fragment was cloned into the EcoRI-SmaI site of pGEX-6P2. The plakoglobin C-terminal tail (amino acids 667–745) was obtained cutting pGEX-6P1-plakoglobin with EcoRI and inserting the 1.1-kilobase fragment in the SmaI site of pGEX-6P2. All hTcf-4, plakoglobin, or β-catenin mutants described in Piedra et al. (9) and inserted in pGEX-6P2 plasmids were expressed in E. coli. The different domains of the recombinant proteins used in this work is shown in Fig. 1.*

*Catenin-Tcf-4 Binding Assays—Binding assays were performed using purified recombinant proteins as described previously (9, 14). Pull down assays were performed using cytosolic extracts of SW-480 cells, an intestinal cell line that contains abundant β-catenin and plakoglobin.*

**FIG. 1. Diagram of the recombinant proteins used in this work.**

The different domains on the three proteins examined, β-catenin, plakoglobin, and Tcf-4 are shown. The darker part of Tcf-4 catenin binding domain corresponds to the sequence that has been shown to be involved in β-catenin association (42). The deletion mutants used in this article are depicted, indicating which part of the molecule they comprise.
The presence of bound proteins in the complexes was analyzed by Western blot with specific mAbs against β-catenin, plakoglobin (both from Transduction Laboratories, Lexington, KY), or hTcf-4 (clone 6H5-3, from Upstate Biotechnology, Lake Placid, NY or anti-N termi

In Vivo Analysis of Catenins-Tcf-4 Association—Epithelial pancreas cancer RWP1 cells were chosen for these experiments because they are transduced with high efficiency. Cells were grown in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal calf serum (Invitrogen). 50% confluent cells were transfected with the indicated Eagle’s medium (Invitrogen) supplemented with 10% fetal calf serum (Invitrogen). 50% confluent cells were transfected with the indicated plasmids using LipofectAMINE (Invitrogen) according to the instructions of the manufacturer. After transfection, cells were incubated for 48 h in Dulbecco’s modified Eagle’s medium plus 10% fetal calf serum. Cells were lysed in 200 μl of 50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 5 mM MgCl₂, 0.1% Nonidet P-40, 1 mM dithiothreitol, 10 mM sodium orthovanadate, 10 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin on ice for 30 min. Lysates were centrifuged at 13,000 rpm in a microcentrifuge for 15 min at 4°C. 150 μg of extract were incubated in a final volume of 0.3 ml with 20 μl of a 50% (w/v) suspension of nickel nitritotriacetic acid-agarose (Qiagen, Hilden, Germany) for 1 h at 4°C. Beads were washed with lysis buffer, and bound proteins were eluted with electrophoresis sample buffer and analyzed by Western blot with the anti-Xpress™ antibody. Blots were stripped and re-analyzed with mAbs against plakoglobin or β-catenin.

Reporter Gene Assays—MDCK cells, a cell line that contain low basal β-catenin-mediated transcription, were cotransfected as above with wild type or mutant Tcf-4 forms and a plasmid containing three copies of the Tcf-4 binding site upstream a firefly luciferase reporter gene (plasmid TOP-FLASH) (28). The activity of the product of the Renilla luciferase gene under the control of a constitutive thymidine kinase promoter (Promega) was used as a control. Assays were always performed in triplicate; the average of the results of 3 independent transfections ± S.D.

Tcf-4-DNA Binding Assays—Two oligonucleotides (sense and antisense) corresponding to the Tcf-4 binding site TBE2 (5′-CTCTTGTGCAAAACCCGGCCG-3′) within the myc promoter were synthesized, labeled with 32P, bound to immobilized oligonucleotides with 200 μg of transfected MDCK total cell extract for 45 min at 4°C. Incubations were performed in the presence of 20 mM HEPES, pH 7.6, 150 mM KCl, 3 mM MgCl₂, 10% glycerol, and 0.3 mg/ml bovine serum in a final volume of 200 μl. 20 μg of poly(dI-dC) were used as a non-specific competitor. Cells were transfected with the indicated plasmids as mentioned above, and cell extracts were prepared as described. Protein complexes were isolated by incubation with 40 μl of a 50% (w/v) suspension of streptavidin-agarose (Sigma) for 30 min at 4°C. Beads were collected by spinning in a microcentrifuge and washed three times with binding buffer. Samples were separated by SDS-PAGE, and the presence of bound proteins in the complex was analyzed by Western blot with specific mAbs against β-catenin, plakoglobin, or Tcf-4 N terminus.

RESULTS

Protein kinase CK2 has been found to phosphorylate many transcription factors (41). Recently, it has been described to phosphorylate two members of the Wnt signaling pathway, Dishevelled and β-catenin (39). Furthermore, consensus sequence analysis indicated multiple sites in the N-terminal do

main of hTcf-4 that fit the classical potential phosphorylation motif SXX(E/D) for CK2 phosphorylation. Therefore, the ability of purified CK2 to phosphorylate in vitro both β-catenin and the β-catenin binding domain of hTcf-4 was tested on recombinant-GST fusion proteins. Whereas CK2 was found to efficiently phosphorylate the N-terminal domain of hTcf-4, neither β-catenin nor GST alone were directly phosphorylated by CK2 (Fig. 2A, right panel). Coomassie Blue staining of the gel confirmed that the same amounts of protein were used in the assay (Fig. 2A, left panel). The stoichiometry of this phosphorylation was investigated. 50 milliunits units of CK2 were able to incorporate up to 1.4 pmol of phosphate/pmol of Tcf-4 (1–80) (Fig. 2B). This stoichiometry indicated that more than one site was being phosphorylated. The identification of the modified Ser/Thr residues in the N-terminal region of hTcf-4 was pursued. No CK2 phosphorylation was detected on two shorter N-terminal Tcf-4 deletion fragments comprising amino acids 1–30 and 1–49 (Fig. 2C, bottom panel). This result indicates that phosphorylation was confined to the hTcf sequence 50–80. The best candidates for phosphorylation were three Ser residues in positions 58–60. Therefore, these three Ser residues were replaced by Ala, and the triple mutant GST-Tcf-4(1–80) S58A/S59A/S60A was

In Vitro Phosphorylation Assays—In vitro assays were performed in a final volume of 25 μl in the following conditions: 0.5 mM EGTA, 9 mM MgCl₂, 0.55 mM EDTA, 27.8 μg/ml β-glycerophosphate, 1 mM diithothreitol, 0.1 mM [γ-32P]ATP (1000 cpm/pmol), 1 μg of β-catenin, GST-Tcf-4(1–80), GST-Tcf-4(1–80) point mutants, or GST as a control, and 50 milliunits of protein kinase CK2, purified from rat liver as previously described (40). One unit of CK2 is defined as the amount that catalyzes the transfer of 1 nmol of phosphate from [γ-32P]ATP to 1 mg/ml of β-casein/min. Reactions were performed at 30°C for 10–150 min. Samples were analyzed by SDS-PAGE and autoradiography. Quanti

Fig. 2. Phosphorylation of the N-terminal domain of hTcf-4 by protein kinase CK2. Panel A, Coomassie Blue-stained gel (left panel) and autoradiogram (right panel) of the in vitro kinase assay of GST-Tcf-4(1–80), β-catenin, or GST as a control. 1 μg of recombinant protein was phosphorylated in the presence of [γ-32P]ATP by 50 milliunits of CK2 under conditions indicated under “Experimental Procedures.” Panel B, the amount of [32P]phosphate incorporated in the GST-Tcf-4(1–80) was determined in a scintillation counter and represented at different times of reaction. Panel C, 1 μg of either GST-hTcf-4(1–80) or the point mutants and equimolar concentrations of the shorter fragments GST-hTcf-4(1–30) or GST-hTcf-4(1–51) were phosphorylated with 50 milliunits of purified CK2 as described and analyzed by SDS-PAGE (upper panel) and autoradiography (bottom panel). WT, wild type.
indicate the densitometric values in arbitrary units, referred to the rose and analyzed by SDS-PAGE and Western blot with anti-cells. Protein complexes were affinity-purified with glutathione-Sepharose, and the presence of β-catenin in the beads was analyzed by Western blot with a specific mAb. As shown in Fig. 3A, CK2 phosphorylation of Tcf-4(1–80) did not affect β-catenin-Tcf-4(1–80) association.

In an attempt to explore the functional significance for the phosphorylation of serines 58–60 in hTcf-4, we investigated the possibility that this modification was affecting the interaction with other hTcf-4 binding partners, such as plakoglobin, which also interacts within the N-terminal domain of hTcf-4. Phosphorylation of GST-Tcf-4(1–80) by CK2 significantly decreased its interaction with plakoglobin in pull-down experiments, although as mentioned above, it did not influence its association with β-catenin (Fig. 3B). To further ensure the specific effect of serine 58–60 phosphorylation, the GST-Tcf-4 mutant in which the three Ser residues 58–60 were replaced by Ala, was used. Equivalent plakoglobin binding was obtained with the triple GST-Tcf-4(1–80) mutant, either incubated or not with CK2 under phosphorylation conditions, and the unphosphorylated wild-type form of GST-Tcf-4(1–80) (Fig. 3B).

The same results were found when in vitro binding of recombinant plakoglobin and Tcf-4 was determined (not shown).

To verify the hypothesis that hTcf-4 phosphorylation within serines 58–60 by CK2 is relevant for the decrease in plakoglobin binding, these three Ser residues were individually replaced by Glu to mimic the effects of phosphorylation. Among them, Ser-58 with 2 Ser residues at positions +1 and +2 and an Asp at position +3 and Ser-60 with Asp and Ser residues at positions +1 and +2 and a Glu at +3 had the best fit for CK2 phosphorylation. Ser-60 is much more conserved in the LEF1 family members than Ser-58. Therefore, these two Ser residues, 58 and 60, were mutated to Glu, and the two mutant proteins were expressed, purified, and tested for phosphorylation experiments. As shown in Fig. 2C, the GST-hTcf-4(1–80) mutant S58E was even more efficiently phosphorylated by CK2 than the wild-type fragment, probably due to a new phosphorylation site created at Thr-54. Therefore, this mutant was discarded for further experiments. In contrast, the S60E mutant was phosphorylated to a much lower extent compared with the wild-type form. Thus, these results suggest that CK2 at least catalyzes phosphorylation of Ser-60. The capacity of this GST-Tcf-4(1–80) mutant S60E to bind β-catenin and plakoglobin was assayed in vitro and compared with that of wild-type GST-Tcf-4(1–80). Whereas the S60E hTcf-4 associated to β-catenin identically as the wild-type form, this mutant bound plakoglobin less efficiently than the wild-type hTcf-4 fragment (Fig. 3B).

This difference was also evidenced in vitro. RWP1 cells were transfected with wild-type or hTcf-4(1–80) mutants S60E and S58A/S60A labeled with polyhistidine and the X-Press® tag to facilitate their purification and identification. Transfected hTcf-4 forms were purified by nickel nitrioltriacetic acid-agarose chromatography, and the amount of bound plakoglobin was determined. As shown in Fig. 3C, the amount of plakoglobin copurified with the S60E hTcf-4 mutant was substantially decreased compared with the wild-type form. No differences could be detected between the wild-type form and the S58A/S60A hTcf-4 mutant. This fact is probably due to poor phosphorylation of wild-type hTcf-4 in these cells. As illustrated in

![Diagram](http://www.jbc.org/)

**Fig. 3. Phosphorylation of the N-terminal domain of Tcf-4 by CK2 does not modify β-catenin-Tcf-4 interaction but inhibits plakoglobin-Tcf-4 association.** Panel A, 1.2 pmol of control and CK2-phosphorylated GST-Tcf-4(1–80) were incubated with 0.64 pmol of β-catenin in a final volume of 200 μl as described. Protein complexes were purified with glutathione-Sepharose and analyzed by SDS-PAGE and Western blot with anti-β-catenin mAb. Known amounts of β-catenin were included as reference (St). The numbers below the lanes indicate the amount of bound β-catenin calculated by densitometry. Panel B, GST or GST-Tcf-4(1–80) fusion proteins were phosphorylated by CK2 under the conditions indicated under “Experimental Procedures.” Pull-down assays were performed incubating 12 pmol of the GST proteins with 50 μg of total cell extracts prepared from SW480 cells. Protein complexes were affinity-purified with glutathione-Sepharose and analyzed by SDS-PAGE and Western blot with anti-β-catenin mAb. Membranes were stripped and re-analyzed with anti-p-catenin. Protein complexes were purified with glutathione-Sepharose and analyzed by SDS-PAGE and Western blot with anti-β-catenin mAb. Known amounts of β-catenin were included as reference (St). The numbers below the lanes indicate the amount of bound β-catenin calculated by densitometry.

Panel C, affinity-purified GST-Tcf-4(1–80) complex was incubated with 1.2 pmol of GST-Tcf-4-(1–80), either phosphorylated or not by CK2. Complexes were bound to glutathione-Sepharose, and the associated plakoglobin was analyzed by Western blot with a specific mAb. Membranes were stripped and re-analyzed against plakoglobin.
In all the panels, the autoradiograms were scanned in a densitometer, and the results were compared with standards. The numbers below the autoradiograms indicate the amount of bound protein.

Panel A, 0.6 pmol of plakoglobin was incubated with 0.8 pmol of either GST-Tcf-4-(1–80) or GST in a final volume of 200 μl. 0.4 or 0.8 pmol of β-catenin were added to displace plakoglobin from the complexes. Samples were analyzed by SDS-PAGE and Western blot with anti-plakoglobin mAb. To verify that β-catenin was associated in the complexes, membranes were stripped and re-analyzed with mAb against β-catenin. 0.025 pmol (2 ng) of plakoglobin or 0.055 pmol (5 ng) of β-catenin were included as internal references (St).

Panel B, 1.2 pmol of GST or GST-plakoglobin fusion proteins were incubated with 1.2 pmol of Tcf-4-(1–80) in the presence or absence of two different concentrations of β-catenin (1 and 2 pmol). The amount of associated Tcf-4 or β-catenin was determined using mAbs specific for these two proteins. 0.06 pmol of Tcf-4-(1–80) was included as internal reference (St). Panel C, 1.2 pmol of GST or GST-plakoglobin deletion mutants were incubated with 1.2 pmol of Tcf-4-(51–110). The amount of bound Tcf-4 was analyzed with a specific mAb against Tcf-4. 0.05 pmol of Tcf-4-(51–110) was included as internal reference (St). The numbers below the autoradiograms indicate the amount of bound protein.

At this point, we investigated whether binding of plakoglobin and β-catenin to the N-terminal tail of hTcf-4 was mutually exclusive or whether both could simultaneously interact with Tcf-4. Several experiments were performed. As shown in Fig. 4A, the addition of β-catenin was unable to displace plakoglobin from a complex with GST-Tcf-4-(1–80) even if β-catenin was added in excess compared with plakoglobin. Thus, association of plakoglobin to Tcf-4-(1–80) was not competed by β-catenin. To verify this conclusion, Tcf-4-(1–80) was bound to GST-plakoglobin in the absence or presence of β-catenin. As shown in Fig. 4B, association of Tcf-4-(1–80) to GST-plakoglobin was not affected by the addition of an excess of β-catenin. The result of this experiment also indicates that β-catenin is retained by the complex GST-plakoglobin-Tcf-4-(1–80) (Fig. 4B, lower panel). Additional binding experiments using GST-β-catenin conclusively demonstrate that β-catenin and plakoglobin associate only in the presence of Tcf-4 (Fig. 4C). Because both catenins bind to different hTcf-4 sites, we generated several N-terminal deletion mutants of Tcf-4 to characterize the plakoglobin binding domain within Tcf-4. Different from β-catenin, which interacted mainly with Tcf-4 fragment 10–51 (Fig. 5A), plakoglobin bound preferentially to Tcf-4-(51–80) (Fig. 5B). Comparison of these two experiments provided evidence that the affinity of Tcf-4-(51–80) for plakoglobin is 2.5-fold lower than that of Tcf-4-(1–50) for β-catenin (see Figs. 5, A and B).

The binding site for Tcf-4-(51–110) in plakoglobin was also mapped using different plakoglobin deletion mutants. Neither the N-tail (amino acids 1–113) nor the C-tail (667–745) bound to Tcf-4-(51–110) (Fig. 5C). The plakoglobin armadillo domain (amino acids 113–667) interacted with Tcf-4-(51–110) slightly but reproducibly better than full-length plakoglobin, suggesting that, as in the case of binding of E-cadherin and TBP to β-catenin (9), the N- and C-tails restrict the association of Tcf-4 to the central domain. As shown in Fig. 5C, binding of Tcf-4-(51–110) to the plakoglobin armadillo domain requires only the first six armadillo repeats (amino acids 111–385), since Tcf-4-(51–110) interacted similarly with plakoglobin fragments corresponding to amino acids 111–672 and 111–385. On the con-
Plakoglobin and β-Catenin Bind to Different Sites on Tcf-4

**FIG. 6. Absence of plakoglobin from hTcf-4-DNA complexes.** MDCK cells were transfected with 5 μg of pcDNA3-His Tcf-4 (wild type (WT) or S60E mutant). 200 ng of biotinylated oligo containing the binding sequence for Tcf-4 in the c-myc promoter (Oligo PrMyc) was incubated with 200 μg of cell lysates prepared from transfected MDCK cells. The biotinylated oligo was purified by chromatography on streptavidin-agarose, and associated Tcf-4 was analyzed by Western blot with polyclonal antibody against Tcf-4. Membranes were stripped and reanalyzed with mAb against β-catenin and plakoglobin. Input, a sample corresponding to 30% of the total extract Tcf-4 used for the assay; cont, no specific oligo added.

**DISCUSSION**

Protein kinase CK2 is present both in the cytoplasm and in the nucleus. CK2 has been postulated to contribute to tumorigenesis since its activity is up-regulated in human tumors and in proliferating cells (37, 38). As indicated in the Introduction, two elements of the Wnt pathway have been suggested to be directly phosphorylated by this kinase. It has recently been reported that, upon transfection of Wnt-1 into the mouse mammary epithelial cell line C57MG, CK2 and β-catenin co-precipitate with Dishevelled (39). These authors indicated that β-catenin was phosphorylated by CK2 in the Dishevelled immunoprecipitates. We can confirm that CK2 and a β-catenin kinase can be pulled down by β-catenin from cell extracts (data not shown). However, a highly purified CK2 was unable to directly phosphorylate recombinant β-catenin (Fig. 2). These possible discrepancies can be explained in different ways: 1) β-catenin is not a direct substrate of CK2 but of another kinase associated to this enzyme; 2) β-catenin has to be pre-phosphorylated by another enzyme in order for CK2 to be able to act on this protein; or 3) interaction of β-catenin with other cell components alters its conformation and renders the phosphorylation sites accessible to CK2. In either case, the role of CK2-catalyzed phosphorylation of β-catenin in the function of this protein still has to be determined.

We show here that CK2 also efficiently phosphorylates another member of the Wnt pathway: hTcf-4. The residue most efficiently phosphorylated in this protein is Ser-60, a residue placed close to the β-catenin binding domain. Modification of this residue is relevant since it decreases binding to plakoglobin and the ability of hTcf-4 to stimulate transcription. However, in the assays of β-catenin-mediated transcription, we have not detected differences in the activity of wild-type and S58A/S59A/S60A Tcf-4 forms, suggesting that transfected Tcf-4 is not phosphorylated. It is then possible that this modification does not play an important role in the control of Tcf-4-mediated transcription in this cell line. However, it should be considered that the ability of CK2 to phosphorylate Tcf-4 is greatly inhibited by binding of β-catenin or plakoglobin; only unbound Tcf-4 is phosphorylated with high activity. This effect has to be taken in consideration when the phosphorylation status of Tcf-4 is analyzed.

In any case, phosphorylation of Tcf-4 by CK2 has allowed us to identify two different binding sites on this protein for β-catenin and plakoglobin. The two catenin binding domains were allocated to different sequences, 1–51 for β-catenin and 51–80 for plakoglobin, and they do not interfere because hTcf-4 can simultaneously bind both proteins. This result suggests that the negative effect of plakoglobin on β-catenin-mediated transcription is unlikely to be a consequence of competition for binding to Tcf-4. It is more plausible that, as suggested by Ben-Ze’ev and co-workers (36), association of plakoglobin might hinder the DNA binding domain of Tcf-4 independently of its interaction to β-catenin. These authors have also suggested that plakoglobin N- and C-tails are involved in this repression; it is possible that the little sequence similarity between these two tails of β-catenin and plakoglobin (44) might be related to this opposite effect on Tcf-4-DNA binding. Because the armadillo domains of β-catenin and plakoglobin are quite similar, it is also possible that the differences in specificity for Tcf-4

**TABLE I**

**Stimulation of transcription by different Tcf-4 forms**

| Transfected plasmid | Relative luciferase activity (fold compared with control) |
|----------------------|----------------------------------------------------------|
| pcDNA3.1HisC         | 1                                                        |
| pcDNA3.1HisC-Tcf-4 (wt) | 1.16 ± 0.10                                             |
| pcDNA3.1HisC-Tcf-4 (S58,59,60A) | 1.20 ± 0.12                                           |
| pcDNA3.1HisC-Tcf-4 (S60E) | 2.70 ± 0.10                                             |

**Note:** Values are the average ± S.D. of three experiments performed in triplicate. wt, wild type.
sequences are due to the action of the tails, which specifically interfere with the binding to the other Tcf-4 subdomain. At this respect, negative effects of the two β-catenin tails on the binding of the TBP and E-cadherin to the armadillo repeats has been previously demonstrated (9).

In line with results from other groups (35), our data indicate that plakoglobin inhibits β-catenin-mediated transcription by blocking the interaction of Tcf-4 to DNA. It is also interesting to remark that the presence of plakoglobin can render Tcf-4 complexes non-functional. These complexes might contain other transcriptional factors required for β-catenin activity, like TBP. We have noticed that plakoglobin has a higher ability than β-catenin to pull-down TBP. Therefore, the complexes containing Tcf-4 and plakoglobin could function as active inhibitors of this pathway, sequestering β-catenin and TBP; thus, decreasing the amount of these factors that can mediate transcription.

Our results indicate that plakoglobin inhibits Tcf-4-mediated transcription. However, these conclusions have to solve the apparent contradiction reflected by the fact that plakoglobin can also activate β-catenin transcriptional activity in some cell lines (34). Nevertheless, this result has been explained by the ability of plakoglobin to compete with β-catenin for binding to the proteosome degradation system (35); according to these suggestions, plakoglobin would increase the half-life of β-catenin and augment the amount of this protein available to be transported to the nucleus. Moreover, plakoglobin seems not to be degraded as actively as β-catenin by the destruction complex, and therefore, in some cells expression of plakoglobin is even more active than β-catenin in promoting β-catenin-Tcf-4 transcription (43). If this is the only mechanism by which plakoglobin can exert a positive effect, its role should be restricted to cells in which the proteosome-dependent degradation of β-catenin is active; thus, in colon cell lines, only in those few with wild-type adenomatous polyposis coli. On the other hand, plakoglobin would promote an inhibitory action on cell lines corresponding to advanced steps in the process of epithelial tumorigenesis, that is, when cells present elevated levels of nuclear β-catenin. Therefore plakoglobin would exert a positive or negative action on β-catenin-mediated transcription depending on its localization in the cytosol or nucleus, respectively. However, additional points of control of this pathway by both catenins can be imagined. The amount of cytosolic and plakoglobin available to be transported to the nucleus might be very variable among different cell lines, depending on their total levels and their grade of association with other proteins, for instance members of adherens junctions or desmosomes. As in the case of β-catenin (14), tyrosine phosphorylation of plakoglobin can affect this interaction. The mechanism of transport to the nucleus of these two proteins, still unknown, might have a different efficiency for both catenins. In addition, when in the nucleus, the extent of phosphorylation of Tcf-4 might favor binding to β-catenin or to plakoglobin. Moreover, the ability of β-catenin, and presumably of plakoglobin as well, to interact with components of the basal transcription machinery is regulated by tyrosine phosphorylation. Therefore, it is possible that a raise in tyrosine phosphorylation of specific plakoglobin residues would increase its association to TBP and sequester this protein to a non-functional complex, decreasing gene transcription. All these points indicate that additional research must be performed to fully understand the comparative role of β-catenin and plakoglobin on Tcf-4-mediated transcription.

In general terms, plakoglobin and β-catenin play opposite roles in epithelial cell growth. For instance, contrary to what happens to β-catenin, plakoglobin overexpression decreases tumorigenicity of several highly transformed cells (45). Transgenic expression of β-catenin and plakoglobin acts inversely on hair growth; whereas β-catenin induces epithelial tumors (46), plakoglobin suppresses epithelial proliferation (47). Data from these last authors indicate that growth inhibition by plakoglobin is not related to decreased β-catenin levels in epidermis. Moreover, plakoglobin expression has been reported to be down-regulated in several epithelial tumors, contrary to β-catenin (48, 49). Our data showing that plakoglobin binding to β-catenin-Tcf-4 complexes blocks interaction to DNA indicate a plausible explanation for these results.

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Plakoglobin and β-Catenin Bind to Different Sites on Tcf-4

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The transcriptional factor Tcf-4 contains different binding sites for β-catenin and plakoglobin.

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Several concerns were raised about Fig. 2, A and C, and Fig. 3B. Because the original data were no longer available, the experiments shown in these figures were repeated. The repeated experiments are shown and do not affect the results or conclusions of this work. The authors regret the inconvenience these errors may have produced.

FIGURE 2. Phosphorylation of the N-terminal domain of hTcf-4 by protein kinase CK2. Panel A, 1 μg of recombinant proteins (GST-Tcf-4-(1–80), β-catenin, or GST as a control) were phosphorylated in the presence of non-radioactive (left panel) or [γ-32P]ATP (right panel) by 50 milliunits of CK2 under conditions indicated under “Experimental Procedures” and analyzed by Coomassie staining or autoradiography.

Panel C, 1 μg of either GST-hTcf-4-(1–80) or the point mutants and equimolar concentrations of the shorter fragments GST-hTcf-4-(1–30) or GST-hTcf-4-(1–51) were phosphorylated with 50 milliunits of purified CK2 as described and analyzed by Coomassie staining (upper panel) or autoradiography (bottom panel). WT, wild type.

FIGURE 3. Phosphorylation of the N-terminal domain of Tcf-4 by CK2 does not modify β-catenin-Tcf-4 interaction but inhibits plakoglobin-Tcf-4 association. Panel B, GST or GST-Tcf-4-(1–80) fusion proteins were phosphorylated by CK2 under the conditions indicated under “Experimental Procedures.” Pull-down assays were performed incubating 12 pmol of the GST proteins with 500 μg of total cell extracts prepared from SW480 cells. Protein complexes were affinity-purified with glutathione-Sepharose and analyzed by SDS-PAGE and Western blot with anti-β-catenin mAb. Membranes were stripped and reanalyzed against plakoglobin. The numbers below the lanes indicate the densitometric values in arbitrary units, normalized to the value obtained for non-phosphorylated Tcf-4 (wild-type) lane.

Authors are urged to introduce these corrections into any reprints they distribute. Secondary (abstract) services are urged to carry notice of these corrections as prominently as they carried the original abstracts.