Protein kinase CKII regulates the interaction of β-catenin with α-catenin and its protein stability

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Summary

β-Catenin is a multi-functional cellular component and a substrate for several protein kinases. Here we investigated the interaction of protein kinase CKII (casein kinase II) and β-catenin. We show that CKII phosphorylates the N-terminal region of β-catenin and we identified Ser29, Thr102, and Thr112 as substrates for the enzyme. We provide evidence that CKII regulates the cytoplasmic stability of β-catenin and acts synergistically with GSK-3β in the multi-protein complex that controls the degradation of β-catenin. In comparing wild-type and Ser/Thr-mutant β-catenin, a decreased affinity of the mutant protein to α-catenin was observed. Moreover, kinase assays in vitro demonstrate a CKII-dependent increase in the binding of wild-type β-catenin with α-catenin. In line with that, cells expressing Ser/Thr-mutant β-catenin exhibit an increased migratory potential, which correlates with an enhanced cytosolic localization and a reduced association with the cytoskeleton of the mutant protein. From these results we conclude that CKII regulates the function of β-catenin in the cadherin adhesion complex as well as its cytoplasmic stability.

Key words: Casein kinase II, β-Catenin, α-Catenin, Protein kinase, Caderin adhesion complex

Introduction

β-Catenin was originally identified in association with the cell adhesion molecule E-cadherin (Vestweber and Kemler, 1984; Ozawa et al., 1989; Nagafuchi and Takeichi, 1989), but has also recently won increasing interest as an important mediator in the Wnt signaling pathway (for a review, see Polakis, 2000). In the cadherin cell-cell adhesion complex β-catenin plays a central role since it binds to both the cytoplasmic domain of E-cadherin and to α-catenin, which in turn binds directly to F-actin and other actin-associated proteins (Ozawa et al., 1990; Stappert and Kemler, 1994; Huber and Weis, 2001). This association with the actin-based cytoskeleton is important for the adhesion function of all classical cadherins, as best demonstrated for the E-cadherin-catenin complex in adherens junctions of epithelial cells (for a review, see Aberle et al., 1996a). Although the respective binding sites between E-cadherin and β-catenin and between α- and β-catenin have been characterized in detail (Stappert and Kemler, 1994; Aberle et al., 1996b; Huber et al., 1997; Pokutta and Weis, 2000; Yang et al., 2001), recent evidence underlines the importance of post-translational modifications in modulating the function of this adhesion complex. Phosphorylation of E-cadherin by casein kinase II (CKII) and glycogen synthese kinase-3β (GSK-3β) increases the affinity of E-cadherin for β-catenin and thus enhances E-cadherin-mediated cell adhesiveness (Huber and Weis, 2001; Lickert et al., 2000). In contrast, reduced cell adhesion has been reported upon tyrosine phosphorylation of β-catenin (Ozawa and Kemler, 1998; Hazan and Norton, 1998; Gaudry et al., 2001) and an association of β-catenin with receptor-tyrosine kinases, EGF-R, IGF-R and c-Met (Hoschützky et al., 1994; Hiscox and Jiang, 1999; Playford et al., 2000) as well as with c-src (Kinch et al., 1995), has been shown. In comparison, little is known about the role of serine/threonine (Ser/Thr) phosphorylation of β-catenin in modulating the function of the E-cadherin adhesion complex. It is known that phosphorylation of β-catenin by GSK-3β determines its availability as a transducer of Wnt signals. In the absence of Wnt, phosphorylation by GSK-3β targets β-catenin for degradation (Yost et al., 1996; Aberle et al., 1997; Orford et al., 1997). Phosphorylation of β-catenin by GSK-3β occurs in a multiprotein complex that includes the tumor suppressor Adenomatous Polyposis Coli (APC) (Munemitsu et al., 1995), Axin (Zeng et al., 1997), or Conductin (Behrens et al., 1998). The composition of this multiprotein complex and the integration of β-catenin depend on the phosphorylation of each component by GSK-3β (reviewed by Seidensticker and Behrens, 2000; Dominguez and Green, 2001). Wnt inhibits GSK-3β-mediated phosphorylation of β-catenin and under these conditions β-catenin enters the nucleus and forms complexes with transcription factors of the LEF/Tcf family, resulting in specific transcriptional activation (reviewed by Roose and Clevers, 1999; Hecht and Kemler, 2000).

β-catenin is implicated in quite different cellular processes, which requires a fine-tuned regulation of its function, so it is very likely that β-catenin is a substrate for other yet to be identified protein kinases. Indeed, it was recently reported that CKII phosphorylates β-catenin (Song et al., 2000), and that CKII and β-catenin co-immunoprecipitate with Dvl proteins, the mammalian homologues of *Drosophila* Dishevelled (Dsh).

From these results it was concluded that CKII participates in Wnt signaling and may act as a positive regulator in this pathway although the underlying molecular mechanisms are at present poorly understood.

CKII exists as a constitutively active tetramer that contains two catalytic (α or α′) and two regulatory (β) subunits (Pinna and Meggio, 1997; Allende and Allende, 1995). Although more than 160 substrates have been identified to date, the regulation of this ubiquitously expressed pleiotrophopic kinase remains unclear. A nuclear shift of CKII-α′ during G_1-phase and in proliferating cells (Seldin and Leder, 1995; Keliher et al., 1996; Landesman-Bollag et al., 1998; McKendrick et al., 1999; Ahmed, 1994) points towards a role of CKII in mitotic control and proliferation. However, due to the broad subcellular distribution, it is generally assumed, that CKII is controlled by different interaction partners and in different subcellular compartments (for a review, see Faust and Montenarh, 2000).

In a search for protein kinases that use β-catenin as substrate we confirmed that CKII also phosphorylates β-catenin. We have now identified amino acid (aa) residues in β-catenin phosphorylated by CKII and performed a mutational analysis to obtain first insights into the biological function of this post-translational modification. By comparing wild-type (wt) and Ser/Thr-mutated (Ser/Thr-mutant) β-catenin in kinase assays in vitro and in vivo we provide evidence here that CKII regulates the best studied functions of β-catenin (i.e. its central role in the E-cadherin adhesion complex and its tight control of cytoplasmic stability), which is a prerequisite for the canonical Wnt signaling pathway.

Materials and Methods

Cell culture
Mouse epithelial carcinoma CMT cells (ATTC, CCL-223) and human kidney epithelial 293 cells (ATTC, CRL-1573) were grown at 37°C/10% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS) and 2 mM L-glutamine. 293 cells stably expressing either hemagglutinin (HA)-tagged wt or Ser/Thr-mutant β-catenin were selected in the presence of 100 μg/ml Zeocin (Invitrogen).

Antibodies and reagents
Mouse monoclonal antibodies against α-catenin, β-catenin, Protein kinase A (PkA), and GSK-3β were obtained from Transduction Laboratories (Lexington, KY), rabbit polyclonal antibodies against CKII were from Santa Cruz Biotechnologies, and rat monoclonal antibodies against hemagglutinin (HA) (clone 3F10) were from Roche. Anti-GST antibodies were from Sigma, and rabbit polyclonal anti-Ki67 was from Novocastra Laboratories (Newcastle). Anti-myc antibodies (Evan et al., 1985) were purified from supernatants of hybridoma clone 9E10 grown in DMEM containing 3% FCS. Fluorescein-conjugated secondary antibodies were purchased from Dianova. 3000 mCi/ml [γ-^{32}P]ATP was from Amersham Pharmacia Biotech.

Immunofluorescence
293 cells grown on collagen- or poly-L-lysine-coated coverslips were washed in PBS, pH 7.4, and fixed in 3% paraformaldehyde/PBS, pH 7.4 at room temperature (RT) for 20 minutes. Free aldehyde groups were blocked with 1 M glycine/PBS, pH 8.5, for 5 minutes. Cells were permeabilized with 0.5% Triton X-100 for 5 minutes. Incubation was performed with primary antibodies at 2 μg/ml for 1 hour at 37°C and with fluorescein-conjugated secondary antibodies (Dianova) for 1 hour at 37°C in the dark. Cells were kept in a mounting solution (50% glycerol; 50% PBS; 100 μg/ml 1,4-diazabicyclo-[2,2,2]octone) and digital images were taken with a computer-controlled digital C4880 camera (Hamamatsu, Japan) on an Axioskop microscope (Zeiss, Jena). Camera and microscope were controlled by the computer program Openlab (Improvision, Coventry, UK).

Immunobiochemistry
Cells grown to 80% confluency were washed with PBS and lysed in 500 μl CSK buffer [150 mM NaCl, 10 mM PIPES (pH 6.8), 3 mM $MgCl_2$, 0.5% Triton X-100, 10 mM NaF, 1 mM sodium vanadate, 10 μg/ml leupeptin, 10 μg/ml phenylmethylsulfonyl fluoride (PMSF)] on ice for 20 minutes. Cell debris was removed by centrifugation at 16,000 **g** for 10 minutes. The amount of total protein was measured using the BCA-Kit (Pierce) and equal amounts of total protein were used for each analysis. Immunoblot analysis was performed as described (Aberle et al., 1996b), but using PVDF membranes (Millipore). Immunoprecipitation experiments were done as described (Hoschützky et al., 1994) with the following modifications: lysis and precipitations were performed in CSK buffer and the immunocomplexes were incubated overnight at 4°C.

Sequential detergent extraction was performed as described (Ramsby and Makowski, 1999). Briefly, after washing the cells three times in PBS, cytosolic and soluble cytoskeletal proteins were released with 0.015% digitonin, prior to the extraction of membrane and organelle-components with 0.5% Triton X-100. Nuclear and cytoskeletal proteins were subsequently solubilized with 1% SDS, prior to 10 fold dilution with normal CSK-buffer. All other steps were carried out on ice.

For transient transfection experiments 1×10^6 293 cells cultured in 9-cm culture dishes for 5-7 hours were incubated overnight with a calcium-phosphate coprecipitate containing 5 μg wt or Ser/Thr-mutant β-catenin DNA; 4 μg DNA for mouse Axin-1 fused to a Myc tag; 2 μg Myc-GSK-3β DNA; 2 μg Myc-tagged CKII DNA, or 2 μg Myc-tagged ERK2. Cells were washed three times with PBS and cultured for 36 hours before analysis.

For pulse-chase experiments 2.5×10^5 293 cells stably expressing either wt or Ser/Thr-mutant β-catenin were cultured in 3.5-cm culture dishes for 6 hours. Cells were then washed twice with PBS, starved for 1 hour in cysteine- and methionine-free medium, incubated with [^{35}S]cysteine/[^{35}S]methionine (150 Ci/ml) for 1 hour, washed and chased for 5 hours. Cells were lysed in 100 μl CSK buffer containing 0.5% SDS and boiled for 10 minutes. The lysates where then diluted 10-fold and equal amounts of incorporated radioactivity were subjected to immunoprecipitation experiments. Precipitates were resolved by SDS-PAGE and the intensities of the products were quantified using the phospho-imager.

Precipitation and elution of β-catenin associated kinase-activity
CMT cells grown to 80% confluency in 9-cm dishes were lysed in 500 μl CSK buffer and immunoprecipitations were usually done overnight at 4°C with anti-β-catenin antibodies coupled to protein A-Sepharose (Amersham-Pharmacia Biotech). Precipitates were washed three times in ice-cold PBS containing 0.5% Triton X-100, 150 mM NaCl, 10 mM NaF, 2 mM sodium molybdate, 1 mM sodium vanadate. Kinase activities present in the immunocomplexes were eluted by applying ascending salt concentrations (200-1000 mM NaCl) in distilled water containing phosphatase inhibitors (10 mM NaF, 10 mM sodium molybdate, 1 mM sodium vanadate). After brief centrifugation the supernatants were diluted to physiological salt concentrations (100 mM NaCl) and the volumes were adjusted to 400 μl. In control assays cell lysates were incubated with 1 μg mouse IgG of unrelated specificity.

Kinase assays

For in vitro kinase assays with eluted kinase activity, the indicated GST fusion proteins immobilized on glutathione-Sepharose 4B (Amersham Pharmacia) were incubated with 100 μl of the eluate at RT for 30 minutes. After three washes with PBS containing 0.5% Triton X-100, 150 mM NaCl, 10 mM NaF, 2 mM sodium molybate and 1 mM sodium vanadate, kinase assays were performed in 50 μl kinase buffer [20 mM Tris-HCl (pH 7.4), 10 mM MgCl$_2$, 1 mM CaCl$_2$, 1 μM ATP] including phosphatase inhibitors and 7 μCi [γ-^{32}P]ATP at RT for 20 minutes. Reactions were stopped by adding 20 μl of stop buffer [10 mM EDTA (pH 8.0), 10 mM sodium phosphate (pH 8.0), 10 mM Na$_4$P$_2$O$_7$] and washed three times with cold PBS containing 0.5% Triton X-100, 1000 mM NaCl, 10 mM NaF, 2 mM sodium molybdate, 1 mM sodium vanadate. Radioactive gels were Coomassie Blue-stained and radioactivity was quantified with a BAS 1000 Bioimaging Analyzer (Fuji), while Coomassie Blue staining was quantified using the NIH Imager program version 1.59. CKII was specifically inhibited with the following peptides: (Arg)$_3$ Ala Asp Asp Ser (Asp)$_5$ (competitor); and (Arg)$_3$ Ala Asp Asp Ala (Asp)$_5$ (control).

Pre-phosphorylation experiments

To pre-phosphorylate recombinant β-catenin with CKII, 2 μg of GST β-catenin linked to GSH beads was incubated in CKII kinase buffer (20 mM Tris-HCl, 50 mM KCl, 10 mM MgCl$_2$, pH 7.5) with 20 mM non-radioactive ATP and 1 U of recombinant CKII (New England Biolabs) at 25°C for 20 minutes. Reactions were stopped by adding 50 μl stop-buffer (see above). To remove all residual kinase-activity, the reactions were extensively washed 5 times with 1 ml ice-cold PBS containing 1000 mM NaCl, 0.5% Triton X-100 and phosphatase-inhibitors. The NaCl concentration was reduced by 2 additional washing steps with PBS containing 100 mM NaCl. Pre-phosphorylation with GSK-3β was done in the same way, but including 5 mM DTT in the assay-buffer, using 0.1 U of recombinant GSK-3β (New England Biolabs), and performing the reaction at 30°C.

Expression and purification of recombinant proteins

All GST fusion proteins were expressed in *E. coli* BL21 (DE3). Affinity purification on GSH-Sepharose beads (Amersham Pharmacia) was carried out as described (Aberle et al., 1996b). Proteins were dialyzed against 20 mM HEPES (pH 8.0). For β-catenin fragments including only aa residues 302-535 or 535-683, concentration with Centricon ultrafiltration cartridges was required.

After purification from *E. coli* BL21 (Amersham Pharmacia Biotech) GST-Axin was loaded on a PD10 column to get rid of some major degradation products. The eluted and pooled fractions were concentrated as described above. β-catenin-His$_6$ was expressed in *E. coli* BL21 (DE3) and affinity purified on Talon-Beads (Clontech) as described (Aberle et al., 1996b). All recombinant proteins were aliquoted and stored at –20°C.

GST pull-down assays

If not indicated otherwise, 2 μg of GST-protein linked to GSH-beads was incubated with recombinant His$_6$-tagged protein pre-absorbed with GSH-beads. Reactions were carried out in 500 μl association-buffer (20 mM Tris-HCl, 10 mM MgCl$_2$, 0.1% Triton X-100, 100 mM KCl) at RT for 30 minutes. Protein complexes were collected by brief centrifugation (1 minute, 800 *g*, 4°C), and washed three times with cold PBS containing 0.5% Triton X-100, 160 mM NaCl, 10 mM NaF, 2 mM sodium molybdate, 1 mM sodium vanadate.

Constructs

GST expression constructs pGEX4T1MMBC, pGEX4T1BNTERM (coding for β-catenin in residues 1-119) and pGEX4T1BCTERM (aa

683-781) were previously described (Aberle et al., 1996b). To obtain pGEX4T1B1-302, pSKβtot was digested with *Bam*HI/*Bgl*II and the resulting 906 bp-fragment coding for the first 302 aa residues of β-catenin was cloned into *Bam*HI-cleaved pGEX4T1. PGEX4T1B302-535 was obtained by cloning the 1176-bp *Bgl*II-fragment into *Bam*HI-cleaved pGEX4T1. The resulting plasmid coding for residues 302-781 was digested with *Spe*I/*Xho*I and blunt-end ligated to obtain a construct coding for β-catenin missing aa residues 535-781.

For construction of pGEX4T1B535-683 a 741 bp-fragment coding for residues 535-781 was amplified from pGEX4T1MMBC by PCR, using the following primers: β535ff (5′-GCGGGATCCCGAC-TAGTTCAGCTG-3′) and βctermrev (5′-TTACAGGTCAGTAT-CAAACCAGGC-3′). The fragment was cloned into *Bam*HI/*Eco*RV digested pGEX4T1BΔCTERM (Aberle et al., 1996b).

The plasmids coding for Myc-tagged mouse Axin 1 and Myc-tagged ERK2 where kindly provided by L. Zeng (Columbia University, New York, NY) and P. E. Shaw (The Beatson Institute for Cancer Research, Glasgow, UK).

For the generation of C-terminal HA-tagged β-catenin, oligonucleotides encoding the influenza virus HA-tag were inserted into pCS^{2+} after *Eco*RI restriction of the vector. cDNA coding for β-catenin aa residues 1-781 was then cloned in-frame in front of the HA-tag region.

Site-directed mutagenesis was performed as described (Higuchi, 1990). For all three mutants the same wt primers were used: β1ff:5′-ATGGCTACTCAAGCTGACC-3′, and β1875rev: 5′-GGCCTCT-GCAGCCTCCTTGTCC-3′.

For generation of the single mutants the following mutagenic primer pairs were used: M1ff: 5′-CTGGCAGCAGCAGGCT-TACTTGGATTCTGG-3′ and M1rev: 5′-CCAGAATCCAAGT-CAGCCTGCTGCTGCCAG-3′. M2: M2ff: 5′-ATGTTCCCTGAG-GCTCTAGATGAGGGC-3′, and M2rev: 5′-GCCCTCATCTAGAGC-CTCAGGGAACAT-3′. M3: M3ff: 5′-GGCATGCAGATCCCATC-CCAGCAGTTTGACGCT-3′, and M3rev: 5′-AGCGTCAAACTG-GTCGGATGGGATCTGCATGCC-3′.

After *Nco*I digestion, the PCR products were cloned into pCS^2+β-catenin-HA. To generate the Ser/Thr-mutant, HA-β-catenin-M3 was first subcloned after into HA-β-catenin-M2 after *Sph*I/*Spe*I digestion of the vector. A *Hin*cII/*Spe*I-fragment containing both mutations, M2 and M3, was then cloned into HA-β-catenin-M1. HA-tagged wt β-catenin and Ser/Thr-mutant constructs were cloned into the pBUD CE4 vector (Invitrogen) under control of the EF-1α promoter. For stable transfection of 293 cells the calcium-phosphate method was used.

Results

CKII phosphorylates the N-terminal region of β-catenin

It has been reported that GSK-3β (Yost et al., 1996; Aberle et al., 1997; Orford et al., 1997), and the protein kinase casein kinase II (CKII) (Song et al., 2000) phosphorylate β-catenin, although for the latter the exact Ser/Thr residues phosphorylated are not known. In a screen for Ser/Thr kinases that phosphorylate β-catenin, we also observed phosphorylation of β-catenin by CKII. In this approach, kinase activities associated with β-catenin in vivo were co-precipitated with anti-β-catenin antibodies from cell lysates and were eluted with an increasing salt gradient. Each fraction was tested for phosphorylation of recombinant β-catenin in vitro and the fractions with the highest activities were tested for CKII inhibition. Heparin, a known CKII inhibitor decreased the amount of phosphorylated GST-β-catenin (not shown). To further proof the specifity of this inhibition, kinase activities were assayed with a competitor peptide containing CK II consensus motifs (Fig. 1A).

Phosphorylation of β-catenin was clearly reduced in the presence of CKII-specific competitor peptides (compare Fig. 1A, lanes 3 and 4), but not with control peptides containing mutated CKII motifs (Fig. 1A, co). These results provide evidence that the heparin-mediated inhibition of β-catenin-phosphorylation is CKII-specific, and therefore CKII activity was measured in immunocomplexes collected with anti-β-catenin from cell lysates. The results indicated further that CKII and β-catenin are associated in vivo. Consistent with that, specific in vivo associations between the two proteins were found in 293- and NIH 3T3 cells in immunoprecipitations with anti-CKII-α antibodies and subsequent immunoblots with anti-β-catenin (Fig. 1B, lanes 2,4). Conversely, when Myc-tagged CKII-α was transiently expressed in 293 cells, CKII could also be co-precipitated with anti-β-catenin antibodies from cell lysates, whereas an unrelated kinase could not (Fig. 1B, lanes 7,8). This association might well be a direct interaction, since recombinant CKII is able to interact directly with GST-β-catenin in vitro (Fig. 1C, lane 5). This interaction becomes enhanced in the presence of ATP (Fig. 1C, lane 6), similar to previous observations for the association of CKII with other target proteins (Muslin et al., 1996; Pawson, 1995). Taken together, these results provide good evidence that CKII interacts with and phosphorylates β-catenin.

To determine which part of β-catenin is phosphorylated by CKII, various β-catenin deletion constructs were expressed and the purified GST fusion-proteins were subjected to in vitro kinase assays (Fig. 2A). Heparin was included in parallel as inhibitor of CKII phosphorylation. As shown in Fig. 2B, strong

phosphorylation was observed in the N-terminal (aa residues 1-119/1-302) and C-terminal (aa residues 683-781) regions of β-catenin. Remarkably, in contrast to the C-terminal region, the N-terminal phosphorylation was efficiently inhibited by heparin, indicating that CKII preferentially phosphorylates the N-terminal part of β-catenin. These results are consistent with


Fig. 1. CKII interacts with and phosphorylates β-catenin. (A) β-catenin immunoprecipitates were collected from cell lysates of epithelial CMT cells, and associated kinases were eluted and tested for activity against GST β-catenin in kinase assays in vitro. Competitor peptides containing CKII-consensus motifs significantly decreased phosphorylation of GST β-catenin compared with control-peptides (co), both at 0.5 mM (compare lanes 4 and 6). Other controls included unrelated mouse IgG in the initial immunoprecipitation (lane 1) and GST (lane 2). The Coomassie-stained gel of the autoradiography is shown. (B) Association of CKII and β-catenin in vivo. CKII-α was immunoprecipitated from cell lysates of 293 and NIH 3T3 cells and in each case β-catenin was detected by immunoblotting of the immunoprecipitates (lanes 2,4). To demonstrate the specifity of the association, protein kinase A (PkA) was immunoprecipitated and blotted against β-catenin in both cases (lanes 1,3). β-catenin could not be detected in the PkA-precipitates. (C) In a reverse experiment, Myc-tagged CKII-α or the Myc-tagged version of ERK-2 were transiently expressed in 293 cells and after 48 hours immunoprecipitates were collected with anti-β-catenin and probed with anti-myc antibodies. Transfected or control lysates were tested in immunoblot for the correct expression of Myc-ERK2 (lane 1) or CKII (lane 2). Whereas Myc-ERK2 was not found in β-catenin immunoprecipitates (lane 3), Myc-CKII co-precipitates with β-catenin in these experiments (lane 4). (D) GST pulldown assays with recombinant β-catenin and recombinant CKII-α were performed in combination as indicated. CKII-α was pre-adsorbed with GSH-beads before use; the overall amount of CKII-α is shown in lane 1 (input). Proteins where allowed to associate in the presence or absence of ATP under phosphorylation-conditions described in Materials and Methods. GST β-catenin was precipitated with GSH-beads and associated CKII-α was detected by immunoblotting. Direct binding of CKII-α to β-catenin is enhanced in the presence of a phosphate-donor (compare lanes 5 and 6).
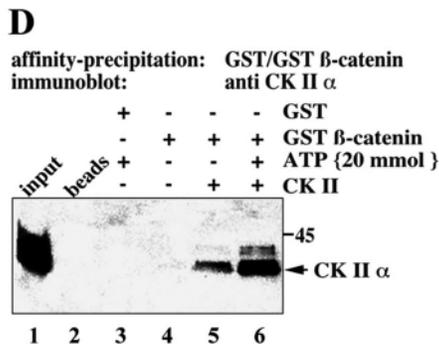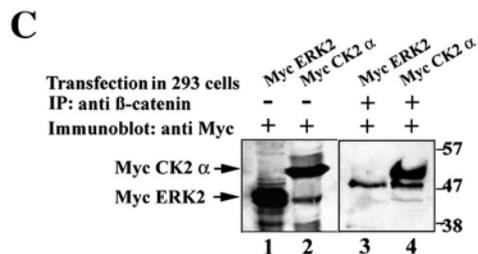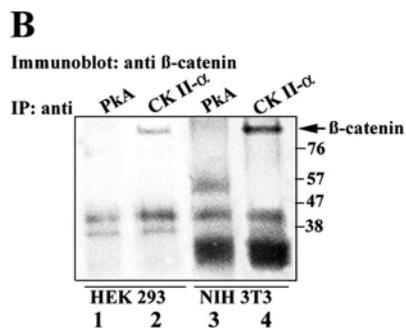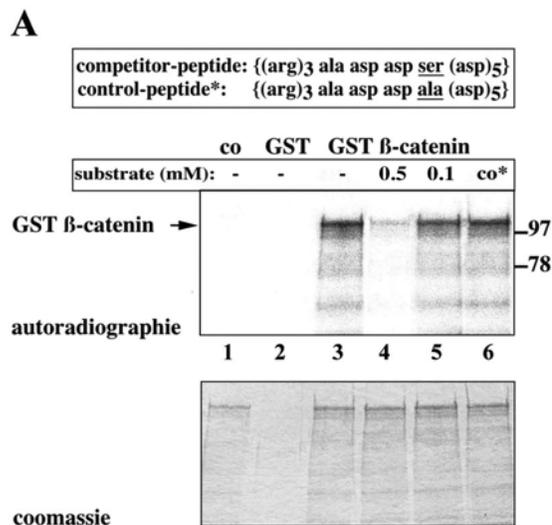
the localization of three conserved CKII-consensus motifs in the N-terminal region of β-catenin around the GSK-3β recognition motifs (Fig. 2C).
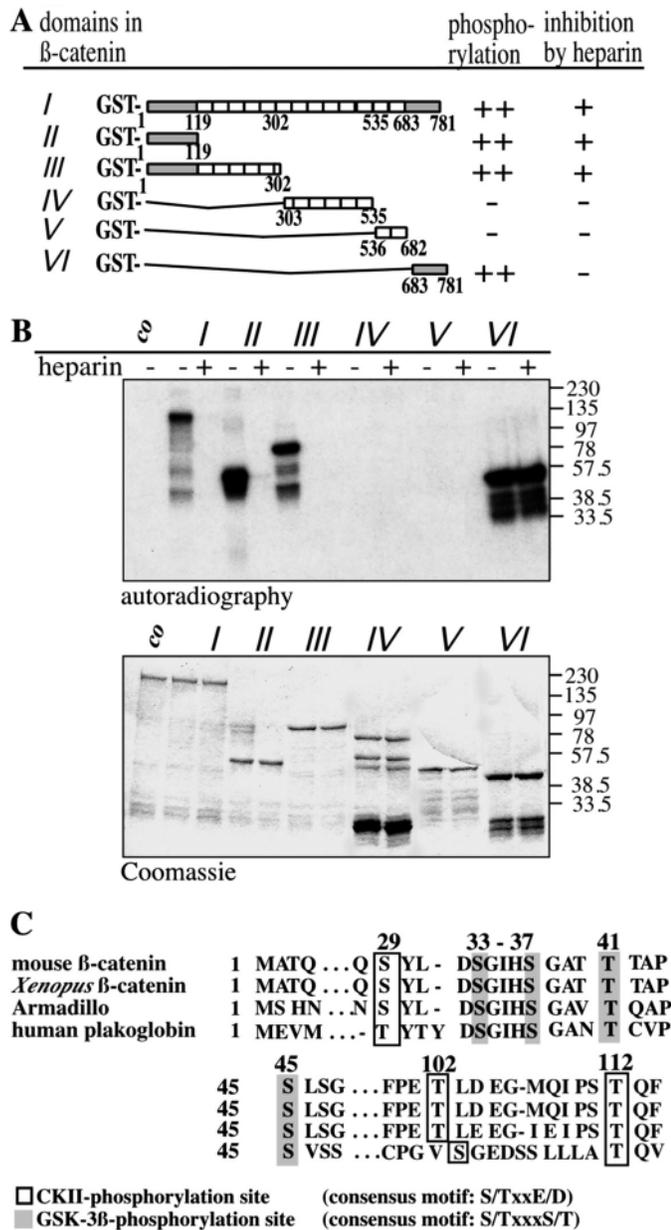

Fig. 2. CKII phosphorylates the N-terminus of β-catenin. (A) β-catenin wt and deletion constructs expressed as GST-fusion proteins and tested in kinase assays in vitro. (B) The proteins were subjected to kinase assays as described in Fig. 1A, but with or without heparin (5 μg/μl) as an inhibitor of CKII-activity. Phosphorylation is efficiently reduced by heparin at the N-terminus (aa 1-119, 1-302) but not at the C-terminus of β-catenin. In the control-lane (co), unrelated mouse IgG in the initial immunoprecipitation was used and incubated with full-length GST β-catenin. The Coomassie-stain provides the loading control for the respective fusion-proteins. (C) Three conserved CKII consensus motifs can be found in the N-terminus of β-catenin (aa 1-119). These sites are located in close proximity to the GSK-3β consensus motif (Aberle et al., 1997). No additional CKII sites are located in the extended N-terminal region (aa 120-302) of β-catenin (not shown).

CKII regulates the cytoplasmic stability of β-catenin

In the following the phosphorylation of β-catenin by CKII was investigated with respect to its biological functions, the best studied of which are its central role in the E-cadherin-catenin adhesion complex and as a Wnt signaling component. In these experiments mutant forms of β-catenin were included where one or more of the three CKII consensus motifs were destroyed by single or combined aa substitutions (i.e. aa S29→A; T102→A; T112→Q) (Fig. 3A). GST fusion proteins with either single or combined mutations were tested in kinase assays in vitro. No phosphate-incorporation was observed when β-catenin mutated in all three CKII consensus motifs (Ser/Thr-mutant) was tested with recombinant CKII (Fig. 5D, lane 7), and single aa substitutions drastically reduced phosphorylation (not shown).

The cytoplasmic turn-over of wt and mutant β-catenin was compared in pulse-chase experiments with 293 cells stably expressing HA-tagged wt or Ser/Thr-mutant β-catenin (Fig. 3).
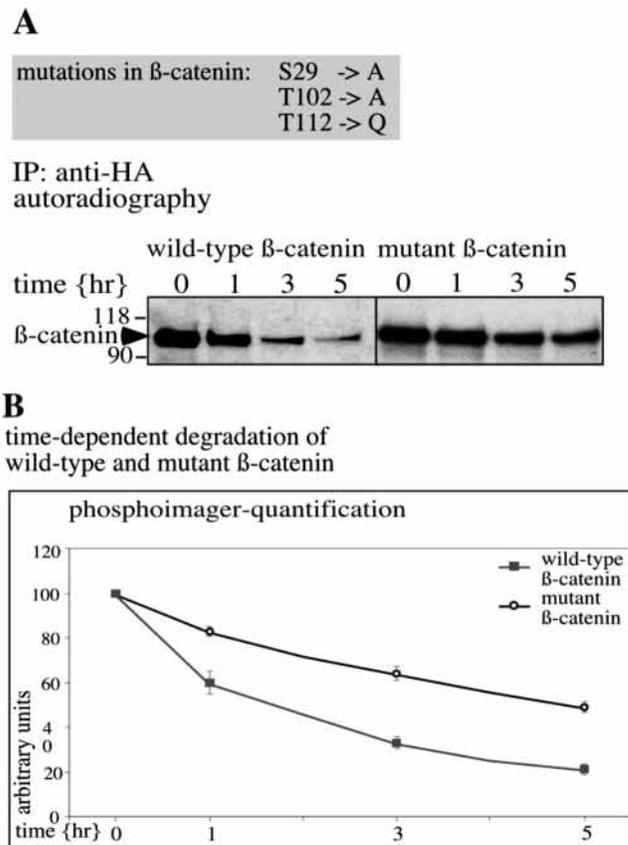
Fig. 3. Mutations in CKII consensus-motifs stabilize β-catenin. (A) 293 cells stably transfected with either wt or Ser/Thr-mutant HA-β-catenin were subjected to pulse-chase experiments as described in Materials and Methods. The triple-mutant used contained the three mutations indicated in panel A. Equal amounts of incorporated radioactivity were immunoprecipitated with anti-HA antibodies at the time points indicated and the autoradiographs were quantified by phospho-imager analysis. The half-life of Ser/Thr-mutant β-catenin was approximately two- to threefold that of wt β-catenin. (B) Quantitative measurements of band intensities from three independent experiments, using MacBas image analyzing software (circles, wild-type β-catenin; squares, mutant β-catenin).

At each time-point, HA-tagged β-catenin was immunoprecipitated from cell lysates containing comparable amounts of incorporated radioactivity and quantitated. The autoradiograph in Fig. 3A shows that the time-dependent degradation of mutant β-catenin was clearly delayed as compared to the wt form. Three independent pulse-chase experiments were quantified using the phospho-imager (Fig. 3B). The half-life of HA-tagged wt β-catenin is similar to that previously determined for endogenous β-catenin (Aberle et al., 1997). In comparison, Ser/Thr-mutant β-catenin showed a significant stabilization by more than two-fold (Fig. 3B). From these results it is concluded that functional CKII-motifs are required for the control of the cytoplasmic amount of β-catenin. These results suggested further that CKII is part of the multi-protein complex which controls the cytoplasmic amount of β-catenin. To test this possibility Myc-tagged Axin, or Myc-GSK3β were transiently expressed in 293 cells, endogenous CKII was immunoprecipitated and the precipitates were probed with anti-Myc antibodies (Fig. 4A). Both, Myc-Axin (Fig. 4A, lane 2) and Myc-GSK3β (Fig. 4A, lane 4) were found associated with endogenous CKII suggesting that CKII is a component of the β-catenin degradation machinery. It is well established that phosphorylation of β-catenin by GSK3β enhances binding of β-catenin to Axin and APC. A similar notion can be taken from the results depicted in Fig. 4B, since β-catenin mutated in its CKII-motifs can only poorly associate with Axin. In these experiments HA-tagged wt or Ser/Thr-mutant β-catenin and Myc-Axin were transiently expressed in 293 cells, comparable amounts of immunoprecipitates were

collected with anti-Myc antibodies (Fig. 4B, lanes 4-6), and precipitates were probed for β-catenin (Fig. 4B, lanes 1-3). The introduction of one mutation in β-catenin already decreased the affinity to Axin (Fig. 4B, lane 2) and binding was further decreased when all three CKII-motifs were mutated (Fig. 4B, lane 3). These results demonstrate that β-catenin mutated in its CKII-motifs can poorly associate with Axin and suggested that both GSK3β and CKII act synergistically in controlling the degradation of β-catenin. To address this question, wt or Ser/Thr-mutant GST-β-catenin was phosphorylated with either CKII, GSK-3β, or both in vitro. The reaction was stopped with a high-salt wash (500 mM NaCl, 0.5% Triton X-100), and bound kinases were eluted and examined by immunoblot analysis with antibodies as indicated (Fig. 5). CKII bound to wt β-catenin, but binding was clearly reduced with mutant β-catenin (compare Fig. 5A, lanes 3 and 7).

Importantly, enhanced binding of GSK-3β to β-catenin was observed when β-catenin was pre-phosphorylated by CKII (Fig. 5B, compare lanes 4 and 5). Pre-phosphorylation of Ser/Thr-mutant β-catenin with CKII did not enhance binding of GSK-3β to mutant β-catenin (Fig. 5B, lane 9) and binding of GSK-3β to wt and Ser/Thr-mutant β-catenin was comparable (Fig. 5B, lanes 4,8). In Fig. 5D the $[\gamma^{-32}P]$ATP-incorporation in these experiments is depicted, demonstrating enhanced phosphate incorporation in wt β-catenin when both kinases were used (lane 5) and the lack of incorporated phosphate in the CKII mutant (lane 7). Comparable amounts of wt and Ser/Thr-mutant β-catenin were used in these experiments, as monitored with anti-GST antibodies (Fig. 5C).

Several conclusions can be drawn from these results. Most notably, CKII and GSK-3β bind and phosphorylate wt β-catenin synergistically and pre-phosphorylation by CKII enhances binding of GSK-3β. In the Ser/Thr-mutant CKII can still bind, although to a reduced amount, but no phosphate incorporation is observed here. Thus, the CKII consensus motifs in β-catenin are substrates for the enzyme. Mutations in the CKII motifs do not affect binding and phosphorylation by GSK-3β, but the enhanced binding in the wt when both kinases were used is not observed for the mutant form. Altogether, a sequential action of CKII and GSK-3β in phosphorylation of β-catenin is suggested, and the role of CKII in controlling the protein turnover is underlined.

CKII regulates the interaction of β-catenin with α-catenin

Since Ser/Thr-mutant β-catenin becomes stabilized, it was of interest to examine how the subcellular distribution of mutant β-catenin compares to wt protein. For this, 293 cells stably expressing HA-tagged wt or Ser/Thr-mutant β-catenin were subjected to sequential detergent extractions to separate the cytosolic, membrane-organelle and cytoskeletal fractions of cells. Immunoblot analysis with anti-HA antibodies revealed the expected subcellular distribution of transfected HA wt β-catenin, with the major content in the membrane-organelle fraction, similar to that of endogenous wt β-catenin in untransfected 293 cells (Fig. 6A). In comparison, the cytoplasmic pool of Ser/Thr-mutant β-catenin was drastically increased and little mutant protein was detected in the cytoskeletal fraction (Fig. 6A). In these preparations the HA antibody crossreact most likely with c-terminal degradation products from HA β-catenin, whereas a 65 kDa-band from the
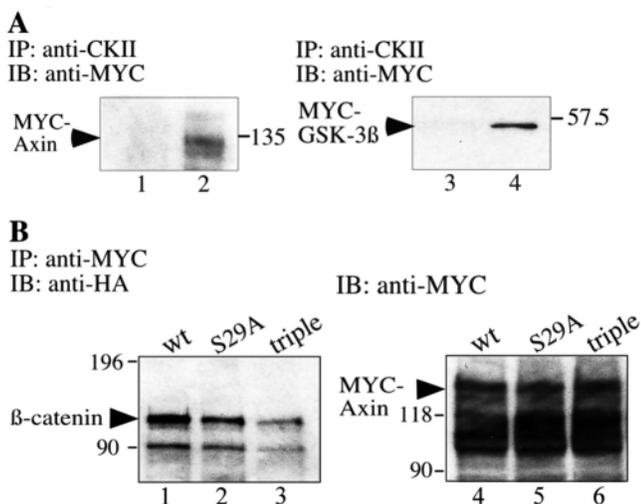
Fig. 4. CKII associates with components of the cytoplasmic degradation complex and modulates the β-catenin-Axin interaction. (A) 293 cells were transiently transfected with Myc-tagged Axin or with Myc-GSK-3β (empty vectors for control, lanes 1 and 3) and endogenous CKII was immunoprecipitated from comparable amounts of whole-cell lysates. Both Myc-Axin (lane 2) and Myc-GSK-3β (lane 4) were detected in the immunoprecipitates. (B) HA-tagged wt or Ser/Thr-mutated (single or triple mutation) β-catenin were transfected together with Myc-tagged Axin into 293 cells. The expression of Myc-Axin was controlled by immunoblot (lanes 4-6). Comparable amounts of Myc-Axin were immunoprecipitated with anti-Myc antibodies and the immunoprecipitates were probed with anti-HA. The association of HA-β-catenin to Myc-Axin is reduced to an extent that depends on the number of introduced mutations in the CKII consensus motifs (lanes 1-3).
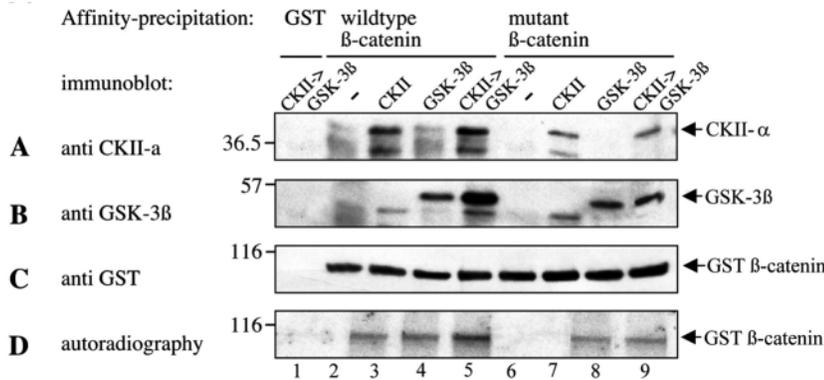
Fig. 5. Synergistic binding of CKII and GSK-3β to β-catenin. GST-fused wt or Ser/Thr-mutant β-catenin coupled to GSH-Sepharose (500 ng each lane) was phosphorylated with either recombinant CKII or GSK-3β, or both, according to the instructions of the manufacturers, and bound kinases were detected in immunoblots with anti-CKII (A) or anti-GSK-3β-antibodies (B). Equal amounts of recombinant β-catenin were used as shown with anti-GST antibodies (C). In parallel experiments (D) the reaction mixture contained ^{32}P-ATP and gels were autoradiographed for 8 hours. CKII binds efficiently to wt GST-β-catenin (A, lanes 3,5), whereas binding to the Ser/Thr-mutant protein is reduced (A, lanes 7,9). GSK-3β bound equally well to wt and Ser/Thr-mutant β-catenin (B, lanes 4,8). However, binding of GSK-3β to wt β-catenin is clearly enhanced when β-catenin was pre-incubated with CKII (B, lanes 4,5). The lower molecular weight bands appearing in lanes 3, 5 and 7 are probably due to crossreactivity of the GSK-3β antibody with CKII-α. Phosphorylation of wt β-catenin is significantly enhanced when both kinases were consecutively used (D, lane 5). Ser/Thr-mutant β-catenin is not phosphorylated by CKII (D, lane 7) and no difference in phosphate-incorporation is observed in lanes 8 and 9.

membrane-organelle fractions is of unrelated specifity. The reduced amount of Ser/Thr-mutant β-catenin in the cytoskeletal fraction was of particular interest and pointed to the possibility of an altered interaction of the mutant form with α-catenin.

Therefore, in vitro association experiments were carried out with recombinant α- and β-catenin (Fig. 6B,C). Wild-type β-catenin bound to increasing amounts of α-catenin. However, little α-catenin interacted with Ser/Thr-mutant β-catenin (Fig. 6B). Interestingly, pre-phosphorylation of wt β-catenin with CKII in vitro enhanced binding of α-catenin, but this was not the case for the mutant form of β-catenin (Fig. 6C). These results provide strong evidence that phosphorylation of β-catenin by CKII regulates the interaction between α- and β-catenin. They further explain the reduced amount of Ser/Thr-mutant β-catenin in the cytoskeletal fraction and its increase in the cytosol as seen in Fig. 6A. This difference in sub-cellular localization could influence cell behaviour. To test this possibility, wound-healing experiments were performed with 293 cells stably expressing wt and Ser/Thr-mutant β-catenin (Fig. 7A). Remarkably, the wound closure was much faster in 293 cells expressing Ser/Thr-mutant β-catenin compared to those expressing the wt form. The enhanced wound closure is very unlikely due to differences in cell proliferation between the two cell types, as monitored with the proliferation marker Ki-67 (Fig. 7B). Instead, these results suggest that 293 cells expressing mutant β-catenin exhibit higher migratory potential.

This is also underlined by the subcellular distribution of HA-tagged wt or Ser/Thr-mutant β-catenin in these stably transfected cells (Fig. 7C). In comparison to HA wt β-catenin, which is strongly localized at cell-cell-contact sites, Ser/Thr-

mutant β-catenin shows a more intense and diffuse staining in the cytoplasm. Since the switch of β-catenin from the membrane to the cytoplasm is required for cell-migration, this also reflects the higher migratory potential of the cells expressing Ser/Thr-mutant β-catenin.

A similar distribution can also be seen for α-catenin in both cell lines. However, significant amounts of α-catenin in mutant cells are still localized at the membrane due to the association with endogenous β-catenin. In line with such a view, an increased staining for the mesenchymal marker vimentin in cells expressing Ser/Thr-mutant β-catenin could be observed (S.B., unpublished).
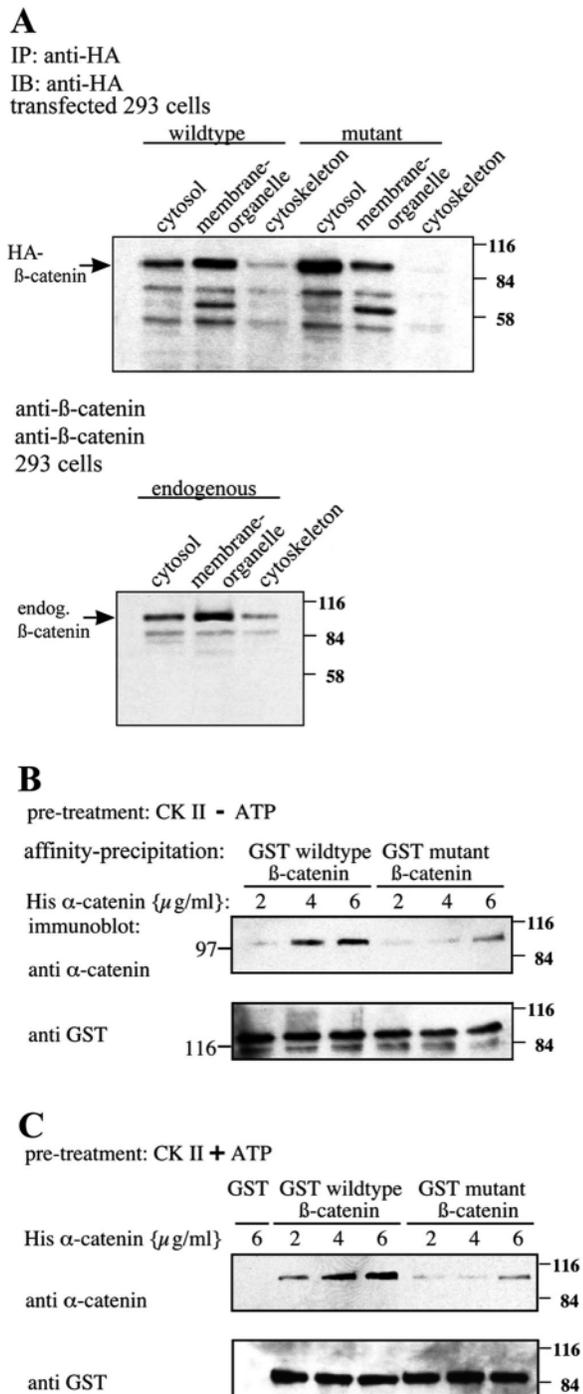
Discussion

In our attempts to identify kinases which use β-catenin as substrate we immuno-precipitated β-catenin from cell lysates and tested the precipitates for kinase activities in vitro. We found that CKII associates with and phosphorylates β-catenin and also identified GSK-3β in the β-catenin-associated precipitates (not shown). Even more interestingly, we detected a yet unknown kinase activity which specifically phosphorylates the C-terminal region (aa 683-731) of β-catenin (Fig. 2B) and which is distinct from CKII and GSK-3β as judged with specific inhibitors for both. It will be of future interest to identify and characterize this kinase. CKII phosphorylates preferentially the N-terminal region of β-catenin and this phosphorylation was specifically inhibited with a peptide containing CKII consensus motifs. We identified aa residues Ser29, Thr102, and Thr112 in the N-terminal region of β-catenin as target-sites for CKII and mutated these aa residues to compare this Ser/Thr-mutant and wt β-catenin in subsequent analysis.

CKII regulates β-catenin stability

It is well established that GSK-3β regulates the cytoplasmic turnover of β-catenin in a multiprotein complex and that the stability and composition of this complex is highly dynamic and depends on phosphorylation (Rubinfeld et al., 1996; Hart et al., 1998; Ikeda et al., 1998; Fagotto et al., 1999; Kawahara et al., 2000; Strovel et al., 2000). Here we propose that CKII participates together with GSK-3β in controlling the cytoplasmic stability of β-catenin and is part of the multiprotein complex. We show that CKII can associate with GSK-3β and Axin and that Ser/Thr-mutant β-catenin binds less efficiently to Axin and GSK-3β. Moreover, Ser/Thr-mutant β-catenin has a longer half-life (Fig. 3) as has been observed with β-catenin which is not phosphorylated by GSK-3β (Aberle et al., 1997). A synergistic phosphorylation by CKII and GSK-3β was observed when wt and Ser/Thr-mutant β-catenin were compared in in vitro kinase assays (Fig. 5). Pre-phosphorylation by CKII enhanced binding of GSK-3β and increased phosphorylation of β-catenin. The CKII-dependent higher binding and phosphorylation capabilities of
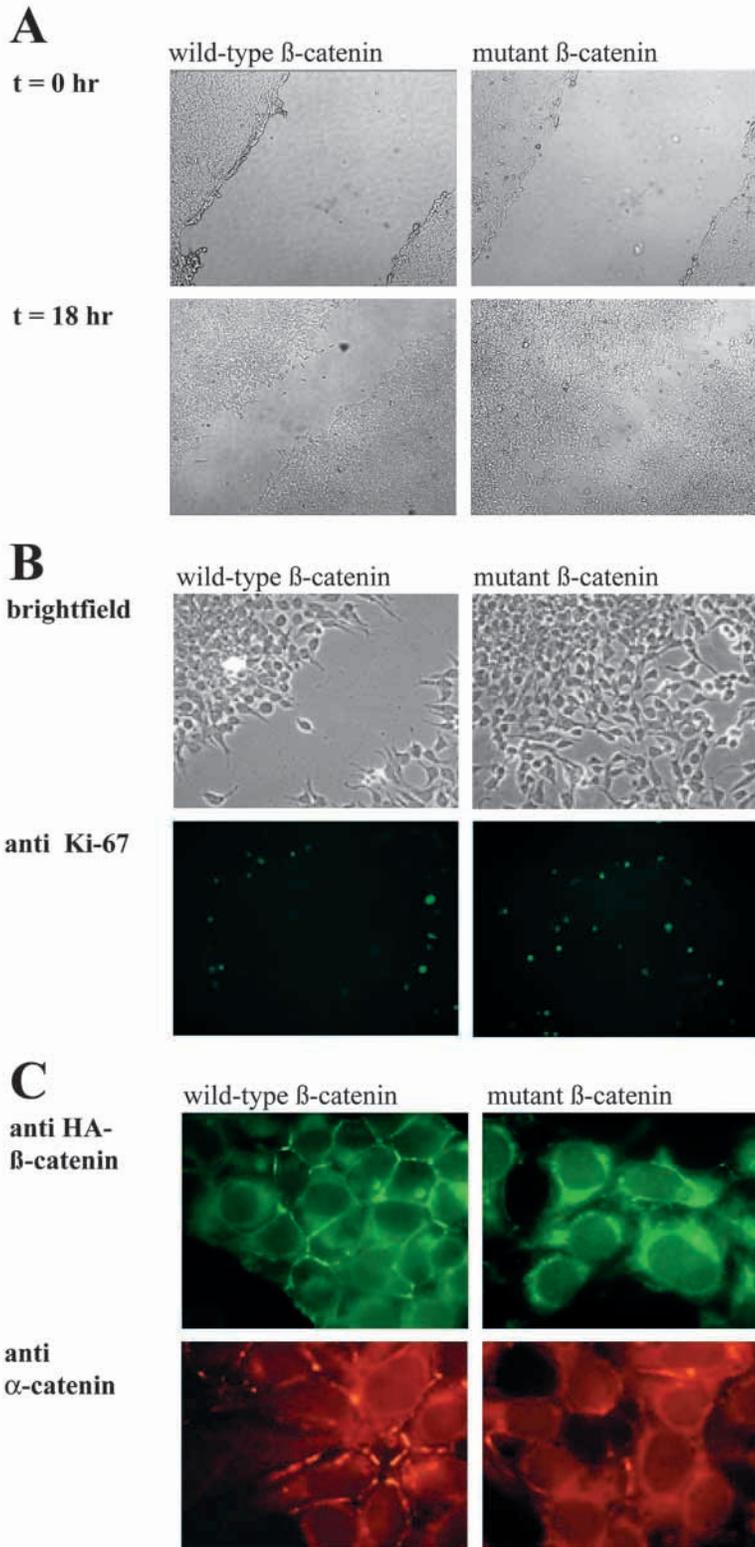
A

IP: anti-HA
IB: anti-HA
transfected 293 cells

anti-ß-catenin
anti-ß-catenin
293 cells

B

pre-treatment: CK II – ATP

C

pre-treatment: CK II + ATP

Fig. 6. CKII regulates binding of β-catenin to α-catenin. (A) 293 cells stably expressing wt or Ser/Thr-mutant HA-tagged β-catenin were grown to 80% confluency and, for subcellular fractionation, cells were subjected to sequential detergent extractions (see Materials and Methods). The Ser/Thr-mutant form of β-catenin contained all three mutations indicated in Fig. 3A. From each fraction HA-β-catenin immunoprecipitates were immunoblotted with anti-HA-antibodies. The same procedure was performed with untransformed 293 cells, which express only endogenous β-catenin. Wt β-catenin from these preparations was immunoprecipitated and immunoblotted with anti β-catenin antibodies. Compared with wt β-catenin and endogenous β-catenin, more Ser/Thr-mutant β-catenin was found in the cytosolic fraction, whereas the amount in the insoluble cytoskeletal fraction was significantly reduced. (B) 500 ng of either GST wt or Ser/Thr-mutant β-catenin coupled to GSH-Sepharose was incubated with recombinant CKII in the absence of ATP. After several washes His_6-tagged α-catenin was added under association-conditions (see Materials and Methods) and bound proteins were probed for α-catenin or GST in immunoblots. The affinity of α-catenin to Ser/Thr-mutant β-catenin clearly decreased compared with wt β-catenin. (C) GST-proteins were pre-phosphorylated with CKII in the presence of 20 mM ATP prior to the incubation with α-catenin. Binding of His_6-tagged α-catenin to pre-phosphorylated wt β-catenin was significantly increased but no phosphorylation-dependent difference for binding of α-catenin was observed with Ser/Thr-mutant β-catenin.

Using the Wnt1-expressing mouse mammary epithelial cell line C57MG, a Wnt1-dependent increase of CKII expression resulting in stronger phosphorylation of β-catenin. Association of both proteins with Dvl proteins (the mammalian homologues of *Drosophila* Dishevelled) was previously observed, and it was suggested that CKII acts as a positive regulator of Wnt signaling (Song et al., 2000). Our results do not support such a view, but one cannot exclude that CKII activity on β-catenin at the membrane and in the cytoplasm vary in different cell types, which raises the question how this can be achieved by a widely distributed and nearly always active kinase.

Possible explanations come from GSK-3β, which is differentially regulated by insulin and Wnt signaling. As a consequence of different post-translational modifications (e.g. phosphorylation on serine-9) and complex formation (e.g. the APC-Axin-Frat/GBP-Dvl-complex), wnt-signals are able to accumulate β-catenin in the cytoplasm, whereas insulin-signals do not (Ding et al., 2000). Although no extracellular signal are confirmed to regulate CKII-activity, similar mechanisms can be discussed for CKII-mediated regulation of β-catenin. CKII-phosphorylation of β-catenin also might be regulated through the interaction with different protein-complexes in the respective subcellular compartments. In line with this, CKII-activity can be regulated through the interaction with p53 in the nucleus and with p47(phox) (Guerra et al., 1997; Kim et al., 2001).

GSK-3β are not observed in mutant β-catenin, suggesting a sequential action of CKII and GSK-3β in phosphorylating β-catenin. Taking this together with the biochemical data, it is concluded that CKII phosphorylation of β-catenin stabilizes its binding to Axin and GSK-3β and thus enhances the activity of GSK-3β. Our findings are in line with the demonstrated involvement of CKII in the proteolytic degradation of other proteins, e.g. IkBα and lens connexin 45.6 (Bren et al., 2000; Yin et al., 2000). Altogether it is concluded that a combined action of CKII and GSK-3β controls the cytoplasmic turnover of β-catenin.

CKII regulates the interaction between α- and β-catenin

Ser/Thr-mutant and wt β-catenin exhibit different sub-cellular distributions when stably expressed in 293 cells. The relative amount of Ser/Thr-mutant β-catenin in the cytosolic fraction is higher, consistent with the prolonged half-life of the mutant protein. Significantly, only little mutant β-catenin distributes to the cytoskeletal fraction, indicating that mutant β-catenin does

A

t = 0 hr | wild-type ß-catenin | mutant ß-catenin

t = 18 hr

B

brightfield | wild-type ß-catenin | mutant ß-catenin

anti Ki-67

C

anti HA-
ß-catenin | wild-type ß-catenin | mutant ß-catenin

anti
α-catenin

Fig. 7. Ser/Thr-mutant β-catenin enhances cell migration. (A) Stable transfectants of 293 cells expressing HA-tagged wt or Ser/Thr-mutant β-catenin containing all three mutations, were subjected to wound healing experiments. Wound closure was much enhanced in cells expressing Ser/Thr-mutant β-catenin suggesting an increased migratory potential of the cells. (B) Cells were treated as described in A, but stained with antibodies for the proliferation marker Ki-6. The morphology of the cells is documented with brightfield-photography. No significant difference in cell proliferation was observed between the two cell types. (C) Stable transfectants of 293 cells expressing HA-tagged wt or Ser/Thr-mutant β-catenin were stained for HA β-catenin and α-catenin with indirect immunofluorescence. Cells expressing HA wt β-catenin showed a stronger staining at cell-cell-contact sites compared with cells expressing HA Ser/Thr-mutant β-catenin. The same is true for the distribution of α-catenin in both cell-lines. However, significant amounts of α-catenin in mutant cells are still localized at the membrane due to the association with endogenous β-catenin.

catenin might be reduced. To test this possibility protein interaction assays were performed with recombinant proteins. Binding of E-cadherin to wt or Ser/Thr-mutant β-catenin was unchanged (as was the binding of another β-catenin interaction partner, LEF-1; S.B., unpublished). However, mutant β-catenin bound less efficiently to α-catenin and, even more importantly, pre-phosphorylation of wt β-catenin with CKII enhanced its binding to α-catenin (Fig. 6B,C). These results provide strong evidence that CKII regulates binding of β-catenin to α-catenin and thus add a new molecular mechanism to modulate the E-cadherin-catenin cell adhesion complex. In 293-cells the catenins are propably sequestered to another cadherin, since these cells do not express E-cadherin. This view is supported by immunoprecipitation-experiments in ^{35}S-labeled 293-cells. After separation of the anti β-catenin immuno-complex, we could detect three bands running at the size of E-cadherin, α- and β-catenin, as could be observed in cells containing E-cadherin (S.B., unpublished). Again, the biochemical data are underlined by the distribution of α-catenin, which shows significant similarity with the staining of HA-tagged wt or Ser/Thr-mutant β-catenin in immunofluorescence analysis. However, significant amounts of α-catenin in mutant cells is still localized at the membrane due to the association with endogenous β-catenin.

From the N-terminal aa-sequence of β-catenin, a amphipathic α-helix can be predicted, which has been confirmed for the aa 134-161 (Graham et al., 2000). However for formation of the β-catenin-α-catenin heterodimer a change in the overall secondary structure of β-catenin is necessary (Huber and Weis, 2001). The introduction of negatively charged phosphate-residues in neighbouring sequences from β-catenin might help to provide the surface for this hydrophobic interactions. Whereas Tyr 142 in β-catenin is absolutely needed for the interaction with the hydrophobic core of the binding region (Aberle et al., 1996b; Huber et al., 1997; Pokutta and Weis,

not fulfil properly its role in the cadherin-catenin complex. Such a view is supported by our immunofluorescence data showing that Ser/Thr-mutant β-catenin is preferentially distributed in the cytoplasm whereas wt β-catenin is mostly localized to the cell membrane. This suggested that the affinity of Ser/Thr-mutant β-catenin to either E-cadherin and/or α-

2000), the residues targeted by CKII might regulate the strength of the interaction.

Related to the interaction with α-catenin, a hydrophobic binding pocket within the armadillo repeats 3 and 4 in β-catenin, is critical for Axin-binding (Graham et al., 2000; von Kries et al., 2000). N-terminal phosphorylation of β-catenin by CKII might provide the structure of the hydrophobic interaction surface. Binding domains for E-cadherin, APC and LEF-1/Tcf in β-catenin are not or are only partially located in this region. Consequently, the binding to these proteins was not influenced after CKII phosphorylation of β-catenin in vitro (S.B., unpublished).

Our biochemical results provide convincing evidence that phosphorylation of β-catenin by CKII modulates the biological function of β-catenin. The wound-healing experiments additionally substantiate this. Cells expressing Ser/Thr-mutant β-catenin exhibit higher migratory potential as compared to cells expressing the wt protein. An increase in cell proliferation is unlikely to account for the enhanced wound healing; instead, cells expressing mutant β-catenin appear to have adopted a more mesenchymal phenotype. The enhanced migratory potential of cells expressing Ser/Thr-mutant β-catenin is likely due to the inefficient binding of mutant protein to α-catenin, which could affect the cytoskeletal architecture of the cells. Although other explanations are possible, this view is supported by the subcellular distribution of HA-tagged wt or Ser/Thr-mutant β-catenin and α-catenin. Mutations in aa residues in β-catenin which are targets for CKII could thus be relevant in tumorigenesis. Notably, aa residue Ser29, which we found to be phosphorylated by CKII, is mutated in some gastric-cancer cell lines, and results in an accumulation of β-catenin (Polakis, 2000; Park et al., 1999).

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