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FAST TRACK

Basic Region of Residues 228–231 of Protein Kinase CK1α Is Involved in its Interaction With Axin: Binding to Axin Does Not Affect the Kinase Activity

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Abstract Protein kinase CK1, also known as casein kinase 1, participates in the phosphorylation of β-catenin, which regulates the functioning of the Wnt signaling cascade involved in embryogenesis and carcinogenesis. β-catenin phosphorylation occurs in a multiprotein complex assembled on the scaffold protein axin. The interaction of CK1α from *Danio rerio* with mouse-axin has been studied using a pull-down assay that uses fragments of axin fused to glutathione S transferase, which is bound to glutathione sepharose beads. The results indicate that the three lysines present in the basic region of residues 228–231 of CK1α are necessary for the binding of CK1 to axin. Lysine 231 is particularly important in this interaction. In order to define the relevance of the axin-CK1α interaction, the effect of the presence of axin on the phosphorylating activity of CK1α was tested. It is also evident that the region of axin downstream of residues 503–562 is required for CK1α interaction. The binding of CK1α to axin fragment 292–681 does not facilitate the phosphorylation of β-catenin despite the fact that this axin fragment can also bind β-catenin. Binding of CK1α to axin is not required for the phosphorylation of axin itself and, likewise, axin does not affect the kinetic parameters of the CK1α towards casein or a specific peptide substrate. J. Cell. Biochem. 94: 217–224, 2005. © 2004 Wiley-Liss, Inc.

Key words: Casein kinase 1; Wnt signaling; β-catenin; docking sites; scaffold proteins

The multiple phosphorylation of β-catenin in its amino terminal region is a key factor in the control of the Wnt signal cascade that is known to have an important role in embryonic development and carcinogenesis [Peifer and Polakis, 2000; Polakis, 2002]. Recent evidence has demonstrated that protein kinase CK1 is responsible for the phosphorylation of serine 45 of β-catenin, which primes the subsequent phosphorylation of Thr 41, Ser 37, and Ser 33 by GSK-3β [Amit et al., 2002; Liu et al., 2002; Sakananak, 2002]. This hierarchical cooperative phosphorylation, which targets β-catenin for degradation, occurs in a multiprotein complex assembled on the axin protein that functions as a scaffold and that brings together β-catenin, GSK-3β, CK1 protein, and adenomatous polyposis coli (APC) protein among other proteins [Polakis, 2002].

Several laboratories have presented evidence that demonstrates that axin can bind various isoforms of protein kinase CK1 [McKay et al., 2001; Rubinfeld et al., 2001; Amit et al., 2002]. Protein kinase CK1, also known as casein kinase 1, is a serine-threonine kinase that catalyzes the phosphorylation of many proteins that play important roles in cell division, differentation, circadian rhythms, and metabolic control [Gross and Anderson, 1998; Vielhaber and Virshup, 2001]. CK1 is present in all eukaryotes from yeast to man. Analysis of the kinome in the organisms whose genome has been deciphered indicates that CK1 is a separate branch in the great family of protein kinases and that all organisms contain several CK1 isoforms [Manning et al., 2002]. CK1 isoforms have only 25% similarity with kinases in other branches of

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the kinome but share a 70% similarity among themselves. In vertebrates, there are apparently seven CK1 isoforms (α, β, γ1, γ2, γ3, δ, and ε) some of which have specific physiological functions [Rowles et al., 1991; Tapia et al., 1994; Fish et al., 1995]. Some isoforms, such as CK1α, have several splicing variants that have different biochemical and cellular properties [Burzio et al., 2002].

In this article, results are presented which demonstrate that a basic lysine-rich region which is localized in residues 228–231 of CK1α is necessary for the binding of this enzyme to axin. It is further demonstrated that the region between axin residues 503 and 684 is necessary for its interaction with CK1α and that binding to axin does not significantly affect the phosphorylation of β-catenin, of axin itself, or of several other substrates by CK1α.

MATERIALS AND METHODS

Materials

Oligonucleotides were prepared by the Oligopeptido Core Facility of the University of Chile. Peptide substrates for CK1 and glutathione agarose were purchased from Sigma Chemical Co (St Louis, MO). Ni-NTA sepharose was from Novagen (EMD Biosciences, affiliate of Merck, Darmstadt, Germany). [γ-^{32}P] ATP was from New England Nuclear-Perkin Elmer (Boston, MA). Monoclonal antibody against 6(His) tag was from CLONTECH (BD Biosciences, San Diego, CA).

Glutathione transferase (GST)-mouse-axin (292–681) and GST-mouse-axin (503–681) were kind gifts from Dr. F. Constantini (Columbia University) and Dr. W. Hsu (University of Rochester) [see Zeng et al., 1997].

The numbering of amino acids in axin is as in NCBI protein accession number NP_033863.

DNA Clones and Mutants

The cDNA clone for the β-catenin of *Danio rerio* was previously described [Marin et al., 2003]. The cDNA clone of CK1α from zebrafish (*D. rerio*) with an NH2-terminal 6(His) tag in a pT 7-7 vector was previously published [Burzio et al., 2002].

Mutant CK1α (K228A, K229A, K331A) was prepared using the overlapping PCR method [Ho et al., 1989]. For the forward primer the oligonucleotide was 5′-G A A G G C T G C C A C A G C G G C A C A G G C T T A T G A G A A G-3′ and for the reverse: 5′-C T T C T C A T A A G C C T G T G C C G C T G T G G C A G C C T T-3′ was used.

For the mutant CK1α (K21A, R22A) the forward primer was: 5′-C A A G C T C G T T G C T G C A A T C G G A T C T G G-3′ and the reverse primer: 5′-C C A G A T C C G A T T G C A G C A 17 C G A G C T T G-3′.

For the CK1α (1–301) carboxyl deletion mutant, which was cloned in pT7-7, the forward primer in the vector was: 5′-T A A T A C G A C T C A C T A T A G G G A G-3′ and the reverse: 5-′ A T A T A T G A A T T C C T A C A G C A T G G T C C A G-3′.

For the CK1α (90–325) amino terminal deletion mutant, which was cloned in a pAGA2 vector, the forward primer was: 5′-T A T A T A C C A T G G A C C T G C-3′ and the reverse: 5′-T A T A T A G G T A C C T T A G A A A C C-3′.

The axin fragment 567–684 was constructured from the full length mouse axin donated by Dr. Constantini using forward primer: 5′-T T T T T G T C G A C T G G G G C C C A G A A A C A C A T G G-3′ and reverse primer: 5′-A A A A A A G C G G C C G C T C A G A C A G A A T T C C G A A G C T G A G C C-3′.

All mutants were checked by DNA automatic sequencing of the clone constructs.

Bacterial Expression and Purification of Proteins

The various plasmids were used to transform *Escherichia coli* BL21 (DE3). Cells were grown at 37°C to an absorbance at 600 nm of 0.4–0.6. At this point protein expression was induced by adding IPTG to a final concentration of 500 μM. In the case of CK1α, induction was carried out overnight at 22°C while for the other proteins the temperature was maintained at 37°C for 3 h. Afterwards the cells were pelleted at 3,000*g* for 20 min at 4°C and cell pellets were suspended in a buffer containing 50 mM HEPES, pH 8.0, 500 mM NaCl, and 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 2 μg/ml each of leupeptin, aprotinin, pepstatin A, antipain. Lysozyme (1 mg/ml) was added and cells were lysed for 30 min at room temperature. Lysates were sonicated and centrifuged at 39,000*g* for 30 min. In the case of 6(His) tagged proteins, the recombinant proteins were purified using Ni-NTA-agarose columns, which were washed and subsequently eluted with a buffer containing 50 mM HEPES, pH 7.5, 200 m NaCl, 1% Triton X-100 with 250 mM imidazole. In the case of GST-fusion proteins, these were purified using a column of glutathione agarose

beads and eluted with 20 mM glutathione dissolved in PBS.

CK1α Activity Assays

The assay has been described in detail previously [Burzio et al., 2002]. Briefly, substrates were phosphorylated by incubation in a 30 μl volume containing 50 mM Tris-HCl buffer (pH 7.5), 10 mM $MgCl_2$, 100 mM NaCl, and 100 μM [γ-^{32}P] ATP (specific radioactivity 500–1,000 cpm/pmol). Usually the assay contained enzyme capable of introducing 10–20 pmol of ^{32}P into casein. The amount of protein or peptide substrates varied and is specified in each experiment. The reactions were incubated for 10 min at 37°C and stopped by cooling in ice and absorption on phosphocellulose paper. Papers were washed three times with 75 mM phosphoric acid, dried and counted in a scintillation counter. The kinetic data were analyzed using the program Kaleidagraph (Synergy Software, Reading, PA). Initial rate data were fit to the Michaelis–Menten equation to obtain V_{max} and K_m values. For β-catenin phosphorylation, 0.005–2 μM recombinant full length β-catenin from *D. rerio* [Marin et al., 2003] was phosphorylated for 15 min at 37°C using the above standard conditions except that the specific radioactivity of [γ-^{32}P] ATP was 8,000-10,000 cpm/pmol. The reaction was stopped by the addition of fivefold concentrated Laemmli buffer and submitted to SDS–polyacrylamide gel electrophoresis and autoradiography.

In Vitro Transcription and Translation and ^{35}S-Labeling of CK1α Constructs

CK1α cDNA and mutant constructs were subcloned in 6(His) pT7-7 or pAGA vectors. The proteins were expressed in the TNT-coupled transcription/translation reticulocyte lysate assay (Promega Corp., Mason, WI). The reactions (50 μl) were performed according to the manufacturer's instructions using [^{35}S]-methionine (Amersham Biosciences Argentina, Buenos Aires, Argentina). Incubations were for 90 min at 30°C.

Autophosphorylation of CK1α

Approximately 20 pmol of recombinant CK1α of *D. rerio* were incubated with the same buffer described for CK1α catalytic activity except that 5 μM [γ-^{32}P] ATP (sp.act. 100,000 cpm/pmol) was used. Incubations were for 30 min at 30°C. The autophosphorylated CK1α was used immediately for the pull-down assay or kept frozen at −80°C.

Pull-Down Experiments

Assays were performed by incubating 10–20 μg of GST-axin fragment fusion proteins during 1 h with glutathione sepharose 4B beads in a buffer containing 20 mM HEPES pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, 1% glycerol, and 0.1 % Triton X-100. The complex was washed three times with the same buffer and subsequently the beads were incubated with radioactive CK1α proteins labeled either with ^{35}S using the in vitro transcription/translation system or with ^{32}P through autophosphorylation.

RESULTS

Regions of Axin and of CK1α Necessary for Interaction

The in vitro interaction of CK1α with axin can be demonstrated by a pull-down assay in which fragments of mouse-axin protein are fused to GST and the interacting CK1α molecules are bound to glutathione sepharose beads dependent on the presence of the interacting axin fragments fused to GST. Radioactive CK1α used in these assays was produced by ^{35}S labeling through an in vitro transcription-translation system or by allowing CK1α to autophosphorylate with ^{32}P. Alternatively, the presence of active CK1α bound to axin on the sepharose beads can be assayed by determining its capacity to phosphorylate a specific peptide substrate.

With regards to axin fragments, it is important to note that there is confusion in the literature about the numbering of residues in the mouse-axin protein. The original numbering system described by Zeng et al. [1997] included 124 amino acid residues upstream of the first methionine. Later, these amino acids were not included in the numbering of mouse-axin. In addition, there seems to be consensus that most of the translation of axin 1 starts in a second methionine, which is five residues downstream. In this publication, we will use the numbering system of amino acid residues of mouse-axin 1 that appears in the NCBI protein data bank under accession number NP_033863.

Figure 1A shows the results obtained by the pull-down assay performed with increasing amounts of mouse-axin fragment (292–681)

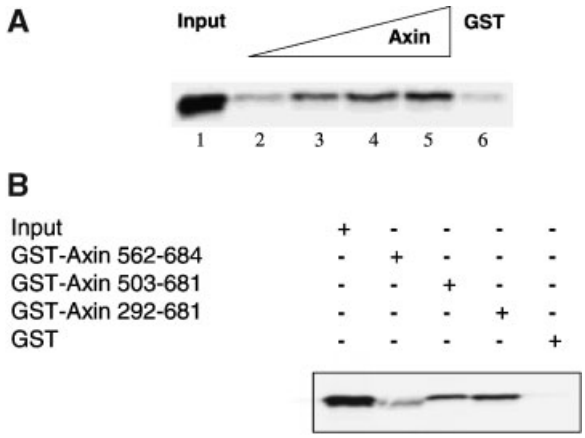

Fig. 1. Interaction of CK1α with different concentrations and different fragments of glutathione transferase (GST)-axin. **A**: The retention of [^{35}S] CK1α on glutathione sepharose was tested in the presence of increasing concentrations of GST-axin (292–681). **Lane 1**: 10% of input [^{35} S] CK1α, **lane 2** with 15 pmol, **lane 3** with 50 pmol, **lane 4** with 100 pmol, and **lane** 5 with 150 pmol of GST-axin (292–681). **Lane 6**: 100 pmol of GST protein was used as a control. The ''pull-down'' assay was carried out as detailed in "Materials and Methods." Autoradiography shows the radioactive CK1α bound. **B**: The effect of different axin fragments fused to GST on the retention of [^{35}S] CK1α. Approximately 75 pmol of each GST-axin fragments were used to test the interaction with the same amount of [^{35}S] CK1α, using the pull-down assay described in ''Materials and Methods.'' Autoradiography shows the radioactive CK1α bound.

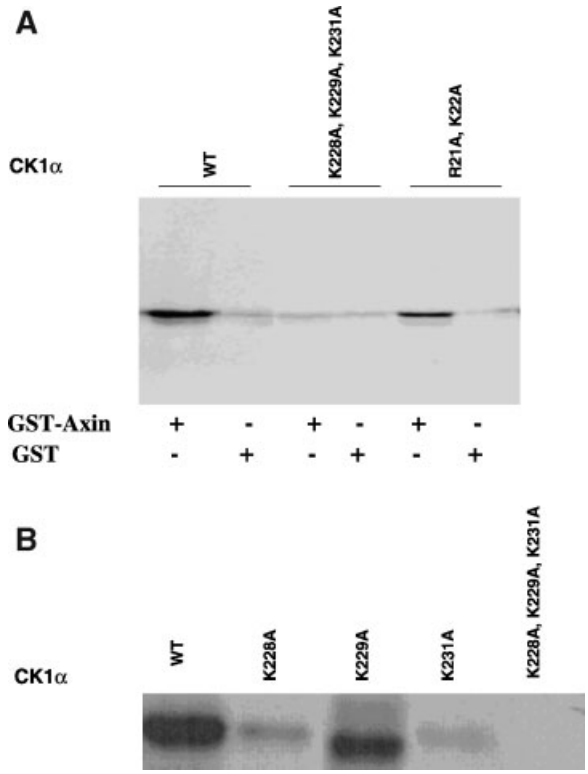

Fig. 2. The effect of mutations of CK1α on its capacity to interact with GST-axin (503–681). The pull-down assay was used to test the binding of GST-axin (503–681) to radioactive CK1α and mutants of the kinase. **A**: Samples were incubated with approximately 100 pmol of GST or GST-axin (503–681) as indicated and with WT (unmutated) or mutant [^{35}S] CK1α. Similar amounts of radioactive CK1α were used. CK1α (K228A, K229A, K231A) represents a triple mutant in which the specified lysines were changed to alanines. CK1α (R21A, K22A) represents a double mutant in which Arg 21 and Lys 22 were mutated to ala. **B**: Binding of [^{32}P] CK1α WT and mutants to 100 pmol of GST-axin (503–681). [^{32}P] CK1α labeled by autophosphorylation was used to test the interaction with GST-axin (502–681) as described in ''Materials and Methods.'' As indicated the recombinant CK1α used was the WT and single mutants replacing the indicated lysines to alanines and the triple mutant as in 2A. Inputs of 10–20 pmol of radioactive CK1α in the different preparations were tested previously to give the same amount of radioactivity.

fused to GST and incubated with a constant amount of the [^{35}S] full length CK1α from *D. rerio* [Burzio et al., 2002]. In lanes 2–5, it is possible to see that the radioactive enzyme binds to the GST-axin fusion protein in a concentration-dependent fashion. Lane 1 shows the migration of the CK1α input for the pull-down assay and lane 6 shows that GST without axin binds a very small amount of CK1α.

Using this assay, it is also possible to define further the region of axin, which is necessary for its interaction with CK1α. Figure 1B shows that the axin fragment that contains residues 503–681 is able to bind CK1α almost as efficiently as the fragment 292–681. However, when the axin fragment (562–684) fused to GST is tested, the binding of CK1 is greatly diminished. This result indicates that the region including residues 503–562 plays an important role in the binding of axin to CK1.

It has also been possible to test the effect of different mutations of CK1α on its interaction with axin. Figure 2A shows that mutation of three lysines (K^{228}, K^{229}, K^{231}) to alanine (mutant CK1α K228A, K229A, K231A) completely abolishes interaction with axin while a mutation that changes two different basic residues (R^{21}, K^{22}) to alanine, CK1α (R21A, K22A), has very little effect on the interaction. In a separate experiment, single mutations of each lysine, 228, 229, or 231 to alanine were assayed (Fig. 2B). It is clear that the lysine 231 mutation causes the most severe reduction in the binding to axin. It has also been determined that the elimination of the amino terminal region of CK1α to generate a catalytically inactive fragment of CK1α (90–325) or the truncation of the carboxyl end to yield CK1α (1–301), which is still catalytically active does not significant-

ly diminish their capacity to bind axin (not shown).

Effect of Axin Binding on CK1α Activity

The question of whether the binding of CK1α to GST-axin had any effect on the enzymatic activity of the kinase was addressed by several experiments.

The apparent K_m values of CK1α for three substrates that do not interact with axin-α-casein, β-casein, and specific peptide substrate, were tested with free CK1α and with CK1α complexed to GST-axin (503–681) fragment and bound to glutathione beads. The results presented in Table I indicate that the binding of CK1α to axin does not significantly modify the apparent K_m of the kinase for these substrates.

Since CK1α is known to phosphorylate axin [Gao et al., 2002], the question arose as to whether the capacity of CK1α to bind axin was related to its capacity to phosphorylate this scaffold protein. In Figure 3, we see that mutant CK1α (K228A, K229A, K231A), which cannot interact with axin can very efficiently phosphorylate the GST-axin fragments 292–681, 503–681, and even 562–684 which in addition lacks the CK1 binding site.

Finally, the effect of the axin fragment (292–681) was tested on the phosphorylation of β-catenin by CK1. This axin fragment contains binding sites for the enzyme, as seen above, and also for the β-catenin substrate [Liu et al., 2002].

Figure 4 shows the results obtained when a constant amount of CK1α in the presence or absence of GST-axin (292–681) was incubated with different concentrations of β-catenin. The presence of axin appears to inhibit the phosphorylation of β-catenin at low concentrations of this protein. This may be due to the fact that axin and some contaminants peptides are substrates for CK1α and as such compete with β-catenin. This competitive effect is supported by the observation that at higher concentrations of β-catenin, the phosphorylation of axin, and other proteins disappears.

Similar experiments were run with different concentrations of CK1α and with different axin concentrations. In all these experiments we failed to see a stimulation of CK1α phosphorylation of β-catenin due to the presence of axin (not shown).

TABLE I. Apparent K_m Values of Recombinant CK1α With Different Substrates Either Free in Solution or Complexed With Axin

Substrate	K_m, μM (free)	K_m, μM (axin-bound complex)
β-casein	37 ± 6	38 ± 6
α-casein	37 ± 11	23 ± 5
Peptide	355 ± 33	413 ± 15

The phosphorylations were carried out as described in "Materials and Methods" for 10 min at 37°C. The axin-bound complex was prepared by incubating CK1α with glutathione transferase (GST)-axin (503–681) under the similar conditions as in the pull-down assays described in "Materials and Methods." The CK1α activity bound to the glutathione beads (that had been washed five times but not eluted with GSH) was then incubated with different concentrations of the above substrates to determine the apparent K_m values.

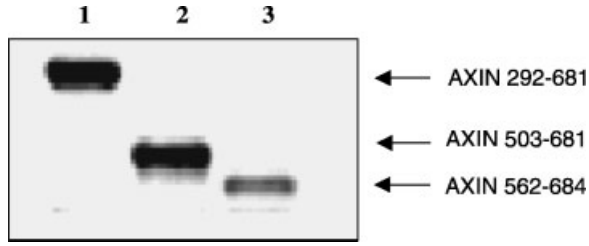

Fig. 3. The mutant CK1α (K228A, K229A, K231A) phosphorylates axin fragments. The triple mutant CK1α (K228A, K229A, K231A) (approximately 1 pmol) was incubated with approximately 2 pmol of each GST-axin fragment under the conditions for protein phosphorylation detailed in "Materials and Methods." The radioactive products were subjected to SDS–polyacrylamide gel electrophoresis and autoradiography. (**Lane 1**) GST-axin (292–681), (**lane 2**) GST-axin (503–681), and (**lane 3**) GST-axin (562–684).

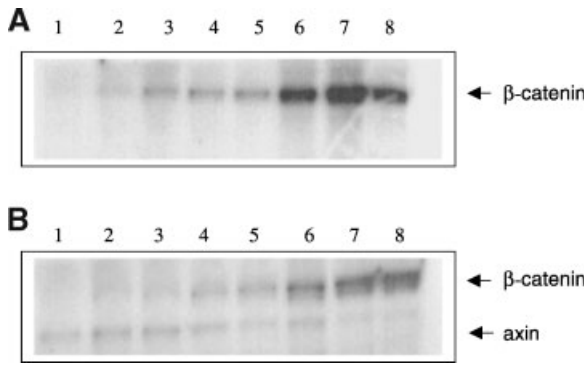

Fig. 4. Phosphorylation of β-catenin by CK1α in the presence or absence of axin fragment (292–681) Recombinant β-catenin from *D. rerio* at different concentrations was incubated with CK1α (0.3 pmol) under the protein phosphorylation conditions described in "Materials and Methods." **A**: In the absence of GST-axin (292–681) and (**B**) in the presence of 0.2 μM of the GST-axin (292–681). The amounts of fusion protein β-catenin added were: (**lane 1**) 5 nM, (**lane 2**) 10 nM, (**lane 3**) 20 nM, (**lane 4**) 50 nM, (**lane 5**) 100 nM, (**lane 6**) 0.2 μM, (**lane 7**) 1 μM, and (**lane 8**) 2 μM. After 15 min of incubation, the phosphorylated products were analyzed by SDS–polyacrylamide gel electrophoresis followed by autoradioagraphy.

DISCUSSION

The work presented above confirms previous work regarding the interaction of CK1α with axin [McKay et al., 2001; Rubinfeld et al., 2001]. Additionally, the results obtained in this work significantly extend our knowledge about the requirements for this interaction. Using a triple mutant of CK1α that changes lysines 228, 299, and 231 to alanines, it was demonstrated that this basic region of the kinase is necessary for this interaction with axin. Single mutants in each lysine residue demonstrated the critical importance of lysine 231 in the interaction with the scaffold protein. This basic interacting region is specific since a mutation that also changes two basic residues in another region of the kinase (R21, K22) to alanine does not affect the binding to axin.

The basic region of CK1α found to interact with axin is the same region of the molecule that was found to interact with the protein centaurin α1 [Dubois et al., 2001]. A peptide representing the residues 217–233 of CK1α, which include this interacting loop was found to bind HMG1, importin, catalytic subunit of protein phosphatase 2A, tubulin, and actin [Dubois et al., 2002]. It is significant that this region is highly conserved in all the isoforms of the CK1 family as well as that other laboratories have shown that axin can interact with all the isoforms of CK1 tested [Rubinfeld et al., 2001]. There was a previous report [Sakanaka et al., 1999], however, indicating that the carboxyl tail region of CK1ε was necessary for binding to axin and that CK1α, which lacks that carboxyl extension, was not able to interact with the scaffold protein. Our results do not agree with those findings.

The interacting region of CK1α is conserved in CK1δ. In the three-dimensional structure of CK1δ this region is present in an exposed loop between helices E and F that was proposed, by the group that determined the crystallographic structure of CK1δ, to be a strategically situated to mediate interactions between the enzyme and other proteins [Longenecker et al., 1996]. This loop was also proposed to mediate substrate recognition, especially the selection of substrates that contain phosphoamino acids that are effective primers for second site phosphorylation by CK1α [Xu et al., 1995]. This proposal was based on the observation that a tungstate ion or a sulfate ion was bound to a lysine residue occupying an equivalent site in the crystal structure of CK1 of *S. pombe* and of CK1δ from mammals as Lys 231 in CK1α [Xu et al., 1995; Longenecker et al., 1996].

Using several constructs of axin, it was possible to determine that the region between residues 503 and 562 in the scaffold protein is necessary for the optimal binding of CK1α. The present finding is in agreement with previous reports that indicated that region 508–712 was important for CK1α binding. Several other proteins, including MEKK4 and PP2A and Smad3, have been found to bind to this same region of axin [Luo and Lin, 2004].

Scaffold proteins that bind enzymes and protein substrates are thought to increase the catalytic efficiency of the enzymes by increasing the local concentration of both interacting protein partners. This appears to occur in the case of axin, which binds GSK3β and β-catenin and increases the phosphorylation of the latter protein by GSK3β 20,000-fold [Kikuchi, 1999; Dajani et al., 2003]. Since axin also binds CK1α, which likewise phosphorylates β-catenin in a dual kinase partnership with GSK-3β, it has been presumed that binding to the scaffold protein might likewise increase the efficiency of CK1 phosphorylation of serine 45 in β-catenin. In vivo results [Liu et al., 2002] performed in *Xenopus* embryos actually demonstrated that serine 45 phosphorylation of β-catenin was greatly enhanced by the presence of axin containing the β-catenin and the CK1 binding sites.

However, the in vitro results described above do not support a direct effect of the presence of axin on the phosphorylation of β-catenin, even though the fragment of axin used was demonstrated to contain functional binding sites for both the enzyme and the β-catenin protein substrate.

There are many possible explanations for this discrepancy of in vivo and in vitro results. Obviously, one is the fact that a level of phosphorylation of a protein in vivo is the result of the balance of the activity of kinases and protein phosphatases that act on that protein as a substrate. Axin could make β-catenin unavailable to protein phosphatases. As a matter of fact, the work of Li et al. [2001] has demonstrated that protein phosphatase 2A binds to axin to a very similar region that binds CK1α and Gao et al. [2002] have presented data that demonstrates that CK1ε can displace PP2A from the axin complex. Additionally, it has been postulated

that diversin, a protein with ankyrin repeats, is responsible for facilitating the binding of CK1ε to the axin complex and stimulating its phosphorylation of β-catenin [Schwartz-Romond et al., 2002]. The absence of diversin in our in vitro work might explain the discrepancy.

Another complexity of the in vivo system is the fact that the APC protein also plays an important role in the destruction complex with axin. APC is also phosphorylated by CK1 [Rubinfeld et al., 2001; Marin et al., 2003] and this phosphorylation increases its affinity for β-catenin and for axin.

As this article was in preparation, a publication appeared [Ha et al., 2004], which tested the effect of axin on in vitro phosphorylation of β-catenin by CK1ε. Axin seemed to enhance β-catenin phosphorylation by this CK1 isoform but these authors tested only one concentration of axin and β-catenin and do not show the relative phosphorylation of β-catenin and axin when both components are present. The use of a different isoform of CK1 may also explain the discrepancy. It is interesting to note that CK1α has a very high affinity for β-catenin in the absence of axin. The apparent K_m of CK1α for this protein was found to be 0.1 μM [Marin et al., 2003]. The affinity of CK1α for full length axin or for its fragments has not been determined but if this affinity of CK1α for axin was found to be less than for β-catenin, one would expect that the presence of axin may not facilitate its phosphorylation.

From the results of the present work, it is also evident that the binding of CK1α to axin does not influence its capacity to phosphorylate the axin protein itself. It is also demonstrated that the binding to axin does not affect the intrinsic activity of the enzyme with respect to substrates that are not part of the axin complex. It is interesting that the binding of CK1α to α-centaurin, which involves the same "interaction loop" as is required for this enzyme to interact with axin, likewise does not affect the catalytic properties of the kinase [Dubois et al., 2001].

REFERENCES

Amit S, Hatzubai A, Birman Y, Andersen JS, Ben-Shushan E, Mann M, Ben Neriah Y, Alkalay I 2002. Axin-mediated CK1 phosphorylation of β-catenin at Ser45: A molecular switch for the Wnt pathway. Genes Dev 16: 1066–1076.

Burzio V, Antonelli M, Allende CC, Allende JE 2002. Biochemical and cellular characteristics of the four splice variants of protein kinase CK1α from zebrafish (*Danio rerio*). J Cell Biochem 86:805–814.

Dajani R, Faser E, Roe SM, Yco M, Good VM, Thompson V, Dale TC, Pearl LH 2003. Structural basis for recruitment of glycogen synthase kinase 3β to the axin-APC scaffold complex. EMBO J 22:494–501.

Dubois T, Kerai P, Zemlickova E, Howell S, Jackson TR, Venkateswarlu K, Cullen PJ, Theibert AB, Larose L, Roach PJ, Aitken A 2001. Casein kinase I associates with member of the centaurin-α family of phosphatidylinositol 3,4,5-triphosphate-binding proteins. J Biol Chem 276: 18757–18764.

Dubois T, Howell S, Zemlickova E, Aiken A 2002. Identification of casein kinase 1α interacting protein partners. FEBS Lett 517:167–171.

Fish KJ, Cegielska A, Getman ME, Landes GM, Virshup D 1995. Isolation and characterization of human casein kinase Iε (CK1), a novel member of the CKI gene family. J Biol Chem 270:14875–14883.

Gao WH, Seeling JM, Hill V, Yochum A, Virshup DM 2002. Casein kinase I phosphorylates and destabilizes the β-catenin degradation complex. Proc Acad Sci USA 99: 1182–1187.

Gross SD, Anderson RA 1998. Casein kinase I: Spatial organization of a multifunctional protein kinase family. Cell Signal 10:699–711.

Ha N-C, Tonuzaka T, Stamos JL, Choi H-J, Weis WI 2004. Mechanism of phosphorylation-dependent binding of APC to β-catenin and its role in β-catenin degradation. Mol Cell 15:511–521.

Ho S, Hunt H, Horton R, Pullen J, Please L 1989. Site-directed mutagenesis by overlap extension using the polymerase chain reaction. Gene 77:51–59.

Kikuchi A 1999. Roles of axin in the Wnt signaling pathway. Cell Signal 11:777–788.

Li X, Yost HJ, Virshup DM, Seeling JM 2001. Protein phosphatase 2A and its B56 regulatory subunit inhibit Wnt signaling in xenopus. EMBO J 20:4122–4133.

Liu C, Li Y, Semenov M, Han C, Baeg G-H, Tan Y, Zhang Z, Lin X, He X 2002. Control of β-catenin phosphorylation/degradation by a dual-kinase mechanism. Cell 108:837–847.

Longenecker KL, Roach PJ, Hurley TD 1996. Three-dimensional structure of mammalian casein kinase 1: Molecular basis for phosphate recognition. J Mol Biol 257:618–631.

Luo W, Lin S-C 2004. Axin: A master scaffold for multiple signaling pathways. Neurosignals 13:99–113.

Manning G, Whyte DB, Martinez R, Hunter T, Sudarsanam S 2002. The protein kinase complement of the human genome. Science 298:1912–1934.

Marin O, Bustos VH, Cesaro L, Meggio F, Pagano MA, Antonelli M, Allende CC, Pinna LA, Allende JE 2003. A non-cannonical sequence phosphorylated by casein kinase 1 in β-catenin may play a role in casein kinase 1 targetting of important signaling proteins. Proc Natl Acad Sci USA 100:10193–10200.

McKay RM, Peters JM, Graff JM 2001. The casein kinase I family in Wnt signaling. Dev Biol 235:388–396.

Peifer M, Polakis P 2000. Wnt signalling in oncogenesis and embryogenesis—A look outside the nucleus. Science 287: 1606–1609.

Polakis P 2002. Casein kinase 1: A Wnt'er of discontent. Curr Biol 12:R499–R501.

Rowles J, Slaughter C, Moomaw C, Hsu J, Cobb MH 1991. Purification of casein kinase I and isolation of cDNAs encoding multiple casein kinase I like enzymes. Proc Natl Acad Sci USA 88:9548–9552.

Rubinfeld B, Tice DA, Polakis P 2001. Axin-dependent phosphorylation of the adenomatous polyposis coli protein mediated by casein kinase 1ε. J Biol Chem 276: 39037–39045.

Sakanaka C, Leong P, Xu L, Harrison S, Williams L 1999. Casein kinase 1ε in the Wnt pathway: Regulation of β-catenin function. Proc Natl Acad Sci USA 96:12548–12552.

Sakananak C 2002. Phosphorylation and regulation of β-catenin by casein kinase Iε. J Biochem 132:697–703.

Schwartz-Romond T, Asbrand C, Bakkers J, Kuhl M, Schaeffer H-J, Huelskin J, Beherens J, Hammerschmidt M, Birchmeier W 2002. The ankyrin repeat protein diversin recruits casein kinase I epsilon to the beta-catenin degradation complex and acts in both canonical Wnt and Wnt/JNK signaling. Genes Dev 16:2073–2084.

Tapia C, Featherstone T, Gomez C, Taillon-Miller P, Allende CC, Allende JE 1994. Cloning and chromosomal localization of the gene coding for human protein kinase CK1. FEBS Lett 349:307–312.

Vielhaber E, Virshup DM 2001. Casein Kinase I: From obscurity to center stage. IUBMB Life 51:73–78.

Xu RM, Carmel G, Sweet RM, Kuret J, Chen X 1995. Crystal structure of casein kinase 1, a phosphate-directed protein kinase. EMBO J 14:1015–1023.

Zeng L, Fagotto F, Zhang T, Hsu W, Vasicek TJ, Perry WL III, Lee JJ, Tilghman S M, Gumbiner BM, Constantini F 1997. The mouse fused locus encodes axin, an inhibitor of the Wnt signaling pathway that regulates embryonic axis formation. Cell 90:181–192.