Chemical Dissection of the APC Repeat 3 Multistep Phosphorylation by the Concerted Action of Protein Kinases CK1 and GSK3†

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ABSTRACT: A crucial event in machinery controlled by Wnt signaling is the association of β-catenin with the adenomatous polyposis coli (APC) protein, which is essential for the degradation of β-catenin and requires the multiple phosphorylation of APC at six serines (1501, 1503, 1504, 1505, 1507, and 1510) within its repeat three (R3) region. Such a phosphorylation is believed to occur by the concerted action of two protein kinases, CK1 and GSK3, but its mechanistic aspects are a matter of conjecture. Here, by combining the usage of variably phosphorylated peptides reproducing the APC R3 region and Edman degradation assisted localization of residues phosphorylated by individual kinases, we show that the process is initiated by CK1, able to phosphorylate S1510 and S1505, both specified by non-canonical determinants. Phosphorylation of S1505 primes subsequent phosphorylation of S1501 by GSK3. In turn, phospho-S1501 triggers the hierarchical phosphorylation of S1504 and S1507 by CK1. Once phosphorylated, S1507 primes the phosphorylation of both S1510 and S1503 by CK1 and GSK3, respectively, thus completing all six phosphorylation steps. Our data also rule out the intervention of CK2 despite the presence of a potential CK2 phosphoacceptor site, S^{1510}LDE, in the R3 repeat. S1510 is entirely unaffected by CK2, while it is readily phosphorylated even in the unprimed peptide by CK1δ but not by CK1γ. This discloses a novel motif significantly different from non-canonical sequences phosphorylated by CK1 in other proteins, which appears to be specifically recognized by the δ isoform of CK1.

In recent years, it has become clear that important regulatory proteins undergo multiple phosphorylations in special regions rich in serines and threonines. These multiple phosphorylations can determine the fate of several of these proteins, notably targeting the protein for degradation by the proteasome, preventing the protein from entering the nucleus where it can act as a transcription factor, or allowing the protein to interact with a second protein or protein complex.

Intriguingly, these multiple phosphorylation events often involve a special group of kinases known as phosphate directed or primed kinases, which participate in hierarchical phosphorylation (*1*). Potentially primed kinases are CK1,[1] CK2, GSK-3, and G-CK (*2*). Protein kinase CK1 (also known as casein kinase 1) recognizes canonical phosphoacceptor sites specified by a $p(T/S)(X)_{2-3}S^*/T^*$ consensus, where p(T/S) indicates a phosphoserine or phosphothreonine, X any other amino acid, and S* the target serine or threonine that will receive the phosphoryl group from ATP. The phosphorylated amino acid, however, can be replaced by an aspartic acid at position $n - 3$ (*3*) and even more efficiently by a stretch of acidic amino acids (*4*). Protein kinase CK2 has a phosphate directed recognition pattern that is a mirror image of that of CK1, S*/T*XXp(S/T), but CK2 only seldom is a primed kinase, its consensus sequence generally relying on a carboxylic side chain at position $n + 3$ (*5*). GSK-3β is reminiscent of CK2, except that it targets serines or threonines that are at position −4 from the phosphorylated amino acid, which cannot be replaced by a carboxylic residue. Thus, GSK-3 appears to be the most strict among primed kinases in requiring a phosphorylated amino acid as a specificity determinant. The Golgi apparatus casein kinase in fact recognizes the consensus sequence S*-X-E/pS, where glutamic acid and phosphoserine are equally effective (*6*). The priming requirement means that the initial phosphorylation within a serine-/threonine-rich sequence can trigger additional phosphorylation(s) by one or more of these kinases.

In principle, the priming kinase should not be itself a phosphate directed one. Thus, the phosphorylation of the protein cubitus interruptus (Ci) is initiated by PKA, which subsequently leads to multiple phosphorylations by the hierarchical intervention of the primed kinases CK1 and GSK-3β (*7*). Multiple phosphorylation of Ci leads to the degradation of the Ci155 form (transcriptional activator) to the Ci75 form (a transcriptional repressor). Other examples where

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[1] Abbreviations: CK1, protein kinase CK1 (casein kinase 1); CK2, protein kinase CK2 (casein kinase 2); GSK3, glycogen synthase kinase-3; G-CK, Golgi apparatus casein kinase; PKA, protein kinase A; NFAT4, nuclear factor of activated T cells 4; APC, adenomatosis polyposis coli; Fmoc, 9-fluorenylmethoxycarbonyl; TFA, trifluoroacetic acid; ATZ, anilinothiazolinone.

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PKA primes CK1 and GSK-3 cooperatively, leading to multiple phosphorylations, are glycogen synthase itself (*8*) and Smoothened (*9*).

However, there are some cases of multiple phosphorylations in which the two kinases involved, notably GSK3 and CK1, are both potentially primed enzymes. The question therefore arises as to how the initial phosphorylation is accomplished since the sequences involved also do not contain the CK1 consensus specified by acidic residues replacing the phosphorylated determinant. Studies with peptides derived from NF-AT4 and β-catenin allowed us to discover a non-canonical sequence present in these proteins that can be recognized by CK1. This sequence can be depicted as S*LS$(X)_{n-2-5}(D/E)_{2-5}$, in which the serine that is targeted for phosphorylation is the first of a SLS triplet followed two to five residues downstream by a cluster of acidic amino acids (*10*, *11*). Mutations in the peptide and in full-length β-catenin demonstrated the importance of both the SLS motif and the acidic stretch. Further work showed that different regions of CK1α are involved in the recognition of the two types of substrates, canonical and non-canonical, respectively (*12*), and that high affinity phosphorylation of β-catenin requires, in addition to the non-canonical sequence mentioned, the integrity of an N-terminal domain including both the phosphoacceptor region and the first armadillo repeat present in the substrate protein (*13*). Computer aided analysis of other proteins containing the non-canonical consensus sequence (*11*) yielded among others APC, which had already been shown to be indeed phosphorylated by CK1ϵ (*14*).

More recent works (*15*, *16*) demonstrated that the β-catenin binding repeats present in APC can be multiply phosphorylated by the combined intervention of CK1 and GSK-3β. These authors also demonstrated the relevance of these phosphorylations to potentiate the binding of APC to β-catenin, an event that competes against axin binding, thus leading to the release of phosphorylated β-catenin from the destruction complex (*17*).

In the present work, a rational stepwise procedure was devised to study and dissect the sequence of events and the main actors that intervene in the multiple phosphorylations of this region of APC.

EXPERIMENTAL PROCEDURES

Materials. Solvents and coupling reagents for peptide synthesis and protein sequencing were from Applied Biosystems (Foster City, CA). Building blocks for solid-phase peptide synthesis were purchased from Novabiochem (Laufelfingen, Switzerland). All other analytical grade reagents were from Sigma-Aldrich (St. Louis, MO). [γ33P]-ATP (3000 Ci/mmol) was purchased from Amersham Pharmacia Biotech (Cleveland, OH).

CK1 Isoforms Cloning, Expression, and Purification. The cDNA clone of CK1γ1 from rat was provided for Dr. Peter Roach (Indiana University, Bloomington, IN). It was subcloned in the vector pQE80L using the primers 5′-TATATAGTCGACTTATGTGGGTGCTGGTGGGTGGGA-3′ and 5′-TATATAGGATCCGACCATTCTAATAGAGAAAAGG. CK1δ1 full-length, the zebrafish homologue to human CK1δ 2, was cloned between the sites Bgl II and Not I of the plasmid pT7HX, using the primers 5′-TATATAAGATCTATGGAGCTACGAGTTGGAAAC-3′ and 5′-ATATAGCGGCCGCCTACTTGCCGTGGTGATCG-3′. The clone of CK1δ from zebrafish, deleted in residue 317 to prevent autophosphorylation, was subcloned into the plasmid pQE80L using the primers 5′-TATATAGTCGACGAATTAAGAGTAGGAAACC-3′ and 5′-TATATAGGATCCTCACCCTTGCCGTATCCTTTCC-3′. The clone of CK1α was obtained as described previously (*18*). All the isoforms of CK1 contained six histidines in the N-terminus to facilitate the purification.

Protein Expression and Purification. The plasmids of CK1α and CK1γ were used to transform *Escherichia coli* BL21(DE3) cells. The plasmid of CK1δ was used to transform *E. coli* B-834. Cells were grown at 37 °C to an OD_{600} of 0.4−0.6. At this point, protein expression was induced by adding IPTG (isopropyl β-D-thiogalactoside) to a final concentration of 100 μM. In the case of CK1α, induction was carried out overnight at 22 °C, while for CK1δ and CK1γ, the temperature was maintained at 37 °C for 3 h. Afterward, the cells were pelleted at 3000*g* for 20 min at 4 °C, and cell pellets were resuspended in lysis buffer (50 mM Tris/HCl, pH 8.0, 500 mM NaCl, 1% Triton X-100, protein inhibitors from Sigma). Cells were lysed using a French press. After centrifugation at 10 000*g* for 30 min, the supernatant was applied to Ni-NTA−agarose columns that were washed and subsequently eluted with a buffer containing 50 mM Tris/HCl, pH 7.5, 0.2 M NaCl, and 1% Triton X-100 with 250 mM imidazole.

Human recombinant α and β subunits of CK2 were expressed in *E. coli*, and the holoenzyme was reconstituted and purified as described previously (*19*). Purified GSK3-β was purchased from Upstate (Lake Placid, NY). For the sake of comparison, the activities of CK2 and GSK3 were normalized to those of CK1 isoforms using the specific peptide substrates RRRADDSDDDDD and YRRAVPPSPSLSRHSSPHQpSEDEEE, respectively.

Peptide Synthesis. Anchored Peptides. The peptide substrate DGFSASSSLSLDEPFIQ corresponding to APC repeat three (R3) and its phosphorylated derivatives were synthesized by solid-phase peptide synthesis as permanently resin-anchored peptides on the solid support amino-PEGA resin (Novabiochem, Bad Soden, Germany). The syntheses were performed using an automatized peptide synthesizer (model 431-A, Applied Biosystems). The 9-fluorenylmethoxycarbonyl (Fmoc) strategy was used throughout the peptide chain assembly (*20*). The building blocks were as follows: Fmoc-Ala-OH, Fmoc-Asp(*t*-butyl)-OH, Fmoc-Gln(trityl)-OH, Fmoc-Glu(*t*-butyl)-OH, Fmoc-Phe-OH, Fmoc-Gly-OH, Fmoc-Ile-OH, Fmoc-Leu-OH, Fmoc-Pro-OH, Fmoc-Ser(*t*-butyl)-OH, and Fmoc-Ser-[PO(O-benzyl)OH]-OH. Coupling was performed with a single reaction for 40−50 min by a 0.45 M solution in *N*,*N*-dimethylformamide (DMF) of 2-(1-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and *N*-hydroxybenzotriazole (HOBt) in the presence of *N*-ethyldiisopropylamine (DIEA) following the manufacturer's protocols. The correct amino acid incorporation into nascent peptide chains was evaluated by monitoring the absorbance at 301 nm of Fmoc removal at every synthesis cycle. At the end of the synthesis, the side chain protecting groups were cleaved by reacting the peptidyl resins with TFA/H_2O/thioanisole/1,2-ethanedithiol/phenol (10 mL/0.5 mL/0.5 mL/0.250 mL/750 mg) for 2−2.5 h (*21*). Then, the petidyl resin was filtered and extensively washed with water,

10% aqueous TFA, DMF, diethyl ether, and methanol and dried.

Soluble Peptides. The synthesis of the peptides RRRDGFSASSSLSLDEPFIQ and RRRDGFSASSALSLDEPFIQ was performed using the same Fmoc strategy previously described for anchored peptides except for the solid support that was the HMPA PEGA resin (Novabiochem, Bad Soden, Germany). Additionally, Fmoc-Arg(2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl) was used as a building block amino acid to assemble the arginyl triplet at its N-terminal side. Such a basic motif was essential for the protein kinase assay based on phosphocellulose-paper substrate absorption. Cleavage of the peptides was performed by reacting the peptidyl resins with a mixture containing TFA/H_2O/thioanisole/ethanedithiol/phenol (10 mL/0.5 mL/0.5 mL/0.25 mL/750 mg) for 2.5 h. Crude peptides were purified by preparative reversed-phase HPLC. Molecular masses of the peptides were confirmed by mass spectroscopy with direct infusion on a Micromass ZMD-4000 mass spectrometer (Waters-Micromass, Manchester, U.K.). The purity of the peptides was about 95% as evaluated by analytical reversed-phase HPLC.

Peptide Phosphorylation Assay. Synthetic peptide substrates derived from the APC R3 sequence and covalently bound to the resin beads (0.5 mg, ~200 nmol) were phosphorylated by incubation in a medium (50 μL final volume) containing 50 mM Tris/HCl buffer (pH 7.5), 10 mM $MgCl_2$, 100 mM NaCl, and 100 μM [γ33P]ATP (specific radioactivity 1500−2000 cpm/pmol). The reaction was started with the addition of protein kinases CK1α, CK1δ, and CK1γ1 normalized against a common peptide substrate, namely, the one derived from inhibitor-2 of protein phosphatase-1 (I-2) RRKHAAIGDDDDAYSITA (*22*). More precisely, each amount of CK1 isoform displayed on peptide I-2 a specific activity of 2.5 pmol of phosphate transferred per minute. The reactions were incubated for the indicated time at 37 °C and stopped by ice cooling and the addition of 50 μL of TFA (5% final concentration). The phosphorylated peptidyl resin was separated from the reaction mixture by filtration and washed exhaustively with 3% phosphoric acid solution, water, 1 M NaCl, and methanol. To evaluated the radioactivity incorporated, each sample was mixed with 2 mL of scintillation cocktail and counted for 1 min in a liquid scintillation counter. Results are representative of at least three independent experiments.

Phosphorylation of soluble peptides was performed in solution in the same incubation medium used for immobilized peptides. The concentration of the peptides was 1 mM. The radioactivity incorporated was quantified by the phosphocellulose-paper procedure as previously described (*11*), taking advantage of the arginyl triplet added on the N-terminal side of the peptides.

Solid-Phase Edman Sequencing. Aliquots (0.5 mg) of synthetic peptide substrates covalently bound to the beads employed for solid-phase synthesis were phosphorylated in the presence of [γ33P]ATP as described previously and exhaustively washed. Subsequently radiolabeled peptidyl resin was resuspended in methanol, and a few beads, corresponding to 1000−10 000 cpm, were spotted onto a micro-TFA filter (Applied Biosystems, Foster City, CA) placed inside the cartridge of a Procise HT 491 protein sequencer (Applied Biosystems). After solvent evaporation, the samples were subjected to N-terminal sequencing analysis utilizing a modified Cartridge chemistry cycle (*23*) to isolate ATZ amino acids accordingly to the manufacturer. No flask cycle or HPLC gradient cycles were loaded. At every cycle, the removed ATZ amino acids were quantitatively transferred to an external fraction collector connected to the ATZ port, using 90% methanol/10% water as the solvent (S1). The collected fractions (600 μL) were mixed with 2 mL of scintillation cocktail and counted for 1 min in a liquid scintillation counter. The recovered 33P radioactivity at every cycle was plotted against the primary sequence of the peptide substrate. All reported data are representative of multiple experiments.

Table 1. Peptides Derived from APC R3 Repeat Used in the Present Study[a]

Immobilized peptides	
R3	DGFSASSSLSALSLDEPFIQ
R3 DE/AA	DGFSASSSLSALSL<u>AA</u>PFIQ
R3 pS1505	DGFSASS<u>**pS**</u>LSALSLDEPFIQ
R3 pS1510	DGFSASSSLSAL<u>**pS**</u>LDEPFIQ
R3 pS1501-1505	DGF<u>**pS**</u>ASS<u>**pS**</u>LSALSLDEPFIQ
R3 pS1501-1504-1505	DGF<u>**pS**</u>AS<u>**pSpS**</u>LSALSLDEPFIQ
R3 pS1507	DGFSASSSL<u>**pS**</u>ALSLDEPFIQ
Soluble peptides	
RRR-R3	**RRR**DGFSASSSLSALSLDEPFIQ
RRR-R3 S1505/A	**RRR**DGFSASS<u>**A**</u>LSALSLDEPFIQ

[a] Parent peptide R3 APC encompasses the R3 motif with a four residue extension on the C-terminal side. Underlining denotes substituted residues. Phosphoserine replaced for serine is indicated in bold as **pS**.

RESULTS

CK1 Is Priming APC R3 Phosphorylation. A 20 amino acid peptide reproducing the R3 region of APC (residues 1498−1517, see Table 1) with Cys 1502 substituted by alanine to avoid side reactions such as the oxidation of the thiol group and the formation of disulfide bridges, and with a triplet of arginines added to its N-terminus to make applicable the phosphocellulose-paper kinase assay (see Experimental Procedures), was synthesized and assayed for its susceptibility to phosphorylation by a number of kinases implicated in the Wnt signaling, namely, three isoforms: CK1, GSK3, and CK2. The R3 region was chosen for this study first because it is the tightest binding site of APC to β-catenin (*24*) and second because the crystal structure of its complex with β-catenin has been solved, revealing the importance of individual phosphorylated residues (*15*, *16*). This peptide (RRR-R3) as well as its resin immobilized congener deprived of the arginyl triplet (R3), on display in Table 1 together with a number of (phospho)derivatives used in this study, contains six seryl residues at positions 1501, 1503, 1504, 1505, 1507, and 1510 whose phosphorylation is required for high affinity association with β-catenin. The phosphorylation assays were performed in the presence of equivalent amounts of the kinases, in terms of units, defined using the specific peptide substrate of each kinase (see the Experimental Procedures). As can be seen in Figure 2A, where the results of a time course experiments are reported, the peptide is readily phosphorylated by CK1γ1 and CK1δ and more slowly by CK1α; in contrast, it was entirely unaffected by GSK3β and CK2.

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R1[1265-1284] EDTPICFSRCSSLSSLSSAE
R2[1378-1397] QETPLMFSRCTSVSSLDSFE
                    * *** *  *
R3[1494-1513] ESTPDGFSCSSSLSALSLDE
R4[1645-1664] EGTPINFSTATSLSDLTIES
R5[1850-1869] EGTPYCFSRNDSLSSLDFDD
R6[1957-1976] ENTPVCFSHNSSLSSLSDID
R7[2015-2034] EDTPVCFSRNSSLSSLSIDS
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FIGURE 1: Sequence alignment of the seven 20-amino acid repeats [R1−R7] of human APC (accession no. REFSEQ NP_000029). Seryl residues that were found phosphorylated in the APC R3 repeat/β-catenin complex (*15*, *16*), denoted by an asterisk (∗), are S1501, S1503, S1504, S1505, S1507, and S1510. Conserved residues are underlined.

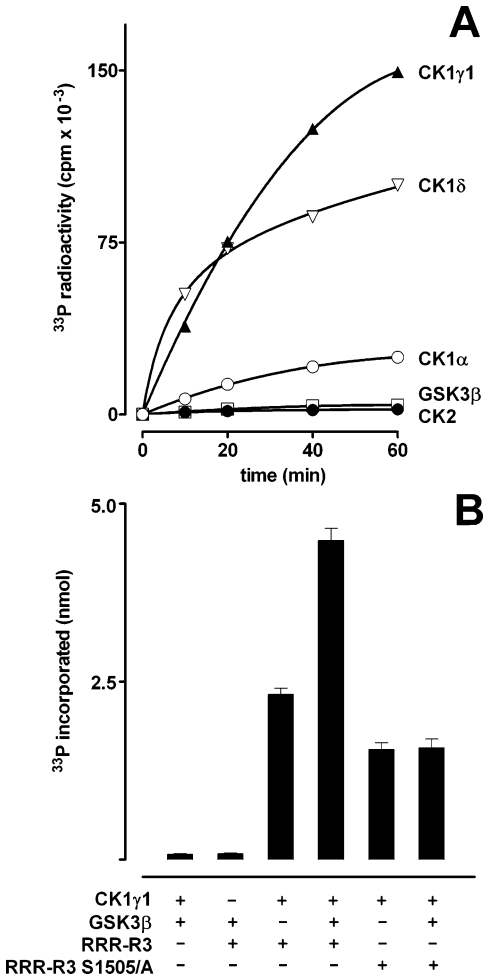

FIGURE 2: Phosphorylation of R3 APC peptides by various protein kinases. Cooperative phosphorylation by GSK3 is abrogated by S1505 to Ala substitution. Phosphorylation was performed in solution taking advantage of the non-immobilized peptides with the N-terminal arginyl triplet (see Experimental Procedures and, for nomenclature, Table 1). For the time course, phosphorylation (A) conditions were those described in the Experimental Procedures (2.5 units of each protein kinase added), and the concentration of the R3 peptide was 1 mM. Results represent the means of triplicate experiments. In panel B, experimental conditions were the same except for incubation time (2 h) and amount of protein kinases (10 units). Results are shown as the means ± SEM of three determinations.

To check if phosphorylation by CK1 could prime subsequent phosphorylation by GSK3, the experimental conditions were modified to increase the stoichiometry of phosphorylation by CK1, which in the experiment of Figure 2A was

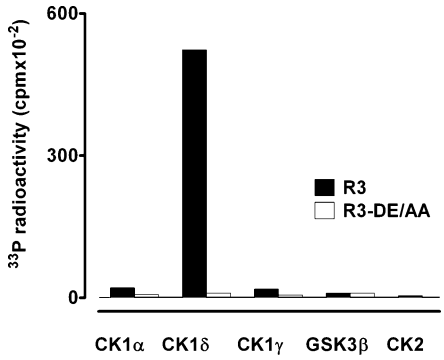

FIGURE 3: Phosphorylation of non-primed parent R3 peptide by various protein kinases: role of D1512/E1513. Phosphorylation of 0.5 mg of the parent peptide R3 either as such (DGFSASSSLSALSLDEPFIQ) or with D1512 and E1513 replaced by alanines (DGFSASSSLSALSLAAPFIQ) anchored to a solid support was performed in the presence of the indicated protein kinases (equivalent in terms of units) by 60 min incubation as detailed in the Experimental Procedures.

negligible (0.2% or less). By prolonging incubation up to 2 h and increasing the amount of CK1γ1, we could approach a phosphorylation stoichiometry around 0.05 mol of phosphate per mol of peptide. Under these conditions, a clear indication that CK1 primes further phosphorylation by GSK3 was obtained, as illustrated in Figure 2B, showing a cooperative effect of adding GSK3 together with CK1 on the R3 peptide phosphorylation. Interestingly, as also shown in Figure 2B, substitution of S1505 by alanine abolishes such a synergistic effect, while also significantly decreasing the extent of phosphorylation by CK1 alone. These data would indicate that although S1505 is not the only target of CK1, it is nevertheless essential for priming subsequent phosphorylation by GSK3.

Identification of the Residues Phosphorylated by CK1 in the APC R3 Peptide. To identify the residues phosphorylated by CK1, the R3 peptide was synthesized immobilized to resin beads to make it amenable to automated solid-phase Edman degradation (see the Experimental Procedures). In this case, the N-terminal arginyl triplet was unnecessary and was omitted (peptide R3 in Table 1). The immobilized peptide was subjected to phosphorylation by the same protein kinases previously used to phosphorylate the soluble peptide. In that case, the activities of the kinases were normalized against their specific peptide substrates (see the Experimental Procedures), and the same amount of each kinase, in term of units, was used in the phosphorylation experiments. These were run by varying the incubation time and amount of immobilized peptide, without noting substantial differences as far as the relative susceptibility of the peptide to individual kinases was concerned. The outcome of a typical experiment is illustrated by histograms in Figure 3, showing that, at variance with the soluble peptide, the immobilized peptide is phosphorylated by the δ isoform of CK1 much more readily than by the γ1 isoform (compare in relative terms Figure 3 with Figure 2A). Although a direct comparison between the data of Figures 2A and 3 is hampered by the impossibility to precisely quantify the immobilized peptide, these data would indicate that immobilization is detrimental to phosphorylation by CK1γ1 but not, or much less by, CK1δ. Note, however, that phosphorylation by CK1α and CK1γ1 is also quite significant, while posphorylation by

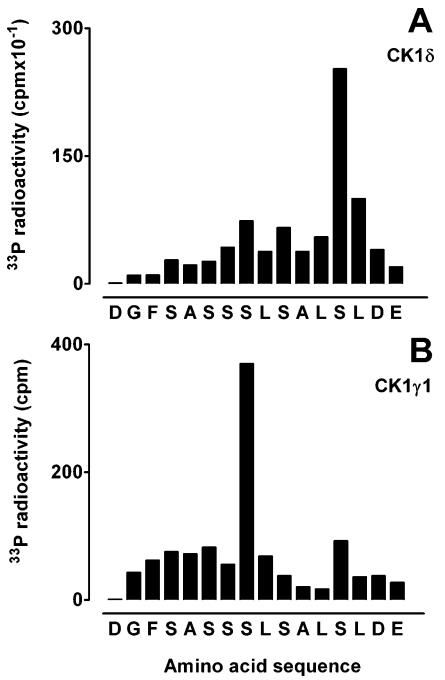

FIGURE 4: Localization of phosphoradiolabeled residues within R3 APC phosphorylated by either CK1δ (A) or CK1γ1 (B) by solid-phase Edman sequencing. The parent unprimed peptide was phosphorylated as in Figure 2 and then subjected to solid-phase Edman sequencing as detailed in the Experimental Procedures. For the experiment shown in panel A, the truncated CK1δ deleted at residue 317 was used. A similar Edman profile was obtained by using full-length CK1δ (not shown).

GSK3 and CK2, as in the case of the soluble peptide, is nearly undetectable. The latter result came as a surprise since S1510 fulfils the consensus for being a CK2 phosphoacceptor site (S^{1510}LED, the minimum consensus being S−X−X−D/E). Interestingly, as also shown in Figure 3, the two acidic residues (D1512 and E1513) that fail to promote phosphorylation by CK2 are essential for CK1 since their replacement with alanines suppresses the phosphorylation of the R3 peptide by either the δ or the α and γ isoforms of the kinase. This finding corroborates the view that CK1 mediated phosphorylation of the APC R3 repeat is dictated by non-canonical consensus, similar to the one determining the phosphorylation of β-catenin S45 (*11*). These non-canonical CK1 phosphoacceptor sites in fact crucially rely on acidic residues located at a certain distance downstream from the target residue (*10*, *11*), instead of upstream, as in the case of canonical sites. In the case of β-catenin, moreover S45 belongs to a triplet, S^{45}LS, whose central hydrophobic residue was also shown to act as an important determinant since its replacement with alanine nearly suppressed phosphorylation (*11*). Both features are indeed displayed in the R3 peptide by S1505, whose replacement by alanine actually decreases phosphorylation by CK1 and, more importantly, abrogates the cooperative effect of adding GSK3 together with CK1 (see Figure 2B).

To identify the residue(s) phosphorylated by CK1 in the R3 peptide, its phosphoradiolabeled product was subjected to automated Edman degradation. As shown in Figure 4A, radioactive phosphate incorporated by CK1δ is mainly recovered in S1510 and, to a lesser extent, in S1505. The opposite is observed using CK1γ1, whose main target is S1505 (Figure 4B). CK1α phosphorylates S1505 and S1510 to aproximately the same extent (not shown). While the

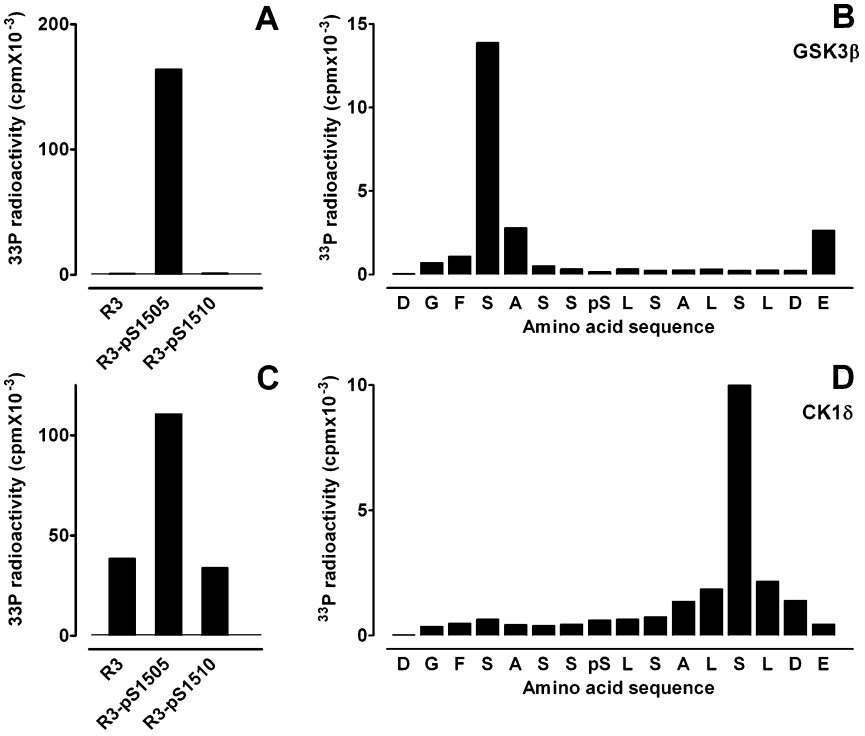

FIGURE 5: Phospho-S1505 but not phospho-S1510 primes S1501 phosphorylation by GSK3. In panel A, phosphorylation of the immobilized phosphopeptides R3 pS1505 and R3 pS1510 by GSK3β was performed and compared to that of the non-primed parent peptide. In panel B, the phosphopeptide R3 pS1505 phosphoradiolabeled by incubation with GSK3β (as in panel A) was subjected to automated Edman degradation to identify the radiolabeled residues. In panel C, phosphorylation of the immobilized phosphopeptides R3 pS1505 and R3 pS1510 by CK1δ was performed and compared to that of the non-primed parent peptide. In panel D, the phosphopeptide R3 pS1505 phosphoradiolabeled by incubation with CK1δ (as in panel C) was subjected to automated Edman degradation.

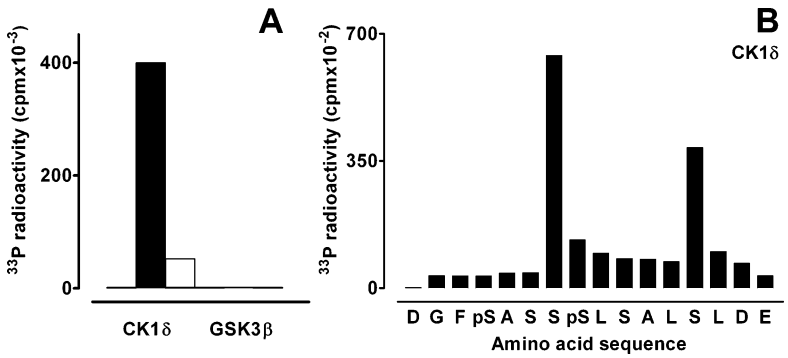

FIGURE 6: Bis-phosphorylated R3 pS1501−pS1505 is an excellent substrate for CK1, which specifically phosphorylates Ser1504. In panel A, the phosphoradiolabeling of the bis-phosphorylated R3 pS1501−pS1505 peptide by either CK1δ or GSK3β is compared to that of the parent unphosphorylated peptide. In panel B, the residues radiolabeled by CK1δ in the bisphosphorylated peptide are localized by automated Edman degradation. For details, see the Experimental Procedures.

phosphorylation of S1505 was expected, as it conforms to the typical features of non-canonical CK1 sites, the phosphorylation of S1510 was not since in this case the SLS motif is partially altered (S^{1510}LD), and the C-terminal acidic residues that are held as hallmarks of non-canonical CK1 sites are closer to the target residue than they are in NF-AT4 and in β-catenin. Note in this respect that phosphorylation of S1510, besides S1505, accounts for the failure of the S1505 to Ala mutation to suppress phosphorylation by CK1 (see Figure 2B).

Phospho-S1505 Primes S1501 Phosphoryaltion by GSK3. Once it was established that CK1 isoforms are variably able to phosphorylate the R3 peptide at both S1505 and S1510, we wanted to see if these phosphoresidues were able to prime the intervention of GSK3. To this aim, we synthesized two phosphopeptides with either S1505 or 1510 replaced by phosphoserine, and we tested their ability to serve as substrates for GSK3β. As shown in Figure 5A, the R3 pS1510 peptide, similar to the parent peptide R3, was unaffected by GSK3, proving that phosphorylation of S1510 is not priming the phosphorylation of any of the five seryl residues upstream. In contrast, the pS1505 peptide was readily phosphorylated. Automated Edman degradation provided the demonstration that the whole radioactivity incorporated by GSK3 is accounted for by S1501 (Figure 5B), whose phosphorylation by GSK3 is indeed expected to be primed by a phosphoresidue at position $n + 4$ as it is phospho-S1505.

We also wanted to check if phospho-S1505 and phospho-S1510 could prime the phosphorylation of additional seryl residues by CK1. To this purpose, the phosphorylation of the two phosphopeptides R3 pS1510 and R3 pS1505 by CK1 was compared to that of the parent peptide (R3). As shown in Figure 5C, the phosphopeptide R3 pS1505 but not R3 pS1510 is phosphorylated by CK1δ more efficiently than the parent peptide, consistent with a priming effect of phospho-S1505 but not of phospho-S1510. The radiolabeled phosphate incorporated by CK1δ into R3 pS1505 was entirely recovered into S1510 (Figure 5D), denoting a hierarchical phosphorylation specified by a phosphoresidue at position $n - 5$. In summary, the data on display in Figure 5 show that while primary phosphorylation of S1510 by CK1 has no priming effects, CK1 phosphorylation of S1505 primes phosphorylation of both S1510 by CK1 and, more importantly, of S1501 by GSK3.

Phospho-S1501 Triggers the Processive Phosphorylation of S1504 and S1507 by CK1. Phospho-S1501, generated by the priming of GSK3 by phospho-S1505, should be in the proper position for the sequential priming of CK1 to phosphorylate S1504 and subsequently S1507. To validate these predictions, we first synthesized a bis-phosphorylated peptide with both S1505 and S1501 replaced by phosphoserines, and we tested it as a substrate for either GSK3 or CK1δ. As shown in Figure 6A, the bis-phosphorylated peptide is unaffected by GSK3 while readily phosphorylated by CK1. The main phosphorylated residue was found to be S1504 with some radioactivity incorporated in S1510 as well (Figure 6B), which, however, also can be phosphorylated in the parent peptide (see Figure 4).

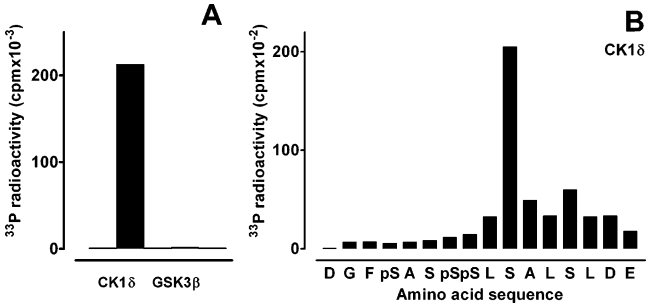

FIGURE 7: R3 pre-phosphorylated at serines 1501, 1504, and 1505 is further phosphorylated by CK1 at Ser1507. In panel A, the phosphoradiolabeling of the tris-phosphopetide R3 pS1501−pS1504−pS1505 by either CK1δ or GSK3β is shown. In panel B, the residues radiolabeled by CK1δ are localized by automated Edman degradation. For details, see the Experimental Procedures.

Next, we synthesized the triply phosphorylated peptide with S1504, besides S1501 and S1505, replaced by phosphoserine, and we showed that again it is an excellent substrate of CK1δ, while unaffected by GSK3 (Figure 7A), with radioactivity almost exclusively incorporated into S1507 (Figure 7B). In this series of experiments, CK1δ could be efficiently replaced by the isoforms γ and α with substantially identical results (not shown).

Phospho-S1507 Primes the Phosphorylation of S1510 and S1503 by CK1δ and GSK3, Respectively. In summary, at this stage, we were able to account for the phosphorylation of five out of six Ser residues in the R3 peptide: S1505 and S1510 primarily phosphorylated by CK1, S1501 phosphorylated by GSK3 as a consequence of S1505 phosphorylation, and S1504 and S1507 as the result of a hierarchical process primed by GSK3 and catalyzed by CK1 isoforms. The only residue left is S1503, whose position is such to make predictable its hierarchical phosphorylation by GSK3 once

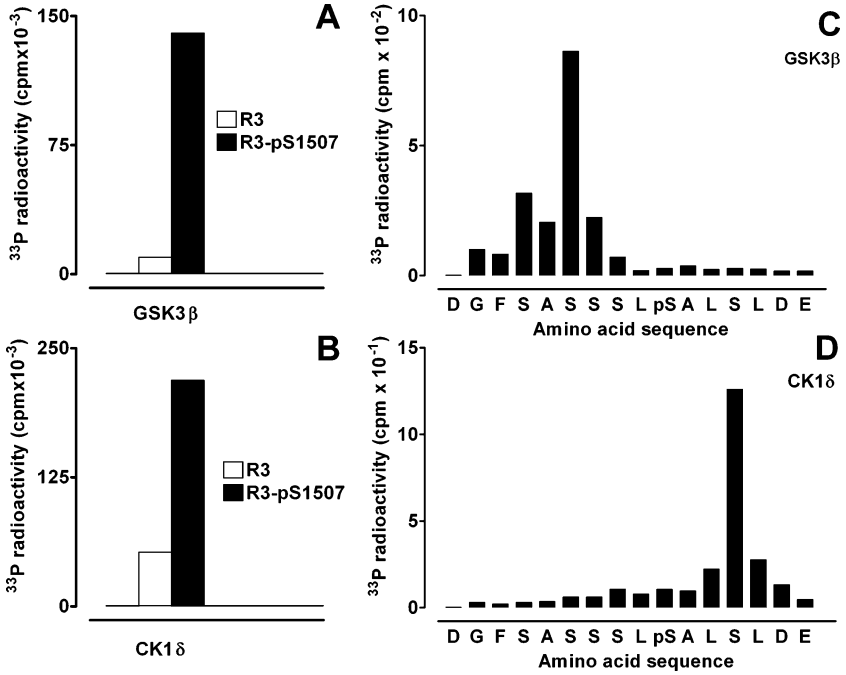

FIGURE 8: Phospho-S1507 primes the phosphorylation of S1510 and S1503. Phosphoradiolabeling of phosphopeptide R3 pS1507 by either GSK3β (A) or CK1δ (B) is compared to that of the parent peptide. In panels C and D, the residues radiolabeled by incubation of the phosphopetide with GSK3β or CK1δ, respectively, are localized by automated Edman degradation. For details, see the Experimental Procedures.

S1507 has been phosphorylated by CK1. Note that once phosphorylated, S1507 also has the potential to prime the phosphorylation of S1510 by CK1, although S1510 has been shown previously to be phosphorylated by CK1 through two additional routes: non-primed phosphorylation (Figure 4A) and phosphorylation primed by phospho-S1505 (Figure 5D).

To validate our predictions, a phosphorylated derivative of the R3 peptide with S1507 replaced by phosphoserine was synthesized and its phosphorylation by either GSK3 or CK1δ compared to that of the unphosphorylated peptide. As shown in Figure 8A,B, the phosphopeptide is phosphorylated more readily than the parent peptide by both kinases, consistent with a priming effect of phospho-S1507 in both cases. As shown in Figure 8C,D, however, the residues whose phosphorylation is primed by phospho-S1507 are different depending on the phosphorylating kinase: S1503 is the main target of GSK3, while CK1 exclusively phosphorylates S1510. The data reported were obtained with CK1δ, but the other CK1 isoforms behaved identically in this respect (not shown).

DISCUSSION

Multiple phosphorylation of the APC protein at its third 20-mer repeat (R3) is required for high affinity association with β-catenin (*24*), which in turn is a crucial step to accelerate the displacement of phosphorylated β-catenin from the destruction box and its commitment to ubiquitination and degradation (*25*, *26*) through a mechanism that still is a matter of conjecture. Interestingly, the same protein kinases, CK1 and GSK3, appear to be committed to the synergistic phosphorylation of both β-catenin and APC, although the CK1 isoforms implicated may be different: CK1α appears to be responsible for β-catenin phosphorylation (*27*, *28*), whereas the isoforms responsible for APC phosphorylation in vivo are unknown, but in vitro APC can be phosphorylated by CK1δ and by the closely related ϵ isoform (*29*). Likewise, by using a concentrated mixture of CK1ϵ and GSK3β, Ha et al. (*15*) were able to incorporate up to six phosphates into a peptide reproducing the R3 APC region and to solve the structure of the complex between this phosphopeptide and the armadillo repeat region of β-catenin, disclosing the role of individual phosphoserines in tightening the interactions between the two proteins (*15*). By analogy with non-canonical sequences phosphorylated by CK1 in β-catenin (*11*), these authors speculated that also in the case of R3 APC, CK1 was acting as the priming kinase by first hitting Ser1505 that displays the SLS motif five to ten residues upstream from acidic residues also typical of the non-canonical CK1 sites in NF-AT4 and β-catenin. Phosphorylation of S1505 would generate a consensus for subsequent phosphorylation of S1501 by GSK3, triggering a cascade of hierarchical phosphorylation steps leading to the complete phosphorylation of R3.

The data presented here, obtained by using a series of R3 APC peptides and phosphopeptides designed to dissect the individual steps of this complicated reaction orderly catalyzed by CK1 and GSK3, and schematically sumarized in Figure 9, substantially confirm the mutual priming hypothesis of Ha et al. (*15*). In particular, it is clear that the original priming kinase must be CK1, which is able to phosphorylate the unphosphorylated R3 APC stretch, while GSK3 fails to do that. It is also clear that one of the residues targeted by CK1δ (and the main one affected by CK1γ), S1505, once phosphorylated, primes the phosphorylation of R3 APC by GSK3 at the expected S1501 residue and that now the bis-phosphorylated peptide (R3 pS1501−1505) becomes an excellent substrate for CK1, able to carry out the stepwise phosphorylation of S1504 (primed by phospho-S1501), S1507 (primed by phospho-S1504), and S1510 (primed by phospho-S1507). We also show that once phosphorylated (by CK1),

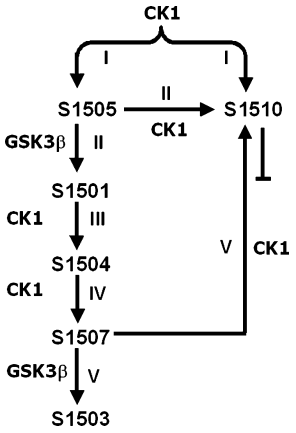

FIGURE 9: Schematic representation of R3 APC multistep phosphorylation by the concerted action of CK1 and GSK3. Phosphorylation is initiated by CK1, which phosphorylates the non-canonical sites S1505 and, especially in the case of the CK1δ isoform, S1510 as well. Phospho-S1505 primes the second phosphorylation step, catalyzed by GSK3, which targets S1501. Phosphorylation of S1501 primes the third step consisting of the canonical phosphorylation of S1504 by CK1, which in turn primes the phosphorylation of S1507 by the same kinase (fourth step). Phospho-S1507 triggers the last step (fifth), leading to the phosphorylation of both S1503 and S1510 by either GSK3 or CK1, respectively. The CK1 isoforms δ, γ, and α are interchangeable at all steps, although unprimed phosphorylation of S1510 is optimally performed by CK1δ alone (see Figure 4). S1510 phosphorylation also can be primed by phosphorylation of either S1507 or S1505. By contrast, its phosphorylation is unproductive as it does not prime further intervention of either GSK3 or CK1.

S1507 not only primes the phosphorylation of S1510 (by CK1) but also that of S1504 by GSK3, thus fully accounting for the stoichiometry of six phosphates incorporated into the R3 peptide by exhaustive incubation with CK1 and GSK3 in the presence of a high ATP concentration (1 mM) (*15*).

Another point that has been assessed by our data concerns the non-canonical mode by which CK1 recognizes unphosphorylated R3 APC: while in fact all subsequent reactions catalyzed by CK1 on variably phosphorylated R3 APC are dictated by the canonical consensus generated by a phosphorylated side chain upstream, none of the seryl residues present in unphosphorylated R3 APC display a canonical consensus, either of this type or relying on acidic side chain(s) that sometimes can efficiently surrogate the phosphorylated residue (*2*−*4*). Rather, S1505 typically displays the SLS motif and the presence of two acidic residues (D1512 and E1513) at positions $n + 7$ and $n + 8$, which are reminiscent of non-canonical sequences phosphorylated by CK1 in β-catenin (*11*) and NF-AT4 (*10*). As in the cases of β-catenin and NF-AT4, moreover, these C-terminally located acidic residues act as important specificity determinants as their replacement by alanine fully abrogates phosphorylation by CK1 (see Figure 3). This also means that the whole R3 APC phosphorylation would be compromised by this substitution given the crucial priming role of phospho-S1505 for the subsequent phosphorylation of the other residues. Interestingly, the number and position of these C-terminal acidic residues are variable throughout the seven APC 20-amino acid repeats (see Figure 1), suggesting that the susceptibility to phosphorylation of individual repeats may also be quite different despite the conservation (or conservative replacement) of most of the seryl residues in them. On the other hand, it may be worth noting that our mechanistic approach clearly demonstrates the feasibility of the hierarchical phosphorylation by GSK3 of S1503 (see Figure 8), which is unique to R3, being replaced in all the other repeats by non-phosphorylatable residues. Note in this respect that R3 is the one responsible for the tightest binding of APC to β-catenin (*24*).

An unexpected outcome of our study was the unprimed phosphorylation of S1510 by CK1δ in the R3 parent peptide. This residue is one of those whose phosphorylation is critical for association with β-catenin (*15*), and its hierarchical phosphorylation can be primed by phospho-S1507 (see Figure 8B) and also by phospho-S1505 (see Figure 5D). Nevertheless, it is also the residue that is more efficiently phosphorylated by CK1δ in unprimed R3 APC, even better than S1505 itself (see Figure 4A), although its phosphorylation, unlike that of S1505, fails to prime the phosphorylation of the R3 peptide by either GSK3 or CK1 (see Figure 5). Inclusion of S1510 among non-canonical sequences phosphorylated by CK1 comes as a surprise first because it only partially fulfils the SLS motif and second because the acidic determinants that in the previously known sequences are generally located rather far away on the C-terminal side, here are very close, at positions $n + 2/n + 3$. This should make S1510 a good target for CK2, whose consensus is S/TX−XE/D, but, intriguingly, CK2 is completely unable to phosphorylate it (see Figures 2 and 3). We have to assume therefore that the structural features underlying non-canonical CK1 substrate recognition need to be re-evaluated at least for the CK1δ isoform. In the case of S1510, it is clear that the two acidic residues close to its C-terminus act as specificity determinants since their replacement with alanines abrogates its phosphorylation (see Figure 3). It is not clear, however, as to which the other structural features that make S1510 prone to CK1δ phosphorylation are, while unsuitable to CK2 mediated phosphorylation, also considering that many CK2 peptide substrates whose sequence is very similar to the one encompassing APC S1510 are totally unaffected by CK1. We have recently described that the efficiency in the recognition of the non-canonical motif that accounts for the S45 phosphorylation of β-catenin by CK1α depends on the presence of the first armadillo repeat in that protein that dramatically decreases the K_m value of the reaction (*13*). Since APC also contains armadillo repeats, it is likely that one or more of these repeat structures may also increase the affinity and efficiency of phosphorylation of the peptide sequences that we have described previously. Indeed, the affinity of the unprimed R3 peptide for CK1 is very low as judged from K_m values >1 mM (data not shown).

Phosphorylation of S1510 and , in general, of R3 also reveals differences in substrate specificity among different CK1 isoforms. Remarkably, phosphorylation of the R3 parent peptide that is mainly accounted for by S1510 in the case of CK1δ, is predominantly accounted for by S1505 if the γ1 isoform of CK1 is used (see Figure 4) and is equally distributed between S1505 and 1510 by using CK1α (not shown). Given the crucial role of S1505 in priming all subsequent phosphorylation steps, one may argue that perhaps CK1γ, rather than CK1δ/ϵ, is the physiological APC kinase. On the other hand, we must take into account that if the activities of CK1 isoforms are normalized against a common canonical peptide substrate, CK1δ and CK1γ1 are equally efficient to phosphorylate the soluble R3 APC

peptide (see Figure 2). Indeed, the data of Gao et al. (*29*) would suggest that the δ/ϵ isoforms are the main agents of APC phosphorylation. Our data would indicate that CK1 catalyzed phosphorylation of S1510, implicated in the high affinity binding of β-catenin, does not need to be primed by GSK3, which is generally held as the mediator of Wnt signaling. This may raise the question as to whether CK1 might be also directly controlled by Wnt signaling, independently of GSK3. Among CK1 isoforms, CK1δ is the one for which more stringent evidence for a down-regulation through phosphorylation of its C-terminal domain has been reported (*30*). It is generally held that such a phosphorylation is an autocatalytic event, but the implication of other protein kinase(s) cannot be ruled out either, considering that none of the residues potentially implicated (*30*) displays the features of a primary CK1 site.

The cooperative, mutual priming action of CK1 and GSK-3β in achieving the hyperphosphorylation of serine-/threonine-rich regions of proteins seems to be a rather widespread phoenomenon. The axin scaffold holds these two kinases together in the cases of β-catenin and APC phosphorylations. Similar physical associations may be present in other instances where these and other enzymes work in a concerted fashion.

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