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Autophosphorylation of Carboxy-Terminal Residues Inhibits the Activity of Protein Kinase CK1α

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ABSTRACT

CK1 constitutes a protein kinase subfamily that is involved in many important physiological processes. However, there is limited knowledge about mechanisms that regulate their activity. Isoforms CK1δ and CK1ε were previously shown to autophosphorylate carboxy-terminal sites, a process which effectively inhibits their catalytic activity. Mass spectrometry of CK1α and splice variant CK1αL has identified the autophosphorylation of the last four carboxyl-end serines and threonines and also for CK1αS, the same four residues plus threonine-327 and serine-332 of the S insert. Autophosphorylation occurs while the recombinant proteins are expressed in *Escherichia coli*. Mutation of four carboxy-terminal phosphorylation sites of CK1α to alanine demonstrates that these residues are the principal but not unique sites of autophosphorylation. Treatment of autophosphorylated CK1α and CK1αS with λ phosphatase causes an activation of 80–100% and 300%, respectively. Similar treatment fails to stimulate the CK1α mutants lacking autophosphorylation sites. Incubation of dephosphorylated enzymes with ATP to allow renewed autophosphorylation causes significant inhibition of CK1α and CK1αS. The substrate for these studies was a synthetic canonical peptide for CK1 (RRKDLHDDEEDEAMS*ITA). The stimulation of activity seen upon dephosphorylation of CK1α and CK1αS was also observed using the known CK1 protein substrates DARPP-32, β-catenin, and CK2β, which have different CK1 recognition sequences. Autophosphorylation effects on CK1α activity are not due to changes in Km_{app} for ATP or for peptide substrate but rather to the catalytic efficiency per pmol of enzyme. This work demonstrates that CK1α and its splice variants can be regulated by their autophosphorylation status. J. Cell. Biochem. 106: 399–408, 2009.

KEY WORDS: CASEIN KINASE 1; PHOSPHATASE ACTIVATION; KINASE REGULATION; PSEUDOSUBSTRATE INHIBITION; CK2β PHOSPHORYLATION

Protein kinase CK1 is a subfamily of serine/threonine kinases in the large kinome family of protein kinases [Manning et al., 2002]. There are six genes that codify members of this subfamily in vertebrates: CK1α, CK1γ_1, CK1γ_2, CK1γ_3, CK1δ, and CK1ε [Vielhaber and Virshup, 2001; Knippschild et al., 2005]. In addition, alternate splicing generates several variants of some of these genes. For instance, CK1α mRNA processing can generate the insertion of two segments, denominated S (short, 12 amino acids) and L (long, 28 amino acids) in the CK1α protein, producing the variants CK1α, CK1αS, CK1αL, and CK1αLS, all of which have been identified in many species [Fu et al., 2001; Burzio et al., 2002] and that are differentially expressed during embryogenesis [Albornoz et al., 2007].

CK1 subfamily members are ubiquitous in eukaryotes and are known to phosphorylate a large number of cellular proteins [Zhu et al., 1998; Vielhaber et al., 2000; Knippschild et al., 2005]. Two modes of substrate sequence recognition have been described for CK1. The canonical sequence recognition mode requires that position −3 (or −4) from the target serine or threonine be occupied by a phosphorylated serine or threonine as expressed by the model sequence pT/pS X X S*/T*, where pT and pS are phosphothreonine and phosphoserine, X is any other residue and S* and T* are the target residues for CK1. Alternatively in this canonical mode, the same −3 position might be occupied by one or several acidic residues [(D/E)n X X S*/T*; Marin et al., 1994; Pulgar et al., 1999]. Some other proteins are recognized in a non-canonical mode that is

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characterized by an SLS sequence followed downstream by an acidic cluster [S*LS(X)n(D/E)n] [Marin et al., 2003]. Mutational experiments indicate the different regions of the enzyme that participate in the recognition of these two types of substrate sequences [Bustos et al., 2005].

CK1 phosphorylation has been involved in a number of key physiological regulatory processes such as Wnt signaling [Liu et al., 2002a], nuclear import of NF-AT factors [Zhu et al., 1998], circadian rhythms [Ebisawa, 2007], and the development of neurodegenerative diseases [Kannanayakal et al., 2008]. This involvement raises the question as to the existence of mechanisms that may regulate the activity of CK1 itself. Earlier studies have presented evidence regarding the inhibitory effect of autophosphorylation of carboxy-terminal residues of CK1δ and CK1ε [Graves and Roach, 1995; Cegielska et al., 1998; Gietzen and Virshup, 1999]. These CK1 isoforms have longer carboxyl extensions than the CK1α and CK1γ isoforms [Knippschild et al., 2005]. Although CK1α also has been known to autophosphorylate, detailed studies concerning the sites that are autophosphorylated have not been reported and also no evidence has been presented in relation to the effects this autophosphorylation might have on the catalytic activity of this isoform.

The present work carried out with CK1α, CK1αS, and CK1αL demonstrates that all of these variants contain common sites of autophosphorylation in their carboxyl end and that CK1αS has two additional autophosphorylation sites in the S segment. Additionally, the results obtained demonstrate that autophosphorylation causes a significant inhibition of the catalytic activity of these CK1α variants.

MATERIALS AND METHODS

Restriction enzymes and Taq DNA polymerase were purchased from Fermentas Inc. (Glen Burnie, MD). Sodium orthovanadate, ATP, protease inhibitors ABESF, benzamidine, and aproteinin were obtained from Sigma Chemical Co. (St Louis, MO). Pepstatin A, leupeptin, lysozyme, and imidazole were from US Biological Inc. (Swamscotte, MA). [^{32}P-γ] ATP was from Perkin Elmer (Waltham, MA). Nickel agarose resin (Ni^{++}-NTA His-Bind Resin) was purchased from Novagen. Recombinant lambda protein phosphatase (PPase) and the synthetic peptide CK1 specific substrate RRKDLHDDEEDEAMSITA were purchased from Calbiochem. Novagen and Calbiochem are brands of EMD Chemicals Inc., an affiliate of Merck KGaA (Darmstadt, Germany). The anti-His monoclonal antibody was from Clontech (Mountain View, CA). The β-catenin derived synthetic peptide was synthesized and donated by Dr. Oriano Marin, University of Padova, Italy:

RRRGATTTAPSLSGKGNPEDEDVDTNQVLYEWEQGFSQSFTQDQVA Oligonucleotides were synthesized at the Oligonucletide Synthesis and Sequencing Facility, Faculty of Medicine of University of Chile:

P1: 5′ TATATACCATGGAGTTTATAGTCGG 3′
P2: 5′ TTAAATCCATGGCCAGCAGCAGCGGCT 3′
P3: 5′ TTTTAACTCGAGGAAACCTGTGGGGG 3′
P4: 5′ AAAAAACTCGAGGAAACCTGCGGGGGCTTGTGCCTG 3′
P5: 5′ TATATACTCGAGCAGCATGGTCC 3′
P6: 5′ GCAAGCGGCCGCCGCAGG 3′

SUBCLONING OF CK1α, CK1αS AND CONSTRUCTIONS OF CK1α MUTANTS

The clones for CK1α, CK1αS, and CK1αL from *Danio rerio* (zebra fish), in pT7.7H6 vector [Burzio et al., 2002] were subcloned in pET21d+ vector (giving a carboxy-terminal His6-tag) by PCR using the clones in pT7.7H6 as template. For the amplification of CK1α and CK1αL, P2 and P3 oligonucleotides were used as forward and reverse, respectively. For CK1αS amplification, oligonucleotides P2 and P5 were used as forward and reverse, respectively.

Substitution mutant $CK1\alpha^{SSTT/A}$ was prepared in two steps. The cDNA of CK1α in pT7.7H6 was used as template to generate the mutant $CK1\alpha^{T321A,T323A}$ with P2 as forward and P4 as reverse primer. In the second step, the $CK1\alpha^{T321A,T323A}$ cDNA was used as template to create a megaprimer at the carboxy end with P6 as forward and P4 as reverse primers, and this megaprimer was later used as reverse oligonucleotide with P2 to create the tetramutant $CK1\alpha^{SSTT/A}$.

Deletion mutant $CK1\alpha^{\Delta 1-9}$ was generated by PCR using cDNA from $CK1\alpha^{WT}$ as template with oligonucleotide P1 as forward and oligonucleotide P3 as reverse.

Deletion and substitution mutant $CK1\alpha^{\Delta 1-9,SSTT/A}$ was generated using cDNA from $CK1\alpha^{SSTT/A}$ as template with P1 as forward and P4 as reverse primers.

All constructions were cloned or subcloned in pET21d+ in *Nde*I and *Xho*I restriction sites. All recombinant constructs were confirmed by complete sequencing.

The clone from β-cantenin for *D. rerio* and CK2β from *Xenopus laevis* have been described by Marin et al. [2003] and Jedlicki et al. [1992], respectively. CK2β was subcloned in pQE80-L to provide a His-tag instead of the GST fusion protein.

PROTEIN EXPRESSION AND PURIFICATION

The kinases were expressed in *E. coli* BL 21 (DE 3) pLysS cells. Cells were grown at 37°C to an absorbance at 600 nm of 1.0. Expression of His-tagged proteins was induced overnight at 20°C in the presence of 0.1 mM isopropyl-β-D-thiogalactoside (IPTG). Then cells were pelleted at 3,000*g* for 20 min at 4°C and were resuspended in buffer A (50 mM Hepes, pH 8.0, 500 mM NaCl, and 20% glycerol) containing 1% TritonX-100, 1 mM aminoethylbenzene sulfonyl fluoride, and 2 μg/ml each of leupeptine, aprotinin, pepstatin A, and antipain. Lysozyme (1 mg/ml) was added and cells were lysed for 20 min on ice. Lysates were sonicated and centrifuged at 39,000*g* for 30 min at 4°C. Supernatants were loaded onto nickel columns (Ni^{++}-NTA Purification System, Novagen) and affinity chromatography was performed at 4°C and according to the manufacturer's instructions. Eluted proteins were analyzed using 10% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE), and specifically identified in Western blots using an anti-His monoclonal antibody and densitometrically quantified by means of the UN-SCAN-IT program and from a standard protein concentration curve also containing a His-tag.

AUTOPHOSPHORYLATION ASSAY

For this assay 1–2 pmol of each recombinant enzyme were incubated in kinase buffer containing 25 mM HEPES, pH 7.5, 100 mM NaCl, 10 mM $MgCl_2$, and 50 μM [^{32}P-γ] ATP (4,000–8,000 cpm/pmol). Incubation was for 30 min at 30°C and stopped by adding 5× concentrated Laemmli buffer. The radioactive proteins were resolved on 10% SDS–PAGE. The CK1 proteins were detected by Western blots (WB) using a monoclonal anti-His as primary antibody and anti-IgG of mouse conjugated to alkaline phosphatase as secondary antibody. The incorporation of ^{32}P-phosphate into proteins was analyzed by autoradiography using X-ray films or using a PhophorImager and Image-Quant software. The Western blots and the radioactive bands were quantified by densitometry using the UN-SCAN-IT program.

DEPHOSPHORYLATION ASSAY OF RECOMBINANT PROTEINS WITH PHAGE PROTEIN PHOSPHATASE

Aliquots of 10 pmol of recombinant enzymes were incubated in 100 μl solution containing 50 mM HEPES pH 7.8, 5 mM DTT, and 2 mM $MnCl_2$ in the presence of 500 U of λ phosphatase for 30 min at 30°C. The reaction was stopped by the addition of 150 mM sodium orthovanadate (Na_3VO_4). Control proteins were incubated in the same way but phosphatase was replaced by water. Aliquots of 10 μl of these preparations were then used immediately for further study.

PROTEIN KINASE ASSAY USING SYNTHETIC CK1 SPECIFIC PEPTIDE OR RECOMBINANT PROTEIN SUBSTRATES

For this assay, 0.5–1.0 pmol of bacterially expressed protein kinase preparations were incubated for 10 min in a 30 μl volume containing 50 mM HEPES pH 7.5, 100 mM NaCl, 10 mM $MgCl_2$, 250 μM specific CK1 peptide substrate (RRKDLHDDEEDEAMSITA), or 1–2 pmol of protein substrate and 50 μM [^{32}P-γ] ATP (2,000–4,000 cpm/pmol). The reaction was stopped by absorption on phosphocellulose paper (Whatman P81), which was washed for 30 min with 3× 75 mM phosphoric acid, dried, and counted in a liquid scintillation counter.

ENZYMATIC ACTIVITY ASSAY OF AUTOPHOSPHORYLATED RECOMBINANT PROTEINS

The re-autophosphorylation of CK1 enzymes was performed using 0.5–1 pmol of previously dephosphorylated protein incubated in 15 μl of kinase buffer in presence of 50 μM non-radioactive ATP for 30 min at 30°C. The activity of these enzymes was assayed in 30 μl of reaction buffer with the final ATP concentration of 50 μM (2,000–4,000 cpm/pmol). The reaction was started with the addition of the 15 μl of pre-incubated proteins in the condition described in each case (+/−ATP). The reaction was stopped by absorption on phosphocellulose paper as described above.

DETERMINATION OF AUTOPHOSPHORYLATION SITES BY MASS SPECTROMETRY

Approximately 20–30 pmol of CK1α, CK1αL, and CK1αS recombinant enzymes, cloned in pT7.7-H6 vector, were submitted to an autophosphorylation assay in 30 ml of reaction buffer for 30 min in the presence of 200 μM of non-radioactive ATP. The samples were run in 10% SDS–PAGE and the bands corresponding to the autophosphorylated enzymes were excised from the gels and analyzed for autophosphorylated residues by mass spectrometry (Taplin Mass Spectrometry Facility, Harvard Medical School). In all of the six samples of CK1α and its variants that were analyzed by mass spectrometry the peptides corresponding to the amino terminal sequence (MASSSGS-) were not present in the proteolytic products and therefore the phosphorylation state of these fragments was not determined in any of the samples. A deletion mutant lacking this nine amino acid end-terminal fragment was prepared in order to evaluate the possible phosphorylation of this segment.

RESULTS

DELAYED MOBILITY OF RECOMBINANT VARIANTS OF CK1α EXPRESSED IN *E. COLI*

When either CK1α or CK1αS are expressed in *E. coli* and the proteins are analyzed using Western blots and antibody against the $(His)_6$ tag of the recombinant proteins, it is possible to observe two bands (Fig. 1A, B, lane 1). The lower bands correspond to the estimated molecular mass of the protein. When these preparations of CK1α and CK1αS are treated with λ phosphatase the slower migrating band is eliminated (Fig. 1A, B, lane 4). In lanes 1 and 3, both enzyme variants were incubated with [^{32}P-γ] ATP under conditions to allow autophosphorylation. In the Western blots, it can be seen that in lane 3 a slower but intermediate moving band appears that is not present in lane 4. That is, neither in the case of CK1α or CK1αS were those bands of retarded mobility as slow moving as the ones present in the original protein obtained from the bacteria (lanes 1). In parts C and D of Figure 1 we see the corresponding autoradiographs of these preparations. With CK1α, the labeling in both lanes 1 and 3 is of similar intensity, showing that even without phosphatase treatment, there is very active autophosphorylation. In the case of CK1αS (Fig. 1D) the intensity of the autophosphorylation with the preparation not pre-treated with phosphatase incorporated less phosphate (lane 1) than the previously dephosphorylated enzyme (lane 3).

Essentially similar results were obtained with CK1αL (not shown). It may be concluded that autophosphorylation in the bacteria or in vitro is responsible for the appearance of retarded species of the protein in SDS–PAGE.

THE TIME COURSE OF AUTOPHOSPHORYLATION

The autophosphorylation as a function of time for a previously dephosphorylated CK1α is shown in Figure 2. The results from densitometric measurements obtained from the re-phosphorylation reaction during 20 min are given. These measurements are presented as the ratio of the intensities obtained for ^{32}P-phosphate incorporation and protein levels (WB) versus time. Maximum autophosphorylation under these conditions is achieved after 8 min of incubation.

THE PRESENCE OF SUBSTRATE INHIBITS AUTOPHOSPHORYLATION

Figure 3 shows the effect of addition of a specific CK1 peptide substrate on the autophosphorylation of CK1α and CK1αS, an experiment similar to that shown in Figure 1. In this case, the

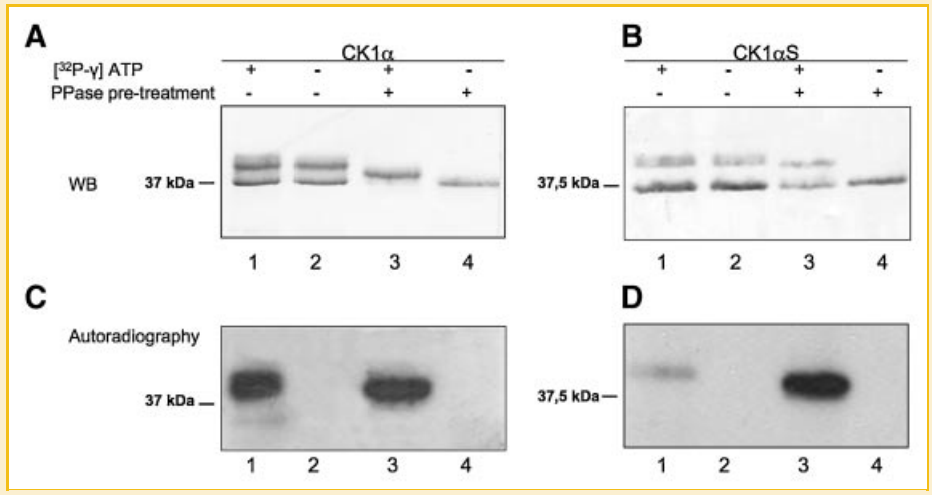

Fig. 1. Autophosphorylation and the effect of protein phosphatase pre-treatment. The recombinant enzymes His-CK1α (A, C) and His-CK1αS (B, D) were pre-treated or not treated with λ phosphatase (PPase) and then submitted to autophosphorylation in the presence of [γ-^{32}P] ATP as described in Materials and Methods Section. In A, B shown are the samples analyzed by Western blot using an anti-His antibody. In C, D shown are the autoradiographs of Western blot membranes showing the ^{32}P-phosphate incorporation. These results are representative of at least four separate experiments.

enzymes not pre-treated with λ phosphatase showed negligible autophosphorylation (C, D; lanes 1). However, with the phosphatase treated preparations of both CK1α and CK1αS, it can be observed in the Western blot (Fig. 3A, B) and in the autoradiography (C, D) that the presence of the peptide substrate greatly reduces the mobility shift (lanes 4) and the autophosphorylation as compared to that obtained in the absence of substrate (lanes 3).

DETERMINATION OF AUTOPHOSPHORYLATION SITES

Mass spectrometry analysis was carried out on the CK1α proteins that were expressed in bacteria and subsequently incubated with non-radioactive ATP for 30 min under autophosphorylation conditions.

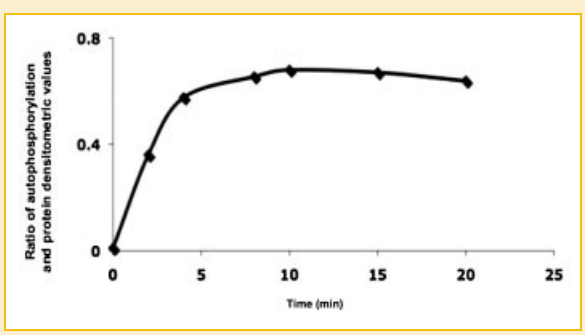

Fig. 2. Time course of the autophosphorylation of CK1α pre-treated with protein phosphatase. Recombinant His-CK1α was dephosphorylated with λ phosphatase for 40 min at 30°C. Aliquots were incubated with 50 μM [γ-^{32}P] ATP for 0–20 min and analyzed by Western blot using an anti-His antibody. The Western blot membrane was used for autoradiography. The ratio of the densitometric values obtained from the autoradiography and Western blots are presented as a function of reaction time. These results are representative of two separate experiments.

Figure 4A shows a schematic representation of the sequences in the carboxy-terminal region of the three variants where phosphoamino acid residues were detected. The bold letters show the hydroxyamino acids that are phosphorylated.

The four amino acids autophosphorylated in CK1α and CK1αL are the same for both variants (note that the numbering varies due to the insertion of the 24 amino acid segment in the catalytic domain of CK1αL). In the case of CK1αS in which the S-inserted segment is very close to its carboxy-terminal end, two additional autophosphorylation sites, T327 and S332, are present.

These results indicate that all the hydroxyl residues present in the last 20 amino acids for CK1α and CK1αL, and also present in the last 32 residues of CK1αS are autophosphorylated.

These mass spectrometry determinations account for the whole peptide complement of the proteins analyzed except for the N-terminal seven amino acids peptide that is linked to the $(His)_6$ tag (see commentary in Material and Methods Section).

MUTANTS DESIGNED TO ELIMINATE AUTOPHOSPHORYLATION SITES

he amino-terminal regions of these proteins were not analyzed by mass spectrometry, possibly due to the formation of a post-translational modification of the His-tagged recombinant structures (Yan et al., 1999; Kim et al., 2001). Considering that this six amino acid sequence could serve as an autophosphorylation site since it contains serines inserted in a sequence very similar to one of the carboxyl region sequences shown to be autophosphorylated (M^1ASSSG^6- and QAAS^{311}S^{312}G-), a deletion mutant was prepared that lacks the first nine N-terminal amino acids of CK1α, designated CK1α$^{\Delta 1-9}$ (Fig. 4B). Other mutants that were generated replaced the four residues shown to be autophosphorylated in CK1α for alanine, CK1α$^{SSTT/A}$ or deleted the amino terminal nine amino acids together with the tetra mutation of hydroxyl residues of the

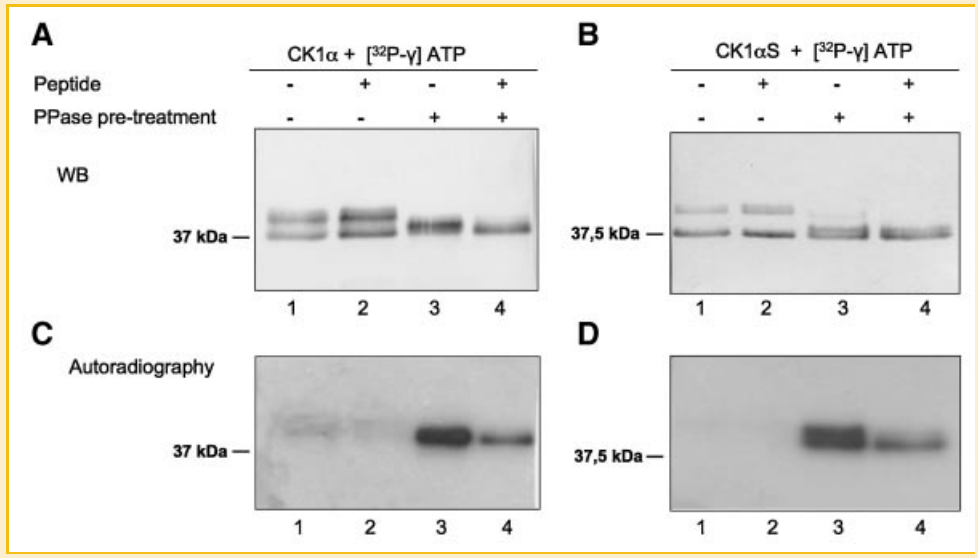

Fig. 3. Autophosphorylation in the presence or absence of substrate peptide. The recombinant enzymes His-CK1α (A, C) and His-CK1αS (B, D) were pre-treated or not with λ phosphatase and then submitted to autophosphorylation with [γ-^{32}P] ATP in the presence or absence of 250 μM CK1 specific peptide substrate as described in Materials and Methods Section. A, B: The samples were analyzed by Western blot using anti-His antibody. C, D: Autoradiography of Western blot membranes showing the ^{32}P-phosphate incorporation. These results are representative of three separate experiments.

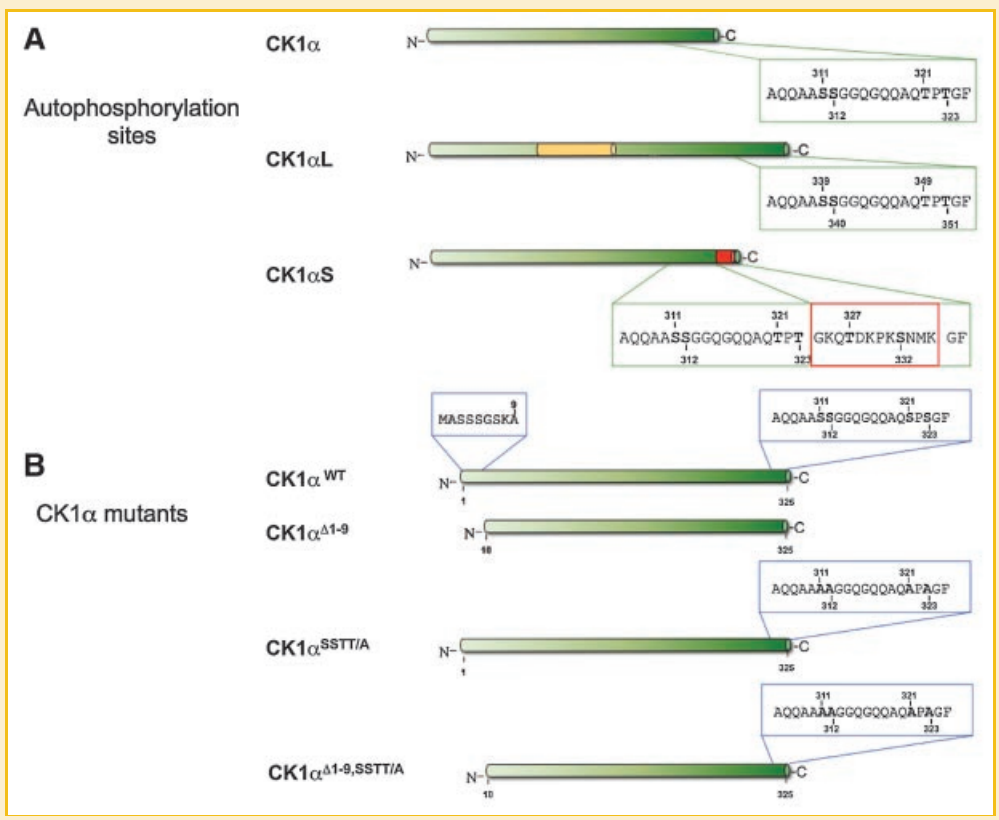

Fig. 4. Schematic description of the autophosphorylation sites of CK1α variants and of the CK1α mutants used. A: Autophosphorylated recombinant enzymes CK1α, CK1αL y CK1αS were analyzed by SDS–PAGE, excised from gels as described in Materials and Methods Section and the phosphorylated residues determined by mass spectrometry. The amino acid sequence of the carboxy-terminal regions is shown boxed and phosphorylated residues indicated in bold. The L and S inserts are shown in orange and red, respectively. B: Wild type CK1α is shown (top) with boxed regions that were subsequently mutated. CK1α$^{\Delta 1-9}$ mutant is lacking the first nine N-terminal amino acids, CK1α$^{SSTT/A}$ has serine and threonine residues in the C-terminal autophosphorylation sites changed to alanine and the mutant CK1α$^{\Delta 1-9,SSTT/A}$ is lacking the first nine N-terminal amino acids and also has the C-terminal autophosphorylation sites changed to alanine.

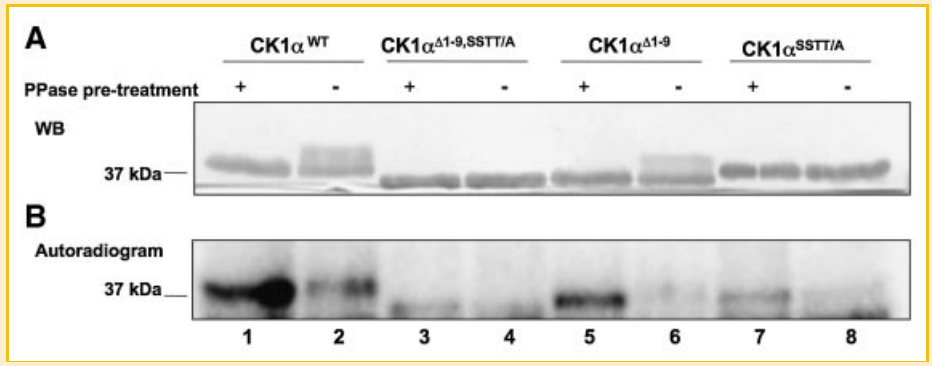

Fig. 5. The effect of protein phosphatase pre-treatment on the autophosphorylation of CK1α mutants. The CK1α^{WT} and the mutants CK1α$^{\Delta 1-9}$, CK1α$^{SSTT/A}$, CK1α$^{\Delta 1-9,SSTT/A}$ were dephosphorylated with λ phosphatase (where indicated) as given in Materials and Methods Section and then submitted to the autophosphorylation reaction in the presence of [γ-^{32}P] ATP. A: The samples were analyzed by Western blot using an anti-His antibody. B: Autoradiogram of Western blot membranes showing the ^{32}P-phosphate incorporation to the enzymes. These results are representative of five separate experiments.

carboxy-terminus to give CK1α$^{\Delta 1-9,SSTT/A}$. The mutant proteins were all catalytically active and were tested for autophosphorylation and for the effect of phosphatase pre-treatment. The results obtained are presented in Figure 5. The effect of pre-treatment with λ phosphatase on the migration of the enzymes in the Western blots is shown (A). It can be observed that only the wild type CK1α (Fig. 5A, lane 2) and the CK1α$^{\Delta 1-9}$ (lane 6) not treated with protein phosphatase show slower migrating forms of the enzymes as expected. In Figure 5B, the capacity of these forms to autophosphorylate is shown. It is again clear that the forms that contain the carboxyl end hydroxyl amino acids and that have undergone dephosphorylation by the phosphatase treatment are autophosphorylated more intensely (Fig. 5B, lanes 1 and 5). The mutants that lack the hydroxyl amino acids in their carboxyl-end show much less incorporation of radioactive phosphoryl groups, even when they have been treated with phosphatase (Fig. 5B, lanes 3 and 7). These results show that CK1α has other residues that can be autophosphorylated beside the mutated carboxyl-end residues, albeit with much lower intensity than the carboxy-end serines and threonines.

AUTOPHOSPHORYLATION INHIBITS THE CATALYTIC ACTIVITY OF CK1α AND CK1αS

Treatment of autophosphorylated CK1α, CK1α mutants, and CK1αS with λ phosphatase significantly increases the catalytic activity of both CK1α and CK1αS (Fig. 6). In the case of CK1α, the stimulation of the activity is 80–100% while in the case of CK1αS the stimulation caused by dephosphorylation is threefold. As a comparison, CK1δ was used as it is known to be inhibited by autophosphorylation [Graves and Roach, 1995] and it was fivefold stimulated by the phosphatase treatment.

In order to determine which of the autophosphorylation sites were involved in this inhibitory effect, a similar analysis, also shown in Figure 6, was carried out with mutants CK1α$^{SSTT/A}$, CK1α$^{\Delta 1-9}$, and CK1α$^{\Delta 1-9,\ SSTT/A}$. The results obtained demonstrate that the mutation of the four carboxyl terminal hydroxyl residues to alanine (CK1α$^{SSTT/A}$) or this same mutation with the deletion of the N-terminal nine amino acid residues (CK1α$^{\Delta 1-9,\ SSTT/A}$) greatly reduces or eliminates the stimulating effect of phosphatase treatment. On the other hand, the CK1α$^{\Delta 1-9}$ mutant is activated by the phosphatase treatment to about the same degree as wild type CK1α.

In order to further demonstrate the inhibitory effect of autophosphorylation, dephosphorylated CK1α and CK1αS were incubated with unlabelled ATP under conditions that allow autophosphorylation. Figure 7 shows that re-phosphorylation of these preparations causes inhibition of 40–50% of CK1α, CK1α$^{\Delta 1-9}$, and CK1αS, which is similar to the inhibition observed with CK1δ under the same treatment. On the other hand, the mutant CK1α$^{SSTT/A}$ that lacks the carboxyl end hydroxyl residues shows practically no effect of the pre-incubation with ATP.

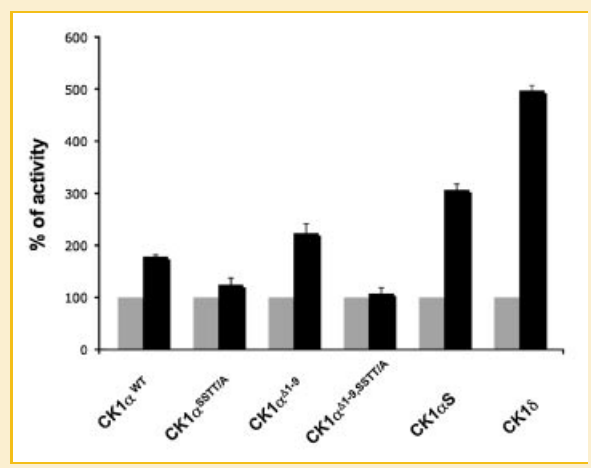

Fig. 6. Relative enzymatic activity of CK1α and mutants with or without phosphatase pre-treated enzymes. The effect of phosphatase pre-treatment on the enzymatic activity of CK1α^{WT}, CK1α$^{SSTT/A}$, CK1α$^{\Delta 1-9}$, CK1α$^{\Delta 1-9,SSTT/A}$, CK1αS, and CK1δ was evaluated for catalytic activity using [γ-^{32}P] ATP and a specific peptide as substrates (black bars). The kinase assay was performed using P-81 phosphocellulose filters as described in Materials and Methods Section and the results are expressed as percent of activity of the enzymes incubated without λ phosphatase pre-treatment (gray bars). The results shown are the mean ± SD of three experiments.

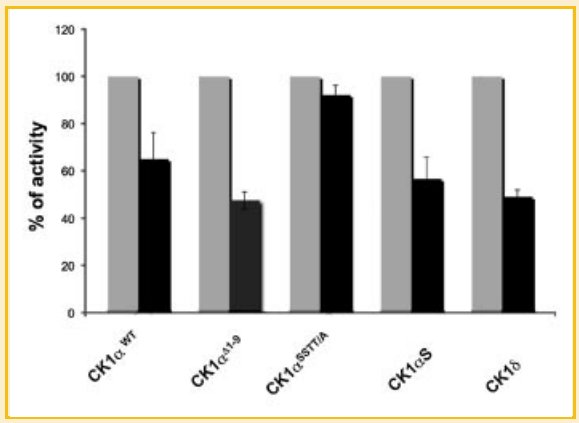

Fig. 7. Inhibition caused by autophosphorylation of dephosphorylated enzymes. The CK1α^{WT}, CK1α$^{\Delta 1-9}$, CK1α$^{SSTT/A}$, CK1αS, and CK1δ were dephosphorylated with λ phosphatase and then incubated in the presence of unlabelled ATP in the conditions of autophosphorylation. The effect of autophosphorylation on enzymatic activity was evaluated in an assay using [γ-^{32}P] ATP and a specific peptide as substrate. The results are expressed as percent of activity of the enzymes incubated without (controls, gray bars) and with unlabelled ATP (black bars). The results shown are the mean ± SD of three experiments.

THE ACTIVATION OF CK1α AND CK1αS BY DEPHOSPHORYLATION MEASURED USING NATURAL PROTEIN SUBSTRATES

Since the previous assays had been carried out using a synthetic peptide substrate, it was pertinent to test whether the activation by phosphatase treatment of CK1α and CK1αS could also be observed when known natural protein substrates were used.

Figure 8 shows the results obtained with DARPP-32, the dopamine and cyclic AMP regulated neuronal phosphoprotein [Desdouits et al., 1995], using CK1α and the mutants described in this work. It can be seen that the phosphatase treatment of the wild-type CK1α and of CK1α$^{\Delta 1-9}$, both of which retain the phosphorylatable carboxyl-end hydroxyl amino acids, causes activation in the phosphorylation of DARPP-32 (Fig. 8B, compare lanes 1 and 2 and lanes 7 and 8). On the other hand, a similar treatment does not affect the phosphorylation of DARPP-32 by the mutants that have these residues changed to alanine (compare lane 3 and 4 and lane 5 and 6).

Using CK1αS, we have also tested the effect of phosphatase treatment using three different substrate proteins that can be phosphorylated by CK1 but that have different modes of recognition by the enzyme. Figure 9 shows that CK1αS is also greatly activated by dephosphorylation when substrate DARPP-32 is used (Fig. 9C). It is interesting to note that in this case phosphorylation of DARPP-32 generates several additional radioactive bands of delayed mobility (Fig. 9B, left).

Recent studies in our laboratory have shown that CK1α can phosphorylate the regulatory beta subunit of protein kinase CK2. This CK2β subunit is phosphorylated in residues 205 and 213 (C. Pérez, unpublished work), neither of which are inserted in canonical or non-canonical CK1 recognition sequences [Bustos et al., 2005]. CK1αS pre-treatment with phosphatase also increases the phosphorylation of CK2β (Fig. 9B center and C).

Very similar results are seen with phosphorylation of β-catenin when the kinase is pre-treated with phosphatase (Fig. 9B right and C).

KINETIC STUDIES WITH CK1α, CK1αS AND CK1α MUTANTS

The apparent K_m values of CK1α^{WT} and CK1α$^{SSTT/A}$ mutant for ATP and for the CK1 specific peptide substrate were determined. The apparent K_m for ATP was 29 μM and 17 μM for CK1α and CK1α$^{SSTT/A}$, respectively. The apparent K_ms for the specific peptide substrate were 765 μM and 619 μM for CK1α and CK1α$^{SSTT/A}$. These values are not significantly different for the two enzymes. The k_{cat} for CK1α^{WT} and CK1α$^{SSTT/A}$ mutant were 178 min^{-1} and 372 min^{-1}, respectively, indicating that the catalytic efficiency (k_{cat}/K_m) is twice for the mutant lacking the carboxyl-end autophosphorylation sites. Moreover, when the activities of the various enzymes were measured and expressed as pmol of ^{32}P-phosphate incorporated per pmol of

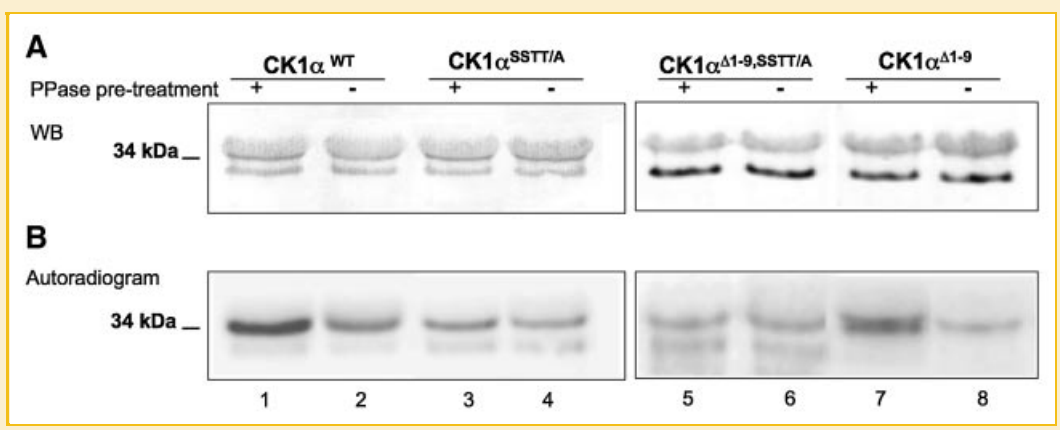

Fig. 8. Activation of DARPP-32 phosphorylation by phosphatase pre-treatment of CK1α and its mutants. The effect of phosphatase pre-treatment on the enzymatic activity of CK1α^{WT}, CK1α$^{\Delta 1-9}$, CK1α$^{SSTT/A}$, CK1α$^{\Delta 1-9,SSTT/A}$ was evaluated in an assay using [γ-^{32}P] ATP and His-tagged DARPP-32 protein (32–34 kDa) as substrates. A: The samples were analyzed by Western blot using an anti-His antibody. B: Autoradiogram of Western blot membranes showing the ^{32}P-phosphate incorporation to the substrate DARPP-32. These results are representative of at least five separate experiments.

enzyme per minute using the peptide substrate, the differences were evident as shown in Figure 10. The results obtained indicate that the enzymes that contained carboxy-terminal sites for autophosphorylation (CK1α, CK1αS, and CK1α$^{\Delta 1-9}$) are considerably less active than the mutants that lack those sites (CK1α$^{SSTT/A}$ and CK1α$^{\Delta 1-9,SSTT/A}$). In addition, the least active enzyme of those tested was CK1αS, which has the highest number of carboxy-terminal autophosphorylation sites.

DISCUSSION

This work demonstrates that CK1α and its splice variants undergo autophosphorylation and that this modification can regulate the catalytic activity of these kinases. The CK1α isoform is similar to the CK1δ and CK1ε isoforms that have previously been reported to inhibit their own activity through autophosphorylation [Graves and Roach, 1995; Cegielska et al., 1998; Gietzen and Virshup, 1999].

Through mass spectroscopy analysis, it was determined that the major sites of autophosphorylation were serines 311 and 312 and threonines 321 and 323 in CK1α. In the case of CK1αL, these residues correspond to serines 339 and 340 and threonines 349 and 351. However, in the case of CK1αS, the number of residues autophosphorylated increases to six: serines 311, 312, and 332 and threonines 321, 323, and 327. The additional residues are within the S insert. It is interesting that in this latter case, the differential splicing affects this important property of the enzyme.

Regarding the autophosphorylated sequences, it is important to note that in CK1α and CK1αL, none of the four residues targeted are part of the canonical or non-canonical sequences, which are known to be recognized by CK1 [Bustos et al., 2005].

On the other hand, in CK1αS, the phosphorylation of threonine 327 would comply with a canonical recognition signal if threonine 323 is previously autophosphorylated. In turn, phosphorylation of threonine 327 and the presence of the aspartic 328 may provide a canonical site for serine 332 phosphorylation. It is also noteworthy that a phosphoproteome analysis of the human mitotic spindle identified threonine 321 of protein kinase CK1 [Nousiainen et al., 2006]. This result confirms that this site, which corresponds to one of the autophosphorylation sites detected in this work, is also autophosphorylated in vitro in human cells.

The results obtained demonstrate that, although these residues in the carboxyl end of the enzyme molecule are the major sites of phosphorylation, there are other sites of autophosphorylation in other parts of the CK1α molecule. However, the cluster of autophosphorylation residues in the carboxyl end seems to be responsible for the appearance of the slower migrating forms of the enzyme in SDS gel electrophoresis. It is interesting that the in vitro autophosphorylation cannot fully reproduce the shift in electrophoretic mobility that is attained when the enzyme is expressed in the bacteria. This observation could be explained if some of the autophosphorylation sites might be only accessible when the protein is nascent. There is a report that indicates that this is the case with in vitro autophosphorylation of dual-specificity tyrosine-phosphorylation-regulated kinases (DYRKs) [Lochhead et al., 2005].

An important finding reported here is the observation that autophosphorylation causes a significant inhibition of the catalytic activity of CK1α and its splice variants. This observation indicates that this isoform, which has been involved in a number of important cellular processes, can be regulated by its phosphorylation state and by its interplay with protein phosphatases. Phosphatase treatment of CK1α, CK1αS, and CK1αL (L form, not shown) causes a significant stimulation of their catalytic activity. The stimulation caused by phosphatase treatment of CK1αS is greater than that observed with the other two splice variants, a finding that may be explained by the fact that CK1αS autophosphorylates in two additional sites present in the S insert. Mutation of the serines and threonines that autophosphorylate in the C-terminus also eliminates or greatly reduces the capacity of phosphatase to stimulate CK1α activity.

In agreement with the observation that autophosphorylation inhibits the catalytic activity of CK1α and its splice variants, it was found that incubation of the dephosphorylated enzyme with ATP to allow re-autophosphorylation causes a significant decrease in the activity. The decrease, in the case of CK1αS, was not as large as could have been expected from the activation caused by phosphatase treatment. This result might be explained by the previous observation that autophosphorylation in vitro is only partial in regard to what occurs in vivo. Again, as shown in Figure 7, incubation with ATP to allow autophosphorylation with the mutant that lacks the carboxyl-terminal sites has very little effect on the activity.

Purification of bacterial expressed CK1α, CK1αS, and of the mutants that lack the carboxy-end autophosphorylation residues (CK1α$^{SSTT/A}$) allowed us to compare their catalytic effectiveness on a per pmol of enzyme basis. This measurement clearly agreed with the other results presented here that demonstrate that the enzymes autophosphorylate as they are expressed in the bacteria and that this modification in their carboxy-terminal residues inhibits their activity. The activity of the mutants that lack autophosphorylation sites in the carboxyl-end were at least twice as active as the forms that undergo autophosphorylation. The least active enzyme was CK1αS, which is the isoform most altered by autophosphorylation.

The difference in the activities of the enzymes that are or are not autophosphorylated in the carboxy-terminal residues is not due to changes in the app. K_m values for either ATP or the synthetic peptide substrate but are probably related to the greater catalytic efficiency of the particular enzyme.

CK1α recognizes different amino acid sequences in its protein substrates. Canonic substrates contain either phosphoamino acids or an acidic cluster in positions −3 or −4 upstream from the target serine or threonine. In non-canonic protein substrates like β-catenin, APC, or NF-AT4 [Bustos et al., 2005], CK1 recognizes a serine that is part of an S*LS sequence followed downstream in position +2 to +5 by an acidic cluster. It has been shown that mutation of lysine 232 in CK1α causes a great reduction of the activity against canonical substrates but has little effect on its activity toward non-canonical substrates. These results indicate that different parts of the enzyme molecule are involved in the recognition of these sequences. It has also been proposed that the carboxyl-end phosphoserines or phosphothreonines generated by autophosphorylation might be inhibitory by acting as pseudosub-

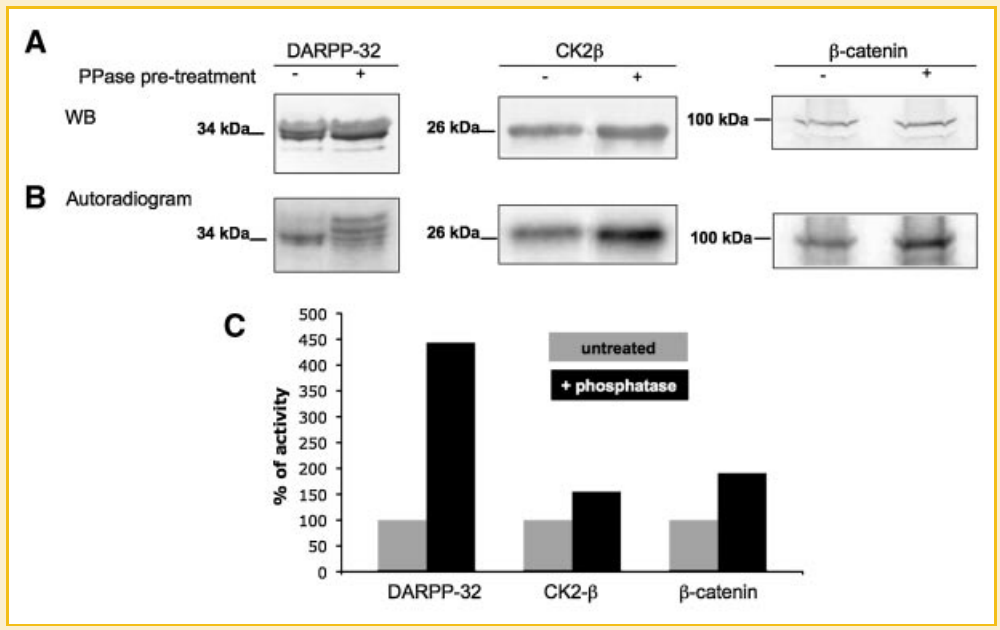

Fig. 9. Activation of CK1αS caused by dephosphorylation using different cellular proteins as substrates. The effect of phosphatase pre-treatment on the enzymatic activity of CK1αS was evaluated using [γ-^{32}P] ATP and His-tagged DARPP-32 (left), CK2β (center), or β-catenin (right) as substrates. A: The samples were analyzed by Western blot using an anti-His antibody. B: Autoradiogram of Western blot membranes showing the ^{32}P-phosphate incorporation to the substrates. C: Graphical representation of the results shown in A, B determined by densitometric analysis, expressed as percent of activity of the enzymes per unit of protein present in A for enzyme treated with phosphatase (black bars) compared to activity without λ phosphatase (gray bars). These results are representative of at least three separate experiments.

strates of the canonical phosphate-directed recognition sequences and blocking access to the bonafide substrates [Cegielska et al., 1998]. The competition between autophosphorylation and a canonical peptide phosphorylation observed in Figure 2 is not surprising and agrees with the idea of a common binding site.

In this light, it became important to determine whether autophosphorylation of CK1α and CK1αS also affected the phosphorylation of proteins, which are natural substrates of CK1α and whether the canonical or non-canonical nature of the surrounding amino acid sequence had any relation to the effect of autophosphorylation.

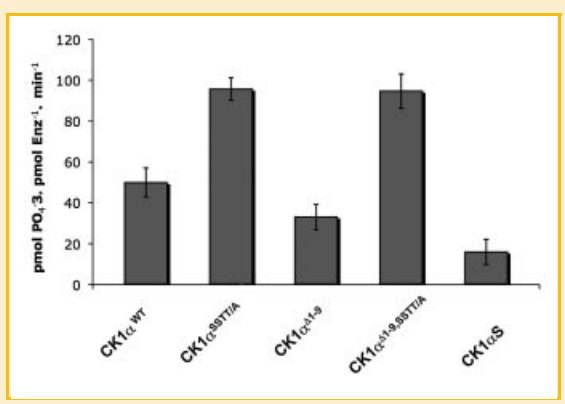

Fig. 10. Comparison of activity of bacterially expressed wild type enzymes and mutants. The recombinant proteins, CK1α^{WT}, CK1α$^{Δ1-9}$, CK1α$^{SSTT/A}$, CK1α$^{Δ1-9,SSTT/A}$, and CK1αS were submitted to activity assays using [γ-^{32}P] ATP and a specific peptide as substrates. The results are expressed as pmol of ^{32}P-phosphate incorporated to the peptide substrate per pmol of enzyme per minute using the P-81 phosphocellulose filter assay as given in Materials and Methods Section. The results shown are the mean ± SD of two experiments.

Using the protein DARPP-32 which has a strong canonical sequence [Desdouits et al., 1995] and that is phosphorylated by CK1 on serine 137, it was determined that phosphatase treatment of CK1α or of CK1α$^{Δ1-9}$ increased significantly the phosphorylation of this substrate, while no such enhancement was observed with mutants that lack the carboxy-end autophosphorylation sites. These results demonstrate that the effect observed with the synthetic peptide also occurs with a protein that is a natural canonical substrate.

In addition to DARPP-32, two other proteins: β-catenin and CK2β were also studied with CK1αS. β-catenin is phosphorylated in the non-canonical sequence serine 45 by CK1 [Marin et al., 2003] and recent results in our laboratory have demonstrated that CK1α phosphorylates CK2β, the regulatory subunit of protein kinase CK2, on serine 205 and threonine 213. The surrounding sequences of the CK2β phosphorylation sites do not conform to either the canonical or non-canonical sequences. With all three of these proteins substrates, CK1αS dephosphorylation through phosphatase treatment stimulates their phosphorylation. However, the stimulation observed with the canonical substrate DARPP-32 is considerably more pronounced than that obtained with the other proteins that do not contain the canonical sequence. However, synthetic peptides containing either canonical or the β-catenin type of non-canonical sequences were both stimulated to the same extent by dephosphorylation of CK1αS (not shown). Obviously, more detailed work is necessary to establish whether this regulatory mechanism can indeed discriminate between the different types of substrates.

However, the above results do indicate that the activity of CK1α and its variants might be regulated by protein phosphatases. In earlier work, using CK1α isolated from Drosophila, it was described that phosphatase treatment stimulated the activity of this enzyme [Santos et al., 1996]. Some phosphatases can be regulated by second messengers who could then regulate CK1α indirectly. Such a mechanism was proposed by Greengard and his collaborators [Liu et al., 2002b] who postulated that stimulation of group 1 metabotropic glutamate receptors could activate CK1ε via Ca^{+2} dependent stimulation of calcineurin, a protein phosphatase, which would dephosphorylate inhibitory carboxy-terminal autophosphorylation sites in that isoform. Recently, Bedri et al. [2007] presented evidence that hydrogen peroxide might trigger cell proliferation of mammalian cells by causing the dephosphorylation of CK1αLS, which would activate the phosphorylation of heterogenous ribonucleoprotein C. It is not clear how hydrogen peroxide can activate this phosphorylation.

Obviously, another mechanism for regulating the activity of CK1α and its splice variants could be through partial proteolysis that would cleave the carboxyl end of the enzyme that contain the autophosphorylation sites, as reported for the CK1ε [Cegielska et al., 1998]. In this sense it is interesting that a carboxy terminal deletion mutant, $CK1\alpha^{1-301}$, prepared in this laboratory (data not shown), which eliminates the entire autophosphorylatable segment has a slightly higher specific activity than the $CK1\alpha^{WT}$.

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