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FAST TRACKS

Cell Cycle Regulatory Protein $p27^{KIP1}$ Is a Substrate and Interacts With the Protein Kinase CK2

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Abstract The protein kinase CK2 is constituted by two catalytic (α and/or α′) and two regulatory (β) subunits. CK2 phosphorylates more than 300 proteins with important functions in the cell cycle. This study has looked at the relation between CK2 and $p27^{KIP1}$, which is a regulator of the cell cycle and a known inhibitor of cyclin-dependent kinases (Cdk). We demonstrated that in vitro recombinant *Xenopus laevis* CK2 can phosphorylate recombinant human $p27^{KIP1}$, but this phosphorylation occurs only in the presence of the regulatory β subunit. The principal site of phosphorylation is serine-83. Analysis using pull down and surface plasmon resonance (SPR) techniques showed that $p27^{KIP1}$ interacts with the β subunit through two domains present in the amino and carboxyl ends, while CD spectra showed that $p27^{KIP1}$ phosphorylation by CK2 affects its secondary structure. Altogether, these results suggest that $p27^{KIP1}$ phosphorylation by CK2 probably involves a docking event mediated by the CK2β subunit. The phosphorylation of $p27^{KIP1}$ by CK2 may affect its biological activity. J. Cell. Biochem. 91: 865–879, 2004.

Key words: docking; phosphorylation; cell cycle regulation; casein kinase 2; protein–protein interaction

The protein kinase CK2 (formerly named "casein kinase-2") is a ubiquitous enzyme in the eukaryotic world, being found from yeast to human. This enzyme is composed of a catalytic subunit (α or α′) and a regulatory subunit (β), forming a holoenzyme found in the combinations $\alpha_2\beta_2$, $\alpha'_2\beta_2$ or $\alpha\alpha'\beta_2$ [for reviews see Allende and Allende, 1995, 1998; Guerra and Issinger, 1999]. It has been found that CK2 phosphorylates more than 300 proteins that participate in cellular processes as diverse as replication, transcription, translation, signal transduction, and cell death [Meggio and Pinna, 2003]. It has been suggested that CK2 may have an important regulatory role in these processes [Ahmed et al., 2002; Litchfield, 2003]. Moreover, it has been observed that CK2 translocates from the cytoplasm to the nucleus when the cell is committed to division, behaves as an oncogene when overexpressed, and induces neoplastic growth [Guerra and Issinger, 1999].

The cell cycle regulatory protein $p27^{KIP1}$ constitutes together with $p21^{CIP1}$ and $p57^{KIP2}$ the kinase inhibitor protein (KIP) family of cyclin-dependent kinase (Cdk) inhibitors [Gu et al., 1993; Xiong et al., 1993; Polyak et al., 1994; Toyoshima and Hunter, 1994; Matsuoka et al., 1995]. These proteins contribute to development and cell growth in two ways: the direct inhibition of the Cdk activity [Sherr and Roberts, 1999; Xu et al., 1999] and the inhibition of Cdk activation by Cdk-activating kinase or

Abbreviations used: CK2, casein kinase-2; KIP, kinase inhibitor protein; Cdk, cyclin-dependent kinase; PCR, polymerase chain reaction; SDS–PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis; GST, glutathione-S-transferase; CD, circular dichroism; SPR, surface plasmon resonance.

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CAK [Rank et al., 2000]. Importantly, $p27^{KIP1}$ expression appears to have prognostic value in certain cancers associated with high morbidity and mortality [Slingerland and Pagano, 2000; Chiarle et al., 2001] and its loss strongly synergizes with myc overexpression in distinct lymphoma models [Martins and Berns, 2002].

The molecular basis for the inhibitory function of KIP proteins on Cdks is not fully understood. However, the structure of human $p27^{KIP1}$, encompassing the N-terminal inhibitory domain complexed with the cyclin A-Cdk2 dimer [Russo et al., 1996], has provided new insights into this phenomenon and it has also been used as a model for the structural and functional analysis of other KIP protein relatives. Despite the fact that the unbound inhibitory domain is largely unfolded in solution [Flaugh and Lumb, 2001], this fragment is active as the full-length p27 [Bienkiewicz et al., 2002] and the crystal structure shows a well-ordered conformation comprising an amphipathic α-helix, an amphipathic β-hairpin, a β-strand, and a 3_{10} helix [Russo et al., 1996]. The function of the C-terminal segment, called the QT domain, remains largely unknown although it contains both a nuclear localization signal and a Cdk phosphorylation site [Polyak et al., 1994; Matsuoka et al., 1995; Bienkiewicz et al., 2002].

Biochemical studies with $p27^{KIP1}$ have demonstrated different roles for the secondary structure regions observed in the cyclin A-Cdk2-p27 complex. Mutagenesis studies showed that stabilization of the partially preformed α-helix in the free p27 inhibitory domain significantly decreases the rate of cyclin A-Cdk2 inhibition [Bienkiewicz et al., 2002]. Similarly, the putative 3_{10} helix might account for the inhibition of cyclin E-Cdk2 and cyclin A-Cdk2 mediated by p57 [Hashimoto et al., 1998].

The regulatory effect of covalent modification on the biological function of $p27^{KIP1}$ is well-established. For example, it is known that the subcellular localization of $p27^{KIP1}$ is controlled through phosphorylation of serine-10 by the recently described human kinase interacting stathmin, hKIS [Rodier et al., 2001; Boehm et al., 2002], while the ubiquitination and subsequent degradation by the proteasome are dependent on Cdk2-phosphorylation of threonine-187 [Sheaff et al., 1997].

$p21^{CIP1}$ has been shown to be a substrate of protein kinase CK2 and also to interact in vitro and in vivo with the regulatory CK2β subunit [Götz et al., 1996; Korn et al., 1999; Romero-Oliva and Allende, 2001]. A deletion study mapped the region in CK2β that could sustain p21 binding to 40 amino terminal residues [Romero-Oliva and Allende, 2001]. To date there are no reports on interaction of CK2 with other inhibitory KIP proteins. The present study shows that $p27^{KIP1}$ is also an in vitro substrate of CK2 and interacts with its β subunit. Pull down assays and surface plasmon resonance (SPR) studies demonstrate that $p27^{KIP1}$ interacts through two domains with the CK2β subunit. Circular dichroism (CD) analysis demonstrates that the phosphorylation of $p27^{KIP1}$ by CK2 affects its secondary structure, suggesting that this modification may affect the in vivo activity of this regulatory protein.

MATERIALS AND METHODS

Materials

Pfu DNA polymerase and T4 DNA ligase were purchased from MBI Fermentas (Hanover, MD). Primers were purchased from Oligopeptido (University of Chile core facility) and Invitrogene (Frederick, MD). [γ-^{32}P]ATP (6,000 Ci/mmol), glutathione-sepharose and superdex 200 (HR 10/30) were from Amersham Pharmacia Biotech (Piscataway, NJ). Monoclonal antibody anti-His was from Clontech (BD, Palo Alto, CA). Ni^{2+}-NTA-Agarose was from Qiagen (Valencia, CA). Monoclonal antibody anti-CK2β was purchased from Calbiochem (San Diego, CA). BioMax MS film and BioMax Transcreen HE intensifying screen were from Kodak (Rochester, NY). CM5 biosensor chip, amine activation kit and HBS buffer were from BIAcore (Uppsala, Sweden).

Cloning and Design of Mutants

Human cDNA for $p27^{KIP1}$ cloned in a suitable vector was a kind gift from Dr. Silvio Gutkind (NIH/NIDR, Bethesda, MD). The cDNA codifying for full-length p27 (amino acids 1–198) was amplified by PCR using Pfu DNA polymerase and the primers 5′-ATATATAGATCTATGTCAAACGTGCG-3′ (BGP27) and 5′-ATATATGTCGACTTACGTTTGACGTCTTC-3′ (P27SAL) in order to incorporate the restriction sites Bgl II and Sal I, respectively. The amplicon was double-digested and further purified from

agarose gels, ligated into the pT7HX and modified pGEX-4T3 vectors using T4 DNA ligase. The p27 deletion mutants were prepared using the pT7HX-$p27^{FL}$ construct as the template and the primers 5′-CGACTCACTATAGGGAGACC-3′ (JT77) and 5′-ATATATGTCGACTTACCGCGGGGGTCT-3′ (JTDC) for the $p27^{NT}$ mutant (N-terminal half, amino acids 1–93) and 5′-ATATATAGATCTCCCCCCAAAGGT-3′ (JTDN) and 5′-TTTGACAGCTTATCATCG-3′ (T7INV) for the $p27^{CT}$ mutant (C-terminal half, amino acids 94–198). Full-length $p27^{S83A}$ site-directed mutant was obtained using a standard PCR overlapping method as published [Ho et al., 1989], using the forward JT77 and reverse T7INV primers, together with the mutagenic primers 5′-GAGAAGGGCGCCTTGCCCGAG-3′ (JTAF) and 5′-CTCGGGCAAGGCGCCCTTCTC-3′ (JTAR). All p27 mutants were subcloned into both pT7HX and pGEX-4T3 modified vectors as mentioned above. Design, expression, and purification of the GST-$CK2\beta^{S2,3G}$ mutant, a form not phosphorylated by CK2α, has been described before [Hinrichs et al., 1993]. Also the expression and purification of *Xenopus laevis* CK2α and β subunits tagged with $(His)_6$ have been previously described [Tapia et al., 2002]. All construct sequences were confirmed by automatic sequencing using an ABI Prism DNA Sequencer (Perkin Elmer, Boston, MA).

Expression and Purification of Recombinant CK2α and β Subunits and p27

His-tagged proteins were expressed in *E. coli* strain BL21 (DE3) pLysS and further purified by Ni^{2+}-NTA-agarose affinity chromatography as given in manufacturer's instructions. All GST fusion proteins were expressed in *E. coli* strain DH5α and affinity chromatography purification was performed using glutathione-agarose resin as described by the manufacturer's procedure. For gel-filtration chromatography, recombinant protein samples were dialyzed into a buffer containing 50 mM Tris-Cl pH 8.0 and 150 mM NaCl, and applied to a calibrated Superdex 200 (HR 10/30) column at 4°C. The column was eluted with the same buffer at a flow-rate of 0.5 ml/min and 0.5 ml fractions were collected. Eluted proteins were analyzed using 12% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE), specifically identified using monoclonal antibodies and densitometrically quantified using bovine seroalbumin as protein standard. Proteins were aliquoted and stored at −80°C.

Protein Concentration Determination

Concentration of all proteins for activity and pull down assays were densitometrically determined from SDS–PAGE gels using bovine seroalbumin as protein standard. Protein concentration of GST-free full-length p27 (22,073 Da), $p27^{NT}$ (11,172 Da), $p27^{CT}$ (10,919 Da), and His-$CK2\beta^{wt}$ (25,783 Da) for BIAcore and CD studies was estimated from the absorbance at 280 nm in 50 mM Tris pH 8.0, 100 mM NaCl, using an extinction coefficient value of 15,460, 15,340, 120, and 28,380 $M^{-1}cm^{-1}$, respectively.

Kinase Activity Assays

Standard assay for CK2 kinase activity and the determination of kinetic constants were carried out essentially as described [Tapia et al., 2002]. Briefly, reactions were carried out in 30 μl containing 100 μM [γ-^{32}P]ATP (2,500–3,000 cpm/pmol), 10 mM Hepes pH 7.5, 10 mM $MgCl_2$, 0.5 mM DTT, 50 mM NaCl, and recombinant $p27^{FL}$, $p27^{NT}$ or $p27^{CT}$ as substrate. Two pmol of catalytic CK2α subunit was used in the absence or presence of an increasing amount of regulatory CK2β subunit. In this latter case 100 mM NaCl was used for the assay. After incubation at 30°C for 30 min, the reaction was stopped in ice, mixed with 7.5 μl 5× loading buffer, and heated for 5 min at 100°C. The mixture was subjected to 12% SDS gel electrophoresis and the phosphorylated band visualized by autoradiography using an intensifying screen after 1 h. The extent of protein phosphorylation was evaluated by densitometric analysis. For CD studies using p27 in the phosphorylated form, the reaction was carried out in the same buffer but containing 100 μM of non-radioactive ATP and 0.1 μM of CK2 holoenzyme for 1 h.

Pull Down Assays

Glutathione-sepharose (6 μl) was mixed with 150 pmol of the bait protein fused to glutathione-S-transferase (GST) in a total volume of 300 μl interaction 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, and incubated for 1 h at 4°C with gentle agitation. After 2 min of 15,000*g* centrifugation at 4°C, the resin with bound GST-protein was washed twice with 200 μl of interaction buffer. The

pellet was mixed with 25–50 pmol of the target His-protein in a total volume of 200 μl interaction buffer plus protease inhibitors, and incubated by 1.5 h at 4°C with gentle agitation. After centrifugation, the pelleted complex was washed twice with 200 μl incubation buffer, once with 500 μl PBS, and mixed with 30 μl of 1.5× loading buffer. Finally, the mixture was subjected to 12% SDS gel electrophoresis, transferred to PVDF membrane and visualized by blotting with a specific monoclonal antibody.

Far-UV CD

Protein species were dialyzed in 50 mM phosphate buffer, pH 8.0, and their concentrations adjusted to 0.3 mg/ml. Far-UV CD spectra were recorded at 20°C on a Jasco J-720 spectropolarimeter (Jasco Ltd., Great Dunmow, UK) using a 1 mm path length quartz cell (Hellma, Essex, UK). Eight scanning acquisitions, from 205 to 260 nm, in 0.2 nm steps were accumulated and averaged, yielding the final spectrum after blank subtraction. CD signals are expressed as mean residue ellipticity.

Biosensor Surface Preparation

Formation and dissociation of p27–CK2β complexes was monitored by SPR with a BIAcore 2000 apparatus (BIAcore AB). Preparations of 4 μM His-CK2β^{wt} and 30 μM each GST-p27^{FL}, GST-p27^{NT}, and GST-p27^{CT} were immobilized on a CM5 biosensor chip by amine coupling chemistry as follows: after activation with a freshly prepared mixture of *N*-hydroxysuccinimide (50 mM in water) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (195 mM in water) for 4 min (flow rate 10 μl/min), purified p27 and CK2β proteins were diluted 1:2 with 10 mM sodium acetate buffer (pH 5.0) containing 50 mM NaCl, and 40 μl of each sample mixture covalently bound to the dextran matrix of CM5 biosensor chips via their free amino groups at a flow-rate of 10 μl/min for 10 min. A second CM5 biosensor chip was prepared with no GST-tagged p27^{FL} or p27^{NT} or p27^{CT} immobilized onto its surface. In one line pure GST was immobilized to serve as a control. The amount of protein bound to each biosensor chip was monitored by the change in refractive index. In order to allow the measurement of kinetic parameters, the matrix was controlled to make sure it was not saturated. A total of 10,000 resonance units (RU) of each protein sample were immobilized by this method. Remaining activated carboxylic groups were deactivated by injection of 40 μl of 1 M ethanolamine hydrochloride (pH 8.6) for 7 min at flow-rate of 10 μl/min. After coupling, the buffer was changed to HBS (10 mM HEPES pH 7.4, 150 mM NaCl, 3.4 mM EDTA, and 0.005% surfactant P20) as the running buffer. Binding experiments were performed at 25°C in HBS at a flow-rate of 10 μl/min. After each run, the biosensor chip was regenerated by a 60 s injection of 0.1 M HCl. The binding interaction between the different p27 constructs and CK2β were detected and displayed as sensorgrams by plotting the RU against time. Detected RU changes represent the association and dissociation of p27-CK2β complexes.

Acquisition of Kinetic Binding Data

Decreasing concentrations of analytes between 7 and 0.5 μM were injected during 5 min (10 μl/min), with HBS as the running buffer. The dissociation phase, initiated by passage of HBS alone, was carried out over a period of 5 min at the same flow-rate. Samples were injected in duplicate in random order in at least two separate experiments. All binding curves were corrected for background and bulk refractive index contribution by subtraction of the reference flow cells.

Analysis of Experimental Data

In order to interpret protein interactions recorded on the biosensor chips, the data analysis was performed with the interactive software BIAevaluation v3.0 (BIAcore, Uppsala, Sweden), which combines numerical integration and nonlinear global curve fitting routines. The dissociation (k_d) and association (k_a) rate constants were measured following each single analyte injection. The equilibrium binding constant (K_D) was defined as the ratio k_d/k_a. The model used to fit the data sets and for a single concentration run was the Simple Bimolecular Reaction where the analyte forms a 1:1 complex with its ligand.

RESULTS

p27^{KIP1} Is a Substrate of CK2

In vitro phosphorylation analysis was used to determine the capacity of p27^{KIP1} to serve as a substrate of the recombinant protein kinase CK2 from *X. laevis*. For this and other experiments described in this study, a nonphosphor-

ylatable CK2β recombinant mutant subunit, GST-CK2β^{S2,3G}, was used to distinguish the phosphorylation of the p27 substrate from the autophosphorylation of the regulatory CK2β subunit, since both p27 and CK2β exhibit a similar molecular mass on SDS–PAGE gels. The GST-CK2β^{S2,3G} mutant has properties nearly identical to the native phosphorylatable form [Hinrichs et al., 1993].

Recombinant full-length His-p27^{KIP1} (p27^{FL}) was efficiently phosphorylated in the presence of purified recombinant CK2α^{wt} and β^{S2,3G} mutant (Fig. 1A, lanes 2–5). Similar phosphorylation levels were obtained with the fusion protein GST-p27^{FL} as substrate (Fig. 1B). In these experiments, it was observed that p27 is phosphorylated only in the presence of the regulatory CK2β subunit. These results contrast with the fact that most other CK2 substrates previously described are readily phosphorylated by the constitutively active catalytic subunit alone, albeit at lower levels than in the presence of the holoenzyme. In the latter case, the activity is typically stimulated in the range of 4 to 10-fold [Marin et al., 1994, 2000; Meggio et al., 1994; Seger et al., 1998]. A kinetic analysis of the phosphorylation reaction using His-p27^{FL} as variable substrate showed

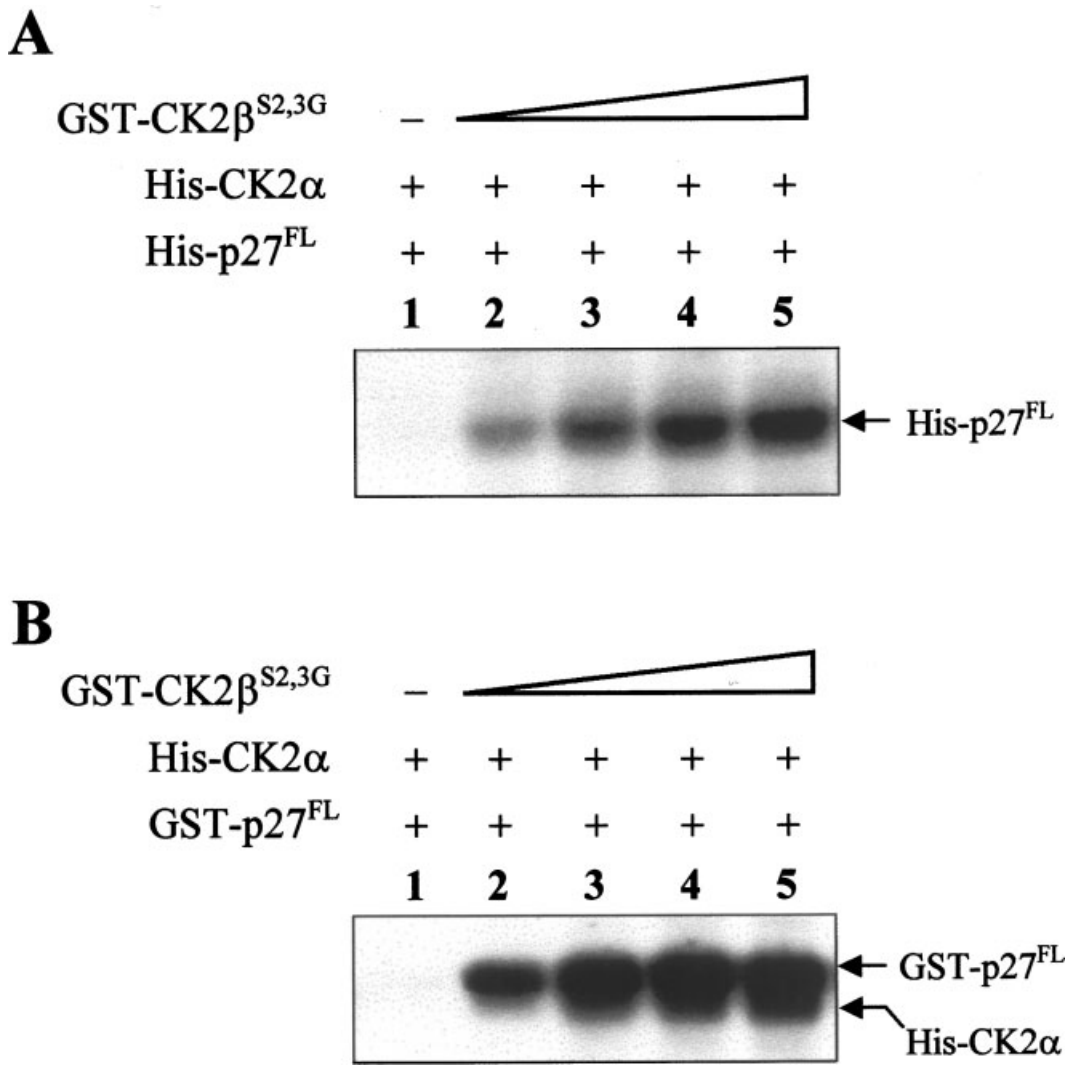


Fig. 1. Recombinant human p27^{KIP1} is a substrate of protein kinase CK2. **A**: Phosphorylation reactions were carried out using 2 pmol of catalytic His-CK2α subunit in the presence of 130 pmol of His-p27^{FL} and either in the absence (**lane 1**) or in the presence of 0.5, 1, 2, and 4 pmol of a nonphosphorylable β subunit, GST-CK2β^{S2,3G} (**lanes 2–5**). The standard kinase assay was used followed by gel electrophoresis and autoradiography as given in ''Materials and Methods.'' **B**: Phosphorylation of 90 pmol of GST-p27^{FL} was assayed using different levels of GST-CK2β^{S2,3G} (lanes 2–5) as given in A. The autophosphorylation of the catalytic α subunit is also shown.

an apparent K_M of 0.467 ± 0.075 μM, a value that is somewhat lower than those reported for other CK2 protein substrates [Marin et al., 1994, 2000; Meggio et al., 1994; Seger et al., 1998; Tapia et al., 2002].

Serine-83 in p27 Is the Major Phosphorylation Site for CK2

The amino acid sequences of p21 and p27 from mouse, rat, and human are compared in Figure 2A. As shown there, the most conserved region among these species is the amino-terminal, which includes the inhibitory domain, while the carboxy-terminal region, which contains the QT domain, highly diverges [Polyak et al., 1994; Matsuoka et al., 1995; Bienkiewicz et al., 2002]. Moreover, a sequence analysis of $p27^{FL}$ indicates that serine-83, which is conserved in mammals, conforms to the known substrate consensus of the CK2 enzyme since it

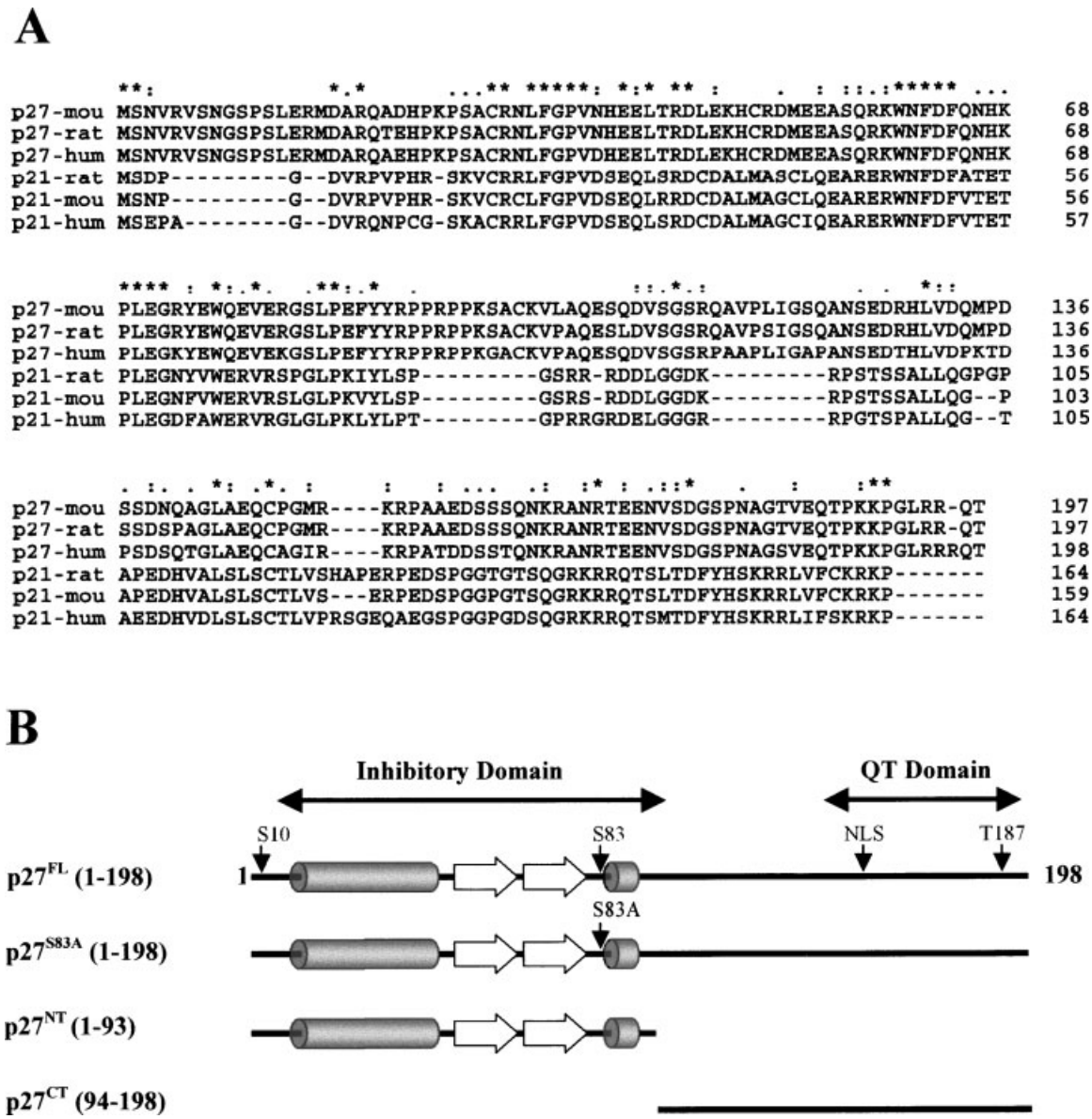


Fig. 2. Human $p27^{KIP1}$ sequence comparison and structure of mutants. **A**: The aligment of sequences between $p27^{KIP1}$ of several species and $p21^{CIP1}$ shows some conserved regions mainly in their amino-terminal half. The aligment was obtained by the ClustalX software, where the asterisks indicate identity. **B**: Depicted is a schematic view for the full-length p27 and different mutants generated in this work as well as the inhibitory and QT domains, the main phosphorylation sites for several kinases, and the nuclear localization sequence (NLS). Cylinders and arrows correspond to α-helixes and β-strands, respectively, according to the structure of cycline A-Cdk2-p27 complex [Russo et al., 1996].

contains an acidic residue in the n + 3 position [Pinna, 2002; Meggio and Pinna, 2003]. In contrast, two other sites, Ser-125 and Thr-170 located in the C-terminal half, do not present a strict CK2 consensus sequence. The human $p27^{FL}$ sequence also shows an additional non-canonical site, Thr-157, which is not present in the other species (Fig. 2A).

As a result of this analysis, deletion mutants corresponding to the amino- and carboxy-terminal segments, $p27^{NT}$ and $p27^{CT}$, respectively were cloned and expressed in *E. coli* (Fig. 2B). As observed in Figure 3, both $p27^{NT}$ and $p27^{CT}$ are substrates of CK2 and, as in the case of $p27^{FL}$, phosphorylation is only observed in the presence of the reconstituted holoenzyme $CK2\alpha_2\beta_2$, but is not observed in the presence of the catalytic CK2α subunit alone. As expected, the effect of increasing levels of the regulatory $CK2\beta^{S2,3G}$ subunit is seen both in an incremented phosphorylation of p27 derivatives as well as in the autophosphorylation of the CK2α subunit.

In addition, these results show that the extent of phosphorylation of $p27^{NT}$ was stronger than that seen with the C-terminal half.

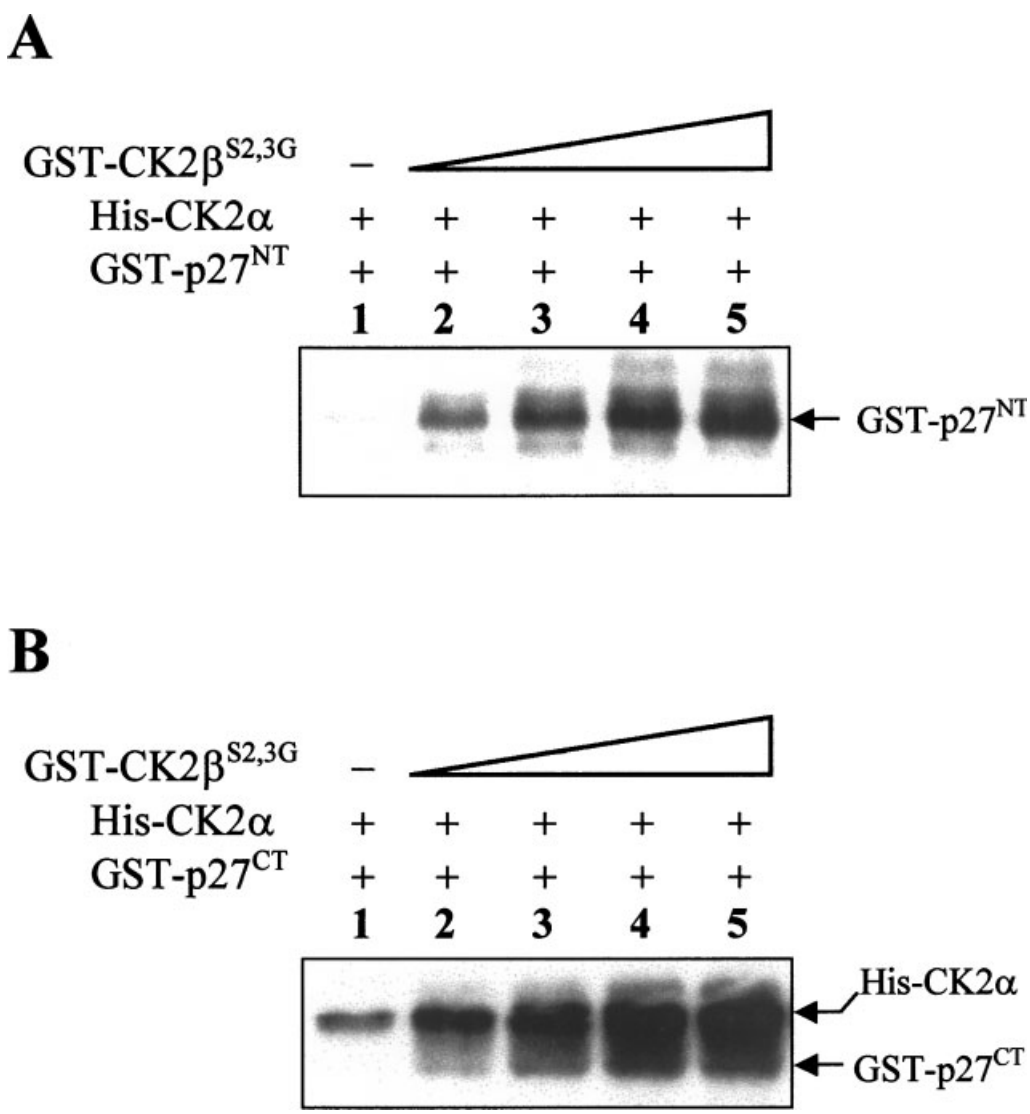


Fig. 3. Amino- and carboxyl-terminal fragments of $p27^{KIP-1}$ contain phosphorylation sites for CK2. Two pmol of catalytic CK2α subunit were used in the in vitro kinase assay in the presence of 50 pmol GST-$p27^{NT}$ (**A**) or GST-$p27^{CT}$ (**B**). The phosphorylation of each p27 fragment was assayed in the absence (**lane 1**) or presence of 0.5, 1, 2, and 4 pmol of GST-$CK2\beta^{S2,3G}$ (**lanes 2–5**). In B was used a higher exposure time than A due to the proximity of the autorradiography bands corresponding to the catalytic α subunit and GST-$p27^{CT}$ fragment.

Densitometry measurements indicate that $p27^{NT}$ phosphorylation is at least 6-fold higher than that observed for $p27^{CT}$. In order to explore the possibility that Ser-83 is the major p27 phosphorylation site, the mutant $p27^{S83A}$ (serine-83 changed to alanine) was cloned and expressed in *E.coli*. Figure 4 shows the phosphorylation assay for this mutant compared to $p27^{FL}$ in the presence of increasing amounts of $CK2\beta^{S2,3G}$. In this assay, phosphorylation of $p27^{S83A}$ was observed to a much lesser extent compared to the wild type protein (Fig. 4, compare lane 3 vs. lane 6, and lane 4 vs. lane 7). A comparison of phosphorylation levels using equal amounts of $CK2\beta^{S2,3G}$ and p27 substrates indicate that substitution of serine-83 results in an 80% decrease in phosphorylation by CK2 (data not shown). These results strongly suggest that in p27, serine-83 is the major phosphorylation site of protein kinase CK2.

Phosphorylation by CK2 Affects the Secondary Structure of p27

The effect of CK2-dependent phosphorylation of p27 was studied using Far-UV CD. The CD spectrum of GST-free $p27^{FL}$ before incubation with CK2 (i.e., the unphosphorylated state) was typical of an unfolded protein, as indicated by the minimum observed at 205 nm (Fig. 5). Nevertheless, a shoulder at 220 nm was also detected and it is indicative of a marginally stable secondary structure. Indeed, the inflexion point around 222 nm, which corresponds to alpha-helix n-π transitions [Bolanos-Garcia et al., 1998], also indicates the presence of residual alpha helix. Moreover, the temperature dependence of the CD signal at 222 nm indicated that the helix is only marginally stable (data not shown).

Interestingly, the CK2-dependent phosphorylation of $p27^{FL}$ resulted in a further decrease of its secondary structure content. Similar results have been observed for the deletion mutant $p27^{NT}$ while for the $p27^{CT}$ deletion the effect was negligible (manuscript in preparation). These results might be correlated with the degree of phosphorylation observed for each protein, as discussed below. The mean residue ellipticity of phosphorylated $p27^{FL}$, estimated at 222 nm ($[\theta]_{222}$), was independent of protein concentration through the range of 80–200 μg/ml. Thus, this observed secondary structure content was not due to protein aggregation. In fact, it has been reported that under conditions that promote molecular crowding, nonphosphorylated $p27^{FL}$ showed essentially the same secondary

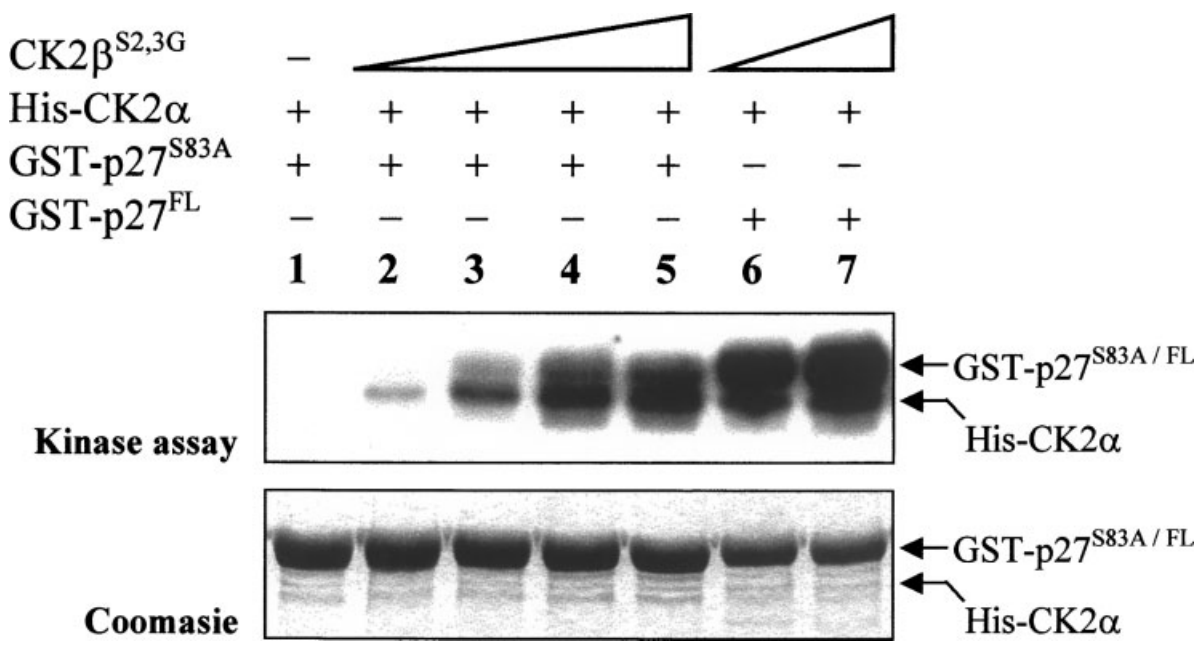


Fig. 4. Serine-83 of p27 is a preferred phosphorylation site of protein kinase CK2. Hundred pmol of mutant GST-$p27^{S83A}$ were assayed using 2 pmol of CK2α subunit in the absence (**lane 1**) or presence of 0.5, 1, 2, and 4 pmol of $CK2\beta^{S2,3G}$ (**lanes 2–5**). For comparison, phosphorylation of 50 pmol of GST-$p27^{FL}$ is shown in the presence of 1 and 2 pmol of $CK2\beta^{S2,3G}$ (**lanes 6**, **7**). As a control of amounts of p27 species loaded, the gel stained with Coomasie blue R-250 is depicted below.

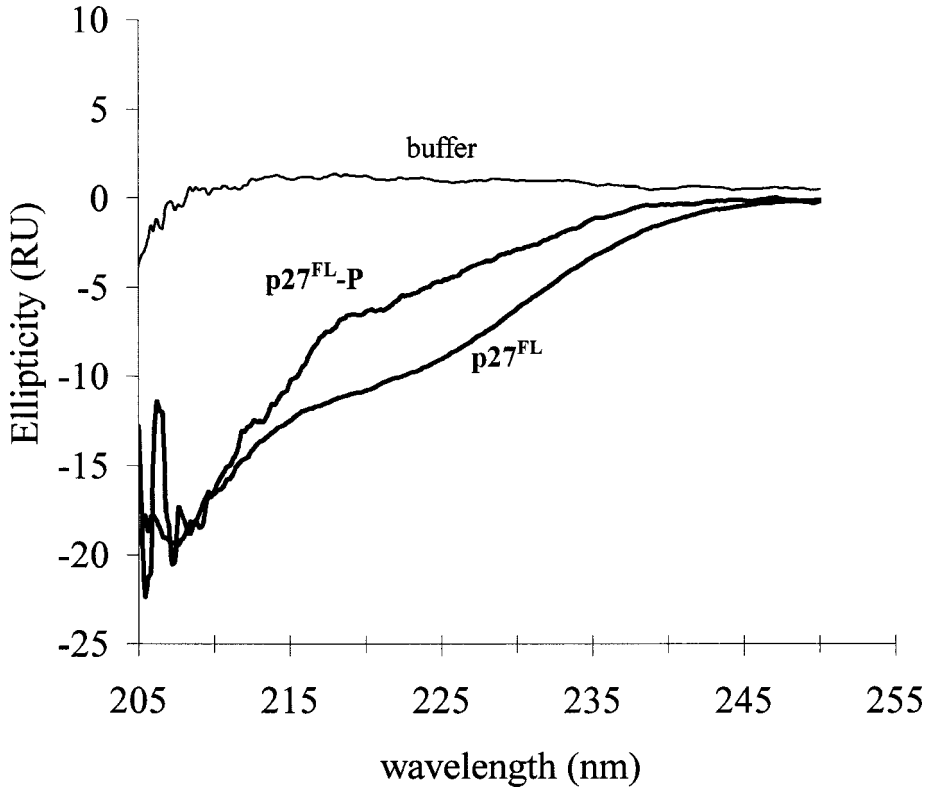


Fig. 5. Secondary structure of $p27^{KIP1}$ is affected by CK2-dependent phosphorylation. Recombinant human GST-free full-length p27 was phosphorylated in the presence of 0.1 μM holoenzyme CK2. Phosphorylated and nonphosphorylated species were dialysed and their concentrations adjusted to 0.3 mg/ml. CD spectra before and after CK2 treatment were recorded from 205 to 250 nm as given in "Materials and Methods."

structure content than fairly more diluted solutions [Flaugh and Lumb, 2001].

Finally, assays were carried out in order to test whether phosphorylation of $p27^{FL}$ or $p27^{NT}$ by CK2 could alter the capacity of these proteins to inhibit the activity of cyclin A-Cdk2. No significant effect of phosphorylation was detected in these in vitro assays (data not shown).

Interaction of $p27^{KIP1}$ With the Regulatory CK2β Subunit

The interaction between $p27^{KIP1}$ and CK2β was initially studied, using an in vitro pull down assay. The protein GST-$p27^{FL}$ bound to glutathione-sepharose was used as bait and the association of the His-$CK2\beta^{wt}$ subunit to the p27 protein was determined by Western blot using a specific anti-$(His)_6$ antibody (Fig. 6A, lane 2). As positive control the bait GST-$CK2\beta^{wt}$ bound to the resin was incubated with the CK2α subunit since these proteins are known to interact strongly (Fig. 6A, lane 3). Similarly, when using GST-$CK2\beta^{wt}$ as the bait to determine its association with three levels of concentration of His-$p27^{FL}$, interaction was also detected (Fig. 6B) and complex formation seems to be dependent on the concentration of $p27^{FL}$ used.

The capacity of CK2β to interact with the p27 deletion mutants $p27^{NT}$ and $p27^{CT}$ was also demonstrated based on pull down assays. Each GST-fusion protein was bound to glutathione-sepharose resin and the association with His-$CK2\beta^{wt}$ revealed by Western blotting using an anti-CK2β specific antibody. The results show that both fragments are able to interact with the regulatory CK2β subunit (Fig. 7A). This association was also confirmed by gel filtration chromatography (data not shown). In order to further compare the relative strength of the interaction between $p27^{FL}$, $p27^{NT}$, and $p27^{CT}$ mutants with CK2β, binding studies were performed using SPR on a BIAcore apparatus. Figure 7B presents the interaction results obtained by exposing different concentrations of $p27^{FL}$ to His-CK2β bound to the sensor chip. Similar experiments were carried out with $p27^{NT}$ and $p27^{CT}$ (not shown). The data from runs at increasing concentrations of

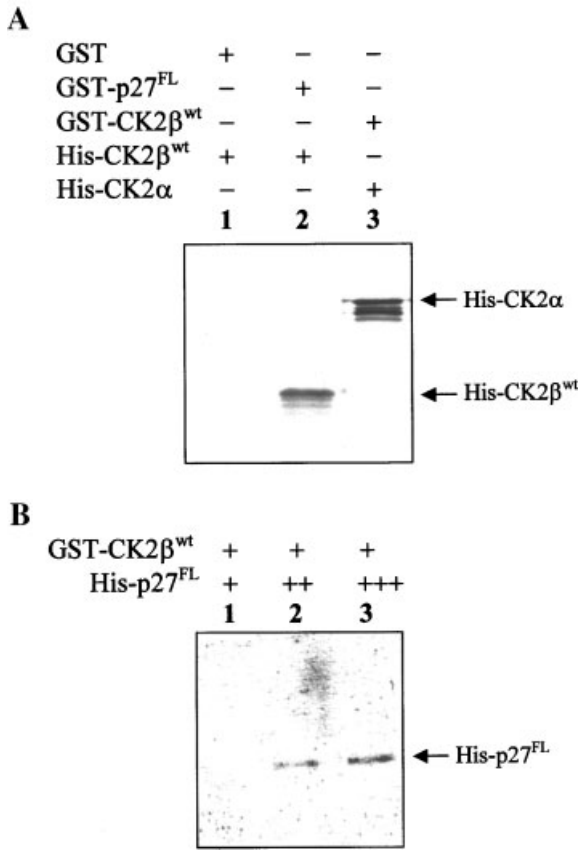


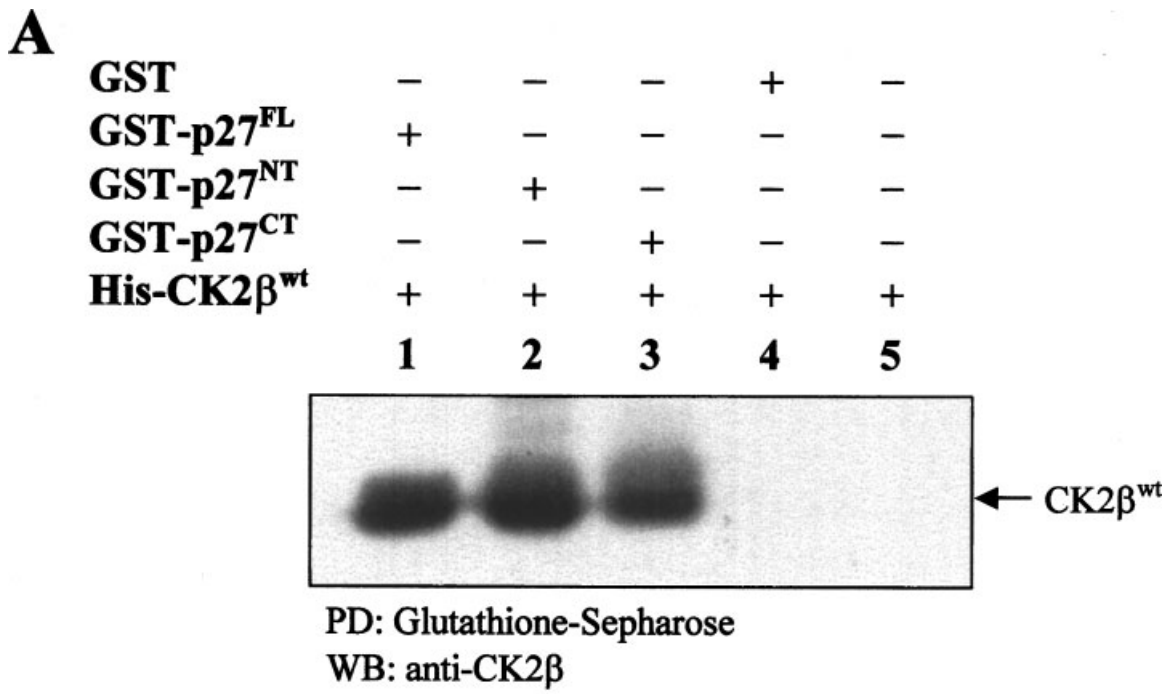
Fig. 6. Human $p27^{KIP1}$ interacts with the regulatory CK2β subunit. **A**: GST, GST-$p27^{FL}$, or GST-$CK2\beta^{wt}$ bound to glutathione-sepharose were incubated with 140 pmol of His-$CK2\beta^{wt}$ (**lanes 1**, **2**) or 200 pmol His-CK2α (**lane 3**) in a standard pull down assay followed by gel electrophoresis and immunoblot analysis using a monoclonal anti-His antibody, as given in "Materials and Methods." **B**: Pull down where GST-$CK2\beta^{wt}$ bound to glutathione-sepharose was incubated with 25, 50, or 100 pmol of His-$p27^{FL}$ (lanes 1–3). Western analysis was done at the same conditions as given in A.

analyte p27 were used to calculate the association and dissociation rate constants (k_a and k_d, respectively) and, subsequently, the equilibrium binding constant (K_D) of the interaction between CK2β and p27 species. K_D values of $1.32 \pm 0.63 \times 10^{-6}$, $0.67 \pm 0.30 \times 10^{-6}$, and $2.35 \pm 0.35 \times 10^{-6}$ M were calculated for $p27^{FL}$, $p27^{NT}$, and $p27^{CT}$, respectively. Therefore, these results indicate that full-length p27 regulatory protein might have at least two domains that interact with CK2β, with $p27^{NT}$ showing the stronger interaction.

DISCUSSION

In this report, the in vitro interaction of the protein kinase CK2 with the cell cycle regulatory protein $p27^{KIP1}$ has been demonstrated using a variety of techniques. One interesting finding is that p27 is a substrate of CK2 only in the presence of the regulatory CK2β subunit. This absolute dependency is surprising since it has been observed that the catalytic CK2α subunit is constitutively active in the absence of CK2β for the vast majority of substrates reported to date. However, it has also been reported that phosphorylation of the CK2 substrate human immunodeficiency virus protein HIV-1 Rev, is strictly dependent on the regulatory CK2β subunit [Marin et al., 1994]. Moreover, this protein seems to modulate some properties of CK2 through the regulatory CK2β subunit, namely autophosphorylation, substrate specificity, and susceptibility to polycationic effectors [Meggio et al., 2001].

Although several putative phosphorylation sites for CK2 are present in p27, most of them do not present the canonical consensus site described for CK2 (S/TXXE/D) where the phosphorylatable serine or threonine is followed by an aspartic or glutamic acid at position n + 3 [Pinna, 2002; Meggio and Pinna, 2003]. All serine/threonine residues located in the C-terminal half of p27 are non-canonical sites and have been shown to act as very poor substrates. In contrast, serine-83 is the unique canonical site in p27 and is located in its N-terminal half. Not surprisingly, the results obtained with the S83A mutant indicate that it is the in vitro preferred site for CK2 phosphorylation. Of special importance is the fact that serine-83 is only present in p27 while in other members of the KIP family the residues topologically equivalent to Ser-83 and Glu-86, which conform to the CK2 consensus site, are replaced by Gly-71 and Lys-74 or Ser-75 and Ala-78, in $p21^{CIP1}$ and $p57^{KIP2}$, respectively [Gu et al., 1993; Xiong et al., 1993; Polyak et al., 1994; Toyoshima and Hunter, 1994; Matsuoka et al., 1995]. Furthermore, the serine-83 is located at the beginning of a 3_{10} helix, which is essential for the inhibitory mechanism of p57 by mimicking ATP inside the catalytic cleft of Cdk2 [Hashimoto et al., 1998]. Although an important function for the 3_{10} helix in p27 or p21 has not been described, the presence of this unique canonical phosphorylation site in p27 strongly suggests that may be involved in a specific, fine-tuned regulatory mechanism through a phosphorylation-dephosphorylation cycle.



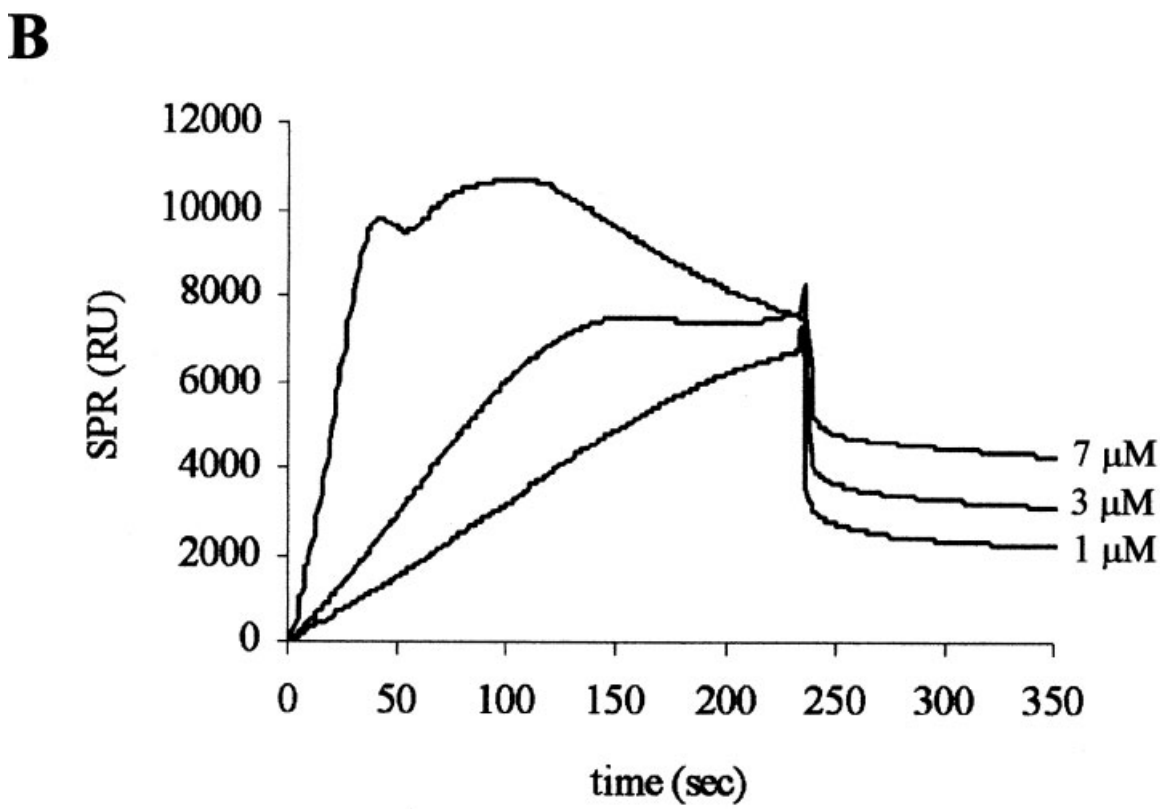


Fig. 7. The amino- and carboxyl-terminal fragments of p27 interact differentially with regulatory CK2β subunit. **A**: A pull-down assay was used in which 500 pmol each of GST-p27^{FL} (**lane 1**), GST-p27^{NT} (**lane 2**) or GST-p27^{CT} (**lane 3**) were bound to glutathione-sepharose and subsequently incubated with 380 pmol of His-CK2β^{wt} and processed as given in ''Materials and Methods.'' The presence of associated His-CK2β^{wt} was revealed by Western blot using monoclonal anti-CK2β antibody. The non-specific interaction of His-CK2β^{wt} with GST bound to resin (**lane 4**) or the resin alone (**lane 5**) were also tested as controls. **B**: Sensorgram obtained from a experimental run where the indicated concentrations of GST-p27^{FL} were assayed with His-CK2β bound onto the biosensor chip. The signals displayed correspond to the interaction-dependent resonance observed at each condition as given in ''Materials and Methods.''

On the other hand, CD studies indicate that phosphorylation of p27 by CK2 has direct structural and functional effects. These results may be interpreted by postulating that p27 in solution behaves like a naturally unfolded protein type II, according to the classification proposed by Zetina [Zetina, 2001]. This author has also suggested the existence of a helix motif that promotes protein unfolding. Interestingly, such a motif seems to correspond to the consensus site for the protein kinase CK2. Therefore, the phosphorylation of p27 by CK2 might play a role in the regulation of total or local protein unfolding/folding depending of whether the substrate site is phosphorylated or not. The change in the CD spectrum of p27 after phosphorylation by CK2 provides experimental evidence to support Zetina's proposal [Zetina, 2001], as the marginal secondary structure content observed was disrupted after that covalent modification. Thus, the incorporated phosphate group indeed seems to disrupt this marginal helix.

Although in experiments carried out in vitro no effect of phosphorylation by CK2 on the inhibitory activity of $p27^{KIP1}$ or cyclin A-Cdk2 was observed (data not shown), it is tempting to speculate that its phosphorylation by the CK2 holoenzyme may have other functional implications. One possibility is that protein kinase CK2 is involved in the regulation of p27 stability through the ubiquitin-dependent proteolytic degradation by the proteasome [Hershko and Ciechanover, 1998; Voges et al., 1999]. This possibility is supported by previous observations where CK2 was found to associate with this large multiprotein complex [Schwechheimer and Deng, 2001]. More recently, it was shown that diverse proteins or protein complexes, which are directly involved in proteasomal degradation of p27, are also substrates or interact with CK2. For example, mammalian E2 ubiquitin-conjugating enzyme, Cdc34, is phosphorylated by CK2 in proliferating cells, resulting in a translocation from cytoplasm to the nucleus [Block et al., 2001].

Of particular interest is the link between p27 degradation and a novel mediator of protein degradation, the COP9/CSN signalosome [Schwechheimer and Deng, 2001; Yang et al., 2002]. This nuclear multiprotein complex is highly conserved in all eukaryotes from budding and fission yeasts to humans and contains eight subunits which share significant sequence and structural homology with the subunits of the proteasome LID subcomplex [Voges et al., 1999; Kapelari et al., 2000]. Nevertheless, the precise role of COP9 signalosome in protein degradation is unclear, although its subunits interact with a number of transcription factors, receptors and cell cycle regulators, as $p27^{KIP1}$ [Schwechheimer and Deng, 2001]. In the particular case of p27, this protein interacts specifically with the subunit Jab1/CSN5 which functions as negative regulator by promoting its degradation [Tomoda et al., 1999]. Another Jab1-interacting protein is HY5, a basic leucine zipper transcription factor that promotes photomorphogenesis in *Arabidopsis* and, surprisingly, whose stability and activity are regulated by CK2-dependent phosphorylation in its COP1 binding domain [Hardtke et al., 2000].

Moreover, either the COP9 itself or a signalosome-associated protein functions as a protein kinase for the phosphorylation of several of its subunits and also of signalosome-interacting proteins [Schwechheimer and Deng, 2001]. Besides, it has been more recently shown that the protein kinases CK2 and PKD/PKC-μ are recruited by COP9 to differentially phosphorylate some of its subunits and protein substrates [Uhle et al., 2003]. Therefore, it is plausible that protein kinase CK2 is involved in protein degradation, possibly forming part of the proteasome or playing an intermediate role in an alternative proteasome-linked proteolytic pathway. In the particular case of p27, this pathway may be the same proposed by Malek et al. [2001] who suggested that a second degradation-dependent regulation of p27 must be activated by mitogens in order to control its protein level exclusively during the G_1 stage of the cell cycle. This new degradation pathway would be independent of the typical Cdk2-mediated phosphorylation on threonine 187 [Sheaff et al., 1997]. Whether the protein kinase CK2 is effectively involved in the in vivo proteasome-dependent degradation of $p27^{KIP1}$ is an aspect that also requires further study.

Two regions in $p27^{KIP1}$ that mediate the interaction with the CK2β subunit have been identified in this work, one located in its N-terminal half and the other in the C-terminal fragment. BIAcore experiments indicated that the two p27 fragments contribute differently to the total p27-CK2β association, the p27 amino-terminal half making the most impor-

tant contribution to this association. The determination of the subset of amino acid residues involved in direct contact between p27 and CK2β is under way.

It is likely that both $p27^{NT}$ and $p27^{CT}$ deletions lead to a dramatic conformational change, affecting the extent to which the CK2β-p27 interaction takes place. As a result of this conformational change, the ability of p27 to sample both local helical and unfolded structures, as observed for the p27 Cdk-inhibition domain, might be modified. In this respect, p27 exhibits a behavior contrary to random-coil ensemble proteins, which show no local conformational preferences [Bienkiewicz et al., 2002]. Moreover, there are several examples of proteins able to sample local secondary and unfolded structures such as peptide fragments with native-like residual structure [Sayers et al., 2000], denatured proteins [Dyson and Wright, 1996; Lee et al., 2000; Kisselev and Downs, 2003], and globular proteins where folding is importantly governed by kinetic stability [Bolanos-Garcia and Nunez Miguel, 2003].

It is worth emphasizing that the $p27^{NT}$ deletion mutant contains the cyclin and the Cdk binding regions, which are both necessary and sufficient to inhibit the cyclin A-Cdk2 or cyclin E-Cdk4 activity [Polyak et al., 1994; Toyoshima and Hunter, 1994; Bienkiewicz et al., 2002]. Other in vitro studies have defined the interaction regions between the CK2β subunit and another KIP family member, namely $p21^{CIP1}$ [Götz et al., 1996, 2000; Romero-Oliva and Allende, 2001]. It remains to be elucidated whether these regions constitute the same domains involved in the interaction between the CK2β subunit and p27.

The complete dependence of the phosphorylation of p27 on the presence of the CK2β subunit and the interaction of p27 with CK2β suggests that the phosphorylation reaction is preceded by the docking of p27 on the regulatory subunit. Indeed, the results from kinetic and BIAcore experiments showed $appK_M$ for $p27^{FL}$ phosphorylation by CK2 and the K_D for the CK2β and $p27^{FL}$ interaction yield, values are similar, which strongly suggest the existence of a docking event. This type of phenomenon has been described for other kinases, such as p38 [Chang et al., 2002], $p90^{RSK}$ [Kusk et al., 1999], and Cdks [Russo et al., 1996; Schulman et al., 1998]. In the particular case of Cdks, a specific region in the cyclin appears to be involved in the docking of substrates. It is pertinent to mention that the catalytic CK2α subunit and the *Cdk2* genes are phylogenetically related, and their expression products share the property of using accessory subunits to regulate their activity. Indeed, one of the functions assigned to CK2β is to confer specificity for substrates and inhibitors to the catalytic CK2α subunit [Allende and Allende, 1995; Pinna, 2002]. Thus, the role of the β subunit of protein kinase CK2 in substrate docking with other substrates is an aspect that requires further investigation.

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