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FAST TRACK

Protein p21$^{WAF1/CIP1}$ is Phosphorylated by Protein Kinase CK2 In Vitro and Interacts With the Amino Terminal End of the CK2 Beta Subunit

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Abstract Protein kinase CK2 is a ubiquitous protein that phosphorylates multiple substrates and is composed of catalytic (α, α′) and regulatory (β) subunits. Abundant evidence relates CK2 to the regulation of cell division. p21$^{WAF1/CIP1}$ is a potent inhibitor of cyclin-dependent kinases and of DNA replication and acts as a key inhibitor of cell cycle progression. In this work we examine the relation between these two important proteins. The interaction between the CK2β regulatory subunit of CK2 and p21$^{WAF1/CIP1}$ has been confirmed. Using a pull-down assay and fusion constructs of glutathione transferase with fragments of CK2β and other mutants, it was possible to define that the N-terminal (1-44) portion of CK2β contains a p21$^{WAF1/CIP1}$ binding site. CK2 reconstituted from recombinant α and β subunits can phosphorylate p21$^{WAF1/CIP1}$ in vitro. This phosphorylation is greatly enhanced by histone H1. p21$^{WAF1/CIP1}$ can inhibit the phosphorylation of substrate casein by CK2. This inhibition, however, seems to be due to competition by p21$^{WAF1/CIP1}$ as an alternate substrate since in order to observe inhibition it is necessary that the concentration of p21 be of the same order of magnitude as the casein substrate concentration. This competition is not related to the binding of p21$^{WAF1/CIP1}$ to CK2β because it can also be observed when, in the absence of CKβ, CKα is used to phosphorylate casein in the presence of the p21. J. Cell. Biochem. 81:445–452, 2001.

Key words: p21$^{WAF1/CIP1}$; cyclin-dependent protein kinase inhibitor; casein kinase 2; cell-cyle regulation; GST-fusion fragments of CK2β; histone H1 stimulation

The protein p21$^{WAF1/CIP1}$ plays a key role in the control of the cell cycle. It has been established that the transcriptional induction of p21$^{WAF1/CIP1}$ is one of the mechanisms through which the tumor suppressor protein p53 acts to block the progression of cells through their cell division cycle [El-Deiry et al., 1993]. The function of protein p21$^{WAF1/CIP1}$ is to inhibit the activity of the cyclin-dependent kinases (CDKs), a set of enzymes that drives cells through the different transition points that regulate cell division. P21$^{WAF1/CIP1}$ has been found to be a universal inhibitor of the CDKs [Xlong et al., 1993; Hengst et al., 1998] and in addition, it has been shown to bind PCNA inhibiting DNA replication but not the repair of the genetic material [Shen and Roberts, 1999].

Protein kinase CK2 is ubiquitous in eukaryotes and is analogous in structure to the CDKs, especially CDK2 [Allende and Allende, 1995; Pinna and Meggio, 1997; Guerra and Issinger, 1999]. CK2 is normally a heterotetramer composed of catalytic (α and α′) and regulatory (β) subunits. Similar to the regulatory role of cyclins in the case of the CDKs, the CK2β subunit greatly stimulates the catalytic activity of the α subunit with most protein substrates and promotes discrimination of specific substrates.

There is considerable evidence that relates CK2 to cell division. A CK2α transgene expressed under the control of immunoglobulin promoters increases the incidence of lymphomas in mice and in this action it is complemen-

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ted by a c-*myc* transgene [Seldin and Leder, 1995]. Overexpression of CK2α and α′ can complement *Ras* in generating foci of transformed cells in culture [Orlandini et al., 1998]. A decrease in CK2 activity obtained by antisense treatment or antibody microinjection causes the cell to stop in the G_1/S and the G_2/M transition points [Pepperkok et al., 1994]. Similar results are observed when yeast cells have been manipulated to contain a temperature-sensitive CK2α gene and are shifted to the nonpermissive temperature [Hanna et al., 1995]. CK2 activity and protein content have been shown to be higher in tumor and proliferating cells [Issinger, 1993], and the enzyme has been shown to phosphorylate a large number of proteins that are important regulators of cell division [Allende and Allende, 1995].

For the above reasons, the report by Götz et al., 1996, which demonstrated that $p21^{WAF1/CIP1}$ binds to the β subunit of CK2 and that this binding caused CK2 inhibition, created much interest in the possibility of a broader role for p21 in protein kinase regulation and stimulated us to examine this interaction in more detail. In the present communication, we confirm that CK2β does indeed interact with $p21^{WAF1/CIP1}$. Using a different assay we conclude that the first 44 amino acids in the amino terminal of CK2β contain the region of the molecule that interacts with $p21^{WAF1/CIP1}$. Our studies also indicate that $p21^{WAF1/CIP1}$ is a substrate for CK2 in vitro and that the inhibition of CK2 phosphorylating activity observed by the addition of $p21^{WAF1/CIP1}$ is probably due to its action as a competitive inhibitor of other protein substrates of the enzyme.

MATERIALS AND METHODS

Preparation of the $p21^{WAF1/CIP1}$ Recombinant Protein

The clone of the cDNA coding for the human $p21^{WAF1/CIP1}$ was kindly donated by Götz et al. [1996] and was used initially without further modification. This cDNA is inserted in the pQE-30 expression vector (pQE-30p21) in such a way that the expressed protein is fused at its amino terminal to a 6-histidine tag and also contains a consensus sequence for phosphorylation with cAMP-dependent protein kinase (PKA site) to facilitate detection. This vector construct was used throughout except where mentioned in Figure 3. The recombinant protein was expressed in M15 *E. coli* as described [Götz et al., 1996]. Since most of the expressed protein was insoluble, the pelleted bacterial lysates were treated with 4 M urea in a Tris–HCl buffer pH 7.6 containing 450 mM NaCl, 1 mM 2-mercaptoethanol, 0.05% NP-40 and 1 mM EDTA. The denatured protein was absorbed on a Ni^{2+} chelate nitrilotriacetic acid (NTA)-agarose column which was subsequently washed with a similar buffer but at pH 6.0. Washing continued until the absorbancy at A_{280} was less than 0.01. The $p21^{WAF1/CIP1}$ was then eluted with the same buffer but at pH 4.5. Renaturation of the protein was achieved by gradually reducing urea concentration through dialysis. The final dialysis buffer contained 50 mM Tris–HCl pH7.5, 150 mM KCl, 10 mM $MgCl_2$, 0.5 mM dithiothreitol and 5% glycerol. The activity of the $p21^{WAF1/CIP1}$ protein was tested by assaying its effect on the phosphorylation of histone H1 by CDK2 immunoprecipitated from HeLa extracts 3.7 ng of this $p21^{WAF1/CIP1}$ preparation completely inhibited the phosphorylation of histone H1 by immunoprecipitated CDK2.

To generate the plasmid pQE-$30p21^{AAM}$ in which the linker region on the His-tagged p21 recombinant protein did not include an artifactual CK2 phosphorylation site, the region was amplified by PCR using the primers 5′-TAATATGGATCCGCTGCAATGTCAGAACCG-3′ (sense primer with *Bam*HI site, underlined) and 5′-AACAGGAGTCCAAGCTCAGC-3′(antisense). The fragment was digested with *Bam*HI and *Hind*III, ligated to the vector pQE-30 to give pQE$p21^{AAM}$ and the protein expressed in *E. coli* as given above. The construct was confirmed by DNA sequencing. The overall changes made were the mutation of an aspartic acid and a serine residue to alanines and the insertion of the p21 first methionine residue.

Recombinant CK2 Subunits and Mutants

The recombinant CK2α and CK2β subunits from *Xenopus laevis* were expressed in *E.coli* and purified as described previously [Hinrichs et al., 1993; Cosmelli et al., 1997]. The CK2β subunit was produced as a fusion protein with glutathione transferase (GST) [Hinrichs et al., 1993] while the CK2α subunit contained a histidine tag to facilitate purification. A number of the CK2β mutations used in this work were previously prepared by the method of Ho et al. [1989] and described [Hinrichs et al., 1995]. These CK2β mutants include: $\beta G^{2,3}$, in which

autophosphorylation sites at serines 2 and 3 are replaced by glycines; βA^{58}, in which proline 58 is replaced by alanine; βA^{59-61}, in which aspartic acid 59 and glutamic acid residues 60 and 61 are replaced by alanines; $\beta A^{151-153}$, in which three histidines are changed to alanines; and β^{1-179}, in which 36 amino acids of the carboxyl end of the molecule are deleted.

Additional mutants of CK2β generated for this work were prepared. Several fragments of CK2β fused to GST in the pGEX-2T vector were generated by amplification with PCR using the original clone [Hinrichs et al., 1993]. $CK2\beta^{1-44}$ which contains the first 44 amino acids from the amino end, was generated using the sense primer 5′-AATATTAGAATTCCTATGGGACC-TGCTC-3′ and pGEX-2T vector primer 1 (5′-TA-CGAATTCAAATGAGTAGCTCG-3′);$CK2\beta^{1-106}$ which contains the amino half of the CK2β molecule was generated by using the sense primer 5′-AATATTAGAATTCCTAAAAATCT-CCCTG-3′ and the pGEX-2T vector primer 1; $CK2\beta^{40-106}$ was prepared by PCR amplification from the mutant $CK2\beta^{1-106}$ using sense primer 5′-TAATATTGAATTCAAAATGAGCAGGTCC-3′ and pGEX-2T vector primer 2 (5′-GGGGAA-TTCTCAACGCATGGTCT-3′).

All the mutations were confirmed by complete sequencing of DNA coding for the desired protein in an automatic Applied Biosystems Sequencer.

Protein kinase assays. Assays for both PKA and CK2 contained in a total volume of 30 μl, kinase buffer (50 mM HEPES pH 7.8, 150 mM NaCl, 10 mM $MgCl_2$, 3 μM ATP, and 0.5 mM dithiothreitol), protein substrate and kinase where indicated in each case. Reactions were started with the addition of [γ-^{32}P]ATP, specific activity of 85000 cpm/pmol. For the PKA assays, the substrate was the recombinant tagged $p21^{WAF1/CIP1}$ protein obtained using pQE-30 p21 which contained the sequence Arg.Arg.Ala.Ser.Val corresponding to the PKA consensus site located in the position −10 to −6 relative to the second amino acid of the p21 sequence and contiguous to the 6 histidine tag residues of this construct [Gotz et al., 1996]. For CK2 assays, p21, with or without an artifactual CK2 site and surrounding "linker" amino acids as described in Results, and/or casein was used as substrate as given in each case. Histone H1 and polylysine were used where indicated at 0.08 and 8.6 mg/ml, respectively. Following incubation for 30 min at 30°C, the samples were processed as given below for the pull down assay and resolved using 12% SDS-gel electrophoresis and autoradiography of the dried gels.

Assay for the interaction of $p21^{WAF1/CIP1}$ with GST-CK2β: pull-down assay. GST-CK2β or mutant forms were incubated with $p21^{WAF1/CIP1}$ recombinant His-tagged protein in the buffer used for the kinase assay as given above except with 250 mM NaCl and added 0.1% Tergitol NP-40, 0.1% Triton X-100, and 10% glycerol. After 30 min at 30°C, GSH-Sepharose beads were added and incubation continued for 45 min at 4°C. Binding of non-specific components was reduced by washing three times with 40 volumes of wash buffer (PBS pH 7.6, 0.1% Tergitol NP-40, and 1% Triton X-100) and once with kinase buffer. GSH-Sepharose beads were resuspended in 25 μl kinase buffer at 100 mM NaCl, 3 μM [γ-^{32}P]ATP and 3 units of PKA and incubated for 30 min at 30°C for the labeling of p21 in the PKA site present in the recombinant protein. The beads were washed three times as given above to remove the kinase reactants. Finally, interacting proteins were eluted with 70 μl of 25 mM glutathione. Gel-loading buffer [Laemmli, 1970] was added to the eluate and samples were run on 12% SDS-polyacrylamide gels. Gels were dried and exposed to autoradiographic film.

Materials

Oligonucleotides for PCR amplifications were prepared by Oligopeptido (the Core Facility of the University of Chile). cAMP-dependent protein kinase (PKA), histone H1, and polylysine 29 kDa (average) were purchased from Sigma Chemicals (St. Louis, MO). [γ-^{32}P]ATP was from ICN. The antibody anti-CDK2 (M2) was purchased from Santa Cruz.

RESULTS

Interaction of $p21^{WAF1/CIP1}$ With the β Subunit of CK2

In the original report of Götz et al. [1996] in which the interaction of p21 with CK2β subunit was described, this interaction was detected by a Far-western assay. In the present study, a "pull-down" assay was used in which GST-CK2β fusion protein or mutants of the β subunit were used and any complex formation with p21 could be detected by the retention on GSH-agarose beads. Using pQE-30p21 in which a PKA site

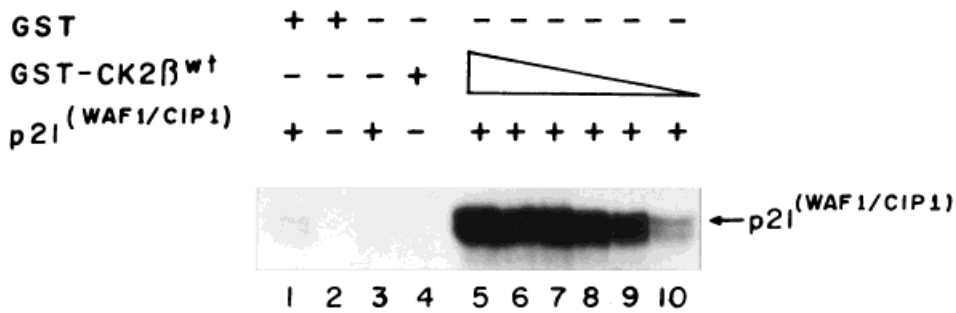

Fig. 1. Concentration-dependent interaction of GST-CK2β and p21. Pull-down assays were performed as described in Materials and Methods, using 49 pmol of p21 (indicated +) and, also where indicated, different concentrations of GST-CK2β. The amounts of GST-CK2β used were 57 pmol (**lanes 4** and **5**) and 46, 34, 23, 11, and 4 pmol (**lanes 6** through **10**). Phosphorylation of a PKA site inserted in the recombinant p21 was used to detect p21 resolved by SDS-gel electrohoresis followed by autoradiography. GST (93 pmol) was used as control (**lanes 1** and **2**).

forms part of the N-terminal sequence of the His-tagged recombinant p21, the bound p21 protein was subsequently labeled by incubation with PKA and [γ-^{32}P]ATP. After extensive washing, bound proteins were eluted with glutathione and analyzed by SDS-gel electrophoresis and autoradiography. Figure 1 shows that the binding of $p21^{WAF1/CIP1}$ is proportional to the amount of CK2β present in the assay. The use of GST alone (in large excess compared to that used as fusion protein) in control experiments shows the binding of a negligible amount of $p21^{WAF1/CIP1}$.

Similar experiments were performed using a constant amount of CK2β and increasing the amount of $p21^{WAF1/CIP1}$ in the incubation (not shown) and demonstrated that complex formation increases until saturation is reached with an approximate molar stoichiometry of 1:1. Using this same assay, a number of mutants and fragments of GST-CK2β were tested for their capacity to bind $p21^{WAF1/CIP1}$. Figure 2 shows the results obtained in these experiments. In Figure 2A, it is shown that a truncated form of CK2β, which lacks the carboxyl amino acids 180–215 ($CK2\beta^{1-179}$) binds $p21^{WAF1/CIP1}$ as efficiently as the wild-type full length protein. This mutant binds CK2α very poorly [Hinrichs et al., 1995]. Several replacement mutations that eliminate functional or structurally relevant sites of CK2β did not result in a reduction of the binding of $p21^{WAF1/CIP1}$ to β. Among the mutants tested were: $CK2\beta G^{2,3}$, which eliminates the autophosphorylation site of CK2β [Hinrichs et al., 1993]; $CK2\beta A^{59-61}$, and $CK2\beta A^{58}$, both of which hyperstimulate the catalytic activity of CK2α [Hinrichs et al., 1995]. It is also observed that $CK2\beta A^{58}$ shows stronger association with $p21^{WAF1/CIP1}$; however, in similar separate experiments, this mutant was only as efficient as the wild-type CK2β.

In Figure 2B the results obtained with other truncated forms of GST-CK2β are observed. It is clear that the amino terminal half of the molecule ($CK2\beta^{1-106}$) binds $p21^{WAF1/CIP1}$ very efficiently and that the extreme amino terminal ($CK2\beta^{1-44}$) can interact very well. On the other hand, the more central portion of the amino segment ($CK2\beta^{40-106}$) binds very poorly and compares to the control levels.

Recombinant $p21^{WAF1/CIP1}$ is a Substrate for CK2

Götz et al. [1996] reported that $p21^{WAF1/CIP1}$ was not significantly phosphorylated by CK2. Our results differ in that, as observed in Figure 3A, we find that the recombinant $p21^{WAF1/CIP1}$ obtained from the same clone used by these authors is phosphorylated by recombinant CK2. This phosphorylation catalyzed by CK2α is greatly enhanced by the presence of CK2β (lane 2). Interestingly the presence of low concentrations of histone H1 greatly stimulates the phosphorylation of $p21^{WAF1/CIP1}$ by the CK2 holoenzyme but not by CK2α alone (lanes 3 and 4). As this study was in progress, sequence analysis of the vector pQE-30p21 demonstrated that the structure of the recombinant p21 protein contained not only the His tag and PKA site sequences, but also that this linker region generated a possible CK2 site. Following the 6-histidine tag, the amino end linker sequence contains, as shown below, a PKA site (italics, underlined) and two serines, and two acidic amino acids that could form a poor CK2

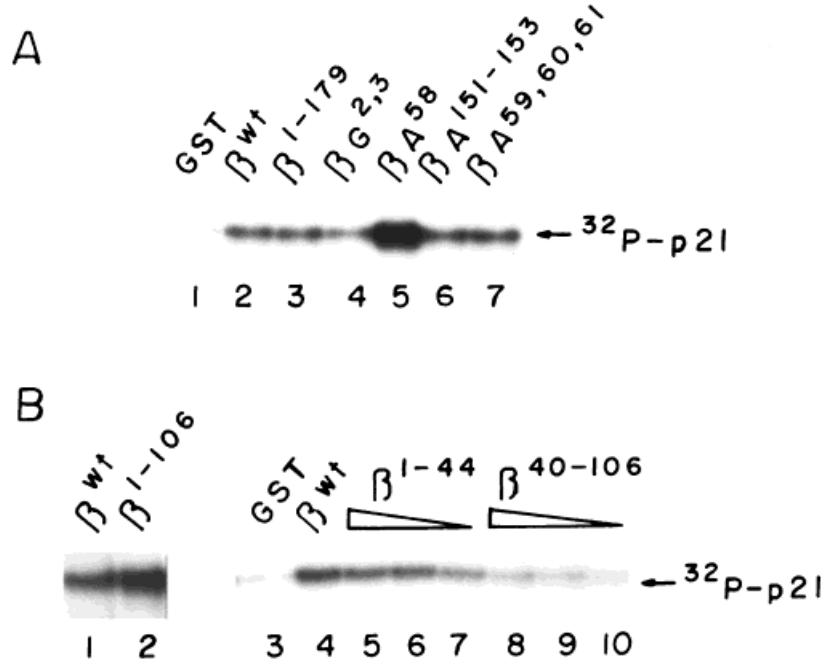

Fig. 2. Interaction of mutants of GST-CK2β with p21. **A**: 10 pmol GST-CK2β or mutant forms were incubated with 30 pmol of p21 using the pull-down assay and resolved as given in Figure 1. GST is a control that lacks CK2β (278 pmol, **lane 1**). **B**: The same as in part A except that for mutants β^{1-44} and β^{40-106} the amounts were 20 pmol (**lanes 5** and **8**), 10 pmol (**lanes 6** and **9**) and 5 pmol (**lanes 7** and **10**), respectively.

site (double underlined) in the context of the p21 sequence that follows (dashed line underlined)

pQE-30p21 ....M R G S $(H)_6$ G S *R R A S V* A G S D S S E P A G....

In order to remove this possible phosphorylation site from the linker region, an aspartic acid and serine residue were both mutated to alanine and in addition, a methionine was inserted, as shown below, to give vector pQE-30p21^{AAM} as described in Materials and Methods.

pQE-30p21^{AAM} M R G S $(H)_6$ G S *R R A S V* A G S A A *M S E P A G*....

In Figure 3B it is seen that the recombinant p21$^{WAF1/CIP1}$ in which these amino acids have been removed is still phosphorylated (lanes 3 and 4) but at a lower level. The phosphorylation is also greatly enhanced by the presence of histone H1.

Inhibition of CK2 Activity Caused by the Presence of p21$^{WAF1/CIP1}$

The p21$^{WAF1/CIP1}$ protein is a potent general inhibitor of CDKs. In their publication Götz et al. [1996], reported that p21$^{WAF1/CIP1}$ was also a strong inhibitor of CK2 phosphorylation activity. In our initial results we failed to observe inhibition of CK2 phosphorylation of casein by adding p21$^{WAF1/CIP1}$ at levels that completely inhibited CDK2-cyclin A. Only when we lowered the casein substrate concentration to levels similar to those of p21$^{WAF1/CIP1}$ were we able to observe the inhibitory effect (Fig. 4). In Figure 4, we also see that the addition of the p21$^{WAF1/CIP1}$ expressed from the original clone of pQE-30p21 causes inhibition of casein phosphorylation by CK2 (compare lanes 2 and 3). The inhibition is competitive since when casein concentration is increased, the inhibition caused by p21$^{WAF1/CIP1}$ tends to disappear (Fig. 4A, lanes 4 and 5). Densitometry of the bands corresponding to casein phosphorylation at 1 and 0.2 μg levels showed 10 and 95% inhibition, respectively, in the presence of p21. Similar results were obtained with p21$^{WAF1/CIP1}$ expressed from pQE-30 p21^{AAM} (not shown).

The inhibition of CK2 activity is related most probably to the capacity of p21$^{WAF1/CIP1}$ to act as a competitive substrate and is not related to the interaction with CK2β. This conclusion is supported by the results shown in Figure 4B in which a similar inhibition of casein phosphorylation by p21$^{WAF1/CIP1}$ is observed (compare lanes 1 with 2 and 4 with 5) when the CK2α catalytic subunit is used.

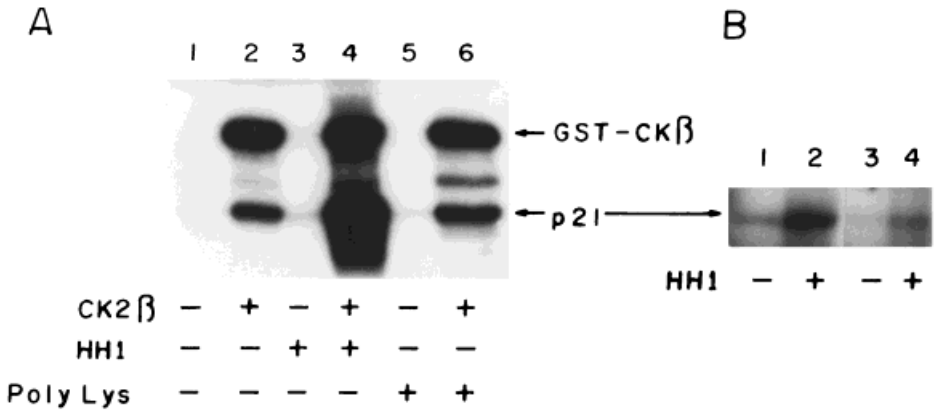

Fig. 3. $p21^{WAF1/CIP1}$ is a substrate of CK2. **A**: Phosphorylation of 16 pmol p21 (prepared from expression vector pQE-30p21) was carried out with CK2α (**lanes 1, 3, 5**) or CK2 holoenzyme (CK2α + β, **lanes 2,4,6**) in the absence (−) or presence (+) of 0.08 μg/ml of histone H1 or 8.6 μg/ml of polylysine. **B**: Phosphorylation of 20 pmol of p21 (prepared from pQE-30p21, **lanes 1** and **2**, or from pQE-30$p21^{AAM}$, **lanes 3** and **4**) using 1 pmol of CK2 holoenzyme and where indicated with 0.06 μg/ml of histone H1.

DISCUSSION

The results presented above contribute to increasing our understanding of the relationship existing between two proteins that play important roles in cell division: $p21^{WAF1/CIP1}$ and protein kinase CK2.

The work described here confirms an observation previously reported by Götz et al. [1996] regarding the binding of CK2β to $p21^{WAF1/CIP1}$. In this report, using a different assay, we have defined that the region encompassing amino acids 1–44 of CK2β contains a binding site for $p21^{WAF1/CIP1}$. Previously, we had also demonstrated that immunoprecipitation of CK2α and β subunits, overexpressed in COS-7 cells through transfection, resulted in the co-precipitation of $p21^{WAF1/CIP1}$ that had been co-transfected in these cells [Korn et al., 1998]. When the present work was in progress, a second report by Götz et al. [2000] appeared in which, using truncated constructs of CK2β and synthetic peptides corresponding to parts of the protein, three regions of CK2β corresponding to amino acids 9–31, 49–63 and 201–215 were defined as being responsible for binding $p21^{WAF1/CIP1}$. Their first region (9–31) coincides very well with the findings described in our work. The second region (49–63) was detected by them through the incubation of the corresponding pentadecapeptide. This finding does not agree with our observation that the GST-CK2β fragment 46–106 is not efficient in pulling down $p21^{WAF1/CIP1}$. The region (201–215) was likewise not found through our analysis although our observation that is relevant in this respect is that CK2β (1–179), which lacks the region 201–215 is as efficient as wild-type CK2β in the interaction with $p21^{WAF1/CIP1}$ in our soluble assay system even at 250 mM NaCl. Obviously, the different methods used may be the source of the discrepancy. Further study is required to resolve this point. One interesting question that arises from the results of Götz

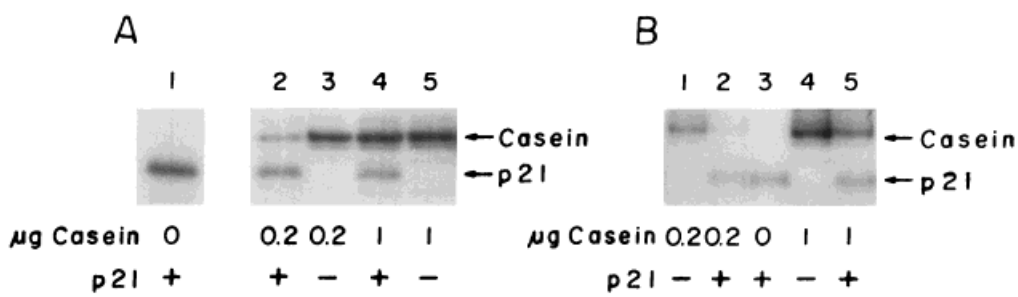

Fig. 4. Competitive phosphorylation of $p21^{WAF1/CIP1}$ and casein substrates. The effect of p21 (2 μg) on casein phosphorylation was studied using reconstituted CK2 holoenzyme (0.1 pmol) (**A**) or CK2 catalytic subunit α (approx. 0.2 pmol) (**B**), and casein in the amounts indicated.

et al. [2000] is whether the same region of the $p21^{WAF1/CIP1}$ can interact with all three regions of CK2β or whether different regions, or different molecules of $p21^{WAF1/CIP1}$ are binding to these different parts of CK2β which the three-dimensional structure analysis indicates are relatively distant [Chantalat et al., 1999]. Our results obtained on the stoichiometry of the interaction would suggest that only one mole of $p21^{WAF1/CIP1}$ binds to each mole $CK2\beta^{WT}$.

Since CK2β is an abundant protein in most cells and since the amount of the CK2 subunits is greatly increased in proliferating cells, it is possible that the interaction of CK2β with $p21^{WAF1/CIP1}$ may represent a mechanism for sequestering the CDK inhibitor. This withdrawal of free $p21^{WAF1/CIP1}$ may stimulate proliferation.

On the other hand, the binding of CK2β of $p21^{WAF1/CIP1}$ is another example of a CK2 substrate that binds the regulatory subunit. Similar interactions with the β subunit have been observed with p53, topoisomerase II, CD5, and Nopp 140. It is interesting, however, that different regions of CK2β molecule are involved in these interactions. With p53, the binding region of CK2β has been reported to be included between amino acids 71 and 149 [Appel et al., 1995]. In the case of Nopp140 the first 20 amino acids of the amino terminus of CK2β have been suggested as responsible for the binding [Li et al., 1997]. In the case of topoisomerase II, the region of CK2β that binds the substrate protein is a central region (amino acids 51–110) [Leroy et al., 1999]. The amino half of the CK2β (amino acids 2–132) was reported to bind CD5 [Raman et al., 1998].

The interaction with substrates through docking sites that differ from the catalytic center of the enzyme may be a general phenomenon of kinases, representing a mechanism through which the enzyme increases its selectivity for protein substrates [Schulman et al., 1998]. The great promiscuity of CK2β in the interaction with many proteins may partly explain the large number of substrates that are known to be phosphorylated by CK2 [Allende and Allende, 1998].

The work of this report demonstrates that CK2 can phosphorylate $p21^{WAF1/CIP1}$. The original construct of Götz et al. [1996] which we used to produce recombinant p21 tagged with a PKA site also produced an artifactual CK2 site that was responsible for part of the phosphorylation that we observed. Removal of that site reduced but did not eliminate the phosphorylation of $p21^{WAF1/CIP1}$ by CK2 (Fig. 3). The intensity of the phosphorylation of $p21^{WAF1/CIP1}$ was greatly enhanced by the presence of the CK2β subunit as is seen with most CK2 substrates. A rather unusual observation is the finding that addition of histone H1 greatly stimulates $p21^{WAF1/CIP1}$ and that this effect is much stronger than that observed with polylysine. The preparation of polylysine used in these experiments stimulated twofold the phosphorylation of casein by CK2. No physiological meaning can be deduced from the observation that histone H1 can greatly stimulate $p21^{WAF1/CIP1}$ phosphorylation but it is interesting that such an abundant cellular protein has this effect. There have been several reports that claim that $p21^{WAF1/CIP1}$ is phosphorylated in vivo [Erhardt and Pittman, 1998; Scott et al., 2000] but the site of those phosphorylations or the kinase involved have not been clearly defined.

The observation that $p21^{WAF1/CIP1}$ can inhibit CK2 phosphorylation of protein substrates as described by Götz et al. [1996] has also been confirmed in this report. However, the concentrations of $p21^{WAF1/CIP1}$ necessary to observe the inhibition are very high, indicating amounts of inhibitor close to two orders of magnitude more than the amount of the enzyme. This finding together with our finding that $p21^{WAF1/CIP1}$ is a substrate, albeit a poor one, for CK2, would indicate rather that the inhibition observed could be explained by simple competitive inhibition by an alternative substrate. This supposition is strengthened by the observation that the inhibition of casein phosphorylation caused by a constant amount of $p21^{WAF1/CIP1}$ is reduced when the concentration of casein is increased and that the amounts of $p21^{WAF1/CIP1}$ required to see inhibition are similar, equal, or greater than the amount of the casein substrate. In their experiments Götz et al. [1996] used 1 μg of casein and 0.5 μg of $p21^{WAF1/CIP1}$ to observe inhibition for 20 ng of CK2.

Our results further demonstrate that the inhibition by $p21^{WAF1/CIP1}$ of the phosphorylation of casein by CK2 is also observed when the assay is carried out in the presence of CK2α alone and in the absence of CK2β. These results clearly separate the binding of CK2β to $p21^{WAF1/CIP1}$ from the inhibition of casein phosphorylation caused by large concentrations of $p21^{WAF1/CIP1}$.

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