Journal of Cellular Biochemistry 81:172–183 (2001)

Wild-Type p53 Inhibits Protein Kinase CK2 Activity

Norbert Schuster, Claudia Götz, Michael Faust, Eberhard Schneider, Alexandra Prowald, Andreas Jungbluth, and Mathias Montenarh*

Medical Biochemistry and Molecular Biology, University of the Saarland, D-66424 Homburg, Germany

Abstract The growth suppressor protein p53 and the protein kinase CK2 are both implicated in cellular growth regulation. We previously found that p53 binds to protein kinase CK2 via its regulatory β-subunit. In the present study, we analyzed the consequences of the binding of p53 to CK2 for the enzymatic activity of CK2 in vitro and in vivo. We found that the carboxy-terminus of p53 which is a potent transforming agent stimulated CK2 activity whereas full length wild-type p53 which is a growth suppressor inhibited the activity of protein kinase CK2. Inhibition of protein kinase CK2 by p53 was dose-dependent and was seen for various CK2 substrates. Experiments with heat-denatured p53 and the conformational mutant $p53_{R175H}$ revealed that an intact conformation of p53 seemed to be necessary. Transfection of wild-type and of mutant p53 into $p53^{-/-}$ cells showed that the inhibition of p53 on CK2 activity was also detectable in intact cells and specific for wild-type p53 indicating that the growth suppressing function of p53 might at least be partially achieved by down-regulation of protein kinase CK2. J. Cell. Biochem. 81:172–183, 2001. © 2001 Wiley-Liss, Inc.

Key words: growth supperessor; p53; protein kinase; phosphorylation

The growth suppressor protein p53 is a key regulator of the cell cycle and cell proliferation [Prives and Hall, 1999]. Usually, levels of p53 in the cell are low due to the short half life of the protein. However, in cases of DNA damage or deregulation of the nucleotide pool in the cell p53 is stabilized and induces a G_1- or G_2-phase arrest of the cell cycle or even causes cell death [for reviews see Levine, 1997; Bates and Vousden, 1999]. In order to fulfil these various functions in the cell p53 is a target for extended post-translational modifications. p53 is a substrate for various protein kinases such as dsDNA-activated protein kinase [Lees-Miller et al., 1990], cyclin H/cdk7/Mat1 [Ko et al., 1997; Lu et al., 1997], protein kinase CK1 [Knippschild et al., 1997], protein kinase CK2 [Meek et al., 1990; Herrmann et al., 1991], protein kinase C [Baudier et al., 1992], JNK1, JNK2, JNK3 [Hu et al., 1997] and $p34^{cdc2}$ [Bischoff et al., 1990]. In some cases it is known that phosphorylation by these kinases leads to altered activities of p53 [for review see Jayaraman and Prives, 1999]. In addition to these post-translational modifications, binding of cellular and viral proteins to p53 modifies its activities. Binding of mdm2 to p53 results in an inactivation of p53 by a translocation of p53 from the nucleus to the cytoplasm followed by degradation, or inhibition of the transactivation function of p53 [Prives, 1998]. Under non-physiological conditions binding of the p53-specific monoclonal antibody PAb421 to the carboxy-terminus of p53 modifies the DNA binding activity of p53 at least in vitro [Hupp, 1999]. On the other hand, only little is known of how binding of p53 to cellular proteins alters their activity. Binding of p53 to ERCC2 and ERCC3 results in an inhibition of the activity of these two helicases [Wang et al., 1996]. Binding of p53 to RAD51 is directly implicated in the regulation of recombination processes [Buchhop et al., 1997]. It was recently shown that binding of p53 to the cyclin H/cdk7/Mat1 complex leads to an inhibition of the kinase activity of this CAK complex which results in a lower T-loop phosphorylation of cyclin-dependent kinases and in an inhibition of the CTD phosphorylation of RNA polymerase II [Schneider et al., 1998]. Binding of the regula-

Grant sponsor: Deutsche Forschungsgemeinschaft; Grant numbers: Mo 309/11-1; Mo 309/11-3.

*Correspondence to: Dr. M. Montenarh, Medical Biochemistry and Molecular Biology, University of the Saarland, Building 44, D-66424 Homburg, Germany.
E-mail: tm13mm@rz.uni-sb-de

Received 21 March 2000; Accepted ? ? ? ?

This article published online in Wiley InterScience, January XX, 2001.

tory β-subunit of protein kinase CK2 to p53 leads to a reduction in the DNA binding activity [Prowald et al., 1997] and to a reduction in the transactivation function of p53 [Schuster et al., 1999]. Protein kinase CK2 is a ubiquitous serine/threonine protein kinase which is composed of two regulatory β- and two catalytic α- or α′- subunits [Singh et al., 1985; Pinna and Meggio, 1997]. Although its precise function in the cell is still unclear there is ample evidence that CK2 plays an important role in the regulation of cell proliferation. The activity of CK2 is elevated in tissues with a high proliferation rate, such as tumors and embryonic tissue [Prowald et al., 1984; Münstermann et al., 1990]. By microinjection experiments using CK2 antibodies or antisense oligonucleotides it was shown that cells arrest in certain phases of the cell cycle [Pepperkok et al., 1994]. Thus, it is an interesting question how p53 might regulate the activity of CK2. Therefore, in the present study we analyzed the effect of full length wild-type p53 on the enzymatic activity of CK2 in an in vitro assay and under in vivo conditions. We found that full length p53 efficiently inhibits the activity of protein kinase CK2 whereas mutant p53 is inactive. Monoclonal antibody PAb421 which is known to activate the DNA binding activity of p53 in vitro has no influence on the inhibition of the enzymatic activity of CK2 by p53. Furthermore, heat-denaturing of p53 results in a loss of the inhibition function of p53 indicating that a native conformation of p53 is necessary for its inhibiting activity on protein kinase CK2. This finding is substantiated by the conformational mutant $p53_{R175H}$, which is also defective for the inhibitory function of p53 on CK2 activity in vitro and in vivo.

MATERIALS AND METHODS

Plasmids and Recombinant Baculovirus

Human CK2 holoenzyme was bacterially expressed from a bicistronic expression vector [Shi et al., 1994]. For the bacterial expression of wild-type p53, mutant $p53_{R175H}$ and the C-terminal fragment of p53, we used previously published constructs cloned into pET19b (Novagen) or pQE (Qiagen) vector, respectively [Appel et al., 1995]. Cyclin H and mdm2 were expressed in bacteria using previously described constructs [Guerra et al., 1997; Schneider et al., 1998]. Plasmids pCMV30, $pRcCMVp53_{R175H}$ and pRcCMVp53 for transfection of mammalian cells, and the baculovirus expression construct were described earlier [Prowald et al., 1997; Schuster et al., 1999].

Expression and Purification of Bacterially Expressed Proteins

CK2 holoenzyme was expressed and purified as described earlier [Prowald et al., 1997]. Plasmids for pQE-cyclin H, pQE-$CT_{264-393}$ and pQEmdm2 were transformed into *E. coli* M15 strain whereas pET19b-p53wt and pET19b-$p53_{R175H}$ were transformed into *E. coli* strain BL21 (DE3). Clones were incubated overnight in 50 ml LB-medium. The following morning 1 liter LB-medium was inoculated with the overnight culture, grown to early log phase and induced with 1 mM isopropyl-β-D-thiogalactoside (IPTG) for 6 h at 30°C. Cells were harvested by centrifugation and resuspended in 6 M guanidine hydrochloride, 0.1 M sodium phosphate, pH 8.0 and lysed overnight at 4°C. The lysate was cleared by centrifugation and loaded onto a pre-equilibrated Ni^{2+}-chelate agarose column and incubated for 1 h at room temperature. The column was washed with 10 vol of lysis buffer, followed by 10 vol of lysis buffer pH 6.0, and finally with lysis buffer pH 8.0 containing 20 mM imidazole. Proteins were eluted with lysis buffer containing 300 mM imidazole and subsequently dialyzed overnight against dialysis buffer A (20 mM Tris–HCl, pH 7.5, 100 mM KCl, 5 mM $MgCl_2$, 0.1% Tween 20).

Purification of Nucleolin

Nucleolin was purified according to a protocol published by Belenguer et al. [1990] with minor modifications. HeLaS3 cells from twenty 15 cm dishes were lysed in low ionic strength buffer (10 mM MES, pH 6.2, 10 mM $MgCl_2$, 10 mM NaCl, 10% glycerol). Nuclei were pelleted and extracted with extraction buffer (25 mM Tris–HCl, pH 10.5, 0.5 M NaCl, 1 mM EDTA, 5 mM DTT, 0.5% Triton X-100). The neutralized extract was loaded onto a hydroxyapatite column and washed with an extraction buffer. Nucleolin was eluted in 300 mM phosphate buffer and subsequently precipitated with 65% ammonium sulfate. The pellet was resuspended in buffer A 175 (175 mM NaCl, 25 mM Tris–HCl, pH 8.0, 2 mM EDTA, 10 mM β-mercaptoethanol, 7.5% glycerol) and

loaded onto a heparin sepharose column. Nucleolin was eluted with buffer A 500 (500 mM NaCl, 25 mM Tris–HCl, pH 8.0, 2 mM EDTA, 10 mM β-mercaptoethanol, 7.5% glycerol).

Cell Culture and Transfection

$p53^{-/-}$ fibroblasts were maintained in DMEM supplemented with 10% foetal calf serum (FCS). Cells were grown to subconfluence in 10 cm dishes and transfected with either pCMV30, $pRcCMVp53_{R175H}$, and pRcCMVp53 using superfect transfection reagent (Qiagen). Transfection was carried out according to the instruction manual. Cells were harvested after 6 h and washed three times with PBS and lysed with extraction buffer (100 mM Tris–HCl, pH 7.5, 100 mM NaCl, 0.5 NP40). After determination of the protein content, equal amounts of protein were applied either to SDS-polyacrylamide gel electrophoresis followed by Western blot or immunoprecipitation.

Antibodies and Western Blot Analysis

For the detection of protein kinase CK2 we used monoclonal antibodies 1AD9 or 1A5 against the α-subunit and monoclonal antibody 6D5 against the β-subunit. Polyclonal rabbit serum #32 was raised against a peptide corresponding to the last 10 amino acids of the β-subunit. For the detection of p53 we used either monoclonal antibody DO-1 or PAb421. The preparation of cell extracts, SDS-polyacrylamide gel electrophoresis, and blotting procedure were described earlier [Schuster et al., 1999]. For detection we used the ECL system (Amersham).

Far Western Blot Analysis

Equal amounts of p53 were subjected to SDS-polyacrylamide gel electrophoresis. The gel was incubated for 1 h at 4°C in 10× concentrated PBS for renaturation of the protein. The protein was transferred onto a PVDF membrane and blocked for 1 h in binding buffer (50 mM Tris–HCl, pH 7.4, 150 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, 0.1% Tween 20) containing 5% dry milk. One part of the filter was preincubated for 30 min with PAb421. Subsequently, both filters were incubated with autophosphorylated protein kinase CK2 in binding buffer for 2 h. After intensive washing with binding buffer filter strips were subjected to autoradiography.

Immunoprecipitation

For immunoprecipitation we used a polyclonal serum (serum #32) raised against the C-terminus of protein kinase CK2 β-subunit. A protein A/G-sepharose mixture was preincubated for 1 h with 50 μl serum #32 and washed three times with phosphate-buffered saline, pH 7.3 (PBS). One milligram of cell extract was preincubated with a mixture of protein A- and protein G-sepharose (Pharmacia) to remove non-specifically bound proteins. The supernatant was applied to the preincubated sepharose antibody matrix and incubated for 1 h. The supernatant was removed and the antibody matrix washed three times with PBS. The immunoprecipitate was split into two equal aliquots and subjected either to SDS-polyacrylamide gel electrophoresis, followed by Western blotting, or to CK2 protein kinase assay.

In Vitro Phosphorylation by Protein Kinase CK2 and PKA

Protein kinase CK2 (10 pmol) was incubated for 1 h on ice to allow complex formation with p53 in different amounts as indicated in the experiments (10–30 pmol). Thereafter, 5 μg of the substrate in 10 μl kinase buffer (50 mM Tris–HCl, pH 7.5, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT) containing 3 μCi $^{32}P\gamma ATP$ per vial was added and incubated at 37°C (the total reaction volume was 40 μl). Subsequently, 5 × concentrated SDS-sample buffer (65 mM Tris–HCl, pH 6.8, 0.01% bromophenol blue, 5% β-mercaptoethanol, 10% glycerol, 2% SDS) was added, samples were boiled at 95°C for 5 min and subjected to SDS-polyacrylamide gel electrophoresis followed by autoradiography. Tubulin is commercially available (Molecular Probes, Leiden, Netherlands), the other substrates were purified from bacteria or HeLa cells as described. For PKA reactions (commercially available catalytic subunit from Sigma) the same experimental procedures were performed.

Peptide Phosphorylation

Protein kinase CK2 was preincubated with increasing amounts of p53 or mutant p53 in a total volume of 20 μl. Subsequently, 30 μl kinase buffer containing the CK2-specific peptide (0.32 mM peptide RRRDDDSDDD) and $^{32}P\gamma ATP$ were added and the kinase reaction was performed for 10 min at 37°C (the final reaction

volume was 50 μl). Thirty microliters of the reaction mix were spotted onto P11 cation exchange paper (Whatman), washed three times with 10 mM phosphoric acid and once with ethanol. The papers were dried and phosphate incorporation was measured in a scintillation counter.

RESULTS

We have recently shown that mdm2 is efficiently phosphorylated by protein kinase CK2 and moreover that the C-terminus of p53 ranging from amino acid 264 to amino acid 393 can stimulate the phosphorylation of mdm2 by CK2 [Guerra et al., 1997]. The C-terminus of p53 is basic [Soussi et al., 1990] and therefore might resemble polyamines which are known to stimulate the activity of CK2 [LeRoy et al., 1995]. To understand the interaction of p53 with CK2 it is necessary to analyze the effect of full length wild-type p53 on the activity of CK2 since this form is the physiologically relevant form of the protein. First of all, we wanted to compare the influence of full length p53 with that of the C-terminus of p53 on the activity of CK2. For these experiments we used recombinant human CK2 and performed kinase reactions in the presence of increasing amounts of bacterially expressed purified full length p53 or the C-terminus of p53 in equal molar amounts. Using the recently discovered new substrate for CK2, namely mdm2 and in agreement with our previous report [Guerra et al., 1997] CK2 kinase activity was stimulated in the presence of the C-terminal fragment of p53 (Fig. 1A) in a dose-dependent manner. In contrast, equal molar amounts of full length p53 showed an inhibitory effect on the phosphorylation of mdm2 and this inhibition was also dose-dependent (Fig. 1B). Thus, we conclude that wild-type full length p53 is a potent inhibitor of protein kinase CK2 at least with regard to the phosphorylation of mdm2 whereas under the same experimental conditions the C-terminus of p53 is a potent activator of CK2 activity.

From a number of other studies it is known that p53 can adopt a so-called latent form which can be activated by various factors, such as monoclonal antibody PAb421 [Hansen et al., 1996] at least with respect to the DNA binding and transactivation function of p53. Therefore, we wanted to analyze whether PAb421 might influence the inhibiting function of p53. First,

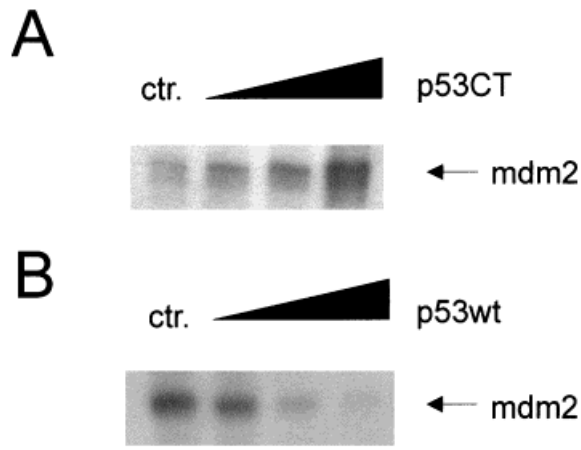


Fig. 1. Influence of the p53 C-terminus (p53CT) and p53 wild-type protein (p53wt) on the activity of protein kinase CK2. Protein kinase CK2 was incubated for 1 h on ice with control buffer, 10, 20, or 30 pmol of either p53CT or wild-type p53. After the incubation period, the substrate (mdm2) and $^{32}P\gamma ATP$ was added and a kinase reaction performed as described in Section 2. Samples were subjected to SDS gel electrophoresis followed by autoradiography. (**A**) Shows the influence of p53 CT on CK2 kinase activity. (**B**) Shows the influence of wild-type p53 on CK2 kinase activity. Ctr.: mdm2 phosphorylation by CK2 in the absence of p53.

we had to analyze whether PAb421 had an influence on the interaction of CK2 with p53. p53 was separated on an SDS-polyacrylamide gel and transferred to a PVDF filter. The filter was incubated with 100 pmol of autophosphorylated CK2 without or in the presence of PAb421 in comparable amounts, which we used for kinase reactions (Fig. 2B). As shown in Figure 2A PAb421 did not inhibit binding of CK2 to p53. Thus, in the next step we analyzed the influence of PAb421 binding to p53 for its inhibitory function on the enzymatic activity of CK2. The kinase assay was repeated as in Figure 1 but in the presence of PAb421. As shown in Figure 2B mdm2 is efficiently phosphorylated by protein kinase CK2 (lane 1). In the presence of full length p53 phosphorylation of mdm2 was considerably reduced and simultaneously p53 was phosphorylated (lane 2). The presence of PAb421 had no influence on the reduction of the phosphorylation of mdm2 by CK2 in the presence of p53 but there was a clear reduction in the phosphorylation of p53 in the presence of PAb421 (lane 3). This result is in agreement with a previous observation showing that PAb421 led to a reduction of the phosphorylation of p53 by a p53-associated protein kinase, which was later on identified as CK2 [Kraiss et al., 1990]. Furthermore, this

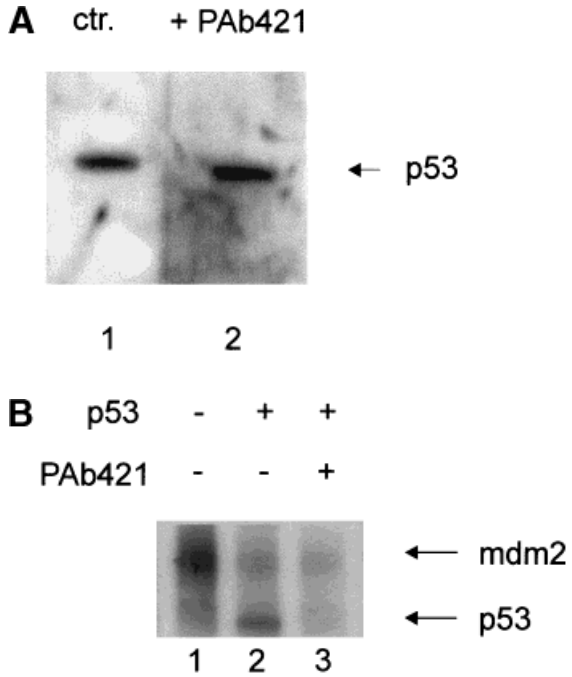


Fig. 2. Influence of monoclonal antibody PAb421 on the interaction of p53 with protein kinase CK2. (**A**) Far Western analysis of complex formation between p53 and protein kinase CK2. Purified p53 was separated on a SDS-polyacrylamide gel, renatured and transferred onto a PVDF membrane. One lane was preincubated for 30 min with binding buffer without (ctr.), the other with PAb421 (+PAb421). Subsequently, membranes were incubated with autophosphorylated CK2. Bound proteins were visualized by autoradiography. (**B**) p53 was preincubated for 30 min with PAb421 and then added to protein kinase CK2 and further incubated for 1 h to allow complex formation (lane 3). As a control CK2 was incubated in dialysis buffer (lane 1) or with p53 which was not preincubated with PAb421 (lane 2). Kinase reaction was performed with mdm2 as a substrate. Samples were separated on a 10% SDS-polyacrylamide gel. Phosphorylated mdm2 was visualized by autoradiography.

result demonstrated that the reduction in the phosphorylation of mdm2 by CK2 in the presence of p53 is not due to a competing phosphorylation of p53 since the inhibitory effect is even maintained when p53 is not phosphorylated simultaneously.

We described mdm2 as a new substrate for CK2 and a stimulating effect of the C-terminus of p53 toward mdm2-phosphorylation [Guerra et al., 1997]. Therefore, the experiments described so far were performed with mdm2 as a substrate. In addition, a great number of other substrates for CK2 are known such as a synthetic peptide with the sequence RRR DDD SDDD [Schneider et al., 1988]. This synthetic peptide with a consensus phosphoacceptor site for CK2 was widely used as a substrate [Sarno et al., 1997] and therefore, we analyzed the effect of full length p53 on the phosphorylation of this substrate by CK2. The kinase reaction described above was performed in the presence of increasing concentrations of full length p53 or heat-inactivated p53 as a control. Incorporation of ^{32}P-phosphate in the synthetic peptide was measured by Cerenkov counting in a scintillation counter. As shown in Figure 3 the presence of full length p53 led to a reduction in the phosphorylation of the peptide to about 46%. Heat-denatured full length p53 was no longer active in inhibiting the CK2 kinase activity as shown by the phosphorylation of the synthetic peptide by CK2 (p53wt ha).

Next, we analyzed whether full length p53 might also influence the phosphorylation of other known CK2 substrates such as nucleolin and tubulin. Recently, we discovered that cyclin H was also a substrate for CK2 [Schneider

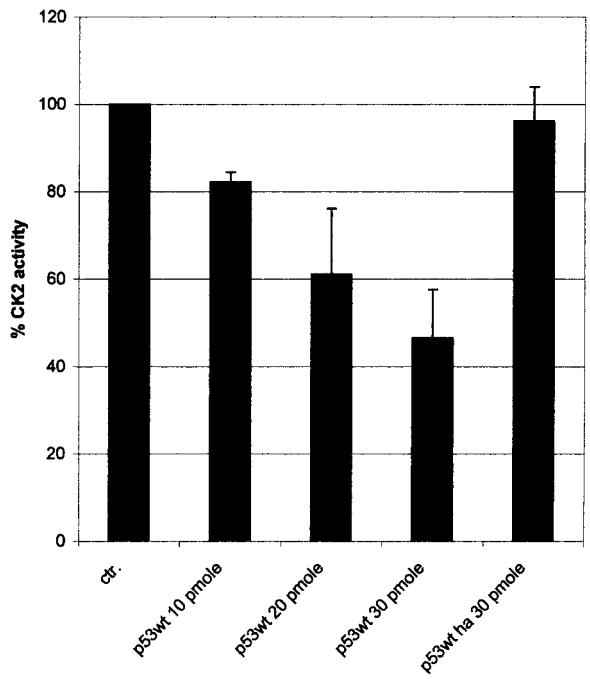


Fig. 3. Phosphorylation of the synthetic peptide RRRDDD-SDDD by CK2 in the absence or presence of wild- type p53. Protein kinase CK2 was incubated for 1 h with dialysis buffer or increasing amounts of wild-type p53 or heat-denatured p53 (p53wt ha) (10, 20, 30 pmol). The synthetic substrate and ^{32}PγATP were added and a kinase reaction was performed. Incorporation of radioactive phosphate was measured in a scintillation counter. The results from three independent experiments are shown (error bars represent standard deviation).

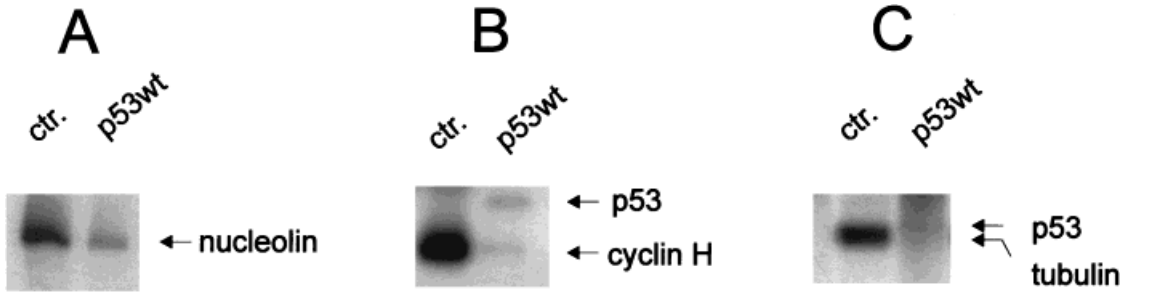


Fig. 4. Phosphorylation of nucleolin, cyclin H and tubulin by CK2 in the absence or presence of wild-type p53. Kinase reaction was performed in the absence (ctr.) or in the presence of wild-type p53. Proteins were separated by SDS-polyacrylamide gel electrophoresis and visualized by autoradiography. (**A**) nucleolin; (**B**) cyclin H; (**C**) tubulin.

et al., in preparation]. We repeated the kinase reaction described above in the absence or in the presence of full length p53 and with nucleolin, cyclin H, and tubulin as substrates. The phosphorylated proteins were subsequently analyzed on an SDS-polyacrylamide gel followed by autoradiography. As shown in Figure 4A–C in all three cases full length p53 led to a reduction of phosphate incorporation into these different substrates. Thus, these experiments showed that full length p53 inhibited the phosphorylation of a variety of different known substrates of CK2 indicating that this property is not restricted to mdm2 phosphorylation but most likely a general phenomenon.

In order to verify further the specificity of the inhibitory effect of p53 on the activity of protein kinase CK2 we next analyzed whether p53 might also inhibit other kinases. For these experiments we used protein kinase A from the heart muscle which is commercially available. As a substrate we used a yeast protein termed vip1 [Jungbluth et al., in preparation] which harbors a synthetic PKA phosphorylation site. Protein kinase A was incubated with vip1 and $^{32}P\gamma ATP$ in the absence or presence of increasing concentrations of full length p53. The phosphorylated vip1 protein was subsequently analyzed by SDS-polyacrylamide gel electrophoresis followed by autoradiography. As shown in Figure 5 there is no significant reduction in the phosphorylation of vip1 by protein kinase A in the presence of increasing concentrations of p53. Thus, p53 does not inhibit the enzyme activity of protein kinase A although we used the same concentrations which were sufficient for an efficient inhibition of CK2 activity.

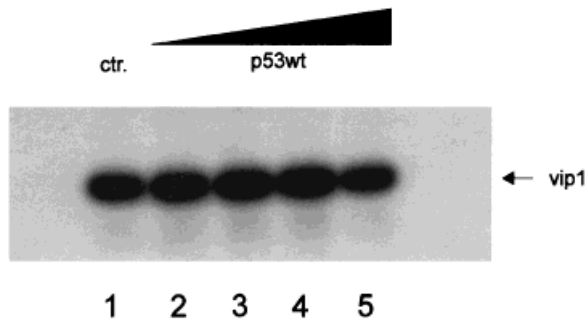


Fig. 5. Phosphorylation of vip1 by protein kinase A in the presence of wild-type p53. Kinase reaction was performed with protein kinase A and bacterially expressed and purified vip1 protein, harboring a synthetic PKA phosphorylation site as substrate. Control (lane 1) and increasing amounts of wild-type p53 (5, 10, 20, 30 pmol, lanes 2–5). Proteins were separated on a 12.5% SDS-polyacrylamide gel and visualized by autoradiography.

Next, we asked whether the native conformation of p53 is necessary for its effect on CK2 activity. Therefore, full length p53 was heat-denatured for 10 min and then incubated with protein kinase CK2. Enzyme activity of CK2 was measured by phosphorylation of the mdm2 protein. As shown in Figure 6 (lane 4) heat-denatured p53 is no longer active as an inhibitor of the CK2 with regard to the phosphorylation of mdm2. Thus, besides binding of p53 to the β-subunit of CK2, an intact native conformation of p53 is necessary for its inhibiting function on the CK2 activity.

Another control for the specificity of the p53-mediated effect on CK2 activity would be the use of $p53_{R175H}$, which is frequently found in a variety of human tumors. The tumor mutant $p53_{R175H}$ was applied in increasing amounts

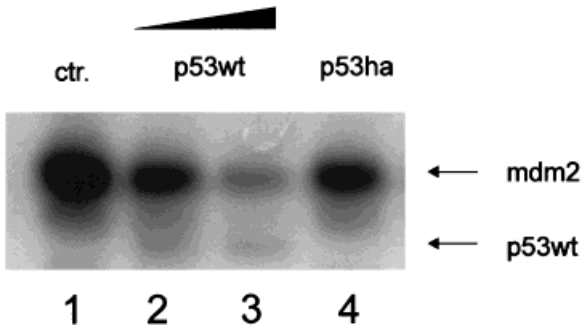


Fig. 6. Influence of heat denatured wild-type p53 on the CK2 kinase activity. Protein kinase CK2 was incubated for 1 h either with dialysis buffer (lane 1), p53wt (10 pmol, 25 pmol, lanes 2 and 3), heat-denatured p53 (25 pmol p53ha, lane 4). Kinase reaction was performed with mdm2 as substrate. Proteins were separated on a 10% SDS-polyacrylamide gel and visualized by autoradiography. ctr: phosphorylation of mdm2 by CK2 in the absence of p53.

corresponding to wild-type p53 protein using cyclin H as CK2 substrate. The experiment which is depicted in Figure 7 shows no reduction in CK2 activity when $p53_{R175H}$ (lanes 5–7) was used instead of wild-type p53 (lanes 2–4). This further control shows again, that the conformation of p53 is important for the observed inhibitory effect. Moreover, it clearly shows that the effect is not mediated by a co-purified protein as both wild-type and mutant p53 were bacterially expressed in *E. coli* and purified by the same protocol.

Though we properly defined the inhibitory effect of p53 on the protein kinase activity in vitro we now raised the question whether we could show this effect under in vivo conditions also. For these experiments we used $p53^{-/-}$ mouse fibroblasts which were transfected either

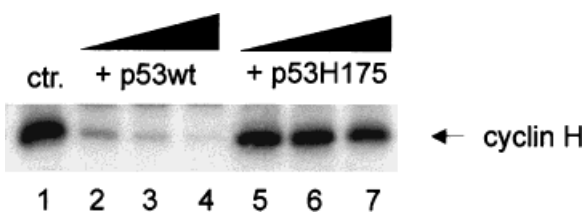
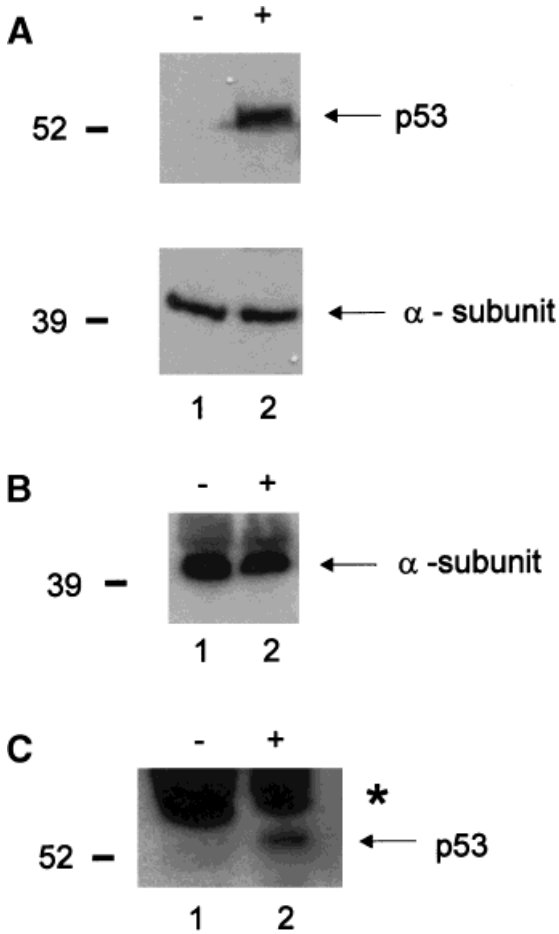


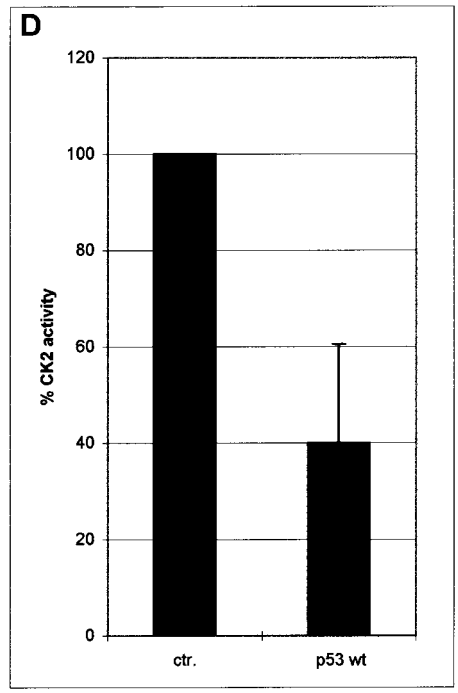
Fig. 7. Influence of $p53_{R175H}$ mutant on protein kinase CK2 activity. Kinase reaction was performed with protein kinase CK2 and cyclin H as substrate in the absence (ctr.) or in the presence of increasing amounts wild-type p53 or $p53_{R175H}$ mutant (10–30 pmol). Proteins were separated by SDS-polyacrylamide gel electrophoresis and visualized by autoradiography.

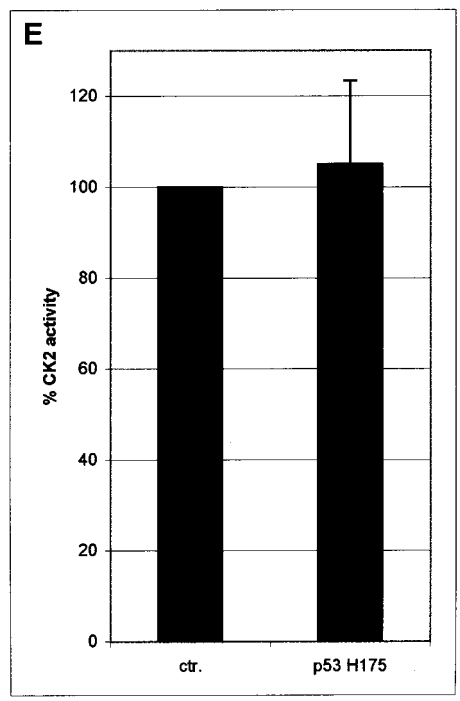
with a eukaryotic expression vector coding for human wild-type p53 or as a control with the same vector without insert. Six hours after transfection cells were harvested and lysed. An aliquot of the lysate was analyzed on a SDS-polyacrylamide gel followed by Western blot. The membrane was either incubated with PAb421 to detect p53 or 1AD9 to detect the α-subunit of CK2. As shown in Figure 8A after transfection of p53(+) the p53 protein is clearly detectable in the cell extract. Furthermore, the amount of CK2 α in the cell extract is not influenced by the presence of p53. The cell lysate was also used to immunoprecipitate CK2 with the CK2 β-specific antiserum #32. Immunoprecipitates were washed and divided into two equal aliquots. One aliquot was analyzed on an SDS-polyacrylamide gel followed by a Western blot. The filter was incubated with 1AD9 to detect the α-subunit of CK2 (Fig. 8B) and with PAb421 to detect p53 (Fig. 8C). The same amounts of CK2 α-subunit were present in the immunoprecipitates with the CK2 β-subunit-specific serum #32 either in the absence or in the presence of p53. Thus, we conclude that p53 in the transfected cells did not change the amount of CK2 but it clearly led to a reduction in the specific activity of protein kinase CK2. The other aliquot was incubated with the CK2-specific substrate peptide and $^{32}P\gamma ATP$. Radioactivity incorporated into the substrate was measured by Cerenkov counting. Figure 8D shows the outcome of five independent experiments. Thus, the presence of p53 in the transfected cells led to a reduction in the phosphorylation of the peptide substrate to about 40% of the phosphorylation which was observed in the absence of p53. To analyze the specificity of the inhibitory effect for wild-type p53 we repeated the same experiment with mutant $p53_{R175H}$ which was transfected into $p53^{-/-}$ fibroblasts. Figure 8E shows that overexpression of $p53_{R175H}$ had no inhibitory effect on CK2 kinase activity as measured by peptide substrate phosphorylation, though equal amounts of wild-type and mutant protein were detected when equal amounts of plasmid were used for transfection (Fig. 8F).

DISCUSSION

Protein kinase CK2 is pleiotropic, ubiquitously expressed, and as known so far is constitutively active. CK2 phosphorylates over 160







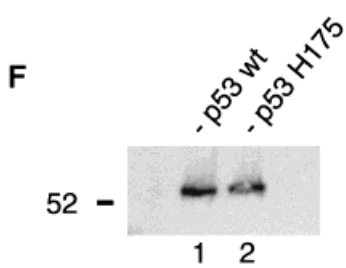


Fig. 8. Inhibition of protein kinase CK2 activity in vivo. $p53^{-/-}$ fibroblasts were transfected with control vector (pCMV30) or p53 (pRcCMV-p53) and harvested after 6 h. Cell lysates were separated by SDS-gel electrophoresis and blotted onto a PVDF membrane. (**A**) p53 protein and CK2 were detected with monoclonal antibodies PAb421 and 1A5, respectively. Molecular weight markers: 52 and 39 kDa are from Benchmark (prestained protein ladder, Gibco BRL). (**B**) One milligram of cell lysate was immunoprecipitated with serum #32 raised against the β-subunit of protein kinase CK2. Immunoprecipitates were split into two aliquots and one aliquot was subjected to SDS-polyacrylamide gel electrophoresis followed by Western blot analysis with monoclonal antibody 1A5 to show the presence of CK2 α-subunit. (**C**) The membrane was also incubated with PAb421 to detect p53 in the CK2 immunoprecipitate. The second aliquot was used to perform a kinase reaction with the CK2 specific peptide. Asterisk: IgG heavy chain. (**D**) Shows CK2 kinase activity from $p53^{-/-}$ fibroblasts, transfected either with control vector or p53 wild-type (results of five independent experiments; error bars represent standard deviation). (**E**) CK2 kinase activity from $p53^{-/-}$ fibroblasts, transfected either with control vector or $p53_{R175H}$ mutant (results of three independent experiments; error bars represent standard deviation). (**F**) Western blot analysis of $p53^{-/-}$ fibroblasts transfected with p53 wild-type vector (lane1) and $p53_{R175H}$ mutant (lane 2).

known substrates involved in signal transduction, transcriptional control, cell cycle control, metabolism etc., and therefore it seems to be obvious that this protein kinase plays a key role in the regulation of cellular processes [Allende and Allende, 1995; Pinna and Meggio, 1997]. The growth suppressor protein p53 also plays a key role in the regulation of cell proliferation particularly during cellular stress situations [Levine, 1997; Bates and Vousden, 1999]. p53 is phosphorylated by CK2 at the penultimate C-terminal amino acid [Meek et al., 1990]. In addition, p53 binds tightly to the regulatory β-subunit of CK2 but not to the catalytic α-subunit [Appel et al., 1995]. We have previously shown that the free CK2 β-subunit inhibits the DNA binding activity [Prowald et al., 1997] and the transactivation function of p53 [Schuster et al., 1999]. In addition to this regulatory effect of free CK2 β-subunit on activities of p53, it was shown that a C-terminal fragment of p53, spanning from amino acid 264 to 393, could stimulate the kinase activity of the CK2 holoenzyme [Guerra et al., 1997]. This stimulatory effect was measured using mdm2 which was identified as a new substrate for protein kinase CK2. In the present study we addressed the question whether wild-type p53 protein might also have a regulatory influence on the activity of protein kinase CK2. We show that in contrast to the C-terminal p53 fragment full length wild-type p53 had an inhibitory effect on the mdm2 phosphorylation by protein kinase CK2. This effect is not due to a competitive phosphorylation as the observed amounts of radioactive incorporation into wild-type p53 were far too small to account for the observed decrease of phosphate incorporation of the investigated substrates. Phosphorylation of p53 is very weak, probably because the wild-type form of p53 efficiently inhibits its own phosphorylation under these experimental conditions. Our finding that not only phosphorylation of mdm2 but also of many other substrates, including cyclin H, nucleolin, tubulin or the synthetic substrate peptide, is inhibited by p53, shows that this effect is not restricted to a single CK2 substrate, but seems to be a general regulatory property. This is underlined by our observation that a native conformation of p53 is necessary to mediate the inhibitory effect on the kinase activity, since heat-denatured p53 as well as mutant $p53_{R175H}$ had lost the inhibitory activity. Our experiments with p53-transfected cells clearly demonstrate, that upon overexpression of p53, CK2 kinase activity is reduced. Furthermore, this reduction can be correlated with the presence of p53 protein in the CK2 immunoprecipitate which was used for phosphorylation experiments. The experiments with transfected cells confirm the in vitro results. Furthermore, the inhibitory effect on the CK2 activity seems to be restricted to wild-type p53 since mutant $p53_{R175H}$ is defective for CK2 inhibition in vitro and in vivo. This also demonstrates that our in vitro experiments are reliable and can be reproduced in a cellular environment.

It is known that the C-terminal region of p53 which was shown to stimulate CK2 is a potent transforming agent [Shaulian et al., 1992]. Similar to various mutant p53 the C-terminus of p53 can transform primary cells in collaboration with an activated *ras* gene. Elevated levels and activities of CK2 were found in transformed cells when compared to normal cells [Münstermann et al., 1990]. Thus, it seems possible that cell transformation mediated by the C-terminus of p53 might at least be accompanied by elevated CK2 activities. In contrast, wild-type p53 is a growth suppressor and in agreement with the hypothesis CK2 activity is down-regulated. Thus, up- and down-regulation of the activity of CK2 by p53 correlates perfectly with transforming or growth suppressing activity of p53. This finding is in line with our previous report, that wild-type p53 can down-modulate the activity of the cyclin H/cdk7/Mat1 kinase, a kinase which is also implicated in the regulation of the cell cycle and transcription. Binding of p53 to cyclin H/cdk7/Mat1 down-regulates the phosphorylation of cdk2 and of the C-terminal domain of RNA polymerase II. Phosphorylation of cdk2 by cyclin H/cdk7/Mat1 (CAK activity) is necessary for progression through the G_1-phase of the cell cycle whereas phosphorylation of the C-terminal domain of RNA pol II is necessary for the switch from the initiation to the elongation state of RNA pol II-dependent transcription [Akoulitchev and Reinberg, 1998]. By inhibition of the kinase activity of cyclin H/cdk7/Mat1 both processes are stopped and cells are growth-arrested. Thus, binding of p53 to the regulatory β-subunit of CK2 and binding of p53 to the regulatory cyclin H subunit of the cyclin H/cdk7/Mat1 complex have the same effect on

cell proliferation. As with CK2 p53 binds to the regulatory subunit of the CAK-complex namely cyclin H [Schneider et al., 1998]. Cyclin H binds to a C-terminal region of p53, between amino acids 315 and 340. A very similar region between amino acids 330 and 339 of p53 was also found to be required for binding of CK2 β-subunit [Götz et al., 1999].

Binding of monoclonal antibody PAb421 leads to an altered conformation of p53 which goes along with an altered DNA binding activity. As shown here this alteration of the conformation does not change binding of the CK2 β-subunit to p53 and the down-regulation of the CK2 activity by p53. In agreement with earlier observations binding of PAb421 to p53 inhibits to some extent phosphorylation of p53 by CK2 [Kraiss et al., 1990] which demonstrates that binding of p53 to the CK2 β-subunit and phosphorylation of p53 by CK2 are two independent events.

Levels of p53 are low in normal non-transformed cells but elevated in a variety of tumor cells. The increased levels of p53 are mostly due to a mutation or a functional inactivation by viral or cellular oncogenes. According to our present results $p53_{R175H}$ with a mutant conformation is unable to down-regulate CK2 activity. Consistent with this observation in tumor cells a higher expression and higher activity of CK2 is found compared to normal cells. Levels of wild-type p53 are enhanced up to 10-fold in response to genotoxic stress like DNA damage, depletion of the ribonucleotide pool or hypoxia and these levels are probably similar to those obtained by transfection. As the absolute level and kinetics vary according to the genotoxic agent it will be difficult to find the right time window for the inhibition of CK2 activity. Moreover, since p53 and CK2 are located in various compartments of the cell it might also be important to find the right place where both molecules meet. Thus, these will be attractive and informative studies to discover the right meeting point of both proteins at the right time. Studies to address all these questions are in progress.

Since wild-type p53 inhibits not only the CK2 activity but also the activity of cyclin H/cdk7/Mat1 one might argue that p53 might in general be an inhibitor of protein kinases. Testing PKA kinase activity in the absence and presence of p53 we found no reduction in PKA kinase activity in the presence of p53. Since PKA does not bind to p53 together with our other results we can conclude that binding of p53 to the kinase is necessary to mediate its inhibitory effect. Therefore, we assume that the inhibitory effect of wild-type p53 is specific for CK2, CAK, and possibly other kinases binding to the same C-terminal region of p53.

Since it is known from studies in yeast and mammalian cells that CK2 activity is necessary at G_1/S- and G_2/M-borders, CK2 regulation at G_2/M transition could be a critical step in cell cycle progression [Pepperkok et al., 1994; Glover, 1998]. This speculation is strengthened by another finding from yeast. Toczyski and coworkers found an important function of the CK2 β-subunit during the adaptation process after DNA damage. When the CK2 β-subunit was defective or deleted, DNA-damaged cells could not reenter the cell cycle [Toczyski et al., 1997]. This is an important finding since it shows that functional CK2 holoenzyme or free CK2 β-subunit are necessary for entry into cell cycle from a DNA damage checkpoint, which implicates that this kinase must be down-regulated when this checkpoint is active.

The plethora of known CK2 substrates together with our present finding of a potential regulation of CK2 activity by p53 implicate an important role of CK2 during processes, which generally inactivate cellular proliferation or will eliminate damaged cells from a cell population. It is an intriguing question if protein kinase CK2 could participate in the decision which determines cell fate for apoptosis or further proliferation.

ACKNOWLEDGMENTS

We thank W. Nastainczyk for peptide synthesis and help with the production of polyclonal sera and G. Brandner for $p53^{-/-}$ fibroblasts, J. Iselborn, S. Kartarius and P. Scholtes for technical assistance and all members of the lab for support. The authors like to thank also P. Wagner for continuous and helpful discussions. MM is supported by SFB 399, B4 and MM and CG are supported by grant Mo 309/11-1 and Mo 309/11-3 from the Deutsche Forschungsgemeinschaft.

REFERENCES

Akoulitchev S, Reinberg D. 1998. The molecular mechanism of mitotic inhibition of TFIIH is mediated by phosphorylation of CDK7. Genes Dev 12:3541–3550.

Allende JE, Allende CC. 1995. Protein kinase CK2: an enzyme with multiple substrates and puzzling regulation. FASEB J 9:313–323.

Appel K, Wagner P, Boldyreff B, Issinger O-G, Montenarh M. 1995. Mapping of the interaction sites of the growth suppressor protein p53 with the regulatory β-subunit of protein kinase CK2. Oncogene 11:1971–1978.

Bates S, Vousden KH. 1999. Mechanisms of p53-mediated apoptosis. Cell Mol Life Sci 55:28–37.

Baudier J, Delphin C, Grunwald D, Khochbin S, Lawrence JJ. 1992. Characterization of the tumor suppressor protein p53 as a protein kinase C substrate and a S100b-binding protein. Proc Natl Acad Sci USA 89:11627–11631.

Belenguer P, Caizergues-Ferrer M, Labbe JC, Doree M, Amalric F. 1990. Mitosis-specfic phosphorylation of nucleolin by p34cdc2 protein kinase. Mol Cell Biol 10:3607–3618.

Bischoff JR, Friedman PN, Marshak DR, Prives C, Beach D. 1990. Human p53 is phosphorylated by p60-cdc2 and cyclin B-cdc2. Proc Natl Acad Sci USA 87:4766–4770.

Buchhop S, Gibson MK, Wang XW, Wagner P, Stürzbecher HW, Harris CC. 1997. Interaction of p53 with the human Rad51 protein. Nucleic Acids Res 25:3868–3874.

Glover CVC. 1998. On the physiological role of casein kinase II in Saccharomyces cerevisiae. Prog Nucleic Acid Res Mol Biol 59:95–133.

Götz C, Scholtes P, Schuster N, Prowald A, Nastainczyk W, Montenarh M. 1999. Protein kinase CK2 binds to a multiprotein binding domain of the growth suppressor protein p53. Mol Cell Biochem 191:111–120.

Guerra B, Götz C, Wagner P, Montenarh M, Issinger O-G. 1997. The carboxy-terminus of p53 mimicks the polylysine effect of protein kinase CK2-catalyzed MDM2 phosphorylation. Oncogene 14:2683–2688.

Hansen S, Hupp TR, Lane DP. 1996. Allosteric regulation of the thermostability and DNA binding activity of human p53 by specific interacting proteins. J Biol Chem 271:3917–3924.

Herrmann CPE, Kraiss S, Montenarh M. 1991. Association of casein kinase II with immunopurified p53. Oncogene 6:877–884.

Hu MCT, Qiu WR, Wang YP. 1997. JNK1, JNK2 and JNK3 are p53 N-terminal serine 34 kinases. Oncogene 15:2277–2287.

Hupp TR. 1999. Regulation of p53 protein function through alterations in protein-folding pathways. Cell Mol Life Sci 55:88–95.

Jayaraman L, Prives C. 1999. Covalent and noncovalent modifiers of the p53 protein. Cell Mol Life Sci 55: 76–87.

Knippschild U, Milne DM, Campbell LE, DeMaggio AJ, Christenson E, Hoekstra MF, Meek DW. 1997. p53 is phosphorylated in vitro and in vivo by the delta and epsilon isoforms of casein kinase 1 and enhances the level of casein kinase 1 delta in response to topoisomerase-directed drugs. Oncogene 15:1727–1736.

Ko LJ, Shieh SY, Chen XB, Jayaraman L, Tamai K, Taya Y, Prives C, Pan ZQ. 1997. p53 is phosphorylated by CDK7-cyclin H in a $p36^{MAT1}$-dependent manner. Mol Cell Biol 17:7220–7229.

Kraiss S, Barnekow A, Montenarh M. 1990. Protein kinase activity associated with immunopurified p53 protein. Oncogene 5:845–855.

Lees-Miller SP, Chen Y-R, Anderson CW. 1990. Human cells contain a DNA-activated protein kinase that phosphorylates simian virus 40 T antigen, mouse p53, and the human Ku autoantigen. Mol Cell Biol 10:6472–6481.

LeRoy D, Schmid N, Behr J-P, Filhol O, Pares S, Garin J, Buurgarit J-J, Chambaz EM, Cochet C. 1995. Direct identification of a polyamine binding domain on the regulatory subunit of the protein kinase casein kinase 2 by photoaffinity labeling. J Biol Chem 270:17400–17406.

Levine AJ. 1997. p53, the cellular gatekeeper for growth and division. Cell 88:323–331.

Lu H, Fisher RP, Bailey P, Levine AJ. 1997. The CDK_7-cycH-p36 complex of transcription factor IIH phosphorylates p53, enhancing its sequence-specific DNA binding activity in vitro. Mol Cell Biol 17:5923–5934.

Meek DW, Simon S, Kikkawa U, Eckhart W. 1990. The p53 tumour suppressor protein is phosphorylated at serine 389 by casein kinase II. EMBO J 9:3253–3260.

Münstermann U, Fritz G, Seitz G, Yiping L, Schneider HR, Issinger O-G. 1990. Casein kinase II is elevated in human tumours and rapidly proliferating non-neoplastic tissue. Eur J Biochem 189:251–257.

Pepperkok R, Lorenz P, Ansorge W, Pyerin W. 1994. Casein kinase II is required for transition of G_0/G_1, early G_1, and G_1/S phases of the cell cycle. J Biol Chem 269: 6986–6991.

Pinna LA, Meggio F. 1997. Protein kinase CK2 ("casein kinase-2") and its implication in cell division and proliferation. Progress in Cell Cycle Research 3:77–97.

Prives C. 1998. Regulation of p53 function by phosphorylation. Jpn J Cancer Res 89:COV2–COV2.

Prives C, Hall PA. 1999. The P53 pathway. J Pathol 187:112–126.

Prowald K, Fischer H, Issinger OG. 1984. Enhanced casein kinase II activity in human tumour cell cultures. FEBS Letters 176:479–483.

Prowald A, Schuster N, Montenarh M. 1997. Regulation of the DNA binding of p53 by its interaction with protein kinase CK2. FEBS Letters 408:99–104.

Sarno S, Vaglio P, Marin O, Issinger OG, Ruffato K, Pinna LA. 1997. Mutational analysis of residues implicated in the interaction between protein kinase CK2 and peptide substrates. Biochemistry 36:11717–11724.

Schneider HR, Issinger O-G. 1988. Nucleolin (C23), a physiological substrate for casein kinase II. Biochem Biophys Res Commun 156:1390–1397.

Schneider E, Montenarh M, Wagner P. 1998. Regulation of CAK kinase activity by p53. Oncogene 17:2733–2742.

Schuster N, Prowald A, Schneider E, Scheidtmann K-H, Montenarh M. 1999. Regulation of p53 mediated transactivation by the β-subunit of protein kinase CK2. FEBS Lett 447:160–166.

Shaulian E, Zauberman A, Ginsberg D, Oren M. 1992. Identification of a minimal transforming domain of p53: negative dominance through abrogation of sequence-specific DNA binding. Mol Cell Biol 12:5581–5592.

Shi Y, Brown ED, Walsh CT. 1994. Expression of recombinant human casein kinase II and recombinant heat shock protein 90 in *Escherichia coli* and characterization of their interactions. Proc Natl Acad Sci USA 91: 2767–2771.

Singh K, Carey M, Saragosti S, Botchan M. 1985. Expression of enhances levels of small RNA polymerase III transcripts encoded by the B2 repeats in simian virus 40-transformed mouse cells. Nature 314: 553–556.

Soussi T, Caron de Fromentel C, May P. 1990. Structural aspects of the p53 protein in relation to gene evolution. Oncogene 5:945–952.

Toczyski DP, Galgoczy DJ, Hartwell LH. 1997. CDC5 and CKII control adaptation to the yeast DNA. Cell 90:1097–1106.

Wang XW, Vermeulen W, Coursen JD, Gibson M, Lupold SE, Forrester K, Xu GW, Elmore L, Yeh H, Hoeijmakers JHJ, Harris CC. 1996. The XPB and XPD DNA helicases are components of the p53-mediated apoptosis pathway. Genes Dev 10:1219–1232.