Regulation of the DNA binding of p53 by its interaction with protein kinase CK2

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Abstract Some of the numerous functions of the growth suppressor protein p53 are regulated by its interaction with viral and cellular proteins. C-terminal sequences of p53 are implicated in binding to the regulatory β-subunit of protein kinase CK2. Using a p53-specific DNA binding element we found that the β-subunit of CK2 inhibited the DNA binding of p53 whereas the α-subunit had no influence. The CK2 holoenzyme consisting of two α- and two β-subunits led to a supershift in DNA binding of p53 similar to the p53-specific monoclonal antibody PAb421 as well as the C-terminus of p53. Thus, our results showed an individual role of the free β-subunit of CK2 on the DNA binding activity of p53.

Key words: Growth suppressor protein; Protein kinase CK2; DNA binding; Protein–protein interaction

1. Introduction

The growth suppressor protein p53 plays an important role governing the integrity of the human genome. Upon DNA damage it can induce growth arrest, DNA repair or apoptosis (for review see [1]). Some of the p53 functions are performed by sequence-specific DNA binding [2], by interaction with different transcription factors such as TBP (TATA-box binding protein) [3], TAFII40 and TAFII60 [4], TAFII31 [5], SP1 [6] and E2F [7]. So far a number of p53-responsive elements has been identified some of which are up-regulated upon DNA damage-induced accumulation of p53 and which appear to be involved in cell cycle arrest such as $p21^{WAF1/CIP1}$ [8], some in DNA replication and DNA repair such as PCNA or GADD45 [9,10], some are involved in apoptosis such as bcl-2 and ba×1 [11] or in a self-regulating loop such as mdm2 [12].

The p53 protein is phosphorylated by several protein kinases at multiple sites which are clustered in the N-terminus and in the C-terminus (for review see [13]). The polypeptide chain of p53 can be divided into several functional domains: the N-terminus which seems to be involved in transcriptional activation [14], the central core domain which is responsible for the sequence-specific DNA binding activity [15] and a C-terminal region. The C-terminus of p53 harbors the oligomerization domain [16], nuclear localization signals [17], the binding sites for the fission yeast tms1 protein [18], for DnaK [19], TBP, XPB and RPA [20], the regulatory β-subunit of protein kinase CK2 [21] and protein kinase $p34^{cdc2}$ (Wagner et al., submitted). The carboxy-terminal region is able to bind non-specifically to DNA [22] and to influence the specific binding of p53 to DNA. Specific DNA binding of p53 is strongly activated (i) by binding of monoclonal antibody PAb421 to its C-terminal epitope [19], (ii) when a C-terminal 30 amino acid long sequence is deleted or (iii) when human p53 is phosphorylated by protein kinase CK2 at residue 392 [19]. Recent results have shown that O-glycosylation in the C-terminus also regulates DNA binding activity of p53 [23]. C-terminal regions of the p53 polypeptide are also involved in recognizing insertion–deletion mismatches [24] and in DNA reannealing activity [25].

We have recently defined a new functional domain in the C-terminus where the fission yeast tms1 protein [18], the regulatory β-subunit of protein kinase CK2 [21] and the protein kinase $p34^{cdc2}$ (Wagner et al., submitted) bind. Furthermore, we could show that binding of the C-terminus of p53 to the regulatory β-subunit of protein kinase CK2 regulates CK2 phosphotransferase activity [26]. CK2 is an ubiquitous serine/threonine kinase found in both the cytoplasm and the nucleus [27] of eukaryotic cells. It is a highly conserved Ca^{2+}- and nucleotide-independent tetrameric enzyme which phosphorylates a broad spectrum of substrates including nuclear oncoproteins, transcription factors and DNA binding proteins [28]. In the present paper we have analyzed whether the regulatory β-subunit, the catalytic α-subunit of CK2 or the CK2 holoenzyme might influence the DNA binding of p53. We found that the CK2 holoenzyme led to a supershift of the p53/DNA complexes. The regulatory β-subunit reduced the DNA binding activity of p53 whereas the α-subunit of CK2 did not influence DNA binding of p53 at all.

2. Materials and methods

2.1. Cell extracts

Sf9 cells were grown in Sf900 medium supplemented with 10% fetal calf serum (FCS) at 27°C. Cells were infected with recombinant baculovirus pAchup53wt (kindly provided by M. Hoever, Freiburg, Germany) or wild-type baculovirus *Autographa californica*. Three days after infection cells were harvested, washed with PBS (phosphate-buffered saline) and resuspended in lysis-buffer (10 mM Hepes, pH 7.9, 400 mM NaCl, 20% (v/v) glycerol, 1 mM EDTA). After three cycles of freezing and thawing cells were centrifuged (4°C, 10 min 13000×*g*). For each cell extract the protein concentration was determined and equal amounts of protein were used for further experiments.

2.2. Labeling of cells

Three days after infection with pAchup53wt Sf9 cells were labeled with [^{35}S]methionine for 10 h. Cells were washed with PBS, resuspended in extraction buffer (100 mM Tris-HCl, pH 9, 100 mM NaCl, 0.5% NP-40) and incubated at 0°C for 1 h. After centrifugation the cell extract was pre-incubated 3 times with *S. aureus*. The supernatant was incubated with the p53-specific monoclonal antibody PAb1620 [29]. Immunoprecipitates were washed 2 times in washing buffer C (50 mM Tris-HCl, pH 8, 500 mM LiCl, 1 mM dithiothreitol, 1 mM Na_3-EDTA, 1% (v/v) Trasylol), 3 times in NET buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM Na_3-EDTA, 5% sucrose)

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containing 1% NP-40 and 2 times in 50 mM NH_4HCO_3. Proteins were eluted from the *S. aureus* complexes with sample buffer (65 mM Tris-HCl, pH 6.8, 5% (v/v) β-mercaptoethanol, 2% (w/v) sodium dodecyl sulphate, 10% (v/v) glycerol, 0.01% (w/v) bromophenol blue). The samples were analyzed on a 10% SDS–polyacrylamide gel and visualized by fluorography as described earlier [30].

2.3. Purification of proteins

Recombinant CK2 holoenzyme (kind gift from Y. Shi, New York, USA) [31], the α- and β-subunits of CK2 (kindly provided by O.-G. Issinger, Odense, DK) [32] were expressed in *E. coli* (BL21 (DE3)). The bacterial pellet was lysed and after incubation on ice for 30 min, the lysate was sonicated 2 times and centrifuged (10000×*g*, step 1) The pellet was resuspended in buffer P1500 (20 mM Tris-HCl, pH 8, 7 mM β-mercaptoethanol, 1 mM phenylmethyl sulfonylchloride (PMSF) and 1.5 M NaCl). Proteins were extracted overnight, pooled with the supernatant from the first step, and dialysed against buffer P300 (20 mM Tris-HCl, pH 8, 7 mM β-mercaptoethanol, 1 mM PMSF and 300 mM NaCl). The lysate was loaded onto a P11 column pre-equilibrated with buffer P300 and eluted with a linear gradient from 0.3–1.5 mM NaCl. Fractions containing active holoenzyme, α- or β-subunit were dialysed against P300 buffer, concentrated and loaded onto a Superose-6 column (Pharmacia, Freiburg, Germany). After gel filtration the peak fractions were collected.

2.4. DNA binding

The DNA consensus sequence 5′-CCGGGCATGTCCGGGCATGTCCGGGCATGT-3′ [33] (kindly provided by M. Mundt, Freiburg, Germany) was labeled in the presence of [α-^{32}P]dCTP. A binding reaction (20 μl vol.) contained binding buffer (final concentration: 50 mM Tris-HCl, pH 8, 4% (v/v) glycerol, 5 mM dithiothreitol, 1 mM $MgCl_2$ and 0.05% (v/v) Triton X-100), labeled DNA (45 ng), 12.5 μg tRNA and 1.25 μg poly[d(AT)] as competitors, 100 ng PAb421 when indicated and 9 μg of p53 containing cell extract or of control cell extract. We added the α-subunit or the β-subunit or the holoenzyme of CK2 to p53 up to a final molar ratio of 1:1. Proteins were premixed (antibody was added at last) before DNA was added. After 30 min at room temperature the mixture was analyzed on a 4% nondenaturing polyacrylamide gel and visualized by autoradiography.

3. Results

The wild-type p53 protein binds DNA in a sequence-specific manner. El-Deiry et al. [34] defined a consensus p53 binding site consisting of two copies of the 10 bp element 5′-PuPuPuC(A/T)(T/A)GPyPyPy-3′. Although the central region of p53 is clearly implicated in direct contact to DNA a contribution of C-terminal amino acids to its non-specific DNA binding as well as to its specific DNA binding is well established [15,33]. We have recently defined a new functional domain on the polypeptide chain of p53 spanning from amino acids 287–340 where several cellular proteins bind [18,21]. Among these proteins that bind to this C-terminal domain is the regulatory β-subunit of protein kinase CK2 [21,35]. Now, we wanted to analyze the influence of the CK2 holoenzyme and its individual subunits on the DNA binding activity of p53. In accordance with several other studies [23,36] binding assays were performed using insect cells infected with a recombinant p53 expressing baculovirus pAchup53wt. As control we used wild-type baculovirus (*Autographa californica*) infected cells in order to exclude unspecific DNA binding reactions.

In order to show the protein expression in insect cells, Sf9 cells were infected with baculovirus pAchup53wt or wild-type baculovirus. Three days after infection cells were labeled with [^{35}S]methionine. After lysis cell extracts were immunoprecipitated with antibody PAb1620 and *S. aureus*. The washed immunoprecipitates were separated on an SDS–polyacrylamide gel (Fig. 1A). As shown in lane 4, insect cells infected with pAchup53wt expressed p53, while this protein is absent in insect cells infected with wild-type baculovirus *A. californica* (lane 2) and in control immunoprecipitations (lanes 1 and 3).

The protein kinase CK2 holoenzyme consists of two catalytic α-subunits and two regulatory β-subunits. The functionally active holoenzyme and the α- and β-subunit of CK2 were expressed in bacteria and purified as described in Section 2. Purified proteins were analyzed on an SDS–polyacrylamide gel. Fig. 1B–D shows a Coomassie blue stain of the α-subunit, β-subunit and the CK2 holoenzyme, respectively, on the SDS–polyacrylamide gel.

Having shown the specificity and purity of the proteins we now started to analyze the DNA binding of p53 in the presence or absence of protein kinase CK2. For band shift assays we used a DNA consensus sequence for p53 described by El-Deiry et al. [34]. ^{32}P-labeled oligonucleotides were analyzed on a polyacrylamide gel in the presence or absence of p53 and purified protein kinase CK2 holoenzyme. Lane 1 of Fig. 2 shows the migration of the ^{32}P-labeled oligonucleotide. Addition of a p53 containing cell extract from Sf9 cells led to a band shift (lane 9). In agreement with published results addition of the p53-specific monoclonal antibody PAb421 resulted in a supershift (lane 8) [19]. In the absence of PAb421 but in the presence of the CK2 holoenzyme p53–DNA complexes were also supershifted (lane 11). The supershift is in the same range which was obtained with PAb421 although not identical. Addition of PAb421 to p53 and CK2 holoenzyme resulted in a stronger DNA binding with no additional shift (lane 10). Thus, the CK2 holoenzyme seems to have a very similar effect on p53 as monoclonal antibody PAb421. Control experiments showed that the holoenzyme did not bind to the p53 consensus sequence (lane 2) nor did the p53-specific antibody PAb421 interfere with CK2 (lane 3). The incubation of the control cell extract with CK2 holoenzyme and DNA did not differ from control cell extract alone with DNA (lane 7 and lane 5, respectively). Lane 4 shows the control cell extract incubated with antibody PAb421 and DNA and lane 6 shows the control cell extract incubated with antibody PAb421 and CK2 holoenzyme. Some very weak bands represent the unspecific DNA binding of the cellular proteins and are different from those obtained with p53-containing cell extract.

Next, we wanted to analyze the effect of the CK2 α-subunit on the DNA binding properties of p53. Therefore, we repeated the experiments described above but instead of the holoenzyme we added only the α-subunit of CK2. Lane 1 of Fig. 3 shows the migration of the labeled DNA. As shown in lane 7 p53 alone binds to DNA, and addition of PAb421 to p53 led to a supershift of the p53–DNA complexes (lane 8). The presence of the α-subunit of CK2 did not change the mobility of the bands on the gel regardless of whether PAb421 was present (lanes 10 and 9). Thus, we conclude that the α-subunit of CK2 does not influence the DNA binding activity of p53, which is in agreement with the observation that the α-subunit of CK2 does not bind to p53 [21,35]. The α-subunit of CK2 does also not bind to the consensus DNA-sequence (lane 2). Lane 3 shows the control cell extract incubated with DNA. Addition of PAb421 to the control cell extract and DNA (lane 4) or to control cell extract, DNA and CK2 α-subunit (lane 5) showed no specific DNA binding. Lane 6 shows the control cell extract with DNA, α-subunit and PAb421.

Next, we repeated the experiment described above, but in-

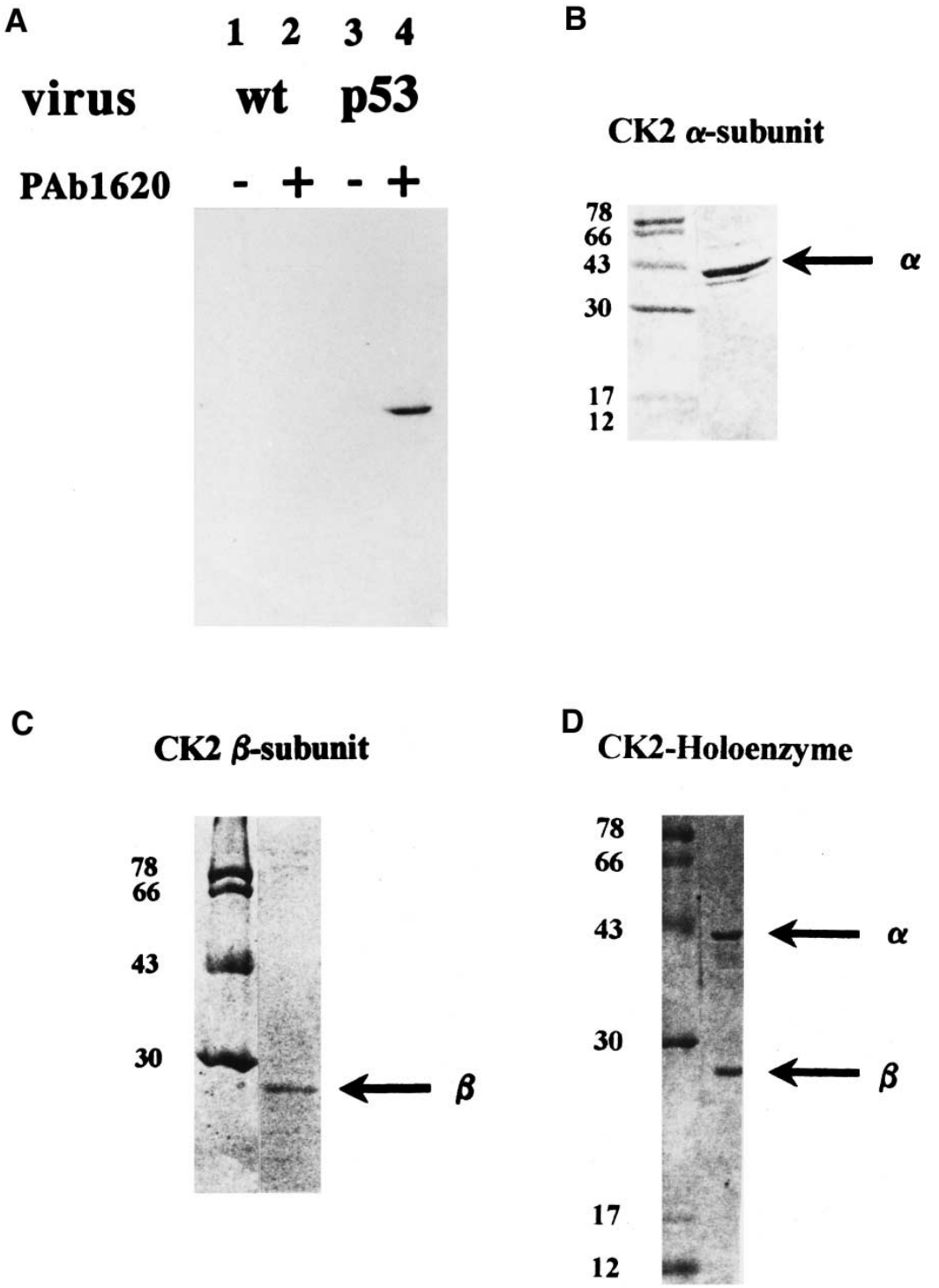

Fig. 1. A: Expression of human p53 in insect cells infected with wild-type baculovirus *Autographa californica* or with recombinant pAchup53wt baculovirus. 72 h after infection insect cells labeled with [^{35}S]methionine were extracted and the cell extract incubated with *S. aureus* alone (−) or with the p53-specific monoclonal antibody PAb1620 (+). Immunoprecipitates were analyzed on a 10% SDS–polyacrylamide gel. Protein bands were visualized by autoradiography. Lanes 2 and 4 show the immunoprecipitates with antibody PAb1620 and *S. aureus*. Lanes 1 and 3 show the control precipitate with *S. aureus* alone. B–D: Bacterially expressed α-subunit, β-subunit and holoenzyme of protein kinase CK2. The α-subunit, β-subunit as well as the bicistronic construct of the holoenzyme of CK2 were expressed in bacteria. Purified proteins were analyzed on a 10% SDS–polyacrylamide gel. Gels were stained with Coomassie blue. Molecular mass markers are ovotransferrin 780 00 Da (78), bovine serum albumin 66 000 Da (66), ovalbumin 43 000 Da (43), carboanhydrase 30 000 Da (30), myoglobin 17 000 (17) and cytochrome *c* 12 000 Da (12).

stead of adding the α-subunit of CK2 we added the β-subunit. For these experiments we used cell extracts with β-subunit of CK2 expressed in insect cells or alternatively purified bacterially expressed β-subunit of CK2. Both experiments led to the same results. As shown in Fig. 4 lane 2, addition of p53 to the labeled oligonucleotide (the migration of the DNA is shown

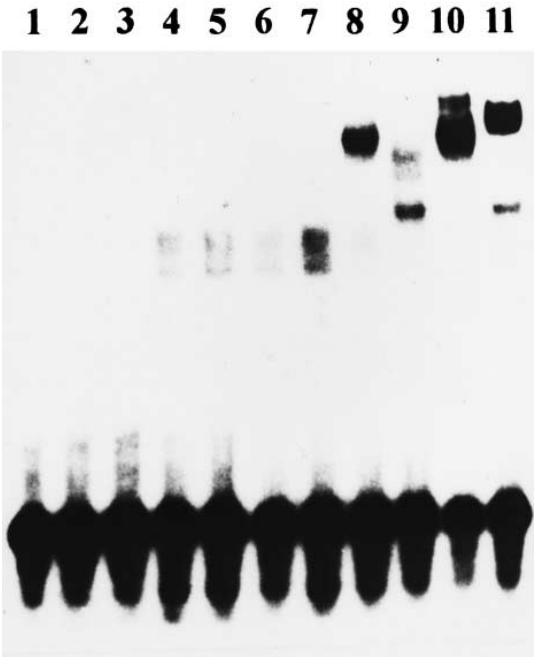

Fig. 2. DNA binding activity of p53 in the presence of the CK2 holoenzyme. The ^{32}P-labeled oligonucleotide containing the p53 consensus sequence was incubated under DNA binding conditions with cell extracts (p53 or control, respectively) in the absence (lanes 1, 4, 5, 8, 9) or presence (lanes 2, 3, 6, 7, 10, 11) of CK2 protein. In addition lanes 3, 4, 6, 8 and 10 contain monoclonal antibody PAb421. After an incubation time of 30 min the probes were analyzed on a 4% polyacrylamide gel followed by autoradiography of the gel. Lane 1: DNA. Lane 2: DNA with CK2. Lane 3: DNA with CK2 and PAb421. Lane 4: Control cell extract with DNA and PAb421. Lane 5: Control cell extract with DNA. Lane 6: Control cell extract with DNA, CK2 and PAb421. Lane 7: Control cell extract with DNA and CK2. Lane 8: p53 cell extract with DNA and PAb421. Lane 9: p53 cell extract with DNA. Lane 10: p53 cell extract with DNA, CK2 and PAb421. Lane 11: p53 cell extract with DNA and CK2.

in lane 1) resulted in a shift in mobility and the band was supershifted in the presence of monoclonal antibody PAb421 (lane 7). Addition of the β-subunit of CK2 abolished DNA binding of p53 (lane 11). However, addition of PAb421 to p53 and the β-subunit resulted in a supershift (lane 8) as in the absence of the β-subunit (lane 7) indicating that PAb421 has a higher affinity for p53 than the β-subunit of CK2. Lane 3 shows the control cell extract with DNA and β-subunit and lane 4 control cell extract with DNA, β-subunit and PAb421. The unspecific DNA binding of the cell extract is shown in lanes 5 and 6 (with and without PAb421, respectively). Control experiments showed that the β-subunit of CK2 did not bind to the p53 oligonucleotide neither in the presence (lane 10) nor in the absence of monoclonal antibody PAb421(lane 9). Thus, we conclude that the interaction of the regulatory β-subunit of CK2 with p53 reduces the DNA binding activity of p53 considerably.

In order to analyze the DNA binding of p53 in the presence of the β-subunit of CK2 in more detail we repeated the experiment described above but in the presence of increasing amounts of the β-subunit of CK2. As shown in Fig. 5 lane 6, p53 binds to DNA. However, binding is reduced in the presence of a molecular ratio of p53/β-subunit of 8:1, 6:1, 4:1 and 2:1 of the β-subunit of CK2 (lanes 5–2). Lane 1 shows the endlabeled oligonucleotide in the absence of p53. Thus at a molar ratio of 4:1 the β-subunit of CK2 is already a potent inhibitor of the DNA binding activity of p53.

4. Discussion

The polypeptide chain of p53 has three major functional domains. The N-terminus is involved in transcriptional activation. The central core domain confers a sequence-specific DNA binding property on wild-type p53. The C-terminus of p53 is involved in various protein–protein interactions leading to the formation of p53 homotetramers as well as p53 hetero-oligomers. In addition the C-terminus of p53 plays a major regulatory role for the specific DNA binding activity of the core domain (for review see [13]). A motif within the C-terminal 30 amino acids negatively controls p53 function as deletion of these amino acids constitutively activates p53 for DNA binding [19] and abolishes the DNA reannealing activity of p53 [25]. The C-terminus is also the target for post-translational modifications such as phosphorylation by different protein kinases namely p34^{cdc2} [37], protein kinase C [38] and protein kinase CK2 [39]. Phosphorylation of p53 by CK2 on its penultimate amino acid has been shown to activate sequence-specific DNA binding of p53 at least in vitro [39].

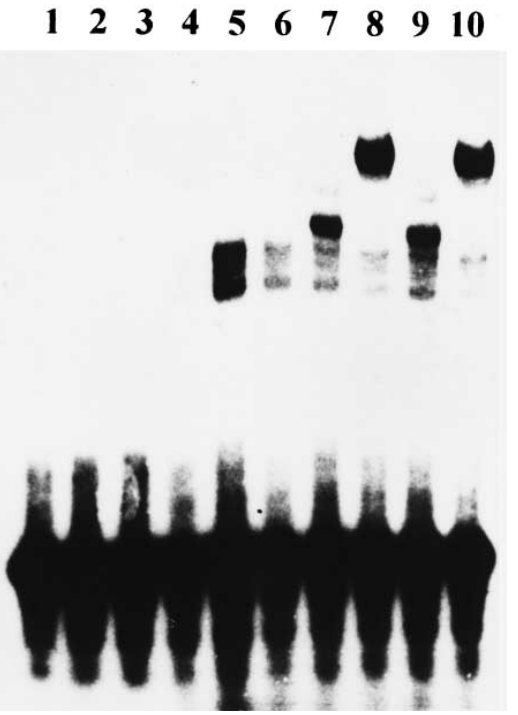

Fig. 3. DNA binding of p53 in the presence of the α-subunit of CK2. The ^{32}P-labeled oligonucleotide containing the p53 consensus sequence was incubated under DNA binding conditions with cell extracts (p53 or control, respectively) in the absence (lanes 1, 3, 4, 7, 8) or presence (lanes 2, 5, 6, 9, 10) of α-subunit of CK2. In addition lanes 4, 6, 8 and 10 contain monoclonal antibody PAb421. After an incubation time of 30 min the probes were analyzed on a 4% polyacrylamide gel, followed by autoradiography of the gel. Lane 1: DNA. Lane 2: DNA with α-subunit. Lane 3: Control cell extract with DNA. Lane 4: Control cell extract with DNA and PAb421. Lane 5: Control cell extract with DNA and α-subunit. Lane 6: Control cell extract with DNA, α-subunit and PAb421. Lane 7: p53 cell extract with DNA. Lane 8: p53 cell extract with DNA and PAb421. Lane 9: p53 cell extract with DNA and α-subunit. Lane 10: p53 cell extract with DNA, α-subunit and PAb421.

It has been shown that binding of monoclonal antibody PAb421 to p53 influences phosphorylation of p53 by CK2. Phosphorylation of p53 by CK2 seems to be important for the growth suppressor function of p53 because it was shown that an alanine-389 mutant of mouse p53 is no longer able to suppress cell growth [40] while the DNA binding function is not inhibited for this mutated p53 [41]. An alanine–392 mutant of human p53 has no transforming capability [42]. Moreover, a serine-392 to alanine exchange has no effect on the transactivation activity of p53 whereas the transrepression activity of p53 on the *c-fos* promotor is inhibited [43]. In addition to the phosphorylation of p53 by CK2 it was shown that p53 binds to the regulatory β-subunit of protein kinase CK2 by C-terminal sequences spanning amino acids 287–340 [21]. In the present paper we described that in addition to the phosphorylation of p53 by protein kinase CK2 also binding of the regulatory β-subunit to p53 influenced the DNA binding activity of p53. DNA binding of p53 is reduced by the β-subunit of CK2 whereas the α-subunit of CK2 had no influence. The CK2 holoenzyme seems to activate the DNA binding properties of p53 similar to monoclonal antibody PAb421 [39] and similar to a C-terminal peptide of p53 spanning amino acids 264–393 (data not shown).

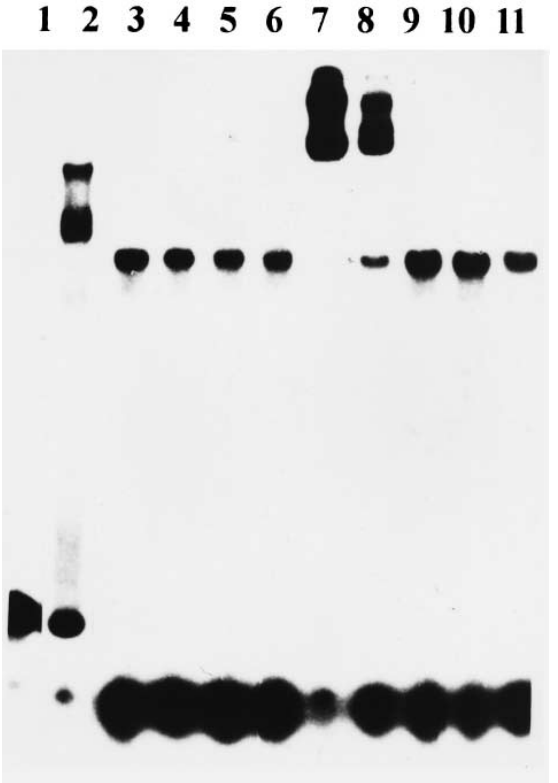

Fig. 4. DNA binding of p53 in the presence of the β-subunit of CK2. The ^{32}P-endlabeled oligonucleotide containing the p53 consensus sequence was incubated under DNA binding conditions with cell extracts (p53 or control, respectively) in the absence (lanes 1, 2, 5, 6, 7) or presence (lanes 3, 4, 8, 9, 10, 11) of β-subunit of CK2. In addition lanes 4, 5, 7, 8 and 10 contain monoclonal antibody PAb421. After an incubation time of 30 min the probes were analyzed on a 4% polyacrylamide gel followed by autoradiography of the gel. Lane 1: DNA. Lane 2: p53 cell extract with DNA. Lane 3: Control cell extract with DNA and β-subunit. Lane 4: Control cell extract with DNA, β-subunit and PAb421. Lane 5: Control cell extract with DNA and PAb421. Lane 6: Control cell extract with DNA. Lane 7: p53 cell extract with DNA and PAb421. Lane 8: p53 cell extract with DNA, β-subunit and PAb421. Lane 9: DNA with β-subunit. Lane 10: DNA with β-subunit and PAb421. Lane 11: p53 cell extract with DNA and β-subunit.

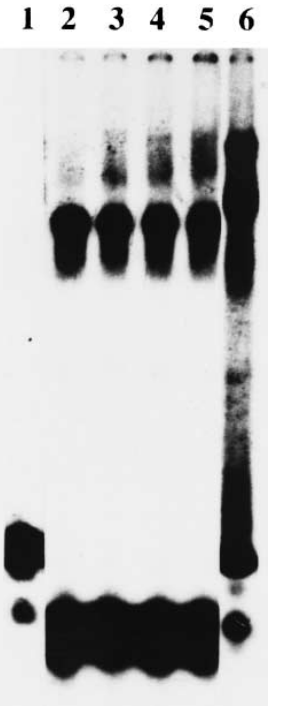

Fig. 5. DNA binding of p53 in the presence of increasing amounts of the β-subunit of CK2. The ^{32}P-endlabeled oligonucleotide containing the p53 consensus sequence was incubated under DNA binding conditions with p53 in the absence (lanes 1 and 6) or presence of increasing amounts of the β-subunit of CK2 (lanes 5–2). After incubation for 30 min. samples were analyzed on a 4% polyacrylamide gel followed by autoradiography. Lane 1: DNA. Lane 2: p53 cell extract and β-subunit molar ratio 2:1 and DNA. Lane 3: p53 cell extract and β-subunit molar ratio 4:1 and DNA. Lane 4: p53 cell extract and β-subunit molar ratio 6:1 and DNA. Lane 5: p53 cell extract and β-subunit molar ratio 8:1 and DNA. Lane 6: p53 cell extract with DNA.

Wild-type p53 promotes the rapid renaturation of complementary RNA and DNA strands [44]. Furthermore, the C-terminus of p53 recognizes DNA with mismatches and moreover it has the capacity to form stable complexes with damaged DNA [24]. Complex formation of p53 with the β-subunit of CK2 as well as binding of monoclonal antibody PAb421 to p53 results in loss of the p53 annealing activity [45].

There is some controversy about the subcellular localization of CK2. Depending on the cell lines used for the analysis, the individual subunits of CK2 are found in the cytoplasm, nucleus or in the nucleoli [28,46], intracellular localizations where also p53 has been found [47,48]. Moreover, in addition to the holoenzyme of CK2 free α- and free β-subunits are found within the same cell [49]. In the present paper we demonstrated an individual role of the free β-subunit compared to the β-subunit in a CK2 holoenzyme. The β-subunit of CK2 reduced DNA binding activity of p53 whereas the holoenzyme led to a supershift of p53/DNA complexes. The α-subunit had no influence on the DNA binding activity of p53.

The fibroblast growth factor-2 [50] and the C-terminus of p53 [26] also bind to the β-subunit of CK2 which lead to an increase in the enzymatic activity. In addition to this direct effect of p53 on the CK2 activity there seems to be an indirect

effect. Upon DNA damage the intracellular level of p53 increases [51]. This elevated level of p53 leads to a transcriptional activation of the p21WAF1 gene expression [52]. The p21 WAF1 protein was shown to inhibit the cyclin dependent kinases [53] and in addition also protein kinase CK2 at least in vitro [30].

Several years ago it was shown that purified protein kinase CK2 associates with double stranded DNA in vitro [54]. This DNA binding is mediated by the α-subunit of CK2. Recently, it was shown that the α-subunit binds to the promoter of the CK2 β-subunit gene thereby regulating its transcription [55]. However, in the present report we demonstrated that the α-subunit neither bound to the p53-specific oligonucleotide nor influenced the binding of p53 to DNA.

Thus, from all of these data it is evident that the β-subunit is the target for the regulation of the activity of the CK2 holoenzyme. However, the free α- and free β-subunits also have individual roles in regulation of various cellular activities.

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