Casein kinase 2 inhibits the renaturation of complementary DNA strands mediated by p53 protein

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Considerable effort is currently being devoted to understand the functions of protein p53, a major regulator of cell proliferation. The protein p53 has been reported to catalyse the annealing of complementary DNA or RNA strands. We report that this activity is inhibited in the presence of the serine/threonine protein kinase CK2. It is shown that this inhibition can be explained by the occurrence of a high-affinity molecular association between p53 and CK2. The molecular complex involves an interaction between the C-terminal domain of p53 and the β subunit of the oligomeric kinase. Accordingly, the isolated α subunit of the kinase was without effect. In addition, after phosphorylation by CK2, phosphorylated p53 lost its DNA annealing activity. Because the C-terminal domain of p53 is both involved in the association with CK2 and phosphorylated by it, our results suggest that either protein–protein interaction or phosphorylation of this domain might control the base pairing of complementary sequences promoted by p53 in processes related to DNA replication and repair.

INTRODUCTION

Wild-type p53 protein has been established as a major tumour suppressor and its functional alterations through deletion or point mutations are believed to play a crucial role in the development of a number of tumours in humans [1–5]. The protein seems to be involved in the cellular response to DNA damage, resulting in growth arrest in G1/S triggering apoptosis [6]. Considerable effort is thus being devoted to the understanding of its mechanism of action at the molecular level. p53 is a DNA-binding protein that acts as a transcriptional activator of gene products directly involved in cell growth control, such as p21-waf, which leads to the inhibition of cyclin-dependent kinases cdks [7,8]. Biochemically, p53 presents three major domains. The N-terminal portion of the protein seems to be involved in transcriptional activation whereas the central core domain confers a sequence-specific DNA-binding property on the intact p53 [9]. The C-terminal domain seems to confer on p53 the ability to oligomerize [10] and is believed to play a major regulatory role in specific DNA-binding activity of the core domain [8]. It is subjected to post-translational modifications by protein kinase C, $p34^{cdc2}$ and CK2 [11–13]. Protein kinase CK2 phosphorylates p53 on its penultimate amino acid residue [13]. This phosphorylation has been shown to activate sequence-specific DNA binding of p53 *in vitro*, although its significance *in vivo* has not yet been clearly established [13,14]. In addition to p53 protein phosphorylation by protein kinase CK2, a molecular complex between the two partners in living cells was suggested by the observation that CK2 and p53 in SV40-transformed 3T3 cells were co-purified throughout several chromatographic steps [13] and were co-immunoprecipitated in SV40-transformed 3T3 cells [15].

CK2 is a ubiquitous serine/threonine kinase found in both the cytoplasm and the nucleus of eukaryotic cells [16]. The enzyme exists as a heterotetramer composed of two subunits, i.e. an α subunit of 35–44 kDa and a β subunit of 24–29 kDa, which associate to form a native $\alpha_2\beta_2$ structure [16,17]. The α polypeptide has been identified as the catalytic subunit. The β subunit, which is believed to play a regulatory role, becomes phosphorylated in an intramolecular autophosphorylation reaction [16,18]. Considerable interest in CK2 has arisen recently because (1) the enzyme was reported to accumulate in nuclei of actively growing cells [17], (2) CK2 has a broad substrate specificity, which includes nuclear oncoproteins such as Myc, Myb, Fos, the adenovirus E_1A protein, the human papillomavirus E_7 protein, the SV40 large T antigen and the p53 protein [19], (3) abolition of CK2 catalytic activity yielded to cell death in yeast [20], and (4) the α subunit gene of CK2 has been proposed to act as an oncogene [21].

In addition to its function as a transcriptional regulator, p53 has been shown to bind single-stranded (ss) DNA [22] and to catalyse renaturation (annealing) of complementary single strands as well as strand transfer [23,24]. This property has been ascribed to the C-terminal domain of p53 [25]. This ability of p53 to favour hybridization of ssDNA ends was proposed to explain its DNA helicase inhibitory property [26] and might contribute to the inhibition of replicative DNA biosynthesis by blocking DNA strand separation. The C-terminal domain of the protein has also been shown to recognize insertion deletion mismatches on DNA and to form stable complexes with such modified DNAs [27]. The p53 domain involved in ssDNA binding has recently been confirmed to be contained in the C-terminal part of the protein, and to overlap with the antibody PAb421 epitope, represented by the 370–378 amino acid sequence [28].

We have recently reported that recombinant p53 and oligomeric protein kinase CK2 form both *in vitro* and in the living cell a stable complex that requires the β subunit of the enzyme [29]. However, the biological significance of such a molecular complex in cell regulation remains to be defined. The present work shows that the stoicheiometric molecular association of CK2 to the C-terminal domain of p53 prevents the p53-catalysed annealing of complementary ssDNA. The complex between the two proteins

Abbreviations used: ds, double-stranded; ss, single-stranded.

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dissociates when p53 becomes phosphorylated by the kinase. In addition, p53 lost its ssDNA-annealing activity after phosphorylation *in vitro* by protein kinase CK2.

EXPERIMENTAL

Materials

[γ-^{32}P]ATP (3000 Ci/mmol) was purchased from Amersham (Buckinghamshire, U.K.). Recombinant *Drosophila* oligomeric CK2 and its isolated α or β subunits expressed in Sf9 cells were purified to homogeneity as previously described [30]. Purified recombinant human CK2 β subunit was a gift from Dr. W. Pyerin (Heidelberg, Germany). Recombinant murine p53 expressed in insect cells was purified as described [11]. Recombinant human p53 expressed in *Escherichia coli* was purified as described by Hupp et al. [14] with some modifications. BL21 (DE3) cells harbouring pETp53WT were grown, induced in Luria–Bertani medium containing 0.5 M NaCl. Washed, frozen cells were lysed by the addition of NaCl to a concentration of 0.68 M and sonication followed by centrifugation for 30 min at 4 °C and 40000 ***g***. Proteins were precipitated with 30 % (w/v) ammonium sulphate. The pellet was resuspended and applied to a heparin–Sepharose column. p53 derived from this step was further purified on a Pharmacia Superose 6 column and the 90 % pure protein was stored in 10 % (v/v) glycerol, 50 mM Tris/HCl, pH 7.5, 0.2 M NaCl, 10 mM dithiothreitol, 0.1 mM EGTA, 0.1 % Nonidet P40, 0.1 mM PMSF, 0.1 mM $ZnCl_2$ at −80 °C. Complementary DNA strands used in the DNA annealing reactions were obtained by heat denaturation of a ^{32}P-end labelled DNA fragment (specific radioactivity 10^9 c.p.m./mg), 99 bp in length, obtained by *Sac*I and *Kpn*I restriction of plasmid pBlueScript SK (Stratagene).

DNA annealing assay

The DNA annealing activity of p53 was assayed by preincubating the p53 protein (50 ng) in the absence or presence of the indicated amounts of CK2 or its isolated subunits for 10 or 30 min at 25 °C in 9 μl of renaturation buffer composed of 40 mM Tris/HCl, pH 7.5, 1.5 mM dithiothreitol, 100 mM NaCl, 1 mM EDTA and 20 mg/ml BSA. The reaction was started by the addition of approx. 0.1 ng (1 μl) of radiolabelled ssDNA. After incubation at 37 °C for 10–30 min, the reaction was stopped by the addition of 0.1 volume of 3 % (w/v) SDS and 150 mM EDTA, after which the mixture was analysed by native PAGE [15 % (w/v) gel] followed by autoradiography. Signals were detected and measured with a Phosphorimager (Molecular Dynamics).

Phosphorylation assay

The human recombinant p53 protein (50 ng) expressed in *E. coli* was phosphorylated by purified recombinant CK2 (2.5 ng) on incubation with [γ-^{32}P]ATP (3000 Ci/mmol) and different concentrations of unlabelled ATP in renaturation buffer containing 1 mM $MgCl_2$ for 15 min at 22 °C followed by 5 min at 30 °C. The reaction was stopped by the addition of Laemmli sample buffer and the mixture was analysed by SDS/PAGE (12 % gel). Phosphate incorporation into p53 was assayed with the Phosphorimager and expressed as pmol of phosphate per pmol of p53.

RESULTS

p53 DNA annealing activity in the presence of CK2

It has been shown recently [23] that human wild-type p53 promotes the rapid renaturation of complementary RNA and DNA strands and that this p53 activity is not dependent on the base composition or the length of complementary strands. We first checked whether purified murine wild-type p53 was also able to promote annealing of complementary DNA strands. Figure 1(A) shows the renaturation of 99 nt complementary DNA strands in the absence or in the presence of recombinant murine p53 protein. We observed that 50 ng of p53 promoted annealing of approx. 40 % of DNA within 10 min at 37 °C. No double-stranded (ds) DNA formation was observed in the absence of p53 protein or in the presence of 50 ng of CK2, clearly showing that the DNA annealing activity was due to the presence of p53. Moreover the preincubation of p53 with CK2, at a 1:1 molar ratio, resulted in a complete inhibition of p53-mediated DNA annealing. To better characterize this inhibition, increasing con-

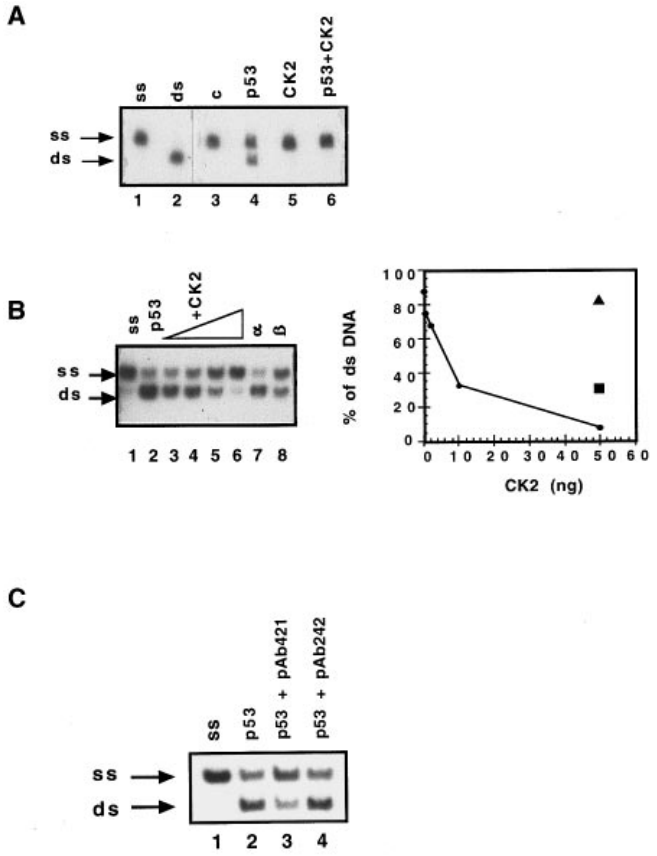

Figure 1 Inhibition of DNA annealing activity of p53 by its interaction with CK2

(**A**) A 99 nt heat-denaturated ^{32}P-labelled DNA was used as a substrate for p53 annealing activity. Proteins were preincubated for 10 min at 25 °C and the annealing reaction was started by the addition of DNA for 10 min at 37 °C. At the end of the incubation, samples were analysed by native gel electrophoresis and autoradiography. The position of ss and ds fragments are shown in lanes 1 and 2 respectively. Lane 3, no protein; lane 4, 50 ng of p53; lane 5, 50 ng of CK2; lane 6, 50 ng of p53 plus 50 ng of CK2. (**B**) Effect of increasing concentrations of CK2 on the DNA-annealing activity of p53. The reactions were performed at 37 °C for 30 min without (lane 1) or with (lanes 2–8) 50 ng of p53 in the presence of 0.4, 2, 10 and 50 ng of CK2 (lane 3, 4, 5 and 6 respectively) or 50 ng of α subunit (lane 7) or 50 ng of β subunit (lane 8). The left panel shows the autoradiograph of the gel that was measured with a Phosphorimager (right panel; ▲, α subunit; ■, β subunit). (**C**) Effect of pAb421 and pAb242 antibodies on the DNA annealing activity of p53. The p53 protein (50 ng) was preincubated for 30 min at 4 °C in the absence (lane 2) or the presence of 50 ng of either pAb421 (lane 3) or pAb242 (lane 4). The annealing reaction was started by the addition of ssDNA for 30 min at 30 °C. At the end of the incubation, samples were analysed by native gel electrophoresis and autoradiography. Lane 1 corresponds to ssDNA that was incubated without addition for 30 min at 30 °C.

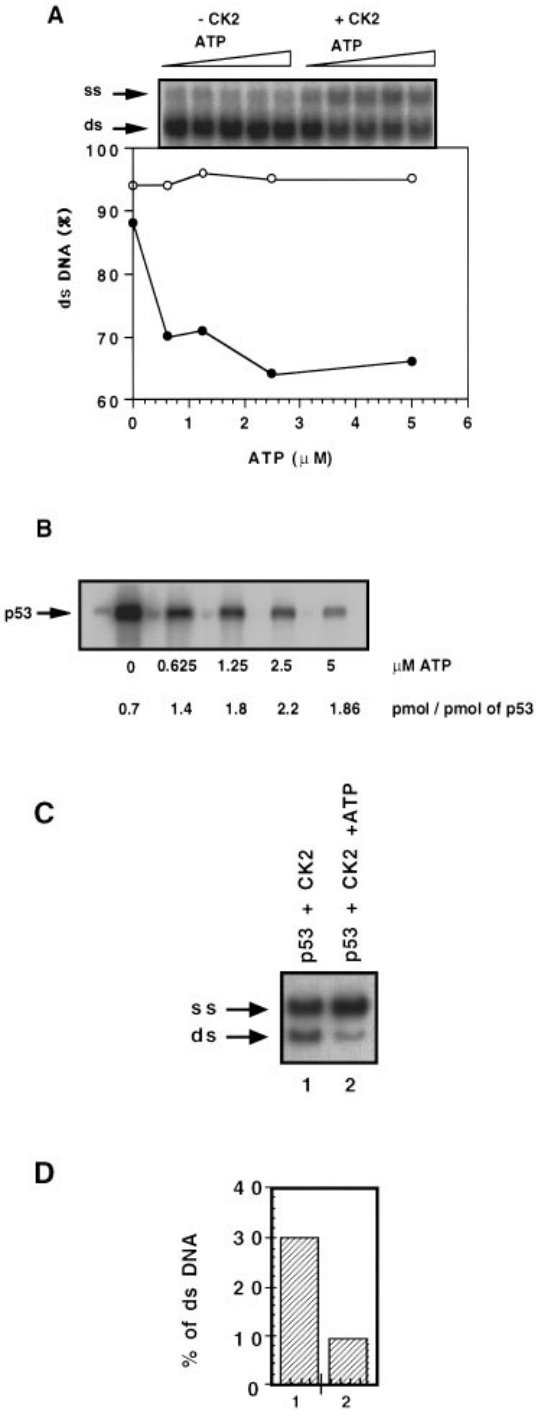

Figure 2 Inhibition of DNA annealing activity of p53 by its phosphorylation by CK2

(**A**) Quantification of the effect of p53 phosphorylation on its DNA annealing activity. The reaction of phosphorylation was performed at 22 °C for 15 min without (○) or with (●) 2.5 ng of CK2 in the presence of 50 ng of human recombinant p53 and increasing amounts of ATP (0–5 μM) as described in the Experimental section. The annealing reaction was started by the addition of DNA for 5 min at 30 °C. At the end of the incubation, samples were analysed by native gel electrophoresis and autoradiography. The percentage of dsDNA was measured with a Phosphorimager. (**B**) Phosphorylation of p53 by CK2. The phosphorylation of human p53 was performed under the same conditions as in (**A**) in the presence of [γ-^{32}P]ATP and increasing concentrations of unlabelled ATP. At the end of the incubation, samples were analysed by SDS/PAGE. Phosphorylated protein was revealed by autoradiography and the stoicheiometry of phosphorylation was determined with a Phosphorimager. (**C**) Effect of p53 phosphorylation by immobilized CK2 on its DNA annealing activity. Immunoprecipitated CK2 was incubated with 1 μg of p53 in the absence (lane 1) or presence of 100 μM ATP-Mg^{2+} for 30 min at 25 °C. The samples were then adjusted to 0.5 M NaCl and p53 was recovered after centrifugation of the Protein A–Sepharose beads. Aliquots (2 μl) of the supernatants were used for determination of p53-mediated DNA annealing activity. (**D**) Quantification of the effect of p53 phosphorylation of (**C**) with a Phosphorimager.

centrations of CK2 were preincubated with p53 before performing the annealing assay. Figure 1(B) shows that the DNA renaturation activity of p53 was strongly inhibited in the presence of increasing amounts of CK2. The IC_{50} for this inhibition was obtained with 5–10 ng of the protein kinase. We have previously reported that p53 protein and CK2 form a stable molecular complex that requires the presence of the β subunit of the kinase [29]. We therefore tested the effect of the isolated CK2 subunits of the enzyme on the p53-mediated DNA annealing activity. As illustrated in Figure 1(B) (lanes 7 and 8), this experiment shows that the isolated α subunit of CK2 had no detectable effect on p53 annealing activity, whereas preincubation of p53 with 50 ng of CK2 β subunit led to more than 50% inhibition of p53 activity. The epitope of anti-p53 pAb421 antibody is localized on the C-terminal part of the protein and we have previously shown that the recognition of p53 by this antibody inhibits its interaction with CK2 [29]. Figure 1(C) shows that the preincubation of p53 with the pAb421 antibody blocked the DNA annealing activity of p53. In contrast, this activity of p53 was not affected by the presence of the pAb242 antibody (Figure 1C, lane 4) or an excess of large T antigen, which are known to bind to p53 in its N-terminal part (results not shown).

Effect of p53 phosphorylation by CK2 on its DNA annealing activity

In addition to its molecular association with CK2 in the absence of ATP, p53 is efficiently phosphorylated by CK2 when a source of phosphate is provided. We thus examined the effect of this phosphorylation on the DNA annealing activity of p53. However, because the presence of a stoicheiometric amount of CK2 is by itself inhibiting (see Figure 1B), the experimental design required either the use of a catalytic amount of CK2 in the annealing assay in the presence of ATP-Mg^{2+}, or the isolation of p53 after its phosphorylation by the kinase. With the first approach, p53 was incubated with increasing concentrations of ATP, in the absence or presence of a catalytic amount of CK2 (a molar ratio of p53/CK2 of 10), for 15 min to allow p53 phosphorylation before starting up the annealing reaction (Figure 2A). The phosphorylation of p53 was monitored by using [γ-^{32}P]ATP under the same reaction conditions and analysed by 12% SDS/PAGE as illustrated in Figure 2(B). In this experiment the stoicheiometry of p53 phosphorylation was calculated and revealed 2 pmol of phosphate incorporated per pmol of p53 in the presence of an excess of ATP. Under these conditions the DNA annealing activity of p53 was inhibited by approx. 30%. This inhibition was not due to the presence of the low CK2 concentration used because in the absence of ATP p53 retained its DNA annealing activity. These results were confirmed with the second approach. As we have previously shown that p53 protein is poorly phosphorylated by the recombinant isolated catalytic CK2 α subunit [29], we used the immobilized oligomeric kinase to phosphorylate p53. We immobilized CK2 by immunoprecipitation with an antiserum directed against the C-terminal end of the β subunit of the enzyme. p53 protein was then incubated with the immobilized kinase in the absence or presence of 100 μM ATP-Mg^{2+}. At the end of the incubation, extraction at high salt concentration (0.5 M NaCl) allowed the recovery of both unphosphorylated and phosphorylated p53, which were

assayed for their DNA annealing activity. Using this approach, the effect of p53 phosphorylation could be examined without any contribution of p53–CK2 complex formation. Whereas the DNA annealing activity of unphosphorylated p53 was detectable, the activity of p53 previously phosphorylated by CK2 was virtually abolished (Figures 2C and 2D).

DISCUSSION

Owing to its involvement in the regulation of basic functions of the normal cell such as the cell cycle or the triggering of apoptosis, and because of its frequent alteration in human cancer, the p53 protein is the subject of extensive research work to understand its mechanisms of action and possibly pave the way to new anticancer therapeutic approaches.

Wild-type p53 protein binds in a sequence-specific manner to dsDNA and behaves as a transcriptional regulator [7]. The specific DNA-binding property has been ascribed to the central domain of the protein and this DNA interaction is strongly inhibited by the SV40 large T antigen, which forms a stable complex by binding to this part of the protein [31]. The MDM2 gene product has been shown to inhibit p53–DNA interaction by a similar mechanism [32].

Covalent modification of p53 by phosphorylation seems to represent another major process regulating its transcriptional activity. A number of different protein kinases, including casein kinase 1 [33], p34 cdc2 [12], DNA-activated kinase [34], protein kinase C [11], Raf-1 kinase [35], JNK1 [36] and CK2 [13], are able to introduce phosphate at different sites in the N- and C-terminal domains. A protein kinase CK2 site has been identified at Ser-389, near the C-terminal part of p53. Although not in the DNA-specific binding domain, this phosphorylation was shown to activate p53 transcriptional activity *in vitro* [31,37]. Replacement of Ser-389 by alanine resulted in the loss of p53 growth-suppressive activity in living cells [38], although this observation has been questioned [39,40].

The C-terminal domain of p53 has also been shown to be required for several functional properties of p53 such as oligomerization [10] and nuclear import [41]. Recently, p53 protein has been shown to act as a catalyst in promoting the renaturation (annealing) of complementary ssDNAs (and RNAs) [23]. This property has been attributed to its C-terminal domain and is of interest because it represents a possible mechanism where p53 can block replication by inhibiting DNA strand separation [24,28].

The fact that p53, through its C-terminal part, recognizes and binds to DNA mismatches adds to the idea that p53 might participate in the recognition of DNA lesions and subsequently blocks replication during DNA repair, acting as a guardian of the genome [6,27,42].

We have previously shown that p53 protein and the protein kinase CK2 associate in a stable complex requiring the β subunit of the kinase [29]. The molecular association of the two purified partners *in vitro* exhibited a high affinity (K_d 70 nM) and was demonstrated to occur in living cells expressing p53 and CK2 or its β subunit [29]. The present work shows clearly that the p53–CK2 association results in the loss of p53 annealing activity. This was confirmed by the observation that the anti-p53 antibody pAb421, which is directed toward a C-terminal domain epitope of the protein, also abolished the annealing activity of p53 [28]. Although the mechanism of this catalytic activity is not yet clearly understood, it may be suggested that p53 oligomerization may contribute to bringing together complementary ssDNA bound to p53 monomers. Association of p53 with large molecules such as CK2 (140 kDa) or an antibody might impair this activity.

When ATP-Mg^{2+} is provided to the p53–CK2 complex, p53 becomes phosphorylated. We have previously shown that p53 phosphorylation results in the dissociation of the p53–CK2 molecular complex [29]. The experimental set-up used in the present study allowed examination of the annealing activity of isolated CK2-phosphorylated p53, in the absence of the kinase. This clearly disclosed that CK2-phosphorylated p53 had lost its ssDNA renaturation promoting activity. It may be noted that under our conditions, phosphorylation by CK2 resulted in the covalent introduction of 2 mol of phosphate per mol of p53. This would suggest that in addition to Ser-389 [14,37], another CK2 phosphorylation site is accessible on the p53 protein.

The present study confirms that p53 protein can efficiently promote renaturation of complementary ssDNA strands. This p53 activity seems to be drastically affected by the protein kinase CK2 in two possible ways: (1) by forming a molecular complex through its β subunit, resulting in a tight association involving the p53 C-terminal domain, and (2) by phosphorylation of p53, which lost its annealing activity after this covalent modification.

The biological significance of these observations in the regulation of cell functions remains to be understood. Both p53 protein and CK2 are major components of the nuclear compartment and are believed to act in the control of the nuclear machinery.

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