Activation of the DNA-binding ability of latent p53 protein by protein kinase C is abolished by protein kinase CK2

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p53 is one of the most important regulators of cell proliferation and differentiation and of programmed cell death, triggering growth arrest and/or apoptosis in response to different cellular stress signals. The sequence-specific DNA-binding function of p53 protein can be activated by several different stimuli that modulate the C-terminal domain of this protein. The predominant mechanism of activation of p53 sequence-specific DNA binding is phosphorylation at specific sites. For example, phosphorylation of p53 by PKC (protein kinase C) occurs in undamaged cells, resulting in masking of the epitope recognized by monoclonal antibody PAb421, and presumably promotes steady-state levels of p53 activity in cycling cells. In contrast, phosphorylation by cdk2 (cyclin-dependent kinase 2)/cyclin A and by the protein kinase CK2 are both enhanced in DNA-damaged cells. We determined whether one mechanism to account for this mutually exclusive phosphorylation may be that each phosphorylation event prevents modification by the other kinase. We used non-radioactive electrophoretic mobility shift assays to show that C-terminal phosphorylation of p53 protein by cdk2/cyclin A on Ser^{315} or by PKC on Ser^{378} can efficiently stimulate p53 binding to DNA *in vitro*, as well as binding of the monoclonal antibody Bp53-10, which recognizes residues 371–380 in the C-terminus of p53. Phosphorylation of p53 by CK2 on Ser^{392} induces its DNA-binding activity to a much lower extent than phosphorylation by cdk2/cyclin A or PKC. In addition, phosphorylation by CK2 strongly inhibits PKC-induced activation of p53 DNA binding, while the activation of p53 by cdk2/cyclin A is not affected by CK2. The presence of CK2-mediated phosphorylation promotes PKC binding to its docking site within the p53 oligomerization domain, but decreases phosphorylation by PKC, suggesting that competition between CK2 and PKC does not rely on the inhibition of PKC–p53 complex formation. These results indicate the crucial role of p53 C-terminal phosphorylation in the regulation of its DNA-binding activity, but also suggest that antagonistic relationships exist between different stress signalling pathways.

Key words: activation, cdk2/cyclin A, DNA binding, electrophoretic mobility shift assay (EMSA), p53, phosphorylation.

INTRODUCTION

The p53 tumour suppressor protein (reviewed in [1,2]) is a potent transcription factor, playing a key role in cell cycle regulation and differentiation. p53 protein is activated in response to a variety of cellular stress signals, including DNA damage, hypoxia, metabolic changes, heat shock, pH changes and oncogene activation, and triggers cell cycle arrest and/or apoptosis to prevent cells from undergoing tumorigenic alterations [3]. The p53 protein has been structurally and functionally divided into four domains, two of which are involved in its DNA-binding function. The central region, known as the core domain (residues 102–290 in human p53), is responsible for DNA binding in a sequence-specific manner, whereas the C-terminal negative regulatory domain (residues 364–393) is necessary for sequence-independent binding and regulates the binding capability of the core domain [4].

Wild-type p53 protein is structurally flexible, and can reversibly adopt a latent conformation, which does not bind DNA, or an active conformation, which binds DNA in a sequence-specific manner. The activation of latent p53 to enable it to bind DNA can be induced by events that target the C-terminal regulatory domain, e.g. by deletion of the last 30 amino acids, or by interaction with specific peptides [5,6], short single-stranded DNAs [7] or monoclonal antibodies [8,9], that can influence both sequence-specific and non-specific DNA-binding functions [10]. The regulatory mechanism of core domain sequence-specific DNA binding via the C-terminal domain involves the ability of the C-terminus to destabilize folding of the core DNA-binding domain [11]. Phosphorylation of the C-terminus neutralizes this destabilizing effect and prevents core domain unfolding, thus activating DNA binding [12]. After DNA binding is activated, p53 can be acetylated in a DNA-dependent manner by the transcriptional co-activator p300 [13]. This acetylation clamps p300 to the p53–DNA complex and presumably enhances recruitment of chromatin remodelling factors. Further, a post-DNA-binding role for acetylation was shown recently by the demonstration that the proline-repeat domain binds directly to p300 and that this binding overcomes the conformational constraint to acetylation in the p53 tetramer [14]. Thus a phosphorylation and acetylation cascade can play a role in activating DNA binding and in clamping p300 to the p53–DNA complex.

Abbreviations used: ATM, ataxia telangiectasia mutated; cdk, cyclin-dependent kinase; Chk, checkpoint kinase; DNA-PK, DNA-dependent protein kinase; DTT, dithiothreitol; EMSA, electrophoretic mobility shift assay; GOPS, glycidoxypropyltrimethoxysilane; MDM2, murine double min clone 2 oncoprotein; PKC, protein kinase C.

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Thus, critical events for p53 protein stabilization and activation in response to cellular stress are post-translational modifications, including phosphorylation, acetylation, glycosylation [15] or conjugation with the small ubiquitin-like protein SUMO-1 (small ubiquitin-related modifier-1) [16]. p53 is phosphorylated by different cellular kinases on several serine and threonine residues within the N- and C-terminal regions [17].

The protein kinase CK1 phosphorylates Ser^{6} and Ser^{9} of p53 *in vitro*, and DNA-PK (DNA-dependent protein kinase) and ATR (ataxia telangiectasia-related) kinase phosphorylate Ser^{15} and Ser^{37}. Phosphorylation at Ser^{15}, which is important for transactivation properties and protein stability, is also mediated by ATM (ataxia telangiectasia mutated) kinase in response to UV- and ionizing radiation-mediated damage. The checkpoint kinases Chk1 and Chk2 can phosphorylate Thr^{18} and Ser^{20} by an allosteric activation step involving Chk2 docking to the DNA-binding domain of p53 [18]. This phosphorylation stabilizes p300 docking to the phosphorylated p53 transactivation domain and promotes the DNA-dependent acetylation of p53 [14,19]. Ser^{33} is phosphorylated by JNK (c-Jun kinase N-terminal kinase) and by cdk7/cyclin H/p36 kinase [20,21]. The N-terminal modifications contribute to p53 regulation by affecting the interaction of p53 with the negative regulatory protein MDM2 (murine double min clone 2 oncoprotein) and with transcriptional co-activators [22].

Several sites of potential modification are clustered within the C-terminus of human p53 and are important for its regulatory function. Lys^{320}, Lys^{373} and Lys^{382} are modified by acetylation with PCAF (p300/CBP-associated factor) and p300/CBP [CREB (cAMP response element-binding protein)-binding protein] [23], and although these modifications have been shown to enhance the DNA-binding activity of p53 *in vitro*, acetylation is conformationally constrained and is a post-DNA-binding event *in vivo* [13,19]. Ser^{315} of p53 is targeted by cdk1 and cdk2 (cyclin-dependent kinases 1 and 2). Ser^{378} is phosphorylated by PKC (protein kinase C) [24], and its *in vitro* dephosphorylation by phosphatases 1 and 2A leads to the restoration of reactivity with monoclonal antibody PAb421 and regeneration of p53 latency [25]. In contrast with Ser^{378}, which was shown to be phosphorylated in irradiated cells, Ser^{376} is dephosphorylated in the presence of ATM as a response to genotoxic stress, resulting in the association of p53 with the 14-3-3 adaptor protein [26]. PKC subunits α and ζ were also shown to phosphorylate Ser^{371} *in vitro* [27]. The experimental evidence that modification of p53 by PKC can be responsible for the induction of the G_1/S growth arrest of transformed cells harbouring a functional p53 allele relies on the observation that the PKC activator PMA can induce this G_1/S growth arrest [27a,27b]. Although these data provide evidence that PKC is involved in the activation of p53 in cells, it does not distinguish between the 12 existing isoforms of PKC, nor other enzymes that may potentially modify serine residues at these sites. The supporting evidence for a role of calcium- and diacylglycerol-dependent PKC isoforms in p53 modification in cells comes from reports describing activation of PKC using stress stimuli, such as UV-C radiation and gamma radiation, that can also activate the p53 pathway [28,29]. Ser^{392} is phosphorylated by the protein kinase CK2 after both UV and ionizing radiation treatment *in vitro* and *in vivo* [30,31]. Phosphorylation on Ser^{392} has been shown to enable the transcriptional activation of the p53 protein *in vitro* [5] and also seems to be important for p53-mediated transactivation *in vivo* [32]. CK2 is composed of two catalytic subunits, α and α', and two β regulatory subunits, and regulates p53 activity not only by phosphorylation on Ser^{392} but also by binding of the β subunits to the p53 oligomerization domain (residues 325–344). The β subunits were also shown to influence the DNA-binding activity of p53 [33] and, vice versa, wild-type p53 can inhibit CK2 kinase activity [34]. The region where the CK2 β subunit binds to p53 also functions as a docking site for several other kinases, including cdks and PKC [35,36] (Figure 1).

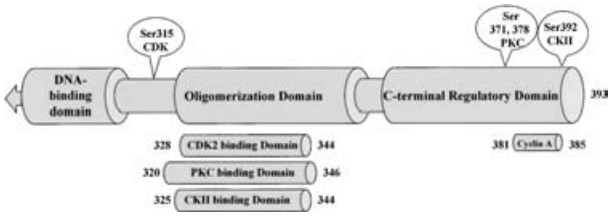

Figure 1 Key phosphorylation sites of human p53 protein and their localization within the protein's functional domains, and binding sites of protein kinases targeting the C-terminus of p53

The importance of different C-terminal modifications for the activation of the sequence-specific DNA binding of the latent form of p53 has still not been successfully explained, namely binding to response elements localized within the long DNA molecules. Here we have studied the mechanisms of p53 protein activation by various C-terminal domain phosphorylations, the role of the C-terminal-directed kinases CK2, PKC and cdks in this process and their relationship with respect to p53 protein binding. The fact that cdk2/CK2 and PKC exhibit mutually exclusive phosphorylations of p53, depending on the level of DNA damage, provides evidence that such a mechanism may operate *in vivo* and form a biochemical basis for the effects observed.

EXPERIMENTAL

Expression of p53 protein in bacteria and in insect cells

Human p53 protein was expressed in *Escherichia coli* DH5α cells carrying the pT7-7 (p53) plasmid, by isopropyl β-D-thiogalactoside induction (0.5 mM for 4 h), or in Sf9 insect cells infected with recombinant baculovirus. Bacterial cells were harvested and lysed according to the standard protocol. Sf9 cells were grown in suspension at 27 °C in Ex-cell 400 medium (JRH Bioscience) with 5 % (v/v) fetal bovine serum and 2 mM glutamine. At 3 days after baculovirus infection, Sf9 cells were washed twice in cold PBS, scraped, pelleted by centrifugation at 300 ***g*** for 5 min and lysed in the lysis buffer for 30 min on ice.

Purification of p53 protein

Cell lysates (from bacteria or insect cells) were centrifuged at 15 000 ***g*** for 30 min and the supernatant was diluted 5-fold in low-salt purification buffer [15 % (v/v) glycerol, 15 mM Hepes/KOH, pH 8.0, 0.04 % Triton X-100, 5 mM DTT (dithiothreitol), 2 mM benzamidine and 1 mM β-glycerophosphate], filtered and loaded on to a 5 ml heparin–Sepharose column (Amersham Biosciences). The p53 protein was eluted by a 0–1 M KCl gradient, and the peak fractions that eluted at approx. 0.5–0.6 M KCl were pooled, dialysed against low-salt purification buffer for 12 h at 4 °C and loaded on to an anion exchange HQ column of the BioCad Sprint perfusion chromatography system (PerSeptive Biosystems, Inc.).

Expression and purification of cdk2 and cyclin A

Human cdk2 and cyclin A were expressed in Sf9 insect cells using recombinant baculoviruses (kindly provided by Dr K. Ball, Cancer Research UK Laboratories, Department of Surgery and Molecular Oncology, University of Dundee Medical School, Dundee, U.K.) grown in suspension at 27 °C in Ex-cell 400 medium (JRH

Bioscience) containing 5 % (v/v) fetal bovine serum and 2 mM glutamine. Each recombinant protein was purified according to the methods described in [36].

In vitro phosphorylation of p53 protein

Purified bacterial p53 protein was phosphorylated *in vitro* by CK2 (New England Biolabs), PKC (Promega), calcium- and diacylglycerol-dependent PKC consisting of α, β and γ isoforms, DNA-PK (New England Biolabs) or cdk2 complexed with cyclin A, expressed in the baculoviral system. Purified glutathione S-transferase–Chk1 fusion protein was a gift from Dr J. Hutchins (University of Dundee) [37]. The kinase reaction mixture contained 20 mM Tris/HCl, pH 7.5, 50 mM KCl, 10 mM $MgCl_2$, 1 mM DTT, 1 mM $CaCl_2$, 100 μg/ml phosphatidylserine, 20 μg/ml dioleoyl-*sn*-glycerol, 1 mM CHAPS and 0.2 mM ATP, and the reaction was performed at 30 °C for 30 min. Samples were analysed by Western blotting and by EMSA (electrophoretic mobility shift assay) for DNA-binding activity.

In vitro radiolabelling of p53 protein

The purified bacterial p53 protein was phosphorylated *in vitro* as described above by the addition of [γ-^{32}P]ATP (37 kBq; 1 μCi; Amersham Biosciences). The kinase reaction was performed at 30 °C for 30 min and analysed by SDS/PAGE on 10 % (w/v) polyacrylamide gels. The gels were stained, dried and analysed by phosphorus screen autoradiography on a STORM optical scanner (Molecular Dynamics). The level of phosphorylation was evaluated quantitatively using densitometric tracing (ImageQuant; Molecular Dynamics).

Western-blot analysis and antibodies

The protein was analysed using SDS/10 %-PAGE according to the standard protocol. Monoclonal antibodies were purified from ascites using Protein A columns. The final antibody concentration, as determined by Bradford protein assay, was 1 μg/ml in all cases. The antibodies used in this study were as follows. (a) DO-1 monoclonal antibody, directed towards the epitope S^{20}DLWKL^{25} within the p53 N-terminal region [38]. (b) CM1 polyclonal antibody, which recognizes many epitopes on the human p53 protein [39]. (c) PAb421 monoclonal antibody, which recognizes the epitope S^{371}KKGQSTSRH^{380} within the p53 C-terminal region [40] and displays a strong preference for the non-phosphorylated epitope [9]. (d) FPS315 phospho-specific monoclonal antibody, which recognizes p53 phosphorylated on Ser^{315} [41]. (e) S-P-3 phospho-specific monoclonal antibody, which recognizes the C-terminus of p53 protein phosphorylated on Ser^{392} [42]. (f) Ica9 monoclonal antibody, which recognizes the epitope E^{388}GPDSD^{393} within the p53 C-terminal region [43] and displays a strong preference for the non-phosphorylated epitope [5].

Sequence-specific DNA probes

For radioactive EMSAs, we used 20 bp oligonucleotides containing a single p53 recognition sequence (AGACATGCCTAGACATGCCT), and a p53 non-recognition sequence (GCATCATAGCGCATCATAGC) as a negative control. Complementary oligonucleotides were hybridized and end-labelled with [γ-^{32}P]ATP using T4 kinase. In non-radioactive EMSAs, we used the plasmid pPGM1 (2987 bp; derived from pBluescript II SK) containing one copy of the 20-mer consensus sequence (AGACATGCCTAGACATGCCT) cloned into the *Hin*dIII restriction site [44]. As a negative control, we used the plasmid pBluescript II SK (2961 bp). Both plasmids were digested with the restriction endonuclease *Pvu*II (MBI Fermentas), producing two restriction fragments: 2513 bp without the consensus sequence and 474 bp containing the consensus sequence (from pPGM1), or 448 bp without the consensus sequence (from pBluescript II SK).

EMSA using PAGE

p53 protein (100 ng) was diluted in activation buffer (5 mM Tris/HCl, pH 7.8, 0.5 mM EDTA, 50 mM KCl, 0.01 % Triton X-100). Then 5 ng of radiolabelled oligonucleotide with (or without) the p53 consensus sequence was added to the reaction mixture and incubated on ice for 30 min. Samples were then analysed by native PAGE on 4 % gels in 0.5 × TBE buffer (89 mM Tris/borate, 2 mM EDTA) at 200 V for 120 min at 4 °C.

EMSA using agarose gel electrophoresis

p53 protein (100 ng) was diluted in activation buffer (as above) and incubated for 30 min at 30 °C with 2 μg of specific monoclonal antibody. A 1 μg sample of DNA restriction fragment, with or without the p53 consensus sequence, was added to the reaction mixture and incubated on ice for 30 min. Loading buffer was added to the reaction mixture and samples were immediately loaded on to a 1.2 % native agarose gel. Electrophoresis was performed in TBE buffer at 10 V/cm for 200 min at 4 °C. After electrophoresis, gels were stained with ethidium bromide (1 μg/ml) for 30 min, rinsed in distilled water, photographed and evaluated quantitatively using densitometric tracing.

Measurement of protein–protein interactions using the piezoelectric biosensor

Piezoelectric biosensor methodology was used to study the interaction of p53 with kinases in real time. The biosensor reflects the changes in the resonant frequency of the crystal bearing the covalently immobilized p53 protein, which are directly proportional to the amount of biomolecules bound to the crystal surface. Piezoelectric crystals (10 MHz, AT cut, gold electrodes) were obtained from International Manufacturing Company (Oklahoma City, OK, U.S.A.). For immobilization of p53 protein, the crystals were washed carefully with acetone and activated further with GOPS (glycidoxypropyltrimethoxysilane) according to the standard protocol. The crystals were incubated for 1 h in 10 % (w/v) GOPS solution (in 95 % ethanol) at 20 °C and dried for 6 h at 50 °C. The GOPS-modified crystal was inserted into the flow-through cell (internal volume 40 μl), connected to the oscillator circuit and the output frequency was determined using a UZ 2400 counter (UTES/Grundig, Brno, Czech Republic). The resonant frequency of the crystal was recorded and displayed using a custom LabTools program in MS Windows. The cell was connected to a Minipuls MP3 peristaltic pump (Gilson, Villeurbanne, France), silicone tubes (internal diameter 0.16 mm) were used for all connections, the flow rate was kept constant at 25 μl/min and all experiments were carried out at 20 °C. Purified p53 protein was added to the flowing solution at a concentration of 5 μg/ml and allowed to bind for 15 min in the presence of 50 mM phosphate buffer containing 1 mM DTT, pH 9.5. Crystals with covalently immobilized protein p53 thus obtained were used for further experiments. The carrier buffer was 20 mM Tris/HCl, pH 7.5, 50 mM KCl, 10 mM $CaCl_2$, 10 mM $MgCl_2$ and 1 mM DTT. To study interactions of p53-modified crystals with protein kinases, solutions of either CK2 or PKC dissolved in the carrier solution were allowed to interact with the crystals; when indicated, 0.2 mM ATP was present in the solution. The binding curve was recorded for 8–10 min, and then the dissociation of the formed p53–kinase affinity complex was determined in the presence of carrier buffer only.

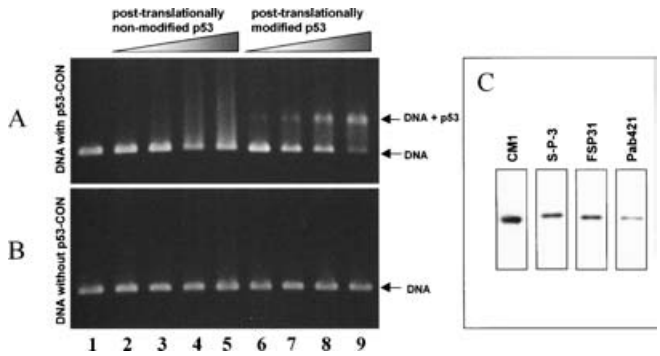

Figure 2 DNA-binding activity of post-translationally modified and non-modified p53 protein

Increasing amounts of post-translationally modified p53 expressed in Sf9 insect cells or bacterially expressed non-modified p53 were incubated with either (**A**) the pPGM1 plasmid carrying the p53 consensus sequence (p53-CON; 474 bp fragment) or (**B**) the pBluescript plasmid lacking the p53 consensus sequence (448 bp fragment). The reaction was performed in the presence of a linear plasmid DNA fragment as a non-specific competitor. The DNA–p53 complexes were separated by electrophoresis on a 1.2 % (w/v) agarose gel as described in the Experimental section. Lane 1, DNA fragment only (250 ng); lanes 2–5, bacterial p53 protein (0.25, 0.5, 1.0 and 1.5 μg respectively), lanes 6–9, baculoviral p53 protein (0.25, 0.5, 1.0 and 1.5 μg respectively). (**C**) p53 expressed in Sf9 insect cells was subjected to 10 % denaturing PAGE. Specific phosphorylation was detected by immune Western blotting using CM1 antibody and the indicated phospho-specific monoclonal antibodies.

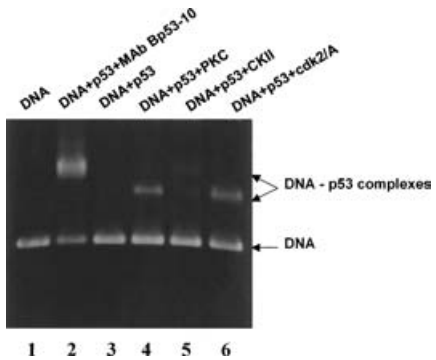

Figure 3 Activation of sequence-specific DNA binding of latent p53 protein by monoclonal antibody binding and phosphorylation

Samples of 100 ng of bacterially expressed p53 (non-modified, phosphorylated *in vitro* or bound with C-terminal anti-p53 monoclonal antibody Bp53-10) were incubated with a 474 bp fragment of the pPGM1 plasmid carrying the p53 consensus sequence. EMSA for DNA–p53 complex detection was performed in a 1.2 % agarose gel. Lane 1, DNA pPGM1/*Pvu*II (fragment of 474 bp carrying the p53 consensus sequence); lane 2, DNA + p53 protein (bacterial) + activating monoclonal antibody (MAb) Bp53-10; lane 3, DNA + p53 (bacterial); lane 4, DNA + p53 phosphorylated by PKC; lane 5, DNA + p53 phosphorylated by CK2 (CKII); lane 6, DNA + p53 phosphorylated by cdk2/cyclin A.

RESULTS

Binding of non-modified and post-translationally modified p53 to DNA fragments containing the consensus response element

The p53 tumour suppressor protein is known to be regulated via C-terminal phosphorylation by cdk2, PKC and CK2. In order to investigate the differential phosphorylation of p53 by these kinases and their effects on the DNA-binding activity of p53, we used the EMSA technique to show the extent of p53 protein–DNA interactions, together with piezoelectric sensor measurements to detect p53 protein–kinase interactions.

The DNA-binding activity of post-translationally modified (expressed in Sf9 insect cells) and non-modified (expressed in *E. coli*) p53 protein using agarose gel EMSA is shown in Figure 2. The post-translationally modified protein (the 'active' fraction phosphorylated on Ser^{315}, Ser^{378} and Ser^{392} and recognized by phospho-specific monoclonal antibodies FPS315 and S-P-3, but partially non-recognized by PAb421, as detected by immunoblotting in Figure 2C) displayed significant sequence-specific DNA-binding activity, resulting in the formation of a retarded band of the p53–DNA complex in the presence of non-specific linear DNA as a competitor. In contrast, the non-post-translationally modified p53 protein did not show any sequence-specific DNA binding (Figure 2A). The same DNA restriction fragment, but without a p53 consensus response element, was used as a negative control (Figure 2B) to demonstrate the specificity of the reaction. Analogous data have been obtained previously in EMSAs of oligonucleotide p53 response elements and by DNase I footprinting, highlighting the importance of post-translational protein modifications for the sequence-specific DNA-binding properties of p53.

Activation of the sequence-specific DNA binding ability of p53 protein by C-terminal monoclonal antibodies and C-terminal phosphorylation

The sequence-specific DNA-binding activity of latent p53 protein can be stimulated by C-terminal-specific monoclonal antibodies such as PAb421 (recognizing an epitope comprising residues 371–380), Bp53-6 (epitope 381–390) and Bp53-10 (epitope 371–380). The previously reported activation of latent p53 protein to a form that binds DNA by monoclonal antibody Bp53-10 is shown in Figure 3 (lane 2), where the retarded band of the p53–DNA–antibody complex is shown. Here we show that phosphorylation of the C-terminal part of p53 protein could also activate its DNA-binding function and induce p53 sequence-specific binding not only to oligonucleotides but also to consensus sequences localized within longer DNA molecules. The retarded band of the p53–DNA complex occurred after phosphorylation by PKC on Ser^{378} or by cdk2/cyclin A on Ser^{315} (Figure 3, lanes 4 and 6); phosphorylation by CK2 on Ser^{392} resulted in only very low activation of p53 that was hardly detectable by EMSA (Figure 3, lane 5). The phosphorylation of the N-terminal domain of p53 by DNA-PK or Chk1 did not influence the DNA-binding activity of p53 (results not shown). Kinase reactions performed without the addition of ATP, which provided the best negative control, did not result in any activation of DNA binding, suggesting that protein phosphorylation was responsible for this effect.

The efficiency of *in vitro* phosphorylation was demonstrated by radiolabelling of p53 using [γ-^{32}P]ATP. This method showed that phosphorylation of the p53 protein reached an equilibrium with all kinases used (i.e. cdk2/cyclin A, CK2 and PKC) within 30 min of kinase reaction. The equilibrium was not due to kinase inactivation, but to the saturation of the p53 phosphorylation sites, as indicated by the following: (a) addition of kinase to the reaction for a second incubation (e.g. for another 30 min) did not increase the level of p53 phosphorylation, and (b) addition of non-modified p53 protein to the reaction mixture for a further 30 min proportionally increased the ^{32}P response, confirming the stability of the kinases. These data allowed us to conclude that all three phosphorylation sites (Ser^{378}, Ser^{315} and Ser^{392}) were modified stoichiometrically. To verify this assumption further, we performed Western blot analysis with two monoclonal antibodies, S-P-3 and FPS315, which recognize phosphorylated (but not unphosphorylated) Ser^{392} or Ser^{315} respectively. With both antibodies we observed strong bands after a 15 min incubation with the respective kinase – CK2 or cdk/A (Figures 4A and 4B) – on our Western blots. The intensity of these bands was not changed after a

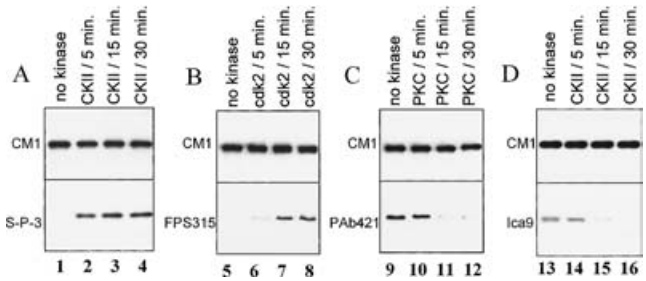

Figure 4 Detection of p53 C-terminal phosphorylation using phospho-specific monoclonal antibodies

Bacterially expressed p53, phosphorylated *in vitro* by CK2, PKC or cdk2/cyclin A, was subjected to 10 % denaturing PAGE. Specific phosphorylation was detected by immune Western blotting using phospho-specific monoclonal antibodies. Lanes 1–4, CK2 (CKII)-phosphorylated p53 detected with S-P-3 (specific for p53 phosphorylated on Ser^{392}); lanes 5–8, cdk2/cyclin A-phosphorylated p53 detected with FPS315 (specific for p53 phosphorylated on Ser^{315}); lanes 9–12, PKC-phosphorylated p53 detected using PAb421 (preferentially recognizes p53 protein not phosphorylated on Ser^{378}); lanes 13–16, CK2-phosphorylated p53 detected with Ica9 (preferentially recognizing p53 protein not phosphorylated on Ser^{392}). Kinase reactions were performed for 5, 15 and 30 min as indicated.

30 min incubation, similar to the results of ^{32}P radiolabelling. It is well known that phosphorylation may occur on a residue that is a part of an epitope for a particular monoclonal antibody and that this may result in a decrease in immunoreactivity, to the extent that the antibody fails to bind to the phosphorylated target protein. For these reasons, phosphorylation of p53 at Ser^{392} was analysed additionally using Ica9 antibody, which prefers binding to the non-phosphorylated epitope $E^{388}GPDS^{393}$ of p53. Our results show (Figure 4D) that, after a 15 min incubation of p53 with CK2, almost all p53 was phosphorylated, and after a 30 min interval there was no non-phosphorylated epitope available for antibody Ica9. The intensity of the band with the control CM1 polyclonal antibody, which recognizes many epitopes of both phosphorylated and non-phosphorylated p53 protein, was not influenced by kinase treatment (Figure 4). p53 phosphorylation by PKC was monitored by the PAb421 antibody, which also preferentially recognizes p53 only when not phosphorylated on Ser^{378}. After a 15 min incubation of p53 with PKC, the PAb421 band almost disappeared (Figure 4C), suggesting that almost all Ser^{378} was phosphorylated. The above data thus confirmed our assumption that practically all serine residues were phosphorylated under the given conditions (Figure 4) by each of the three kinases used in our experiments.

Figure 5(A) shows the phenomenon of p53 activation by C-terminal phosphorylation in more detail. Sequence-specific DNA-binding activity of p53 was mainly stimulated by cdk2/cyclin A and by PKC, whereas phosphorylation by CK2 did not have such a marked effect, as shown by the weak intensity of the retarded band. In addition, CK2 phosphorylation-induced p53–DNA complexes were significantly more retarded than cdk2/cyclin A- and PKC-phosphorylated p53–DNA complexes, suggesting that they have a higher molecular mass (Figure 5A, lanes 2–4; Figure 5B, lane 5). Incubation with monoclonal antibody DO-1 provided a supershift of the p53–DNA complexes and demonstrated the presence of p53 protein in the complex (Figure 5A, lanes 4, 8 and 12). A parallel experiment showed the binding of phosphorylated p53 to an oligonucleotide consensus response element (20-mer; AGACATGCCTAGACATGCCT) using PAGE (Figure 5B). Phosphorylation by all three kinases for 30 min resulted in the formation of a retarded band; in addition, the electrophoretic mobility of the CK2-phosphorylated p53–DNA complex was lower than that of those due to phosphorylation by PKC and cdk2/cyclin A.

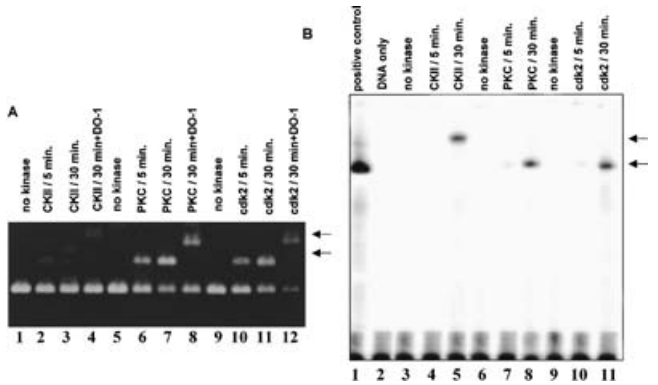

Figure 5 Activation of p53 by phosphorylation by CK2, PKC and cdk2/cyclin A

(**A**) Samples of 100 ng of bacterially expressed p53 protein were phosphorylated *in vitro* by CK2, PKC or cdk2/cyclin A, which target the C-terminal domain. Kinase reactions were performed for 5 or 30 min at 30 °C in the presence or absence of the anti-p53 monoclonal antibody DO-1. The DNA–p53 complexes were separated on a 1.2 % agarose gel as indicated by the arrows. Lanes 1, 5 and 9, non-modified p53 + consensus DNA; lanes 2 and 3, p53 phosphorylated by CK2 (CKII) (5 and 30 min respectively) + consensus DNA; lane 4, CK2-phosphorylated p53 (30 min) + consensus DNA + DO-1; lanes 6 and 7, PKC-phosphorylated p53 (5 and 30 min respectively) + consensus DNA; lane 8, PKC-phosphorylated p53 (30 min) + consensus DNA + DO-1; lanes 10 and 11, cdk2/cyclinA-phosphorylated p53 (5 and 30 min respectively) + consensus DNA; lane 12, cdk2/cyclin A-phosphorylated p53 (30 min) + consensus DNA + DO-1. (**B**) Samples of 100 ng of non-modified or *in vitro* phosphorylated p53 protein were incubated with radioactively labelled 20-mer DNA consensus sequence and the p53–DNA complexes were separated on 4 % non-denaturing PAGE and detected by autoradiography. The baculovirus-expressed p53 protein was used as a positive control (lane 1).

These results, which correlate well with the results obtained by agarose gel shift in Figure 5(A), suggest similar DNA-binding behaviour of the protein towards both oligonucleotides and long DNA molecules. The small quantitative difference between the proportion of CK2-induced DNA-binding activity of p53 to the oligonucleotide and to a 474 bp fragment indicates possible negative influences of non-specific DNA sequences on the sequence-specific DNA-binding activity of CK2-phosphorylated p53 protein.

Multiple phosphorylation of the C-terminus of p53 does not increase its DNA-binding activity significantly

The effects of multiple phosphorylation of p53 at C-terminal modification sites on its DNA-binding activity are presented in Figure 6. p53 was incubated with cdk2/cyclin A, PKC and CK2 either separately or in different combinations (CK2 + PKC, CK2 + cdk2, PKC + cdk2 and CK2 + PKC + cdk2), as indicated in Figure 6. We noted that: (a) phosphorylation by cdk2/cyclin A activated p53 protein more efficiently than did that by PKC or CK2, (b) double phosphorylation by cdk2/cyclin A and PKC did not result in a significant increase in p53 activity compared with that after phosphorylation with either kinase alone, (c) phosphorylation by CK2 was only a very weak activator of p53 DNA-binding activity, and (d) preincubation with CK2 inhibited the activatory effect of PKC, but not that of cdk2/cyclin A.

Phosphorylation by CK2 prevents PKC-induced p53 protein activation

To characterize further the phenomenon of competition between CK2 and PKC, which can play an important role in the co-operation of cell signalling processes, p53 was incubated in the presence of CK2, PKC or a combination of both kinases (Figure 7). The retarded bands in lanes 2 and 3 show p53 activation

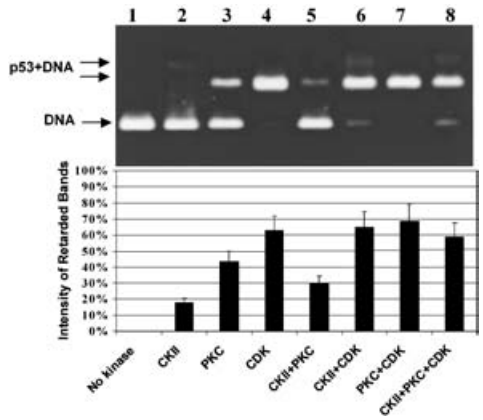

Figure 6 Kinase co-operation and competition during p53 phosphorylation

Samples of 100 ng of bacterially expressed p53 protein were phosphorylated *in vitro* with CK2, PKC, cdk2/cyclin A or different combinations of these kinases added to the reaction at the same time. DNA–p53 complexes were separated by EMSA on a 1.2 % agarose gel. Lane 1, 474 bp pPGM1/*Pvu*II DNA fragment carrying the p53 consensus sequence + non-phosphorylated p53 protein; lane 2, DNA + CK2 (CKII)-phosphorylated p53; lane 3, DNA + PKC-phosphorylated p53; lane 4, DNA + cdk2/cyclin A-phosphorylated p53; lane 5, DNA + p53 phosphorylated with CK2 and PKC; lane 6, DNA + p53 phosphorylated by CK2 and cdk2/cyclin A; lane 7, DNA + p53 phosphorylated by PKC and cdk2/cyclin A; lane 8, DNA + p53 phosphorylated by CK2, PKC and cdk2/cyclin A. Lower panel: quantification of super-shifted DNA–p53 complexes by densitometric tracing (representative measurement of three independent experiments). The total intensity of all bands within each lane was taken as 100 %.

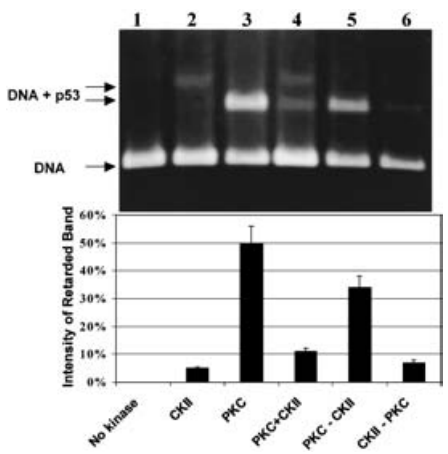

Figure 7 Competition between CK2 and PKC: the presence of CK2 prevents phosphorylation of p53 protein by PKC

Samples of 100 ng of bacterially expressed p53 protein were phosphorylated in the presence of CK2 (CKII) or PKC for 30 min either alone or in combination. When CK2 and PKC were used, they were added to the reaction either at the same time or subsequently (15 min after the beginning of the first kinase reaction). DNA–p53 complexes were separated by EMSA on a 1.2 % agarose gel. Lane 1, 474 bp pPGM1/*Pvu*II DNA fragment carrying the p53 consensus sequence + non-phosphorylated p53 protein; lane 2, DNA + CK2-phosphorylated p53; lane 3, DNA + PKC-phosphorylated p53; lane 4, DNA + p53 phosphorylated by CK2 + PKC (added simultaneously); lane 5, DNA + p53 protein preincubated with PKC (15 min) and then phosphorylated by CK2 (30 min); lane 6, DNA + p53 protein preincubated with CK2 (15 min) and then phosphorylated by PKC (30 min). Formation of the DNA–p53 complexes was indicated by super-shifted bands, and the intensity of the bands was quantified using densitometric tracing. Representative measurements of three independent experiments are shown. The total intensity of all bands within each lane was taken as 100 %.

induced by CK2- and PKC-mediated phosphorylation respectively. The sample in lane 4 was phosphorylated by both CK2 and PKC added to the reaction mixture at the same time. The intensity of the retarded band corresponding to the PKC-phosphorylated p53–DNA complex was significantly decreased (approx. 4-fold), suggesting an inhibitory effect of phosphorylation by CK2 on PKC-induced p53 activation. This was not observed if the p53 protein was preincubated with PKC for 15 min in the presence of 0.2 mM ATP prior to phosphorylation by CK2 (lane 5). In contrast, preincubation of p53 protein with CK2 and 0.2 mM ATP for 15 min prior to phosphorylation by PKC resulted in the complete abolition of p53 DNA-binding activity (lane 6). This effect was not observed in the absence of ATP. These results indicate a direct inhibitory effect of p53 phosphorylation by CK2 at Ser^{392} on the phosphorylation of human p53 by PKC. The kinase reactions performed in the absence of ATP did not stimulate p53 activity, demonstrating that phosphorylation rather than kinase binding or other conformational changes is responsible for p53 activation.

The inhibitory effect of the phosphorylation of p53 by CK2 on phosphorylation by PKC was also detected using radioactive ATP labelling, and similar results were obtained. Phosphorylation of p53 by CK2 and PKC when the kinase reactions were started at the same time led to a mild increase in total protein phosphorylation, as measured by the ^{32}P signal. However, preincubation of p53 with CK2 in the presence of [γ-^{32}P]ATP led to the complete abolition of p53 phosphorylation by PKC (the ^{32}P signal did not change further due to subsequent phosphorylation by PKC).

The kinetics of the binding of CK2 and PKC to p53 docking sites and the role of this interaction in their competition were studied further using the piezoelectric biosensor system. This system, a convenient tool for the characterization of bioaffinity interactions [45], reflects changes in the resonant frequency of a crystal which are directly proportional to the amount of biomolecules bound to the sensing surface, and enables the affinity interaction to be followed in real time. Here a decrease in the resonant frequency of a p53-modified crystal corresponds to the interaction of p53 with another molecule, i.e. CK2 or PKC dissolved in kinase buffer. The interactions of p53-modified crystals with these kinases were studied in the presence (Figure 8, left panel, curves b and d) or absence (curves a and c) of ATP. PKC easily bound to p53 (curves a and b), and addition of the carrier buffer resulted in the complete dissociation of PKC (the resonant frequency returned to its former level). Subsequent addition of CK2 induced partially irreversible binding of the kinase, which did not dissociate from the p53 protein. The CK2 β subunits that bind to the p53 oligomerization domain in the region between amino acids 325 and 344 (Figure 1) seem to be responsible for this effect. This finding also explains the results shown in Figures 3 and 5, where the CK2-phosphorylated p53 complexes with DNA displayed a lower electrophoretic mobility than the p53–DNA complexes induced by other kinases. Surprisingly, binding of CK2 β subunits to the docking site on p53 shared with several C-terminal kinases, including PKC, did not prevent PKC from binding to p53 in either the presence or the absence of ATP (Figure 8, left panel, curves c, d). In addition, phosphorylation of p53 by CK2 promoted the ability of PKC to bind to p53 (curve d), but not to phosphorylate the p53 protein, inducing its DNA-binding activity. The mass change due to phosphorylation of the immobilized p53 was too small to be visible by the piezoelectric biosensor. However, the effect of p53 phosphorylation by CK2 was clearly demonstrated by the subsequent 3-fold higher binding of PKC (curve d). From these results we can conclude that phosphorylation by cdk2/cyclin A and PKC, rather than by CK2, activates the latent ability of p53 to bind specific DNA sequences. Co-operation between different signalling pathways is mutually antagonistic, acting via a hierarchal mechanism, and probably has a profound influence on one of the crucial functions of the p53 protein, i.e. its DNA-binding activity.

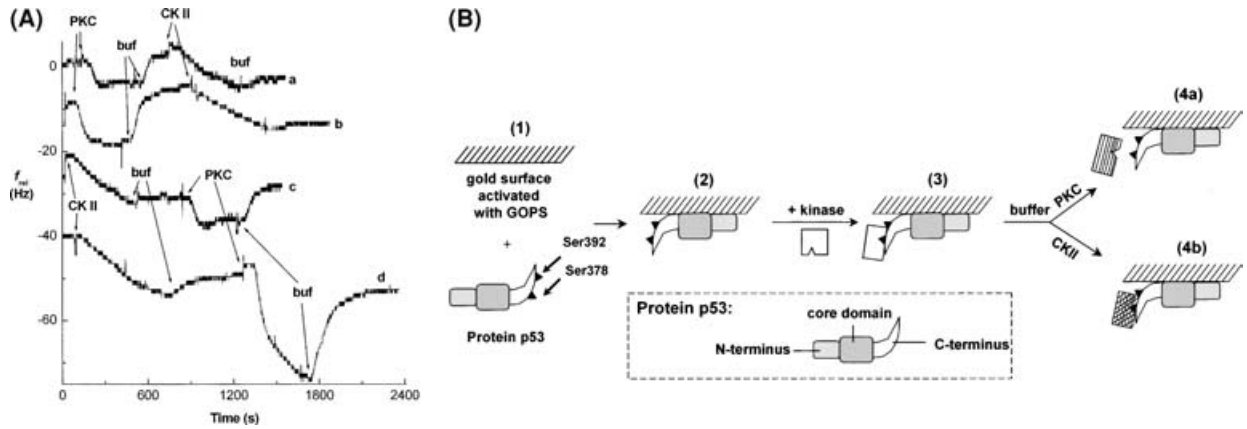

Figure 8 Interactions of p53 with kinases measured using a piezoelectric biosensor

(**A**) The p53-modified piezoelectric crystal was used in flow-through mode. Binding curves obtained during affinity interactions are shown as plots of relative frequency against time. The associations of p53 with either PKC or CK2 (CKII) are presented. Injection of kinases was carried out in either the absence (curves a, c) or the presence (curves b, d) of ATP (0.2 mM). Dissociation of affinity complexes formed at the sensing surface was also studied in the presence of carrier buffer only (buf). The individual traces have been vertically shifted for the sake of clarity. (**B**) Scheme of interactions of two kinases (CK2 and PKC) with p53 protein as measured by the piezoelectric sensor. Steps (1) and (2), p53 protein is covalently bound to the activated gold surface; (3), abundant PKC or CK2 kinases are added to the flow-through system, binding specifically to Ser^{378} or Ser^{392} respectively; this binding is reflected by a decrease in the resonant frequency; (4a), PKC molecules dissociate from p53 after the enzyme in solution is replaced by a blank buffer, as indicated by the return of the resonant frequency almost to its original value; (4b), with CK2 almost no changes in resonant frequency are observed, suggesting that this kinase remains bound to p53 under the same conditions.

DISCUSSION

Post-translational modifications have been shown to play a crucial role in the activity of the p53 protein. Higher levels of p53 phosphorylation and acetylation, together with specific p53 phosphorylation patterns, have been found in human tumour tissues [46]. p53 protein has two regions that are subject to multiple phosphorylations as well as other post-translational modifications (Figure 1). The N-terminal modifications influence the transactivation capabilities of p53 and regulate its interaction with the MDM2 oncoprotein. Modifications within the C-terminal region of p53 were suggested to play an important role in the regulation of its DNA-binding activity towards specific responsive elements, and the presence of an unmodified C-terminus was shown to prevent its specific transactivation function [47].

Several studies concerning the activation of p53 binding to consensus DNA sequences by C-terminal phosphorylation have been reported [5,25], mostly taking into account modifications by a single kinase. In contrast, here we directly compare for the first time the combined effects of phosphorylation by various kinases targeting the p53 C-terminal domain, such as CK2, PKC and cdk2 (either alone or in combination) on the activation of p53 DNA-binding to a specific consensus sequence. The binding sequences were presented not only in the form of oligonucleotides, but also localized in longer DNA molecules to reflect possible effects of neighbouring DNA sequences, including their conformation. Binding activity towards the consensus sequence was not observed in the case of p53 protein lacking post-translational modifications (Figure 2), which confirms the previously observed necessity for activation of the p53 transcription factor function [5]. Modified p53 protein expressed in a eukaryotic expression system provided retarded bands of protein–DNA complexes in EMSA gels. The non-radioactive EMSA technique that we used seems to be more convenient for these studies compared with the radioactive approach, as it enables more sensitive quantification of different protein–DNA complexes (Figures 5 and 6).

We have previously reported the ability of the C-terminus-specific monoclonal antibody Bp53-10 to activate the sequence-specific binding function of p53 [9,48]. Phosphorylation of the C-terminal region of p53 had the same effect as monoclonal antibody binding in an agarose EMSA gel, with the difference in the molecular size of the complexes being due to the presence or absence of the antibody molecule. Our results show that modification of Ser^{315}, residing just outside the oligomerization domain, has the strongest stimulatory effect on p53 activation. We have also detected phosphorylation of the same amino acid *in vivo* after UV irradiation of MCF7 and A375 cells, co-inciding with elevated p53-dependent transcription [41]. These findings suggest an important role for this pathway and for Ser^{315} phosphorylation in the stimulation of p53 function.

In contrast, we show that phosphorylation of Ser^{392} by CK2 stimulates the DNA-binding ability of p53 only very weakly. The first studies of this phenomenon [8] showed the *in vitro* activation of p53 DNA binding by purified CK2 from rabbit muscle, and also described the allosteric inhibition of the DNA-binding activity of p53 by the Ica9 antibody, which recognizes the CK2 target site [43]. There is also evidence that Ser^{392} phosphorylation increases the association constant for reversible tetramer formation nearly 10-fold, while phosphorylation of Ser^{315} and Ser^{378} had only a small effect on tetramer formation [49]. In contrast, results from Fiscella et al. [50] based on studies with the Ser^{392} mutant showed that neither phosphorylation of nor RNA attachment to Ser^{392} is required for the ability of p53 to suppress cell growth or to activate transcription *in vivo*, and that Ser^{392} phosphorylation has no discernible effect on p53 function. With respect to these discrepancies concerning the role of Ser^{392} phosphorylation in p53 protein activity, we attempted here to elucidate the role of CK2 in p53 function.

We also considered the possibility that not only Ser^{392} phosphorylation but also interaction of kinases with p53 protein could influence its activity. The CK2 regulatory β subunits have been shown to bind stably to the kinase docking region situated between amino acids 325 and 344 [51], which may explain the lower electrophoretic mobility of p53–DNA complexes phosphorylated by CK2 compared with complexes phosphorylated by other kinases (Figures 3 and 5). It has been shown not only that the

stable interaction of CK2 β subunits with p53 has implications for p53 behaviour, but also that p53 influences the enzymic activity of CK2. The p53 C-terminus stimulates CK2 activity, but conversely full-length wild-type p53 inhibits the activity of CK2 [34]. The importance of CK2 for p53 protein activation therefore seems to be controversial. Thus we also concentrated on the effect of phosphorylation by CK2 on the modifications and activation of p53 protein by other C-terminal kinases. Results shown in Figures 6 and 7 suggest that the CK2-mediated phosphorylation of p53 has no effect on phosphorylation by cdk2, but strongly inhibits phosphorylation by PKC and subsequent stimulation of p53 DNA-binding activity. CK2 added to the kinase reaction mixture together with PKC, in the presence of ATP, significantly decreased the phosphorylation of p53 by PKC, and CK2 added 15 min before PKC completely abolished phosphorylation by PKC. These results were obtained both using the EMSA experiments shown in Figures 6 and 7 and by radioactive ATP phosphorylation measurements, where no changes in the phosphorylation level due to PKC were detected in case of p53 preincubation with CK2 in the presence of ATP. These findings can explain the results obtained by Schuster et al. [33], where co-expression of CK2 with p53 protein in mammalian cells inhibited the sequence-specific DNA binding of p53. We suggest that this inhibitory effect of CK2 is caused by the abolition of phosphorylation by PKC, which is a strong activator of the DNA-binding function of p53 (Figures 3 and 5).

In order to test whether CK2 prevents PKC from binding to its docking site on p53 or whether it allows PKC binding but prevents PKC-mediated phosphorylation, we developed a new binding assay based on piezoelectric biosensors, which are able to detect very sensitively the presence of kinase–p53 complexes. Using this approach we found that binding and phosphorylation of p53 by PKC did not affect the binding or phosphorylation of p53 by CK2 (PKC molecules dissociated completely from p53). In contrast, CK2 bound irreversibly to p53 in the presence or absence of ATP, but surprisingly this interaction did not influence PKC binding to p53, in spite of the docking site being common to both kinases. In addition, the presence of CK2 phosphorylation promoted the binding of PKC to p53 (Figure 8). Nevertheless, phosphorylation by PKC was dramatically blocked in the presence of phosphorylation by CK2, suggesting that even increased PKC binding to p53 protein cannot mediate the phosphorylation reaction.

We can conclude that cdk2/cyclin A and PKC both have a stimulatory role in the activation of sequence-specific DNA binding by latent p53, both in the oligonucleotide form and when localized within DNA molecules. The phosphorylation of p53 by CK2, which is apparently constitutively active, evokes an antagonistic effect with respect to p53 activation. In fact, Ser^{392} phosphorylation partially activates the DNA-binding function of p53, but it blocks the kinase activity of PKC, a strong p53 activator. Generally, we can say that our results support an allosteric rather than a competitive model of p53 protein latency. The allosteric model assumes that the interaction of the non-modified C-terminal domain of p53 blocks the core domain with respect to sequence-specific binding and promotes the latent state of the molecule, while modification of the C-terminus activates the binding of p53 to target DNA sequences [8,52]. Further studies should concentrate on elucidating the possible interactions of individual cell signalling pathways with regard to p53 activation and on details of the specific molecular interaction patterns.

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