Activation of the cryptic DNA binding function of mutant forms of p53

Ted R.Hupp, David W.Meek[1], Carol A.Midgley and David P.Lane*
Cancer Research Campaign Laboratories, [1]MRC Protein Phosphorylation Unit,
Department of Biochemistry, University of Dundee, Dundee DD1 4HN, UK

ABSTRACT

Wild type p53 assembles into a latent multiprotein complex which can be activated for sequence-specific DNA binding *in vitro* by proteins targeting the carboxy-terminal domain. Using an optimized system coupling the post-translational modification of wild type p53 to activation of sequence specific DNA binding, we examined the affects of common mutations on the cryptic DNA binding function of p53. Two mutant forms of p53 were shown to be efficiently converted from the latent state by PAb421 and DnaK, but were defective in activation by casein kinase II, indicating that mutant p53 may not be receptive to allosteric regulation by casein kinase II phosphorylation. A reactive sulfhydryl group is absolutely required for DNA binding by wild type and mutant forms of p53 once converted to the activated state. Together, these data show that some mutant forms of p53 harbour the wild-type machinery required to engage in sequence-specific DNA binding and define a signalling pathway whose inactivation may directly result in a loss of p53 function.

INTRODUCTION

Mutation of p53 is a very common genetic alteration in human cancers (1). Loss of the p53 tumour suppressor activity is co-incident with a loss of G1-S checkpoints following DNA damage (2), increases in genomic instability and selectable gene amplification (3, 4). Mutant forms of p53 also appear to acquire a dominant growth promoting function (5, 6). The region responsible for this transforming activity has been localized to a small C-terminal domain (7). Mice null for the wild type p53 locus develop normally, yet are susceptible to the development of neoplasia at elevated frequencies (8), indicating that wild type p53 is dispensable for the control over normal development and cell differentiation, but is essential to prevent spontaneous tumour formation. Consistent with this data, inherited germline point mutations in p53 lead to a predisposition to cancer in humans (9).

Wild type p53 protein levels rise dramatically in response to the DNA-damaging agents mitomycin C (56), UV light (10, 11) and γ irradiation (2). Biochemical characterization of wild type protein has shown that p53 can function as a sequence-specific

DNA binding protein (12, 13) and a transcription factor (14−16). In addition, sequence specific DNA binding activity is cryptic, but can be unmasked by enzymes and proteins, including casein kinase II (17). These results support a model for wild type p53 in which its function is activated post-translationally after DNA damage to allow DNA repair by controlling the expression of regulatory gene products (18). These may include the DNA damage inducible gene gadd45 (19) and the host protein with oncogenic properties, mdm-2 (20).

Biochemical characterization of p53 has become possible recently due to the use of protein expression systems which allow for an abundant source of the protein. p53 purified using immunoaffinity chromatography has been shown to be a sequence-specific DNA binding protein which recognizes a motif containing two contiguous monomers of the sequence $(Pu)_3$-$C(A/T)(A/T)G(Py)_3$ (21). Sequence-specific DNA binding activity is manifested in the ability of p53 to bind to the SV40 origin of replication (13), and by its ability to activate transcription in vitro from templates harbouring its DNA binding sequence (22). Mutant forms of p53 are defective in non-specific DNA binding (23), sequence-specific DNA binding (21) and transcriptional activation (14) suggesting that this activity is normally required to suppress tumour formation.

Phosphorylation of nuclear DNA binding proteins is an effective mechanism through which gene expression is controlled in response to environmental cues (24). Multi-site phosphorylation of p53 by protein kinases (25−27) suggests its tumour suppressor activity may be tightly co-ordinated by complex signal transducing pathways. Enzymatic modulation of p53 may provide a framework from which to couple signal transducing pathways with the p53 response to DNA damage and ultimately growth control.

To study the affects of p53 phosphorylation on the activity of the protein, unmodified recombinant p53 was purified by conventional chromatography from bacteria and shown to be a multimeric protein which is latent for sequence specific DNA binding (17). A motif within the C-terminal 30 amino acids negatively controls p53 function as deletion of this domain constitutively activates p53. A set of p53 activating proteins, including rabbit muscle casein kinase II, monoclonal antibody PAb421, and *E.coli* Hsp70, target this C-terminal domain. The

* To whom correspondence should be addressed

structural assembly of this region and the determination of the mechanism of activation have exciting implications for understanding the allosteric regulation of p53 function.

Casein kinase II is a highly conserved calcium and nucleotide independent enzyme which phosphorylates a broad spectrum of substrates, including transcription factors and DNA binding proteins (28). The activity of the kinase is stimulated in cells exposed to a variety of mutagens and growth factors (29). Mouse (25) and human p53 (17) are phosphorylated at the penultimate C-terminal amino acid by casein kinase II in vitro. Mutation of this highly conserved C-terminal serine residue of mouse p53 to an alanine abolishes the growth suppressive function of the protein in mammalian cells, suggesting that phosphorylation at this site is one important modification required to activate the tumour suppressor function of p53 (30). Based on biochemical and physiological data, casein kinase II is the only known enzyme involved in a direct and positive regulation of the activity of p53.

Understanding the regulation of p53 activity is a vital step for the development of therapeutic strategies designed to restore tumour suppressor activity of the protein in transformed cells. Using in vitro systems, it has already been established that wild type p53 activity is negatively regulated in vitro by the viral oncogene, T-antigen (22), and host associated oncogene mdm-2 (31).

We are interested in reconstituting with purified cellular enzymes a homologous system in which the sequence specific DNA binding activity of human p53 can be regulated positively. With such a system in place, understanding the biochemical mechanisms of activation are possible and the effects of mutation on the cryptic activity of p53 can be assessed. Mutant forms of p53 encoded by the hotspot alleles, His175, Trp248, and His273, are defective in sequence specific DNA binding to the p53 consensus DNA site (21). Given that p53 is regulated positively by casein kinase II, it is important to determine if some mutant forms of p53 are inactive in DNA binding due to a defect in conversion from a latent to an activated state.

To this end, mutant forms of p53 encoded by common 'hotspot' alleles were purified and their activities were characterized at the biochemical level. Two mutant forms of p53 encoded by the His273 and Lys285 alleles are not inherently defective in sequence-specific DNA binding, but appear to prefer residence in the latent state. A monoclonal antibody and *E.coli* DnaK are able to promote sequence-specific DNA binding by these mutant forms of p53, but the most potent physiological activator, casein kinase II, is unable to effectively unmask the DNA binding function. A new biochemical class of mutant forms of p53 are thus established which is defective in DNA binding after post-translational modification by the growth controlling enzyme, casein kinase II. This class of mutant p53 defective in the conversion from the latent to the activated state is a prime target for the construction of molecules with the ability to reactivate the tumour suppressor function of mutant p53 and, thus, potentially lead to selective growth arrest and induction of apoptosis in tumour cells (32, 33).

MATERIALS AND METHODS

Enzymes and reagents

Purified fractions of recombinant human wild type p53 and p53Δ30 from a bacterial expression system, p53 specific monoclonal antibodies PAb421 and DO-1 were obtained as described previously (17). Mutant forms of p53 encoded by the Lys285

and His273 alleles were purified from a bacterial expression system using Heparin Sepharose and Gel filtration chromatography as described for wild type p53. Fractions containing mutant p53's eluting at approximately 440 Kd on gel filtration (compared to protein standards) and which could be activated for sequence-specific DNA binding by PAb421 were concentrated using a Centricon-30 (Amersham) to 0.5 mg/ml and stored frozen at −70°C. Mutant forms of p53 purified by this method were greater than 90% pure when stained with Coomasie Blue in an SDS polyacrylamide gel (unpublished data).

The *E.coli* Hsp70 homologue (DnaK) was purified from an overproducing strain by a modification of the published protocol (49). The DnaK overproducing strain was a generous gift of Dr Maciej Zylicz, University of Gdansk, Poland. Active fractions of DnaK eluting from MonoQ (49) were applied to an ATP-Agarose column in Buffer P (10% glycerol, 25 mM HEPES (pH 7.6), 0.1 mM EDTA, and 1 mM DTT) containing 10 mM MgCl$_2$. After a 10 column volume wash in Buffer P containing 1.0 M KCl + 10 mM MgCl$_2$ and a 5 column volume wash with Buffer P containing 10 mM KCl + 10 mM MgCl$_2$, bound DnaK was eluted with Buffer P containing 10 mM MgCl$_2$ + 5 mM ATP. The fractions of DnaK eluting from ATP agarose were dialyzed overnight at 3°C against Buffer P containing 2 mM EDTA and 0.25 M KCl. DnaK was further purified on Superose-12 gel filtration equilibrated in Buffer P containing 0.25 M KCl. NEM and Diamide were from Sigma.

Sources of casein kinase II

Casein Kinase II from rabbit muscle was purified as described (17) with the following modifications; Muscle from a rabbit was ground in 2 liters of homogenization buffer (4 mM EDTA, 2 mM DTT, 1 mM Benzamidine) and centrifuged at 4000×g for 45 minutes. The soluble supernatent containing 27.5 grams of protein was batch adsorbed to 200 ml of phosphocellulose resin in Buffer P for 2 hours. The column was washed with Buffer P containing 0.35 M KCl, and casein kinase II activity was step eluted with Buffer P containing 1.2 M KCl and 0.1% Triton X-100. After Phosphocellulose, fractions containing casein kinase II were applied to a Heparin Sepharose column and eluted with a linear gradient from 0.05 to 1 M KCl in Buffer P containing 0.1% Triton X-100. Active fraction were applied to a MonoQ column and eluted with a linear KCl gradient in Buffer P containing 0.1% Triton X-100. Active casein kinase II was concentrated to 0.75 mg/ml using Centricon-30 and stored at 4°C. Kinase activity was monitored by assaying for radioactive phosphate incorporation into p53 and casein as described (17). Casein and p53 kinase activities were co-incident after Heparin Sepharose and MonoQ chromatography (unpublished data). Recombinant human casein kinase II holoenzyme expressed in *E.coli* was 95% pure and was obtained from Boehringer Mannheim.

Activation of wild type or mutant forms of p53

Activation of the sequence-specific DNA binding function of wild type and mutant forms of p53 were performed using the assay conditions previously described with the following modifications. In a 10 μl reaction containing 20% glycerol, 25 mM HEPES (pH 7.6), 0.05% Triton X-100, 5 mM Mg Cl$_2$, 50 mM KCl, 0.1mM EDTA, 1 mg/ml BSA, 0.1 mg/ml creatine kinase, 20 mM phosphocreatine, 1 mM ATP, the indicated amounts of wild type or mutant p53 and the indicated activating protein, reactions were incubated at 30°C for 30 minutes. A 10 μl aliquot containing

the radiolabeled consensus DNA binding oligonucleotide (5' AG-CTT AGACATGCCT AGACATGCCT A 3' and 5' AGCTT AGGCATGTCT AGGCATGTCT A3') defined by El-Diery (21) with competitor plasmid DNA was added and reactions were incubated at 30°C for 5 minutes. Unless indicated otherwise, PAb421 (400 ng), DnaK (2 μg), Casein Kinase II (240 ng), or DO-1 (400 ng) were added. Reaction products were separated by electropohoresis in a native 4% polyacrylamide gel containing 0.1% Triton X-100 and 0.33× TBE at 3°C.

RESULTS

Phosphorylation of p53 by recombinant human casein kinase II effectively activates DNA binding

Naturally occuring enzymes and a monoclonal antibody are able to regulate positively wild type p53 activity in vitro. Of immense interest was the identification of a mammalian fraction containing casein kinase II from rabbit muscle which could replace *E.coli* Hsp70 (DnaK) or the monoclonal antibody PAb421 in the activation of p53. This reaction was coincident with stoichiometric phosphorylation and was ATP dependent (17). Allosteric modulation of p53 activity by this nuclear kinase in vitro suggests that the tumour suppressor function may require modification through a casein kinase II-related signalling pathway.

It was important to determine if recombinant casein kinase II alone was active towards recombinant bacterially expressed p53. First, a variety of protein kinases from eukaryotic cells post-translationally modify p53 (25−27, 53); these enzymes minimally include casein kinase II, a casein kinase I-like enzyme, DNA activated protein kinase, and protein kinase C. Eukaryotic cells harbor evolutionarily conserved casein kinases, making the use of these expression systems less attractive for the purification of unphosphorylated p53. The use of bacterially expressed p53 presumably gives rise to an unmodified form of the protein, thus allowing for rigorous biochemical analysis of the effects of phosphorylation by unique protein kinases on the activity of p53. Second, it has also been shown that cdc2 kinase phosphorylates the β subunit of Xenopus casein kinase II and stimulates the activity of the enzyme in vitro (50). In addition, there is a 10 fold increase in casein kinase II activity during meiosis in amphibian oocytes (51). Together, these data suggest that casein kinase II activity can be regulated post-translationally in the MPF cascade during the M phase of the cell cycle (28). In light of these findings, the intriguing possibility remained that post-translational modification of casein kinase II in vivo regulates its activity towards p53 and that the endogenous casein kinase II purified from tissue is itself in an 'activated' state.

To begin the reconstitution of the p53 activation reaction with highly purified human proteins, we tested the ability of recombinant human casein kinase II (95% purified holoenzyme containing both the α and β subunits expressed in *E.coli*) to replace rabbit muscle casein kinase II in activation reactions. The addition of 125 ng of recombinant casein kinase II phosphorylated p53 stoichiometrically within 10−20 minutes (Figure 1A). A fraction of highly purified casein kinase II (375 ng) from rabbit muscle also phosphorylated p53 stoichiometrically, although at a 3.5 fold reduced rate (Figure 1A).

Phosphorylation of p53 and subsequent sequence specific DNA binding were performed sequentially to determine if phosphorylation could induce rapid conformational changes leading to high affinity DNA binding by p53. In the first stage, p53 was phosphorylated with either recombinant or rabbit muscle casein
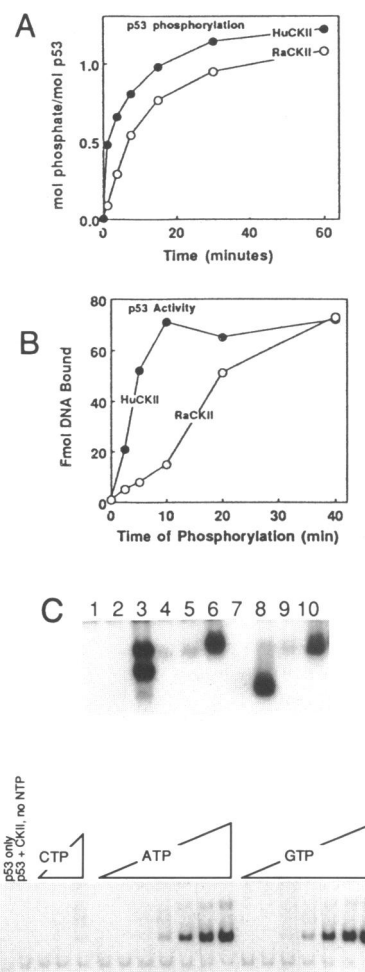

Figure 1. (A). Rate of phosphorylation of p53 by native and recombinant casein kinase II. p53 (50 ng) was phosphorylated at 30°C in DNA binding buffer (in the absence of and ATP regeneration system and DNA) for the indicated times using recombinant human casein kinase II (HuCKII; 125 ng) or native rabbit muscle casein kinase II (RaCKII; MonoQ fraction; 375 ng). Reaction products were separated by SDS gel electrophoresis. Radiolabeled p53 was excised from the gel and radioactive phosphate was quantified by scintillation counting. Activity is expressed as the moles of phosphate incorporated per mole of p53 monomer. **(B).** Rate of activation of p53 DNA binding by rabbit muscle or recombinant casein kinase II. p53 (50 ng) was phosphorylated at 30°C for the indicated times (in the absence of an ATP regeneration system and DNA) using rabbit muscle (RaCKII; MonoQ fraction, 375 ng) or recombinant casein kinase II (HuCKII, 125 ng). Radiolabeled DNA was added and reactions were incubated at 0°C for 5 minutes, followed by gel electrophoresis as described in the Materials and Methods. Radiolabeled p53-DNA complexes were scanned and quantified using a phosphoimager. Activity is expressed as the Fmoles of DNA bound by 50 ng of p53 as a function of the time of phosphorylation by casein kinase II. **(C).** p53 was activated for DNA binding by phosphorylation for 40 minutes with different amounts of rabbit muscle or recombinant casein kinase II as in parts A and B. Reaction products were separated by electrophoresis on a 4% polyacrylamide gel; Lane 1(p53 + 1.2 ng of HuCKII), lane 2 (p53 + 12.5 ng of HuCKII), lane 3 (p53 + 125 ng of HuCKII), lanes 4−6 (as in lanes 1−3, but with the addition of DO-1 after DNA binding), lane 7 (p53 + 37 ng of RaCKII), lane 8 (p53 + 375 ng of RaCKII), lanes 9 and 10 (as in lanes 8 and 9, but with DO-1 added after activation of DNA binding) **(D)** GTP replacement of ATP in the activation of p53. Complete reactions (without an ATP regeneration system and DNA) containing 50 ng of p53 and 375 ng of native casein kinase II were assembled without nucleotide. The indicated nucleotide was added and incubations were at 30°C for 40 minutes. After the addition of the DNA binding mixture, reaction products were analyzed as indicated in the Materials and Methods. Reading from left to right; lane 1 (p53 only, with 1 mM ATP), lane 2 (p53 and CK II, without NTP), lane 3−5 (p53 and CK II, with 62, 250 and 1000 μM CTP, respectively), lanes 6−12 (p53 and CK II, with 0.24, 0.97, 3.4, 15, 62, 250, and 1000 μM ATP, respectively), lanes 13−19 (as in 6−12, but using GTP in place of ATP).

A. PAb421 Activation

B. DnaK Activation

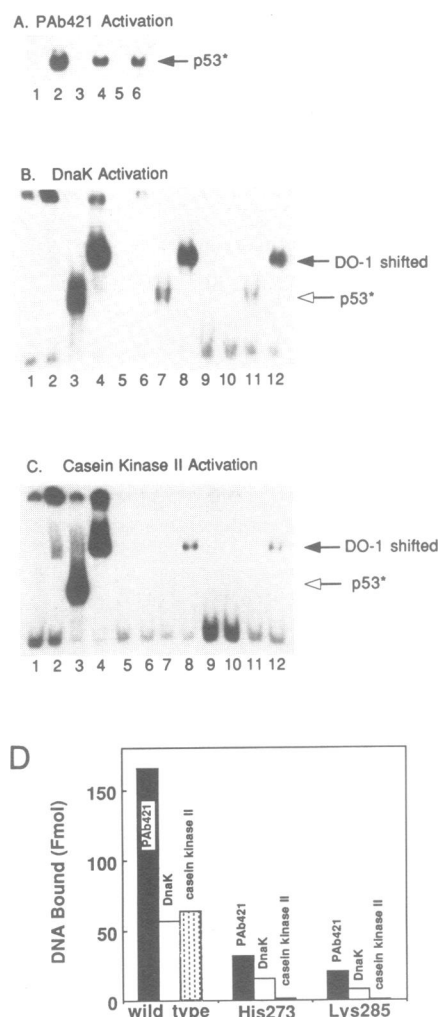C. Casein Kinase II Activation

D

Figure 2. Activation of sequence-specific DNA binding of wild type and mutant p53. Activation reactions were staged in two parts. In the first stage, wild type p53 (60 ng) or mutant forms of p53 (180 ng) was incubated with ATP and the indicated activating protein at 30°C for 30 minutes. In the second stage, the consensus site oligonucleotide was added to the reactions to assay for sequence-specific DNA binding. (A) PAb421; lanes 1, 3 and 5 (p53, p53 His273, and p53 Lys285, respectively), lanes 2, 4 and 6 (PAb421 and p53, p53 His273, and p53 Lys285, respectively). (B) DnaK; lanes 1, 5, and 9 (p53, p53 His273, and p53 Lys285, respectively), lanes 2, 6 and 10 (DO-1 and p53, p53 His273, and p53 Lys285, respectively), lanes 3, 7, and 11 (DnaK and p53, p53 His273, and p53 Lys285, respectively), lanes 4, 8, and 12 (DnaK and p53, p53 His273, and p53 Lys285, respectively, followed by DO-1). (C) Casein Kinase II; lanes 1, 5, and 9 (p53, p53 His273, and p53 Lys285, respectively), lanes 2, 6 and 10 (DO-1 and p53, p53 His273, and p53 Lys285, respectively), lanes 3, 7, and 11 (Casein Kinase II and p53, p53 His273, and p53 Lys285, respectively), lanes 4, 8, and 12 (Casein Kinase II and p53, p53 His273, and p53 Lys285, respectively, followed by DO-1). p53* marks the migration of the activated p53-DNA complex. (D) Specific activity of wild type and mutant p53. PAb421-activated p53 (solid bar), DnaK-activated p53 (open bar), casein kinase II-activated p53 (stippled bar). The specific activities of wild type, His273 and Lys285 are indicated.

kinase II at 30°C for the indicated times (Figure 1B). In the second stage, sequence specific DNA binding was assayed at 0°C for 5 minutes. Maximal sequence specific binding by p53 occured after 10 minutes of phosphorylation by recombinant casein kinase II (Figure 1B). Presumably, this recombinant enzyme represents an unmodified form of the kinase which also lacks unknown co-factors. These results demonstrate conclusively that phosphorylation is accomplished by casein kinase II and this modification alone is sufficient to activate p53 for sequence specific DNA binding. Using rabbit muscle casein kinase II, maximal sequence specific DNA binding by p53 was also observed upon near-stoichiometric phosphorylation (Figure 1B), although at a reduced rate compared to the recombinant enzyme. However, using either preparation of kinase, the data are consistent with the idea that once stoichiometric phosphorylation occurs, subsequent conformational changes giving rise to high affinity DNA binding are rapid.

Although the specific activities of the p53-DNA complexes activated by either recombinant or rabbit muscle kinase were nearly identical (Figure 1B), we have noted an interesting difference in the apparent molecular weight of the species produced during native gel electrophoresis. The molecular size of p53-DNA complexes activated by the rabbit muscle kinase is homogeneous and migrated as a unique species of approximately 600 Kd ((17), Figure 1C, lanes 7 and 8). In contrast, the recombinant enzyme produced a ladder of p53-DNA complexes ranging in size from greater than 800 Kd to a minor species of 600 Kd (Figure 1C, lanes 1−3). Both activated forms of p53 were supershifted by the N-terminal specific monoclonal antibody DO-1 (Figure 1C, lanes 4−6 or 9 and 10, for recombinant and rabbit muscle kinase, respectively). Clearly, the conformational changes occuring during phosphorylation give rise to very differently folded p53 multimers. Possibly, the size difference relates to the faster rate at which the recombinant kinase phosphorylates p53 (Figure 1A), induces folding or rearrangement of the multimers, and activates DNA binding (Figure 1B). Alternatively, the enzyme from tissue may undergo some type of modification that changes its activity towards p53, in comparison with the presumably unmodified recombinant enzyme.

Casein kinase II holoenzyme, consisting of two alpha and two beta subunits, is rare as a kinase in its ability to efficiently utilize GTP as a phosphate donor for the modification of serine residues within its consensus site (34). The nucleotide binding site of the alpha subunit of casein kinase II is adjacent to a highly basic hexapeptide motif, which also is unique to this kinase family of proteins, and is presumably involved in protein recognition. The C-terminus of p53 has a phosphorylation site with weak homology to the known casein kinase II consensus sequence, yet this motif is effectively phosphorylated by the kinase in vitro (25, 17). Other ill-defined structural motifs in this region may be contributing to the casein kinase II specificity, and it was important to see whether casein kinase II could activate p53 using GTP as a phosphate donor.

Low levels of GTP were able to effectively substitute for ATP in activation reactions using rabbit muscle casein kinase II (Figure 1D). The apparent K_m of activation for either nucleotide in this reaction was nearly identical; from 42 μM using ATP and 46 μM using GTP. CTP did not replace GTP or ATP in activation reactions (Figure 1D), consistent with the observations that casein kinase II can only utilize the purine nucleotides. The omission of GTP or ATP prevented activation (Figure 1D).

Activation of mutant forms of p53 by PAb421 and DnaK

It appears that sequence specific DNA binding is one activity of p53 required for its tumour suppressor function. To date, all mutant forms of p53 have been shown to be defective in sequence specific DNA binding (21) and in the activation of transcription from templates harbouring its consensus DNA binding site (14).
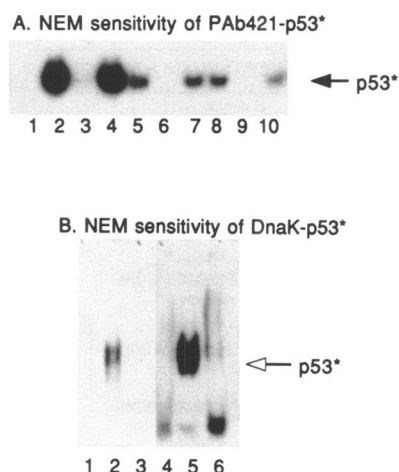
A. NEM sensitivity of PAb421-p53*

1 2 3 4 5 6 7 8 9 10 → p53*

B. NEM sensitivity of DnaK-p53*

1 2 3 4 5 6 ◁— p53*

Figure 3. (A) NEM sensitivity of PAb421 activated p53. After PAb421 activation of wild type p53 (60 ng; lanes 2−4), p53 His273 (60 ng; lanes 5−7), and p53 Lys 285 (60 ng; lanes 8−10) as described in the methods, redox reagents were added (DTT to 2 mM or NEM to 1mM) and incubations were continued at 30°C for 10 minutes. Lane 1 (no protein), lanes 2, 5, and 8 (p53's-PAb421 only), lanes 3, 6, and 9 (p53's-PAb421 and NEM followed by DTT), lanes 4, 7, and 10 (p53's-PAb421 and DTT followed by NEM). Reactions were then incubated with the consensus DNA oligonucleotide to assay for sequence specific DNA binding as described in the methods. (B) NEM sensitivity of DnaK activated p53. After activation of p53 His273 (180 ng; lanes 1−3) and p53 (60 ng; lanes 4−6) as described in the methods, redox reagents were added and incubations were continued at 30°C for 10 minutes. Lanes 1 and 4 (p53's only), lanes 2 and 5 (DnaK activated p53 with DTT, followed by NEM), lanes 3 and 6 (DnaK activated p53 with NEM followed by DTT). Incubations were then continued with the consensus DNA oligonucleotide to assay for DNA binding as described in the methods. p53* marks the position of the activated p53-DNA complex.

The recent identification of the latent biochemical phenotype of wild type p53 (17) supports the possibility that some mutant forms of p53 may be inactive due to a relative defect in activation of DNA binding by a post-translational modification (Figure 5). Identification of such a class of mutant p53 would provide suggestive evidence for the importance of a casein kinase II signalling pathway in the cell and define a class of p53 which could presumably have the capacity to have its tumour suppressor function re-activated by alternate post-translational modifications.

To test this possibility, we have examined the behaviour of mutant forms of p53 in sequence-specific DNA binding assays. Mutant forms of p53 encoded by the His175, Trp248, His273, and Lys285 alleles were purified using Heparin Sepharose and gel filtration chromatography as described for wild type p53. The mutant proteins eluted as a multi-protein complex of approximately 440Kd on gel filtration (unpublished data), similar to that observed with wild type p53 (17). These results indicate that the mutations have not prevented stable associations between monomers.

Wild type p53 did not exhibit sequence-specific DNA binding unless activated by the monoclonal antibody, PAb421 (Figure 2A, lane 2 vs. lane 1). PAb421 binds to the C-terminus of p53 and presumably induces a conformational change that neutralizes the function of a negative regulatory domain (Figure 5A). Highly purified mutant forms of p53 encoded by the His273 and Lys285 alleles were also unable to bind DNA sequence-specifically, but were effectively activated for DNA binding by PAb421 (Figure 2A, lanes 4 and 6 vs. lanes 3 and 5). The specific activities of the mutant proteins were 6−8 fold

lower than wild type p53 (Figure 2D). Apparently, these point mutations in p53 do not abolish sequence-specific DNA binding as the cryptic function can be activated (Figure 5B). The other two common mutants studied, His175 and Trp248 could not be activated in DNA binding by PAb421 under these conditions (unpublished data), suggesting that the cryptic activity may be permanently locked into the latent state (Figure 5B).

p53 can bind to DnaK and Hsc70 in vivo (35). Hsc70 binds to the C-terminus of p53 synthesized in reticulocyte lysates (36), suggesting that this family of proteins targets the same domain as PAb421 and casein kinase II (Figure 5A). We have found that this interaction may have functional significance, since purified recombinant DnaK is able to activate sequence specific DNA binding of wild type p53. The His273 and Lys285 mutant p53's were also activated by DnaK to give rise to products similar in mobility to activated wild type p53 (Figure 2B, lanes 3, 7, and 11 vs. lanes 1, 5 and 9). A monoclonal antibody specific for the N-terminus of p53, DO-1, was able to supershift the DnaK activated wild type and mutant p53's (lanes 4, 8, and 12 vs. lanes 3, 7, and 11). DnaK activated mutant p53 bound by DO-1 are 4−6 fold less active than wild type p53 activated by this heat shock protein (Figure 2D). The enhancement of DnaK activated mutant p53 DNA binding function was observed upon the inclusion of DO-1 (Figure 2B). The mutant forms of p53, though in an activated state, may yet favour equilibrium towards the latent state (Figure 5), and the inclusion of DO-1 may help to lock the protein into the high affinity DNA binding conformation.

Mutant forms of p53 are severely defective in activation by casein kinase II

The third protein which we have been using to activate wild type p53, and one which is presently the most physiologically relevant, is casein kinase II. The C-terminal casein kinase II phosphorylation site is required for tumour suppressor function in mammalian cells (30). Covalent modification of wild type p53 by casein kinase II in vitro is GTP or ATP dependent and activates the cryptic sequence-specific DNA binding function of p53 (Figure 1). Although wild type p53 was activated very effectively by casein kinase II, both latent, mutant forms of p53 (Figure 2C, lanes 3, 7, and 11 vs. lanes 1, 5, and 9; Figure 2D) were activated to a very low extent by casein kinase II; this is more noticeable after the addition of DO-1, which supershifted the protein−DNA complexes (lanes 4, 8, and 12 vs. lane 2). Quantification of the products of DNA binding indicates that casein kinase II is 20−30 times less effective than PAb421 in activation of these two mutant forms of p53 (Figure 2D). The mutant proteins were phosphorylated as effectively as wild type p53 by casein kinase II in vitro (unpublished data). This modification was inhibited by PAb421 (unpublished data), indicating that phosphorylation is occuring within the C-terminus as is observed with wild type p53.

Activated wild type and mutant forms of p53 both require a reactive sulfhydryl for sequence-specific DNA binding

Independent biochemical analysis of wild type p53 has shown that it requires a reactive sulfhydryl(s) for effective sequence specific DNA binding (see below) and it was of interest to determine if the altered conformation of activated mutant forms of p53 could express this biochemical phenotype. In staged activation of wild type and mutant forms of p53 by PAb421, the inclusion of NEM after the activation step inhibited sequence specific DNA binding (Figure 3A, lanes 3, 6, and 9 vs. lanes
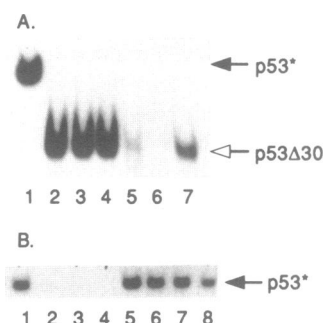
Figure 4. p53Δ30 DNA sequence-specific DNA binding activity is sensitive to sulfhydryl modifying reagents. (A) Sensitivity of p53Δ30 to oxidation. p53Δ30 (60 ng) was first treated with redox reagents: lane 1 (full length p53 activated by PAb421), lane 2 (p53Δ30 only), lane 3 (p53Δ30 and 2 mM DTT), lane 4 (p53Δ30 and 2 mM DTT followed by 1 mM NEM), lane 5 (p53Δ30 and 1 mM NEM followed by 2 mM DTT), lane 6 (p53Δ30 and 0.5 mM Diamide), and lane 7 (p53Δ30 and 0.5 mM Diamide followed by 2 mM DTT). (B) PAb421-activated full length p53 is sensitive to reversible oxidation by diamide. p53 (60 ng) was first activated by PAb421 as indicated in the methods and then treated with the indicated redox reagents: lane 1−4 (0.1 mM, 0.4 mM, 1.6 mM, and 5.4 mM Diamide, respectively) and lanes 5−8 (as in '1−4' but followed by the addition of DTT to 6 mM). After the modifications, p53 was assayed for sequence specific DNA binding as indicated in the Materials and Methods.

2, 5, and 8). As with wild type p53, the prior inclusion of DTT followed by the addition of NEM prevented inhibition of DNA binding of the PAb421 activated mutant forms of p53 (lanes 4, 7, and 10 vs. lanes 3, 6, and 9).

A similar analysis was carried out using DnaK activated p53 His273 and wild type p53 (Figure 3B). After the activation by DnaK (lanes 2 and 5 vs. lanes 1 and 4), NEM inhibited sequence specific DNA binding of both mutant and wild type p53's (lanes 3 and 6), while the control reactions containing an excess of DTT were not affected by NEM (lanes 2 and 5). These results suggest that post-translational modulation of the reactive sulfhydryl group through a redox mechanism will greatly affect p53 function and imply that mutant p53 protein's are not defective in DNA binding due to a propensity of this sulfhydryl group to exist in an oxidized state.

The constitutively active p53Δ30 requires a reactive sulfhydryl for DNA binding

Given the possibility that a reactive sulfhydryl is required at some undetected stage during the activation of wild type or mutant forms of p53, we studied the effects of sulfhydryl modifying agents on p53Δ30 activity. This recombinant enzyme is constitutively active for sequence-specific DNA binding as it lacks the C-terminal 30 amino acids containing the negative regulatory motif which, in an unmodified state, prevents activity. The inclusion of NEM inhibited DNA binding of this protein as it does wild type full length activated p53 (Figure 4A, lane 5 vs. lanes 2−4). Diamide, which unlike NEM, reversibly oxidizes sulfhydryl residues, also inactivated p53Δ30 activity (lane 6 vs. lanes 2−4). The addition of DTT to diamide-oxidized p53Δ30 reactivated the function of the protein (lane 7).

Wild type full length p53 also responded similarly to Diamide. The inclusion of increasing amounts of Diamide inactivated the DNA binding activity of PAb421-activated full length p53 (Figure 4B, lanes 1−4). The subsequent treatment of the reactions with an excess of DTT reversed the oxidation promoted by Diamide (lanes 5−8).
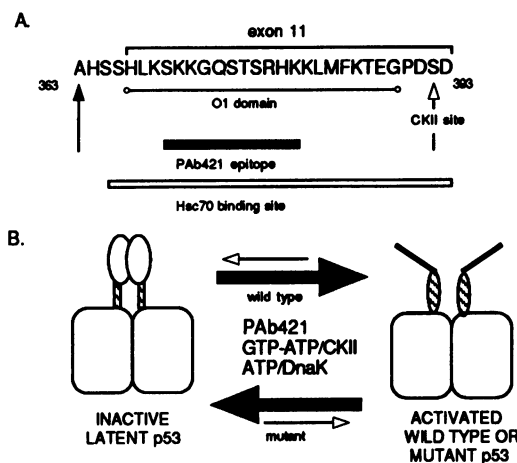

Figure 5. (A). C-terminal binding sites of the p53 binding proteins within exon 11 (45). The 30 C-terminal amino acids from 363 to 393 are listed (46). Important motifs include: The O1 domain required for higher order oligomerization (38); the CKII phosphorylation site (open headed arrow) (47); the PAb421 binding site (48), the Hsc70 binding site (36), and the endpoint of p53Δ30 (closed arrow) (17). (B) Model describing the equilibrium shifts between dimeric p53 molecules in the latent or the activated states. The solid arrow indicates the direction favoured in the equilibrium for wild type or mutant p53's. Wild type p53 can be effectively converted to the active form by casein kinase II, PAb421, or DnaK. Conformational changes which occur after modification by the activating protein relieve the inhibition of DNA binding dependent upon C-terminal amino acids flanking the PAb421 binding site (stippled rectangle or ellipse). This conformational change activates the sequence specific DNA binding function. In contrast to the wild type protein, p53 mutants may be trapped in the latent state, even in the presence of activating proteins. Conditions that favour the interaction of activating proteins with p53 and that induce conformational changes in the C-terminus may activate the tumour suppressor function of mutant forms of p53.

DISCUSSION

Mutant forms of p53 defective in activation by casein kinase II

Using an in vitro system coupling post-translational modification of wild type p53 to the activation of sequence specific DNA binding, the affect of mutation on the latent DNA binding function of p53 was examined. By identifying rate limiting steps in vitro, we hope to reveal mechanistically the stages describing multimer assembly and regulation of p53 activity. Purification and biochemical characterization of mutant forms of p53 from bacterial expression systems demonstrated that a set of mutants share fundamental properties with the wild type protein. Like wild type p53, the purified mutants encoded by the His273 and Lys285 alleles 1) exist as latent multi-protein complexes and can be converted to active DNA binding forms by PAb421 and DnaK, 2) express the N-terminal epitopes recognized by DO-1 in the native p53-DNA complex, 3) require a reactive sulfhydryl(s) to promote sequence-specific DNA binding, and 4) are effectively phosphorylated by casein kinase II in vitro (unpublished data). Thus, these mutants can assemble into latent multi-protein complexes and are receptive to two types of activating proteins in vitro.

There is, however, one noticeable difference between these two mutant proteins and wild type p53. It appears that the mutants are severely defective in the GTP or ATP dependent activation of sequence-specific DNA binding by the cellular enzyme casein kinase II. Given that wild type p53 is regulated positively by phosphorylation, implicating casein kinase II involvement in the p53 pathway (52), it is reasonable to expect that defects in this

modulation would result in the net loss of p53 tumour suppressor activity. This defect could be made manifest in a perturbation of the casein kinase II signal transducing pathway, in which hypo-phosphorylated p53 would be inactive as a tumour suppressor. In the selection for mutation during the process of cellular transformation, there could be a selection for: (1) a p53 mutation in the casein kinase II recognition site at the C-terminus, which would prevent post-translational modification by the kinase, (2) a dominant p53 mutation in exon 11 which would strengthen the activity of the negative regulatory domain, effectively locking p53 into the latent state, or (3) a p53 mutation outside the C-terminal domain which confers an immunity to activation after phosphorylation. The two mutants reported here fall into the latter class. Mutations residing in the C-terminus may exist, but since this region is not highly conserved, little emphasis has been placed on screening p53 alleles for mutation in this negative regulatory domain.

We extend our initial studies by showing that highly purified human recombinant casein kinase II can replace the kinase from rabbit muscle in the activation of p53 in vitro. Given the unusual casein kinase II phosphorylation motif in the C-terminus of p53, it was important to establish that casein kinase II need not be modified by a second mammalian kinase to confer specificity and that phosphorylation alone by this enzyme can rapidly induce the conformational changes in p53 which lead to high affinity DNA binding. The ability of p53 to be activated by rabbit muscle casein kinase II through the use of GTP provides further evidence for the involvement of casein kinase II in the activation of p53. These biochemical results support the physiological evidence (30) that casein kinase II phosphorylation of p53 is one important modification required for its tumour suppressor activity. As p53 is phosphorylated by a variety of protein kinases (25–27, 53), the affects of modification at these alternate sites on the activity of the protein remains to be determined.

Although native gel electrophoresis is not necessarily an accurate method to estimate size of a protein, the native molecular weight of p53-DNA complexes under these conditions was calculated to be approximately 600 Kd. This form is slightly larger than p53 constitutively activated by deletion of the 30 C-terminal amino acids, which is approximately a complex of 250–400 Kd when bound to DNA (17). After translation in vivo, p53 monomers are rapidly converted into higher molecular weight forms (37). Together, these results are consistent with the view that p53 multimers are very stable and are characteristic of biochemically active molecules. Indeed, serial point mutations in the C-terminus of p53 are required to prevent stable complex formation between monomers (38), indicating that severe perturbations are required to potently inhibit multimer formation. The quaternary subunit structure of p53 required for DNA binding activity may be an important regulatory element in its control, as it is important in modulating the expression of conformationally sensitive epitopes (39).

p53 immunoaffinity purified using PAb421 is an unusually shaped tetramer (54). A fraction of this material isolated as a monomer on gel filtration can bind to TBP and inhibit general transcription in vitro (40). The monomeric nature of the p53 protein purified by these methods suggests that some factor may be involved in modulating monomer assembly. Regulation of multimer assembly may channel p53 into biochemically distinct pathways which, simplistically, lead to either (1) assembly of the homomultimer, phosphorylation of p53 by casein kinase II, and activation of DNA binding, or (2) inhibition of multimer

assembly through the formation of heterodimers with proteins including TBP (40) or Sp1 (55), and alteration of the DNA binding function of p53.

Wild type and mutant p53 require a reactive sulfhydryl group for activity

Activated mutant and wild type forms of p53 are sensitive to sulfhydryl modifying reagents NEM or Diamide. These results indicate that a reactive sulfhydryl(s) is essential for sequence-specific DNA binding activity of both mutant and wild type p53. This observation indicates that the mutations do not obscure or promote oxidization of a reactive sulfhydryl and prevent DNA binding of the mutant p53's once in the activated state.

Many nuclear sequence-specific DNA binding proteins require reduced sulfhydryl's for interaction with their respective DNA target sites (41, 42). In some situations, the reactive sulfhydryl can sensitize the protein to oxidation and loss of DNA binding activity in vivo. These results suggest that oxidation of DNA binding proteins may be a relevant mechanism through which the activity of transcription factors are modulated. Based on this premise, a cellular redox protein, Ref-1, was identified and purified from HeLa lysates and was shown to activate oxidized forms of Fos-Jun heterodimers and NF-kB for their DNA binding function (41, 43, 44). This provides strong evidence for a widespread enzymatic redox modulation of transcription factors in vivo.

Essential for p53 function in vitro, is the maintenance of a highly reduced environment. The removal of reducing agents by dialysis completely inactivates p53 DNA binding function, indicating that it is very rapidly oxidized under certain conditions (unpublished data). Transformed cells may be subjected to the stresses of oxidized environments which places an additional negative constraint on the tumour suppressor function of p53.

CONCLUSION

From our studies, we have identified a class of mutant p53 which is defective in a GTP or ATP dependent post-translational activation of its sequence-specific DNA binding function by casein kinase II. However, these mutants can be converted to activated states by a distinct set of proteins, including PAb421 and *E. coli* Hsp70. Two important criteria appear necessary for this conversion. These modifications presumably will be relevant in the cell and include 1) a specific, high affinity binding of an 'activating' polypeptide involved in neutralization of the C-terminal negative regulatory domain and 2) a highly reduced environment which can maintain p53 in an activated state. Activation of mutant p53 from its cryptic state by a monoclonal antibody and a heat shock protein has tremendous therapeutic implications. Given the clear association between the DNA binding activity and tumour suppressor functions of p53, these results imply that in many tumour cells there are high levels of mutant p53 that can potentially be activated to restore significant wild type function.

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