Novel phosphorylation sites of human tumour suppressor protein p53 at Ser[20] and Thr[18] that disrupt the binding of mdm2 (mouse double minute 2) protein are modified in human cancers

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The ability to separate the isoforms of human tumour suppressor protein p53 expressed in insect cells using heparin–Sepharose correlates with differences in the isoelectric point of p53, demonstrating that p53 can be heterogeneously modified and providing support for the use of insect cells as a model system for identifying novel signalling pathways that target p53. One p53 isoform that was reduced in its binding to the monoclonal antibody DO-1 could be stimulated in its binding to DO-1 by prior incubation with protein phosphatases, suggesting the presence of a previously unidentified N-terminal phosphorylation site capable of masking the DO-1 epitope. A synthetic peptide from the N-terminal domain of p53 containing phosphate at Ser[20] inhibited DO-1 binding, thus identifying the phosphorylation site responsible for DO-1 epitope masking. Monoclonal antibodies overlapping the DO-1 epitope were developed that are specific for phospho-Thr[18] (adjacent to the DO-1 epitope) and phospho-Ser[20] (within the DO-1 epitope) to determine whether direct evidence could be obtained for novel phosphorylation sites in human p53. A monoclonal antibody highly specific for phospho-Ser[20] detected significant phosphorylation of human p53 expressed in insect cells, whereas the relative proportion of p53 modified at Thr[18] was substantially lower. The relevance of these two novel phosphorylation sites to p53 regulation in human cells was made evident by the extensive phosphorylation of human p53 at Thr[18] and Ser[20] in a panel of human breast cancers with a wild-type p53 status. Phospho-Ser[20] or phospho-Thr[18] containing p53 peptides are as effective as the phospho-Ser[15] peptide at reducing mdm2 (mouse double minute 2) protein binding, indicating that the functional effects of these phosphorylation events might be to regulate the binding of heterologous proteins to p53. These results provide evidence *in vivo* for two novel phosphorylation sites within p53 at Ser[20] and Thr[18] that can affect p53 protein–protein interactions and indicate that some human cancers might have amplified one or more Ser[20] and Thr[18] kinase signalling cascades to modulate p53 activity.

Key words: kinase, monoclonal antibody, signalling, transcription.

INTRODUCTION

The tumour suppressor protein p53 is a key co-ordinator of a DNA-damage cell-cycle checkpoint pathway whose action prevents the propagation of permanently damaged cells by the induction of apoptosis or growth arrest [1]. The biochemical activity of p53 most closely associated with tumour suppression is its function as a stress-activated transcription factor that drives the expression of a large class of gene products implicated in DNA repair, cell-cycle arrest, redox regulation and protein degradation [1]. Although most research has focused on identifying the 'downstream' signalling pathways that are regulated by p53, more recent approaches have been centred on identifying 'upstream' factors that control p53 activity by post-translational modification.

p53 is composed of at least four functional domains that regulate its function as a stress-activated sequence-specific DNA-binding protein and transcription factor. The N-terminus of p53 contains the transactivation domain through which p53 interacts with components of the transcriptional machinery [2] and a smaller highly N-terminal conserved Box 1 domain of p53 (BOX-I) domain, which directs the binding of p53 to mdm2 (mouse double minute 2) protein. p53 has a short half-life in proliferating cells; the binding of mdm2 protein to p53 can mediate the degradation of p53 through the ubiquitin-dependent degradation machinery [1]. Phosphorylation of p53 after DNA damage within BOX-I by either DNA-activated protein kinase (DNA-PK) [3,4] or one or more ataxia–telangiecstasia mutated (ATM)-associated protein kinases [5,6] might activate p53 via both an inhibition of mdm2-dependent degradation [7] and a stimulation of the binding of cAMP-response-element-binding-protein ('CREB')-binding protein ('CBP') or p300 [8]. Alternatively, phosphorylation of p53 within BOX-I by either DNA-PK or ATM might also inhibit p53 via the inhibition of binding of transcription factor IID ('TFIID') [9]. Thus both functional and regulatory domains reside within the N-terminus of p53, whose modification seems to be cell-specific.

The central core domain of p53 contains the sequence-specific DNA-binding domain, which is highly conserved in vertebrates and in two recently identified human homologues: p73 and p63^{KET} [10–12]. A C-terminal tetramerization domain flanks the conserved core-sequence-specific DNA-binding domain and this

Abbreviations used: ATM, ataxia–telangiecstasia mutated; BOX-I, N-terminal conserved Box 1 domain of p53; CK2, casein kinase 2; DNA-PK, DNA-activated protein kinase; DTT, dithiothreitol; ECL, enhanced chemiluminescence; HRP, horseradish peroxidase; PKC, protein kinase C; PP1, protein phosphatase type 1; PP2A, protein phosphatase type 2A; Sf9, *Spodoptera frugiperda* cell line; TMB, 3,3′,5,5′-tetramethylbenzidine.

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motif is required to assemble p53 into a fully competent, tetrameric transcription factor [13,14]. Point mutations in the p53 gene found in over 50 % of human cancers usually map to the core-specific DNA-binding domain or the tetramerization domain and can either inactivate p53-sequence-specific DNA binding or prevent tetramerization [15–18]. Either of these types of mutation will decrease the activity of p53 as a transcription factor and compromise the cellular response to damage.

Flanking the tetramerization domain of p53 at the extreme C-terminus is a negative regulatory domain whose modification by phosphorylation at the casein kinase 2 (CK2) site activates the latent specific DNA-binding function of p53 *in vitro* [19–21]. Recent work has also established that phosphorylation at the CK2 site is increased in cells after damage to DNA; enhanced steady-state phosphorylation is correlated with enhanced p53-dependent transcription [22]. Together, these results suggest a role for at least two kinase signalling pathways in activating p53 after cellular damage by a concerted mechanism: (1) the N-terminal DNA-PK/ATM pathway decreases the mdm2-dependent degradation of p53 and stimulates p53 interaction with components of the transcriptional machinery, and (2) the C-terminal CK2 kinase pathway activates the specific DNA-binding function of p53.

Because it is likely that pathways that are as yet unidentified have a role in modulating p53 function, cellular models need to be developed to identify novel enzymic pathways that regulate p53 activity. We have previously used insect cell expression systems as a model with which to begin to identify novel signalling pathways that target p53 [23], because this eukaryotic cell contains many of the conserved kinase and phosphatase signalling cassettes likely to target p53. In addition, the overproduction of p53 in this cell line can be uncoupled from p53 degradation, permitting the purification of relatively large amounts of post-translationally modified p53 for biochemical characterization. The first novel signalling pathway targeting p53 that was identified by using this cell model was a UV-dependent pathway that resulted in phosphorylation at the protein kinase C (PKC) site in the C-terminal negative regulatory domain of p53 [24,25]. Phosphorylation at the PKC site might have important regulatory functions, as this modification can both activate the sequence-specific DNA-binding function of p53 [23,26] and modulate the binding of 14-3-3 proteins to p53 [27].

To continue our studies with the insect cell expression system as a model with which to identify unique post-translationally modified isoforms of human p53, we present an immunochemical approach that has identified two novel phosphorylation sites (at Ser^{20} and Thr^{18}) that overlap the mdm2-binding site of human p53. The development and characterization of monoclonal antibodies against these phosphorylation sites has shown that extensive phosphorylation can occur at the sites in recombinant expression systems and in human breast cancers. In addition, the ability of these phosphorylation events to decrease mdm2 binding suggests that they might regulate the binding of heterologous proteins to BOX-I. These studies provide phospho-specific monoclonal antibody reagents with which to study BOX-I signalling cascades that target p53 and also highlight the need to identify the Ser^{20} and Thr^{18} kinases that might modulate p53-dependent growth arrest and apoptosis.

MATERIALS AND METHODS

Enzymes, cells and reagents

Sf9 (*Spodoptera frugiperda*) cells expressing human p53 were grown in medium as described previously [24]. *Escherichia coli* harbouring the plasmid pT7.7 containing the human gene for mdm2 was treated with IPTG to express mdm2 protein, which was subsequently purified from bacterial pellets with heparin–Sepharose, as described for p53 [23]. The epitopes for the monoclonal antibodies DO-1, DO-12 and PAb421 have been mapped previously [28,29]; the antibodies were raised against full-length p53. Phosphopeptides from the N-terminal domain of p53 (residues 13–27 of human p53, with a phosphate at Ser^{15}, Thr^{18} or Ser^{20}, or phosphates at both Thr^{18} and Ser^{20}) were synthesized by Dr. Graham Bloomberg (University of Bristol, Bristol, U.K.). Biotinylated peptides and the corresponding phosphopeptides from residues 13–27 of human p53 were obtained from Chiron Mimotopes. Streptavidin was obtained from Vector Laboratories (Peterborough, Cambs., U.K.) and potato acid phosphatase (60 units/ml) was obtained from Boehringer Mannheim (Mannheim, Germany). Monoclonal antibodies against phospho-BOX-I epitope peptides were developed by standard methods, as described previously [23]. Phosphopeptides were coupled to keyhole limpet haemocyanin before immunization; IgG produced by hybridoma cell lines after the fusion was first screened by immunoblotting with a panel of phosphopeptides, then tested for specificity by surface plasmon resonance with a Pharmacia Biacore (results not shown) and ELISA (see Figure 5).

Isoelectric focusing and immunoblotting

Human p53 was expressed in Sf9 cells; isoforms were separated by heparin–Sepharose chromatography, as described previously [24]. The monoclonal antibody DO-1 was used to localize the peak fraction of p53 after SDS/PAGE and immunoblotting (see Figure 7A). The peak fractions containing p53 (1 μl) that were eluted from a heparin–Sepharose column (fractions 20–25) were dissolved in 10 μl of solubilization buffer [8 M urea/1 % (v/v) Triton X-100/40 mM Tris base/10 mM dithiothreitol (DTT)], applied to a Pharmacia Immobiline Dryplate (pH 4.0–7.0) equilibrated in denaturation buffer [8 M urea/0.05 % (v/v) Triton X-100/0.1 M DTT/0.5 % (v/v) Pharmalyte (pH 3–10)] and focused for 7 kV·h with a circulating-water cooling unit set at 15 °C.

Separated proteins were transferred to a nitrocellulose membrane and the immunoblots were developed overnight with affinity-purified DO-1 (or other indicated monoclonal antibodies at 1 μg/ml) in PBST3M [PBS: 140 mM NaCl/2.6 mM KCl/10 mM Na_2HPO_4/1.7 mM KH_2PO_4, containing 3 % (w/v) milk powder and 0.1 % (v/v) Tween 20]. DO-1 (or the indicated antibody) bound to p53 was detected by incubating the membranes with horseradish peroxidase (HRP)-conjugated anti-mouse IgG in PBST3M. The immune complexes were detected with enhanced chemiluminescence (ECL) solution (Amersham).

Assay for p53-dephosphorylation with two-site capture ELISA

A quantitative two-site capture ELISA was used to determine whether recombinant human p53 was phosphorylated within the PAb421 and DO-1 epitopes by analysing PAb421 or DO-1 epitope expression with or without protein phosphatase treatment. The monoclonal antibodies DO-1, PAb421 or FPS20 (as indicated) were coated overnight at 100 ng per well (in 50 μl of 0.1 M carbonate buffer, pH 9.0) in 96-well ELISA plates. The non-specific protein binding was blocked by incubating each well with 200 μl of PBST3B [PBS containing 3 % (w/v) BSA and 0.1 % (v/v) Tween 20] for 2 h. p53 (as indicated, 5 μl of the peak fractions from a heparin–Sepharose column) was added to 50 μl of protein phosphatase buffer [10 % (v/v) glycerol/50 mM KCl/25 mM Hepes (pH 7.6)/1 mM DTT/20 mM $MgCl_2$/1 mM benzamidine] with or without 0.4 unit of protein phosphatase 1

(PP1) plus 0.2 unit of protein phosphatase 2A (PP2A) and incubated for 30 min at room temperature, before transfer to ELISA wells. Immunoreactive p53 was detected with a 1:5000 dilution of p53-specific CM5 polyclonal anti-serum and detected with HRP-conjugated secondary anti-rabbit IgG, followed by either ECL solution or complete 3,3′,5,5′-tetramethylbenzidine (TMB) substrate (Sigma), in accordance with the manufacturers' instructions. Quantification was performed with an ECL-based ELISA plate reader or colorimetric plate reader (450 nm), as indicated in the figure legends. For p53 addition and subsequent steps, the dilutions were made in PBST5M [as PBST3M but containing 5 % (w/v) dried milk] and applied at 50 μl per well. All incubations were for 1 h at room temperature and included three washes with 200 μl per well of PBST [PBS buffer containing 0.1 % (v/v) Tween 20] between incubations.

Assay for DO-1 and mdm2 protein binding to synthetic peptides with the use of the ELISA format

ELISA wells were coated with streptavidin (5 μg/ml) in 50 μl of double-distilled water; non-reactive sites were blocked with PBST3B. Synthetic biotinylated, unphosphorylated or phosphorylated peptides containing the core sequence biotin-linker-(SGSG)-EPPLSQETFSDLWK were applied at 25 ng per well, then diluted in PBST3B containing 50 mM NaF and 12 nM okadaic acid to preserve phosphorylation. A second blocking step was performed by incubating in each well 200 μl of PBST5M, followed by the addition to the respective ELISA wells of fixed amounts of DO-1 (5 ng) or full-length mdm2 protein (40 ng) diluted in PBST5M containing 50 mM NaF and 12 nM okadaic acid. In experiments in which the phospho-Ser^{20} peptide was treated with phosphatase before incubation with antibody, 100 ng of peptide was incubated with 0.06 unit of potato acid phosphatase at 30 °C for 1 h in Mes buffer [10 % (v/v) glycerol/100 mM KCl/50 mM Mes (pH 6.0)/1 mM DTT/1 mM benzamidine] before addition to ELISA wells. DO-1 bound to the peptides was detected by incubating with HRP-conjugated anti-mouse IgG, as summarized above. mdm2 protein binding to the peptides was detected with two different mdm2-specific monoclonal antibodies (2A10 or 4B2 at 1 μg/ml) followed by HRP-conjugated anti-mouse IgG, as summarized above. Results are represented as A_{450} measured with a Dynatech ELISA plate reader. All incubations and washes were as described previously for the two-site capture ELISA [24].

Lysis of human breast cancers

Lysates of frozen human breast tissue previously defined with respect to p53 status and defined as wild-type [30] were prepared as indicated [31] by incubating the tissues in urea lysis buffer [8 M urea/1 % (v/v) Nonidet P40/50 mM Hepes (pH 7.6)/5 mM DTT/0.4 M KCl/1 mM benzamidine/50 mM NaF/120 nM okadaic acid] to preserve the phospho-epitopes; the protein in lysates was quantified with the method of Bradford [32].

RESULTS

Distinct isoelectric variants of p53 are synthesized in Sf9 cells

As a unique model with which to identify novel upstream factors regulating the p53 response to radiation (see Figure 1), the insect cell expression system has been used to produce at least three isoforms of p53 that can be separated chromatographically with heparin–Sepharose [24]. These three isoforms differ in their specific activities in sequence-specific DNA binding reactions and their degrees of phosphorylation at the C-terminal PKC site within the PAb421 epitope [24,25]. The differential affinities of these isoforms for heparin–Sepharose are presumably due to differences in post-translational modifications on p53. To address this issue, the peak fractions of p53 were subjected to isoelectric focusing to determine whether changes in the net charge of p53 are correlated with its differential elution from the heparin column. Compared with the peak of p53 as determined by SDS/PAGE (see the control in Figure 7A, fractions 20–25), p53 from fractions 20–25 can be resolved into predominantly acidic and basic isoforms after isoelectric focusing (Figure 2). The acidic isoforms were eluted earlier and coincided with activated p53 (fraction 20), whereas the more basic isoforms were eluted later and coincided with the elution of the latent form of p53 (fraction 25). The immunoreactivity of p53 in fractions 20–25 after SDS/PAGE (see Figure 7A) establishes that one major protein band is observed and confirms the well-established specificity of DO-1 for p53. These data provide a chemical basis for explaining the separation of p53 isoforms with heparin–Sepharose and demonstrate that substantial post-translational modification occurs in this cell system.

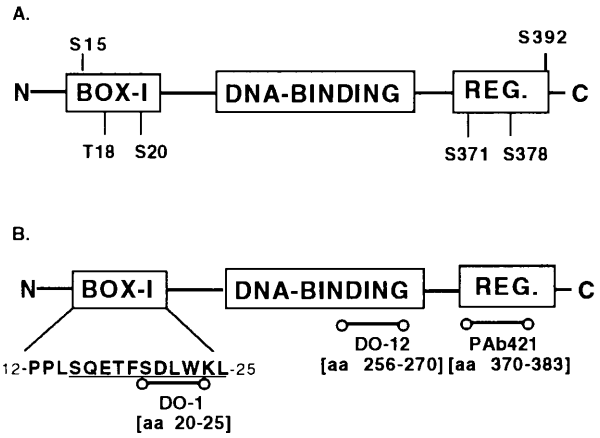

Figure 1 Domain structure of p53

(**A**) Novel phosphorylation sites identified on human p53 with the use of insect cell expression systems. The locations of the BOX-I regulatory domain, the central sequence-specific DNA-binding domain and the C-terminal negative regulatory domain are indicated by 'BOX-I', 'DNA-binding' and 'Reg.' respectively. The Ser^{15} (DNA-PK) and Ser^{392} (CK2) phosphorylation sites regulate the biochemical activity of human p53 *in vitro*; both of these phosphorylation sites were originally mapped by using standard ^{32}P-labelling techniques [4,50]. Three additional phosphorylation sites that were originally identified *in vivo* using the insect cell system expressing human p53 include two sites at Thr^{18} and Ser^{20} (the present study) and an additional C-terminal PKC phosphorylation site at Ser^{371} whose phosphorylation masks the PAb421 epitope [24]. (**B**) Map of the positions of monoclonal antibody epitopes and functional domains of p53. The monoclonal antibody epitope of DO-1 is residues 20–25 within BOX-I [29], that of DO-12 is residues 256–270 within the core DNA-binding domain [27], and that of PAb421 is residues 370–383 within the C-terminal PKC phosphorylation site [28]. Abbreviation: aa, amino acid residues.

Phosphorylation of recombinant p53 *in vivo* within the C-terminal PAb421 epitope and the N-terminal DO-1 epitope

One novel signalling pathway targeting p53 that has been identified by using insect cells [24,25] and has since been shown to function in human cells [27] is the PKC site pathway whose phosphorylation of p53 masks the epitope for the monoclonal antibody PAb421. Thus the masking of the PAb421 epitope via PKC phosphorylation has been a convenient assay for quantifying the extent of PKC-site phosphorylation of p53 [23,24]. To begin to purify a PKC-site phosphatase, we first needed to purify

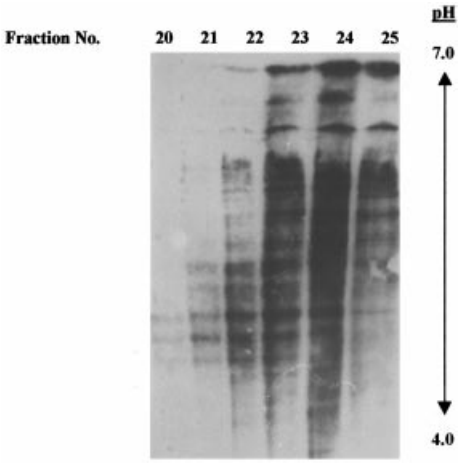

Figure 2 Multiple isoelectric variants of human p53 are produced in a eukaryotic expression system

Human p53 was expressed in Sf9 cells and functional isoforms were separated by heparin–Sepharose chromatography as described previously [24]. The peak fractions of p53 that were eluted from a heparin–Sepharose column (fractions 20–25) were applied to a Pharmacia Immobiline Dryplate (pH 4.0–7.0) equilibrated in denaturation buffer and focused for 7 kV·h as indicated in the Materials and methods section. The protein in the gel was transferred to nitrocellulose and blotted with DO-1. The gel is orientated with the top representing more basic isoforms (later-eluting fractions from heparin–Sepharose; fractions 23–25) and the bottom containing a higher proportion of acidic p53 isoforms (earlier-eluting fractions 20 and 21). As a control for DO-1 specificity, p53 from fractions 20–25 was separated into unique bands by using one-dimensional SDS/PAGE (see Figure 7).

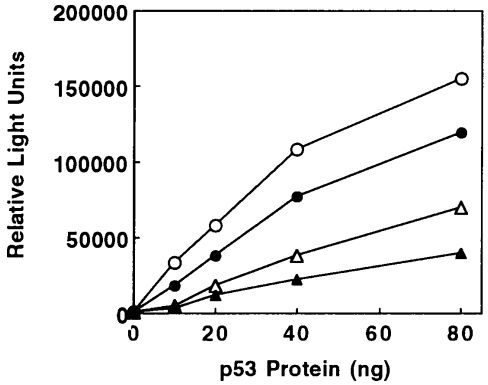

Figure 3 Incubation of recombinant human p53 purified from Sf9 cells with phosphatases stimulates DO-1 binding

A two-site capture ELISA was used to determine whether recombinant human p53 was phosphorylated within PAb421 by analysing PAb421 or DO-1 epitope expression without or with protein phosphatase treatment using a two-site capture ELISA. Different volumes of the p53 peak fraction (number 23; 80 ng/μl of p53 expressed in insect cells and fractionated on heparin–Sepharose) were incubated in a phosphatase buffer as indicated in the Materials and methods section. Immunoreactive p53 captured by the monoclonal antibodies was detected with p53-specific polyclonal antiserum and quantified with HRP-conjugated secondary anti-rabbit IgG as described in the Materials and methods section. Binding of DO-1 epitope (○, ●) and PAb421 epitope binding (△, ▲) without protein phosphatases (●, ▲) or with protein phosphatases (○, △) was quantified with an ECL-based ELISA plate reader and is plotted as relative light units as a function of increasing p53 concentration (peak fraction number 23; see Figures 2 and 7).

biochemical forms of p53 (which were PAb421-non-reactive via PKC-site phosphorylation) to be used as a phosphatase substrate. The main basis for using p53 phosphotetramers as a phosphatase substrate instead of phosphorylated synthetic peptides is that protein phosphatases require the assistance of targeting subunits to direct them to their substrates, implicating a relatively large protein interface for phosphatase interaction [33].

In setting up a quantitative two-site capture ELISA to assay for a phosphatase that targets the PKC phosphorylation site within the PAb421 epitope (Figure 1), increases in PAb421 binding to p53 could be observed on treatment with protein phosphatase (Figure 3). When analysing control reactions, a more pronounced increase in DO-1 binding was observed after incubation with protein phosphatases (Figure 3), suggesting that DO-1 binding to its epitope can also be influenced by phosphorylation within or adjacent to its epitope (Figure 1). Because these results suggested the existence of a previously unreported phosphorylation site that masks the DO-1 epitope, further studies were performed to define this phosphorylation site and to determine its possible role in modulating p53 function.

Ser^{20} phosphorylation preferentially inhibits DO-1 binding to BOX-I peptides

A quantitative phosphopeptide-binding ELISA was subsequently developed to determine which known (or putative) phosphorylation sites could be affecting the affinity of DO-1 for its binding site. Potential phosphorylation sites that could affect DO-1 binding include: (1) the DNA-PK or ATM site at Ser^{15}, which is highly conserved in different species [3]; (2) a potential phosphorylation site at Thr^{18}, which is highly conserved between human p53 and human p73, as well as in p53 from vertebrates and squid [3]; and (3) a potential phosphorylation site at Ser^{20}, which is conserved in mammals.

A series of biotinylated unphosphorylated and phosphorylated peptides were synthesized chemically and used to probe for DO-1 binding to its epitope. Although DO-1 bound with similar affinities to unphosphorylated peptides or peptides with a phosphate at Ser^{15} or Thr^{18}, a phosphate at Ser^{20} completely inhibited DO-1 binding (Figure 4A). Pretreatment of the synthetic phospho-Ser^{20} peptide with potato acid phosphatase restored DO-1 binding (Figure 4B), indicating that a single phosphorylation event can reversibly inhibit DO-1 binding. These results are consistent with the mapping of the DO-1 epitope, in which Ser^{20} and Asp^{21} were shown to be essential components of its binding site (Figure 1) [29]. Further, the ability of DO-1 to bind to phospho-Ser^{15} peptides indicates that the phosphorylation that masks the DO-1 epitope is distinct from the DNA-PK/ATM site at Ser^{15}.

Ser^{20} and Thr^{18} phosphorylation decreases the binding of mdm2 protein

The Ser^{20} and Thr^{18} phospho-epitope might have important regulatory functions, because this region of p53 is an important docking site for mdm2 protein (Figure 1). Studies were performed to determine whether the three phosphopeptides exhibited any differential affinity for mdm2 protein (Figures 4C and 4D). Previous studies had demonstrated that synthetic peptides derived from BOX-I contain the necessary determinants required for mdm2 protein interaction with p53 [34] and that the synthetic-peptide binding assay is a quantitatively accurate method for assessing mdm2 protein binding [35]. In comparison with the unphosphorylated peptide, mdm2 protein binding was

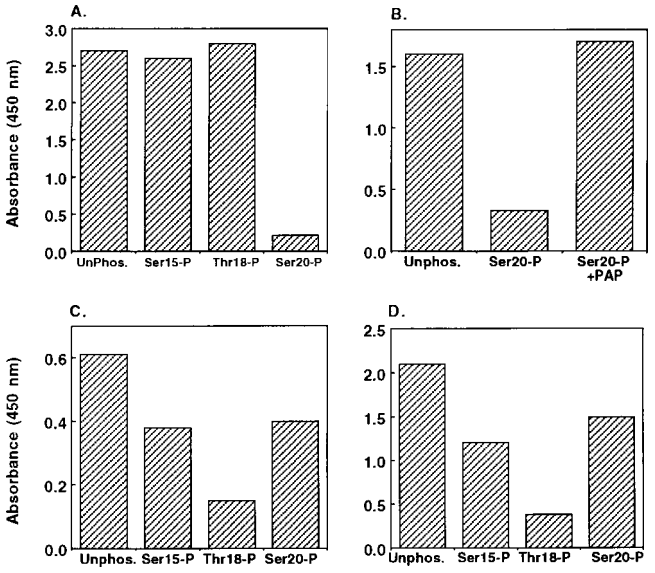

Figure 4 Effects of phosphorylation on DO-1 and mdm2 protein binding to BOX-I phosphopeptides derived from human p53

(**A**) DO-1 binding is inhibited by a phospho-Ser^{20} substitution. (**B**) Phosphatase treatment restores DO-1 binding to the phospho-Ser^{20} peptide. (**C**, **D**) Binding of mdm2 to all three phospho-specific synthetic peptides is decreased with monoclonal antibodies 2A10 (**C**) and 4B2 (**D**) as the primary anti-mdm2 antibodies. ELISA wells were coated with streptavidin and synthetic biotinylated, unphosphorylated or phosphorylated peptides (with the indicated phosphate addition) bound to either 5 ng of DO-1 (**A**, **B**) or 40 ng of mdm2 (**C**, **D**); the amount of protein bound was detected as indicated in the Materials and methods section. The binding results are shown as A_{450} with the TMB colorimetric peroxidase assay.

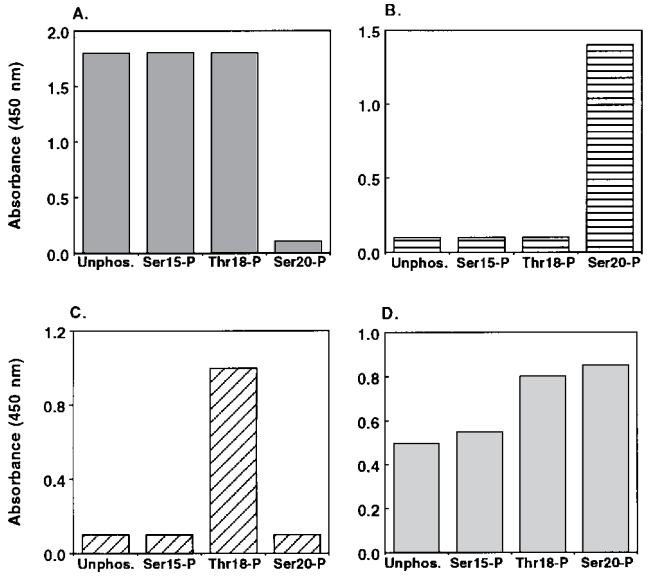

Figure 5 Specificity of phospho-specific monoclonal antibodies for phospho-Thr^{18} and phospho-Ser^{20} with ELISA

ELISA wells were coated with streptavidin and synthetic biotinylated, unphosphorylated or phosphorylated peptides (with the indicated phosphate addition) and the indicated monoclonal antibodies (5 ng of each) were added into ELISA wells as indicated in the Materials and methods section. The antibodies were: (A), DO-1; (B), FPS20; (C), FPT18; and (D), FPT18/20. The binding data are represented as A_{450} against fixed antibody levels (5 ng) using the TMB colorimetric peroxidase assay.

diminished to increasing extents to the phospho-Ser^{20} peptide, the phospho-Ser^{15} peptide and the phospho-Thr^{18} peptide respectively. Although the Ser^{15}, Thr^{18} and Ser^{20} side chains do not make direct contacts at the core interface with mdm2, both the Thr^{18} and Asp^{21} side chains form hydrogen bonds that contribute to the initiation of the helical structure of p53 within the mdm2-binding cleft [34]. Thus the disruption of the hydrogen bonds at Thr^{18} by the substitution of phospho-Thr^{18} might explain why mdm2 binding is more potently inhibited by this substitution than by the phospho-Ser^{15} or phospho-Ser^{20} substitutions. These results (Figure 4) localize the phosphorylation that is most probably involved in blocking DO-1 binding to p53 (Ser^{20}) and indicate that mdm2 protein and DO-1 show overlapping but distinct specificities for phospho-epitopes in BOX-I. In addition, the 40–50 % decrease in mdm2 binding to the phospho-Ser^{15} peptides (Figures 4C and 4D) is quantitatively similar to the decrease in mdm2 protein binding to full-length p53 phosphorylated by DNA-PK [7], further validating the peptide–mdm2 binding assay as an accurate reflection of full-length p53–mdm2 interactions.

Generation of phospho-specific monoclonal antibodies to two phosphorylation sites in BOX-I of human p53

Given the sensitivity of DO-1 to Ser^{20} phosphorylation and the functional effects of mdm2 binding to peptides containing phosphate at positions Thr^{18} or Ser^{20} (Figure 4), potentially novel phosphorylation sites might exist within the BOX-I motif of p53. However, as standard radiolabelling methods have not previously shown unequivocally the existence of novel phosphorylation sites within BOX-I, a different approach was taken. A panel of hybridoma cell lines was generated that produced monoclonal antibodies reacting with two BOX-I phosphopeptides (Thr^{18} and Ser^{20}). The development of such monoclonal antibodies would provide non-invasive reagents for determining whether phosphorylation occurs at these sites *in vivo*. This is especially important in view of recent results [36] showing that the treatment of normal human diploid fibroblasts with [^{32}P]P_i damages cells, resulting in the unmasking of the DO-1 epitope without changes in p53 levels. These results further suggest that reversible phosphorylation at Ser^{20} can occur in normal human cells and that this site might not have been detected previously because [^{32}P]P_i labelling conditions result in cell injury and dephosphorylation at the DO-1 epitope.

Antibodies from hybridoma supernatants were screened first by dot-blotting and then characterized quantitatively in terms of specificity for their epitopes using ELISA (Figure 5). This demonstrated the specificity of FPS20 for the phospho-Ser^{20} peptide and the FPT18 antibody for the phospho-Thr^{18} peptide (Figures 5B and 5C). Intriguingly, the monoclonal antibody FP18/20 was generated against the phospho-Thr^{18} peptide and this antibody exhibited preferential specificity for either phospho-Ser^{20} and phospho-Thr^{18} peptides (Figure 5D). The relative binding affinities of the FPS20 and FPT18 antibodies for their respective phospho-epitopes seem similar to that of DO-1 for its unphosphorylated epitope (Figure 5A vs. Figures 5B–D). In addition, using such a double-site phospho-Ser^{20} and phospho-Thr^{18} peptide, in comparison to the single-site phosphopeptides, it is clear that the monoclonal antibody specific for phospho-Ser^{20} (FPS20) binds to a much smaller extent to the double-site phospho-epitope than to the Ser^{20} phosphopeptide (Figure 6). This contrasts with FPT18, which, although unable to bind to the phospho-Ser^{20} peptide, binds equally well to the phospho-

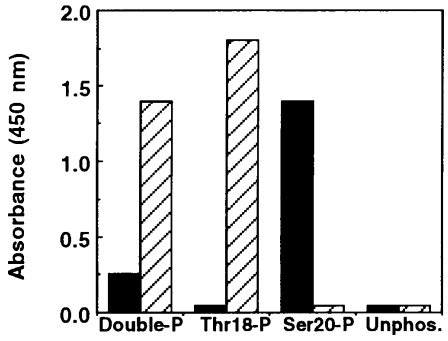

Figure 6 FPS20 is sensitive to a double Thr[18] and Ser[20] phospho-epitope

ELISA wells were coated with streptavidin and synthetic biotinylated, unphosphorylated or phosphorylated peptides with the indicated phosphate additions [double Thr[18] and Ser[20] (Double-P), single Thr[18] (T18-P), single Ser[20] (S20-P) or unphosphorylated] and their binding to FPS20 (black columns) and FPT18 (hatched columns) (5 ng of each) was quantified as indicated in the Materials and methods section. The binding results are shown as A_{450} with the TMB colorimetric peroxidase assay.

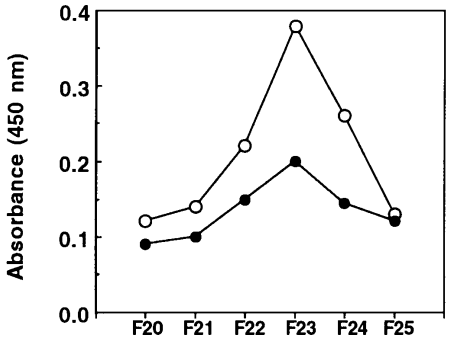

Figure 8 Phosphatase treatment of p53 decreases the binding of FPS20 to its epitope

The peak fractions of FPS20-reactive p53 were left untreated (○) or treated with PP1 and PP2A (●) as indicated in the Materials and methods section and in the legend to Figure 3; p53 from fractions 20–25 (F20 to F25) captured by FPS20 was detected with p53-specific polyclonal antiserum as described in the Materials and methods section. The binding results are shown as A_{450} with the TMB colorimetric peroxidase assay.

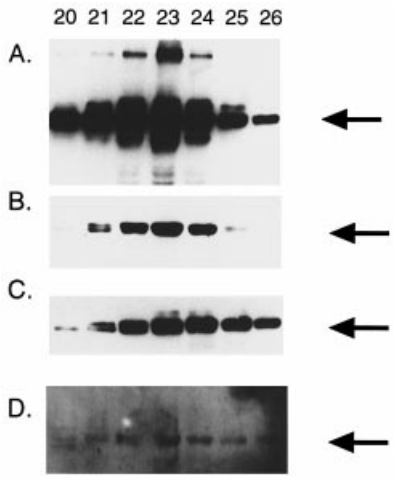

Figure 7 Phospho-Thr[18]- and phospho-Ser[20]-specific monoclonal antibody binds to human p53 expressed in recombinant insect cells

The elution of p53 from a heparin–Sepharose column (fractions 20–26) was detected after SDS/PAGE [10% (w/v) gel] by immunoblotting as indicated in the Materials and methods section with the monoclonal antibodies DO-1 (**A**), FPS20 (**B**), FP18/20 (**C**) and FPT18 (**D**). The arrow marks the position of p53.

Thr[18] peptide and to the double-site phospho-Thr[18]/phospho-Ser[20] peptide (Figure 6).

Monoclonal antibodies specific for phospho-Ser[20] and phospho-Thr[18] bind to p53 expressed in recombinant insect cells

FPT18 and FPS20 were used to determine whether Ser[20] or Thr[18] phosphorylation could be detected on full-length p53 expressed in Sf9 cells, because this system was the original model used to identify DO-1 epitope masking (Figure 3). In comparison with the peak of DO-1 reactive p53 (Figure 7A), FPS20 detected a peak of Ser[20]-phosphorylated p53 (Figure 7B), further suggesting that Ser[20] phosphorylation within the DO-1 epitope can occur in this cell line. FPT18 weakly detected a different subfraction that was eluted more broadly than DO-1-reactive and FPS20-reactive p53 (Figure 7D). The smaller signal obtained with FPT18 was not due to an inherently low affinity of the antibody, because the signal by ELISA was similar to that of the other antibodies (Figure 5) and the antibody can be used to detect significantly high levels of Thr[18] phosphorylation of human p53 in breast cancers (see Figure 9). FP18/20, which binds preferentially to both phospho-Thr[18] and phospho-Ser[20] peptides, detected a subfraction different from that detected by FPS20, FPT18 or DO-1 (Figure 7C).

As a control to establish the phospho-specificity of FPS20 towards full-length p53, a two-site capture ELISA was used for quantification. In comparison with the peak fractions of p53 in which DO-1 (and PAb421) was stimulated in binding to its epitope (Figure 3), FPS20 binding decreased after phosphatase treatment (Figure 8). These results provide further support for a novel phosphorylation site within BOX-I of human p53 expressed in Sf9 cells and validates this cell system as a model with which to reveal the existence of enzymes that might be used to modulate p53 function.

Immunochemical evidence for phosphorylation of human p53 at the DO-1 epitope (Ser[20]) and at Thr[18] in human breast cancers

Apart from the insect cell model that we are using to dissect p53 activation mechanisms, breast cancers with wild-type p53 status are being characterized biochemically in an attempt to understand the mechanism(s) by which a tumour cell bypasses mutation in p53. It is the hope that by studying p53 modification in these cancers, previously uncharacterized signalling pathways that have a role in the p53 response might be revealed.

A panel of human breast cancer tissue was therefore screened to determine whether evidence could be found for the phosphorylation of p53 at Ser[20] or Thr[18], with the immunochemical probes DO-1, FPS20 and FPT18 (representative classes are described in Figure 9). The BOX-I-phospho-specific monoclonal antibodies were utilized by blotting lysates from human breast cancers that had previously been characterized with respect to their p53 status [30]. Relative to p53 levels normalized with DO-12 (Figures 9A and 9D), a significant proportion of the p53 in one class of cancers was predominantly DO-1 reactive (Figure 9B) and a second class of cancers produced p53 in a form that was largely non-reactive towards DO-1 (Figure 9E). Although

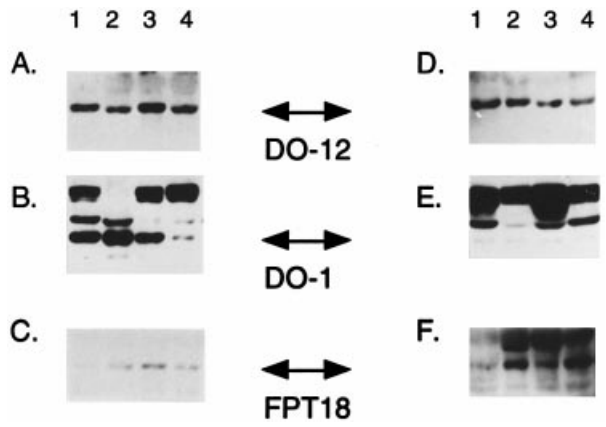

Figure 9 Thr[18] and Ser[20] phosphorylation of p53 in human breast cancers

A human breast cancer archive [30] was lysed in urea lysis buffer as indicated in the Materials and methods section and analysed by immunoblotting to determine whether the Ser[20] or Thr[18] signalling pathways function *in vivo*. (**A**–**C**) One class of cancers in which p53 levels were normalized with DO-12 (**A**, lanes 1–4), p53 was predominantly DO-1-reactive (**B**, lanes 1–3) and p53 was weakly FPT18-reactive relative to p53 levels (**C**, lanes 2–4). (**D**–**F**) A second class of cancers in which p53 levels were normalized with DO-12 (**D**, lanes 1–4), p53 was predominantly non-reactive towards DO-1 (**E**, lanes 1–4) and p53 was more FPT18-reactive relative to p53 levels (**F**, lanes 2–4). The position of p53 is indicated by the arrow.

these results suggest that Ser[20] phosphorylation occurred *in vivo*, the FPS20 did not bind to p53 in any of the breast cancers (results not shown), suggesting that multiple modifications might occlude FPS20's binding to its phospho-epitope. For example, double-site phosphorylation at Ser[20] and Thr[18] precluded FPS20's binding to its epitope (Figure 6).

To determine whether there was in fact phosphorylation at the adjacent Thr[18] residue, lysates from the breast cancers were immunoblotted and shown to possess a significant level of Thr[18] phosphorylation relative to p53 levels normalized with DO-12 (compare Figures 9C and 9F with Figures 9A and 9D). These results provide immunochemical evidence that Thr[18] and Ser[20] phosphorylation occurs *in vivo* in a large majority of human breast cancer biopsies and highlights the utility of the p53–insect cell expression system in identifying novel p53 signalling cascades that function in human cells.

DISCUSSION

Development of phospho-specific monoclonal antibodies against phospho-Thr[18] and phospho-Ser[20] peptides: detection of phosphorylation of BOX-I of human p53 expressed in insect cells

Human cultured cell lines express relatively small amounts of p53 and regulatory post-translational modifications might be short-lived, making it difficult to develop a comprehensive biochemical dissection of p53 regulation. The development of p53 expression systems with the use of bacteria [19,23], yeast [37–40] and insect cells [24] to study p53 regulation has provided a model that permits the dissection of p53 regulation. The utility of these systems relies on the evolutionary conservation of post-translational signalling cassettes in eukaryotes (and chaperones in prokaryotes) that regulate the tumour suppressor activity of p53 in human cells and on the presence of relatively large amounts of p53 that can be overexpressed and purified for biophysical study. In particular, insect cells expressing human p53 have revealed a UV- or serum-dependent signalling pathway that leads to the phosphorylation of p53 at the PKC site contained within the C-terminal PAb421 epitope [24] that was later shown to exist in human cells as well [27].

In our continued characterization of the p53 isoforms synthesized in insect cells, denaturing isoelectric focusing was performed (Figure 2) and a correlation was established between the isoelectric point of p53 and its differential affinity for heparin–Sepharose (Figure 2). This heterogeneous modification of p53 in the insect cell system suggests that some type of compartmentation or selective modification of p53 tetramers occurs *in vivo*. Heterogeneous post-translational modification of p53 can also occur in human cells; up to 11 phosphorylated isoforms have been detected in some instances [41]. In addition, human p53 expressed in baculovirus-infected Sf9 cells displays a two-dimensional isoform pattern very similar to that of wild-type p53 from human cells [42]. Taken together, these results support the utility of the insect cell for dissecting p53 regulatory pathways. It is the continued use of this cell to identify the enzymes implicated in modulating steady-state C-terminal PKC-site phosphorylation that led to our observation of novel phosphorylation sites within the N-terminal BOX-I of human p53.

Our initial rationale for studying BOX-I phosphorylation was based on results showing that a pool of p53 expressed in insect cells was DO-1 non-reactive and that DO-1 binding could be stimulated by the treatment of p53 with protein phosphatases. The same approach has previously been used to show that treatment of PAb421-non-reactive p53 with PP1 or PP2A could restore PAb421 binding to p53 [25] and established that phosphorylation within the PAb421 epitope could occur in cells. Our similar analysis of DO-1 epitope masking established that the most likely phosphorylation site that inhibits DO-1 binding is at Ser[20]. The DO-1 epitope overlaps the mdm2-binding site with the minimal epitope defined with the use of phage-peptide display as Ser[20]-Asp-Leu-Xaa-Lys-Lys[25] [29]. Amino acid residues identified by phage-peptide display that are generally invariant, and presumably are essential for DO-1 binding, include Ser[20] and Asp[21]. These results are consistent with the phospho-Ser[20] sensitivity and the phospho-Thr[18]/phospho-Ser[15] insensitivity of DO-1 binding to synthetic peptides.

Although masking of the DO-1 epitope on p53 (as in Figures 3 and 9) would provide an indirect assay to probe for phosphorylation at Ser[20], the Thr[18] or Ser[20] phosphorylation sites have not previously been mapped on human p53 by using classic ^{32}P-labelling methods *in vivo*. During the development of the present study, it has been shown that treatment of normal human diploid fibroblasts with [^{32}P]P_i induces a p53-dependent growth arrest and results in the unmasking of the DO-1 epitope without changes in p53 levels [36]. These results suggest that Ser[20] phosphorylation can occur in normal proliferating cells, but because dephosphorylation at the DO-1 epitope occurs after the cell damage induced on incubation in standard ^{32}P-labelling medium, this might explain why this site has not been mapped previously. We have therefore developed phospho-specific monoclonal antibodies for use as non-invasive probes to examine the steady-state phosphorylation of these phosphorylation sites. The antibody specific for the phospho-Ser[20] epitope showed an absolute requirement for phosphate at Ser[20] and was relatively intolerant of an adjacent phosphate moiety (Figures 5 and 6). With the Thr[18] phosphopeptide as an antigen, two monoclonal antibodies were acquired: one with an absolute requirement for phosphate at Thr[18] (FPT18) and insensitive to an adjacent phosphate at Ser[20] (Figures 5 and 6), and a second (FPT18/20) that binds equally well to peptides containing a single phospho-Thr[18] or phospho-Ser[20] substitution (Figure 5). FPT18 and

FPS20 can detect recombinant human p53 phosphorylation in insect cells, indicating that this cell type does harbour one or more enzymes that targets novel phosphorylation sites within BOX-I.

Defining the relative specificity of this newly produced panel of phospho-specific monoclonal antibodies was important, because this type of reagent has not previously been well characterized and it remained possible that the antibodies retained specificity more for phosphoamino acids than for phosphopeptide epitopes. Although the results (Figure 5) indicate that the amino acids surrounding the phosphoamino acid moiety also provide an important determinant for the antibody to bind stably to its phospho-epitope, our evidence that one monoclonal antibody, FPT18/20, might not discriminate absolutely between phospho-Thr^{18} and phospho-Ser^{20} places an emphasis on determining whether the phospho-Ser^{15} reagents previously used to study DNA-PK or ATM-site phosphorylation at Ser^{15} [7,9] display absolute specificity for the phospho-Ser^{15} epitope or whether they also react with other phospho-BOX-I epitopes.

Ser^{20} and Thr^{18} are phosphorylated in human cancers and their phosphorylation of BOX-I decreases mdm2 binding

The relevance of phosphorylation at Thr^{18} or Ser^{20} in human cells was investigated by determining whether human cancers expressed p53 in a Thr^{18}- or Ser^{20}-phosphorylated state for two reasons. First, this cancer type has a very high proportion of wild-type p53 and is a good model in which to search for dysregulation in upstream signalling pathways that might disrupt normal cell-cycle ckeckpoint pathways [43]. Secondly, the relevance of these signalling cascades to human cancers would be more evident if we could establish whether the phosphorylations occurred in a large panel of tissues *in vivo*, as opposed to a small number of cell lines cultured *in vitro*. In our panel of over 33 breast cancer biopsies, most showed elevated Thr^{18} or Ser^{20} phosphorylation, indicating that activation of these pathways is widespread in a pathological condition. Most notable was the pronounced masking of the DO-1 epitope of p53 and the substantial reactivity to the antibody that binds to phospho-Thr^{18} (Figure 9). It is interesting to note that although Thr^{18} phosphorylation is detectable at only low levels in insect cells expressing p53 (Figure 7), it is observed at much higher levels *in vivo* (Figure 9). This contrasts with FPS20, which detects a more substantial level of Ser^{20} phosphorylation of p53 in insect cells, but none at all in human cancers *in vivo*. This apparent discrepancy has been resolved by demonstrating that FPS20 does not bind well to a peptide phosphorylated on both Thr^{18} and Ser^{20}, whereas FPT18 can bind well to the doubly phosphorylated peptide (Figure 6). These results provide insight into why FPS20 does not detect the DO-1-non-reactive (i.e. Ser^{20}-phosphorylated) p53 in breast cancers (Figure 9): a double phosphorylation at Thr^{18} and Ser^{20} would block the FPS20 epitope but would be tolerated by FPT18 (Figure 6). These results also suggest why FPS20 can bind to p53 expressed in insect cells: the p53 is only weakly reactive with FPT18 (Figure 7) and is therefore not heavily phosphorylated at the Thr^{18} site, permitting FPS20 binding to its phospho-epitope.

The biochemical significance of Ser^{20} and Thr^{18} phosphorylation of human p53 seems to be the ability to regulate p53 protein–protein interactions, with Thr^{18} phosphorylation showing the most substantial inhibition of mdm2 protein binding (Figures 4C and 4D). Mutagenesis of Leu^{14} or Phe^{19} as well as other amino acids within BOX-I (Leu^{22} and Trp^{23}) has been shown to decrease mdm2 protein binding to p53 [44]. In fact, recent observations that p53 is predominantly DO-1 non-reactive (i.e. Ser^{20}-phosphorylated) in human cancers (Figure 9) and in normal proliferating human fibroblasts [36] suggests that the Ser^{20} kinase pathway might be associated with proliferation pathways. Consistent with this is the observation that the damage induced by ^{32}P labelling of cells results in dephosphorylation at the DO-1 epitope and induces a p53-dependent growth arrest [36]. Therefore the most likely role for Ser^{20} phosphorylation in proliferating cells might be to decrease the rate of mdm2-dependent degradation of p53 and yield a stable, inactive form of p53 that can be activated post-translationally without the need for p53 synthesis.

Although it would be natural to suspect that these BOX-I phosphorylation sites might control the rate of degradation of p53 via inhibition of mdm2 protein function, other protein–protein interactions might also be affected. For example, BOX-I mutant forms of p53 (Leu^{22} and Trp^{23} substitutions) that fail to bind to mdm2 protein do not interact efficiently with RNA polymerase III either [45,46], highlighting a second pathway that might be affected by BOX-I phosphorylation. Thus the role for Ser^{20} phosphorylation in proliferating human cells might be not only to decrease the rate of mdm2-dependent degradation of p53 but also to permit efficient Pol III-dependent gene expression that is normally coupled to effective growth control.

Finally, although most current models indicate a role for p53 in a 'damage' response pathway, early-passage p53-deficient embryo fibroblasts (p53$-/-$) divide faster than normal embryo fibroblasts, achieve higher confluent densities and have a higher fraction of division-competent cells under conditions of low cell density [47]. In addition, proliferating p53-deficient embryo fibroblasts exhibit a markedly different pattern of protein synthesis from that of the wild-type equivalent [48]. Taken together, these results indicate for a role for p53 in regulating protein synthesis and cell growth in proliferating cells and suggest that Ser^{20} phosphorylation might be involved in stimulating p53 transcriptional activity at a set of genes distinct from those involved in the response to damage. In particular, the extensive phosphorylation of p53 at these new sites in breast cancers identifies a model with which to begin to dissect the regulation of p53 via Ser^{20} or Thr^{18} phosphorylation and highlights the need to develop clinically relevant models to study cancer as a biological disease [49].

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