Phosphorylation of Human p53 on Thr-55†

Andrea Gatti, Heng-Hong Li, Jolinda A. Traugh, and Xuan Liu*

Department of Biochemistry, University of California, Riverside, California 92521

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ABSTRACT: The pleiotropic function of p53 is believed to be greatly influenced by phosphorylation, and several sites on p53 are known to be targets for distinct protein kinases. In this study, we observed that affinity-purified p53 from overexpressing cells was phosphorylated by a co-purified protein kinase in vitro. To identify phosphorylation site(s), the resulting phosphorylated p53 protein was subjected to primary and secondary proteolytic cleavage, and phosphopeptides were fractionated by a two-dimensional peptide gel system. Phosphoamino acid analysis and manual Edman degradation of the isolated phosphopeptides enabled us to unequivocally identify Thr-55 as the major phosphorylation site on p53. Furthermore, comparative phosphopeptide mapping data suggest that DNA-PK is not the kinase responsible for this phosphorylation. Significantly, using a phospho-specific antibody for Thr-55, we have shown that Thr-55 is indeed phosphorylated in vivo. These data define Thr-55 as a novel phosphorylation site and for the first time show threonine phosphorylation of human p53.

p53 represents an integration point for a variety of signaling pathways, ultimately leading to diverse cellular responses, as growth arrest and apoptosis (*1*, *2*). The state of p53 activation is known to be dependent on a number of factors, one of which is represented by the interplay of those protein kinases and phosphatases which have p53 as a physiological substrate (*3*, *4*). Indeed, the phosphorylation state of p53 appears to function as a converging target for a wide array of protein kinase cascades. Hence, understanding how phosphorylation of individual sites contributes to the overall state of p53 is of significance.

Analysis of human p53 enabled the identification of a number of phosphorylation sites, most of which were determined under in vitro conditions. A list of relevant protein kinases and respectively targeted residues of human p53 (*3*) includes ATM on Ser-15, DNA-dependent protein kinase (DNA-PK)[1] on Ser-15 and Ser-37, cyclin-activating kinase on Ser-33, cyclin D-associated kinase on Ser-315, members of the protein kinase C family on Ser-371, Ser-376, and Ser-378, and casein kinase II on Ser-392. Furthermore, analysis of murine p53 allowed the identification of additional phosphorylation sites (*4*), some of which are conserved in the human sequence, while others do not correspond to any phosphorylatable residue in human p53. Belonging to the first group and being reportedly phosphorylated by a casein kinase I-like kinase are a couple of serine residues that correspond to Ser-6 and Ser-9 in human p53. Within the second group, Ser-34, Thr-73 and Thr-83 of the murine sequence were found phosphorylated by c-Jun N-terminal protein kinase and mitogen-activated protein kinase, respectively. It is important to note that the majority of phosphorylation sites in human and murine p53 have been identified upon treatment of purified p53 with exogenously added kinases, with only a few of them being characterized as targets of endogenous kinases.

Here, we focused on the phosphorylation of human p53 by endogenous protein kinases. In particular, we used the pAb 421 antibody to purify baculovirus-expressed or vaccinia virus-expressed human p53, followed by phosphorylation in vitro in the presence of [γ-^{32}P]ATP. To analyze p53 phosphorylation, we employed a recently developed methodology that previously enabled us to identify a number of protein phosphorylation sites (*5*, *6*). In this study, however, due to the limited amount of starting phosphoprotein (i.e., p53 being phosphorylated by the co-purified protein kinase), direct automated amino acid sequencing was ineffective, and the identification of the phosphorylation site required manual Edman sequencing of phosphopeptides resulting from primary endoproteinase digestion as well as secondary chemical cleavage. Using this approach, we identified Thr-55 as the major site phosphorylated by the associated protein kinase. This result was also supported by phosphoamino acid analysis. Importantly, using a phospho-specific antibody for Thr-55, we have shown that Thr-55 is indeed phosphorylated in vivo. In addition, phosphopeptide mapping analysis of p53 phosphorylated by exogenously added DNA-PK allowed us to rule out this prominent p53 kinase as responsible for Thr-55 phosphorylation.

EXPERIMENTAL PROCEDURES

Expression, Purification, and Phosphorylation of p53. Sf21 cells were infected with recombinant baculovirus expressing p53 (a gift from Dr. C. Prives, Columbia University) as described by Bargonetti et al. (*7*). One milliliter of nuclear extracts of infected cells (6 mg of protein/mL) was incubated

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* To whom correspondence should be addressed. Tel: 909-787-4350. E-mail: xuan.liu@ucr.edu.

[1] Abbreviations: BPNS-skatole, 2-(2′-nitrophenylsulfonyl)-3-methyl-3-bromoindolenine; DNA-PK, DNA-activated protein kinase; HA, hemagglutinin; IEF, isoelectric focusing; V8, endoproteinase Glu-C.

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for 3 h at 4 °C with 80 μL of packed protein A−Sepharose beads to which pAb 421, a monoclonal antibody specific to p53, was covalently linked. Beads were then washed 3 times with 0.4 M KCl D buffer (20 mM HEPES, pH 7.9, 20% glycerol, 0.2 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride) and once with 0.1 M KCl D buffer. After washing, p53 was eluted from beads with 80 μL of 421 epitope oligopeptide (KKGQSTSRHKK) at 1 mg/mL concentration in 0.1 M KCl D buffer.

To obtain purified p53 from human cells, HeLa cells were infected with recombinant vaccinia virus expressing a hemagglutinin-tagged p53 (HA-p53; *8*). p53 was purified from the nuclear extract of infected cells, either by binding to the matrix of monoclonal antibody (12CA5) specific for the HA tag prior to elution with the epitope peptide as described by Liu and Berk (*9*), or by binding to pAb 421 antibody and subsequent elution as described above. Purified proteins were subjected to SDS−PAGE and detected by silver staining.

In vitro phosphorylation was performed using 0.1−10 μg of purified p53 in 10−100 μL of phosphorylation buffer (25 mM HEPES, pH 7.9, 12.5 mM $MgCl_2$, 100 mM KCl, 0.1 mM EDTA) in the presence of 66 μM [^{32}P]ATP (30 Ci/mmol). When indicated, 300 μg of DNA-PK (Promega) and 2 μg of sheared salmon sperm DNA were included in the reaction mix, and phosphorylation was performed according to the manufacturer's instruction. The reaction was terminated after 30 min incubation at 30 °C by addition of 2× sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, and 100 mM dithiothreitol), prior to SDS−PAGE analysis on 10% polyacrylamide gel and autoradiography.

Phosphopeptide Mapping. After SDS−PAGE, proteins were electrotransferred onto nitrocellulose and stained with 0.5% (w/v) Ponceau in 1% acetic acid prior to detection via autoradiography. The relevant ^{32}P-labeled bands of nitrocellulose-bound p53 were excised and digested with endoproteinase Glu-C (V8, Boehringer Mannheim), essentially as described by Gatti and Traugh (*5*). Aliquots (20−80 μL) of the digested protein were subjected to a two-dimensional (2D) peptide PAGE, consisting of nondenaturing isoelectric focusing in gel tubes (native IEF) followed by electrophoresis on a 40% polyacrylamide alkaline slab gel (40% PAGE), prior to autoradiography. When indicated, phosphopeptides fractionated after primary V8 digestion were subjected to a cleanup procedure (see below) prior to a secondary, chemical cleavage with 2-(2′-nitrophenylsulfonyl)-3-methyl-3-bromoindolenine (BPNS-skatole). After BPNS-skatole cleavage (*10*), the resulting phosphopeptides were further fractionated upon one-dimensional gel electrophoresis either on 16% SDS−PAGE in Tris−Tricine buffer (*11*) or on 40% PAGE (*5*).

Manual and Automated Sequencing. The radioactive phosphopeptides were individually extracted from the 40% gel and subjected to a cleanup procedure through Sep-Pak cartridges (50 mg, Waters), as previously described (*5*). Each phosphopeptide was immobilized on a disk of arylamine membrane (Sequelon-AA, Millipore), as described by the supplier. After washing, the disk was routinely cut into two parts; approximately three-fourths of the disk was applied to an amino acid sequencer (Procise 492, Applied Biosystems), while the remaining one-fourth was subjected to manual Edman degradation as described by Sullivan and Wong (*12*).

Phosphoamino Acid Analysis. After binding to Sequelon-AA, individual phosphopeptides were treated with 6 N HCl for 1.5 h at 100 °C. Following acid hydrolysis, samples were subjected to thin-layer chromatography as previously described (*13*). Unlabeled standards were visualized by spraying the cellulose plates with ninhydrin, while ^{32}P-labeled amino acid residues were visualized by autoradiography with a PhosphorImager.

Generation of Mutant p53 Plasmid. Plasmid T55A, containing a point mutation at threonine 55 with conversion to alanine, was constructed using a PCR-based site-directed mutagenesis method. pCDNAp53 (*14*) was used as template DNA. The sequence of the oligonucleotides used to generate T55A is as follows: T55ATOP, 5′GAACAATGGTTCGCAGAAGACCCAGG3′; T55ABTM, 5′CCTGGGTCTTCTGCGAACCATTGTTC3′. The mutation was confirmed by sequencing analysis.

Analysis of Thr-55 Phosphorylation in Vivo. The p53 peptide spanning Thr-55 residue [acetyl-IEQWF(pT)EDPG-PDC-amide] was injected into rabbits, and phospho-specific antibody for Thr-55, pAb 202, was generated and purified by QCB (Hopkinton, MA).

To detect p53 phosphorylation in vivo, a p53-expressing plasmid (pcDNA-p53, *14*) was transfected into Sao-S2 cells using the calcium phosphate precipitation method. Whole cell extract was prepared 28 h after transfection by lysing the cells in a buffer containing 50 mM Tris-HCl (pH 8.0), 120 mM NaCl, 0.5% NP-40, 1 mM DTT, 2 μg/mL aprotinin, and 2 μg/mL leupeptin. The extract was cleared by centrifugation, and immunoprecipitation assay was performed using 202Ab, a purified anti-phosphoThr-55 antibody. The amount of p53 in the immunoprecipitates was determined by immunoblotting with DO-1, an anti-p53 antibody (Santa Cruz Biochemicals, CA).

Immunoprecipitation. Wild-type and mutant p53 RNAs were synthesized according to conditions recommended by the manufacturer (Promega). The mRNAs were translated in vitro for 1.5 h at 30 °C using the rabbit reticulocyte lysate in the presence of [^{35}S]methionine. Immunoprecipitation was performed using either pAb 202 or DO-1.

RESULTS

As many protein kinases are tightly associated with their substrates, we sought to determine if this was so for p53. Thus, p53 was purified with pAb 421 antibody from nuclear extracts of insect cells infected with a p53-expressing baculovirus (Figure 1A, lane 1). To detect p53 phosphorylation by potentially associated kinases, an in vitro phosphorylation assay was performed in the presence of [γ-^{32}P]-ATP (Figure 1B, lane 1). These results revealed that baculovirus-expressed and purified p53 was indeed phosphorylated by an endogenously associated protein kinase. Since p53 was purified from nuclear extracts and an extensive wash procedure was performed during purification (see Experimental Procedures for details), such a kinase activity is likely to be tightly associated with p53 in vivo. To determine whether this result was peculiar to insect cells and baculovirus infection, human p53 was also purified with pAb 421 antibody from nuclear extracts of HeLa cells infected with a p53-expressing recombinant vaccinia virus

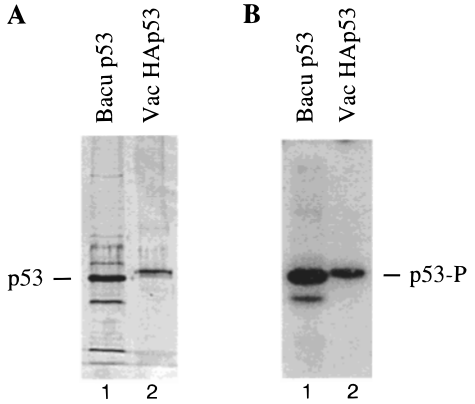

FIGURE 1: Phosphorylation of p53 by a tightly associated protein kinase. p53 purified with pAb 421 antibody from baculovirus-infected insect cells (lane 1) or from vaccinia virus-infected HeLa cells (lane 2) was either directly loaded onto 10% SDS−PAGE and silver stained (panel A) or subjected to in vitro phosphorylation prior to electrophoresis and autoradiography (panel B).

(Figure 1A, lane 2). As with the baculovirus-expressed form, p53 isolated from human cells was found to undergo phosphorylation in vitro (Figure 1B, lane 2). To rule out the possibility that p53 phosphorylation was due to the interaction of the kinase with pAb 421 antibody during the purification, we also carried out the in vitro phosphorylation of HA-tagged p53 purified with 12CA5 antibody, and a similar level of phosphorylation was observed (data not shown). Taken together, these data suggested that a kinase activity is tightly associated with human p53 in vivo and is responsible for its phosphorylation in vitro.

Next, we searched for the resulting phosphorylation site-(s) on p53. For this purpose, baculovirus-expressed human p53 was purified with pAb 421 and subjected to in vitro phosphorylation in the presence of [^{32}P]ATP. When the phosphorylated p53 protein was digested with V8 and analyzed by two-dimensional (2-D) phosphopeptide mapping, four radiolabeled spots were detected, and assigned numbers 1, 2, 3, and 4, according to the increasing size of the corresponding phosphopeptides (Figure 2A).

As spot 2 is the major radiolabeled signal of the resulting 2-D map of V8-digested p53, we focused on the corresponding phosphopeptide (phosphopeptide 2) to sequence its phoshorylation site(s). After extraction from the 2-D peptide gel, phosphopeptide 2 was subjected to cleanup, bound to Sequelon-AA, and subjected to automated and manual amino acid sequencing. The amount of the isolated peptide was not sufficiently pure for an unequivocal identification by the amino acid sequencer (data not shown). Manual Edman degradation (Figure 2B), however, showed a major release of radioactivity after the fourth cycle, thus indicating that the only phosphorylated amino acid is located four residues downstream (4+) from a V8-cleavage site (presumably a glutamic acid). Notably, within the five possible V8 phosphopeptides that exhibit a phosphorylatable residue in position 4+ of a glutamic acid, four have serine residues (Ser-6, Ser-15, Ser-362, and Ser-392) and one has a threonine residue (Thr-55) as a potential target site of phosphorylation (Figure 2B). This prompted us to carry out a phosphoamino acid analysis of phosphopeptide 2. As shown in Figure 2C, the predominant ^{32}P-labeled residue of phosphopeptide 2 is threonine. Taken together, these results suggested that

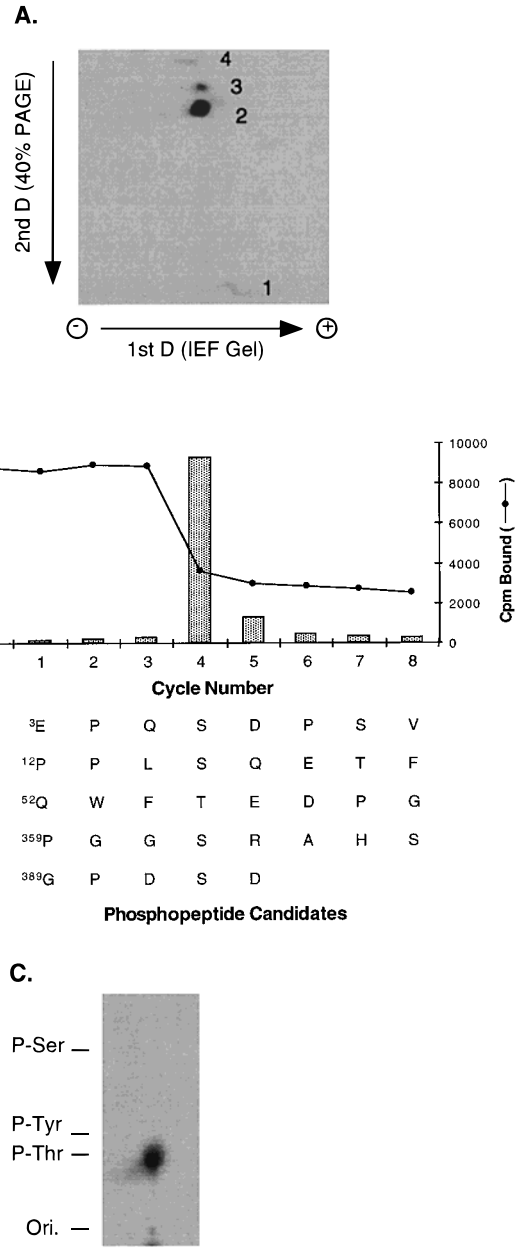

FIGURE 2: Identification of Thr-55 as the major phosphorylation site. (A) Two-dimensional gel separation of V8-digested p53 phosphopeptides. Baculovirus-expressed p53 was purified with pAb 421 antibody, and in vitro phosphorylation was performed in the presence of [^{32}P]ATP. After separation by 10% SDS−PAGE and transfer onto nitrocellulose, p53 was digested with V8, and the resulting phosphopeptides were fractionated by 2-D peptide PAGE. The autoradiogram of the second-dimensional gel is shown. (B) Manual amino acid sequencing of phosphopeptide 2. After isolation from 2-D peptide PAGE, phosphopeptide 2 was purified and bound to a Sequelon-AA disk prior to manual sequencing. The amounts of ^{32}P released and bound to the disk after each cycle of Edman degradation are indicated, together with the p53 sequences of possible candidates for the V8 phosphopeptide. (C) Phosphoamino acid analysis of phosphopeptide 2. After binding of phosphopeptide 2 to Sequelon-AA, the disk was subjected to acid hydrolysis, and the released radioactivity was resolved by thin-layer chromatography. Migration of standard phospho residues was assessed by spraying the cellulose plates with ninhydrin.

phosphopeptide 2 consists of the V8 peptide starting with Gln-52 and being phosphorylated on Thr-55.

To verify this conclusion, we carried out a secondary, chemical cleavage of phosphopeptide 2 with BPNS-skatole

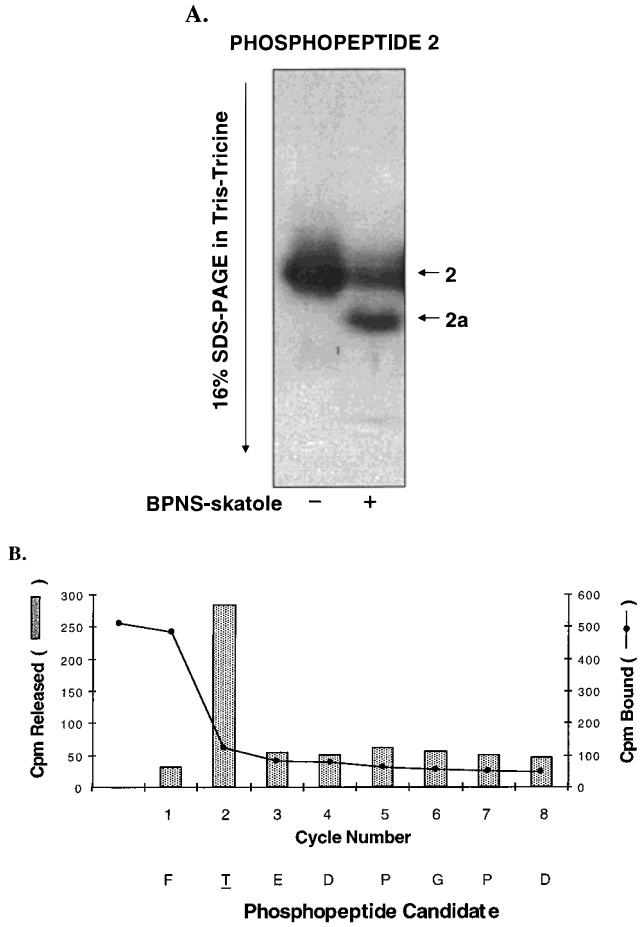

FIGURE 3: Analysis of Thr-55 phosphorylation upon secondary cleavage with BPNS-skatole. (A) Cleavage of phosphopeptide 2 by BPNS-skatole. An aliquot of phosphopeptide 2 isolated from 2-D peptide PAGE was subjected to secondary cleavage with BPNS-skatole, prior to 16% SDS−PAGE in Tris/Tricine buffer (*11*). The autoradiogram of the gel with the BPNS-skatole-dependent generation of phosphopeptide 2a is shown. (B) Manual amino acid sequencing of phosphopeptide 2a. Phosphopeptide 2a resulting from cleavage of phosphopeptide 2 with BPNS-skatole was isolated from one-dimensional 40% PAGE, purified, and bound to the Sequelon-AA disk, prior to manual Edman degradation. The amounts of ^{32}P released and bound to the disk after each cycle are indicated, together with the sequence of the only possible phosphopeptide candidate.

(Figure 3), an agent that exclusively cleaves at the C-terminus of tryptophan residues (*10*). To accomplish that, we isolated phosphopeptide 2 from V8-digested p53 and subjected it to a cleanup via Sep-Pak, prior to treatment with BPNS-skatole. To confirm the occurrence of the secondary cleavage, an aliquot of the reaction mix was analyzed by one-dimensional 16% SDS−PAGE in Tris−Tricine buffer (*11*). Despite an incomplete cleavage, generation of a smaller phosphopeptide (designated 2a) in the BPNS-skatole-treated sample (Figure 3A, lane 2) was clearly evident. To isolate phosphopeptide 2a for manual sequence analysis, we employed the same experimental procedures previously described, except that the BPNS-skatole-treated sample was fractionated by 40% PAGE. The data from manual Edman degradation of phosphopeptide 2a revealed a major release of radioactivity after the second cycle (Figure 3B), which indicated that the only phosphorylation site was in position 2+ from a tryptophan residue. Consequently, the phosphorylation on Thr-55 would account for these observations as the Thr-55-containing peptide is the only one among the five V8 phosphopeptides (Figure 2B) which is capable of generating a smaller peptide phosphorylated at position 2+ followed by BPNS-skatole cleavage. This was entirely consistent with the previous results from phosphoamino acid analysis. Additional manual sequencing, phosphoamino acid analysis, and chemical cleavage with BPNS-skatole (data not shown) indicated that the phosphopeptides corresponding to spots 3 and 4 of V8-digested p53 (shown in Figure 2A) shared with phosphopeptide 2 the same phosphorylation site. Of note, neither of these phosphopeptides is shorter than 20 amino acid residues, as assessed by comparative phosphopeptide mapping with phosphopeptides of known sizes.

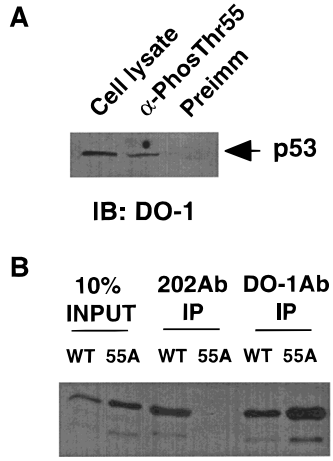

FIGURE 4: Thr-55 phosphorylation in vivo. (A) Sao-S2 cells were transfected with a p53 expression vector (pcDNA-p53), and the whole cell extract was subjected to immunoprecipitation analysis with pAb 202 to determine Thr-55 phosphorylation. The amount of p53 in the immunoprecipitates was determined by immunoblotting with an anti-p53 antibody (DO-1) and compared to the signal detected from direct immunoblotting of whole cell extract. (B) The specificity of pAb 202 as anti-phosphoThr-55 antibody to p53 was determined by immunoprecipitation analysis using ^{35}S-labeled wild-type p53 and mutant p53 (55A).

To test the possibility that Thr-55 is phosphorylated in human cells, we generated a phospho-specific antibody for Thr-55, pAb 202. In particular, a peptide was synthesized comprising 13 amino acids, with the central residue containing phopshorylated Thr-55 and used as antigen to generate pAb 202. The specificity of this antisera was first confirmed by ELISA using phosphorylated and unphosphorylated peptides (data not shown), and then by immunoprecipitation analysis of wild-type and substitution mutant of p53 (Figure 4B). Using this antibody, we investigated the Thr-55 phosphorylation status of p53 in human cells. For this purpose, Sao-S2 cells were transiently transfected with a p53 expression vector. Because pAb 202 preferentially recognizes p53 under native rather than denaturing conditions (data not shown), we first immunoprecipitated p53 with pAb 202 and then determined the presence of Thr-55-phosphorylated p53 in the immunoprecipitates by immunoblotting with the anti-p53 antibody DO-1 (Figure 4A). This result shows that Thr-55 is indeed phosphorylated in human cells.

Having identified Thr-55 as the major site phosphorylated by the p53-associated protein kinase, we tested the effect of DNA-PK on the phosphorylation of p53 (Figure 5), as DNA-PK is known to target multiple residues of the N-terminal region of p53 (*15, 16*). When p53 was incubated with DNA-

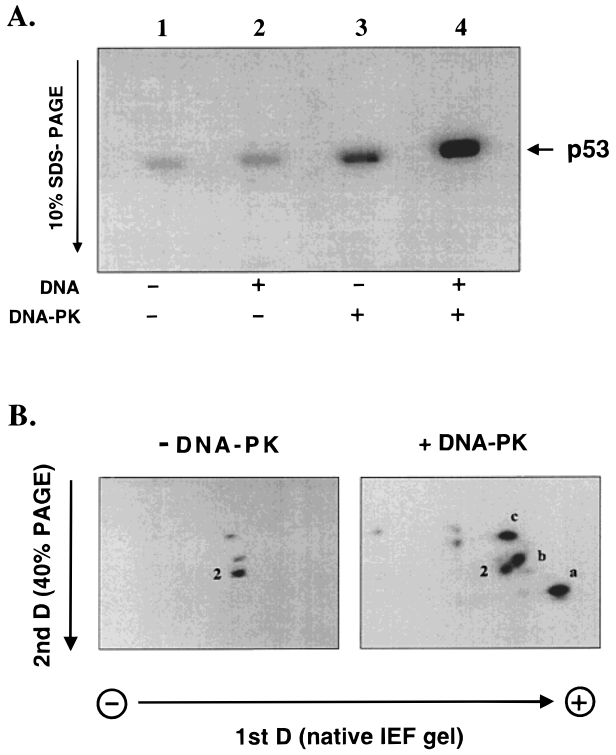

FIGURE 5: Phosphorylation of p53 by DNA-PK and p53-associated protein kinase. (A) SDS−PAGE and resulting autoradiogram of p53 phosphorylated with or without DNA-PK. Baculovirus-expressed p53 was purified with pAb 421 antibody, prior to in vitro phosphorylation in the absence (lanes 1 and 2) or in the presence of exogenously added DNA-PK (lanes 3 and 4), with (lanes 2 and 4) or without (lanes 1 and 3) DNA. (B) Comparative phosphopeptide mapping of p53. After in vitro phosphorylation in the absence (left panel) or in the presence of DNA-PK and DNA (right panel), p53 was digested with V8, and the resulting phosphopeptides were fractionated upon 2-D peptide PAGE.

PK in the presence of DNA, a substantial increase in phosphorylation was observed (Figure 5A, lanes 3 and 4), indicating that the purified p53 could be efficiently phosphorylated by active DNA-PK under the assay conditions. In contrast, the presence of DNA did not significantly enhance p53 phosphorylation by the p53-associated protein kinase (Figure 5A, compare lanes 1 and 2), indicating that such a kinase is unlikely to be DNA-PK.

A 2-D phosphopeptide map of p53 phosphorylated in the presence of active DNA-PK showed a pattern of phosphorylation (Figure 5B, right panel) which differed from that induced by the p53-associated kinase alone (Figure 5B, left panel). Notably, spot a is the most abundant radioactive signal of the 2-D peptide map of DNA-PK-treated p53 and is absent from the map of p53 phosphorylated in the absence of DNA-PK. Furthermore, spot 2, which represents the peptide with phosphorylated Thr-55, did not accumulate upon treatment with DNA-PK. Given the distinctive phosphorylation detected upon addition of DNA-PK, we concluded that DNA-PK is not responsible for Thr-55 phosphorylation.

DISCUSSION

Posttranslational modifications such as protein phosphorylation have been shown to play an important role in p53 regulation. In this paper, we identified Thr-55 as a novel phosphorylation site targeted by a native protein kinase which was found to be tightly associated with human p53. Importantly, using a phospho-specific antibody for Thr-55, we have shown that Thr-55 is indeed phosphorylated in vivo. Furthermore, we concluded that DNA-PK, which is known to phosphorylate several residues within the N-terminal region of p53, is not responsible for Thr-55 phosphorylation in vitro.

Several proteins expressed in baculovirus-infected insect cells are known to be functionally indistinguishable from the corresponding native proteins. In particular, human p53 forms expressed in baculovirus-infected insect cells and in human cells were found to exhibit a similar phosphorylation pattern, as assessed by conventional 2-D PAGE analysis (*17*). Consistent with this observation, our results showed that the p53-associated protein kinase is responsible for phosphorylation of baculovirus and vaccinia virus-expressed human p53, suggesting that phosphorylation on Thr-55 may be of physiological relevance. To support this view, we have shown that Thr-55 is indeed phosphorylated in vivo. This result, together with the finding that the kinase remains tightly associated with p53 during purification, suggests that Thr-55 phosphorylation is likely to play a significant role in vivo.

Two explanations may account for the limited extent of p53 phosphorylation detected in vitro by the p53-associated protein kinase, as compared to phosphorylation by DNA-PK. First, the target site (i.e., Thr-55) of the purified p53 may be already endogenously phosphorylated in baculovirus-infected insect cells to a significant level, thus limiting the incorporation of labeled phosphate. In fact, our data from Sao-S2 cells (Figure 4B) and from vaccinia virus-expressed p53 (data not shown) strongly support this view. Second, the amount or the function of the co-purified p53-associated kinase may not be sufficient to phosphorylate the overexpressed p53 with high stoichiometry.

Of note, a recent study showed that a combined mutation of all potential phosphorylation sites of p53 (Ser-6, Ser-9, Ser-15, Thr-18, Ser-20, Ser-33, Ser-37, Ser-315, Ser-371, Ser-376, Ser-378, and Ser-392) resulted in no detectable phosphorylation in vivo, suggesting that the major phosphorylation sites are likely to be included within these residues (*18*). The discrepancy with our results may be explained by at least two lines of reasoning. First, phosphorylation on Thr-55 may depend on a particular conformation that is disrupted by mutating a combination of N-terminal and C-terminal sites. Second, the assay conditions reported by Ashcroft et al. (*18*) might not be optimal for detecting p53-associated protein kinase activity.

As described above, most of the p53 phosphorylation sites were identified in vitro, and likewise most of the interactions of p53 with protein kinases have been established by in vitro assays. In contrast, the analysis of in vivo phosphorylation of p53 relies on the use of phosphorylation site-specific antibody (*3*). Consequently, there is no direct proof that the kinases used in vitro are indeed responsible for the phosphorylation site(s) identified in vivo. In this study, we focused on the activity of a p53-associated protein kinase and searched for the resulting phosphorylation site(s) on p53. This approach, therefore, may closely reflect phosphorylation by the associated protein kinase in vivo. Further analysis of the Thr-55 phosphorylation should help to characterize the protein kinase and ultimately the functional impact of such a phosphorylation on p53.

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