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Human DNA-Activated Protein Kinase Phosphorylates Serines 15 and 37 in the Amino-Terminal Transactivation Domain of Human p53

SUSAN P. LEES-MILLER,[1]† KAZUYASU SAKAGUCHI,[2] STEPHEN J. ULLRICH,[2] ETTORE APPELLA,[2] AND CARL W. ANDERSON[1]*

Biology Department, Brookhaven National Laboratory, Upton, New York 11973,[1] and Laboratory of Cell Biology, National Cancer Institute, Bethesda, Maryland 20892[2]

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Human DNA-PK is a nuclear, serine/threonine protein kinase that, when activated by DNA, phosphorylates several DNA-binding substrates, including the tumor suppressor protein p53. To identify which p53 residues are phosphorylated, we examined DNA-PK's ability to phosphorylate synthetic peptides corresponding to human p53 sequences. Serines 15 and 37 in the amino-terminal transactivation domain of human p53, and serines 7 and 18 of mouse p53, were phosphorylated by DNA-PK in the context of synthetic peptides. Other serines in these p53 peptides, and serines in other p53 peptides, including peptides containing the serine 315 $p34^{cdc2}$ site and the serine 392 casein kinase II site, were not recognized by DNA-PK or were phosphorylated less efficiently. Phosphorylation of the conserved serine 15 in human p53 peptides depended on the presence of an adjacent glutamine, and phosphorylation was inhibited by the presence of a nearby lysine. Phosphorylation of recombinant wild-type mouse p53 was inhibited at high DNA concentrations, suggesting that DNA-PK may phosphorylate p53 only when both are bound to DNA at nearby sites. Our study suggests that DNA-PK may have a role in regulating cell growth and indicates how phosphorylation of serine 15 in DNA-bound p53 could alter p53 function.

Many cellular functions, including cell growth, cell cycle progression, and transcription, are controlled and coordinated by phosphorylation and dephosphorylation of specific protein factors. Kinases implicated in regulating these processes include the CDK family of kinases, casein kinase II, and casein kinase I (19, 52, 53). We (26) and Carter et al. (6) recently described a nuclear serine/threonine protein kinase, DNA-PK, that requires double-stranded DNA for activity. In vitro DNA-PK phosphorylates several DNA-binding proteins, including the simian virus 40 (SV40) large tumor antigen (TAg), the tumor suppressor gene product p53, and several transcription factors, e.g., Sp1, Oct-1, Fos, and serum response factor (reviewed in reference 25). These observations suggest that DNA-PK may play a role in controlling gene expression and cell growth.

The p53 protein is an important regulator of cell growth (reviewed in references 27 and 54). The p53 tumor suppressor gene is altered or deleted in most solid human tumors (57), and expression of a functional wild-type p53 gene in cells with missing or mutant p53 genes arrests their growth (3, 8, 14, 35). Although the biochemical function(s) of p53 is still unclear, p53 binds to DNA (4, 21, 22, 48), and its amino-terminal region functions as a transcription transactivator when coupled to a heterologous DNA binding domain (15, 40, 41). Induction of wild-type p53 expression inhibits transcription of the proliferating cell nuclear antigen gene, a component of the DNA replication complex (34, 50). These observations suggest that p53 may function as a transcription modulator of genes required for cell cycle progression from G_1 to S phase (28). Like SV40 TAg, p53 is phosphorylated at amino-terminal and carboxy-terminal sites in vivo (30, 43, 51, 56, 58) and by several protein kinases, including DNA-PK (26, 58), in vitro (1, 5, 31, 32, 36).

Previously we identified two sites in the heat shock protein hsp90 (24) and four sites in SV40 TAg (9) that can be phosphorylated in vitro by human DNA-PK. Each site consisted of a threonine or serine that is followed immediately by glutamine; no other common sequence features were noted among these six sites. On the basis of these findings, we suggested that -SQ- and -TQ- might represent substrate sequence motifs recognized by DNA-PK (9, 25). Two serines in the amino-terminal transactivation region of mammalian p53s are followed immediately by glutamine; thus, phosphorylation of these serines, perhaps by DNA-PK, might influence the ability of p53 to activate or inhibit transcription and/or to regulate cell growth.

In this study, we have analyzed the ability of DNA-PK to phosphorylate peptides corresponding to sequences surrounding the six serines in human p53 that are adjacent to glutamine. This approach was chosen because the half-life of wild-type p53 in vivo is short, making direct sequence analysis of p53 phosphorylation sites difficult. One DNA-PK site, Ser-15, was analyzed further by synthesizing additional peptides with small sequence changes; this analysis showed that an adjacent glutamine was important for site recognition by DNA-PK. Analysis of the effect of DNA concentration on the ability of DNA-PK to phosphorylate p53 further suggested that DNA binding also may be required for efficient p53 phosphorylation. We suggest that DNA-PK may phosphorylate serines in the transactivation domain, thereby altering the properties of DNA-bound p53 with

* Corresponding author.
† Present address: Department of Sciences, University of Calgary, Calgary, Canada T2N 1N4.

TABLE 1. Synthetic p53 peptide substrates

Peptide no.	p53 residues[a]	Peptide sequence[b]	K_m (μM)	V_{max} (pmol/min/μg of DNA-PK)	PO_4 incorporated (pmol/min/μg of DNA-PK)[c]
1	1–28	MEEPQSDPSVEPPLSQETFSDLWKLLPE	ND[d]	ND	ND
2	4–31	MEESQSDISLELPLSQETFSGLWKLLPP	ND	ND	ND
3	4–13	MEESQSDISLELP-*YKK*	650	390	94
4	1–24	MEEPQSDPSVEPPLSQETFSDLWK-*K*	210	90	44
5	1–24[e]	MEEPQSDPSVEPPL**A**QETFSDLWK-*K*	—[f]	—	5.3
6	29–44	NNVLSPLPSQAMDDLM-*KK*	470	470	102
7	92–108	PLSSSVPSQKTYQGSYG-*KK*	—	—	3
8	160–175	MAIYKQSQHMTEVVRR	740	200	44
9	306–327	ALPNNTSSSPQPKKKPLDGEY	—	—	0.6
10	371–385	SKKGQSTSRHKKLMF	—	—	0
11	380–393	HKKLMFKTEGPDSD	—	—	0
12	11–24	EPPLSQETFSDLWK-*K*	350	360	130
13	11–19	EPPLSQETF-*KK*	—	—	15
14	11–20	EPPLSQETFSD-*KK*	—	—	12
15	11–24[e]	EPPLSQE**A**F**A**DLWK-*K*	760	380	83
16	11–24[e]	EPPLSQE**A**F**A**DL**L**K-*K*	560	160	36
17	11–24[e]	EPPLS**E**E**A**F**A**DLWK-*K*	—	—	0.4
18	11–24[e]	EPPLS**N**E**A**F**A**DLWK-*K*	—	—	0.1
19	11–24[e]	EPPLS**E**Q**A**F**A**DLWK-*K*	—	—	0
20	11–24[e]	EPPLSQ**K**A**A**F**A**DLWK-*K*	1,000	70	14[g]
21	11–24[e]	EPPQS**L**E**A**F**A**DLWK-*K*	420	310	99
22	11–24[e]	EPPQSQE**A**F**A**DLWK-*K*	290	390	161
23	11–24[e]	EPPL**T**QE**A**F**A**DLWK-*K*	670	460	116

[a] The amino acid positions corresponding to the first and last p53 residues in each peptide are given; all sequences correspond to human p53 except peptides 2 and 3, which correspond to mouse p53.
[b] The sequence is given in the one-letter amino acid code; serines (S) followed by glutamine (Q) are shown in bold type; changes from the wild-type sequence of p53 are underlined; lysines (*K*) added at the carboxy terminus are separated by a dash.
[c] Phosphate incorporated for each peptide at 200 μM.
[d] ND, not determined.
[e] Differs from the wild-type sequence.
[f] —, an appropriate concentration range for determining the indicated value was not achieved.
[g] Obtained at 300 μM peptide.

respect to interactions that control cell cycle progression in vivo.

MATERIALS AND METHODS

DNA-PK, mouse p53, hsp90, and oligonucleotides. DNA-PK was purified from HeLa cells as described previously (26) except that gradient elution from Q-Sepharose Fast Flow was substituted for DEAE-Sepharose chromatography, and gradient elution from S-Sepharose Fast Flow (Pharmacia) was substituted for double-stranded DNA-cellulose chromatography. This procedure achieved a similar DNA-PK specific activity but yielded more activity per cell.

To examine cells for DNA-PK content, extracts were prepared by freeze-thaw lysis of cells harvested from five nearly confluent 9-cm-diameter plates (23). Lysates in low-salt buffer were centrifuged at 10,000 × *g*, and pellets were resuspended in 0.5 M KCl–25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.5)–10 mM $MgCl_2$–1 mM dithiothreitol with sonication. The P10 suspensions were centrifuged at 10,000 × *g* for 10 min, and the supernatants (P10S) were diluted with buffer lacking KCl to give a volume equal to that of the original supernatant (S10). Protein concentrations, determined by using the Bio-Rad assay, were as follows: HeLa S10, 3 mg/ml; HeLa P10S, 1 mg/ml; T98G S10, 3.5 mg/ml; T98G P10S, 1 mg/ml; L-929 S10, 1 mg/ml; and L-929 P10S, 0.3 mg/ml. T98G (ATCC CRL 1690) is a human glioblastoma cell line; L-929 (ATCC CCL 1) was derived from connective tissue of the NCTC 2071 mouse.

Wild-type mouse p53 was purified by immunoaffinity chromatography from Sf9 insect cells infected with the recombinant virus NPVp53 (49). Human hsp90 was purified as described previously (23). Alternating $(dG\text{-}dC)_n$ oligonucleotides of defined length were synthesized on a Milligen/Biosearch 8750 oligonucleotide synthesizer and purified by using an RP-1 cartridge (Glen Research, Inc.); before use, the oligonucleotides were dissolved at 1 mg/ml in 10 mM Tris-HCl (pH 8)–1 mM EDTA–50 NaCl, heated briefly at 100°C, and allowed to cool slowly overnight.

Synthetic peptides. Protected peptide chains were assembled by the stepwise solid-phase method, using an Applied Biosystems 430A automated synthesizer. After removal of the *N*-tert-butoxycarbonyl (tBoc) group with trifluoroacetic acid, cleavage of the peptide from the resin and removal of side chain protecting groups were accomplished with HF in the presence of *p*-cresol. The crude peptide was extracted with 5% acetic acid and chromatographed on Sephadex G-25. Further purification was by reverse-phase high-pressure liquid chromatography (HPLC) on a Vydac C_4 column in 0.05% trifluoroacetic acid–water–acetonitrile. Peptide 16 (Table 1) and the synthetic casein kinase I substrate DDDEESITRR (2) were obtained from Multiple Peptide Systems, Inc. The S6 kinase substrate RRLSSLRA was purchased from Bachem California, Inc.; the casein kinase II substrate RREEETEEE was provided by E. Krebs, University of Washington, Seattle. The purity of each peptide, as

judged from its reverse-phase HPLC profile, was greater than 95%. Purified peptides were dissolved in water and stored at −20°C. Peptide concentrations were determined by quantitative amino acid analysis after hydrolysis for 24 h at 105°C in 6 N HCl.

Phosphorylation reactions. Phosphorylation reactions were performed in a 20- or 40-μl final volume as described previously (9, 26) except that for determinations of kinetic constants, the ATP concentration was increased to 0.5 mM. Incubations were at 30°C.

Assay of synthetic peptides. The transfer of [^{32}P]phosphate to synthetic peptides was quantitated by the phosphocellulose paper binding method (7, 18). Kinase reactions were stopped by adding an equal volume of 30% acetic acid, and portions of the acidified reaction were spotted on 2- by 2-cm squares of P-81 paper (Whatman). Squares were washed four times for 5 min each in 15% acetic acid. The P-81 squares were then transferred to scintillation vials containing 3.5 ml of water, and bound radioactivity was determined from the Cerenkov radiation. Enzyme activity was calculated from triplicate determinations, the specific activity of the ATP, and enzyme concentration as determined by the Bio-Rad dye-binding assay, using bovine serum albumin as the standard. Blank values, obtained without added peptide, gave less than 0.5 pmol of bound phosphate; these blanks were subtracted from assay values with peptides. Control assays indicated that at least 80% of spotted phosphopeptides bound to P-81 squares and that the fraction bound was linear over the concentration range of peptide used. K_m and V_{max} values were calculated from plots of $1/[S]$ versus $1/V$; values reported are averages of at least two independent determinations.

Phosphorylation of peptides that lacked positively charged residues was estimated from gel filtration profiles. Peptides were separated from [^{32}P]ATP by passage through a column (1.5 by 12 cm) of Bio-Gel P4 in 10% acetic acid.

Phosphorylation site identification. Radiochemical sequence analysis was performed by using a Beckman 890M spinning-cup sequencer as described previously (24). The conversion of peptidylphosphoserine to *S*-ethylcysteine and identification of phenylthiohydantoin (PTH)-*S*-ethylcysteine by chemical microsequence analysis using an Applied Biosystems 470 sequencer and 120A PTH-amino acid analyzer has been described elsewhere (23).

RESULTS

p53 peptides phosphorylated by DNA-PK. Previously, we showed that purified SV40 TAg and mouse p53 protein were phosphorylated in vitro on serines by the human DNA-activated protein kinase, DNA-PK (26). We found recently that the 14-residue synthetic peptide corresponding to TAg Thr-661 to Pro-674 was phosphorylated primarily on the serine equivalent to TAg residue 665 by DNA-PK (9). Each serine or threonine that is phosphorylated by DNA-PK in vitro is followed immediately by the amino acid glutamine (25). These observations suggested that similar sequences might be phosphorylated in p53 and that these sites might be recognized if presented in the context of appropriate synthetic peptides.

Wild-type human p53 has six serines (but no threonines) that are immediately preceded or followed by glutamine: serines 6 (-QSD-), 15 (-LSQ-), 37 (-PSQ-), 99 (-PSQ-), 166 (-QSQ-), and 376 (-QST-). To determine whether these sites could be phosphorylated by DNA-PK, we synthesized five peptides corresponding to sequences surrounding these

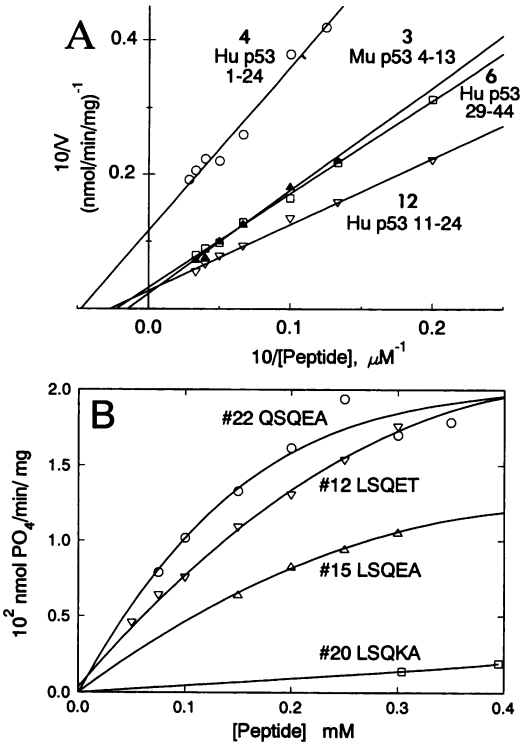

FIG. 1. Phosphorylation of synthetic p53 peptides by DNA-PK. (A) Reciprocal plots for human (Hu) and mouse (Mu) peptides 3, 4, 6, and 12 (for sequences, see Table 1). K_m and V_{max} values were calculated from the coefficients of the linear regression fits. (B) Rates of peptide phosphorylation (picomoles of phosphate incorporated per minute per microgram of kinase preparation) as a function of peptide concentration for wild-type human p53 peptide 12 and three variants, peptides 15, 20, and 22 (for sequences, see Table 1). Reactions were for 10 min at 30°C.

serines, Met-1–Lys-24, Asn-29–Met-44, Pro-92–Gly-108, Met-160–Arg-175, and Lys-372–Lys-381 (peptides 4, 6, 7, 8, and 10; Table 1). In addition, we made a peptide that contained the p34^{cdc2} kinase site at serine 312 (peptide 9) and a peptide that contained the casein kinase II site, serine 392 (peptide 11; Table 1). For most peptides that had fewer than two arginine or lysine residues, we added one or two lysines at the carboxy terminus to facilitate peptide binding to phosphocellulose paper (18). The ability of each peptide to be phosphorylated by calf thymus DNA-activated DNA-PK was then assayed. Only the peptides containing serines 6 and 15, serine 37, and serine 166 were phosphorylated well (Table 1 and Fig. 1). The K_m for peptide 4, Met-1–Lys-24–*Lys* (the lysine added at the carboxy terminus is italicized) was 210 μM, and the V_{max} was 90 pmol/min/μg (Fig. 1A). The K_m for peptide 6 (Table 1), Asn-29–Met-44–*LysLys*, was twice as high, but its V_{max} was five times greater. Peptide 8, Met-160–Arg-175, was a slightly poorer substrate than either N-terminal peptide. At 200 μM, the rate of phosphorylation of peptide 7, Pro-92–[Ser-99]–Gly-108–*LysLys*, was less than 7% of the rate at which the N-terminal peptide 4 was phosphorylated. The other three peptides (peptides 9, 10, and 11; Table 1) were phosphorylated at a negligible rate by DNA-PK. Phosphorylation was completely dependent on the presence of added dsDNA (data not shown).

DNA-PK recognizes serines next to glutamine in p53 peptides. Peptide 8, Met-160–Arg-175, has only one serine; thus,

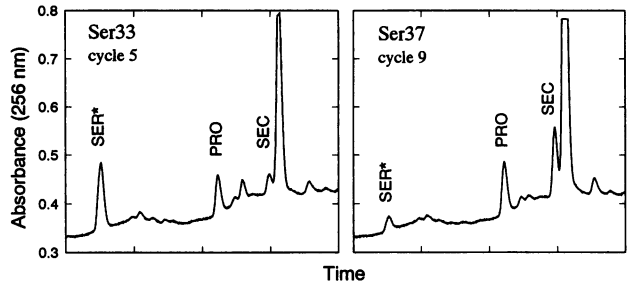

FIG. 2. Identification of the site phosphorylated by DNA-PK in the p53 synthetic peptide Asn-29–Met-44. Peptide 6 (Table 1), Asn-29–Met-44–*LysLys*, was incubated with DNA-PK as described in Materials and Methods, and the major phosphorylated product was isolated by reverse-phase HPLC. After conversion of phosphoserine to *S*-ethylcysteine, the peptide was sequenced; shown are portions of the PTH-amino acid analyses for the two sequencer cycles (5 and 9) for which serine was expected. These serines correspond to residues 33 and 37, respectively, of human p53. The major amino acid identified in cycle 9 was *S*-ethylcysteine (SEC); dehydroalanine (SER*), a by-product of serine, was the major product in cycle 5. The small amount of SEC in cycle 5 results primarily from a minor (10%) cleaved form of the peptide.

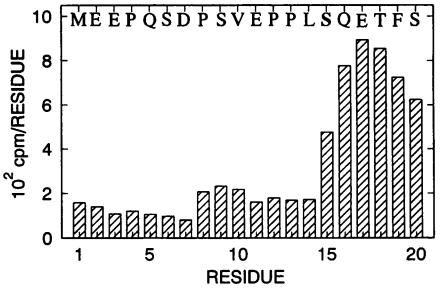

FIG. 3. Identification of the amino-terminal DNA-PK phosphorylation site of human p53. Peptide 1 (human p53 Met-1–Glu-28) (Table 1) was incubated with DNA-PK and desalted by chromatography through Bio-Gel P4. Approximately 75,000 cpm was applied to the spinning-cup sequencer. Shown is the total release of radioactivity after each Edman degradation cycle.

the serine equivalent to p53 residue 166 must be phosphorylated. Peptide 6, Asn-29–Met-44–*LysLys*, has two serines, equivalent to residues 33 and 37 of human p53. To determine which of these were phosphorylated, after incubation with DNA-PK, the peptide was isolated by reverse-phase HPLC, phosphoserine was converted to *S*-ethylcysteine, and the position of the *S*-ethylcysteine was determined by chemical microsequence analysis. Only the ninth residue, i.e., equivalent to serine 37, had a significant amount of PTH-*S*-ethylcysteine, indicating that this serine was phosphorylated by DNA-PK whereas the equivalent to serine 33 was not (Fig. 2).

The amino-terminal peptide, Met-1–Lys-24–*Lys*, has four serine residues. Radiochemical sequence analysis (Fig. 3) showed that the major site of phosphorylation was serine 15. To verify this result, we made a similar peptide with alanine at the position of serine 15. At 200 μM, peptide 5, Met-1–[Ala-15]–Lys-24–*Lys*, was phosphorylated at about 10% of the rate of the wild-type peptide sequence, confirming that serine 15 was the major site of phosphorylation in peptide 4 (Table 1). This conclusion was confirmed (Fig. 1B) with peptide 12, Glu-11–[Ser-15]–Lys-24–*Lys* (and other derivatives; see below), lacking serines 6 and 9. Peptide 12 was phosphorylated at over three times the rate of the Met-1–Lys-24–*Lys* wild-type peptide, but its K_m was 1.5 times higher (Fig. 1 and Table 1).

We also examined a peptide corresponding to mouse p53 residues Met-4 to Pro-31. Serine 7 of mouse p53 is at the position equivalent to proline 4 of human p53 (Fig. 4), and the glutamine at position 8 of mouse p53 is conserved among mammals (47). After phosphorylation, peptide 2 (Table 1) was digested with endoproteinase Glu-C, and the resulting fragments were separated by reverse-phase HPLC. Two major phosphopeptides were obtained, and sequence analyses indicated that one began at Met-1 and the other began at Leu-15 (data not shown). Each peptide was treated to convert phosphoserine to *S*-ethylcysteine; then they were resequenced. Figure 5 shows that *S*-ethylcysteine was present at the positions of Ser-7 and Ser-18 but not at the positions of Ser-9 and Ser-12. Mass spectrographic analysis of the uncleaved, phosphorylated peptide showed the presence of products with masses of 3,176, 3,256, 3,336, as expected for the unphosphorylated peptide and derivatives with one and two phosphates (data not shown). We have not examined the mouse sequence equivalent to peptide 6, Asn-29–Met-44–*LysLys*, that would contain serine 37 of mouse p53. Note that serine 37 of mouse p53 is followed by proline rather than glutamine (Fig. 4).

Recognition of serine 15 by DNA-PK requires the adjacent glutamine. To show directly whether the adjacent glutamine is important for recognition by DNA-PK, we made a series of peptides based on the sequence surrounding serine 15 of human p53; each peptide was then tested for substrate activity (Fig. 1B and Table 1). Starting with the peptide 12, Glu-11–[Ser-15]–Lys-24–*Lys*, we first tried to make a shorter peptide substrate by omitting residues 21 to 23 (Asp-Leu-Trp) or 20 to 23 (Ser-Asp-Leu-Trp). Neither of these peptides (peptides 13 and 14; Table 1) was phosphorylated well by DNA-PK, primarily because of an eightfold increase in K_m compared with the Glu-11–Lys-24–*Lys* peptide.

Next, keeping Glu-11–Lys-24–*Lys* as a base sequence, we changed individual residues. First, Thr-18 and Ser-20 were changed to alanine, leaving Ser-15 as the only phosphorylation site (peptide 15; Table 1). These changes produced only a twofold increase in K_m and no significant change in V_{max}. Therefore, we chose this sequence as a new base for further sequence changes. Changing Gln-16 to either glutamic acid (peptide 17) or asparagine (peptide 18) effectively eliminated the ability of the sequence to act as a DNA-PK substrate. Glutamic acid is the most frequent substitute for glutamine in related proteins (12).

To determine whether the position of glutamine is critical, we switched the positions of Gln-16 and Glu-17. This peptide (peptide 19) also had negligible substrate activity. In contrast, peptide 21, with the positions of Gln-16 and Leu-14 switched, was as good a substrate as the base peptide, suggesting that glutamine must be immediately adjacent to the phosphorylated residue, but it may either follow or precede that residue. Peptide 22, with Leu-14 changed to glutamine, i.e., where the serine was surrounded by glutamines, was only slightly better as a substrate (Fig. 1B). Peptide 23, with threonine substituted at residue 15, was a slightly better substrate than the base sequence (Fig. 1B); this result suggests that DNA-PK does not discriminate against threonine sites in substrates.

Peptide 20 has lysine substituted for Glu-17, but it has an intact -SQ- sequence. At first we were surprised that peptide 20 was a comparatively poor DNA-PK substrate (Fig. 1B);

Residue	1			5		9			15			20			25			30					33		37
Human	M	E	E	P	Q	S	D	P	S	V	E	P	P	L	S	Q	E	T	F	S	D	L	W	K	L	L	P	E	N	N	V	L	-	-	-	-	-	-	S	P	L	P	S	Q	A	M
Monkey	M	E	E	P	Q	S	D	P	S	I	E	P	P	L	S	Q	E	T	F	S	D	L	W	K	L	L	P	E	N	N	V	L	-	-	-	-	-	-	S	P	L	P	S	Q	A	V
Rat	M	E	D	S	Q	S	D	M	S	I	E	L	P	L	S	Q	E	T	F	S	C	L	W	K	L	L	P	P	D	D	I	L	P	T	T	A	T	G	S	P	N	S	M	E	D	L
Mouse	M	T	A	M	E	E	S	Q	S	D	I	S	L	E	L	P	L	S	Q	E	T	F	S	G	L	W	K	L	L	P	P	E	D	I	L	P	-	-	-	-	-	S	P	H	C	M	D	D	L
Residue	1					7					12					18						25				30				35						37		41											

FIG. 4. Alignment of the amino-terminal sequences for primate and rodent p53s. Translated cDNA sequences are shown; these were aligned manually. -SQ- sequences are in bold type. Residue numbers for the human and monkey sequences are shown at the top; mouse p53 residue numbers are given at the bottom. cDNA sequences are from Zakut-Houri et al. (59) (human), Rigaudy and Eckhart (42) (monkey), Soussi et al. (46) (rat), and Pennica et al. (38) (mouse).

however, this result suggested that basic residues near an -SQ- sequence may inhibit recognition by DNA-PK. The closeness of the carboxy-terminal lysines in peptides 13 and 14 to Ser-15 could also explain why these shorter peptides were ineffective substrates (Table 1). Peptides 5, 7, 9, 11, 13, 14, 17, 18, 19, and 20, i.e., all of the poorer substrate peptides, were assayed at concentrations of up to 250 μM with peptide 12 at 100 μM; none significantly inhibited the phosphorylation of peptide 12.

A second determinant for DNA-PK recognition is substrate binding to DNA. DNA-PK is activated by relatively low concentrations of DNA, and as was shown by using calf thymus DNA and hsp90, DNA-PK remains active at DNA concentrations well above the K_m for activation (26). This result was confirmed by using p53 synthetic peptides as DNA-PK substrates (data not shown) and several synthetic oligonucleotides made of alternating dG-dC. The rate of phosphorylation of synthetic peptide 15 (Table 1) was constant at oligonucleotide concentrations of between 5 and >200 μg/ml for dG-dC oligomers of chain length 16 or above (data not shown), and this rate was the same as that obtained with calf thymus DNA.

To determine whether DNA binding affected p53 phosphorylation by DNA-PK, we measured the rate of phosphorylation of recombinant wild-type mouse p53 as a function of DNA concentration. At low calf thymus DNA concentrations (0 to ~10 μg/ml), the initial rate of p53 phosphorylation increased sharply but then decreased as the DNA concentration was further increased; by 100 μg/ml, the rate of p53 phosphorylation had fallen to only ~25% of that at 5 μg/ml (Fig. 6). A similar dependence of phosphorylation rate on calf thymus DNA concentration was observed with SV40 TAg and the human Oct-1 POU domain (data not shown). In contrast, the rate at which wild-type mouse p53 was phosphorylated when short oligonucleotides were used to activate DNA-PK was substantially lower at all oligonucleotide concentrations (0 to 100 μg/ml) assayed, and the rate remained constant at oligonucleotide concentrations above those required for maximal DNA-PK activation (about 5 μg/ml) (Fig. 6). Thus, when DNA-PK was activated by short oligonucleotides, p53 behaved very much like hsp90, a non-DNA-binding substrate. However, when longer calf

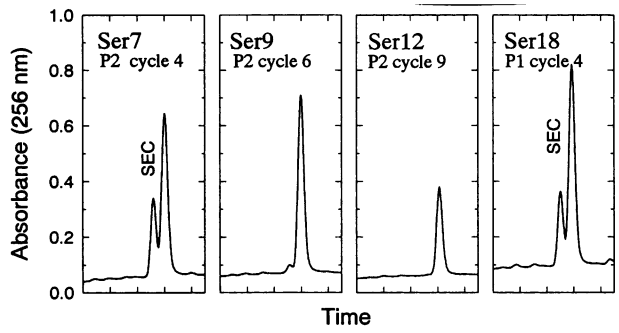

FIG. 5. Identification of the DNA-PK phosphorylation sites at the amino terminus of mouse p53. Peptide 2 (mouse p53 Met-4–Pro-31) was incubated with DNA-PK and then digested with endoproteinase Glu-C; the resulting peptides were separated by C_8 reverse-phase HPLC, and the two major phosphate-containing peaks were collected. These were treated to convert phosphoserine to *S*-ethylcysteine and then sequenced. Cleavage occurred after Glu-15 and produced P1 (LPLSQE) and P2 (MEESQSDISLE); *S*-ethylcysteine was observed in P2 at Ser-7 (cycle 4) and in P1 at Ser-18 (cycle 4) but not at Ser-9 (P2, cycle 6) or Ser-12 (P2, cycle 9). Shown are portions of the PTH-amino acid identification analyses corresponding to the elution position of PTH-*S*-ethylcysteine (see Fig. 2).

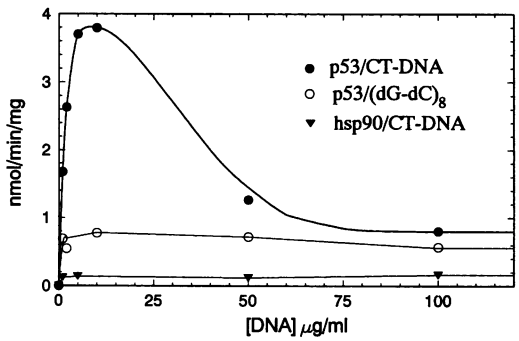

FIG. 6. Effect of DNA concentration on p53 and hsp90 phosphorylation. Approximately equal concentrations (700 nM) of human hsp90 and mouse p53 were mixed and phosphorylated with DNA-PK at the concentrations of calf thymus DNA (CT-DNA) (filled symbols) or $(dG\text{-}dC)_8$ (open symbols) indicated. After incubation for 7 min, reactions were stopped, and a portion of each reaction was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Segments from each gel lane corresponding to the positions of p53 (circles) and hsp90 (inverted triangles) were excised, and the radioactivity in each was determined from the Cerenkov radiation. For the plot shown, the rates of phosphorylation of hsp90 and of p53 by DNA-PK activated with $(dG\text{-}dC)_8$ were multiplied by 20. The oligonucleotide $(dG\text{-}dC)_{12}$ gave a phosphorylation rate profile similar to that for $(dG\text{-}dC)_8$ (data not shown). The rates of phosphorylation of synthetic peptide 15 (Table 1) (and of hsp90) were similar for similar DNA concentrations whether activation was with calf thymus DNA or with oligonucleotide $(dG\text{-}dC)_8$ or $(dG\text{-}dC)_{12}$; activation of DNA-PK by $(dG\text{-}dC)_7$ was substantially poorer at low oligonucleotide concentrations (e.g., 10 μg/ml) (data not shown).

TABLE 2. Phosphorylation of synthetic peptides in crude cell extracts

Cells/ fraction[a]	PO_4 incorporated (nmol/min/ml of extract)[b]			
	Peptide 15 (-LSQE-)		Peptide 19 (-LSEQ-)	
	+	−	+	−
HeLa				
S10	33.8	0.8	0.3	0.0
P10S	28.9	6.5	0.0	0.0
T98G				
S10	22.6	0.2	0.04	0.02
P10S	15.0	1.2	0.0	0.0
L-929				
S10	0.0	0.0	0.0	0.0
P10S	0.005	0.0	0.0	0.0

[a] The soluble fraction (S10) and a salt wash (P10S) of the insoluble fraction, in equal volumes, were prepared from about 2.5×10^7 cells as described in Materials and Methods.

[b] Extracts were incubated for 10 min at 30°C with the indicated peptides (Table 1) at 200 μM and with (+) or without (−) calf thymus DNA at 10 μg/ml; phosphate incorporation was approximately linear for the assay period (data not shown). Background values from reactions without peptide were subtracted.

thymus DNA fragments were used to activate the kinase, the rate of p53 phosphorylation was highly dependent on the concentration of DNA at low DNA concentrations, and at high calf thymus DNA concentrations (above 100 μg/ml), it approached the rate of phosphorylation obtained with short oligonucleotides. These results strongly suggest that the rate of substrate phosphorylation may be enhanced substantially when substrate and kinase bind to the same fragment. Thus, DNA binding may be another factor that influences substrate recognition by DNA-PK.

The peptide EPPLSQEAFADLWKK is a specific DNA-PK substrate. All proteins known to be phosphorylated by DNA-PK in vitro also are in vitro substrates for other protein kinases. Inspection of the consensus recognition sequences for other characterized protein kinases (37) suggested that none were likely to phosphorylate peptide 15. This supposition was tested directly for casein kinase I, casein kinase II, and the catalytic subunit of cyclic AMP kinase; these kinases did not phosphorylate peptide 15 or 19 at significant rates compared with control substrates (data not shown). DNA-PK also did not phosphorylate the casein kinase II substrate peptides RRREEETEEE and RRRD DDSDDD, the casein kinase I substrate peptide DDDEES ITRR, or the S6 kinase substrate RRLSSLRA (data not shown).

We then examined whether peptide 15 would function as a specific indicator of DNA-PK activity in crude cell extracts. Freeze-thaw-lysed S10 and P10S fractions were prepared from HeLa, T98G human glioblastoma, and mouse L cells, and these extracts were assayed for the ability to phosphorylate peptides 15 and 19 with and without added calf thymus DNA (Table 2). The S10 and P10S fractions from the two human cell lines phosphorylated peptide 15 well; furthermore, phosphorylation by the S10 fractions was entirely, and phosphorylation by the P10S fraction was partially, dependent on added DNA. In contrast, peptide 19 was phosphorylated poorly, if at all, by all extracts. Table 2 also shows that DNA-dependent kinase activity was distributed about equally between the S10 and P10S fractions. Western immunoblot analysis, using an antibody against a partial DNA-PK cDNA fusion protein, confirmed that roughly equal amounts of the 350-kDa DNA-PK peptide were present in the two fractions (data not shown).

Peptide 15 was phosphorylated poorly in mouse L-cell extracts, whether or not DNA was added, and this result is consistent with our previous observation that L-cell extracts did not phosphorylate hsp90 in a DNA-dependent manner. L-cell extracts also lacked a 350-kDa polypeptide that reacted with our anti-DNA-PK serum (data not shown). We conclude that mouse L cells have little DNA-PK activity compared with the cultured human cell lines that we have examined. Several other rodent cell lines, including SV40-transformed BALB/c mouse embryo fibroblasts and secondary rat embryo fibroblasts, also had little if any DNA-PK activity (data not shown). Rodent cell extracts do have other kinases, including casein kinases I and II; thus, these results show that peptides 15 and 19 are poor substrates for the common, active, DNA-independent kinases of mammalian cells.

DISCUSSION

Identification and characterization of the elements that govern site recognition by protein kinases is a prerequisite for determining whether specific sites recognized in vitro are recognized by the same kinase in vivo. Many protein kinases recognize as specific substrates small peptides containing simple sequence motifs that are present in protein substrates (37). Synthetic peptides can be used, therefore, to explore sequence elements of site recognition. We show here that DNA-PK phosphorylates small peptides containing the sequence elements -(Ser/Thr)Gln- [or -Gln(Ser/Thr)-] and that a synthetic peptide containing the -SQ- motif can be used to evaluate whether DNA-PK is present in crude cell lysates containing DNA and other kinases. Phosphorylation of the peptide substrate required the presence of a glutamine residue adjacent to the phosphorylated serine or threonine; this is the only sequence element conserved among the nine DNA-PK sites that we have identified. Two DNA-PK phosphorylation sites recently were identified in the carboxy-terminal domain of the serum response factor, and in both cases phosphorylation in vitro required the adjacent glutamine (29). The sequence elements -SQ-, -QS-, -TQ-, and -QT- occur about once per 400 residues in the PIR protein sequence data base, but only a subset of sites with these elements are phosphorylated. Thus, other elements must contribute positively or negatively to site recognition.

Several -(S/T)Q- and -Q(S/T)- sequences in p53, TAg, and hsp90 are not phosphorylated or are phosphorylated poorly by DNA-PK in vitro. Peptide 20, with lysine at the position of Glu-17, was a much poorer substrate than peptides with otherwise similar sequences. Although further studies are required, we suggest that basic residues located near potential DNA-PK recognition sites may provide a negative element that affects recognition by DNA-PK. There is a strong correlation between the presence of one or more nearby basic residues and the inability of DNA-PK to phosphorylate potential (Ser/Thr)Gln sites in the three substrates that have been analyzed. For two of the three -SQ-/-QS- sites in human p53 that are not recognized by DNA-PK in vitro (Ser-99 in peptide 7 and Ser-376 in peptide 10; Table 1), lysines are located within two or five residues on the carboxy-terminal side. In SV40 TAg, a lysine is found six residues carboxy terminal of Ser-639. Ser-639 is in the sequence context -DSQ- and is phosphorylated in vivo, but it was not phosphorylated by DNA-PK in vitro (9). Ser-120 (-DSQ-) of Tag also is phosphorylated in vivo, but this serine is phosphor-

ylated poorly by DNA-PK in the context of a synthetic peptide. The TAg nuclear localization signal, -KKKRK-, starts seven residues distal to serine 120. Residues Thr-195, Ser-211, Ser-416, Ser-453, and Thr-683 of human hsp90α are adjacent to glutamines but are not phosphorylated by DNA-PK; one or more basic residues are found within four residues of each. An apparent exception to the correlation is Ser-166 of p53 (peptide 8, Table 1); Ser-166 is surrounded by Lys-164 and His-168, but it also has adjacent glutamines on both sides. In the synthetic peptide, the surrounding glutamines may protect the site from the negative effects of the nearby basic amino acids; however, there is no indication that Ser-166 of p53 is phosphorylated in vivo. Secondary, tertiary, and quaternary structural changes and other posttranslational modifications also may affect site recognition by DNA-PK in vivo, perhaps permitting a sophisticated control of a substrate's phosphorylation state.

The -SQ- motif may not be the only motif recognized by DNA-PK; thus, we cannot exclude substrate phosphorylation at other sites. DNA-PK, or a DNA-PK-like kinase, phosphorylates the carboxy-terminal domain of eukaryotic RNA polymerase II (39). This domain consists primarily of a repeated seven-amino-acid element, -YSPTSPS-, which is devoid of -SQ-/-QS- or -TQ-/-QT- sequences. DNA-PK phosphorylates the synthetic carboxy-terminal domain repeat peptide Arg-hepta [RRR(YSPTSPS)$_4$] (10), but the K_m for this substrate is poor (25a). Further work will be required to determine whether DNA-PK has a second recognition motif, whether other factors modulate site recognition by DNA-PK, or whether cells encode several similar DNA-activated kinase activities. Several other kinases that phosphorylate the carboxy-terminal domain in vitro have been described (11).

A second positive recognition element for DNA-PK is DNA binding. Most identified DNA-PK substrates are DNA-binding proteins (25), and Jackson et al. (20) found that Sp1 was phosphorylated poorly by DNA-PK unless the DNA used for kinase activation contained the Sp1 GC-box recognition motif. Here we show that the rate of p53 phosphorylation in vitro decreased as the calf thymus DNA concentration increased to concentrations well above the K_m for kinase activation (26); this decrease was not due to inhibition of kinase activity. An effect of DNA concentration on phosphorylation rate was not observed when short oligonucleotides (16 or 24 bp) were used to activate DNA-PK; the average length of the calf thymus DNA was about 300 bp. Our interpretation of this result is that colocalization of kinase and substrate on a DNA fragment may be required for efficient phosphorylation by DNA-PK. p53 has significant sequence-independent (nonspecific) DNA binding activity in vitro (48), and at low DNA concentrations, p53 and DNA-PK should frequently bind the same DNA fragment, effectively increasing locally the concentrations of substrate and kinase. As the DNA concentration is increased, an increasing proportion of DNA-PK and p53 will bind to separate fragments, thereby eliminating the effect of tethering. With short oligonucleotides, colocalization cannot occur, the rate of phosphorylation should be independent of oligonucleotide concentration above the concentration required for kinase activation, and the rate of phosphorylation should be lower than with longer DNAs. This is precisely what was observed with the short dG-dC oligomers. Because no decrease in the rate of p53 phosphorylation was observed as the oligonucleotide concentration was increased, an alternative explanation, that nonspecific DNA binding causes a conformational change in p53 that affects DNA-PK recognition, seems unlikely. We further suggest that DNA-PK may phosphorylate its substrates in vivo only when they are bound to DNA and only when DNA-PK is activated by binding to DNA in the same vicinity. A prediction of this suggestion is that mutations which prevent substrate binding to DNA also will prevent phosphorylation by DNA-PK. For the specific case of p53, we predict that p53 mutants that cannot bind DNA will be underphosphorylated at amino-terminal sites. Although hsp90, casein, phosvitin, and synthetic peptides that do not bind DNA may be phosphorylated by DNA-PK in vitro, the association constants for these substrates are poor. Phosphorylation of these nonphysiological substrates is facilitated in vitro by high substrate concentrations.

p53 has several roles in cell growth regulation (54). Wild-type p53 is required for the reentry of cycling cells into S phase (13, 33), but overexpression of wild-type p53 inhibits induction of proliferating cell nuclear antigen and blocks cells from entry into S phase (34, 35). p53 binds to specific cell and viral DNA sequences (4, 22), probably through its carboxy-terminal domain (17), and the amino-terminal domain of p53 serves as a *trans* activator of transcription (15, 40). These properties suggest that p53 functions as a transcription factor. p53 is present at low levels in normal cells throughout the cell cycle (5); thus, its function as a cell growth regulator must be controlled by posttranslational modifications or by interactions with other factors.

Both the amino- and carboxy-terminal domains of p53 are phosphorylated in vivo, suggesting that phosphorylation is one factor that regulates p53 function in vivo (30, 44, 51, 56, 58). The p34^{cdc2} kinase phosphorylates mouse, monkey, and human p53s at homologous sites (Ser-312/Ser-315) near a nuclear localization element in the carboxy-terminal domain (1, 5, 31, 51). Serine 389 of mouse p53 is phosphorylated in vitro by casein kinase II (32), and this serine may be further modified in vivo by addition of RNA (43, 44); a homologous serine (Ser-392) is phosphorylated in human and monkey p53s (51, 56).

Several phosphorylation sites near the amino terminus of p53 were identified recently. Wang and Eckhart (58) found that mouse p53 is phosphorylated at serines 7, 9, 18, and 37, while Tack and Wright (51) report that monkey p53 is phosphorylated at serines 9, 15, 20, and either 33 or 37. The amino-terminal sequences of the four sequenced mammalian p53s are highly conserved (Fig. 4). Human and monkey p53s are very similar and differ at three of their first 50 residues. The amino termini of rat and mouse p53s also are very similar except for a possible three-residue extension of the mouse amino terminus and a five-residue insertion in rat p53 at residue 34. Rodent and primate p53s, however, have a slightly different arrangement of amino-terminal serines. All four mammalian p53s have six serines in the first 40 residues; five serines, residues 6, 9, 15, 20, and 33 (primate numbering), are at conserved positions (Fig. 4). A sixth serine is found at residue 7 in mouse p53 and at residue 4 in rat p53; this position is occupied by proline in primate p53. In contrast, residue 37 of primate p53 is serine, but this sequence segment is not conserved in rodents (mouse serine 37 is in a context similar to that of primate serine 33). A consequence of this change is that all four p53s have two potential DNA-PK phosphorylation sites, but only one site, serine 15 (primate), is at a conserved position. It is interesting that serines 7 and 18 of mouse p53 and serines 15 and either 33 or 37 of monkey p53 are phosphorylated in vivo (51, 58).

Human p53 mutants with Ser-15 and/or Ser-37 changed to

alanine recently were constructed (55). Mutants having alanine at residue 15 in place of serine as the only change to the wild-type human p53 sequence caused a significant reduction in the steady-state level of p53 in human T98G cells transfected with a mutant-expressing construct. Changing Ser-37 to alanine had no discernible effect on p53 steady-state levels. Preliminary peptide maps of wild-type and mutant p53s expressed in transfected T98G cells are consistent with the conclusion that human p53 is phosphorylated at several amino-terminal sites, including Ser-15 (55). The mechanism responsible for lowering the steady-state level of p53 with the Ser-15-to-Ala-15 mutation, and whether the lowered level stems from an inability of DNA-PK and/or other kinases to phosphorylated amino-terminal p53 residues, currently is under study.

Milne et al. (36) recently reported that several amino-terminal p53 residues can be phosphorylated in vitro by a casein kinase I-like kinase. Casein kinase I preferentially phosphorylates serines or threonines near another phosphorylated residue (16). Thus, we imagine that phosphorylation of the amino terminus of p53 may be the consequence of a kinase cascade that regulates the properties of DNA-bound p53. We suggest that the binding of p53 to appropriate DNA sequences may potentiate the phosphorylation of serines 15 and 37 by DNA-PK; nearby residues may then be phosphorylated by casein kinase I or by other kinases (45). Phosphorylation of additional amino-terminal sites may be essential for p53 function, may further modulate p53 function, or may allow differential p53 function at different chromatin-binding sites. A prediction of the cascade model is that an inability of p53 to bind DNA would result in the underphosphorylation of amino-terminal sites. It is easy to imagine how phosphorylation might influence the interaction of p53 with components of the transcription apparatus or might alter the stability of DNA-bound p53. Our characterization of the requirements for phosphorylation by DNA-PK should allow the putative role of DNA-PK in controlling p53 function (and the functions of other proteins) to be tested by construction of appropriate mutants.

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