Identification of *in vitro* and *in vivo* phosphorylation sites in the catalytic subunit of the DNA-dependent protein kinase

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The DNA-dependent protein kinase (DNA-PK) is required for the repair of DNA double-strand breaks (DSBs), such as those caused by ionizing radiation and other DNA-damaging agents. DNA-PK is composed of a large catalytic subunit (DNA-PKcs) and a heterodimer of Ku70 and Ku80 that assemble on the ends of double-stranded DNA to form an active serine/threonine protein kinase complex. Despite *in vitro* and *in vivo* evidence to support an essential role for the protein kinase activity of DNA-PK in the repair of DNA DSBs, the physiological targets of DNA-PK have remained elusive. We have previously shown that DNA-PK undergoes autophosphorylation *in vitro*, and that autophosphorylation correlates with loss of protein kinase activity and dissociation of the DNA-PK complex. Also, treatment of cells with the protein phosphatase inhibitor, okadaic acid, enhances DNA-PKcs phosphorylation and reduces DNA-PK activity *in vivo*. Here, using solid-phase protein sequencing, MS and phosphospecific antibodies, we have identified seven *in vitro* autophosphorylation sites in DNA-PKcs. Six of these sites (Thr^{2609}, Ser^{2612}, Thr^{2620}, Ser^{2624}, Thr^{2638} and Thr^{2647}) are clustered in a region of 38 amino acids in the central region of the protein. Five of these sites (Thr^{2609}, Ser^{2612}, Thr^{2638}, Thr^{2647} and Ser^{3205}) are conserved between six vertebrate species. Moreover, we show that DNA-PKcs is phosphorylated *in vivo* at Thr^{2609}, Ser^{2612}, Thr^{2638} and Thr^{2647} in okadaic acid-treated human cells. We propose that phosphorylation of these sites may play an important role in DNA-PK function.

Key words: autophosphorylation site, DNA double-strand break repair, non-homologous end-joining.

INTRODUCTION

The non-homologous end-joining (NHEJ) pathway is essential for the repair of ionizing radiation-induced DNA double-strand breaks (DSBs) in human cells (reviewed in [1,2]). Genetic studies have implicated several genes in this pathway, including XRCC7, XRCC5, XRCC6, XRCC4, DNL4 and Artemis [1–5]. These genes encode for the DNA-dependent protein kinase (DNA-PK) catalytic subunit (DNA-PKcs), Ku70, Ku80, XRCC4, DNA ligase IV and Artemis respectively [1–5].

Biochemical studies suggest that the first step in NHEJ is binding of the Ku70/80 heterodimer to the ends of DNA at the DSB. Ku then translocates inwards a few bases, which serves to recruit DNA-PKcs. The protein kinase activity of DNA-PKcs is greatly enhanced by DNA-mediated association with the Ku heterodimer; thus assembly of the DNA-PKcs-Ku complex at the ends of DNA results in formation of a serine/threonine protein kinase holoenzyme, DNA-PK, that is tethered to the ends of the DNA at the site of the DNA DSB [1,2,6]. XRCC4 and DNA ligase IV form a tetrameric complex [7], which is also recruited to the repair complex, and is required for end joining. Artemis has recently been shown to possess both endo- and exo-nucleolytic activities, which may be important for processing DNA ends during NHEJ [5]. Experiments in cell-free extracts have revealed that MgATP is required for DNA-PK-dependent end joining *in vitro* [8,9]. DNA-PK-dependent DNA end joining is also inhibited by the fungal metabolite wortmannin [8,9], an inhibitor of the protein kinase activity of DNA-PK [10,11]. These facts point to an essential role for the protein kinase activity of DNA-PK in NHEJ. Moreover, DNA-PKcs that contains mutations in the protein kinase domain fails to restore efficient DNA DSB repair in cell lines that are deficient for DNA-PKcs, indicating that the protein kinase activity of DNA-PK is essential for DNA DSB repair *in vivo* [12,13]. However, a clear demonstration of the downstream target or targets of DNA-PK has been lacking. Current models predict that DNA-PKcs interacts with Ku at the site of a DNA DSB, and that this interaction results in increased protein kinase activity. Thus, DNA-PK-mediated phosphorylation of proteins that are assembled at the DNA DSB site may play an important role in NHEJ.

We and others have previously shown that, *in vitro*, DNA-PK phosphorylates many protein substrates, for example Hsp90 [14], p53 [15] and c-Jun [16], on serine or threonine residues that are followed by glutamine, i.e. an SQ or TQ motif. DNA-PK also shows a preference for SQ and TQ sequences in the context of synthetic peptides [15,17]. However, DNA-PK can also phosphorylate proteins at 'non-SQ' sites, with a preference for serine or threonine followed by a hydrophobic amino acid [18,19]. DNA-PKcs, Ku70, Ku80, XRCC4, DNA ligase IV and Artemis contain numerous SQ or TQ motifs. In addition, these proteins are intimately involved in NHEJ, and are present at the DNA break sites. They are therefore logical candidates for *in vivo* targets of DNA-PK. Previously, we have shown that DNA-PKcs, Ku70 and Ku80 undergo DNA-PK-dependent phosphorylation *in vitro* [20,21]. Phosphorylation of DNA-PK *in vitro* results in a loss of protein kinase activity and dissociation of DNA-PKcs from DNA-bound Ku [21,22]. Moreover, we have shown that DNA-PKcs, Ku70 and Ku80 are phosphorylated

Abbreviations used: DNA-PK, DNA-dependent protein kinase; DNA-PKcs, DNA-dependent protein kinase catalytic subunit; DSB, double-strand break; MALDI-TOF MS, matrix-assisted laser-desorption ionization–time-of-flight MS; NHEJ, non-homologous end joining.

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in vivo, and that conditions that promote hyperphosphorylation of DNA-PKcs and Ku *in vivo* lead to a reduction in DNA-PK kinase activity, suggesting that autophosphorylation may regulate DNA-PK activity *in vivo* [23]. *In vivo* phosphorylation of DNA-PKcs, Ku70 and Ku80 was enhanced when cells were treated with the protein phosphatase inhibitor okadaic acid, suggesting that DNA-PK phosphorylation *in vivo* is regulated by the activity of protein phosphatases [23].

As a first step towards elucidating the role of protein phosphorylation in NHEJ, we recently mapped DNA-PK phosphorylation sites in Ku70 and Ku80 [19]. Although recombinant Ku70 was phosphorylated at an SQ sequence *in vitro* (Ser^{51}), in the context of the Ku70/80 heterodimer the major phosphorylation site in Ku70 was Ser^{6}, which is located in the amino acid sequence ESSY (phosphorylated amino acid underlined) [19]. Similarly, the major DNA-PK phosphorylation sites in Ku80 are Ser^{578}, Ser^{580} and Thr^{715}, none of which correspond to the 'typical' SQ/TQ motif [19]. Interestingly, the DNA-PK phosphorylation sites in Ku70/80 all lie in unique N- or C-terminal regions outside of the conserved DNA-binding core [24,25]. Here we have identified seven autophosphorylation sites in human DNA-PKcs (Thr^{2609}, Ser^{2612}, Thr^{2620}, Ser^{2624}, Thr^{2638}, Thr^{2647} and Ser^{3205}). Five of these sites correspond to SQ/TQ sites and two occur on serines that are followed by a hydrophobic amino acid. Six of the sites are tightly clustered within a 38-amino-acid region of the 469 kDa DNA-PKcs polypeptide. Furthermore we show, using phosphospecific antibodies, that four of the sites (Thr^{2609}, Ser^{2612}, Thr^{2638} and Thr^{2647}) are phosphorylated *in vivo* in okadaic acid-treated cells.

MATERIALS AND METHODS

Materials

Okadaic acid, microcystin-LR and Zwittergent 3-16 were purchased from Calbiochem. BSA, PMSF, Tris base, EGTA, leupeptin, pepstatin, 4-vinylpyridine, α-cinnamic acid and wortmannin were from Sigma. Calf thymus DNA was purchased from Aldrich. Dithiothreitol was from BDH. [γ-^{32}P]ATP was from Perkin-Elmer Life Sciences.

Identification of *in vitro* autophosphorylation sites on DNA-PKcs

The DNA-PKcs and Ku subunits of DNA-PK were purified from human placenta as described previously [21]. Purified DNA-PKcs (10 μg) and Ku (3.3 μg) proteins were incubated in 50 μl of reaction mixture containing 25 mM Hepes/NaOH, pH 7.5, 100 mM KCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, 0.2 mM EGTA, 0.1 mM EDTA, 0.5 μM microcystin-LR plus 10 μg/ml sonicated calf thymus DNA, and reactions were started by the addition of 0.25 mM ATP containing stabilized [γ-^{32}P]ATP (specific activity 500–1000 d.p.m./pmol). Aliquots were removed at timed intervals and analysed by SDS/PAGE and autoradiography to calculate the stoichiometry of phosphate incorporation and to determine that the reaction had achieved saturation. The reaction was terminated after 60 min by the addition of 1 % SDS and 10 mM dithiothreitol, and the sample was heated at 100 °C for 1 min. After cooling, 4-vinylpyridine was added to a concentration of 0.5 % (v/v) and the samples were placed on a shaking platform for 30 min at room temperature to alkylate cysteine residues. Each reaction was subjected to electrophoresis on an 8 % acrylamide/SDS gel and then analysed by autoradiography. The phosphorylated band corresponding to ^{32}P-labelled DNA-PKcs was excised from the gel and cut into small pieces. The gel pieces were washed sequentially, for 15 min per wash, on a vibrating platform with 1 ml of water, followed by 1 ml of water/acetonitrile (1:1, v/v), 0.1 M ammonium bicarbonate, 0.2 M ammonium bicarbonate/acetonitrile (1:1, v/v) and finally 100 % acetonitrile. The gel pieces were dried in a rotary evaporator and incubated in 0.2 ml of 50 mM ammonium bicarbonate/0.05 % (w/v) Zwittergent 3-16 containing 2 μg of trypsin. After 16 h, the supernatant was removed and the gel pieces were washed for 10 min in 0.2 ml of 50 mM ammonium bicarbonate/0.05 % (w/v) Zwittergent 3-16/0.1 % (v/v) trifluoroacetic acid. The combined supernatants, which contained > 90 % of the ^{32}P radioactivity, were chromatographed on a Vydac C_{18} column as described in the legend to Figure 1 (see below).

Phosphopeptide sequence analysis

^{32}P-labelled peptides were analysed at the MRC Protein Phosphorylation Unit at the University of Dundee, Dundee, U.K., by matrix-assisted laser-desorption ionization–time-of-flight MS (MALDI-TOF MS) on a PerSeptive Biosystems Elite STR mass spectrometer (Framingham, MA, U.S.A), using α-cyanocinnamic acid as the matrix. Spectra were obtained in both linear and reflector modes. Peptide masses obtained from MALDI-TOF mass spectra in the reflector mode are given as monoisotopic masses, while those obtained in the linear mode are given in average mass units. Mass values were used to search against the predicted masses of predicted DNA-PKcs tryptic peptides, and candidate peptides were identified. The site of phosphorylation in each HPLC-purified phosphopeptide was determined by solid-phase Edman degradation of the peptide coupled to Sequelon-AA membrane (Milligen) on an Applied Biosystems 476A sequenator as described previously [26]. Some phosphopeptides were also analysed by electrospray MS on a Q-TOF2 mass spectrometer (Micromass, Altrincham, Cheshire, U.K.). Phosphoamino acid analysis was as described [27].

Antibodies

A monoclonal antibody to the C-terminus of DNA-PKcs (mAb 42-27) was as described previously [28]. Phosphospecific antibodies recognizing DNA-PKcs at Thr^{2609}, Ser^{2612}, Thr^{2620}, Thr^{2638} and Thr^{2647} were raised in sheep against the following peptides: Thr^{2609}, (K)(K)LTPMFVETQAS(R); Ser^{2612}, (K)TQASQGTLQTR; Thr^{2620}, (K)TRTQEGSLSAR; Thr^{2638}, (K)GQIRATQQQHD; and Thr^{2647}, (K)FTLTQTADGRS. The underlined residue in each peptide corresponds to phosphothreonine or phosphoserine. Amino acids in parentheses represent additional lysine or arginine residues that were added to the N- and/or C-terminus of the peptides to promote solubility. The peptide sequences correspond to residues 2602–2612, 2609–2619, 2618–2628, 2633–2643 and 2644–2654 of human DNA-PKcs respectively. Equal amounts of BSA-conjugated peptide and keyhole limpet haemocyanin-conjugated peptides were combined and used for immunization.

Each of the antisera was affinity purified on CH-Sepharose (Amersham Biosciences) to which the corresponding phosphorylated peptide had been covalently coupled. The bound fraction was then passed through a second column of CH-Sepharose to which the corresponding non-phosphorylated peptide had been coupled. Antisera that did not bind to the second column were selected. Secondary antibodies coupled to horseradish peroxidase (Pierce) raised against sheep IgG were used for immunoblotting.

DNA-PK phosphorylation assays

Purified DNA-PKcs (100 ng) and Ku70/80 (30 ng) were incubated in the presence of 50 mM Hepes/NaOH (pH 7.5), 50 mM KCl, 10 mM $MgCl_2$ or $MnCl_2$ (as indicated in Figure 4) and

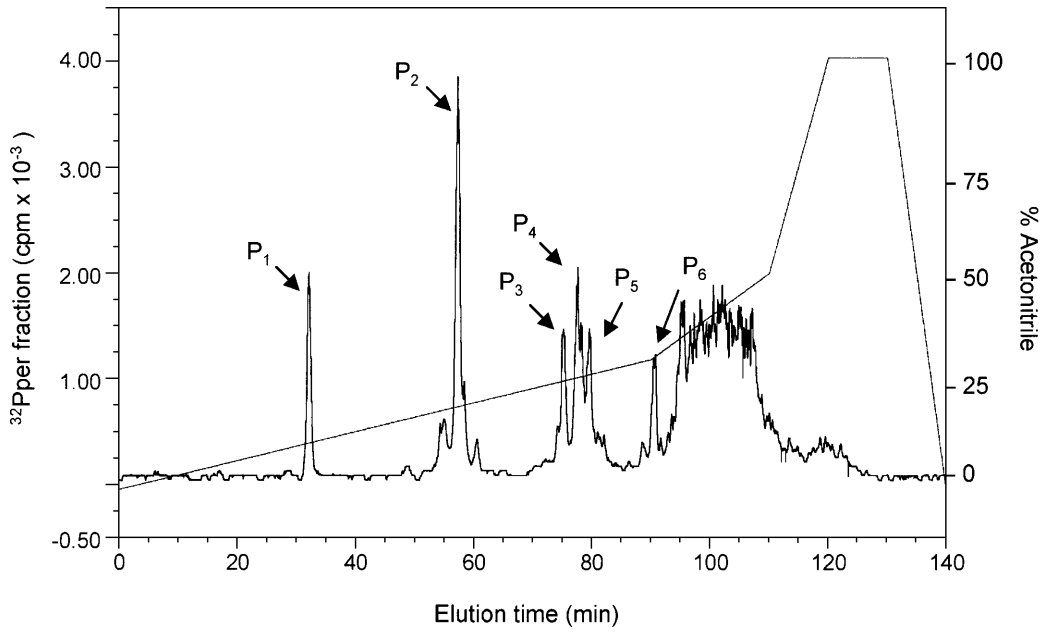

Figure 1 Tryptic peptide map of ^{32}P-labelled DNA-PKcs

Autophosphorylated DNA-PKcs was separated by electrophoresis on an SDS/polyacrylamide gel and then digested with trypsin as described in the Materials and methods section. The resulting ^{32}P-labelled peptides were chromatographed on a Vydac 218TP54 C_{18} column (Separations Group, Hesperia, CA, U.S.A.) equilibrated in 0.1% (v/v) trifluoroacetic acid in water. The column was developed with a linear acetonitrile gradient (diagonal line) at a flow rate of 0.8 ml/min, and fractions of 0.4 ml were collected; $>$ 80% of the radioactivity applied to the column was recovered in the fractions. Similar profiles were obtained in three separate experiments. The positions of the phosphopeptides termed P_1–P_6 are indicated.

0.25 mM unlabelled (non-radioactive) ATP, plus sonicated calf thymus DNA (at 10 μg/ml). Samples were analysed by SDS/PAGE on 8% acrylamide/0.1% bisacrylamide gels followed by immunoblotting as described previously [21], and probed with phosphospecific and non-phosphospecific antibodies as described below.

Cell culture and cell extracts

Human lymphoblastoid cells (BT) were maintained in RPMI 1640 medium (Life Technologies) supplemented with 10% fetal calf serum in an atmosphere of 5% CO_2 [29]. Where indicated, logarithmic-phase cells (1×10^6 cells/10 ml dish) were incubated in media containing okadaic acid at a 1 μM final concentration. Okadaic acid was prepared in DMSO. For control incubations the corresponding volume of DMSO was added to the cells. Cells were harvested by centrifugation, washed twice in PBS, and lysed by incubation on ice for 1 h in lysis buffer (50 mM Tris/HCl, pH 8.0, 0.25 mM EDTA, 0.15 M NaCl and 1% (v/v) Nonidet P-40). Lysates were then centrifuged at 10000 ***g*** for 10 min at 4 °C. The supernatant was removed, snap-frozen in liquid nitrogen and stored at −80 °C. Protein concentrations were determined using a detergent-compatible dye-binding assay (Bio-Rad) with BSA as a standard.

Immunoblotting

For immunoblots of total cell lysates (see Figure 5, below), 100 μg of protein was used. For purified DNA-PKcs (see Figures 3 and 4, below), 100 ng of protein was used. Samples were subjected to SDS/PAGE and transferred to nitrocellulose membrane (Bio-Rad). For the phosphospecific DNA-PKcs antibodies, membranes were incubated in 25 mM Tris/HCl (pH 7.5), 0.5 M NaCl, 0.5% (v/v) Tween-20 and 10% (w/v) skimmed milk powder for 7 h at 4 °C in the presence of 2 μg/ml antiserum. Where indicated, phosphorylated (blocking peptide) or non-phosphorylated (control) peptide, corresponding to the antigen to which the antibody was raised, was included with the primary antibody at a final concentration of 10 μg/ml. After probing with the phosphospecific antibodies, immunoblots were stripped and reprobed using a non-phosphospecific monoclonal antibody (mAb 42-27) [28] to the C-terminus of DNA-PKcs. Detection was performed using horseradish peroxidase-conjugated secondary antibodies and ECL (Amersham Biosciences).

RESULTS

Identification of *in vitro* phosphorylation sites in DNA-PKcs

Purified DNA-PKcs and Ku70/80 heterodimer were incubated with DNA as described in the Materials and methods section. Under these conditions, approx. 7 mol of phosphate were incorporated per mol of DNA-PKcs, suggesting that DNA-PKcs was phosphorylated at several sites *in vitro*. Reactions were terminated after 60 min, and, after reduction and alkylation of cysteine residues, the proteins were separated by SDS/PAGE. Radioactively labelled DNA-PKcs was excised from the SDS/PAGE gel, digested with trypsin and the released phosphopeptides were isolated by HPLC (see the Materials and methods section and legend to Figure 1). Over 90% of the radioactivity was released from the gel pieces by tryspin digestion. Six peaks of radioactivity that fractionated between 5 and 30% acetonitrile were resolved, labelled P_1–P_6 (Figure 1). A cluster of radioactive peaks that eluted from 30–50% acetonitrile was also observed; however, these peptides proved refractory to further purification, and were not studied further at this time. Each of the six phosphopeptides (P_1–P_6) was next analysed by phosphoamino acid analysis, solid-phase Edman degradation and MS.

Table 1 Characterization of HPLC-purified phosphopeptides from a tryptic digest of *in vitro*-phosphorylated DNA-PKcs

DNA-PKcs was phosphorylated and the phosphopeptides generated were purified on reversed-phase HPLC as described in the Materials and methods section. Six major peaks of radioactivity were detected (P_1–P_6; Figure 1). Each phosphopeptide was analysed by phosphoamino acid analysis, solid-phase sequencing and MS as described in the text. Unless otherwise indicated, MS results are from MALDI-TOF MS in reflector mode, and are expressed as monoisotopic mass. In the column where peptide masses are given, the mass determined by MS is shown first, followed, in parentheses, by the theoretical peptide mass. In the final column, phosphorylated amino acids are underlined and the position of the phosphorylated amino acid is given in parentheses. MetSO, methionine sulphone; PAAA, phosphoamino acid analysis.

Peptide	PAAA	Cycle of radioactivity release	Peptide residue numbers, post-translational modifications and peptide mass	Amino acid sequence
P_1	Ser	5	2620–2628 + PO_4; 1028.42* (1028.441)	TQEG<u>S</u>LSAR (Ser^{2624})
P_2	Thr	2 and 11	2637–2653 + PO_4; 1997.8302 (1997.9055)	A<u>T</u>QQQHDFTL<u>T</u>QTAGDR (Thr^{2638} and Thr^{2647})
P_3	Thr	10 and 19	2629–2653 + PO_4/2PO_4; 2910.51/2988.93† (2907.1/2987.1)	WPVAGQIRA<u>T</u>QQQHDFTL<u>T</u>QTAGDR (Thr^{2638} and Thr^{2647})
P_4	Ser/Thr	11 and 14	2599–2619 + PO_4/2PO_4; 2392.74/2472.75† (2392.62/2472.62)	STVLTPMFVE<u>T</u>QA<u>S</u>QGTLQTR (Thr^{2609} and Ser^{2612})
P_5	Ser/Thr	11 and 14	2599–2619 + PO_4 + MetSO; 2390.97 (2391.16)	STVLTPMFVE<u>T</u>QA<u>S</u>QGTLQTR (Thr^{2609} and Ser^{2612})
P_6	Ser	9	3197–3232 + PO_4 + MetSO; 4197.84† (4197.55)	LTPLPEDN<u>S</u>MNVDQDGDPSDRMEVQEQEEDISSLIR (Ser^{3205})

* Mass was detected by electrospray MS on a Q-TOF2 instrument.
† Average mass assigned from MALDI-TOF MS in the linear mode.

Phosphopeptide P_1 contained only phosphoserine (Table 1, and results not shown). When analysed by Edman degradation, radioactivity was released in sequencer cycle 5 (Figure 2A). MALDI-TOF MS revealed an ion of mass 1028.42 (monoisotopic mass), which was consistent with the presence of the peptide TQEGSLSAR containing one phosphate group (Table 1). Further analysis of this peptide by electrospray on a Q-TOF2 mass spectrometer confirmed the presence of this sequence, and indicated phosphorylation at position 5. We conclude that phosphopeptide P_1 corresponds to amino acids 2620–2628 of DNA-PKcs, and is phosphorylated at Ser^{2624} (Table 1). Although Ser^{2624} is not followed by a glutamine and therefore does not conform to the previously identified SQ motif [15], it does resemble the 'S-hydrophobic' consensus identified in DNA-PK phosphorylation sites in Ku70 and Ku80 [19].

Phosphopeptide peak P_2 was phosphorylated only at threonine (Table 1, and results not shown). Solid-phase Edman sequencing resulted in a major burst of radioactivity at cycle 2 and a smaller release at cycle 11 (Figure 2B). Using MALDI-TOF MS, a peptide of mass 1997.8302 (monoisotopic mass) was detected. Together, these data are consistent with the presence of a phosphopeptide corresponding to amino acids 2637–2653 of DNA-PKcs, which is phosphorylated predominantly at Thr^{2638} in the amino acid sequence ATQQQHD (Table 1). The solid-phase Edman sequencing data (Figure 2B) suggest that P_2 also contains a small amount of the same peptide in which Thr^{2647} (at position 11 of the peptide) is phosphorylated (Table 1). Both Thr^{2638} and Thr^{2647} are followed by glutamine, and thus conform to the typical TQ-type DNA-PK phosphorylation consensus.

Phosphopeptide peak P_3 was also phosphorylated only on threonine (Table 1, and results not shown). Solid-phase Edman sequencing revealed a major phosphorylation site at position 10, and a minor site at position 19 (Figure 2C). Two ions of average mass 2910.51 and 2988.93 were detected in MALDI-TOF MS linear mode (Table 1). Together these data are consistent with the presence of amino acids 2629–2653 of DNA-PKcs containing a single phosphorylation site (predicted mass, 2907.1) and a doubly phosphorylated species (predicted mass, 2987.1). From these data we propose that phosphopeptide P_3 corresponds to amino acids 2629–2653 of DNA-PKcs, in which both Thr^{2638} and Thr^{2647} are phosphorylated (Table 1). Thus phosphopeptide P_3 contains the same phosphorylation sites as P_2, but differs from P_2 in that the Arg at position 2636 of DNA-PKcs was not cleaved by trypsin.

Peak P_4 was also analysed by phosphoamino acid analysis, solid-phase Edman sequencing and MS. P_4 contained phosphoserine and phosphothreonine (Table 1, and results not shown), and radioactivity was released predominantly at positions 10, 11 and 14 (Figure 2D). Ions of average mass 2392.737 and 2472.748 were observed by MALDI-TOF MS linear mode (Table 1). A tryptic peptide containing amino acids 2599–2619 of DNA-PKcs and phosphorylated at one site is predicted to have a mass of 2392.62, whereas the doubly phosphorylated form of the same peptide is predicted to have a mass of 2472.62. Together these data are consistent with phosphorylation of Thr^{2609} and Ser^{2612} in the tryptic peptide beginning at amino acid 2599 and ending at amino acid 2619 (Table 1). It is possible that additional phosphopeptides are present in P_4; however, we have not identified them further at this time.

Like P_4, P_5 was also phosphorylated on serine and threonine (Table 1, and results not shown). The major position of radioactivity release following Edman sequencing occurred at cycles 11 and 14 (Figure 2E). MALDI-TOF analysis revealed a peak of mass 2390.97 (Table 1). This corresponds well to a mass of 2391.16 that is predicted to occur from the peptide beginning at amino acid 2599 and ending at amino acid 2619, containing one phosphate group and one modified methionine (Table 1). We conclude that P_5 is also derived from amino acids 2599–2619 of DNA-PKcs, and is phosphorylated predominantly on Thr^{2609}. P_4 and P_5 were poorly resolved on HPLC (Figure 1). It is likely that the radioactivity released at cycle 14 represents phosphorylation of Ser^{2612}, as observed in Figure 2(D) for peptide P_4.

P_6 contained only phosphoserine (Table 1, and results not shown), and radioactive phosphate was released at position 9 (Figure 2F). An ion of average mass 4197.84 was observed on MALDI-TOF MS (linear mode), which is consistent with a peptide corresponding to amino acids 3197–3232 of DNA-PKcs, containing a single phosphate and an oxidized methionine (predicted mass 4197.55; Table 1). We therefore conclude that P_6 corresponds to amino acids 3197–3232 of DNA-PKcs phosphorylated at Ser^{3205} (Table 1).

In summary, analysis of HPLC-purified peptides by solid-phase sequencing and MS suggests that, *in vitro*, DNA-PKcs is autophosphorylated at Thr^{2609}, Ser^{2612}, Ser^{2624}, Thr^{2638}, Thr^{2647} and Ser^{3205}. From the relative abundance of each of the phosphorylated peaks in HPLC (Figure 1), our data suggest that the major site of phosphorylation in DNA-PKcs is Thr^{2638} (P_2), with Ser^{2624} (P_1), Thr^{2647} (P_2 and P_3), Thr^{2609} (P_5), Ser^{2612} (P_4) and

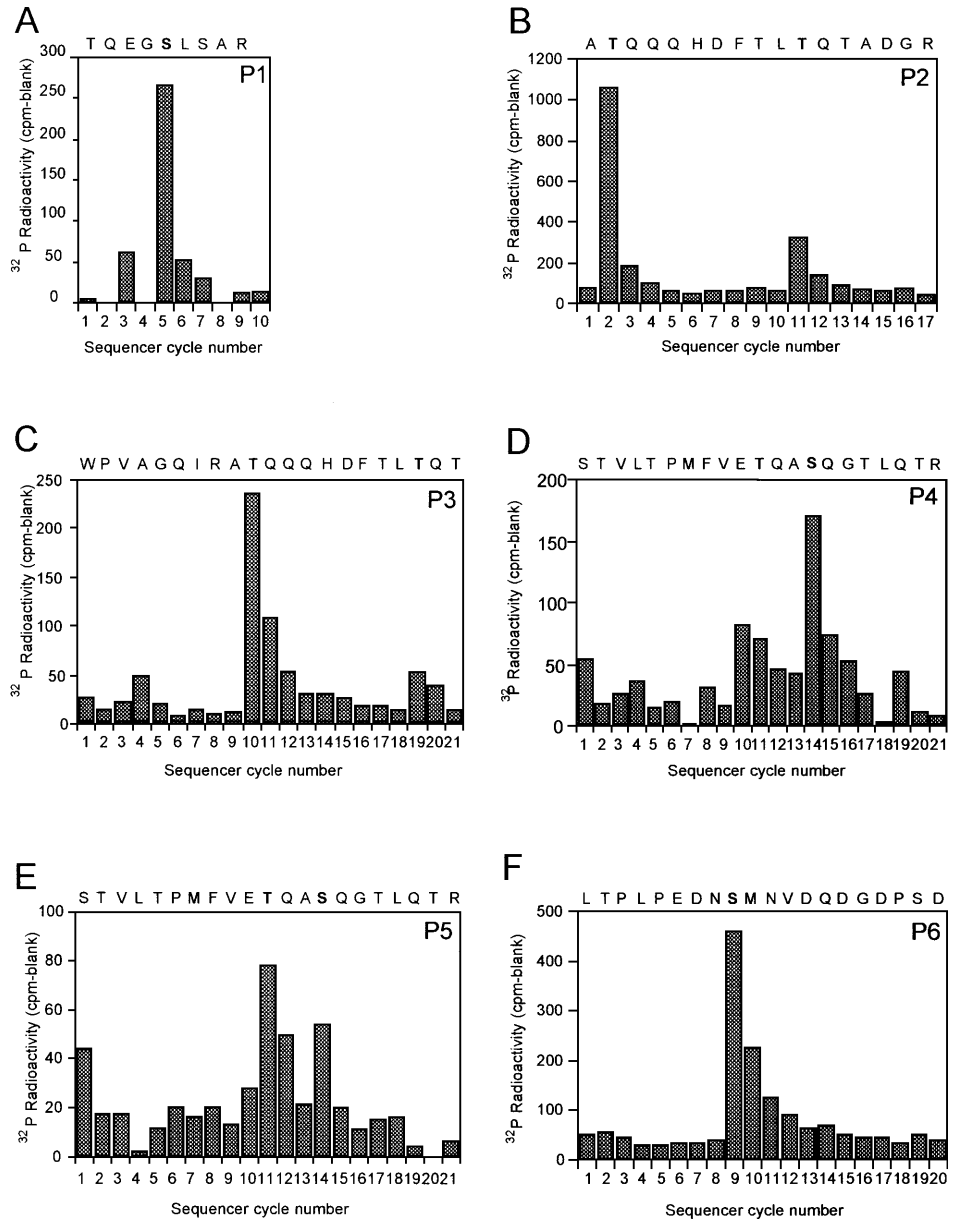

Figure 2 Identification of the autophosphorylation sites in ^{32}P-labelled DNA-PKcs peptides

Aliquots of the major ^{32}P-labelled peptides derived from phosphorylated DNA-PKcs (Figure 1) were covalently coupled to a Sequelon arylamine membrane and analysed on an Applied Biosystems 476A sequenator. ^{32}P radioactivity was measured after each cycle of Edman degradation. In combination with MALDI-TOF MS, database searching against predicted DNA-PKcs tryptic peptides and phosphoamino acid analysis (Table 1); this enabled the identification of the sites of phosphorylation in each of the peptides. The putative amino acid sequence of each peptide, deduced from a combination of phosphoamino acid analysis, MS and solid-phase sequencing, is indicated on the top of each panel. Phosphopeptide peaks P_1–P_6 are as in Figure 1.

Ser^{3205} (P_6) representing less-abundant *in vitro* phosphorylation sites. Interestingly, five of the identified sites lie in a cluster of 38 amino acids corresponding to positions 2609–2647 of DNA-PKcs. This region of the protein is distal to the C-terminal kinase domain, which located in the last 500 amino acids of the 4127-amino-acid polypeptide. The function of this region of the protein is not known; however, it is interesting to note that four of the identified phosphorylated amino acids (Thr^{2609}, Thr^{2638}, Thr^{2647} and Ser^{3205}) are conserved between human, mouse, horse, dog, chicken and *Xenopus laevis* (Table 2). Ser^{2624} is conserved in human, horse, dog and chicken, but not mouse (Table 2). This suggests that these amino acids are important for the function of DNA-PKcs. Although several of the identified sites were present as SQ or TQ motifs (Thr^{2609}, Ser^{2612}, Thr^{2638} and Thr^{2647}), others did not conform to the 'classical' DNA-PKcs consensus (SQ/TQ motif). Rather, these sites, Ser^{2624} (GSLSAR) and Ser^{3205} (DNSMNV), conform to the Ser hydrophobic amino acid DNA-PK sites previously identified in Ku70 and Ku80 [19].

Characterization of phosphospecific antibodies to the identified DNA-PKcs phosphorylation sites

To determine whether any of these sites were phosphorylated *in vivo*, we generated a panel of phosphospecific antibodies corresponding to Thr^{2609}, Ser^{2612}, Thr^{2638} and Thr^{2647} (see the

Table 2 Conservation of *in vitro* and *in vivo* DNA-PKcs phosphorylation sites in vertebrate species

The following DNA–PKcs amino acid sequences were obtained from NCBI: human (accession number P78527); horse (accession number AAL40980); dog (accession number AAL40979); mouse (accession number P97313), chicken (accession number BAB91148); frog (*Xenopus laevis*, accession number BAA36690). The numbering at the top of each block of sequence corresponds to the identified phosphorylation sites in human DNA–PKcs (P78527). Amino acids that are conserved between all 6 species are indicated in bold.

	$T^{2609}S^{2612}$	S^{2624}	T^{2638}	T^{2647}
human	**WRFRS**TV**LTPMF**V**ETQAS**QGTLQTRT**Q**EGSLSARWPVAGQI**RATQ**QQHD**FTLTQ**TADG**R**			
horse	**WRFRS**TV**LTPMF**I**ETQAS**QSALQTRT**Q**EGSLSARGVMTGQI**RATQ**QQYD**FTPTQ**NTDG**R**			
dog	**WRFRS**TV**LTPMF**I**ETQAS**QSTLQTRT**Q**ERSLPAQGVMARQI**RATQ**QQYD**FTPTQ**TADG**R**			
mouse	**WRFRS**TV**LTPMF**I**ETQAS**PSILHTQT**Q**EGPLSDQRQKPGQV**RATQ**QQYD**FTPTQ**ASVE**R**			
chicken	**WRYRS**TM**LTPMF**V**ETQAS**QSTNRNSS**Q**ERSLSISGSVGGRV**RATQ**RQYE**FTPTQ**NVSG**R**			
frog	**WRFRS**SV**LTPMF**V**ETQLS**QSMQRSRA**Q**G-TIEADEPIGGQL**RATQ**QHYQ**FTPTQ**NIGG**R**			

	S^{3205}
human	PLPE-**DNSMNVD**QDGDPSDRME**V**Q
horse	IPPD-**DHSMNTD**GDEDSSDRMK**V**Q
dog	LPLG-**DHSLSMD**EERDSSDKME**V**Q
mouse	APSG-**DHSMSVD**EDEESIDR-E**V**Y
chicken	CDKAN**D**-**S**MEV**D**EESSVGDQME**V**D
frog	PQLV-**DES**MEV**D**DLADGNEAME**V**D

Materials and methods section for details). As discussed above, five of the identified phosphorylation sites reside in a region of 38 amino acids within the 4127-amino-acid sequence of DNA-PKcs. Also present in this region was another TQ motif (Thr^{2620}) that was not identified in the experiments described above. In order to test whether this site might also be phosphorylated in DNA-PKcs, we also generated a phosphospecific antibody to Thr^{2620} (see the Materials and methods section for details). Phosphospecific antibodies to Ser^{2624} and Ser^{3205} are currently being generated.

The phosphospecific antibodies were then used to characterize phosphorylation of DNA-PKcs *in vitro*. The DNA-PK enzyme is only active when assembled on DNA. We would therefore predict that DNA-PKcs would only be phosphorylated in the presence of added DNA. To test this, purified DNA-PKcs and Ku were incubated in the presence of MgATP in either the absence (Figure 3, lane 1) or presence (Figure 3, lanes 2 and 3) of sonicated calf thymus DNA. Samples were then run on SDS/PAGE and probed with phosphospecific antibodies to Thr^{2609}, Ser^{2612}, Thr^{2620}, Thr^{2638} or Thr^{2647} as indicated (Figure 3). The upper immunoblots in each panel correspond to immunoblots that were incubated with the phosphospecific antibodies, while the lower blots in each panel represent the same blots after stripping and reprobing with mAb 42-27. Immunoblots shown on the upper left-hand side of each panel were incubated with the phosphospecific antibody in the presence of the corresponding dephosphopeptide, while those in the upper right-hand panel were incubated with the phosphospecific antibody in the presence of the appropriate blocking phosphopeptide. These results show that DNA-PKcs is phosphorylated *in vitro* at Thr^{2609}, Ser^{2612}, Thr^{2620}, Thr^{2638} and Thr^{2647}, that the antibodies generated are indeed specific for the phosphorylated forms of DNA-PKcs (as the signal is ablated by incubation with the blocking peptide). Additionally, each phosphospecific antibody was specific for its specific phosphorylation site, as the phosphopeptide corresponding to a different site did not ablate the signal (results not shown). Although Thr^{2620} was not identified directly using HPLC and MS, the phosphospecific antibody indicates that it also is an *in vitro* phosphorylation site (Figure 3C). The data also show that phosphorylation at each site is DNA-dependent. As expected, no signal was observed when samples containing DNA-PKcs, Ku and sonicated calf thymus DNA were incubated in the absence of added ATP (results not shown). DNA-PKcs was also phosphorylated at each site in the presence of single-stranded M13 DNA and linearized plasmid double-stranded DNA (results not shown).

DNA-PKcs is a member of the phosphoinositide 3-kinase-like family of serine/threonine protein kinases, and, as such, its protein kinase activity is inhibited by the wortmannin [6,10,11]. When 1 μM wortmannin was added to reactions containing DNA-PKcs, Ku, DNA and MgATP, phosphorylation at each site was abolished (Figure 4, lane 2). Other members of the phosphoinositide 3-kinase-like family of protein kinases are active in manganese, rather than magnesium [30–34], and we have previously shown that DNA-PK is also active in the presence of manganese [33]. We therefore carried out *in vitro* phosphorylation reactions in the presence of manganese instead of magnesium. Phosphorylation at Thr^{2609}, Ser^{2612}, Thr^{2620}, Thr^{2638} and Thr^{2647} also occurred in the presence of manganese, and, as with magnesium, was inhibited by wortmannin (Figure 4, lanes 3 and 4).

We and others have shown previously that highly purified DNA-PKcs has very weak protein kinase activity in the absence of Ku [6,21,35,36]. These low levels are likely due to either traces of Ku in the purified DNA-PKcs or low basal activity of DNA-PKcs. When purified DNA-PKcs was incubated with DNA and MgATP in the absence of Ku, phosphorylation at each of the identified sites was observed, but at a reduced level (Figure 4, lane 5). Thus phosphorylation at Thr^{2609}, Ser^{2612}, Thr^{2620}, Thr^{2638} and Thr^{2647} occurs in the presence of either manganese or magnesium, is inhibited by wortmannin, and is decreased in the absence of Ku.

Phosphorylation of DNA-PKcs *in vivo*

We have previously shown that DNA-PKcs, Ku70 and Ku80 are phosphorylated *in vivo* in cells that have been treated with the protein phosphatase inhibitor okadaic acid [23]. In order to

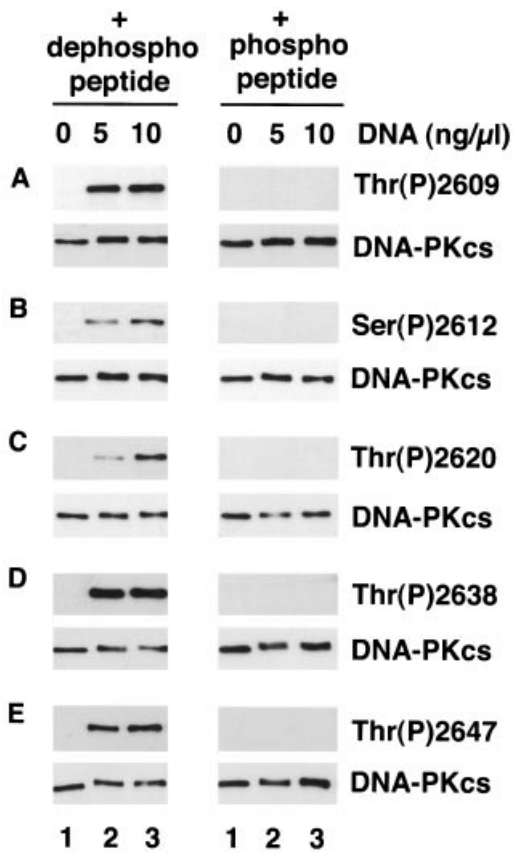

Figure 3 The phosphospecific antibodies recognize autophosphorylated DNA-PKcs *in vitro*

DNA-PKcs (100 ng) and Ku (30 ng) were incubated under standard assay conditions as described in the Materials and methods section, with and without the addition of sonicated calf thymus DNA as indicated. All samples contained MgATP at a final concentration of 2.5 mM. Samples were analysed by SDS/PAGE followed by immunoblot using phosphospecific antibodies raised against phosphorylated Thr^{2609}, Ser^{2612}, Thr^{2620}, Thr^{2638} or Thr^{2647} as indicated in (**A**)–(**E**). For each experiment, immunoblots were either incubated with the phosphospecific antibody in the presence of the respective dephosphopeptide (upper left of each panel), or with the phosphospecific antibody in the presence of the respective phosphopeptide (upper right of each panel). After probing with the phosphospecific antibody, each immunoblot was stripped and incubated with a non-phosphospecific antibody to DNA-PKcs (mAb 42-27) (lower right- and left-hand sections of each panel).

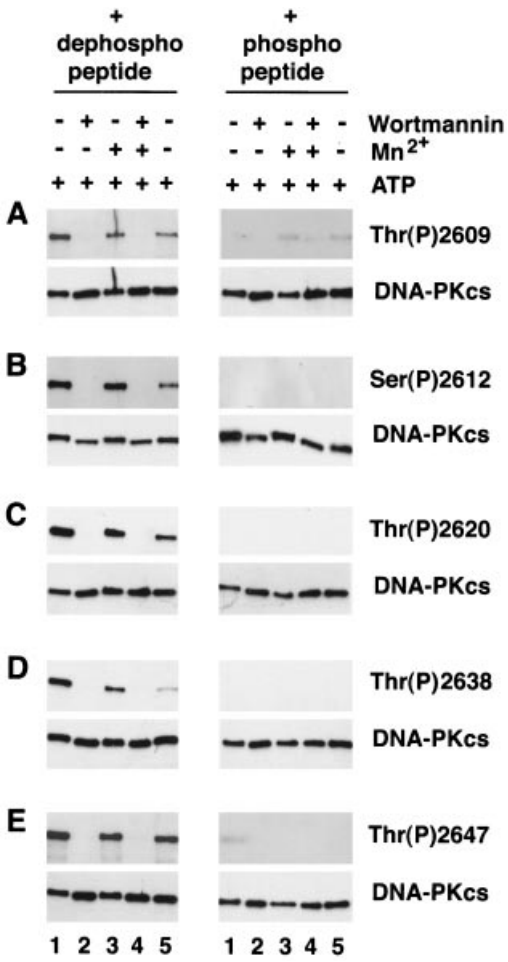

Figure 4 Autophosphorylation at each site is inhibited by wortmannin

DNA-PKcs (100 ng) and/or Ku (30 ng) were incubated and processed exactly as described in Figure 3 except for the following changes. Lanes 2, 1 μM wortmannin was added to samples prior to addition of MgATP; lanes 3 and 4, samples were as in lanes 1 and 2, except that 10 mM Mn^{2+} was added to reactions instead of 10 mM Mg^{2+}. Lane 5, the sample contained DNA-PKcs and MgATP but not Ku. All reactions contained 10 μg/ml sonicated calf thymus DNA.

determine if DNA-PKcs was phosphorylated *in vivo* at the identified *in vitro* phosphorylation sites, cells were treated with okadaic acid for 0, 2 or 3 h, and extracts were prepared and probed by immunoblot using the phosphospecific antibodies as indicated in Figures 3 and 4. DNA-PKcs was found to be phosphorylated at Thr^{2609}, Ser^{2612}, Thr^{2638} and Thr^{2647} in okadaic acid-treated cells (Figure 5). However, no phosphorylation of Thr^{2620} was detected under these conditions (results not shown).

DISCUSSION

Using protein chemistry and MS, we have identified the major autophosphorylation sites in DNA-PKcs. *In vitro*, DNA-PKcs is phosphorylated at Thr^{2609}, Ser^{2612}, Ser^{2624}, Thr^{2638}, Thr^{2647} and Ser^{3205}, with Thr^{2638} likely representing the most abundant site. Phosphorylation at each site was confirmed using phosphospecific antibodies, was dependent on the presence of added DNA, and was inhibited by wortmannin. Using phosphospecific antibodies, Thr^{2620} was also identified as an *in vitro* DNA-PKcs phosphorylation site.

Interestingly, Thr^{2609}, Ser^{2612}, Thr^{2620}, Ser^{2624}, Thr^{2638} and Thr^{2647} are clustered in a 38-amino-acid region, which represents less than 1% of the total amino acids in DNA-PKcs. Also, all of the sites occur distant to the C-terminal catalytic domain of DNA-PKcs. Moreover, Thr^{2609}, Ser^{2612}, Thr^{2638}, Thr^{2647} and Ser^{3205} are conserved in DNA-PKcs from human [10], mouse [37], dog [38], horse [39], chicken [40] and *X. laevis* [41] (Table 2). This high degree of conservation strongly suggests that phosphorylation of DNA-PKcs at these sites is important for DNA-PK function. Although we cannot exclude the possibility that other sites are phosphorylated in DNA-PKcs, this short region of DNA-PKcs appears to contain the major *in vitro* phosphorylation sites.

DNA-PKcs is required for DNA DSB repair via the process of NHEJ, and the catalytic activity of DNA-PKcs is required for its function [12,13]. Biochemical models suggest that DNA-PK assembles at the ends of DNA to form the active protein kinase complex. We therefore propose that the physiological substrates

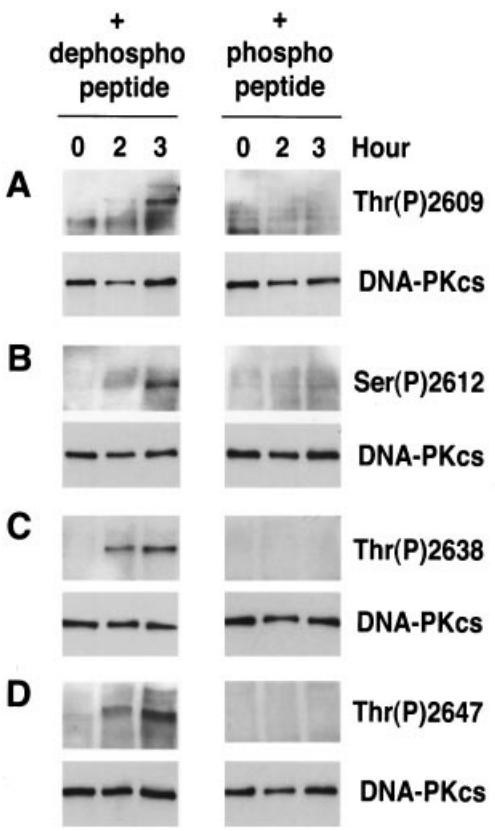

Figure 5 Okadaic acid induces phosphorylation of DNA-PKcs *in vivo*

Human lymphoblastoid cells (BT) were incubated with okadaic acid (1 μM) and harvested after 0, 2 or 3 h. Whole cell extracts were prepared as described in the Materials and methods section and 100 μg of protein was analysed on SDS/PAGE followed by immunoblot using phosphospecific antibodies followed by mAb 42-27, exactly as described in Figure 3.

of DNA-PK are also present at the site of the DNA DSB. We have previously shown that DNA-PKcs, Ku70 and Ku80 undergo autophosphorylation *in vitro*, and that autophosphorylation results in the loss of DNA-PK protein kinase activity and dissociation of DNA-PKcs from the DNA-bound Ku complex [21–23]. We propose that after assembly of the active DNA-PK complex at the DNA DSB, DNA-PK undergoes autophosphorylation, which results in the release of phosphorylated DNA-PKcs from the complex. Another prediction of this model is that protein phosphatases are required to dephosphorylate the phosphorylated inactive DNA-PKcs to allow DNA-PKcs to interact with Ku in a subsequent round of the cycle. We have previously shown that, *in vitro*, protein phosphatases can remove the phosphate groups from autophosphorylated inactive DNA-PKcs and restore DNA-PK protein kinase activity [23]. Moreover, incubation of cells with the protein phosphatase inhibitor, okadaic acid, increased the *in vivo* phosphorylation state of DNA-PKcs and reduced its protein kinase activity [23]. We therefore propose that, by inhibiting protein phosphatases, okadaic acid maintains DNA-PKcs in a more highly phosphorylated, inactive state. Here we show that four of the identified *in vitro* phosphorylation sites in DNA-PKcs (Thr^{2609}, Ser^{2612}, Thr^{2638} and Thr^{2647}) are phosphorylated *in vivo* in okadaic acid-treated cells. As observed previously in cells that were labelled metabolically with $[^{32}P]P_i$, very little *in vivo* phosphorylation of DNA-PKcs was observed in the absence of okadaic acid [23]. These data suggest that DNA-PKcs is rapidly dephosphorylated *in vivo* in the absence of protein phosphatase inhibitors.

From our model, we predict that DNA-PKcs would be phosphorylated *in vivo* only when bound to the site of the DNA DSB. Treatment of cells with DNA-damaging agents, such as ionizing radiation, which produce DNA DSBs, would therefore be expected to induce a transient increase in DNA-PKcs phosphorylation at the sites of the DNA DSBs. To date we have been unable to detect phosphorylation of DNA-PKcs in nuclear extracts from irradiated cells, either by immunoblot using phosphospecific antibodies directly, or by immunoprecipitation with DNA-PKcs-specific antibodies, followed by immunoblot with phosphospecific antibodies. It has been estimated that the number of DNA DSBs produced in human cells by ^{137}Cs ionizing radiation is 2–8 sites/billion base pairs per Gy [42]. Since the human genome contains approx. 3 billion base pairs, 1 Gy of ionizing radiation would be predicted to produce 6–24 DNA DSBs per nucleus. Assuming that two molecules of DNA-PKcs assemble at each DNA DSB, 1 Gy of radiation would be expected to result in no more than 50 phosphorylated DNA-PKcs molecules per nucleus. The number of Ku molecules in the cell has been estimated at approx. 400000 [43]. We estimate that DNA-PKcs is, at most, half as abundant as Ku; thus there are likely to be less than 200000 molecules of DNA-PKcs in the nucleus of a human cell. Thus less than 0.05% of the total population of DNA-PKcs is likely to undergo phosphorylation under these conditions. It is therefore likely that the signal from *in vivo*-phosphorylated DNA-PKcs was below the detection limits of our antibodies. Alternatively, other methods of detection such as immunofluorescence will be required to see DNA-PKcs phosphorylation *in vivo*. Recently, Chen and colleagues [44] identified Thr^{2609} as an *in vitro* and *in vivo* DNA-PKcs phosphorylation site. Moreover, mutation of Thr^{2609} to Ala resulted in radiation sensitivity and impaired rejoining of DNA DSBs [44]. Preliminary results suggest that mutation of other DNA-PKcs phosphorylation identified in this study have a similar phenotype (K. Meek and S. P. Lees-Miller, unpublished work). Thus *in vivo* phosphorylation of DNA-PKcs at one or more of the autophosphorylation sites identified in this study is likely to be required for DNA-PKcs function in NHEJ.

We thank Dr Jane Leitch and Diagnostics Scotland for making the phosphospecific antibodies, Dr Graham Bloomberg for advice on phosphospecific peptides, and Ruiqiong Ye for assistance with cell culture. We are also very grateful to Dr David Chen (Lawrence Berkeley Laboratory, Berkeley, CA, U.S.A.) for communicating his results to us prior to publication. Work in S.P.L.-M.'s laboratory was supported by a grant (no. 13639) from the Canadian Institutes of Health Research. Work in D.R.A.'s laboratory was funded by the Medical Research Council (U.K.), Diabetes UK, Association for International Cancer Research and the pharmaceutical companies supporting the Division of Signal Transduction Therapy Unit in Dundee (AstraZeneca, Boehringer Ingelheim, GlaxoSmithKline, Novo-Nordisk and Pfizer). P.D. was supported by a post-doctoral fellowship of the Alberta Heritage Foundation for Medical Research. A.A.G. is supported by a graduate studentship from the Alberta Heritage Foundation for Medical Research. S.P.L.-M. is a Scientist of the Alberta Heritage Foundation for Medical Research and an Investigator of the Canadian Institutes for Health Research.

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Received 25 June 2002/12 August 2002; accepted 19 August 2002
Published as BJ Immediate Publication 19 August 2002, DOI 10.1042/BJ20020973