DNA-Dependent Protein Kinase Phosphorylation Sites in Ku 70/80 Heterodimer†

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ABSTRACT: Ku antigen is composed of 70 and 82 kDa subunits (Ku70 and Ku80, respectively) that together bind with high affinity to ends of double-stranded DNA and other DNA structures in vitro. When bound to DNA, the Ku 70/80 heterodimer enhances the kinase activity of the catalytic subunit of the DNA-dependent protein kinase, DNA-PKcs. Ku and DNA-PKcs are required for V(D)J recombination and DNA double-strand break repair in vivo and may also play a role in regulation of transcription. Ku is phosphorylated by DNA-PKcs in vitro, and cells that lack DNA-PKcs are deficient in Ku phosphorylation in vitro, suggesting that Ku may be a physiological target for DNA-PK. Here we have identified the sites of DNA-PK phosphorylation in human Ku protein. We find that Ku70 is phosphorylated at a single serine residue, serine 6, located in the putative transcriptional activation domain, and Ku80 is phosphorylated at serines 577 and 580 and at threonine 715. Interestingly, none of the phosphorylation sites identified in Ku correspond to the serine-glutamine consensus for DNA-PK phosphorylation, consistent with previous reports that DNA-PK can recognize additional phosphorylation motifs.

The Ku protein is a heterodimer of 70 and 82 kDa subunits (called Ku70 and Ku80 or Ku86, respectively) that is abundant in the nucleus of human cells (*1*, *2*). Ku was originally identified as an autoantigen due to cross-reaction with immune sera from patients with autoimmune diseases such as lupus and polymyosistis-scleroderma overlap syndrome (*1*). In vitro, Ku binds with high affinity to the ends of double-stranded DNA (*3*, *4*) and, to a lesser extent, to single-stranded DNA (*3*). In addition, Ku interacts with other DNA structures such as hairpins and double-stranded to single-stranded transitions (*5*), and DNA-bound Ku molecules can interact to form looped DNA structures in vitro (*6*). Ku or Ku homologues have also been reported to bind to specific DNA sequences in vitro (*7*−*12*), suggesting that, in addition to binding to DNA ends, Ku can act as a sequence-specific DNA binding protein; however, recent studies on the mechanism of Ku binding to DNA have questioned this interpretation (*13*).

When complexed to double-stranded ends and certain other DNA structures, the Ku heterodimer acts to enhance the kinase activity of the catalytic subunit of DNA-dependent protein kinase, DNA-PKcs[1] (reviewed in *14*, *15*). Although recent studies have shown that DNA-PKcs can bind DNA and function in the absence of Ku (*16*−*18*), in general, the complex formed between DNA-PKcs and Ku in the presence of DNA is referred to as DNA-PK (*14*, *15*, *19*). Murine cells that lack either Ku70 or Ku80 are radiosensitive, defective in DNA double-strand break repair and defective in V(D)J recombination (reviewed in *15*, *19*). In addition, mice that lack Ku70 are susceptible to developing lymphoma, suggesting that Ku70 has tumor suppressor functions in vivo (*20*). Absence or dysfunction of DNA-PKcs in murine, equine, and human cells also confers defective DNA double-strand break repair and V(D)J recombination (reviewed in *14*, *15*, *19*), and loss of DNA-PKcs function contributes to the severe combined immunodeficient (SCID) phenotype in mice and horses (*21*−*23*). Therefore, one of the major roles of Ku and DNA-PKcs is the recognition and/or repair of DNA strand breaks in vivo. The precise function of DNA-PKcs and Ku in DNA double-strand break repair has not been determined; however, it is likely that DNA-PKcs, either alone or in complex with the Ku heterodimer, phosphorylates proteins that may facilitate the end rejoining process. Many in vitro substrates have been identified for DNA-PK (reviewed in *24*−*26*); however, little is known regarding its in vivo substrates. Interestingly, several lines of evidence suggest that Ku may be a relevant physiological target of DNA-PK. For example, Ku is phosphorylated on serine in human cells in vivo (*27*), and phosphorylation of Ku is reduced at least 10-fold in extracts from SCID cells compared to normal murine cells (*28*). In vitro, DNA-dependent phosphorylation of highly purified DNA-PK proteins (Ku80, Ku70, and DNA-PKcs) results in phosphorylation of serine and threonine residues, and phosphorylation correlates with loss of kinase activity, and reduced interaction between phosphorylated DNA-PKcs and DNA-bound Ku (*29*). Others have reported that Ku has ATP-dependent helicase activity (*30*), and it has been suggested that phosphorylation of Ku by DNA-PK may stimulate helicase activity (*15*).

In vitro DNA-PK substrates such as hsp90, SV40 large T antigen, and p53 are phosphorylated on serine or threonine

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[1] Abbreviations: DNA-PK, DNA-dependent protein kinase; DNA-PKcs, catalytic subunit of DNA-PK; SDS−PAGE, SDS−polyacrylamide gel electrophoresis.

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residues that are immediately followed by glutamine, leading to identification of a consensus for DNA-PK phosphorylation that is serine or threonine followed by glutamine, a so-called "SQ" motif (*25*, *26*, *31*). However, other proteins including c-fos and the C-terminal domain of RNA polymerase II are phosphorylated at serine residues (shown below in boldface) that are followed by hydrophobic amino acids, such as valine (R**S**VP) in c-fos and tyrosine (**S**YSPT) in RNA polymerase (*25*, *26*). Some proteins such as the 34 kDa subunit of RPA are phosphorylated at "SQ" and "non-SQ" sites (*32*). These studies clearly show that the consensus motif for phosphorylation by DNA-PK in vitro may be more complicated than was originally suggested. The predicted amino acid sequences of both human Ku70 (*33*, *34*) and Ku80 (*35*, *36*) have canonical "SQ" motifs, for example, serines 51 and 319 in Ku70 in the sequences FESQED and KRSQIY, respectively, and serines 102, 145, 359, and 526 in Ku80 (in the contexts PGSQQAD, SKSQLDI, KSSQVQ, and TKSQIPL respectively). We previously showed that lysine residues adversely affect the ability of DNA-PK to phosphorylate peptide sequences (*31*); therefore, serine 51 in Ku70 and serine 102 in Ku80 could be considered the most likely DNA-PK phosphorylation sites. Recently Jin and Weaver (37) reported that serine 51 of Ku70 is phosphorylated by DNA-PK in vitro, but that serine 51 is not required for the repair of ionizing radiation induced DNA damage. In their study, Ku was immunoprecipitated from a human cell line, and the immunoprecipitated complex was phosphorylated in vitro by the associated polypeptides. Two-dimensional electrophoresis of tryptic digests of phosphorylated Ku70 revealed two polypeptides with high negative charge that the authors concluded represented phosphorylation at serine 51. Since the assignment of serine 51 as the major phosphorylation site in Ku70 was not direct, and since in vitro phosphorylation sites in Ku80 have not been determined, we have examined in detail the DNA-PK phosphorylation sites in Ku70 and Ku80 using direct amino acid sequencing, phosphorylation of synthetic peptides, and mutagenesis of recombinant Ku70 protein. We show that the major sites of phosphorylation in both Ku70 and Ku80 in vitro are not "SQ" sites and that phosphorylation occurs in regions of Ku that have been shown to have transcriptional activation properties or to be required for DNA or protein interaction.

MATERIALS AND METHODS

Protein Purification. DNA-PKcs and Ku were purified from human placenta as described previously (*38*).

Protein Phosphorylation and Generation of Phosphopeptides. Reactions (final volume 100 μL) containing 20 μg of purified Ku and 8 μg of purified DNA-PKcs were incubated with ATP (0.25 mM) containing 10 μCi of [γ -^{32}P]ATP at 30 °C for 1 h as described previously (*29*). The protocol for isolating radiolabeled polypeptides was adapted from Aebersold (*39*). Reactions were stopped by the addition of SDS sample buffer, and proteins were analyzed by electrophoresis on 8% SDS-gels, followed by transfer to nitrocellulose. Proteins were visualized by brief staining with Ponceau S (Sigma) and/or by autoradiography. Membrane containing phosphorylated Ku70 or phosphorylated Ku80 was excised, cut up into approximately 1.5 × 1.5 mm squares, and washed extensively with ddH_2O. Nitrocellulose pieces containing Ku70 or Ku80 from at least 10 reactions (approximately 200 μg or 1.4 nmol of each Ku70 and Ku80) were incubated for 30 min in 1 mL of 0.5% (w/v) PVP-360 (Sigma) dissolved in 100 mM acetic acid. Excess PVP-360 was removed by extensive washing with ddH_2O. The nitrocellulose pieces were rinsed once with 1 mL of digestion buffer (100 mM ammonium bicarbonate, pH 7.8, 2% acetonitrile) and resuspended with the minimal volume of digestion buffer required to submerge the sample pieces (at least 200 μL). Trypsin (sequencing grade, Boehringer Mannheim) was added at approximately 1:50 (wt/wt) protease-to-substrate ratio, and samples were incubated at 37 °C overnight or as indicated. Under these conditions, at least 80% of the ^{32}P label was released into the supernatant. Tryptic phosphopeptides were either further digested by V8, cleaved by CNBr, or analyzed directly by reverse phase HPLC or tricine gel electrophoresis as indicated.

Analysis of Ku70 Phosphopeptides. For CNBr digestion of Ku70, peptides released from in situ tryptic digestion were evaporated to dryness, and then washed with ddH_2O. CNBr (0.1 mg in 100 μL of 70% formic acid) was added, and digestion was allowed to proceed for 16 h in the dark under N_2. Samples were evaporated and washed extensively with ddH_2O and analyzed by tricine gel electrophoresis as described below. Where indicated, Ku70 tryptic phosphopeptides were resuspended in 0.1 M ammonium bicarbonate (pH 8) and bound to Macro-Prep DEAE anion-exchange resin (Biorad) that had been equilibrated in the same buffer. After incubation for 15 min, unbound polypeptides were removed by washing with 500 μL of 50 mM Tris-HCl, pH 8, containing 200 mM KCl. Bound phosphopeptides were eluted by washing the support 3 times with 100 μL of 50 mM Tris, pH 8.0, 800 mM KCl. At least 80% of the radioactivity was recovered from the anion-exchange resin. Samples that eluted from the Macro-Prep DEAE resin were diluted with 50 mM Tris-HCl, pH 8.0, to a final concentration of 100 mM KCl and loaded onto a Mono Q FPLC column (Pharmacia) equilibrated in 50 mM Tris-HCl, pH 8.0, and 100 mM KCl. Phosphopeptides were eluted with a linear gradient of 100−500 mM KCl. A single peak of radioactivity eluted at approximately 400 mM KCl was collected and desalted over a 1 × 25 cm column of Bio-Gel P-2 (BioRad) that had been equilibrated in 50 mM ammonium bicarbonate buffer, pH 7.8. The radioactive sample was further digested or analyzed by amino acid analysis, HPLC, or amino acid sequencing as described below.

Where indicated, Ku70 tryptic phosphopeptides were digested with V8 protease (Sigma) as indicated. The tryptic/V8 peptides were analyzed by tricine gel electrophoresis, or isolated by HPLC and analyzed by amino acid analysis and amino acid sequencing as described below.

Ku80 Phosphopeptides. Ku80 was digested in situ with trypsin as described above and analyzed directly either by reverse phase HPLC or by tricine gel electrophoresis. Where indicated, Ku80 phosphopeptides were further digested with V8 protease in ammonium bicarbonate buffer at 37 °C for 16 h as indicated.

Synthetic Peptides. A peptide corresponding to amino acids 710−729 of human Ku p86 was purchased from SantaCruz Biotechnology Inc. (sc1484p) (amino acid sequence KPSGDTAAVFEEGGDVDDLL). All other peptides were synthesized using f-moc chemistry and HPLC-purified in the

Peptide Synthesis Facility, University of Calgary. Peptides were phosphorylated by highly purified DNA-PK (DNA-PKcs and Ku) under standard assay conditions (*29*) using 0.25 mM final concentration of peptide. Unincorporated ATP was removed by desalting on a 1 × 25 cm column containing Bio-Gel P-2 (BioRad) as described above, and samples were purified by reverse phase HPLC.

Phosphoamino Acid Analysis. Phosphoamino acid analysis was as described (*40*).

Tricine Gel Electrophoresis. Small aliquots of phosphopeptides were removed from the supernatant during digestion at the specified times and analyzed by tricine gel electrophoresis as described by Schagger and von Jagow (*41*) with some modifications. The resolving gel contained 1 M Tris-HCl, pH 8.45, 16.2% acrylamide, 0.45% piperazine diacrylamide (PDA) (BioRad), 0.1% SDS, and 13.3% glycerol. The stacking gel contained 750 mM Tris-HCl, pH 8.45, 3.9% acrylamide, 0.1% PDA, and 0.08% SDS. The cathode running buffer contained 100 mM Tris-HCl, 100 mM Tricine (Sigma), and 0.1% SDS. The anode running buffer was 200 mM Tris-HCl, pH 8.8. 2× tricine sample buffer was purchased from BioRad. Electrophoresis was at 100 V for 100−150 min, and gels were dried without staining and exposed on film at −80 °C overnight. Polypeptide size was estimated using Kaleidoscope markers (Biorad).

Reverse Phase HPLC. Phosphopeptides were generated as described above and acidified by addition of TFA (Pierce) to 10% (v/v) final concentration. Samples were centrifuged at 10000*g* for 10 min and analyzed directly on a 2.0 mm × 150 mm Delta-Pak (Waters) C18 reverse phase column equilibrated in 0.1%TFA/99.9% H_2O. Phosphopeptides were eluted using a linear gradient of 0.1% TFA in 99.9% H_2O to 0.1% TFA in 99.9% acetonitrile at 0.2% acetonitrile per minute. The flow rate was 0.25 mL/min, and 0.25 mL fractions were collected. The column eluent was monitored by absorbance at 214 nm and by Cerenkov emission.

Protein Sequencing and Amino Acid Analysis. Isolated phosphopeptides were analyzed by amino acid analysis on a Beckman 6300 analyzer, or by amino acid sequencing using an Applied Biosystems Model 470A gas phase sequencer in the Protein Sequencing Facility, University of Calgary. For sequencing, ^{32}P-labeled phosphopeptides were covalently attached to Sequelon-AA support (Perceptive Biosystems) by the carbodiimide method according to the manufacturer's instructions. The standard gas phase sequencing cycle was modified to accommodate 90% aqueous acetonitrile as extraction solvent, S3. Product from each sequencing cycle was collected into a sample vial and measured for ^{32}P release by Cerenkov radiation. The same sample was then analyzed for PTH-amino acid release by reverse phase HPLC.

Cloning, Mutagenesis, and Expression of Recombinant Ku70. Recombinant proteins containing the wild-type sequence or mutations at serines 6 and/or 51 were constructed so as to contain an amino-terminal methionine and a carboxy-terminal hexahistidine tag. Nucleotides 1−342 from Ku70.3-II/ puc19 plasmid (a kind gift from Dr. Westley Reeves) were amplified using PCR. Five nucleotides were added to the 5′ end to create an *Nde*I site, and a *Hin*dIII site was built in to the 3′ end. The resulting amplified fragment was digested with *Nde*I and *Hin*dIII, inserted into linearized pET29b(+) vector, and transformed into *E. coli* BL21(DE3). Serines 6 and 51 were mutated using PCR-mediated site-specific mutagenesis as described (*42*). Two mutagenic primers were used in which either T16 or T151 or both were changed to G. The 3′ end of the primer was as used for amplification of the native fragment. All mutations were confirmed by DNA sequencing. Recombinant proteins were expressed, purified over NTA−agarose, and dialyzed into buffer (50 mM Tris-HCl, pH 8.0, 5% glycerol, 100 mM KCl, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF) before use. Recombinant proteins were phosphorylated under standard assay conditions (*29*) using purified DNA-PKcs and Ku. Samples were run on SDS−acrylamide gels and analyzed by autoradiography. Phosphate incorporation was quantitated by phosphoimaging using Fuji MacBas software. Where indicated, phosphorylated recombinant Ku70 proteins were transferred to nitrocellulose and digested with trypsin as described for Ku70/80.

RESULTS

Optimal DNA-PK kinase activity requires the presence of DNA-PKcs, Ku, and a suitable source of DNA ends. Approximately equimolar amounts of DNA-PKcs and Ku are required for maximal kinase activity in vitro (*38*, *43*); however, the stoichiometry of interaction of DNA-PKcs with Ku on DNA is not known. At least two modes of phosphorylation of Ku by DNA-PKcs can be envisioned. Either DNA-PKcs can phosphorylate Ku only when Ku and DNA-PKcs are present as part of a macromolecular (DNA-PK) complex that is assembled on DNA; or DNA-PKcs or DNA-PKcs plus Ku can, in the presence of DNA, phosphorylate Ku molecules that are in solution and that may or may not be bound to DNA. We recently found that chemical cross-linkers or additional proteins can stabilize the formation of a complex containing DNA-PKcs, Ku, and DNA (*44*), suggesting that the interaction between DNA-PKcs and DNA-bound Ku is weak or transient. Therefore, a third possibility exists in which DNA-PKcs associates transiently with DNA-bound Ku heterodimers.

In this study we incubated highly purified human DNA-PKcs with highly purified Ku heterodimer under conditions known to promote phosphorylation of DNA-PKcs and both Ku subunits (*29*). The molar ratio of DNA-PKcs to Ku heterodimer used in this study was 1:10. Thus, the majority of the Ku would not be expected to be part of a DNA-PK complex. Also, an excess of sonicated calf thymus DNA was present as activator, and since Ku binds with high affinity to DNA ends, this suggests that the majority of the Ku in the reactions was associated with DNA. Under these conditions, 0.7 or 0.5 mol of phosphate/mol of protein was incorporated into Ku70 and Ku80, respectively. Increasing the protein concentration or increasing the time of incubation did not significantly increase the amount of phosphate incorporated, suggesting that saturation had been reached. Phosphorylated Ku70 and Ku80 were separated by SDS−PAGE, transferred to nitrocellulose membrane, and digested separately with trypsin in situ as described under Materials and Methods. Phosphoamino acid analysis revealed that Ku70 was phosphorylated only on serine, whereas Ku80 was phosphorylated on serine and threonine (data not shown).

Identification of DNA-PK Phosphorylation Sites in Ku70. Immobilized, phosphorylated Ku70 was incubated with trypsin, and aliquots were removed after 1−16 h and analyzed by tricine gel electrophoresis followed by autora-

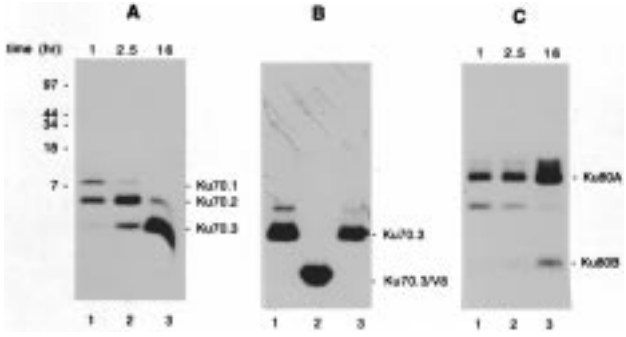


FIGURE 1: Characterization of Ku70 and Ku80 phosphopeptides by tricine gel electrophoresis. The DNA-PK complex was phosphorylated, and Ku70 and Ku80 were digested separately in situ as described under Materials and Methods. Panel A: Nitrocellulose membrane containing phosphorylated Ku70 was incubated with trypsin (1:50 w/w ratio of protease to Ku70) in bicarbonate buffer and acetonitrile as described under Materials and Methods. Aliquots of soluble peptides were removed after 1 (lane 1), 2.5 (lane 2), or 16 h (lane 3) and analyzed on SDS−tricine gels followed by autoradiography as described. Panel B: Tryptic peptides released from immobilized Ku70 were further digested with V8 protease (lane 2) or cleaved with CNBr (lane 3) as described under Materials and Methods. Panel C: Phosphorylated Ku80 was digested in situ with trypsin and analyzed by tricine gel electrophoresis and autoradiography as described above. Aliquots were removed after 1 (lane 1), 2.5 (lane 2), or 16 h (lane 3). Two phosphopeptides, labeled Ku80A and Ku80B, were obtained. The positions of migration of prestained protein markers (in kDa) for all panels (A, B, and C) are shown at the left-hand side of panel A.

diography. At least 80% of the phospholabel was released from immobilized Ku70 by 16 h of incubation. Initially, one major phosphopeptide was observed (called Ku70.1 in Figure 1A). With increasing times of digestion, Ku70.1 was replaced by two faster migrating phosphopeptides, Ku70.2 and Ku70.3 (Figure 1A). The predominant phosphopeptide, Ku70.3, migrated between the 7 kDa marker and radiolabeled ATP (approximately 600 Da) (data not shown and Figure 4B). The tryptic peptide Ku70.3 was sensitive to cleavage with V8 protease, suggesting the presence of acidic amino acids (Figure 1B, lane 2), but was not cleaved by CNBr, suggesting the absence of methionine (Figure 1B, lane 3). Together these data suggest that trypsin cleavage produces a single phosphoserine-containing peptide that contains acidic amino acids and lacks internal methionine.

Several attempts to isolate tryptic phosphopeptide Ku70.3 using reverse phase HPLC were unsuccessful as the phosphopeptide(s) eluted as a broad, poorly resolved peak (data not shown). The presence of acidic amino acids (Figure 1B) suggested that Ku70.3 might bind to anion-exchange resins. Indeed, over 95% of the radioactivity present in Ku70.3 bound tightly to MacroPrep DEAE anion-exchange resin and Mono Q FPLC (see Materials and Methods for details). Ku70.3 tryptic phosphopeptide was therefore purified by anion-exchange chromatography, desalted, and analyzed by Edman degradation and by amino acid analysis. No amino acid sequence was obtained by Edman degradation; however, amino acid analysis was consistent with a polypeptide containing aspartate (or asparagine), threonine, serine, glutamic acid (or glutamine), glycine, alanine, leucine, tyrosine, and lysine (Table 1). Comparison of the amino acid composition with the amino acid sequence of Ku70 revealed a good match with amino acids 2−31 of Ku70 (Table 1). The presence of an excess of glycine in the analysis was likely due to contamination from SDS−PAGE and electroblot transfer buffers. Amino acid analysis of Ku70.3 did not reveal the presence of methionine, consistent with previous observation that Ku70 protein begins with an N-terminal serine residue (*34*). We and others previously found that the amino terminus of full-length Ku70 protein is blocked (*34*, *45*) and the amino terminus of Ku70 protein likely begins with an acetylated serine (*34*). This fact would explain our inability to obtain an amino-terminal sequence from the Ku70.3 phosphopeptide. Taken together, these data suggest that the phosphopeptide Ku70.3 begins with the a modified amino-terminal serine and extends to amino acid 31 in the sequence SGWESYYKTEGDEEAEEEQEENLEASGDYK. This tentative assignment supposes that lysine at position 9 (amino acid 8 in the sequence above) would not be cleaved by trypsin under the conditions used.

Table 1: Amino Acid Composition of the Tryptic Ku70 Phosphopeptide Fraction[a]

amino acid	nmol	composition	predicted composition
Asp/Asn	1.09	3.11	3
Thr	0.39	1.13	1
Ser	1.15	3.29	3
Glu/Gln	3.48	9.94	11
Gly	2.31	6.58	3
Ala	0.87	2.48	2
Leu	0.46	1.322	1
Tyr	0.97	2.75	3
Lys	0.70	2.00	2

[a] Tryptic phosphopeptide Ku70.3 was purified over MonoQ FPLC, desalted over Bio-Gel P2 in ammonium bicarbonate, and analyzed by amino acid analysis. The nanomole yield of each amino acid is shown (nmol). Also shown is the composition of the peptide when normalized for 2 nmol of lysine (composition). The best match between the composition and the known sequence of Ku70 Was to Amino Acids 2−31, in the sequence SGWESYYKTEGDEEAEEEQEENLEASGDYK (*33*, *34*). The amino acid composition of this sequence is shown as "predicted composition". No other sequence of Ku70 was predicted to give a similar composition.

To further identify the phosphopeptide present in Ku70 tryptic peptide Ku70.3, the anion-exchange-purified tryptic phosphopeptide was further digested with V8 protease, and the resulting peptide was analyzed by reverse phase HPLC. V8 protease cleaves after glutamic acid (E) or aspartate (D) and would be expected to cleave the putative amino-terminal phosphopeptide at several sites (see sequence of predicted phosphopeptide, above). HPLC analysis revealed a major peak of ^{32}P-containing peptide that eluted at approximately 22% acetonitrile (Figure 2) that coincided with a homogeneous peak with absorbance at 214 nm (data not shown). Three attempts to obtain an amino acid sequence from this tryptic/V8 phosphopeptide were unsuccessful despite the fact that nanomolar quantities of amino acids were detected by amino acid analysis, again consistent with the presence of a peptide containing a blocked amino terminus. Amino acid analysis was consistent with the presence of a peptide or mixture of peptides containing aspartate (or asparagine), threonine, serine, glutamic acid (or glutamine), glycine, alanine, tyrosine, and lysine (data not shown). No one peptide sequence was consistent with the amino acid analysis; however, our results were consistent with the presence of a series of polypeptides derived from the amino-terminal 31 amino acids of Ku70 that were incompletely cleaved at the series of glutamic acids in the putative amino-terminal sequence.

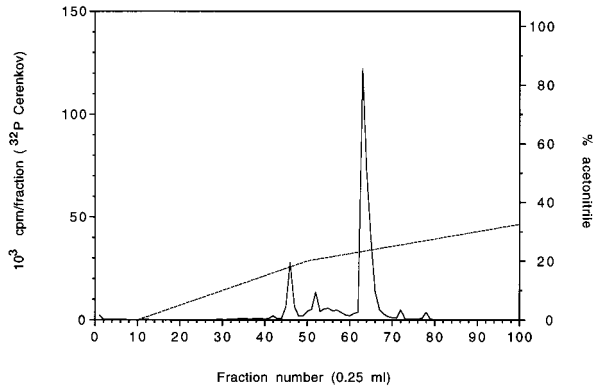


FIGURE 2: Reverse HPLC analysis of Ku70 phosphopeptides. Phosphorylated Ku70 was digested in situ with trypsin, and released peptides were adsorbed onto Macro-Prep DEAE, eluted, and digested with V8 protease as described under Materials and Methods. Tryptic V8 phosphopeptides were isolated by reverse phase C18 HPLC and detected by Cerenkov emission as described. The major peak of radioactivity eluted at fractions 62−64 (solid line) which corresponds to approximately 22% acetonitrile (dashed line).

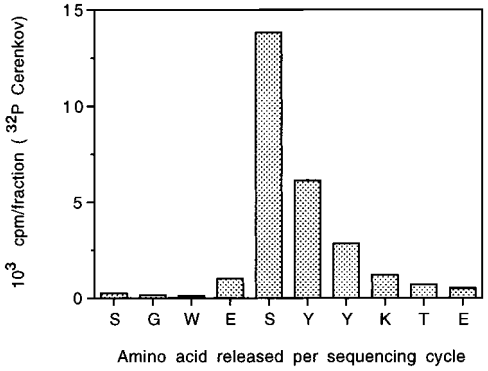
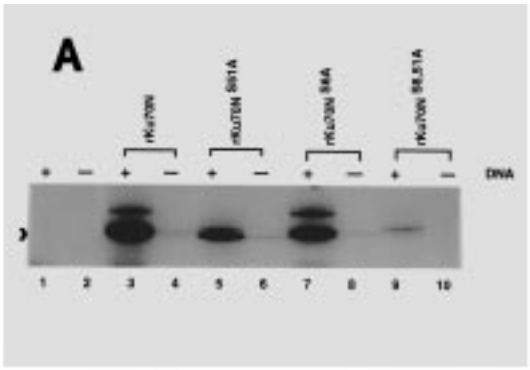


FIGURE 3: Radiochemical sequencing of a phosphorylated synthetic peptide corresponding to Ku70 amino acids 2−14. A synthetic peptide corresponding to amino acids 2−14 of Ku 70 (SGWESYYKTEGDE) was synthesized, phosphorylated by DNA-PK in vitro, and isolated by HPLC as above. The results of gas phase sequencing (*x* axis) and release of radioactive phosphate (*y* axis) in each sequence cycle are shown.

Since the amino-terminal 31 amino acids of Ku70 do not contain a canonical DNA-PK consensus site, i.e., an "SQ" motif, it was important to determine if the N-terminal region of Ku70 could indeed be phosphorylated by DNA-PK. A synthetic peptide corresponding to amino acids 2−14 of Ku70 (SGWESYYKTEGDE) was synthesized and shown to be efficiently phosphorylated by DNA-PK (DNA-PKcs and Ku) only in the presence of DNA (data not shown). Radiochemical sequencing revealed that serine at cycle 5 (corresponding to serine 6 in the predicted amino acid sequence of Ku70) was phosphorylated by DNA-PK (Figure 3).

To further examine phosphorylation at the amino-terminal region of Ku70, we expressed, in bacteria, the amino-terminal 114 amino acids of Ku70 in fusion with a carboxy-terminal hexahistidine tag (rKu70N). This protein was soluble and was phosphorylated by DNA-PK in a DNA-dependent manner (Figure 4A, lanes 3 and 4). Recently, Jin and Weaver (*37*) proposed that Ku70 is phosphorylated at serine 51, which lies in a canonical DNA-PK phosphorylation motif (an "SQ" site). To distinguish between phosphorylation of serine 6 and serine 51, these serines were changed to alanine



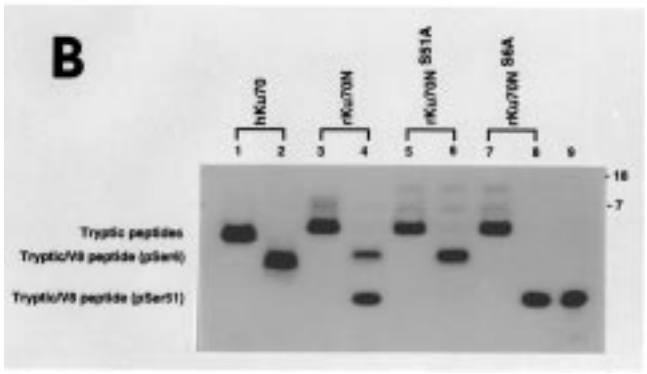


FIGURE 4: Analysis of phosphorylated recombinant Ku70 proteins. Panel A: One microgram each of purified rKu70N, rKu70N^{S51A}, rKu70N^{S6A}, or rKu70N^{S6,51A} was phosphorylated using purified DNA-PK under standard assay conditions. Proteins were analyzed by electrophoresis on 15% acrylamide−SDS gels followed by autoradiography. Samples in odd numbered lanes were incubated with sonicated calf thymus DNA (10 μg/mL) as activator. Samples in even numbered lanes contained an equivalent volume of TE buffer. Lanes 1 and 2 contained DNA-PK alone; lanes 3 and 4 contained rKu70N; lanes 5 and 6 contained rKu70N^{S51A}; lanes 7 and 8 contained rKu70N^{S6A}; and lanes 9 and 10 contained rKu70N^{S6,51A}. Only the polypeptide indicated by the arrowhead cross-reacted with a polyclonal antibody raised to rKu70. The recombinant protein migrated close to the 18 kDa marker. Panel B: Ku70 from purified human Ku70/80 heterodimer (lanes 1 and 2), rKu70N (lanes 3 and 4), rKu70N^{S51A} (lanes 5 and 6), or rKu70N^{S6A} (lanes 7 and 8) was phosphorylated as above and transferred to nitrocellulose. The phosphorylated Ku70 proteins were excised and digested in situ with trypsin as described under Materials and Methods. Polypeptides released by trypsin treatment either were run directly on tricine gels (lanes 1, 3, 5, and 7) or were further digested with V8 protease (0.5 μg, overnight, 37 °C) (lanes 2, 4, 6, and 8). Lane 9 contained [γ-^{32}P]ATP. Equal cpm of ^{32}P were loaded in each lane. Shown is the corresponding autoradiogram. The positions of migration of 18 and 7 kDa Kaleidoscope markers are shown on the right-hand side of the figure.

to create proteins rKu70N^{S51A}, rKu70N^{S6A}, and rKu70N^{S6,51A}. An equal amount of each protein was phosphorylated by DNA-PK, and samples were analyzed by SDS−PAGE followed by autoradiography and quantitation of phosphate incorporation. Removal of serine 51 reduced phosphate incorporation by 61% compared to rKu70N (Figure 4A, lane 5); however, a significant amount of phospholabel remained. Mutation of serine 6 to alanine reduced the phosphate content by approximately 25% compared to rKu70N (Figure 4A, lanes 7 and 8), and mutation of both serines 6 and 51 effectively abolished phosphorylation (Figure 4A, lanes 9 and 10). The small phosphoprotein remaining is likely a contaminating bacterial protein (Figure 4A, lane 9). From these data, we conclude that serine 6 and serine 51 are the

major DNA-PK phosphorylation sites in the context of a soluble, recombinant fragment of the amino-terminal 114 amino acids of Ku70.

To compare the properties of phosphopeptides generated from the recombinant proteins with those generated purified from human Ku70 protein, the purified Ku70/80 heterodimer, rKu70N, rKu70N^{S51A}, and rKu70N^{S6A} were phosphorylated by DNA-PK (DNA-PKcs and Ku) and transferred to nitrocellulose. Tryptic peptides were generated and analyzed either by tricine gel electrophoresis directly or after further digestion by V8 protease as described under Materials and Methods. The major tryptic peptide produced from each of the recombinant proteins migrated at a similar position to that of Ku70.3 produced from the Ku70/80 heterodimer (Figure 4B, lanes 1, 3, 5, and 7). Thus, tryptic peptides containing phosphorylated serine 6 or phosphorylated serine 51 each have characteristics on electrophoresis that are similar to those of the predominant tryptic peptide from Ku70 that had been phosphorylated in the context of the Ku70/80 heterodimer. Further digestion of the tryptic peptide from rKu70N with V8 protease produced two phosphopeptides. One migrated in a similar position to the trypsin/V8 phosphopeptide derived from Ku70/80 heterodimer (Figure 4B, lanes 2 and 4), and one migrated close to the ATP marker (Figure 4B, lanes 4 and 9). Digestion of phosphorylated rKu70N^{S51A} with V8 produced only one phosphopeptide which migrated with the slower migrating peptide from rKu70N and with the trypsin/V8 peptide produced from digestion of Ku70 that was phosphorylated as part of the Ku70/80 heterodimer (Figure 4B, lanes 6, 4, and 2, respectively). From Figure 4A we know that this phosphopeptide is phosphorylated at serine 6. In contrast, digestion of rKu70N^{S6A} with V8 protease generated a single phosphopeptide that migrated close to the ATP marker (Figure 4B, lane 8). Since this phosphopeptide was also present in trypsin/V8 digests of rKu70N, it must represent phosphorylation at serine 51. Phosphorylation at serines 6 and 51 was also confirmed by radiochemical sequencing of HPLC-purified peptides (data not shown). Interestingly, the recombinant rKu70N was accessible to Edman degradation and began with an unblocked serine (data not shown).

To summarize these data, we show that a recombinant protein containing amino acids 1−114 of Ku70 can be phosphorylated at serine 6 and at serine 51. Tryptic peptides that are phosphorylated at either serine 6, serine 51, or serine 6 plus serine 51 are indistinguishable on tricine gel electrophoresis. These phosphopeptides also migrated very close to a tryptic peptide (Ku70.3) derived from Ku70 that had been phosphorylated in the context of the Ku70/80 heterodimer. Phosphorylation at serine 6 and serine 51 in the tryptic peptides can be distinguished by subsequent digestion with V8. V8 digestion of rKu70N produced two phosphopeptides, one phosphorylated at serine 6, and another phosphorylated at serine 51. Only a single phosphopeptide was produced by V8 digestion of Ku70.3, and this migrated with the peptide phosphorylated at serine 6. Taken together, our data strongly suggest that the major site of phosphorylation in native human Ku70 in the context of the Ku70/80 heterodimer is serine 6.

DNA-PK Phosphorylation Sites in Ku80. Digestion of Ku80 with trypsin in situ resulted in the production of two major polypeptides that migrated below the 18 kDa marker

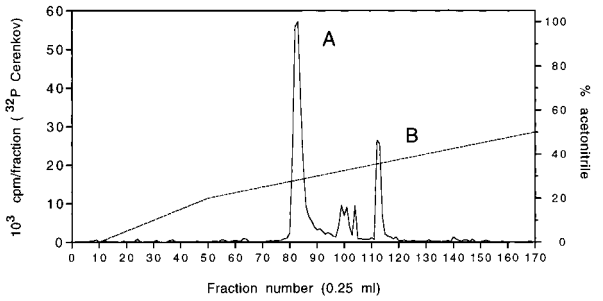


FIGURE 5: Reverse phase HPLC analysis of Ku80 tryptic phosphopeptides. Ku80 was phosphorylated, transferred to nitrocellulose, and digested with trypsin in situ as described under Materials and Methods. Released peptides were purified over reverse phase HPLC, and Cerenkov emissions were monitored as described. Two major peaks of radioactivity eluted at 28 and 36% acetonitrile and are labeled A and B, respectively. ^{32}P label per fraction is shown in solid lines and percent acetonitrile is shown by the dashed line.

on tricine gels (Ku80A and Ku80B in Figure 1C). Analysis of the same tryptic digest by reverse phase HPLC revealed two major peaks of ^{32}P that migrated at 28 and 36% acetonitrile (Figure 5). Electrophoresis of fractions from the first peak from HPLC (peak A) revealed a phosphopeptide that comigrated with Ku80A on tricine gels, while the later eluting peak (labeled B in Figure 5) contained a phosphopeptide that comigrated with Ku80B (data not shown). Phosphoamino acid analysis of the HPLC-purified phosphopeptides indicated that peak A (Ku80A) contained exclusively phosphoserine and peak B (Ku80B) contained exclusively phosphothreonine (data not shown). Both pools were next analyzed for amino acid sequence.

Amino acid sequencing results revealed that phosphopeptide Ku80A contained a mixture of three polypeptides (numbered i, ii, and iii) that were present in approximately equal amounts and began at amino acids 569, 567, and 566, respectively (Figure 6). Peptide Ku80A(i) began at threonine 569 and extended to at least asparagine 593. Sequence Ku80A(ii) began at leucine 567 and was read until alanine 582 at which point low repetitive yield prevented further assignment of amino acids. Sequence Ku80A(iii) began at lysine 566 and was read up to leucine 581. Release of radioactive phosphate was monitored in the same samples at the time of sequencing. Direct comparison of the position of phosphate release with the amino acids present in the same sample allows us to predict which amino acids, in this case serine, were phosphorylated in the peptide sample. Predominant release of phosphate occurred at sequencer cycles 9−16, which corresponded to the presence of a series of serines at positions 9−12 in peptide Ku80A(i), at positions 11−14 of peptide Ku80A(ii), and at positions 12−15 in Ku80A(iii) (Figure 6). The presence of three polypeptides was attributed to incomplete digestion following lysines 565, 566, and 568 despite extended digestion with multiple aliquots of trypsin. These data strongly suggest that serines located between amino acids 577 and 580 (sequencer cycles 9−15) in the sequence SVSSL are phosphorylated by DNA-PK, whereas serines 588 and 591 (Figure 6, Ku80A(i), sequencer cycles 17, 20, and 23) are not.

To confirm that serines at amino acids 577−580 were phosphorylated in Ku80, the HPLC-purified tryptic peptide Ku80A [that contained the three peptides Ku80(i), (ii), and (iii)] was subdigested with V8 protease, which is predicted

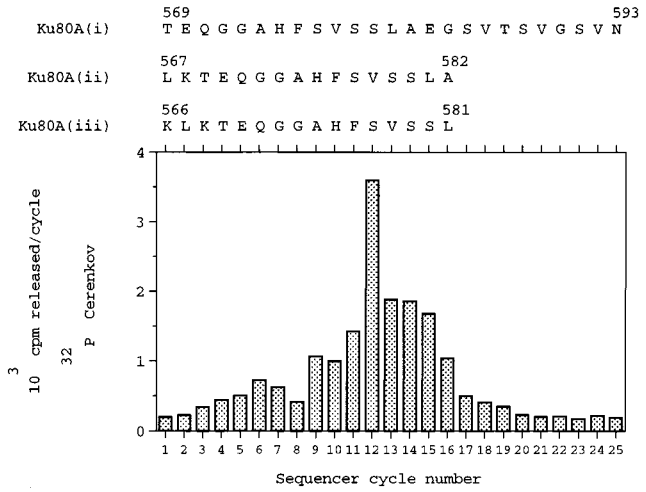


FIGURE 6: Amino acid sequences of phosphopeptide Ku80A (peak A) from tryptic digestion of DNA-PK phosphorylated Ku80. Ku80 was phosphorylated and digested in situ with trypsin as described. Phosphopeptides were purified by HPLC, and the peak Ku80A was analyzed by gas phase amino acid sequencing and for release of radioactive phosphate at each sequencer cycle as described under Materials and Methods. Three polypeptide sequences were read in this fraction [labeled Ku80A (i), (ii), and (iii), upper portion]. Each sequence was present in approximately equal amounts. The initial yield of each polypeptide was between 23 and 28 pmol, and the yield in sequencer cycle number 10 was between 8 and 13 pmol. Sequence Ku80A(i) began at threonine 569 and extended to at least asparagine 593. Sequence Ku80A(ii) began at leucine 567 and was read until alanine 572. Sequence Ku80A(iii) began at lysine 566 and extended past leucine 571. Sequences from peptides Ku80A-(ii) and Ku80(iii) could not be read with confidence past amino acids 582 and 581, respectively. Radioactive phosphate release was measured in each sequencer cycle from the same experiment (see Materials and Methods for details).

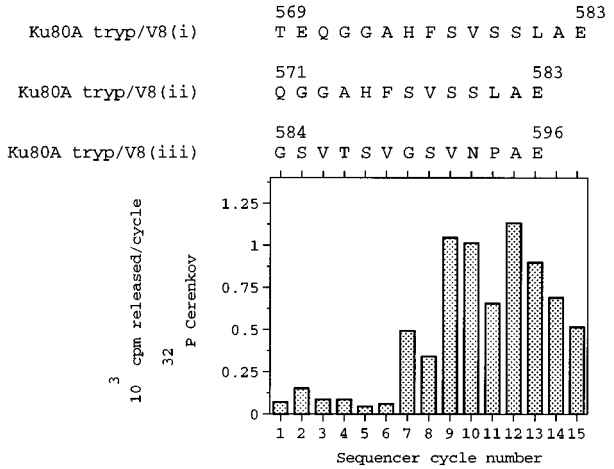


FIGURE 7: Amino acid sequences of phosphopeptide Ku80A (peak A) after V8 digestion. Ku80 tryptic peptide Ku80A (that contained three polypeptides as described in Figure 6) was purified from reverse phase HPLC, digested with V8 protease and repurified by HPLC using a gradient of 0.1% acetonitrile per minute. The peak of radioactivity obtained was analyzed by gas phase sequencing and phosphate release as described in Figure 6. Three amino acid sequences were read from this sample that were present at approximately equal amounts and which began at amino acids 569, 571, and 584 and extended to amino acids 583, 583, and 596, respectively. Each polypeptide was present at approximately 80−100 pmol in cycle 1.

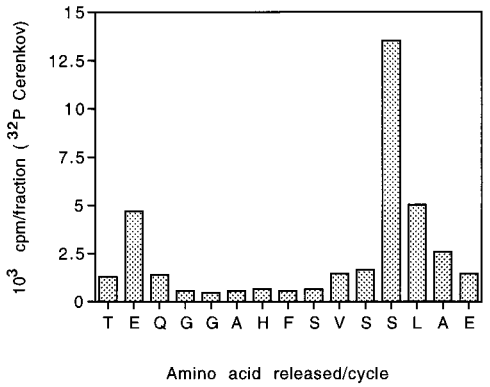


FIGURE 8: Radiochemical sequencing of phosphorylated synthetic peptide corresponding to amino acids 569−583 of Ku80. The synthetic peptide of sequence TEQGGAHFSVSSLAE was synthesized, phosphorylated by DNA-PK in vitro, and purified by HPLC as described under Materials and Methods. The isolated polypeptide was analyzed by gas phase sequencing and phosphate release. Amino acid content and ^{32}P phosphate release at each sequencer cycle are shown.

to cleave after glutamic acids 570 and 583 (Figure 6). HPLC analysis of the sample after V8 cleavage revealed a single broad peak of radiolabel that was analyzed by amino acid sequencing. The results revealed a cluster of three polypeptides beginning at amino acids 569, 571, and 584 (Figure 7). Again, phosphate release was correlated with the positions of multiple serine residues in the peptide sequences. Phosphate release occurred in sequencer cycles 7−15. Serines were present at positions 9, 11, and 12 of phosphopeptide Ku80A tryp/V8(i) and positions 7, 9, and 10 of phosphopeptide Ku80A tryp/V8(ii) (Figure 7). Serines 585, 588, and 591 were present in Ku80A tryp/V8(iii); however, these serines had previously been shown not to be phosphorylated (Figure 6). We conclude that the most likely interpretation of these data from direct amino acid sequencing is that DNA-PK phosphorylates Ku80 protein predominantly at serine 580 (see Figure 6, cycles 12, 14, and 15; and Figure 7, cycles 10 and 12). In addition, our results also strongly suggest that serine 577 is phosphorylated, but to a lesser extent (see Figure 7, cycle 7). However, from our data, we cannot preclude the possibility that serine 579 may also be phosphorylated to some extent.

Since this sequence does not contain a canonical DNA-PK consensus site, a synthetic peptide corresponding to amino acids 569−583 (TEQGGAFHSVSSLAE) of Ku80 was synthesized and incubated with DNA-PKcs and Ku (plus DNA) under conditions that promote phosphorylation. The peptide was efficiently phosphorylated by DNA-PK in vitro in a DNA-dependent manner (data not shown). Amino acid sequencing and phosphate release revealed that the major site of phosphorylation in this peptide was serine 580 in the sequence SVS**S**LAE (Figure 8). Phosphoamino acid analysis revealed that only serine was phosphorylated, suggesting that the small amount of phosphate release in sequencer cycle 2 was due to some loss of peptide from the support.

The phosphothreonine-containing tryptic peptide from Ku80 (Ku80B in Figure 1C and peak B in Figure 5) was analyzed by amino acid sequencing. The only polypeptide present began at amino acid 709 (Figure 9). Ten cycles of sequence were read corresponding to amino acids 709−718 in the sequence DKPSGDTAAV. This sequence possibly extended to the C-terminus of the Ku80 polypeptide since no other lysines or arginines are present in the known sequence (*35*, *36*). Radioactive phosphate release occurred

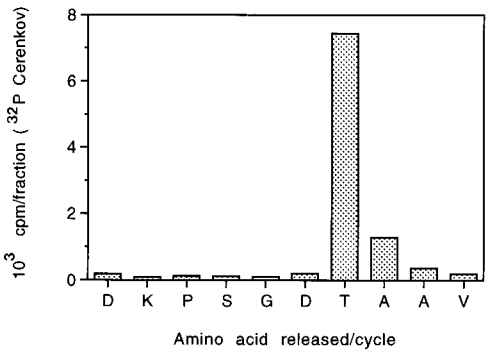


FIGURE 9: Radiochemical sequencing of Ku80 tryptic peptide B. Phosphorylated Ku80 was digested with trypsin in situ and purified by HPLC as described. The isolated polypeptide Ku80B was analyzed by gas phase sequencing and phosphate release. Amino acid content and ^{32}P phosphate release at each sequencer cycle are shown.

at position 7 which corresponds to threonine 715 in Ku80. Also a peptide corresponding to amino acids 710−729 of human Ku (amino acid sequence KPSGDTAAVFEEGGD-VDDLL) was phosphorylated by DNA-PK in vitro on threonine 715 in a DNA-dependent manner (data not shown).

DISCUSSION

We have examined the major sites of phosphorylation of Ku70 and Ku80 after in vitro phosphorylation of the purified human Ku70/80 heterodimer by DNA-PK. Repeated attempts to obtain the amino acid sequence from tryptic and tryptic/V8 phosphopeptides derived from Ku70 were unsuccessful, and amino acid analysis suggested to us that the major phosphopeptide in Ku70 originated from the extreme amino terminus. Previous work from ourselves (*45*) and from others (*34*) has shown that the amino terminus of human Ku70 protein is blocked and therefore inaccessible to Edman degradation. This fact alone does not prove that the phosphopeptides originated from the amino terminus of Ku70 since internal amino acids can become blocked or cyclize during purification of phosphopeptides. However, we also show that both a synthetic peptide and a recombinant protein consisting of the amino-terminal 144 amino acids of Ku70 are phosphorylated at serine 6 in the sequence (M)SGWESYYKTE and this result is consistent with amino acid analysis of the purified phosphopeptide from Ku70. Together these data provide strong evidence that, under the conditions of our study, Ku70 is phosphorylated predominantly at serine 6.

A recent study by Jin and Weaver in which Ku was immunoprecipitated from human cells and phosphorylated in in vitro kinase assays led the authors to conclude that serine 51, which is located in a known DNA-PK consensus motif, was phosphorylated (*37*). This conclusion was based on analysis of the phosphopeptide pattern by two-dimensional electrophoresis and from computer-aided predictions. Interestingly, two phosphopeptides were actually observed on two-dimensional electrophoresis; however, this was not explained further. Our data suggest that although serine 51 can be phosphorylated by DNA-PK in the context of the recombinant Ku70 protein, under the conditions used in our study, where the Ku70/80 heterodimer is present in excess of DNA-PKcs, this residue is not the major site of phosphorylation. With this in mind, it is interesting to note that Ku is present in large molar excess over DNA-PKcs in both human cells (*46*) and rodent cells (*47*), suggesting that in the cell phosphorylation of Ku heterodimer may occur predominantly under conditions in which the majority of the Ku is not present in a complex with DNA-PKcs. During our studies we did not detect a phosphopeptide containing phosphorylated serine 51 either by either tricine gel electrophoresis or by HPLC followed by protein sequencing. However, we cannot exclude the possibility that serine 51 represents a minor site of phosphorylation under the conditions used here (see, for example, Figure 2). It is also possible that serine 51 is fully phosphorylated in purified human Ku heterodimer, and hence is not available for phosphorylation in vitro. However, we think this unlikely since Ku was purified in the absence of phosphatase inhibitors. Phosphorylation at serine 51 is not required for DNA double-strand break repair in vivo (*37*), and we (*29*) and others (*28*) have shown that phosphorylation of Ku by DNA-PK in vitro is not required for binding of Ku to DNA ends in vitro. The possible role of phosphorylation of serine 6 in the function of Ku remains to be determined. It also remains to be determined at what sites Ku is phosphorylated in vivo.

From our biochemical studies we conclude that trypsin followed by V8 released a phosphopeptide that likely spanned amino acids 2−14 of Ku70 in the sequence (M)-SGWESYYKTE. V8 protease would be expected to cleave this sequence after glutamic acid 5; however, this was not observed despite extended digestion. We suspect that the presence of the phosphorylated serine at position 6 or the presence of hydrophobic amino acids (at positions 4, 7, and 8) may have affected the affinity of V8 for this site. Similarly, the amino-terminal tryptic peptide Ku70.3 polypeptide would have been expected to be cleaved by trypsin after lysine 9. We suggest that the highly unusual stretch of acidic amino acids at positions 11−25 of Ku70 may have hindered recognition of this site by trypsin and/or may have formed elements of secondary structure that prevented interaction with the protease.

Serine 6 in Ku70 lies in an acidic amino-terminal region that has been suggested to have transcriptional transactivation properties (*48*). Homologues of Ku70 have been identified in the yeast *Saccharomyces cerevisiae* (*49*), *Drosophila* (*50*), the tick *Rhipicephalus appendiculatus* (*51*), hamsters (accession number 1836065), rat (*52*), and mice (accession number S25149) as well as humans (*33*, *34*). Serine 6 and the acidic amino-terminal region are highly conserved between mammals (Table 2). In contrast, serine 6 is not conserved in Ku70 homologues in the tick, *Drosophila*, and yeast, and there is generally less amino acid conservation in this region (Table 2). Several studies have suggested that DNA-PK and Ku also play a role in transcription by both RNA polymerases I and II (*53*−*55*) and it is possible that Ku and DNA-PK have functions other than those in DNA double-strand break repair. It will therefore be of interest to determine if phosphorylation at serine 6 affects the function of the Ku70/80 heterodimer in DNA repair and transcription. Others have suggested that phosphorylation of Ku by DNA-PK may affect its helicase activity and be important for V(D)J recombination (*15*). The identification of phosphorylation sites in Ku70 and Ku80 will allow these hypotheses to be tested directly.

We also show, by direct protein sequencing, that DNA-PK phosphorylates Ku80 on serine 577 and serine 580 and possibly on serine 579 in the sequence SVSSL as well as

Table 2: Alignment of Amino-Terminal Sequences of Ku70 from Various Species[a]

	1									10										20
human	M	S	G	W	E	**S**	Y	Y	K	T	E	G	D	E	E	A	E	E	E	Q
mouse	M	S	E	W	E	**S**	Y	Y	K	T	E	G	E	E	·	·	E	E	E	E
rat	M	S	E	W	E	**S**	Y	Y	K	T	E	G	E	E	·	·	E	E	E	E
hamster	M	S	G	W	E	**S**	Y	Y	K	T	E	G	D	E	E	A	E	E	E	Q
tick	M	D	Q	P	W	M	R	Q	D	D	D	E	S	D	D	E	S	S	T	V
Drosophila	M	S	T	W	N	P	E	N	D	V	D	L	L	S	G	S	E	D	E	E
S. cerevisiae	M	R	P	V	T	N	A	F	G	N	S	G	E	L	N	D	Q	V	D	E

[a] Sequences were obtained using a "blastp" search of ncbi databases using the amino terminus of human Ku70 (from references *33*, *34*) and were aligned using the GCG program "pileup". The accession numbers for the sequences were human Ku70 (M32865), mouse Ku70 (S25149), hamster (ncbi identification number l836065). the sequence of rat Ku70 was from ref *52*. Also shown are the aminoterminal sequences from the Ku homologues in the tick *Rhipicephalus appendiculatus* (*51*), *Drosophila* (*50*), and the yeast *S. cerevisiae* (*49*). The phosphorylated serine at position 6 is shown in bold.

on threonine 715 in the sequence GDTAAV. Phosphorylation at serine 580 but not serine 577 or serine 579 was confirmed using a synthetic peptide. The lack of phosphorylation at serine 577 and serine 579 in the context of the synthetic peptide may reflect differences between the structure or context of the native protein compared with the synthetic peptide. Interestingly, these phosphorylation sites lie in regions of Ku80 that have been shown by yeast two-hybrid screens to be important for both subunit interaction and DNA binding (*48*). Serines 577, 579, and 580 are conserved in mouse (*56*) and hamster (*57*), while threonine 715 is conserved between human and mouse but not hamster (*57*).

Several interesting observations can be made regarding the assignment of the DNA-PK phosphorylation sites in Ku. First, none of these sites corresponds to the canonical DNA-PK consensus site of serine or threonine followed by glutamine (SQ motif) (*31*). However, we have previously observed several other polypeptides and proteins that are phosphorylated at non-SQ sites including Fos (serine 286 in the sequence R**S**VP) and the CTD of RNA polymerase (SP**S**YSPT, where the phosphorylated serine is shown in boldface) (*25*, *26*). Indeed, these data strongly suggest that DNA-PK can phosphorylate serines (or threonines) that are followed by other amino acids such as tyrosine (RNA polymerase II CTD, and Ku70), valine, leucine, or alanine (fos, and Ku80), suggesting a predisposition for hydrophobic amino acids in the +1 position.

Finally it is perhaps worth noting that DNA-PK appears to have a predisposition for phosphorylation sites at the extreme termini of its substrates. For example, threonines 3 and 5 are phosphorylated in vitro in the α form of the 90 kDa heat-shock protein (*58*), p53 is phosphorylated at amino-terminal sites, also in a putative transcriptional activation domain (*31*), and the amino-terminal 40 amino acids contain all of the multiple DNA-PK phosphorylation sites in the 32 kDa subunit of RPA (*32*). Synthetic peptides containing heptapeptide repeats of the CTD of RNA polymerase II are also phosphorylated by DNA-PK in vitro (*25*, *26*), and the CTD is thought to exist as a relatively unstructured "tail" that extends from the large subunit of the polymerase. We have also shown that the amino-terminal leader sequence of a recombinant protein containing the DNA binding POU domain of the Oct transcription factor is phosphorylated by DNA-PK in vitro (*26*), and here we show that serine 6 in the amino-terminal transcriptional activation domain of Ku70 and threonine 715 at the carboxyl-terminal region of Ku80 are also phosphorylated by DNA-PK in vitro. We speculate that predisposition toward amino-terminal or carboxy-terminal sites perhaps may indicate better accessibility of the substrate to the active site of the large DNA-PK complex.

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REFERENCES

1. Mimori, T., Akizuki, M., Yamagata, H., Inada, S., Yoshida, S., and Homma, M. (1981) *J. Clin. Invest. 68*, 611−620.
2. Mimori, T., Hardin, J. A., and Steitz, J. A. (1986) *J. Biol. Chem. 261*, 2274−2278.
3. Mimori, T., and Hardin, J. A. (1986) *J. Biol. Chem. 261*, 10375−10379.
4. Blier, P. R., Griffith, A. J., Craft, J., and Hardin, J. A. (1993) *J. Biol. Chem.* 268, 7594−7601.
5. Falzon, M., Fewell, J. W., and Kuff, E. L. (1993) *J. Biol. Chem. 268*, 10546−10552.
6. Cary, R. B., Peterson, S. R., Wang, J., Bear, D. G., Bradbury, E. M., and Chen, D. J. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 4267−4272.
7. Knuth, M. W., Gunderson, S. I., Thompson, N. E., Strasheim, L. A., and Burgess, R. R. (1990) *J. Biol. Chem 265*, 17911−17920.
8. May, G., Sutton, C., and Gould, H. (1991) *J. Biol. Chem. 266*, 3052−3059.
9. Messier, H., Fuller, T., Mangal, S., Brickner, H., Igarashi, S., Gaikwad, J., Fotedar, R., and Fotedar, A. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 2685−2689.
10. Jacoby, D. B., and Wensink, P. C. (1994) *J. Biol. Chem. 269*, 11484−11491.
11. Roberts, M. R., Han, Y., Fienberg, A., Hunihan, L., and Ruddle, F. H. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 6354−6458.
12. DiCrce, P. A., and Krontiris, T. G (1995) *Proc. Natl. Acad. Sci. U.S.A 92*, 10137−10144.
13. Bliss, T. M., and Lane, D. P. (1997) *J. Biol. Chem. 272*, 5765−5773.
14. Dynan, W. S., and Yoo, S. (1998) *Nucleic Acids Res. 26*, 1551−1559.
15. Chu, G. (1997) *J. Biol. Chem. 272*, 24097−24100.
16. Yaneva, M., Kowalewski, T., and Lieber, M. R. (1997) *EMBO J. 16*, 5098−5112.
17. Hammarsten, O., and Chu, G. (1998) *Proc. Natl. Acad. Sci. U.S.A. 95*, 525−530.

18. West, R. B., Yaneva, M., and Lieber, M. R. (1998) *Mol. Cell. Biol. 18*, 5908−5920.
19. Jeggo, P. A., Taccioli, G. E., and Jackson, S. P. (1996) *Bioessays 17*, 949−957.
20. Li, G. C., Ouyang, H., Li, X., Nagasawa, H., Little, J. B., Chen, D. J., Ling, C. C., Fuks, Z., and Cordon-Cardo, C. (1998) *Mol. Cell 2*, 1−8.
21. Blunt, T., Gell, D., Fox, M., Taccioli, G. E., Lehmann, A. R., Jackson, S. P., and Jeggo, P. A. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 10285−10290.
22. Kirchgessner, C., Patil, C. K., Evans, J. W., Cuomo, C. A., Fried, L. M., Carter, T., Oettinger, M. A., and Brown, J. M. (1995) *Science 267*, 1178−1183.
23. Wiler, R., Leber, R., Moore, B. B., VanDyk, L., Perryman, L. E., and Meek, K. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 11485−11489.
24. Lees-Miller, S. P. (1996) *Biochem. Cell. Biol. 74*, 503−512.
25. Anderson, C. W., and Lees-Miller, S. P. (1992) *Crit. Rev. Eukaryotic Gene Expression 2*, 283−314.
26. Anderson, C. W., Connelly, M. A., Lees-Miller, S. P.. Lintott, L. G., Zhang, H., Sipley, J. A., Sakaguchi, K., and Appella, E. (1995) in *Methods in Protein Structure Analysis* (Atassi, M. Z., and Appella, E., Eds.) pp 395−406, Plenum Press, New York.
27. Yaneva, M., and Busch, H. (1986) *Biochemistry 25*, 5057−5063.
28. Boubnov, N. V., and Weaver, D. T. (1995) *Mol. Cell. Biol. 15*, 5700−5706.
29. Chan, D. W., and Lees-Miller, S. P. (1996) *J. Biol. Chem. 271*, 8936−8941.
30. Tuteja, N., Tuteja, R., Ochem, A., Taneja, P., Huang, N. W., Simoncsits, A., Susic, S., Rahman, K., Marusic, L., Chen, J., Zhang, J., Wang, S., Pongor, S., and Falaschi, A. (1994) *EMBO J. 13*, 4991−5001.
31. Lees-Miller, S. P., Sakaguchi, K., Ullrich, S., Appella, E., and Anderson, C. W. (1992) *Mol. Cell. Biol. 12*, 5041−5049.
32. Zernik-Kobak, M., Vasunia, K., Connelly, M., Anderson, C. W., and Dixon, K. (1997) *J. Biol. Chem. 272*, 23896−23904.
33. Chan, J. Y. C., Lerman, M. I., Prabhakar, B. S., Isozaki, O., Santisteban, P., Kuppers, R. C., Oates, E. L., Notkins, A. L., and Kohn, L. D. (1989) *J. Biol. Chem. 264*, 3651−3654.
34. Reeves, W. H., and Sthoeger, Z. M. (1989) *J. Biol. Chem. 264*, 5047−5052.
35. Yaneva, M., Wen, J., Ayala, A., and Cook, R. (1989) *J. Biol. Chem. 264*, 13407−13411.
36. Mimori, T., Ohosone, Y., Hama, N., Suwa, A., Akizuki, M., Homma, M., Griffith, A. J., and Hardin, J. A. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 1777−1781.
37. Jin, S., and Weaver, J. T. (1997) *EMBO J. 16*, 6874−6885.
38. Chan, D. W., Mody, C. H., Ting, N. S. Y., and Lees-Miller, S. P. (1996) *Biochem. Cell. Biol. 74*, 67−73.
39. Aebersold, R. (1993) *A practical guide to protein and peptide purification for microsequencing* (Matsudaira, P., Ed.) 2nd ed., pp 103−124, Academic Press, Inc., New York.
40. Boyle, W. J., van der Geer, P., and Hunter, T. (1991) *Methods Enzymol. 201*, 110−149.
41. Schagger, H., and von Jagow, G. (1987) *Anal. Biochem. 166*, 368−379.
42. Hemsley, A., Arnheim, N., Toney, M. D., Cortopassi, G., and Galas, D. J. (1989*) Nucleic Acids Res. 17*, 6545−6551.
43. Morozov, V. E., Falzon, M., Anderson, C. W., and Kuff, E. L. (1994) *J. Biol. Chem. 269*, 16684−16688.
44. Ting, N. S. Y., Kao, P. N., Chan, D. W., Lintott, L. G., and Lees-Miller, S. P. (1998) *J. Biol. Chem. 273*, 2136−2145.
45. Lees-Miller, S. P., Chen, Y.-R., and Anderson, C. W. (1990) *Mol. Cell. Biol. 10*, 6472−6481.
46. Anderson, C. W., and Carter, T. H. (1996) *Curr. Top. Microbiol. Immunol. 217*, 91−111.
47. Ting, N. S. Y., Chan, D. W., Lintott, L. G., Allalunis-Turner, J., and Lees-Miller, S. P. (1998) *Radiat. Res.* (in press).
48. Wu, X., and Lieber, M. R. (1996) *Mol. Cell. Biol. 16*, 5186−5193.
49. Feldmann, H., and Winnacker, E. L. (1993) *J. Biol. Chem. 268*, 12895−12900.
50. Jacoby, D. B., and Wensink, P. C. (1994) *J. Biol. Chem. 269*, 11484−11491.
51. Paesen, G. C., Zanotto, P. M., and Nuttall, P. A. (1996) *Biochim. Biophys. Acta 1305*, 120−124.
52. Yang, S.-H., Nussenzweig, A., Yang, W.-H., Kim, D., and Li, G. C. (1996) *Radiat. Res. 146*, 603−611.
53. Kuhn, A., Gottlieb, T. M., Jackson, S. P., and Grummt, I. (1995) *Genes Dev. 9*, 193−203.
54. Maldonado, E., Shiekhattar, R., Sheldon, M., Cho, H., Drapkin, R., Rickert, P., Lees, E., Anderson, C. W., Linn, S., and Reinberg, D. (1996) *Nature 381*, 86−89.
55. Giffin, W., Torrance, H., Rodda, D. J., Prefontaine, G. G., Pope, L., and Hache, R. J. G. (1996) *Nature 380*, 265−268.
56. Falzon, M., and Kuff, E. L. (1992) *Nucleic Acids Res. 20*, 3784−3788.
57. Errami, A., Smider, V., Rathmell, W. K., He, D. M., Hendrickson, E. A., Zdzienicka, M. Z., and Chu, G. (1996) *Mol. Cell. Biol. 16*, 1519−1526.
58. Lees-Miller, S. P., and Anderson, C. W. (1989) *J. Biol. Chem. 264*, 17275−17280.

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