DNA Ligase I Is an In Vivo Substrate of DNA-Dependent Protein Kinase and Is Activated by Phosphorylation in Response to DNA Double-Strand Breaks[†,‡]

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ABSTRACT: DNA-dependent protein kinase (DNA-PK) phosphorylates several cellular proteins in vitro, but its cellular function and natural substrate(s) in vivo are not established. We reported activation of DNA ligase in cultured normal human epidermal keratinocytes (NHEK) on exposure to the DNA-damaging compound bis-(2-chloroethyl) sulfide. The activated enzyme was identified as DNA ligase I, and this activation was attributed to phosphorylation of the enzyme. Here, we show that the phosphorylation is mediated by DNA-PK and that DNA ligase I is one of its natural substrates in vivo. DNA ligase I phosphorylation-cum-activation is a response specific to DNA double-strand breaks. We also demonstrate that affinity-purified inactive DNA ligase I is phosphorylated and activated in vitro by HeLa Cell DNA-PK confirming the in vivo observations. The findings specify the roles of DNA-PK and DNA ligase I in mammalian DNA double-strand break repair.

In mammals, three distinct DNA ligases have been identified and characterized (*1*). Among these, the ∼120 kDa DNA ligase I has been studied the most. This enzyme is DNA substrate-specific and is involved in DNA repair and replication. DNA ligases III and IV have similar substrate specificity in that they can ligate both DNA−DNA and RNA−DNA substrates. DNA ligase IV is a ∼ 100 kDa enzyme (*2*) and is considered to be specific for V(D)J recombination (*3*), which assembles the variable (V), diversity (D), and joining (J) antigen receptor gene segments within a lymphoid cell, generating a diverse repertoire of receptors in T and B cells.

We previously reported DNA ligase activation (enhancement of DNA ligase activity) in normal human epidermal keratinocyte (NHEK) on exposure to bis-(2-chloroethyl) sulfide (sulfur mustard, SM)[1] (*4*). The results of our experiments using actinomycin D and cycloheximide indicated that this enzyme's activation involves protein modification rather than new protein synthesis. It has been suggested that poly (ADP-ribose) polymerase (PARP) regulates DNA ligase activity and this action is at the ligation step (*5*). Cell-free extract (CFE) from SM-exposed NHEK in which ^{3}H NAD$^+$ was metabolically labeled at adenine by incubation with ^{3}H-adenosine did not show any ^{3}H-labeled DNA ligase on chromatographic purification, suggesting that DNA ligase is not a substrate for PARP (*6*). It is, therefore, of interest to elucidate the nature of the modification and its mechanism for DNA ligase activation following DNA damage. Prignet et al. (*7*) reported that bovine DNA ligase I can be activated by cellular casein kinase II by phosphorylation in vitro. The results of our preliminary studies indicated that phosphorylation could be the mechanism of DNA ligase activation in vivo when the cells are exposed to SM (*6*). Here, we show that in cultured NHEK exposed to the bifunctional alkylating compound SM, DNA ligase I is activated via phosphorylation by DNA-PK.

MATERIALS AND METHODS

Chemicals and Cells. Sulfur mustard **(**SM, >98% pure) was obtained from the U.S. Army Edgewood Chemical Biological Center, Aberdeen Proving Ground, MD, and chloroethyl ethyl sulfide (CEES) was obtained from Aldrich Chemical Co. Milwaukee, WI. Frozen-stock, normal human epidermal keratinocyte (NHEK) culture and keratinocyte growth medium (KGM) were from Cambrex, Walkersville, MD. $^{33}PO_4^{-3}$ (specific activity, 8810 Ci/mmol) and [γ-^{33}P]-ATP (specific activity, 2000 Ci/mmol) were obtained from Perkin-Elmer, Boston, MA. Agarose-ATP, ^{3}H-adenosine, oligo dT cellulose, calf thymus DNA (cat. no. D3664), spermidine, protein-G precipitation kit, and wortmanin were obtained from Sigma-Aldrich, St. Louis, MO. Human DNA-PK and terminal transferase were purchased from Promega, Madison, WI. ^{3}H oligo dT was synthesized according to the procedure described previously (*4*). Bovine DNA ligase I monoclonal antibody was initially a kind gift from Dr. Tomas

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[1] Abbreviations: CEES, chloroethyl ethyl sulfide; CFE, cell-free extract; DNA-PK, DNA-dependent protein kinase; DMAP, 6-dimethyl amino purine; SSB, single-strand break; DSB, double-strand break; SM, sulfur mustard; PARP-1, poly (ADP-ribose) polymerase-1; ATM, ataxia telangiectasis mutated; ATR, ataxia telangiectasis Rad-3 related protein.

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Lindahl, Imperial Cancer Research Fund, U.K., and was later purchased from Sigma-Aldrich, St. Louis, MO. All other chemicals were of the purest grade available.

Cell Culture. Frozen-stock NHEK (passage 2, 5×10^5 cells/vial) were cultured in 150 cm^2 tissue culture flasks (10^5 to 3×10^5 cells/flask) in KGM according to company instructions to initiate the culture. When these monolayer cells became 70−80% confluent, they were subcultured to 100% confluency and used in the experiments. Confluent cells were used as a model system because they do not actively synthesize DNA, allowing focus on unscheduled DNA synthesis and repair mechanisms. NHEK from a single donor and subcultured only to passage 3 were used.

Exposure of NHEK to SM and CEES and Metabolic ^{33}P Labeling of DNA Ligase in NHEK. The experimental and control cells were washed with 37 °C normal saline and then exposed to SM in a phosphate-free medium (148 mM NaCl, 5 mM KCl, 10 mM $NaHCO_3$, 10 mM glucose, 25 mM HEPES, pH 7.4, 326 mOsm/L). Frozen SM stock (4 mM) obtained in normal saline was thawed, dissolved, and then applied to the washed monolayer of NHEK along with $^{33}PO_4^{-3}$. For preparation of CFE, the cells were placed in a 37 °C phosphate-free medium containing 0.5 mCi of $^{33}PO_4^{-3}$ in a total volume of 20 mL per150 cm^2 flask with or without SM in saline. In later experiments (Figure 7 (Wortmanin) and Figure 8), to improve radiolabeling, this protocol was changed by only reducing the incubation volume to 10 mL, other conditions remaining the same. These cells were then set aside for 30 min at room temperature followed by incubation for 2 h at 37 °C in a cell culture incubator. Following this, the medium containing $^{33}PO_4^{-3}$ was removed, and the cells were washed with ice-cold saline and then collected by scraping the monolayers. The cells were extracted in a buffer containing the following: 300 mM NaCl, 50 mM Tris-HCl, pH 7.5, 1 mM each of EDTA and DTT, 0.1% Triton X-100, and 10% glycerol. The following protease inhibitors at the stated concentration were included in the buffer: phenyl methyl sulfonyl fluoride, 1 mM; pepstatin, 5 μg/mL; aprotinin, 2 μg/mL; leupeptin, 1.5 μg/mL; and *N*-α-*P*-tosyl-L-lysine chloromethyl ketone, 0.5 μg/mL (*4*). The cells were harvested after a 2-h incubation, suspended in the extraction buffer, and homogenized by vortexing and by several freeze−thaw cycles. The CFE was obtained by centrifugation at 20 000*g* for 20 min at 4 °C and then used for determination of DNA ligase activity and affinity purification of DNA ligase I.

Metabolic ^3H-Labeling of NAD. NAD^+ in NHEK was labeled by incubating cells with ^3H adenosine 2−8 according to the procedure described by Malanga and Althaus (*8*).

DNA Ligase and Protein Assays. DNA ligase was assayed using poly dA-^3H oligo dT substrate as previously described (*4*). Protein was assayed using Bio-Rad protein assay reagent and γ-globulin as the standard.

DNA Ligase I Affinity Chromatography. The bovine DNA ligase I monoclonal antibody was immobilized using the Pierce amino link kit (Pierce, Rockford, IL). The column contained approximately 1 mg of the conjugated antibody and was stored in PBS containing 0.04% sodium azide. The protocol for DNA ligase I binding from the CFE, washing, and elution are given below.

For DNA ligase I binding, 1 mL CFE was absorbed into the column and left to stand at room temperature for 1 h. The column was then washed first with 14 mL of PBS, followed by 8 mL of 1 M NaCl to remove any weakly bound proteins. Bound DNA ligase I was eluted with 12 mL of Pierce IgG elution buffer at a flow rate of ∼ 1 mL/min. Fractions were neutralized with 1 M Tris-HCl, pH 9.5, when needed. Extra washes with 1 M NaCl were used to reduce radioactivity to background level before elution.

In Vitro DNA Ligase I Phosphorylation by DNA-PK. The following solutions described by the vendor (Promega, Madison, WI) were used: (a) Dulbecco's PBS, PBS containing 0.1 g/L $CaCl_2$ and 0.1 g/L of hydrated $MgCl_2$; (b) DNA-PK reaction premix, 13 mM spermidine, 4 mM $MgCl_2$ in 0.05× Dulbecco's PBS; (c) DNA-PK sample buffer, 25 mM Hepes-KOH, pH 7.5, 12.5 mM $MgCl_2$, 50 mM KCl, 20% glycerol, 1 mM DTT, and 0.1% NP 40. The phosphorylation reaction mixture was constituted as follows: reaction premix, 20 μL; 1 mg/mL DNA, 2 μL; 40 mM ATP, pH 7.0, in water, 2 μL; [γ-^{33}P]ATP, 10 μL (1 μCi/μL); DNA ligase I, 100 μL (∼ 75 ng protein contained in 50 mM Tris-HCl, 1 mM EDTA, and 10% glycerol); DNA-PK sample buffer, 110 μL; and 25 μL of 10 mg/mL acetylated BSA. The reaction mixture was incubated with 5 μL of DNA-PK containing a minimum of 50 units of the enzyme according to the supplier at 30 °C for an hour. Triplicate sets were incubated, then combined and passed through the affinity column to obtain the elution profile shown in Figure 5.

Analysis and Quantitation of DNA Ligase I Phosphorylation. As described above, the phosphorylated DNA ligase I was isolated by affinity chromatography using bovine DNA ligase I monoclonal antibody that cross reacts with human DNA ligase I. Under identical conditions, the extents of phosphorylation in different experiments can be directly correlated to the chromatographic peak areas.

Immunoprecipitation with Protein G. Immunoprecipitation of phosphorylated DNA ligase I was done according to the directions supplied by the vendor. DNA ligase I in the affinity column eluate was coprecipitated with BSA using 70% ammonium sulfate. Immunoprecipitation buffer supplied with the kit was added to this precipitate or cell-free extract, and the mixture was incubated with 5 μg of DNA ligase I monoclonal antibody at 4 °C overnight. Following this incubation, the samples were shaken with protein-G beads overnight at 4 °C and then processed according to the directions provided. The bound protein was either eluted with Pierce IgG elution buffer for assay of enzyme activity or treated with SDS−PAGE sample buffer for use in gel analysis.

Gel Electrophoresis and Autoradiography. Immunoprecipitated DNA ligase I samples were analyzed by SDS−PAGE using pre-cast acrylamide gels (10%), which were coomassie blue-stained and dried. The dried gels were exposed to KODAK X-Omat AR film for 8 days at −80 °C and then developed.

RESULTS AND DISCUSSION

DNA ligase phosphorylation in SM-exposed cells was demonstrated by size fractionation and affinity chromatography. The CFE prepared from NHEK exposed to 1 mM SM in the presence of $^{33}PO_4^{-3}$ was fractionated through a Sephacryl S-200 sizing column. DNA ligase coeluted with ^{33}P radioactivity, suggesting that the enzyme might be

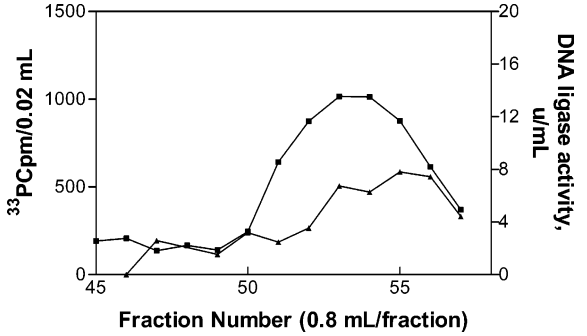

FIGURE 1: Elution profile of phosphorylated DNA ligase I from agarose-ATP column. Cell-free extract (CFE) prepared from NHEK exposed to 1 mM SM in the presence of $^{33}PO_4^{-3}$ was first fractionated using a 1 × 100 cm Sephacryl S-200 column. Fractions containing DNA ligase and coeluting ^{33}P activities were pooled and absorbed onto an agarose-ATP affinity column equilibrated with a column buffer (10 mM NaCl, 5 mM PO_4^{-3}, 10 mM Tris-HCl, 1 mM EDTA, pH 7.5, and 20% glycerol). After a complete wash of unbound radioactivity with the equilibrating buffer, the column was eluted with the same buffer containing 10 mM Mg^{2+} and 1 mM ATP. ■, ^{33}P-DNA ligase I eluting from the column; ▲, enzyme activity.

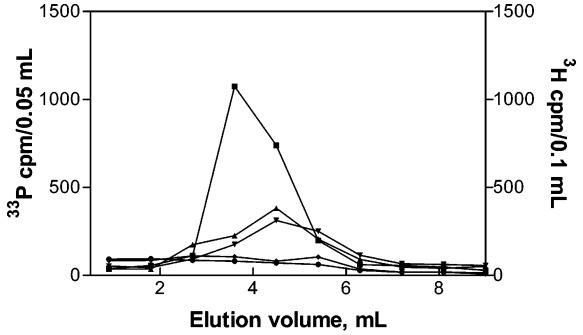

FIGURE 2: Elution profile of radioactivity associated with protein bound to the bovine DNA ligase I monoclonal antibody column. Cell-free extracts (CFEs) prepared under different conditions were absorbed into the column, and the protein components retained after several washes were eluted. NAD^+ represents NHEK in which NAD^+ was metabolically labeled with ^{3}H at adenine. Cells other than NAD^+ were NHEK treated with $^{33}PO_4^{-3}$ and as specified. Cells were exposed to either 1 mM SM or 4 mM CEES. ▼, SM-unexposed NHEK control; ◆, SM-unexposed NAD^+ control; ●, SM-exposed NAD^+; ■, SM-exposed NHEK; ▲, CEES-exposed NHEK.

phosphorylated (data not shown). Since size fractionation is nonspecific, the column fractions containing both ^{33}P label and DNA ligase activity were pooled and analyzed on an agarose−ATP affinity column (Figure 1). The results showed coelution again of both ^{33}P label and DNA ligase activity confirming enzyme phosphorylation. We previously observed that exposure to SM (0.3, 0.5, and 1.0 mM) resulted in a highly reproducible activation of DNA ligase at all SM concentrations, and the maximum was at 1.0 mM SM (*4*), which was used for this study. The radiolabel may be derived from either a DNA ligase−AMP intermediate or from a stable covalent modification of the protein such as phosphorylation. However, this result, though indicative of phosphorylation, lacks the specificity required to unequivocally identify the particular DNA ligase that is being phosphorylated.

Bovine DNA ligase I antibody cross-reacts with human DNA ligase I (personal communication, Dr. Tomas Lindahl, Imperial Cancer Research Fund, U.K.) and, therefore, can be used to specify whether DNA ligase I in SM-exposed NHEK is phosphorylated. CFE prepared from confluent NHEK exposed to SM in the presence of $^{33}PO_4^{-3}$ was passed through a bovine DNA ligase I monoclonal antibody column, and the bound protein was then eluted. The bound human DNA ligase I was found to be ^{33}P-labeled, indicating that the enzyme was phosphorylated (Figure 2). This result confirms our observations based on Sephacryl S-200 sizing gel and agarose−ATP affinity chromatographic analyses described earlier. To eliminate any possibility of DNA ligase activation through concurrent ADP ribosylation and phosphorylation, CFE prepared from SM-exposed NHEK in which NAD^+ was metabolically labeled using ^{3}H-adenosine was similarly bound and eluted through the affinity column. The eluted protein had no excess SM-induced radioactivity over the SM-unexposed control (Figure 2). These results suggested that, in SM-exposed NHEK, DNA ligase I is activated by phosphorylation because dephosphorylated DNA ligase I is inactive (*7*).

DNA-dependent protein kinase (DNA-PK) is activated in response to DNA double-strand break (DSB). In vitro phosphorylation of several functional proteins by DNA-PK is known. However, the in vivo protein substrates are unknown (*9*). Interferon regulatory factor-3 has been shown to be an in vivo substrate (*10*). SM causes intra- and interstrand cross-links, alkyl adducts, and DSBs in DNA (*11*). Therefore, it is possible that DSBs introduced by SM may activate DNA-PK that in turn may phosphorylate DNA ligase I. If any other kinase activity would be involved in this phosphorylation, then all damage and single-strand breaks (SSBs) would also result in phosphorylation of DNA ligase I. To examine such a possibility, CFE was prepared following exposure of NHEK to 2-chloroethyl ethyl sulfide (CEES), a chemical that reacts with cellular DNA to form mono-alkyl adducts and does not introduce DSBs (*11*). Cell-free extracts prepared from both unexposed control and CEES-exposed NHEK cultures containing $^{33}PO_4^{-3}$ were passed through the monoclonal antibody column, and the bound protein was eluted. The elution profiles are shown in Figure 2. Both control and experimental (CEES) profiles are superimposable, indicating that the higher level of phosphorylation observed in CFE prepared from SM-exposed NHEK requires DSBs. The observed low level of phosphorylation in SM-unexposed control cells may be due to the formation of an unstable DNA ligase−AMP intermediate in these cells. A structural study of DNA-PK showed that the enzyme has interacting single- and double-strand DNA binding sites and that single-strand DNAs inhibit kinase activation (*12*). The observed DNA ligase I phosphorylation and activation may, therefore, be attributed to DSBs.

We utilized immunoprecipitation and autoradiography techniques for further confirmation of DNA ligase I phosphorylation in SM-exposed NHEK. For these experiments, DNA ligase I was first enriched by immunoprecipitation from two different sources: (a) purified enzyme from DNA ligase I affinity chromatography and (b) enzyme from in vivo radiolabeled CFE (i.e., from cells exposed to 1 mM SM in the presence of $^{33}PO_4^{-3}$). These enriched samples were analyzed by SDS−PAGE. The dried gel obtained from in vivo radiolabeled CFE was exposed to X-ray film to get an autoradiogram. The autoradiogram was obtained from the CFE because the sample would be freshly labeled. The

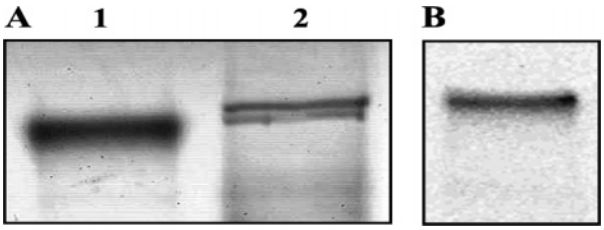

FIGURE 3: Demonstration of DNA ligase I phosphorylation in SM-exposed NHEK by SDS−PAGE (A, lanes 1 and 2 stained with coomassie blue) and autoradiography (B). Immunoprecipitated DNA ligase I samples obtained from both (a) an affinity-purified enzyme preparation and (b) the cell-free extract (CFE) from NHEK exposed to 1 mM SM in the presence of $^{33}PO_4^{-3}$ (in vivo radiolabeling) were used for these analyses. The in vivo radiolabeled DNA ligase I (lane 2) migrated similar to the affinity-purified enzyme (lane 1) and also produced a clear autoradiogram (B).

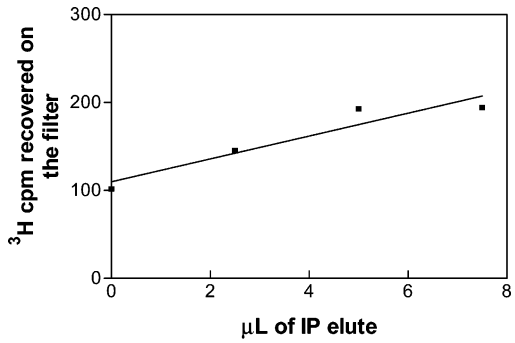

FIGURE 4: Demonstration of DNA ligase activity in in vivo phosphorylated DNA ligase I. Affinity purified in vivo ^{33}P-labeled DNA ligase I was precipitated using 70% ammonium sulfate and 1 mg/mL BSA as co-precipitant. The precipitate was dissolved in immunoprecipitation buffer described earlier, and then the enzyme was immunoprecipitated. The protein G−agarose-bound immunoprecipitate was eluted, and the neutralized eluate (protein concentration < 0.1 μg/mL) was assayed for enzyme activity.

protein-stained gels (panel A) and the autoradiogram (panel B) are shown in Figure 3. A single prominent DNA ligase I band was obtained from the affinity-purified enzyme (lane 1). The immunoprecipitated protein from the radiolabeled CFE comigrated with the purified enzyme (lane 2) and also produced a clear autoradiogram. In control experiments using SM-unexposed cells, immunoprecipitated protein obtained from the CFE showed a protein band corresponding to DNA ligase I by SDS−PAGE, but no discernible autoradiograms (data not shown), which may be explained by very poor enzyme phosphorylation or the formation of an unstable enzyme−AMP complex or both in unexposed cells. These results confirm that, in SM-exposed NHEK, DNA ligase I is phosphorylated.

To demonstrate that the in vivo phosphorylated DNA ligase I is enzymatically active, phosphorylated DNA ligase I that was eluted from the affinity column was concentrated and then immunoprecipitated. The precipitate was eluted with Pierce IgG elution buffer, the same buffer that had been used earlier to elute bound DNA ligase I from the monoclonal antibody affinity column. The neutralized eluate was assayed and found to be enzymatically active (Figure 4).

To test whether DNA ligase I is a DNA-PK substrate in vitro, we purified the enzyme from CFE obtained from confluent SM-unexposed NHEK using the monoclonal antibody column. This purified DNA ligase I was then used as the substrate in a reaction mixture containing commercially

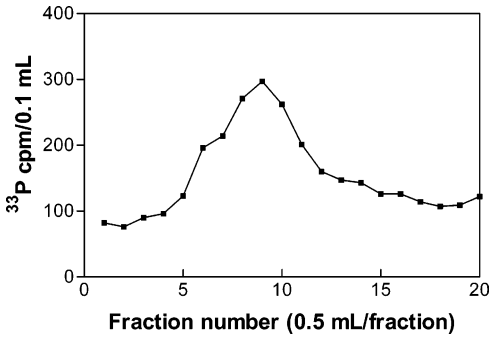

FIGURE 5: Elution profile of ^{33}P radioactivity associated with in vitro DNA-PK-phosphorylated DNA ligase I protein bound to bovine DNA ligase I monoclonal antibody column.

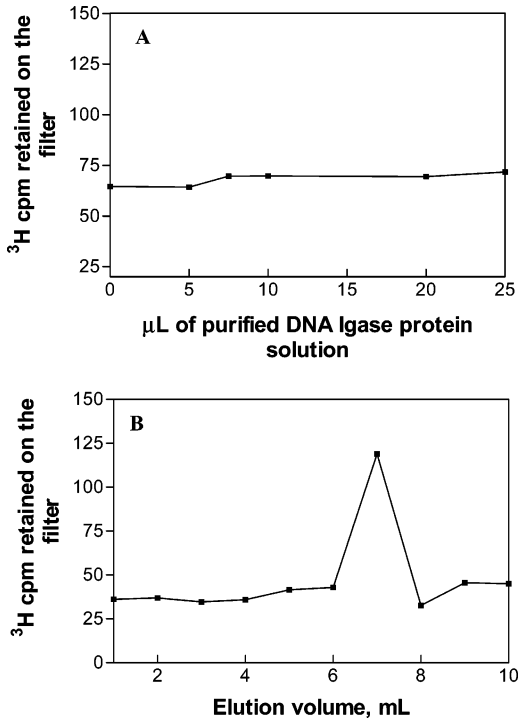

FIGURE 6: In vitro activation of DNA ligase I protein by phosphorylation. DNA ligase I protein was purified from the cell-free extract (CFE) prepared from confluent NHEK using the monoclonal antibody column, then phosphorylated in vitro using HeLa cell DNA-PK and re-purified using the monoclonal antibody column. The eluted fractions were assayed for DNA ligase activity using the ^{3}H oligo dT substrate. (A) Affinity-purified enzyme activity before phosphorylation; (B) affinity-purified enzyme activity after phosphorylation (in vitro kinase treatment).

obtained HeLa cell DNA-PK and [γ-^{33}P]ATP. The reaction mixture was then passed through the antibody column as before. The elution profile shown in Figure 5 indicates that DNA ligase I is ^{33}P-labeled, confirming that DNA ligase I is a substrate of DNA-PK in vitro. In a separate experiment, a control reaction mixture containing all the reactants except DNA-PK showed a typical wash-out profile without a ^{33}P-labeled DNA ligase I peak (data not shown). This observation excludes the presence of any contaminating protein kinase associated with any of the components in the reaction mixture. The affinity-purified DNA ligase I protein that was used in the in vitro phosphorylation experiment was assayed for enzyme activity. No activity was observed (Figure 6A) in conformity with a previous report that the dephosphorylated enzyme is inactive (*7*).

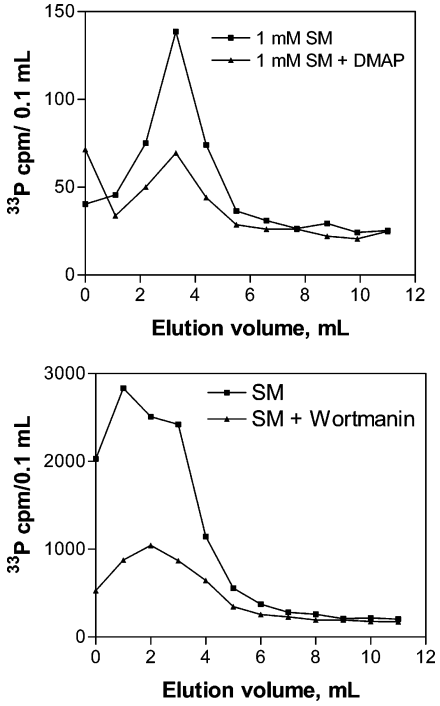

FIGURE 7: Elution profiles from the affinity column of radiolabeled DNA ligase I present in the cell-free extract (CFE) prepared from NHEK exposed to 1 mM SM in the absence or presence of either 0.1 mg/mL 6-DMAP (general protein kinase inhibitor) or 2 μM wortmanin (DNA-PK specific inhibitor).

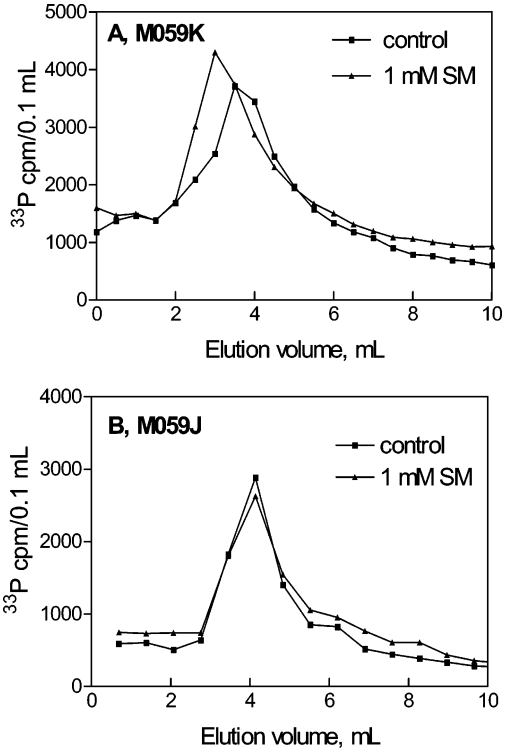

FIGURE 8: Elution profiles from affinity chromatography (DNA ligase I antibody column) of in vivo ^{33}P-labeled DNA ligase I from M059K (DNA-PK$^+$) and M059J (DNA-PK$^-$) cells. (A) M059K; (B) M059J.

To demonstrate that DNA ligase I was activated via phosphorylation, we phosphorylated the purified enzyme protein in vitro in a separate experiment using a low level of ^{33}P to minimize interference with the DNA ligase assay. As shown in Figure 6B, DNA ligase I activity was recovered in one fraction. This confirms that DNA-PK phosphorylation results in enzyme activation. The difference in the elution volumes of the enzyme from the affinity column as noted in different experiments may be due to differences in column behavior and/or sample size.

To confirm our observation that DNA-PK phosphorylates and activates DNA ligase I, we conducted experiments using a general kinase inhibitor, 6-dimethyl amino purine (6-DMAP) (*13*) and a specific DNA-PK inhibitor, wortmanin (IC_{50} of ~250 nM). Wortmanin forms covalent adducts with a highly conserved lysine residue in the active site of DNA-PK, and the formation of such adducts is irreversible (*14*). We compared DNA ligase I phosphorylation in SM-exposed NHEK in the absence or presence of either 6-DMAP (0.1 mg/mL) or wortmanin (2 μM). Both 6-DMAP and wortmanin almost completely abolished DNA ligase I phosphorylation (Figure 7). The small radioactive peak observed in the presence of either 6-DMAP or wortmanin is comparable to that observed in SM-unexposed control (Figure 2), suggesting that this radioactivity is not associated with SM-induced enzyme phosphorylation but could be due to an enzyme−AMP intermediate. Results from preliminary experiments using a higher wortmanin concentration (40 μM) showed that this peak is insensitive to wortmanin. Therefore, the ^{33}P-labeled enzyme fraction represented by this peak is not phosphorylated DNA ligase I, and is inactive.

On exposure of NHEK to SM, poly (ADP-ribose) polymerase (PARP) is activated (*15*). PARPs represent a superfamily of 18 proteins of which PARP-1 is the major component. DNA-PK belongs to the phosphatidyl inositol-3 kinase family of enzymes that include damage responsive kinases, ATM, and related ATR; wortmanin inhibits DNA-PK more effectively than ATM or ATR (*16*). Activated PARP-1 inhibits ATM (*17*). Therefore, the observed inhibition of DNA ligase I phosphorylation by wortmanin can be attributed to the specific inhibition of DNA-PK. In addition, experiments using human cell-free extracts demonstrated that wortmanin inhibits NHEJ that requires DNA-PK (*18*). In this connection, it should be noted that wortmanin sensitizes cells to agents that produce DSBs (*19*, *20*). Above observations strongly support our conclusion that DNA ligase I is an in vivo substrate of DNA-PK.

To obtain some genetic evidence for the involvement of DNA-PK in the regulation of DNA ligase I, we examined the DNA ligase I phosphorylation pattern in the DNA-PK-deficient MO59J vis-à-vis the DNA-PK-proficient MO59K human glioblastoma cell clones. The elution profiles of ^{33}P-labeled DNA ligase I bound to the antibody affinity column are shown in Figure 8, panel A (M059K) and panel B (M059J). In the case of MO59K cells, about 20% excess SM-induced phosphorylation was observed (Figure 8A), whereas in the case of MO59J cells, SM-exposed and unexposed cells show nearly identical profiles (Figure 8B). In these experiments, the radioactivity associated with DNA ligase I possibly represents an enzyme−AMP complex as observed in SM-unexposed NHEK (Figure 2). The higher levels of radioactivity in SM-unexposed M059K and M059J cells compared to SM-unexposed NHEK may be due to (a) a higher concentration (2-fold) of radiolabel used in these experiments, and (b) M059K and M059J cells being transformed cell clones. In experiments using SM-unexposed

NHEK also, increasing ^{33}P concentration by decreasing the volume of radiolabeling medium from 20 to 10 mL as described in Materials and Methods increased the constitutive ^{33}P-label (data not shown). The above observations further support our conclusion that DNA-PK catalyzes the phosphorylation of DNA ligase I in vivo.

DNA-PK acts upstream of p53, and p53 has been shown to be phosphorylated by DNA-PK in vitro (*21*) in response to DNA damage. Therefore, the events leading to the activation of the DNA repair pathway may involve p53 that initiates cell cycle arrest. We investigated whether p53 is also involved in DNA ligase I activation following DNA damage. We observed that DNA ligase I phosphorylation-cum-activation following SM exposure is unaffected in NHEK in the presence of the p53 inhibitor curcumin, as well as in the small cell human lung carcinoma p53-null mutant clone NCI-H1299 (data not shown). These results suggest that during SM-induced DNA damage repair, DNA-PK activates DNA ligase I, a natural substrate, via phosphorylation, which is independent of p53.

Activation of DNA ligase I by phosphorylation suggests that the enzyme activity is regulated by a phosphorylation and dephosphorylation phenomena. The ability of DNA-PK to phosphorylate DNA ligase I derived from confluent NHEK indicates that DNA-PK-specific phosphorylation sites are available on the protein. This is possible only if (a) the enzyme is dephosphorylated following confluence, (b) there is a specific cell cycle-dependent phosphatase(s), and (c) DNA ligase I activity is regulated by phosphorylation and dephosporylation cycles. It has been reported that DNA ligase I is phosphorylated by cyclin B/p34^{cdc2} in X*enopus laevis* during new DNA synthesis and oocyte maturation (*22*), suggesting that the active form of DNA ligase I is phosphorylated. Phosphatase-treated DNA ligase I is inactive (*7*). We observed that affinity-purified DNA ligase I protein from confluent NHEK is inactive and that the kinase-treated protein is active (Figure 6B). The activation probably is responsible for the accelerated rate of DNA DSB repair that is nearly complete in about 4 h in SM-exposed NHEK (*4*) and 5 h in human fibroblasts exposed to ultra-soft X-rays (*23*). This rapid repair would ensure a minimum delay in the cell cycle progression.

In conclusion, we have demonstrated that DNA ligase I is an in vivo substrate of DNA-PK and is activated by phosphorylation in response to DNA double-strand breaks. It has been suggested that DNA-PK is required for DSB repair; however, its function has not been defined. Our results show how DNA-PK may be involved in DSB repair. Recent reports suggest that, besides participating in DNA replication and repair including DSB repair (*24*, *25*), DNA ligase I may have other functions such as recombination (*26*−*28*) and maintenance of genomic stability (*24*, *25*). In HeLa cells, a ∼200 kDa factor, which is dependent on DNA-PK, preferentially increases intermolecular ligation, typical of eukaryotic systems (*27*). In another report (*28*), cells from a patient with severe combined T^-B^- immunodeficiency was found to have defects similar to NHEJ-defective rodent cells, and these cells had dramatically increased radiation sensitivity, decreased DSB rejoining, and reduced fidelity in signal and coding joint formation during V(D)J recombination. However, this patient was not deficient in the known factors required for NHEJ, namely, Ku70, Ku80, DNA-PKcs, Xrcc4, DNA ligase IV, and Artemis. In vitro, addition of DNA ligase I stimulates recombination (*26*). Moreover, DNA ligase I point mutations have been shown to cause replication failure, genomic instability, and increased susceptibility to cancer (*24*). It has also been reported that DNA ligase I-null mutant cells show normal DNA repair activity, but with altered DNA replication and reduced genomic stability (*25*). These observations support our proposition that DNA-PK-activated DNA ligase I is likely to have functions other than DNA replication and repair.

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