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Identification of DNA-PKcs phosphorylation sites in XRCC4 and effects of mutations at these sites on DNA end joining in a cell-free system

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Abstract

Nonhomologous end joining (NHEJ) is the principal mechanism for repairing DNA double-strand breaks in mammalian cells. NHEJ requires at least three protein components: the DNA-dependent protein kinase catalytic subunit (DNA-PKcs), Ku protein, and the DNA ligase IV/XRCC4 (DNL IV/XRCC4) complex. Although DNA-PKcs phosphorylates several sites within itself and these other proteins, the significance of phosphorylation at individual sites is not yet understood. Here we investigate the effects of DNA-PKcs-mediated phosphorylation at two sites in XRCC4. One is a previously described site at serine 260; the other is a newly mapped site at serine 318. XRCC4 bearing mutations at these sites was co-expressed with DNL IV, the resulting complexes were purified, and activity was tested in a cell-free end-joining system reconstituted from recombinant and purified proteins. Substitution of alanine for serine 260 or 318, which prevents phosphorylation at these positions, or aspartate for serine 260, which mimics constitutive phosphorylation, had no significant effect on overall end-joining activity. In the assay system used, DNA-PKcs is not essential, but when present, arrests the reaction until phosphorylation occurs, in effect establishing a reaction checkpoint. Mutations at serines 260 and 318 did not affect establishment or release from the checkpoint. Results demonstrate that DNA-PKcs-mediated phosphorylation of XRCC4 serine 260 and serine 318 does not directly control end-joining under the conditions tested.

Keywords: XRCC4; DNA-PKcs; Phosphorylation; NHEJ; Double-strand breaks

1. Introduction

DNA double-strand breaks (DSBs) are a particularly lethal form of DNA damage. DSBs are generated by ionizing radiation, radiomimetic drugs, and certain recombination nucleases [1,2]. In vertebrates, the same DSB repair proteins participate in repair of radiation-induced DNA damage and V(D)J recombination [3]. Humans or mice with defective DSB repair genes are radiosensitive and immunodeficient [4].

The principal mechanism of DSB repair in human cells is nonhomologous end joining (NHEJ). In humans and other vertebrates, NHEJ requires at least three protein components: Ku, the DNA-dependent protein kinase catalytic subunit (DNA-PKcs), and the DNA ligase IV/XRCC4 (DNL IV/XRCC4) complex [4]. Ku binds DNA ends and recruits DNA-PKcs to form an active protein kinase complex [5,6]. An inactivating point mutation in the kinase domain renders DNA-PKcs unable to rescue the radiosensitive phenotype of a DNA-PKcs-deficient cell line [7,8]. In addition, DNA-PKcs inhibitors block DSB repair in vitro and in vivo [9–11]. Together, these findings suggest that the kinase activity of DNA-PKcs is essential for DSB repair.

Evidence suggests that the key target or targets for DNA-PKcs phosphorylation are local within the repair complex [9]. DNA-PKcs undergoes radiation-induced autophosphorylation at threonine 2609 and several nearby sites [12–15]. Introduction of a point mutation that blocks phosphorylation at position 2609 causes radiosensitivity [13,15]. Although

Abbreviations: NHEJ, nonhomologous end joining; DNA-PKcs, DNA-dependent protein kinase catalytic subunit; DNL IV, DNA ligase IV; GST-X4, glutathione-S-transferase-XRCC4

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the exact mechanism by which autophosphorylation contributes to DSB repair is unknown, autophosphorylation is known to destabilize binding of DNA-PKcs to DNA, which could facilitate access by other proteins to the DNA ends [16,17].

Here we investigate the potential for regulation of end joining by DNA-PKcs-mediated phosphorylation of XRCC4. XRCC4 is an essential DSB repair factor that binds to and increases the activity of DNL IV [18,19]. Phosphorylation of XRCC4 occurs in vivo, in response to ionizing radiation, in a DNA-PKcs-dependent manner [20]. It also occurs in vitro, in the presence of purified DNA-PKcs, Ku, and DNA with free ends [14,19,21,22]. Structural studies indicate that the 334-residue XRCC4 protein is composed of at least three domains: an N-terminal globular domain that may be involved in DNA contacts, a central coiled-coil domain that mediates dimerization and DNL IV interaction, and a domain extending from approximately residue 200 to the C terminus [23,24]. Although the structure of this C-terminal domain has not yet been solved, it appears to be a nexus for potential regulatory sites. It contains a caspase 3 site at residues 265/266, which is subject to cleavage during radiation-induced apoptosis [20], and at least two DNA-PKcs phosphorylation sites [22,25]. We have previously mapped one of these to serine 260 and the other to the region between residues 265 and 334 [25]. We refer to these provisionally as the N-X4 and C-X4 sites, respectively.

Although the XRCC4 C-terminal domain is phylogenetically conserved and contains putative regulatory sites, a deletion mutant lacking the C-terminal domain has been shown to retain its ability to complement repair deficiency in a transient transfection assay [21,22]. These results suggest that the domain is not essential for DNA end joining per se, but instead performs some other function. One hypothesis, consistent with previous findings, is that the nonphosphorylated C-terminal domain has an autoinhibitory function, which sequesters the enzyme in an inactive state until it comes into contact with DNA-PKcs and is phosphorylated at specific sites. An alternative hypothesis is that radiation-inducible phosphorylation of the C-terminal domain influences DSB repair by an indirect mechanism, perhaps by influencing XRCC4 localization or turnover. For example, many DSB repair proteins, including XRCC4, are known to be degraded early in apoptosis [20]; reviewed in [26].

Cell-free assays provide a powerful tool for analysis of the end-joining mechanism. We have recently described a system, based on recombinant and purified proteins, that is strongly dependent on exogenous DNL IV/XRCC4 complex [27]. This reconstituted system has a particular advantage in that reactions can be run in either the presence or absence of DNA-PKcs. Although DNA-PKcs is not essential for end joining, when present it restricts the reaction, establishing what may be termed a “reaction checkpoint”. Checkpoint release is dependent on a DNA-PKcs-mediated phosphorylation event. We are thus able to use the system to evaluate the effect of mutations in XRCC4 on either the basal level of end-joining activity (in the absence of DNA-PKcs) or on the regulation of the reaction by phosphorylation (in the presence of DNA-PKcs). Mechanistic investigation using this reconstituted system provides information that is complementary to that from a companion study using whole cells [28].

In the present study, we show that C-X4 phosphorylation occurs in vitro, in part, at serine 318. We have investigated the importance of both the serine 260 and 318 sites using site-directed mutagenesis. We find that these residues are required neither for basal end-joining activity nor for regulation by phosphorylation. Results thus favor the alternative hypothesis, that the function of XRCC4 phosphorylation, if any, is to influence DSB repair by an indirect mechanism.

2. Materials and methods

2.1. Construction of GST-XRCC4 fusion proteins

To generate glutathione-S-transferase (GST) fusion proteins containing XRCC4 sequences from residue 251 to residues 296, 310, 320, or 323, separate PCR reactions were performed with a common 5′ primer, d(TAGGATCCACCCATATGGTAAGTAAGATGATTCCA), and the following 3′ primers: d(ATAGTCGACTTACTTTTCTTGAAGCTGATTCT), d(ATAGTCGACTTACTCCTTTTTAGACGTCTCA), d(ATAGTCGACTTATTCTAAAGACATGTTTTCA), d(ATAGTCGACTTATCTCAGAGTTTCTAAAGACA). PCR products were cloned into the PCR cloning vector, pCR2.1-TOPO (Invitrogen, Carlsbad, CA). PCR products were digested with *Bam*H I and *Eco*R I, or *Bam*H I and *Sal* I, and transferred into corresponding sites in glutathione-S-transferase fusion protein expression vectors, pGEX2T or pGEX4T, respectively (Amersham Pharmacia Biotech, Piscataway, NJ). The resulting plasmids were introduced into *E. coli* strain Top10 (Invitrogen) for expression.

To generate the XRCC4 S260D, S318A, and S318D point mutants using GST-X4 (251–334) as a template, PCR reactions were performed with complementary primers: d(GATGATTCCATTATTTCAGATCTTGATGTCACTGAT) and d(ATCAGTGACATCAAGATCTGAAATAATGGAATCATC) for S260D, d(CTCAGCTGAAAACATGGCTTTAGAAACTCTGAGAAAC) and d(TCTCAGAGTTTCTAAAGCCATGTTTTCAGCTGAGATG) for S318A, and d(TCAGCTGAAAACATGGATTTAGAAACTCTGAGAAAC) and d(TCTCAGAGTTTCTAAATCCATGTTTTCAGCTGAGATG) for S318D. These mutagenic primers were used to generate the desired mutations at each amino acid. After limited PCR (13 cycles), products were digested with *Dpn* I to eliminate *dam* methylated parental template. The mutated plasmids were transformed into the Top10 bacterial strain.

To generate full-length XRCC4, containing point mutations at serine 260, PCR was performed with pCITE HA-XRCC4 as a template. The same mutagenic primers that were used for constructing GST fusion XRCC4 point

mutations S260A and S260D were used for generating full-length mutant XRCC4. To introduce S318A or S318D mutations in full-length XRCC4, pGEX-4T XRCC4 (S318A) or (S318D) were digested with *Bsu*36 I and *Not* I, and the mutated gene fragments were cloned into the corresponding sites in pCITE HA-XRCC4 to replace wild-type sequences.

2.2. Protein expression and purification

GST fusion proteins were purified as described [29]. Cultures of bacteria containing the GST expression plasmids were induced with 1 mM of isopropyl-β-D-thiogalactopyranoside and further incubated for 6 h. Cells were collected and resuspended in lysis buffer containing 50 mM Tris–HCl, pH 7.9, 12.5 mM $MgCl_2$, 1 mM EDTA, 100 mM KCl, 20 μg/ml PMSF, 1 μg/ml Pepstatin A, 1 μg/ml leupeptin, 1 μg/ml soybean trypsin inhibitor, and 15 mM β-mercaptoethanol. After incubation on ice for 30 min, Triton X-100 was added to a final concentration of 1% and incubation was continued for 10 min. The mixture was sonicated, then centrifuged for 15 min at 6500 × *g*. The supernatants were mixed with 100 μl of glutathione-agarose (1:1 slurry in lysis buffer containing 1% Triton X-100) (Sigma–Aldrich, St. Louis, MO) and the mixture was incubated at 4 °C overnight. Beads were collected and subjected to three cycles of washing alternately with wash buffer 1 (50 mM Tris–HCl, pH 7.9, 1 M NaCl, 15 mM β-mercaptoethanol, and protease inhibitors) and wash buffer 2 (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, 1% Triton X-100, 15 mM β-mercaptoethanol, and protease inhibitors). The GST-X4 fusion proteins were eluted with lysis buffer containing 15 mM glutathione. Purification of recombinant DNL IV and XRCC4 complex was performed as described [29]. Purified recombinant DNL IV/XRCC4 complex was dialyzed against DB 0.1 buffer containing 0.1 M KOAc, 20 mM Tris–HCl, pH 7.9, 0.5 mM EDTA, 1 mM DTT, 20% glycerol, and protease inhibitors.

2.3. In vitro phosphorylation and caspase cleavage

In vitro phosphorylation was performed essentially as described [30]. Phosphorylation reactions contained 25 mM Tris–HCl, pH 7.9, 25 mM $MgCl_2$, 1.5 mM DTT, 50 mM KCl, 10% glycerol, 20 nM pGEM 3Z plasmid digested with *Bam*H I, 0.16 μM [γ-^{32}P]ATP (6000 Ci/mmol), 8 nM DNA-PKcs, 20 nM Ku, and recombinant DNL IV/XRCC4 complex or GST fusion XRCC4 proteins as indicated in the figure legends. The final volume was 10 μl. Reactions were incubated for 30 min at 30 °C. Protein phosphorylation reactions were terminated by addition of SDS-PAGE sample buffer. Products were analyzed by 12% SDS-PAGE and detected by PhosphorImager (Amersham Pharmacia Biotech, Piscataway, NJ) analysis. Relative incorporation of radiolabel was measured by PhosphorImager. For caspase cleavage, GST-XRCC4 or DNL IV/XRCC4 complex (5 μg) was incubated with 20 units of caspase 3 (Upstate Biotechnology, Lake Placid, NY) in 20 μl of reaction buffer (50 mM PIPES, pH 6.5, 2 mM EDTA, 5 mM DTT) for 1 h at 37 °C [31].

2.4. Mass spectroscopy analysis

In vitro phosphorylation and in vitro caspase 3 cleavage of XRCC4 were performed as described above. Phosphorylated, caspase 3-cleaved DNL IV/XRCC4 was subjected to 12% SDS-PAGE. The cleaved C-terminal XRCC4 fragment was visualized by Coomassie staining. The gel slice was excised and dried in vacuo. To remove the stain, the gel slice was subjected to three cycles of vigorous shaking in 100 mM NH_4HCO_3, pH 7.5 for 10 min, followed by acetonitrile for another 10 min. Supernatants from all six extractions were discarded and the gel slice was dried in vacuo. The gel slice was rehydrated in 20 μl of 50 μg/ml trypsin, 25 mM NH_4HCO_3, 2.5 mM $CaCl_2$, 0.5% HOAc. Sufficient 50 mM NH_4HCO_3, 5 mM $CaCl_2$, was added to cover the gel slice (approximately 250 μl), and the mixture was incubated overnight at 37 °C with shaking. The supernatant was collected and residual peptides were extracted by incubation in 20 mM NH_4HCO_3 for 10 min, followed by three 10 min incubations in 50% acetonitrile/5% HCOOH/45% H_2O (v/v). The supernatant and washes were pooled and dried in vacuo. Peptides were resuspended in 10 μl 0.1% trifluoroacetic acid and purified using a C_{18} ZipTip (Millipore, Bedford, MA) according to the manufacturer's protocol. The eluate was dried and resuspended in 1 μl 0.1% trifluoroacetic acid. Approximately half of this sample was mixed with an equal volume of 80 mg/ml 2,5-dihydroxybenzoic acid dissolved in 70% H_2O:30% ACN (v:v) and the mixture was spotted to a MALDI plate. Mass spectra were obtained using the oMALDI source on a QSTAR Pulsar i (MDS Sciex, Concord, ON) in positive ion mode. MS/MS spectra were obtained by collisionally induced dissociation using Ar as a collision gas. The instrument parameters were fine-tuned for each MS/MS experiment. Optimum conditions depended on the ion of interest and varied significantly. The pulsed mode was used during MS/MS acquisitions when necessary to enhance signal-to-noise ratio.

2.5. Two-dimensional thin-layer chromatography

Two-dimensional thin-layer chromatography (2D-TLC) was performed using the Hunter thin-layer electrophoresis system (HTLR 7000) (C.B.S. Scientific Company Inc., Del Mar, CA). In vitro phosphorylated XRCC4 protein was cleaved by caspase 3 as described above. The cleaved XRCC4 protein was subjected to SDS-PAGE, transferred to PVDF membrane, and after visualization by PhosphorImager analysis, the desired band was excised for acid hydrolysis. The membrane was treated with 200 μl of 5.7 M HCl for 1 h at 110 °C. The mixture was centrifuged at 22,000 × *g* for 5 min and transferred for lyophilization on a Speed-Vac

(Savant Instruments, Holbrook, NY). The sample was then resuspended in 10 μl of TLC, pH 1.9 buffer (2.5% formic acid and 7.8% glacial acetic acid). Samples were mixed with loading dye containing a standard phosphoamino acid mixture (1.0 mg/ml each of phosphoserine, phosphothreonine, and phosphotyrosine) and spotted onto a pre-wetted TLC plate (E.M. Science, Gibbstown, NJ) on the HTLR 7000 apparatus. Electrophoresis was at 1.5 kV for 20 min. The TLC plate was dried completely after the first dimension chromatography. The TLC plate was pre-wetted again with TLC buffer, pH 3.8 (0.5% pyridine and 5.0% glacial acetic acid) and replaced on the apparatus with 90° rotation. The second dimension electrophoresis was run at 1.3 kV for 16 min, after which the TLC plate was removed from the apparatus and dried. To detect the amino acid markers, a 0.2% ninhydrin solution in acetic acid was sprayed over the TLC plate, which was incubated at 60 °C until colors developed. Radiolabeled phosphoamino acids on the TLC plate were visualized by PhosphorImager analysis.

2.6. Preparation of recombinant baculovirus encoding mutant XRCC4 and purification of mutant DNL IV/XRCC4 complex

Construction of baculovirus transfer vectors containing full-length XRCC4 was performed as described [29]. To generate viral stocks, transfer vectors encoding DNL IV and XRCC4 were transfected, together with linearized AcNPV baculovirus DNA (Pharmingen, San Diego, CA), into *Sf*9 insect cells using a liposome-mediated method (CELLFECTIN, Life Technologies, Grand Island, NY). Recombinant baculovirus stocks were amplified separately to a titer of at least 5×10^7 pfu/ml. Protein expression was confirmed by immunoblotting using anti-histidine antibody (mAb Tetra-His, Qiagen, Valencia, CA) and anti-HA antibody (mAb 12CA5, Roche, Indianapolis, IN).

2.7. DNA end-joining assay

DNA end-joining assays were performed as described [27]. Reactions contained recombinant Ku and a novel factor obtained by sequential chromatography on heparin agarose, Q-Sepharose (0.3 M KOAc fraction), and Superdex 200 [27]. In the present study, the factor was further purified using Mono S, with elution by a linear KOAc gradient. DNA substrate was prepared by digestion of pUC19 plasmid DNA with *Bam*H I restriction enzyme and labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP. End-joining assays were performed in a 20 μl reaction containing 50 mM triethanolamine, pH 7.5, 20 mM Tris–HCl, pH 7.9, 65 mM KOAc, 0.25 mM EDTA, 0.5 mM DTT, 10% glycerol, 1.0 mM $Mg(OAc)_2$, 100 ng/μl bovine serum albumin, 1 mM ATP. The reaction mixture and purified proteins were incubated for 10 min at 37 °C and continued to incubate for 30 min after adding 0.5 ng/μl substrate DNA. Reactions were terminated by addition of 4 μl of 1% SDS, 30% glycerol, 0.1% bromophenol blue, 0.1% xylene cyanol. Reaction mixtures were incubated for 15 min at 70 °C and subjected to electrophoresis on a 0.1% SDS/0.6% agarose gel. Radiolabeled DNA was visualized by PhosphorImager analysis.

3. Results

3.1. Sequences between amino acids 310–320 are required for utilization of the C-X4 phosphorylation site

There are at least two phosphorylation sites in the C-terminal domain of XRCC4 [22,25]. We previously mapped one in vitro DNA-PKcs phosphorylation site to serine 260 (the N-X4 site) and another in the interval between the caspase 3 site between residues 265/266 and the C terminus (the C-X4 region) [25]. A schematic showing the relative location of these sites is shown in Fig. 1A. To map the C-X4 site more precisely, we expressed and purified GST-XRCC4 (GST-X4) fusion proteins containing

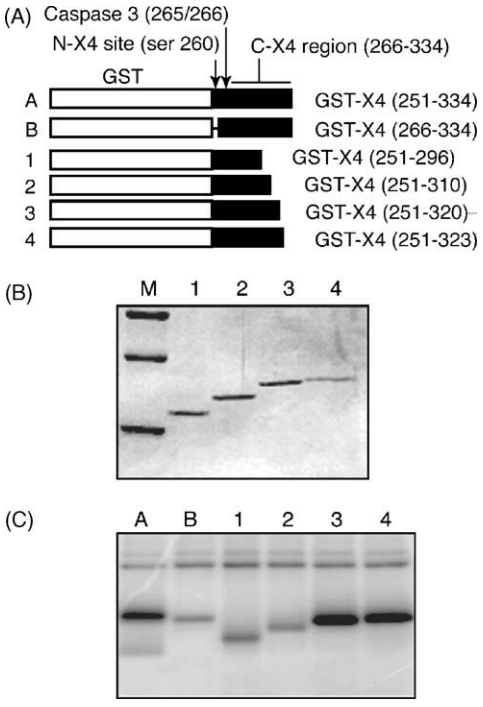


Fig. 1. Construction and in vitro phosphorylation of GST-XRCC4 fusion proteins. (A) Schematic. Open boxes denote GST, filled boxes denote XRCC4. The N-X4 phosphorylation site maps to serine 260 [25], the C-X4 site to the region spanning residues 266–334. Caspase 3 cleaves uniquely between residues 265 and 266. Constructs A and B are from our previous study [25]; constructs 1–4 are new. (B) The 12% SDS-PAGE analysis of purified GST-XRCC4 fusion proteins, with Coomassie blue staining. Comparable analysis of protein expressed from constructs A and B is shown in [25]. (C) In vitro phosphorylation of GST-XRCC4 proteins. Equal amounts of GST-XRCC4 proteins were phosphorylated in vitro by DNA-PKcs in the presence of [γ-^{32}P]ATP and analyzed by 12% SDS-PAGE. Phosphorylated proteins were visualized by PhosphorImager analysis. Lanes represent proteins shown in (A).

various amounts of XRCC4 sequence (Fig. 1B). Purified GST-X4 proteins were phosphorylated by DNA-PKcs in vitro and resolved by SDS-PAGE. Incorporation of radiolabeled phosphate was detected by PhosphorImager analysis (Fig. 1C). GST-X4 (251–334), which contains both the N-X4 and C-X4 sites, was phosphorylated efficiently and serves as a positive control (lane A). GST-X4 (266–334), which lacks the N-X4 site as well as certain N-terminal sequences required for C-X4 utilization [25], was phosphorylated poorly and serves as a negative control (lane B). GST-X4 (251–296) and GST-X4 (251–310) showed only low levels of phosphorylation, whereas GST-X4 (251–320) and GST-X4 (251–323) showed higher levels of phosphorylation similar to the positive control (lanes 3–4). These results suggest that a 10-residue sequence between residues 310–320 is required for efficient C-X4 utilization and may contain the site itself.

3.2. C-X4 phosphorylation occurs at serine 318

There are two potential phosphoacceptor amino acids within the residue 310–320 interval, serine 313 and serine 318. The precise site of phosphorylation was identified by mass spectroscopy. Purified, recombinant DNL IV/XRCC4 complex was phosphorylated in vitro by DNA-PKcs, cleaved with caspase 3, and subjected to preparative SDS-PAGE. A nonphosphorylated control sample was processed in parallel. Caspase 3 releases a unique peptide containing XRCC4 residues 266–334 [25]. The C-terminal caspase cleavage product (residues 266–334), which contains the C-X4 site, was excised and subjected to in-gel trypsin digestion. The resulting tryptic fragments were analyzed by orthogonal MALDI Q-TOF mass spectroscopy. A peptide of *m*/*z* 1629.78, corresponding to the 310–323 tryptic fragment (EHISAENMSLETLR) was detected in the nonphosphorylated sample but reduced in the phosphorylated sample (data not shown). A peptide of *m*/*z* 1709.79, corresponding to the predicted mass of the singly phosphorylated form of this peptide, was detected at low abundance in the phosphorylated sample. In separate experiments, each of these ions was selected and analyzed by MS/MS (Fig. 2). The *m*/*z* 1629.78 ion from the nonphosphorylated sample (panel A) produced a y ion series consisting of y_1–y_8 and y_{10}. The y ion series confirmed the peptide identification. MS/MS analysis of the corresponding *m*/*z* 1709.79 ion from the phosphorylated sample showed that there was extensive neutral loss of 80 and 98 mass units and poor yield of other fragment ions (data not shown), which are characteristics of a phosphopeptide. The lower mass region showed the presence of y_1–y_5, and y_6 to a minor extent. The y_7, y_8, and y_{10} species were shifted by −18 mass units relative to their counterparts in the spectrum of the nonphosphorylated ion. This shift can be explained by neutral loss of phosphoric acid from position 318, leaving a dehydroalanine species in place of phosphoserine. Although the *b* ion series was less complete, it was also consistent with the presence of phosphoserine at position 318. There was no evidence for a doubly phosphorylated form of the 310–323 tryptic fragment or for a singly phosphorylated form with phosphoserine at position 313. These negative results do not exclude the possibility that these species were present but not detectable with the methods and conditions used.

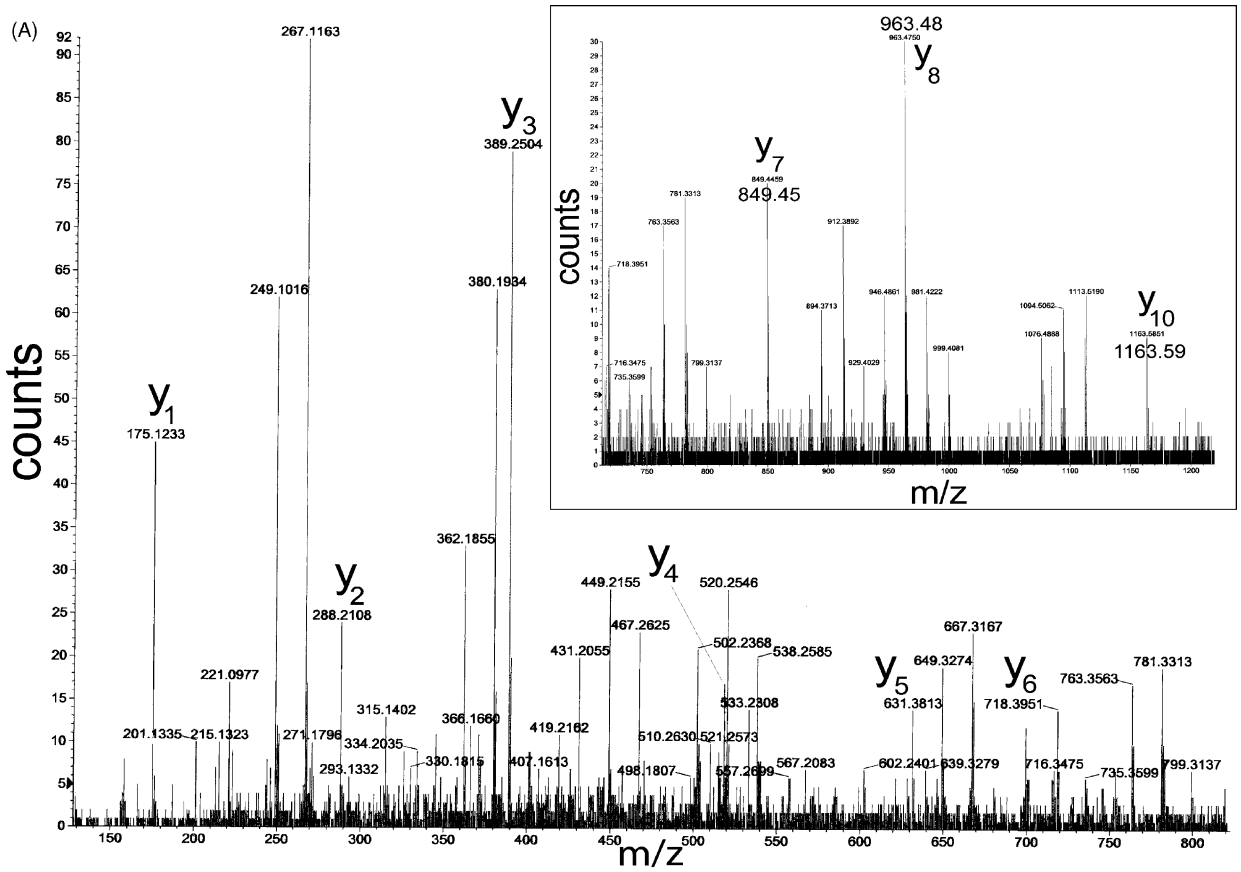
3.3. Substitution of alanine at position 318 reduces C-X4 utilization

To confirm the results of mass spectroscopy, an alanine substitution was introduced at position 318 in the GST-X4 (251–334) context. Protein was purified and tested as substrate in an in vitro phosphorylation assay using purified DNA-PKcs (Fig. 3A). The overall level of phosphorylation of the S318A mutant (right panel) was moderately reduced, relative to wild type (left panel). The lower part of panel A shows that levels of protein substrate in the reaction were similar for mutant and wild type. Phosphorylated products were digested with caspase 3 to separate the N-X4 and C-X4 sites (Fig. 3B). Phosphorylation of the C-X4 site was reduced up to 4.6-fold in the mutant (see figure legend), whereas phosphorylation of the N-X4 site was unaffected, as expected. The presence of residual C-X4 phosphorylation in the S318A mutant could reflect compensatory phosphorylation at nearby sites when serine 318 is lost, or alternatively, the presence of a second phosphoacceptor residue in the C-X4 region. A companion study identifies a number of minor phosphorylation sites in the C-terminal region of XRCC4 that could account for the incorporation of radiolabel in the S318A mutant [28].

A separate experiment was performed in which intact DNL IV/XRCC4 complex, GST-X4 (251–334), and GST-X4 (251–334)(S318A) were phosphorylated in vitro, cleaved with caspase 3, and subjected to preparative SDS-PAGE. The fragment containing the C-X4 site was subjected to phosphoamino acid analysis (Fig. 3C). In all three proteins, phosphoserine was the only phosphoamino acid detected in significant amounts. Consistent with results in panel B, phosphoserine in the S318A mutant (lower panel) was reduced relative to wild type (middle panel), but not eliminated.

3.4. Characterization of DNL IV/XRCC4 complexes with substitution mutations at N-X4 and C-X4 phosphorylation sites

Work described thus far has dealt with serine 318 phosphorylation in the context of GST-XRCC4 fusion constructs, which contain only an isolated portion of the C-terminal domain. To investigate phosphorylation of the native DNL IV/XRCC4 complex, alanine and aspartate substitutions were introduced at the previously described N-X4 site (serine 260) and at the C-X4 site (serine 318) in the context of full-length XRCC4. DNL IV/XRCC4 complexes were obtained from *Sf*9 insect cells co-infected with DNL IV and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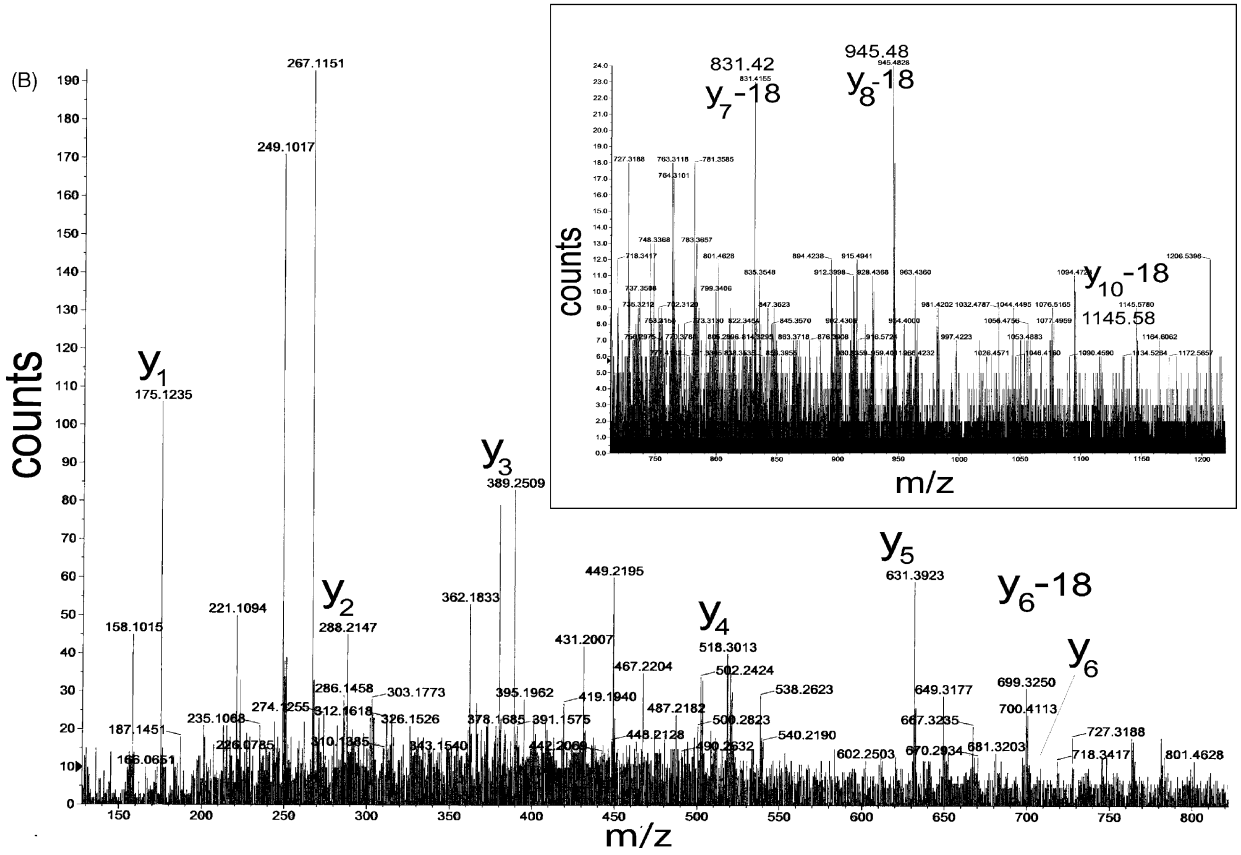
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y7-18
y8-18
y10-18
1145.58
831.42
945.48
267.1151
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391.1575
326.1526
312.1618
310.1885
343.1540
274.1255
235.1068
187.1451
226.0785
166.0651

XRCC4-expressing baculovirus vectors. The complexes were purified by sequential Ni+ NTA-agarose affinity chromatography, Superdex 200 gel filtration chromatography, and Mono Q ion exchange chromatography [29].

Three of the four potential mutants (S260A, S260D, S318A) were obtained in the purified form, as shown in Fig. 4A. The S318D mutant formed protein aggregates in the baculovirus expression system and was not characterized further. The purified wild-type complex and two of the mutant complexes (containing XRCC4 (S260D) and XRCC4 (S318A)) showed an approximately 1:1 ratio of DNL IV to XRCC4, consistent with previous findings that the complex is a mixed tetramer [29,32]. The DNL IV/XRCC4 (S260A) complex clearly contained a reduced level of DNL IV, relative to XRCC4, although the same amount of DNL IV baculovirus vector was used for infection of *Sf*9 cells and intact DNL IV/XRCC4 complexes were size-selected during the purification. The mutation appears to cause some aggregation of free XRCC4, which may have cross-contaminated the size-selected DNL IV/XRCC4 complexes (data not shown). We cannot, however, rule out the alternative possibility that the mutation alters the intrinsic stoichiometry of the DNL IV/XRCC4 complex.

Purified recombinant wild-type and mutant DNL IV/XRCC4 complexes were used as substrates for in vitro phosphorylation. Consistent with results in the GST-XRCC4 system, the S318A mutation reduced phosphorylation of the C-X4 fragment (Fig. 4B, lanes 10–12). We also tested the phosphorylation of the S260A and S260D mutants. The S260D mutant was not phosphorylated at the N-X4 site (lanes 7–9). Unexpectedly, the S260A mutant was phosphorylated in the N-X4 region (lanes 4–6). Previous results have shown that the same mutant is not phosphorylated in the GST-X4 and peptide complex [25]. It is possible that, in the context of the intact DNL IV/XRCC4 complex, contacts with DNA-PKcs are sufficiently strong as to promote compensatory phosphorylation at nearby sites in the absence of serine 260. We note that serine 259 was identified as a minor phosphorylation site in a companion study [28]. This phosphorylation was not seen in the S260D mutant, perhaps because the introduced negative charge eliminates the tendency for this compensatory phosphorylation.

3.5. Substitutions at positions 260 and 318 do not directly affect end-joining activity

To investigate the functional requirement for phosphorylation sites at S260 and S318 in DNA end joining, purified mutant XRCC4 complexes were added to an in vitro DNA end-joining assay. We have recently developed a reconstituted assay system based on recombinant and purified proteins [27]. In this system, end joining is strictly dependent on three protein components: exogenous DNL IV/XRCC4, Ku protein, and a ∼200 kDa factor that has been partially purified from HeLa cell extracts. The physical presence of DNA-PKcs is not required for end joining, but when present, DNA-PKcs restricts the reaction, sensitizing it to the effects of the DNA-PKcs inhibitor, LY294002 [27]. This system is ideally suited for testing the functional significance of potential phosphoacceptor sites. In the absence of DNA-PKcs, mutation of phosphoacceptor sites should be irrelevant. In the presence of DNA-PKcs, mutation of key phosphoacceptor sites to alanine should block the reaction from proceeding, because phosphate transfer cannot occur. By contrast, mutation to aspartate, which mimics constitutive phosphorylation, might relieve inhibition by LY294002.

Results are shown in Fig. 5. Assays were performed with equal mass of enzyme (approximately 100 ng) for each wild-type and mutant XRCC4 complex. The wild type and the three mutant enzymes each stimulated end joining to an approximately equal extent in the absence of DNA-PKcs and LY294002 (lanes 1–5). As expected under these conditions, the addition of LY294002 had no effect, because its target, DNA-PKcs, is not present (lanes 6–10). Consistent with previous results [27], addition of DNA-PKcs caused either no change in activity or a slight decrease (compare lanes 2–5 with 12–15). Addition of LY294002, in combination with DNA-PKcs, inhibited activity with wild type and all three mutants (compare lanes 12–15 with 17–20).

These results demonstrate that neither serine 260 nor serine 318 regulates end-joining under the conditions tested. The S260A and S318A mutants do not arrest the reaction, and the S260D mutation does not relieve LY294002 inhibition. It is important to note that we cannot rule out the possibility that XRCC4 phosphorylation might be required in a more complex system, containing more than the minimal set of proteins used here.

4. Discussion

The C-terminal domain of XRCC4 is phylogenetically conserved and contains several potential regulatory sites, including a caspase cleavage site and sites of DNA-PKcs phosphorylation. However, in vivo studies have failed to identify a specific function for the domain. As a first step toward understanding its function, we have identified the major sites of DNA-PKcs phosphorylation and tested

Fig. 2. Mass spectroscopy analysis of in vitro phosphorylated XRCC4. GST-XRCC4 (251–334) was phosphorylated in vitro with DNA-PKcs, cleaved with caspase 3, and the C-X4 fragment was isolated by preparative SDS-PAGE, subjected to in-gel tryptic digestion, and prepared for analysis by mass spectroscopy as described in Section 2. A nonphosphorylated sample was processed in parallel. (A) MS/MS analysis of ion corresponding to 310–323 tryptic peptide from nonphosphorylated sample (*m*/*z* 1629.78). Fragmentation products beginning at the C terminus (the y ion series) are labeled. Main panel shows region of y_1–y_6, and inset shows region of y_7–y_{10}. (B) Analysis of corresponding phosphopeptide from phosphorylated sample (*m*/*z* 1709.79).

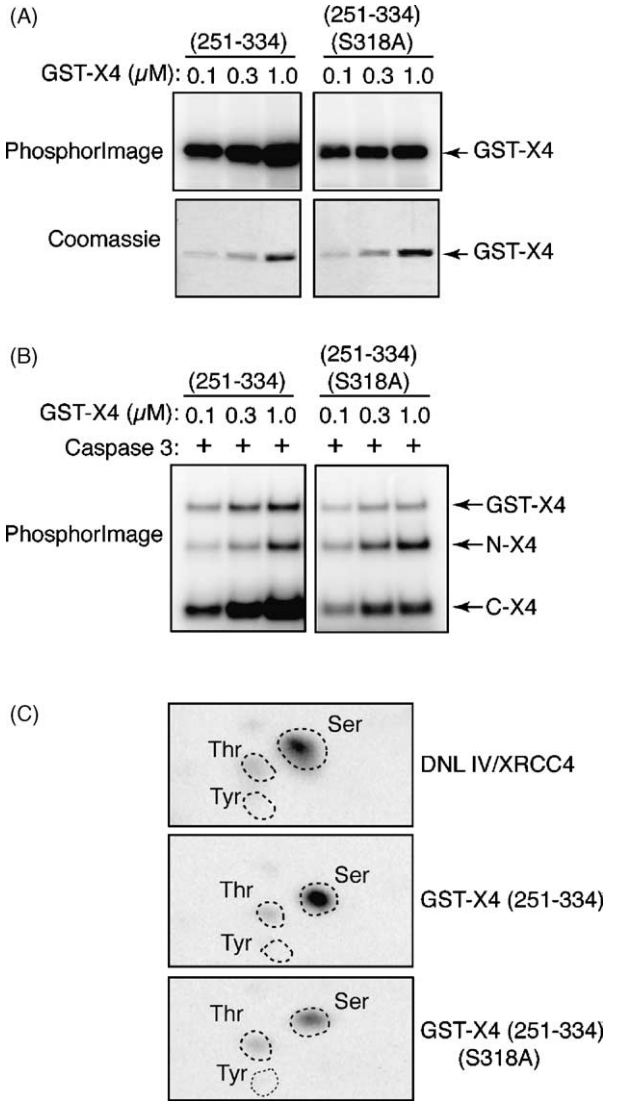

Fig. 3. In vitro phosphorylation assay with GST-XRCC4 point mutants. (A) In vitro phosphorylation was performed as described in Section 2 with DNA-PKcs and the indicated concentrations of GST-XRCC4 (251–334) and GST-XRCC4 (251–334)(S18A). Phosphorylated products were subjected to 12% SDS-PAGE and visualized by PhosphorImager analysis, upper panel. The same gel was also stained with 0.1% Coomassie blue, lower panel. (B) Phosphorylated products from the same reaction mixtures as in panel A were cleaved with caspase 3 to allow separate analysis of N-X4 and C-X4 sites. "N-X4" and "C-X4" denote fragments derived from sequences N-terminal or C-terminal to the caspase 3 cleavage site, respectively. "GST-X4" designates residual uncleaved GST-XRCC4 fusion protein, which is present in all reactions to a variable extent. NIH Image software was used to quantitate the decrease in labeling of the C-X4 species. With 0.1, 0.3, and 1.0 μM GST-X4, labeling decreased by 2.6-, 2.9-, and 4.6-fold respectively. (C) Two-dimensional thin-layer chromatography analysis of XRCC4 phosphopeptides. The indicated proteins were phosphorylated in vitro with DNA-PKcs, cleaved with caspase 3, and the C-terminal fragments were isolated by SDS-PAGE. These were acid hydrolyzed and separated by thin-layer chromatography as described in Section 2. Phosphoamino acid residues were detected by PhosphorImager analysis. Dotted circles indicate phosphoamino acid markers, which were developed using a 2% aqueous ninhydrin solution.

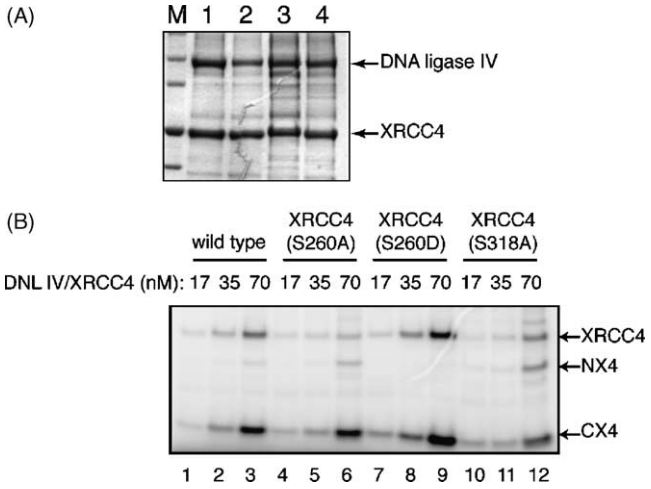

Fig. 4. Purification and analysis of DNL IV/mutant XRCC4 complexes. (A) Proteins were purified from baculovirus vector-infected *Sf*9 cells as described in Section 2, then analyzed by SDS-PAGE with Coomassie blue staining. Lane 1, DNL IV/wild-type XRCC4; lane 2, DNL IV/XRCC4 (S260A); lane 3, DNL IV/XRCC4 (S260D); lane 4, DNL IV/XRCC4 (S318A). Arrows denote DNL IV and XRCC4 polypeptides. (B) Phosphorylation of DNL IV/mutant XRCC4 complexes. In vitro phosphorylation was performed in reactions containing DNA-PKcs and the indicated concentrations of DNL IV/XRCC4 complexes. Products were analyzed by SDS-PAGE and visualized by PhosphorImager analysis. "N-X4" and "C-X4" denote XRCC4 fragments derived from sequences N-terminal or C-terminal to the caspase 3 cleavage site, respectively. "XRCC4" designates residual uncleaved XRCC4 protein, which is present in all reactions to a variable extent.

the effect of mutations at these sites in a cell-free assay system.

In addition to a previously mapped site at serine 260, we identified a major site at serine 318. These sites correspond, respectively, to the N-X4 and C-X4 sites defined in our previous study [25]. These sites are phosphorylated by purified DNA-PKcs both in the context of the isolated XRCC4 C-terminal domain and in the context of the full DNA ligase IV/XRCC4 complex. The latter point is demonstrated by reduced phosphorylation of the N-X4 and C-X4 sites, respectively, in the DNL IV/XRCC4 (S260D) and (S318A) mutant complexes. Consistent with an important biological role, both of these sites, and their surrounding regions, are conserved in mouse XRCC4 (refer to alignment in a companion paper [28]).

Results showed that neither serine 260 nor serine 318 is an essential target of DNA-PKcs-mediated phosphorylation. It is important to recognize several limitations of these studies. Interpretation of the results is complicated by the fact that there may be compensatory phosphorylation at sites near serine 260, as evidenced by residual phosphorylation of the N-X4 site in S260A but not the S260D mutant (Fig. 4). We note that a companion paper identifies the adjacent residue, serine 259, as a minor phosphoacceptor site [28]. Compensatory phosphorylation at nearby sites may blunt the effect of individual mutations. We also cannot rule out the possibility that mutation of serine to alanine disrupts enzyme

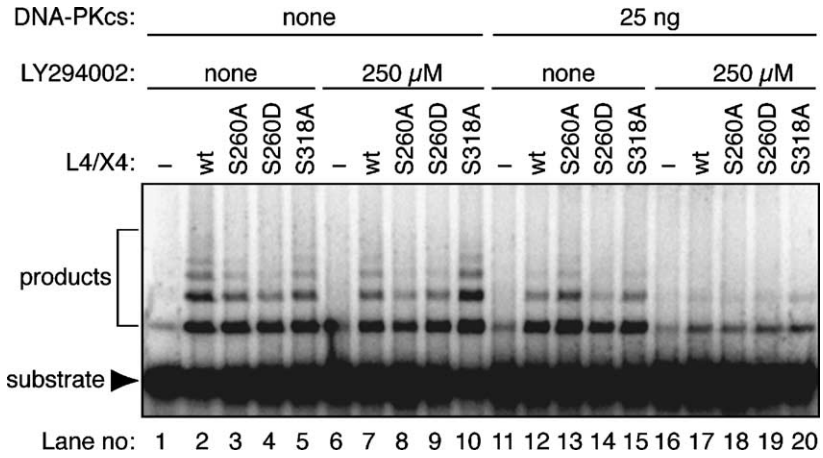

Fig. 5. In vitro DNA end-joining assay (NHEJ). (A) End-joining assays were performed using *Bam*H I-digested, 5′-^{32}P-radiolabeled plasmid substrate. All reactions contained 25 ng of Ku protein and 25 ng (total protein) of a fraction from HeLa cell extracts that is required for end-joining activity. DNA-PKcs, LY294002, and wild type or mutant DNL IV/mutant XRCC4 complexes were present as indicated. Products were analyzed by 0.6% agarose gel electrophoresis and detected by PhosphorImager analysis.

structure in the nonphosphorylated state, rendering phosphorylation unnecessary. The S260A mutation has previously been shown to destroy a dominant human autoimmune epitope in this region [25]. Finally, we characterized the effects of mutation at serines 260 and 318 in a simplified reaction system containing only a limited set of proteins and substrate DNA with cohesive 5′-phosphoryl ends. Phosphorylation of serines 260 and 318 might be required in a more complex system containing additional proteins or where processing of the DNA ends was needed prior to ligation.

A companion paper [28] has independently identified the same major DNA-PKcs phosphorylation sites, serines 260 and 318, as those identified in the present study. A number of more minor sites were also mapped. The in vivo studies in the companion paper failed to identify a specific function for phosphorylation at either site. Our results are therefore in general agreement.

Exclusion of the hypothesis that phosphorylation of serines 260 and 318 directly regulates end-joining, as suggested by our results and the companion study, favors an alternative hypothesis that the function of XRCC4 phosphorylation, if any, is to regulate DSB repair by an indirect mechanism, perhaps influencing localization or turnover. In this respect, the inability to express the S318D mutant in baculovirus is of interest. Introduction of a negative charge at position 318 may destabilize folding of the C-terminal domain, altering levels of protein expression in vivo. We note that S318D was one of few potential mutants not evaluated in the study of Lees-Miller and coworkers [28]. The possibility that phosphorylation of S318 might down regulate DSB repair by an indirect destabilization mechanism therefore remains to be tested.

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