Radiosensitization of MDA-MB-231 breast tumor cells by adenovirus-mediated overexpression of a fragment of the XRCC4 protein

Kara R. Jones,[1] David A. Gewirtz,[1,3] Steven M. Yannone,[4] Shaoming Zhou,[5] David G. Schatz,[5] Kristoffer Valerie,[2,3] and Lawrence F. Povirk[1,3]

Departments of [1]Pharmacology and Toxicology and [2]Radiation Oncology, [3]Massey Cancer Center, Virginia Commonwealth University, Richmond, Virginia; [4]Division of Life Sciences, Department of Molecular Biology, Lawrence Berkeley Laboratory, Berkeley, California; and [5]Howard Hughes Medical Institute, Section of Immunology, Yale University School of Medicine, New Haven, Connecticut

Abstract

Incomplete DNA repair or misrepair can contribute to the cytotoxicity of DNA double-strand breaks. Consequently, interference with double-strand break repair, by pharmacologic or genetic means, is likely to sensitize tumor cells to ionizing radiation. The current studies were designed to inhibit the nonhomologous end joining repair pathway by interfering with the function of the XRCC4/ligase IV complex. A PCR-generated fragment of the *XRCC4* gene, encompassing the homodimerization and ligase IV – binding domains, was inserted into a plasmid vector (pFLAG-CMV-2) expressing the FLAG peptide and the cassette encoding FLAG-tagged XRCC4 fragment was cloned into an adenoviral vector. Both the plasmid and the corresponding adenovirus elicited robust expression of a truncated XRCC4 protein designed to compete in a dominant-negative fashion with full-length XRCC4 for binding to ligase IV. Binding of the XRCC4 fragment to ligase IV *in vivo* was confirmed by immunoprecipitation. Clonogenic survival assays showed that the adenovirus expressing the truncated XRCC4 protein sensitizes MDA-MB-231 breast tumor cells to ionizing radiation, presumably through interference with the functional activity of ligase IV, leading to inhibition of the final ligation step in end joining. These studies support the potential clinical utility of combining radiation therapy with agents that inhibit DNA double-strand break repair. [Mol Cancer Ther 2005;4(10):1541 – 7]

Introduction

Radiation therapy is widely used in the treatment of localized breast cancer (1, 2). A major focus of current radiation research is to elucidate molecular and cellular mechanisms that determine sensitivity or resistance to ionizing radiation (3, 4). During radiotherapy, efficient repair of DNA damage in tumor cells could lead to treatment failure and disease recurrence. Thus, DNA repair proteins are potential targets for anticancer therapies because interference with repair of double-strand breaks can promote tumor cell death. Strategies that selectively target and/or sensitize tumor cells to radiation could ultimately lead to improved patient survival after radiotherapy.

There are at least two independent pathways for repair of double-strand breaks in DNA—nonhomologous end joining (NHEJ) and homologous recombination (5–8). Homologous recombination, a highly accurate form of DNA repair that is dependent on the presence of an intact template, has been shown to occur after DNA replication—in late S and G_2 phases of the cell cycle. NHEJ is the predominant pathway in mammalian cells; however, it is an inherently inaccurate process because two DNA ends are joined directly, usually with no sequence homology (6, 9), often causing the loss of nucleotides from the site of the DNA break (8).

Essential factors in NHEJ include the serine/threonine DNA-dependent protein kinase (consisting of Ku70, Ku80, and the catalytic subunit), the *XRCC4* gene product, and DNA ligase IV (6, 8, 9). Previous work has shown that mutations in any of these key proteins involved in NHEJ cause increased sensitivity to radiation, associated with a demonstrable deficiency in repair of double-strand breaks (10–17). Thus, inhibitors of NHEJ could have potential utility as radiosensitizing agents in cancer treatment.

DNA ligase IV, an ATP-dependent enzyme composed of 911 amino acids, is responsible for the final ligation step of NHEJ (8, 18) and is also required for gap filling on aligned double-strand break ends by DNA polymerases λ and μ (19–21). Human XRCC4 is a 334-amino-acid protein that dimerizes with and binds tightly to one molecule of ligase IV, forming a 2:1 XRCC4-ligase IV trimer (18, 22). XRCC4 stimulates ligase IV end joining activity, possibly by facilitating the recruitment of ligase IV to DNA ends (23, 24). In the current work, we have developed a system for genetic disruption of DNA double-strand break repair through targeting of XRCC4. Based on previous structural

Received 6/13/05; revised 7/13/05; accepted 7/25/05.

Grant support: Department of Defense predoctoral fellowship BC022042, and NIH grants CA40615 and CA72955.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Note: D.G. Schatz is an investigator of the Howard Hughes Medical Institute.

Requests for reprints: Lawrence F. Povirk, Department of Pharmacology and Toxicology, Virginia Commonwealth University, P.O. Box 980230, Richmond, VA 23298. Phone: 804-828-9640; Fax: 804-828-8079. E-mail: lpovirk@gems.vcu.edu

doi:10.1158/1535-7163.MCT-05-0193

data for XRCC4 (22), we created a fragment of the XRCC4 protein comprising amino acids 115 to 292. This fragment does not restore NHEJ function to XRCC4-deficient cells (22) but retains its dimerization domain and should be able to bind to its heterotrimeric partner, ligase IV. Consistent with this prediction, when this fragment was expressed from an adenoviral vector in MDA-MB-231 breast tumor cells, it formed a complex with DNA ligase IV and sensitized the cells to ionizing radiation.

Materials and Methods

Cell Culture

The MDA-MB-231 breast tumor and 293 kidney epithelial cells were obtained from American Type Culture Collection (Manassas, VA). MDA-MB-231 cells were grown as monolayers in RPMI 1640 with L-glutamine supplemented with 5% fetal bovine serum and 5% defined bovine calf serum. The 293 cells were also grown as monolayers in DMEM medium with high glucose and L-glutamine supplemented with 10% fetal bovine serum. Both cell lines were cultured at 37°C in an atmosphere of 5% CO_2/100% humidity. All experimental analyses were done with cells in logarithmic growth. Both cell lines were determined to be free of *Mycoplasma*.

Construction of an Adenovirus Expressing a Fragment of the XRCC4 Protein

Full-length XRCC4 cDNA was amplified by nested PCR from human cDNA of 184B5 mammary epithelial cells, using Pfu ultrahigh-fidelity DNA polymerase (Stratagene, La Jolla, CA) and the primers AACCTTGATCTGTGAAAGCG and GTAAGATACGGTAATAGCGGC (Integrated DNA Technologies, Inc., Coralville, IA; Fig. 1). The unpurified product was diluted 100-fold and subjected to PCR with nested primers GCAAGAATTCACGATGGAGAGAAAAATAAGCA and TGCTCTAGAGCTGCTGACTTGAAATTCTCC; the resulting product (with terminal *Eco*RI and *Xba*I linkers) was gel purified. A fragment of the *XRCC4* gene with terminal *Eco*RI and *Xba*I restriction enzyme sites was generated from the full-length cDNA by PCR with primers GCAAGAATTCGACGAAAGTTGAAAACCCAGCT and TGCTCTAGATTACTGATTCTCCTGAGGAGC. The PCR profile consisted of 95°C for 5 minutes followed by 40 cycles of 95°C for 1 minute, 64°C for 30 seconds, and 72°C for 1.5 minutes. Following cleavage of the terminal linkers with *Eco*RI and *Xba*I, the XRCC4 fragment PCR product was ligated into the pZErO-2.1 expression plasmid vector (Invitrogen, Grand Island, NY). *Escherichia coli* XL1-Blue cells (Stratagene) were transformed with the ligated DNA, and colonies were screened for presence of the XRCC4 fragment insert. The XRCC4 fragment coding sequence was released from pZErO-2.1 using *Eco*RI and *Xba*I, gel purified, and ligated into the *Eco*RI- and *Xba*I-digested pFLAG-CMV-2 expression vector, which expresses the insert with an NH_2-terminal FLAG peptide epitope used for identification purposes (Sigma, St. Louis, MO). *E. coli* XL1-Blue cells were transformed with the ligated DNA and colonies were screened for presence of the XRCC4 fragment insert. A pFLAG-CMV-2 vector expressing full-length XRCC4 was similarly constructed. Both inserts were sequenced and found to be free of mutations.

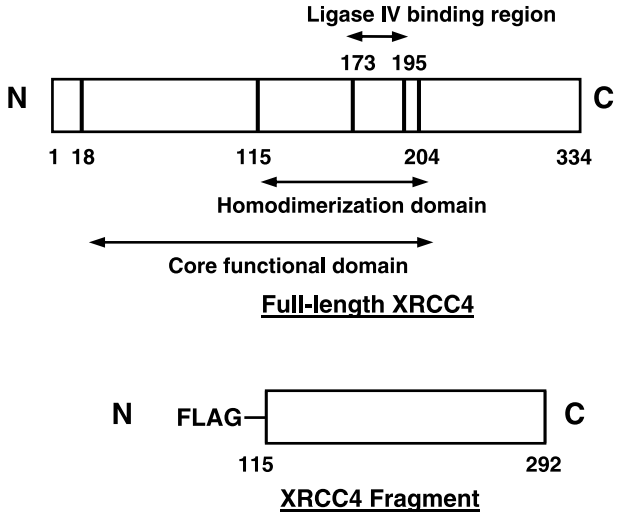

Figure 1. Construction of an adenovirus that expresses a fragment of the XRCC4 protein. Schematic representation of the human XRCC4 protein is shown. The core functional and homodimerization domains of XRCC4 are shown as well as the ligase IV binding region. *Numbers,* amino acid position; *N,* NH_2 terminus; *C,* COOH terminus. Adapted from ref. (22).

A first-generation adenovirus that expresses the FLAG-tagged XRCC4 fragment was prepared by the Massey Cancer Center Virus Vector Shared Resource as previously described with minor modifications (25). Briefly, the FLAG-tagged *XRCC4* gene fragment was released from the plasmid expression vector by cleavage with *Nde*I and *Xba*I, and cloned into the adenovirus transfer plasmid, pZErOtgCMV, a modified version of the plasmid pTG9539 (26) that confers resistance to kanamycin rather than ampicillin. Recombination of the pZErOtgCMV-(FLAG-tagged XRCC4 fragment) expression cassette (obtained using *Pac*I and *Pme*I restriction enzymes) into the *Cla*I-digested adenovirus plasmid, pTGCMV was done. The adenovirus was generated by transfection of the resulting plasmid into 293 cells, and characterized by Western blot analysis. A large amount of crude adenovirus was then prepared from infected 293 cells (25).

Immunoprecipitation Assay

MDA-MB-231 cells near confluence in 100 mm dishes were infected with adenovirus at a multiplicity of ~30 for 2 hours followed by removal of the adenovirus and incubation in fresh complete medium for 3 days. Cells were scraped from the plates and centrifuged to pellet the cells. The cell pellets were washed twice with ice-cold PBS to remove any remaining medium. The cell pellets were resuspended in thrice the cell pellet volume (~500–600 μL) EMSA lysis buffer consisting of 10 mmol/L HEPES (pH 7.8), 60 mmol/L KCl, 1 mmol/L EDTA, 0.5% NP40, 0.5 mmol/L DTT, 0.2 mmol/L phenylmethylsulfonyl fluoride, 1:100 of protease inhibitor cocktail (Sigma), and phosphatase inhibitor cocktails I (P2850) and II (P5726; Sigma) and

incubated on ice for 10 minutes. The lysates were centrifuged at 4°C for 5 minutes at 3,000 rpm. The supernatant containing the cytoplasmic fraction was removed from the pelleted nuclei and stored at −80°C. The nuclei were resuspended in thrice the pellet volume (~500–600 μL) of nuclear extraction buffer containing 20 mmol/L Tris (pH 7.9), 25% glycerol, 0.42 mol/L NaCl, 1.5 mmol/L $MgCl_2$, 0.2 mmol/L EDTA, 0.5 mmol/L dithiotheritol, 0.5 mmol/L phenylmethylsulfonyl fluoride, 1:100 of protease inhibitor cocktail (Sigma), and phosphatase inhibitor cocktails I and II, and incubated on ice for at least 30 minutes. The cells were centrifuged at 4°C for 10 minutes at 14,000 rpm and the supernatant, which contains the nuclear fraction, was removed and stored at −80°C.

The FLAG-tagged XRCC4 fragment was immunoprecipitated from the nuclear and cytoplasmic fractions from MDA-MB-231 cells using EZview Red Anti-FLAG M2 Affinity gel (50% slurry suspension in PBS; Sigma). For 12 samples, ~500 μL of the affinity gel was washed twice with 500 μL EMSA wash buffer (EMSA lysis buffer without 0.5% NP40) and resuspended in ~1 mL EMSA wash buffer. The nuclear and cytoplasmic fractions were thawed and equal volume of 10 mmol/L Tris-HCl–1 mmol/L EDTA with inhibitors was added to the nuclear fractions to lower the salt concentration. Approximately 100 μL of the prepared beads were added to the ~1 mL nuclear and 500 μL cytoplasmic fractions and rotated at 4°C for 3 hours. Following the 3-hour incubation, each tube was centrifuged at 5,000 rpm for 5 minutes at 4°C. The supernatant was removed and stored in microcentrifuge tubes at −80°C. The beads were washed thrice with 1 mL EMSA wash buffer, allowing each wash to equilibrate on ice for 5 minutes. After each wash, the beads were centrifuged and the supernatant was carefully removed without disturbing the beads. Following the final wash, the beads were resuspended in ~50 μL of 10 mmol/L Tris-HCl–1 mmol/L EDTA with inhibitors and 1 to 2 μL (5–10 μg) of thawed FLAG peptide was added to elute the FLAG-tagged fusion proteins from the beads. The beads were allowed to incubate with the peptide overnight at 4°C with gentle agitation. Without touching the beads, as much supernatant as possible was removed from the beads and placed in a clean microcentrifuge tube and stored at −80°C. Approximately half of each sample was mixed with 5× sample buffer and Western blot analysis was done.

Antibodies Used for Western Blot Analysis

An XRCC4 antibody (RB-XRC40) that was raised against full-length XRCC4 and detects the XRCC4 fragment was purchased from GeneTex (San Antonio, TX). An XRCC4 antibody (SC-8285) that detects full-length XRCC4 but does not detect the fragment was purchased from Santa Cruz (Santa Cruz, CA). This antibody was raised against a peptide from the COOH-terminal region of the human XRCC4 protein (amino acids above 300), which was deleted in our XRCC4 protein fragment. The FLAG (F-3165) and actin antibodies were purchased from Sigma.

To generate an antibody against DNA ligase IV, the peptide CSVDKGELQEENQYLL (the 15 COOH-terminal amino acids of murine ligase IV plus NH_2-terminal cysteine) was coupled to keyhole limpet hemocyanin (Pierce, Rockford, IL) with *m*-maleimidobenzoyl-*N*-hydroxsulfo-succinimide ester (Pierce). Peptide/keyhole limpet hemocyanin was mixed with TiterMax-Gold (CYTRX, Norcross, GA) and used to immunize rabbits, with repeated boosts at 2-week intervals and with bleeds done 10 days after boosting. Antibodies were purified from rabbit sera on a peptide affinity column generated by coupling of the peptide to aminobutyl agarose (Sigma) using *N*-succinimidyl 3-(2-pyridyldithio)propionate (Pierce). Specific antibodies were eluted from the affinity

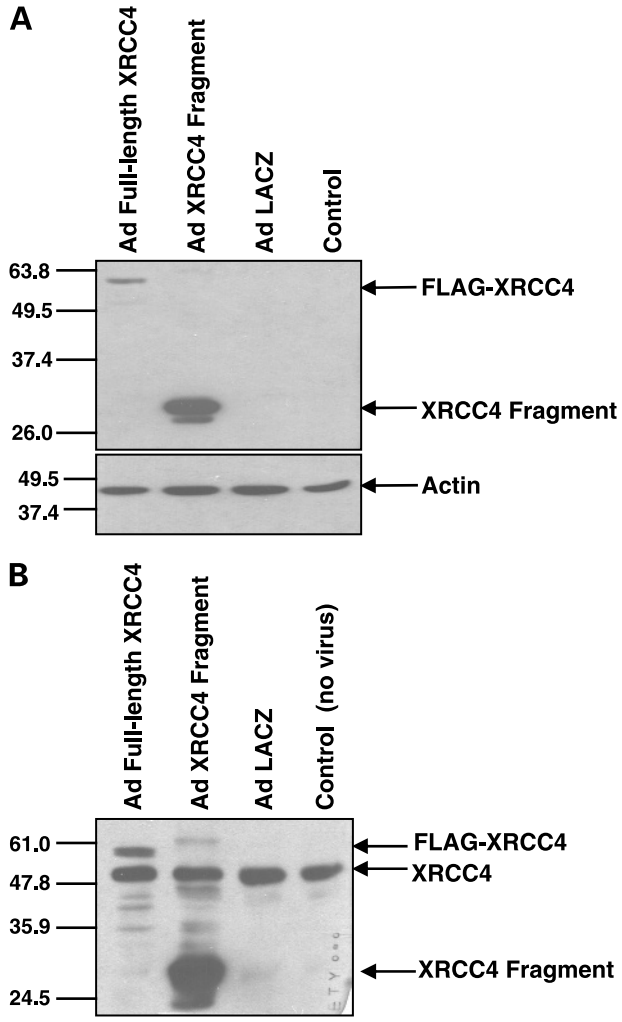

Figure 2. MDA-MB-231 cells express a fragment of the XRCC4 protein from adenovirus. Protein was extracted 3 d after MDA-MB-231 cells were infected at a multiplicity of infection of 30 with either the LACZ or the XRCC4 fragment adenovirus (*Ad*) or transiently transfected with pFLAG-CMV-2 expressing full-length XRCC4. Two microliters from 500 μL of extracted protein were loaded on a 10% polyacrylamide gel and Western blots were probed with anti-FLAG antibody (**A**) or an antibody against full-length XRCC4 (**B**). Actin levels serve as loading controls. *Numbers to the left,* molecular mass in kDa and migration positions of protein size standards.

column using 3.5 mol/L $MgCl_2$, dialyzed against PBS, and concentrated. Recombinant XRCC4/ligase IV complex prepared in baculovirus (27) was used to verify the electrophoretic mobility of XRCC4 and ligase IV.

Clonogenic Survival

Cells 70% to 80% confluent in 60 mm dishes were infected with adenovirus at a multiplicity of infection of 30 for 2 hours and then allowed to incubate in virus-free medium for 3 days. Cells were released from the dish by incubation with trypsin/EDTA, counted, plated in triplicate at low density in 100 mm dishes, allowed to adhere to the surface for 12 hours, and irradiated. Following incubation at 37°C for 10 to 14 days, the cells were fixed with 100% methanol and allowed to air dry. The cells were then stained with 0.1% crystal violet. Colonies (a group of aggregated cells numbering at least 50) were then counted.

Results

Expression of the FLAG-Tagged XRCC4 Fragment in MDA-MB-231 Breast Tumor Cells

Western blotting of protein isolated from MDA-MB-231 breast tumor cells 3 days after infection with the adenovirus, using the FLAG antibody (Sigma) and an XRCC4 antibody (GeneTex) raised against full-length XRCC4, shows that MDA-MB-231 cells express the FLAG-tagged XRCC4 protein fragment (Fig. 2). Uninfected cells and cells infected with the LACZ adenovirus served as negative controls. The XRCC4 fragment (amino acids 115–292; see Fig. 1) is overexpressed compared with endogenous levels of XRCC4 and is, therefore, likely to effectively compete with endogenous full-length XRCC4 for binding to ligase IV. Thus, it seems that the XRCC4 fragment is being expressed properly in MDA-MB-231 cells.

A Fragment of the XRCC4 Protein Expressed from Adenovirus Binds to Ligase IV

Immunoprecipitation assays were done to confirm that this fragment of the XRCC4 protein expressed in the MDA-MB-231 cells retains the ability to homodimerize and bind to ligase IV. Using an affinity resin containing FLAG antibody covalently attached to agarose beads, FLAG-tagged XRCC4 fragment was immunoprecipitated from nuclear and cytoplasmic protein fractions isolated from adenovirus-infected MDA-MB-231 cells (full-length XRCC4 fragment is not seen due the short exposure time required to avoid overexposure of overexpressed XRCC4 fragment; Fig. 3C). Figure 3B shows that, in MDA-MB-231 cells, ligase IV was coimmunoprecipitated from both the nuclear and cytoplasmic fractions with the FLAG-tagged XRCC4 fragment. As expected, beads with FLAG antibody did not precipitate ligase IV from cells infected with a LACZ-expressing adenovirus (*lanes 1* and *3*), indicating that ligase IV does not bind nonspecifically to the beads.

The presence of both FLAG and the XRCC4 fragment in the cytoplasmic supernatant decanted from the FLAG beads after the 3-hour incubation indicates that not all of the FLAG-tagged XRCC4 fragment is bound to the FLAG beads (Fig. 3C). In addition, there is evidence of ligase IV in

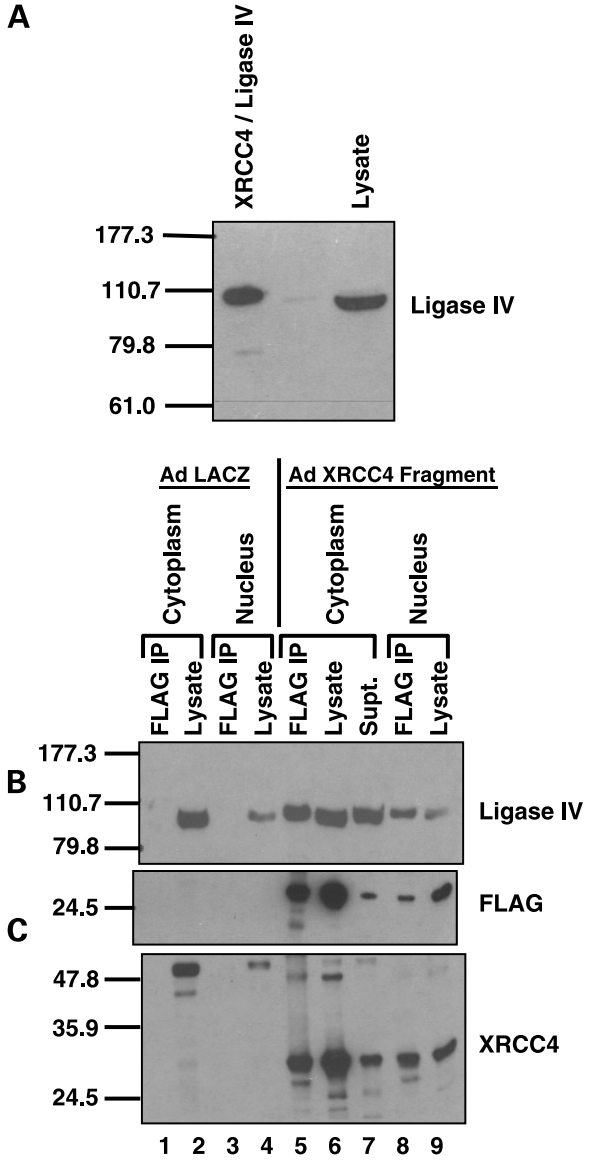

Figure 3. A fragment of the XRCC4 protein binds to ligase IV in MDA-MB-231 cells. **A**, 50 ng of purified recombinant XRCC4/ligase IV complex were electrophoresed along with 5 μL of cell lysate and blots were probed with antibody to ligase IV to verify its mobility. **B**, MDA-MB-231 cells were infected with the LACZ or the XRCC4 fragment adenovirus at a multiplicity of infection of 30 for 2 h, and 3 d later cytoplasmic and nuclear protein fractions were extracted. The FLAG-tagged XRCC4 fragment was immunoprecipitated (*IP*) and the amount of ligase IV bound was determined by Western blot analysis. Approximately 25 μL from 50 μL of the immunoprecipitated samples, 5 μL from 500 μL of cytoplasmic extracts, and 10 μL from 1 mL of nuclear extracts were loaded on a 10% polyacrylamide gel. In addition, 5 μL from 500 μL of the cytoplasmic supernatant, which was removed from the FLAG beads following a 3-h adsorption, was loaded on the gel to determine the proportion of ligase IV that is not bound to FLAG-tagged XRCC4. **C**, levels of FLAG-tagged XRCC4 protein in lysates and immunoprecipitates were also determined using the indicated antibodies to confirm expression and immunoprecipitation of FLAG-tagged XRCC4 fragment. Approximately 1 μL from 50 μL of the immunoprecipitated samples, 1 μL from 500 μL of cytoplasmic crude and supernatant samples, and 10 μL from 1 mL of nuclear crude samples were loaded on a 10% polyacrylamide gel.

the cytoplasmic supernatant. This was expected because the FLAG beads did not precipitate all of the FLAG-tagged XRCC4 fragment out of the lysate. However, the presence of ligase IV in the cytoplasmic supernatant could also represent a portion of ligase IV that is not bound to the XRCC4 fragment but that may be bound to full-length XRCC4. Nevertheless, these data clearly show that the XRCC4 fragment can effectively compete with endogenous full-length XRCC4 for binding to DNA ligase IV.

The XRCC4 Fragment Binds Full-Length XRCC4

Because XRCC4 must homodimerize before binding to ligase IV, it is possible that the XRCC4 fragment can also bind to full-length XRCC4. This possibility was addressed using an antibody against the XRCC4 COOH-terminal domain, which recognizes only full-length XRCC4 and not the fragment (Santa Cruz). This antibody detected two bands on Western blots from both lysates and immunoprecipitates (Fig. 4). The lower band comigrates at ~50 kDa with the band detected by the GenTex antibody and represents full-length XRCC4. The upper band migrating at ~55 kDa apparently represents cross-reacting material as it was never detected by the GenTex antibody (raised against full-length XRCC4) and it appears in samples that should not contain XRCC4, e.g., the FLAG-bound fraction from lysates of Ad-LACZ–infected cells (Fig. 4B, *lanes 1* and *3*). As shown by the presence of full-length XRCC4 in the immunocomplex of the cytoplasmic fraction (*lane 5*), the XRCC4 fragment does bind to full-length XRCC4. As expected, XRCC4 was not immunoprecipitated from the LACZ-expressing cells (*lane 1*). These data are consistent with the formation of heterotrimers comprising one molecule each of XRCC4 fragment, full-length XRCC4, and ligase IV. Figure 4 also suggests a marked decrease in the level of full-length XRCC4 protein in nuclei of cells overexpressing XRCC4 fragment (compare *lane 9* with *lane 4*), consistent with the possibility that formation of the complex between XRCC4 fragment and ligase IV occurred at least partially at the expense of the normal XRCC4/ligase IV complex.

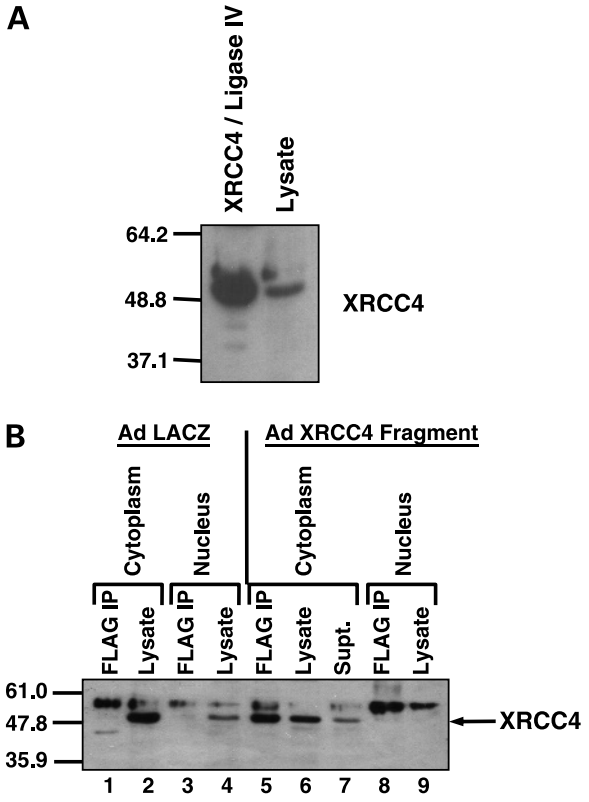

Figure 4. Full-length XRCC4 is complexed with the XRCC4 fragment. **A**, to determine the mobility of XRCC4, 50 ng of purified recombinant XRCC4/ligase IV and 7 μL from 400 μL of cytoplasmic extract from uninfected MDA-MB-231 cells were subjected to electrophoresis and Western blotting with an antibody against the XRCC4 COOH-terminal domain. **B**, MDA-MB-231 cells were infected with the LACZ or the XRCC4 fragment adenovirus at a multiplicity of infection of 30 for 2 h, and 3 d later cytoplasmic and nuclear protein fractions were extracted. The FLAG-tagged XRCC4 fragment was immunoprecipitated using FLAG beads and the amount of full-length XRCC4 bound was determined by Western blot analysis.

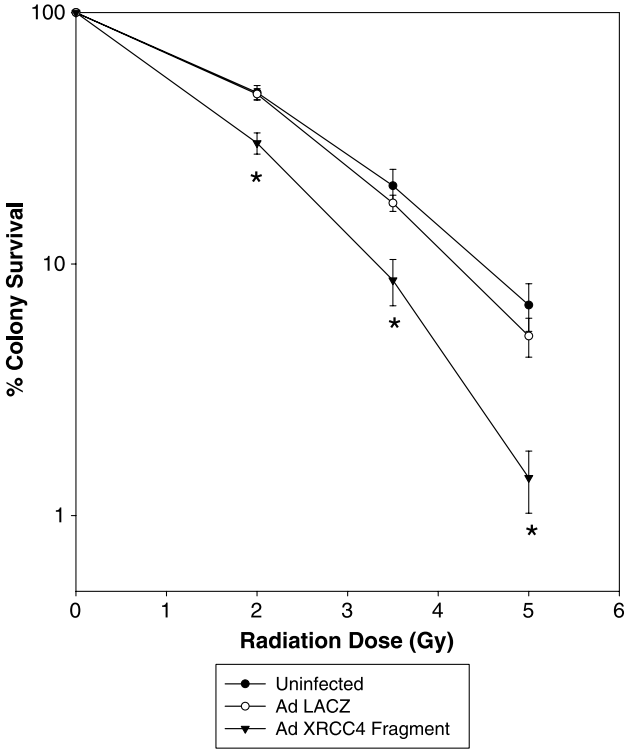

Figure 5. The XRCC4 fragment expressed from adenovirus sensitizes MDA-MB-231 breast tumor cells to ionizing radiation. MDA-MB-231 cells were infected with the LACZ or the XRCC4 fragment adenovirus for 2 h, and 3 d later cells were plated for clonogenic survival studies. Uninfected and infected cells were exposed to 0, 2, 3.5, and 5 Gy ionizing radiation the day after plating. Clonogenic survival was assessed between 10 and 14 d after radiation exposure. Each experiment was done at least four times and triplicates were done for each experiment. *Points,* combined average of all experiments; *bars,* SE between experiments. Statistical analysis was done using either the paired or unpaired Student's *t* test. *, significant difference from uninfected and adenovirus LACZ – infected cells ($P < 0.05$).

The FLAG-Tagged XRCC4 Protein Fragment Expressed from Adenovirus Sensitizes Breast Tumor Cells to Ionizing Radiation

Clonogenic survival assays were done to determine the influence of the XRCC4 protein fragment expressed by the adenovirus on breast tumor cell sensitivity to ionizing radiation. Figure 5 indicates that MDA-MB-231 cells expressing the XRCC4 fragment are more radiosensitive than uninfected and virus controls in terms of clonogenic survival, with a dose-modifying factor of ~1.2-fold. Statistical analysis shows that clonogenic survival in irradiated MDA-MB-231 cells that express the XRCC4 fragment is significantly reduced compared with irradiated control MDA-MB-231 cells. These data confirm that the XRCC4 protein fragment can bind to ligase IV and increase sensitivity to ionizing radiation.

Discussion

Because deficiency in NHEJ confers profound sensitivity to agents that induce double-strand breaks, NHEJ repair proteins, such as DNA-dependent protein kinase and DNA ligase IV, are attractive targets for potentiation of chemotherapy and radiotherapy. However, whereas deficiencies in Ku as well as in catalytically active DNA-dependent protein kinase result in telomere dysfunction as well as defective repair (28–31), the only known effects of deficiencies in XRCC4 and DNA ligase IV are reduced double-strand break repair and lack of V(D)J recombination (the process whereby immunoglobulin and T-cell receptor genes become rearranged; refs. 13, 31–34). Interference with these cellular functions may be tolerable if produced only for short periods during radiotherapy. In addition, because DNA repair proteins function to repair double-strand breaks in cells that have sustained DNA damage, inhibitors of repair proteins would presumably be selectively toxic only to those (tumor) cells with DNA damaged by radiation therapy, having little effect on cells in unirradiated tissue. Furthermore, DNA repair protein inhibitors would act exclusively against the enzymes involved in DNA repair but would not damage DNA directly, unlike other DNA-damaging agents widely used in the treatment of cancer. Consequently, these agents might have fewer adverse effects due to their increased selectivity (35).

The current studies were undertaken to assess the feasibility of sensitizing breast tumor cells to ionizing radiation by inhibiting the NHEJ DNA repair pathway. A number of studies have shown radiosensitization in cells with mutation-based defects in DNA ligase IV (16, 17). In these studies, the defective DNA ligase IV protein was compromised either in its functional (ligation) ability or its ability to form a stable complex with XRCC4 (16, 17). XRCC4/ligase IV complex may be a particularly vulnerable target because experiments with nuclear extracts suggest that it may be a rate-limiting component of NHEJ (36) in contrast to DNA-dependent protein kinase, which is extremely abundant in human cells. In our current study, instead of disrupting DNA ligase IV function directly to inhibit NHEJ, we target its obligate partner, XRCC4.

Based on previous structural and functional data relating to the human XRCC4 protein (22), an adenovirus that expresses a fragment of the XRCC4 protein was constructed. Our data show that MDA-MB-231 cells infected with the adenovirus overexpress the XRCC4 protein fragment. Immunoprecipitation studies indicated that the XRCC4 fragment is able to compete with full-length endogenous XRCC4 for binding to ligase IV in the cytoplasm and nucleus of MDA-MB-231 breast tumor cells. Presumably, the newly synthesized ligase IV associates with the overexpressed XRCC4 fragment (from adenovirus), diluting the new and existing normal endogenous XRCC4/ligase IV complex and disrupting its function.

MDA-MB-231 cells infected with the adenovirus expressing the XRCC4 fragment were significantly sensitized to ionizing radiation compared with uninfected and LACZ adenovirus-infected controls. As the XRCC4 fragment is able to bind ligase IV, it is likely that the XRCC4 fragment causes radiosensitization by interfering with NHEJ. Although MDA-MB-231 cells were sensitized to ionizing radiation by the XRCC4 fragment, we did expect that inhibition of NHEJ would result in a more dramatic sensitization response. Our data showed that full-length XRCC4 was immunoprecipitated from the cytoplasm with the XRCC4 fragment and ligase IV, indicating that the XRCC4 fragment is able to bind full-length XRCC4 as well as ligase IV. It is not known whether it is absolutely necessary for two molecules of the XRCC4 fragment to bind ligase IV to fully inhibit ligase IV function or whether full inhibition of ligase IV function occurs when only one XRCC4 fragment molecule binds ligase IV. It is also not known whether functional XRCC4/ligase IV complexes must be present on both DNA ends being joined or whether a functional complex on one end is sufficient. Thus, it is difficult to predict what extent of substitution of XRCC4 with XRCC4 fragment would be necessary to disrupt NHEJ, but it is possible that efficient repair requires four functionally competent full-length XRCC4 molecules (a dimer on each end) and that replacement of only one of these molecules with XRCC4 fragment would block repair. Initial attempts to directly assess a repair defect by analysis of nuclear 53BP1 foci (a putative measure of unrepaired double-strand breaks) were inconclusive. Although there did seem to be a slightly higher level of foci in cells expressing XRCC4 fragment than in LACZ-expressing cells 2 hours after irradiation, the difference was not statistically significant (data not shown).

The extent of radiosensitization may also be limited due to the presence of preexisting active XRCC4/ligase IV complexes, which may be resistant to disruption by newly synthesized XRCC4 fragment and thus may persist despite XRCC4 fragment overexpression. Consistent with this hypothesis, our data indicate that there may be a significant amount of ligase IV that is not bound to the XRCC4

fragment but that remains bound to full-length XRCC4, as ligase IV was detected in the supernatant following immunoprecipitation of FLAG-tagged XRCC4 fragment from the cytoplasmic extract of MDA-MB-231 cells infected with the XRCC4 fragment adenovirus. However, it should be noted that not all of the FLAG-tagged XRCC4 was precipitated with the beads and that ligase IV in the supernatant could also represent ligase IV bound to the XRCC4 fragment. Regardless, our data show that even incomplete disruption of the XRCC4/ligase IV complex radiosensitizes breast cancer cells and establish that the disruption of this complex is a viable target for radiosensitizing cancer cells. Because the XRCC4 fragment is able to bind ligase IV, it is highly likely that sensitization occurs through inhibition of DNA ligase IV function, ultimately inhibiting DNA repair by NHEJ. Further refinement of this approach, with either small molecules or variations of the XRCC4 fragment, holds promise for clinical application to cancer therapies.

Acknowledgments

We thank Elizabeth Rosenberg and Aaron Randolph for assistance with adenovirus construction.

References

1. Arthur DW, Morris MM, Vicini FA. Breast cancer: new radiation treatment options. Oncology 2004;18:1621 – 30.

2. Violet JA, Harmer C. Breast cancer: improving outcome following adjuvant radiotherapy. Br J Radiol 2004;77:811 – 20.

3. Schmidt-Ullrich RK, Contessa JN, Lammering G, Amorino G, Lin PS. ERBB receptor tyrosine kinases and cellular radiation responses. Oncogene 2003;22:5855 – 65.

4. Jameel JK, Rao VS, Cawkwell L, Drew PJ. Radioresistance in carcinoma of the breast. Breast 2005;13:452 – 60.

5. Valerie K, Povirk LF. Regulation and mechanisms of mammalian double-strand break repair. Oncogene 2003;22:5792 – 812.

6. Karran P. DNA double strand break repair in mammalian cells. Curr Opin Genet Dev 2000;10:144 – 50.

7. Jackson SP. Sensing and repairing DNA double-strand breaks. Carcinogenesis 2002;23:687 – 96.

8. Lees-Miller SP, Meek K. Repair of DNA double strand breaks by non-homologous end joining. Biochimie 2003;85:1161 – 73.

9. El-Khamisy SF, Saifi GM, Weinfeld M, et al. Defective DNA single-strand break repair in spinocerebellar ataxia with axonal neuropathy-1. Nature 2005;434:108 – 13.

10. Khanna KK, Jackson SP. DNA double-strand breaks: signaling, repair and the cancer connection. Nat Genet 2002;27:247 – 54.

11. Jeggo PA. DNA-PK: at the cross-roads of biochemistry and genetics. Mutat Res 1997;384:1 – 14.

12. Gottlieb TM, Jackson SP. The DNA-dependent protein kinase: requirement for DNA ends and association with Ku antigen. Cell 1993; 72:131 – 42.

13. Chernikova SB, Wells RL, Elkind MM. Wortmannin sensitizes mammalian cells to radiation by inhibiting the DNA-dependent protein kinase-mediated rejoining of double-strand breaks. Radiat Res 1999;151: 159 – 66.

14. Taccioli GE, Gottlieb TM, Blunt T, et al. Ku80: product of the XRCC5 gene and its role in DNA repair and V(D)J recombination. Science 1994; 265:1442 – 5.

15. Weaver DT. Regulation and repair of double-strand DNA breaks. Crit Rev Eukaryot Gene Expr 1996;6:345 – 75.

16. Riballo E, Critchlow SE, Teo SH, et al. Identification of a defect in DNA ligase IV in a radiosensitive leukaemia patient. Curr Biol 1999;9: 699 – 702.

17. O'Driscoll M, Cerosaletti KM, Girard PM, et al. DNA ligase IV mutations identified in patients exhibiting developmental delay and immunodeficiency. Mol Cell 2001;8:1175 – 85.

18. Sibanda BL, Critchlow SE, Begun J, et al. Crystal structure of an Xrcc4-DNA ligase IV complex. Nat Struct Biol 2001;8:1015 – 9.

19. Lee JW, Yannone SM, Chen DJ, Povirk LF. Requirement for XRCC4 and DNA ligase IV in alignment-based gap filling for nonhomologous DNA end joining *in vitro*. Cancer Res 2003;63:22 – 4.

20. Lee JW, Blanco L, Zhou T, et al. Implication of DNA polymerase λ in alignment-based gap filling for nonhomologous DNA end joining in human nuclear extracts. J Biol Chem 2004;279:805 – 11.

21. Mahajan KN, Nick McElhinny SA, Mitchell BS, Ramsden DA. Association of DNA polymerase μ (pol μ) with Ku and ligase IV: role for pol μ in end-joining double-strand break repair. Mol Cell Biol 2002;22: 5194 – 202.

22. Mizuta R, Cheng HL, Gao Y, Alt FW. Molecular genetic characterization of XRCC4 function. Int Immunol 1997;9:1607 – 13.

23. Modesti M, Hesse JE, Gellert M. DNA binding of Xrcc4 protein is associated with V(D)J recombination but not with stimulation of DNA ligase IV activity. EMBO J 1999;18:2008 – 18.

24. Grawunder U, Wilm M, Wu X, et al. Activity of DNA ligase IV stimulated by complex formation with XRCC4 protein in mammalian cells. Nature 1997;388:492 – 5.

25. Holmes M, Rosenberg E, Valerie K. Adenovirus expressing p53. Methods Mol Biol 2003;234:1 – 16.

26. Chartier C, Degryse E, Gantzer M, Dieterle A, Pavirani A, Mehtali M. Efficient generation of recombinant adenovirus vectors by homologous recombination in *Escherichia coli*. J Virol 1996;70:4805 – 10.

27. Hsu H-L, Yannone SM, Chen DJ. Defining the interactions between DNA-PK and ligase IV/XRCC4. DNA Repair 2001;1:225 – 35.

28. Hande MP. DNA repair factors and telomere-chromosome integrity in mammalian cells. Cytogenet Genome Res 2004;104:116 – 22.V.

29. Espejel S, Martin M, Klatt P, Martin-Caballero J, Flores JM, Blasco MA. Shorter telomeres, accelerated ageing and increased lymphoma in DNA-PKcs-deficient mice. EMBO Rep 2004;5:503 – 9.

30. Hsu HL, Gilley D, Galande SA, et al. Ku acts in a unique way at the mammalian telomere to prevent end joining. Genes Dev 2000;14: 2807 – 12.

31. Jaco I, Munoz P, Blasco MA. Role of human Ku86 in telomere length maintenance and telomere capping. Cancer Res 2004;64:7271 – 8.

32. Grawunder U, Zimmer D, Kulesza P, Lieber MR. Requirement for an interaction of XRCC4 with DNA ligase IV for wild-type V(D)J recombination and DNA double-strand break repair *in vivo*. J Biol Chem 1998;273: 24708 – 14.

33. Gao Y, Sun Y, Frank KM, et al. A critical role for DNA end-joining proteins in both lymphogenesis and neurogenesis. Cell 1998;95:891 – 902.

34. Jackson SP, Jeggo PA. DNA double-strand break repair and V(D)J recombination: involvement of DNA-PK. Trends Biochem Sci 1995;20: 412 – 5.

35. Shi YQ, Blattmann H, Crompton NE. Wortmannin selectively enhances radiation-induced apoptosis in proliferative but not quiescent cells. Int J Radiat Oncol Biol Phys 2001;49:421 – 5.

36. Huang J, Dynan WS. Reconstitution of the mammalian DNA double-strand break end-joining reaction reveals a requirement for an Mre11/ Rad50/NBS1-containing fraction. Nucleic Acids Res 2002;30:667 – 74.