A Hot Spot for RAD51C Interactions Revealed by a Peptide That Sensitizes Cells to Cisplatin

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Abstract

DNA repair via the homologous recombination pathway requires the recombinase RAD51 and, in vertebrates, five RAD51 paralogs. The paralogs form two complexes in solution, a XRCC3/RAD51C heterodimer and a RAD51B/RAD51C/RAD51D/XRCC2 heterotetramer. Mutation of any one of the five paralog genes prevents subnuclear assembly of recombinase at damaged sites and renders cells 30–100 fold sensitive to DNA cross-linking drugs. Phage display was used to isolate peptides that bind the paralog XRCC3. Sequences of binding peptides showed similarity to residues 14–25 of RAD51C protein. Point mutations in this region of RAD51C altered its interaction with both XRCC3 and RAD51B in a two-hybrid system. A synthetic peptide composed of residues 14–25 of RAD51C fused to a membrane transduction sequence [protein transduction domain 4 (PTD4)], inhibited subnuclear assembly of RAD51 recombinase, and sensitized Chinese hamster ovary cells to cisplatin when added to growth medium. These results suggest that residues 14–25 of RAD51C contribute to a “hot spot” used in both XRCC3-RAD51C and RAD51B-RAD51C interactions. Peptide-based inhibition of homologous recombination may prove useful for improving the efficacy of existing cancer therapies.

Introduction

Studies have demonstrated a dramatic enhancement of cisplatin sensitivity associated with cellular defects in proteins that promote DNA repair via the homologous recombination (HR) pathway (1–4). HR has multiple roles in DNA repair including the repair of DNA double-strand breaks and recovery from the replication blocking lesions formed by DNA-cross-linking agents. Bypass and/or repair of cross-linked DNA by HR proteins are thought to contribute to the resistance of tumor cells to platinum-based chemotherapy. HR requires the recombinase RAD51 and, in vertebrates, five RAD51 paralogs. The paralogs form two complexes in solution, a XRCC3/RAD51C heterodimer and a RAD51B/RAD51C/RAD51D/XRCC2 heterotetramer (5–8). Mutation of any one of the five paralog genes prevents subnuclear assembly of recombinase at damaged sites and renders cells 30–100-fold sensitive to DNA cross-linking drugs (1–4, 9–11). These paralogs are thought to serve as assembly “mediators” for RAD51. Single-strand DNA-binding proteins can inhibit assembly of RAD51 recombinase at sites of damage, and mediator proteins act to overcome this inhibition. The proposal that mammalian paralogs serve as RAD51 mediators is supported by biochemical studies using a partial complex that includes only RAD51B and RAD51C (12).

Screening of random peptide phage display libraries has become a valuable method for developing peptides that bind to particular targets. Interestingly, the peptide motifs resulting from such screens can mimic or identify natural ligands of the target in question. On the basis of these findings, XRCC3-binding peptides were isolated with the goal of inhibiting HR by blocking protein-protein interactions. XRCC3-binding peptides were isolated by phage display, and a subset of these peptides were similar in sequence to an NH2-terminal region of RAD51C. This region of RAD51C was shown to be important for its interaction with the paralog proteins RAD51B and XRCC3. Direct cellular transduction with a synthetic peptide corresponding to this region reduces the efficiency of damage-induced RAD51 focus formation and sensitizes cells to cisplatin.

Materials and Methods

Antibodies and Cell Lines. Rabbit polyclonal anti-hsRAD51 antiserum (provided by A. Shinohara, Osaka University, Toyonaka, Osaka, Japan) was purified with a protein-A column (Amersham Pharmacia, Piscataway, NJ) followed by affinity purification. Antiserum against His-tagged XRCC3 protein was purified via protein-A column (Amersham Pharmacia). Iris1SF cells and a human XRCC3-expressing plasmid (pXR3) were provided by L. Thompson, Lawrence Livermore National Laboratory, Livermore, CA (1). Iris1SF cells were stably cotransfected with pXR3 and the geneticin-resistance plasmid pCB6 or were transfected with pCB6 only. Clones were selected based on resistance to 0.71 μg/ml geneticin (Sigma, St. Louis, MO). The resulting cell lines were subsequently tested for cisplatin resistance. Western blot analysis of nuclear extracts confirmed XRCC3 protein expression in the cotransfected cells (Iris1SF + pXR3 + pCB6), but no detectable expression in the control cells (Iris1SF + pCB6).

Preparation of Proteins. A bacteriophage T7 expression library was created by cloning 6xHis-tagged human XRCC (pET29-XR3) was provided by Larry Thompson. BL21(DE3) lambda bacteria (Novagen, Madison, WI) were transformed with pET29-XR3 and were induced with isopropyl-1-thio-β-n-d-galactopyranoside for 2 h. Cells were disrupted by sonication, and the 6xHis-tagged protein was purified via cobalt affinity chromatography (Clontech, Palo Alto, CA) in a buffer containing 8 M urea. The resulting XRCC3 protein was >95% pure on Coomassie-stained SDS-PAGE. To promote protein renaturation, we reduced the concentration of urea from 8 to 2.5 M by dialysis.

Phage Display. High-binding microtiter wells (Costar) were coated with 6xHis-XRCC3 protein by adding 2.5 μg of the partially renatured protein into 100 μl of PBS. Wells were blocked (Tris-buffered saline, 0.1% Tween 20, and 5% dried milk) and washed five times with Tris-buffered saline/0.1% Tween 20. Wells were then incubated with the random 12-residue Ph.D.-12 phage display library (New England Biolabs, Beverly, MA) and were washed. The XRCC3-binding clones were eluted with anti-XRCC3 polyclonal antibodies (100 μg/ml in Tris-buffered saline/0.1% Tween 20) and were amplified on agar plates.

Yeast Two-Hybrid Assays, Plasmids for two-hybrid analysis, pDS157, pDS138, and pDS151, were provided by David Schild, Lawrence Berkeley National Laboratory, Berkeley, CA; these plasmids encode RAD51C fused to the Gal4-activating domain (AD), XRCC3 fused to the Gal4 DNA-binding domain (DBD), and RAD51B fused to the Gal4-DBD, respectively (13). Alanine substitution mutations were generated in RAD51C with the Transformer Site-Directed Mutagenesis kit (Clontech). Yeast strain P69-4A (MATa, trpl–901, leu2–3,112, ura3–52, his3–200, gal4Δ, gal80Δ, GAL2–3002...
ADE2, LYS2::GAL1-HIS3, met2::GAL7-lacZ, Ref. 14) was transformed with appropriate pairs of the activating domain (AD)- and DNA-binding domain (DBD)-expressing plasmids, and cotransformants were selected on appropriate amino acid omission medium. β-galactosidase quantification was performed using previously published methods (15).

Preparation of Synthetic Peptides. Synthetic peptides were modified by NH2-terminal acetylation and COOH-terminal amidation to reduce proteolytic degradation. Peptides were purified by high-performance liquid chromatography, were analyzed by mass spectroscopy (Genemed Synthesis, San Francisco, CA), and were aliquoted dry for storage at −80°C.

Cytotoxicity Assays. Cytotoxicity was determined by loss of colony-forming ability and were performed in triplicate. Crystal violet-stained colonies were imaged with a charge-coupled device camera and were counted using NIH Image software.

RAD51 Focus Formation Assay. RAD51 focus formation assays were performed, as described previously (11). RAD51 foci were scored by visual examination of microscopic images. Slides were coded to avoid the possibility of scoring bias.

Results and Discussion

An M13 phage display library was used, wherein each phage virion expresses five copies of a random 12-residue peptide. Peptides were selected based on binding to purified 6xHis-XRCC3 and were eluted with anti-XRCC3 polyclonal antibodies. Two consecutive rounds of selection were performed. After the second round, 50 phage were sequenced to identify displayed peptides. Twenty-eight of these sequences contained known polystyrene-binding motifs (e.g., Trp Trp or Trp His) and were eliminated from subsequent analyses. Analysis of the 22 remaining sequences revealed 4 that could be aligned to residues 14–25 of RAD51C protein (Fig. 1A). The alignment was found to be significant (P = 0.0025) using a multiple sequence alignment tool (MACAW, BLOSUM-80 matrix) and a search space that included full length RAD51C and the four peptides. The same analysis did not generate significant alignments when the 22 peptides were compared with other proteins (RAD51, XRCC2, XRCC3, RAD51B, RAD51D, Ku, or BSA).

RAD51C protein is known to interact with XRCC3 and RAD51B. Detection of residues in RAD51C that aligned with those in the peptides raised the possibility that these RAD51C residues might be important for XRCC3 binding and perhaps for RAD51B binding as well. Yeast two-hybrid experiments were performed to test these possibilities using alanine substitution mutations in RAD51C. The two-hybrid system used allows two types of assays, a qualitative growth assay in which the two-hybrid interaction is required for histidine prototrophy, and a quantitative β-galactosidase assay in which the two-hybrid interaction is required for expression of a Pgalα-lacZ fusion gene (14). Two of the RAD51C mutations, P18A and S20A, resulted in reduced XRCC3 interaction in the growth assay (Fig. 2A), and this corresponded to modest reductions in the β-galactosidase assay (Fig. 2B). A double mutant containing both of these alanine substitutions (P18A, S20A) yielded a dramatic effect in the growth assay and a 38.6% reduction in interaction on the β-galactosidase assay. These results are consistent with a recent study by

Fig. 1. Peptide sequences and alignments. A, alignment of XRCC3-binding peptide sequences to RAD51C (amino acids 14–25). Residues in bold, identity. B, the sequences of the synthetic peptides. A three-residue glycine linker (GGG) connects the RAD51C-derived and peptide transduction segments. PTD4, protein transduction domain 4; scram, scrambled (the order of residues 14–25); RAD51c(scram)-PTD4, negative control peptide.
Kurumizaka et al. (16), in which NH2-terminal deletions of the first 6 residues of RAD51C had no significant effect on binding to XRCC3, but deletions of 19 residues reduced binding. The L19A mutant and double mutants that included L19A displayed a strong reduction in RAD51B-RAD51C interaction in both assays. The importance of the RAD51C region for binding to both XRCC3 and RAD51B may explain why RAD51C is the only component shared by both of the two paralog complexes; XRCC3-RAD51C binding and RAD51B-RAD51C binding may be mutually exclusive. These yeast two-hybrid results should be confirmed via coimmunoprecipitation and with functional assays in mammalian cells.

Surprisingly the L19A mutation, although reducing RAD51B-RAD51C interaction, increased XRCC3-RAD51C interaction. This result suggests that L19 is specifically important for RAD51C-RAD51B interaction. Specific differences between the XRCC3-RAD51C interface and the RAD51B-RAD51C interface could provide a means to regulate the relative abundance of the XRCC3/RAD51C and RAD51B/RAD51C/RAD51D/XRCC2 complexes, or allow for dynamic RAD51C interface exchanges like those proposed for RAD51 protein (17).

A synthetic fusion peptide [RAD51C(14–25)-PTD4] was prepared containing residues 14–25 of RAD51C, a three-residue polyglycine linker, and protein transduction domain 4 (PTD4; Fig. 1B). Peptides containing PTD4, have been shown to cross lipid bilayers and to promote direct intracellular transduction of peptides and proteins (18). A negative control peptide [RAD51C(scram)-PTD4] was synthesized wherein the order of residues 14–25 of RAD51C was scrambled (scram). Colony-forming assays were performed to determine the survival of Chinese hamster ovary cells after cisplatin treatment in combination with peptides (Fig. 3). The RAD51C(14–25)-PTD4 peptide sensitized cells to cisplatin with peptide concentrations as low as 125 nM, reducing the yield of viable clonogenic cells ~2-fold relative to treatment with cisplatin alone or cisplatin with scrambled control peptide (Fig. 3, A and B). In the absence of cisplatin, neither RAD51C(14–25)-PTD4 nor the scrambled control peptide was toxic at concentrations up to 2 μM, indicating that the killing effect of the peptide depends on cisplatin-induced damage. In contrast to its effects on Chinese hamster ovary cells that express all five paralogs, RAD51C(14–25)-PTD4 peptide did not enhance the cisplatin sensitivity of a genetically matched XRCC3-deficient Chinese hamster ovary cell line (Fig. 3C). This result indicates that sensitization depends on functional HR.

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RAD51 focus formation assays were performed to test whether RAD51C(14–25)-PTD4 prevents the subnuclear assembly of RAD51 after DNA damage. XRCC3-expressing Chinese hamster ovary (CHO) cells (irs1sf+pXR3+pCB6) were treated with medium containing 25 μM cisplatin (Cis) or with medium alone, followed by various concentrations of peptide. B. XRCC3-expressing CHO cells (irs1sf+pXR3+pCB6) were treated with various concentrations of cisplatin followed by medium containing 0.5 μM peptide. C. XRCC3-deficient CHO cells (irs1sf+pCB6) were treated with various concentrations of cisplatin followed by incubation in 0.5 μM peptide. 51c (14–25), peptide [RAD51C(14-25)-PTD4]; 51c (scram), negative control peptide [RAD51C(scram)-PTD4].
RAD51 recombinase after DNA damage (Fig. 4). Fifty randomly selected nuclei per treatment group were examined. Untreated cells had an average of 3.4 spontaneous RAD51 foci per nucleus, and treatment with cisplatin treatment increased this to 15.9 foci per nucleus. The addition of RAD51C(14–25)-PTD4 peptide after cisplatin treatment reduced the average number of foci by 2.6-fold to an average of 5.6 foci per nucleus, relative to the scrambled control peptide that had essentially no effect (14.7 foci per nucleus). This difference was significant based on a Mann-Whitney rank sum test ($P < 0.0001$). The difference was also significant based on a Fisher exact test if a threshold of 10 foci/nucleus was used to score individual nuclei as “focus-positive” or “focus-negative” ($P = 0.0044$). These results suggest that RAD51C(14–25)-PTD4 sensitizes cells to cisplatin by inhibiting the assembly of RAD51 recombinase at sites of damage.

These findings add to the growing body of evidence that the HR pathway is a useful therapeutic target for overcoming the resistance of cancer cells to DNA-damaging therapies (19–21). The potential advantages of the RAD51C(14–25)-PTD4 peptide over other strategies include its small size, membrane permeability, and effectiveness at low concentrations. Additional studies are required to determine the specific molecular mechanism through which the peptide sensitizes cells to cisplatin. However, the two-hybrid interaction results, together with the observation that cisplatin-sensitizing activity is specific to HR-competent cells, suggests that the peptide acts in cells by disrupting the interaction of RAD51C with XRCC3 and/or RAD51B. Finally, the ability to use peptide transduction to block a particular protein-binding surface in cells may facilitate “separation of function” analyses for proteins found to have two or more separate surfaces that bind different partners. Such analyses may prove particularly valuable for studies of human cells in which functional analysis is currently limited to methods (such as gene silencing with small interfering RNAs) that can only alter the steady-state level of full-length protein.

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