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Mechanism of RAD51dependent DNA interstrand crosslink repair

David T. Long¹, Markus Räschle², Vladimir Joukov³, and Johannes C. Walter^{1,*}

¹Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, MA 02115, USA

²Department of Proteomics and Signal Transduction, Max Planck Institute of Biochemistry, 82152 Martinsried, Germany

³Department of Cancer Biology, Dana-Farber Cancer Institute, Boston, MA 02115, USA

Abstract

DNA interstrand cross-links (ICLs) are toxic DNA lesions whose repair in S phase of eukaryotic cells is incompletely understood. In *Xenopus* egg extracts, ICL repair is initiated when two replication forks converge on the lesion. Dual incisions then create a DNA double-strand break (DSB) in one sister chromatid while lesion bypass restores the other sister. We report that the broken sister chromatid is repaired via RAD51-dependent strand invasion into the regenerated sister. Recombination acts downstream of FANCI-FANCD2, yet RAD51 binds ICL-stalled replication forks independently of FANCI-FANCD2 and prior to DSB formation. Our results elucidate the functional relationship between the Fanconi anemia pathway and the recombination machinery during ICL repair. In addition, they demonstrate the complete repair of a DSB via homologous recombination *in vitro*.

In vertebrate cells, DNA interstrand cross-link repair is coupled to DNA replication and involves structure specific endonucleases, translesion DNA polymerases, recombinases, and numerous proteins mutated in the human disease Fanconi anemia (FA) (1). FA is characterized by genomic instability and cellular sensitivity to DNA interstrand cross-linking agents. A central event in the FA pathway is the ubiquitylation of the FANCI-FANCD2 heterodimer, which activates it for ICL repair (2, 3). Extensive evidence indicates that homologous recombination (HR) is essential for ICL repair (49). However, the precise role of HR in ICL repair remains conjectural, and the functional relationship between the FA and HR pathways is unclear.

Using *Xenopus* egg extracts, we established a cell-free system for replication-dependent repair of a plasmid containing a single, site-specific cisplatin ICL (pICL) (Figure 1A) (2, 10). Upon addition of pICL to egg extracts, replication initiates at a random site and two replication forks converge on the ICL (Figure 1B, i). The leading strand of one fork is then extended to within one nucleotide of the ICL (Figure 1B, ii). Next, dual incisions surrounding the ICL create a DSB in one sister chromatid and translesion DNA synthesis

*To whom correspondence should be addressed. johannes_walter@hms.harvard.edu.

restores the other sister by first inserting a nucleotide across from the adducted base (Figure 1B, iii), followed by strand extension beyond the ICL (Figure 1B, iv). Ultimately, 5–25% of replicated pICL is fully repaired, as measured by regeneration of a SapI site that was originally interrupted by the crosslink (2, 10). In the absence of ubiquitylated FANCD2, DNA incisions, lesion bypass, and SapI site regeneration are greatly diminished (2). Given the established links between HR and ICL repair, and the inefficient removal of the unhooked ICL in egg extracts (10), we postulated that SapI site regeneration involves repair of the broken sister chromatid by recombination with the intact sister (Figure 1B, v).

To look for evidence of HR in ICL repair, pICL or an undamaged control plasmid (pControl) was replicated in egg extract, digested with HincII (Figure 1A), and analyzed by two-dimensional gel electrophoresis (2DGE) (Figure 1C and D) (11). After 5 minutes, both plasmids generated the expected double-Y and bubble replication intermediates (Figure 1C; Figure 1D, i). As replication neared completion, pControl produced unit-sized linear DNA molecules (Figure 1C, red arrowhead; Figure 1D, i), whereas pICL yielded a discrete X-shaped intermediate that results from the convergence of two forks on the ICL (Figure 1C, blue arrowhead; Figure 1D, i). Two simple-Y intermediates also accumulated (3–7% of total signal), suggesting that a small number of converging forks undergo aberrant breakage or cleavage (Figure 1C, green arrowheads; Figure 1D, ii). By 60 minutes, the X-shaped intermediates showed signs of processing, likely due to resection of the nascent lagging strands (10). Even later, the X- and Y-shaped intermediates were replaced by an “X-arc” (Figure 1C, purple arrowhead; Figure 1D, iii). Formation of the X-arc coincided with SapI site regeneration (Figure 1E, red graph), suggesting the X-arc contains intermediates of ICL repair.

X-arcs are generally formed when two unit-sized linear molecules are joined by a mobile branch point, such as a Holliday junction or hemicatenane [Figure 1D, iii; (12)]. Under conditions that promote resolution of mobile junctions through branch migration (see *Methods*), the X-arc intermediates of pICL were resolved primarily into the predicted unit-sized linear molecules, whether Magnesium was present or not (Figure S1A–C). Since Magnesium inhibits migration of Holliday junctions, this implies that the X-arc intermediates contain hemicatenanes. Consistent with this, Xarc intermediates were resistant to cleavage by the Holliday junction resolvase, RuvC (Figure S1D). Exposing replicated pICL to branch migration conditions increased the yield of SapI repair fragments (Figure 1E, purple graph), indicating that X-arcs contained unresolved intermediates of ICL repair.

If repair of pICL involves HR, inhibiting the RAD51 recombinase should block repair. To test this prediction, we used a ~35 amino acid ‘BRC’ peptide derived from BRCA2 that binds RAD51 and, when supplied at high concentrations, inhibits RAD51 nucleoprotein filament formation (13). As recently reported (14), addition of a BRC^{WT} peptide (Figure S2A) to *Xenopus* egg extracts abolished RAD51 loading onto damaged chromatin (Figure 2A). Two variants of the peptide that were mutated in one (BRC*) or three (BRC***) amino acids (Figure S2A) were increasingly compromised for RAD51 binding (Figure 2B) and inhibition of RAD51 loading (Figure 2A), demonstrating that BRC^{WT} specifically targets RAD51. Without affecting DNA replication (Figure S2B), the BRC peptides inhibited X-arc formation proportional to their effect on RAD51 binding and filament formation (Compare

Figures 2A and 2B with Figure 2C). Thus, X-arc intermediates are RAD51- dependent, indicating they represent products of HR. While BRC^{WT} almost completely abolished SapI site regeneration, BRC* caused intermediate inhibition and BRC*** had no effect (Figure 2D). These results demonstrate that replication-coupled ICL repair requires RAD51-dependent HR. Since dual incisions result in a two-ended DSB (Figure 1B, iii), recombination likely first yields a double Holliday junction, which is then converted to a mobile hemicatenane.

We next used BRC peptides to investigate which step in ICL repair involves RAD51. Replicating pICL was digested with AflIII (Figure 1A) and subjected to denaturing gel electrophoresis to examine leading strand products of the rightward and leftward (not shown) replication forks as they converge on the ICL (Figure 3A). In the presence of BRC^{WT}, the arrival of leading strands 20–40 nts from the ICL (Figure 3B and C), the approach of leading strands to the -1 position (Figure 3B and D), and nucleotide insertion across from the adducted base (Figure 3B and E) were all unaffected. When BRC^{WT} samples were analyzed by native 1D and 2DGE, there was no change in the rate at which dual-stalled fork structures disappeared (Figure S3A–D), indicating that DNA incisions were unaffected. In contrast, BRC^{WT} specifically reduced full-length extension products two-fold (Figure 3B and F), which is consistent with an absence of RAD51-mediated restoration of the broken sister chromatid (Figure 1B, v). Timed addition of BRC peptides to ICL repair reactions indicated that RAD51 completes its repair function late, just prior to SapI site regeneration (Figure S4A and B). Additionally, plasmid competition experiments showed that recombination occurred primarily between sister chromatids (Figure S5). Together, the data indicate that RAD51-dependent HR between the broken and intact sister chromatids is a crucial, late step in ICL repair.

We used chromatin immunoprecipitation (ChIP) to examine the binding of RAD51 and RPA to three locations on pICL during repair (Figure 4A). Initially, RPA and RAD51 accumulated near the ICL (Figure 4B; site I). After a delay, they also bound ~700 base pairs from the ICL (Figure 4B; site II), likely due to resection of lagging strands (10), but they never accumulated opposite the ICL (Figure 4B; site III). No RAD51 binding was detected in the absence of replication, on undamaged plasmids, or in the presence of BRC^{WT} (Figure S6A). The timing of RPA binding coincided with the convergence of DNA replication forks at the lesion, followed shortly thereafter by RAD51 binding (Figure 4C, green, blue, red graphs). When RAD51 binding peaked at 40 minutes, greater than 90% of the dual-stalled fork structures remained, indicating that little or no incisions had taken place (Figure 4C, compare red and blue graphs). To rule out that RAD51 binding at this time was limited to a small number of broken fork intermediates, we immunoprecipitated RAD51 from repair reactions and examined the associated DNA intermediates. Fifty-three percent of DNA associated with RAD51 consisted of intact, dual-stalled fork structures (Figure 4D, red arrow). Thus, in the context of ICL repair, RAD51 is loaded onto stalled replication forks early, prior to DSB formation.

We next examined the functional relationship between the FA and HR pathways. In FANCD2-depleted extracts, the X-arc disappeared, and it was rescued by re-addition of recombinant FANCI-FANCD2 (Figure 4E, purple arrowheads). As expected, the dual-

stalled fork structure persisted in the absence of FANCD2 (Figure 4E, blue arrowhead), consistent with a defect in incisions (2). The data indicate that HR acts downstream of FANCI-FANCD2 during ICL repair, explaining previous epistasis experiments (7). Consistent with some analyses of RAD51 foci [reviewed in (8)], RAD51 binding to ICLs was not reduced in the absence of FANCD2 (Figure 4F and Figure S7A, B, C). Conversely, inhibition of RAD51 function using BRC^{WT} peptide had no effect on FANCD2 recruitment or ubiquitylation (Figure 2A and Figure S7D). Together, our data show that FANCI-FANCD2 acts upstream of HR in the context of replication-coupled ICL repair, but that it is not required for RAD51 recruitment to chromatin. Instead, the requirement for FANCI-FANCD2 in promoting HR can be explained by its role in promoting the incisions that underlie DSB formation (2). The FA pathway may also enhance HR via more direct mechanisms, since FA proteins also stimulate HR in the context of preformed DSBs (6, 7, 9, 15, 16).

Here, we report that in the context of replication-coupled ICL repair, the DSB generated in one sister chromatid through the action of FANCI-FANCD2 is fixed via strand invasion into the intact sister (Figure S8). We find that RAD51 binds efficiently to ICLs before a DSB has been generated (Figure S8, ii). While lesion bypass likely displaces RAD51 from one sister chromatid, incisions and resection of the other sister creates a new docking site for RAD51, such that both ends of the DSB are coated with the recombinase (Figure S8, iv). The interaction of RAD51 with ICL-stalled forks before DSB formation may function to prevent fork breakage (17) in favor of regulated incisions and/or to initiate strand invasion as soon as possible once the DSB has been formed.

A major obstacle impeding our understanding of DSB repair has been the absence of cell-free systems. Combined with ChIP and the ability to inactivate or remove essential proteins, the system described here represents a powerful tool to elucidate the complex mechanism underlying DSB repair.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Summary Statement

RAD51 promotes repair of DNA double-strand breaks created by the Fanconi anemia pathway during interstrand cross-link repair.

Figure 1.

The X-arc contains intermediates of ICL repair. **(A)** pICL schematic. **(B)** Model of ICL repair in *Xenopus* egg extracts (2, 10). **(C)** pControl or pICL was replicated in egg extract, digested with HincII, and analyzed by 2DGE. Arrowheads, see main text. **(D)** Cartoon of 2DGE patterns and relevant DNA intermediates. **(E)** ICL repair of samples from (C) was analyzed under normal and branch migration (+BM) conditions. “Background,” SapI fragments from contaminating uncross-linked plasmid. For primary data, see Figure S1E and F. All graphed experiments were performed at least three times, and a representative example is shown.

Figure 2.

ICL repair requires RAD51-dependent HR. **(A)** Cross-linked sperm chromatin was replicated in extract containing buffer (Mock) or the indicated BRC peptide, and chromatin-associated proteins were analyzed by Western blotting. **(B)** BRC peptides immobilized on glutathione sepharose beads were incubated with extract (NPE), pulled down, and the supernatant and pellet blotted for RAD51. **(C)** pICL was replicated in extract containing buffer (Mock) or the indicated BRC peptide. Samples were digested with HincII and analyzed by 2DGE. Purple arrowhead, X-arc position. **(D)** Samples from (C) were analyzed for ICL repair as in Figure 1E.

Figure 3. RAD51-dependent HR functions at a late step in ICL repair. **(A)** Schematic depiction of leading strand intermediates from the rightward fork. **(B)** pICL was replicated in extract containing buffer (Mock) or the indicated BRC peptide. Samples were digested with AflIII and separated on a sequencing gel alongside a ladder generated with primer S [shown in **(A)**]. Nascent strands generated by the rightward fork are indicated at right. The following products from **(B)** were quantified and graphed: **(C)** -20 to -40, **(D)** -1, **(E)** insertion, and **(F)** extension.

Figure 4.

Interplay between the HR and FA pathways. **(A)** Schematic of ChIP primer pairs. **(B)** pICL was replicated and analyzed by RPA and RAD51 ChIP. Controls containing BRC^{WT} peptide, pControl, or lacking DNA replication are shown in Figure S6A. **(C)** Samples from **(B)** were also analyzed for the timing of fork convergence, -1 product accumulation, and repair (raw data in Figure S6B–D). The data were graphed as % of peak value and compared with RPA and RAD51 ChIP at site I [from **(B)**]. **(D)** pICL was replicated in extract for 40 minutes and immunoprecipitated with RAD51 antibodies (see *Methods*). Recovered DNA was digested with HincII and analyzed by agarose gel electrophoresis. Repair intermediates are depicted at left for pICL (blue) and an internal control plasmid, pQuant (gray). **(E)** pICL was replicated in mock-depleted egg extract (Mock), FANCD2-depleted extract (FANCD2^Δ), or FANCD2^Δ extract supplemented with 375 nM FANCI-FANCD2 (FANCD2^Δ+ID). Samples were digested with HincII and analyzed by 2DGE. Arrowheads, see text. See Figure S7E for complete 2D gel time courses. **(F)** Samples from **(E)** were analyzed by ChIP using primer pair I. Primer pairs II and III are shown in Figure S7A.