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Inhibition of topoisomerase I prevents chromosome breakage at common fragile sites

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ABSTRACT

Common fragile sites are loci that preferentially form gaps and breaks on metaphase chromosomes when DNA synthesis is perturbed, particularly after treatment with the DNA polymerase inhibitor, aphidicolin. We and others have identified several cell cycle checkpoint and DNA repair proteins that influence common fragile site stability. However, the initial events underlying fragile site breakage remain poorly understood. We demonstrate here that aphidicolin-induced gaps and breaks at fragile sites are prevented when cells are co-treated with low concentrations of the topoisomerase I inhibitor, camptothecin. This reduction in breakage is accompanied by a reduction in aphidicolin-induced RPA foci, CHK1 and RPA2 phosphorylation, and PCNA monoubiquitination, indicative of reduced levels of single stranded DNA. Furthermore, camptothecin reduces spontaneous fragile site breakage seen in cells lacking ATR, even in the absence of aphidicolin. These data from cultured human cells demonstrate that topoisomerase I activity is required for DNA common fragile site breaks and suggest that polymerase–helicase uncoupling is a key initial event in this process.

1. Introduction

Common fragile sites (CFSs) are loci that demonstrate reproducible, non-random gaps and breaks on metaphase chromosomes when cells are grown under conditions that partially perturb replication, particularly in the presence of low doses of the polymerase inhibitor, aphidicolin (APH) or following folate stress [1,2]. CFSs are large, with breakage occurring over a broad region ranging from hundreds of kilobases to over a megabase. FRA3B at 3p14.2 stands out as the most fragile site in the human genome and can be induced to form gaps or breaks in the majority of treated cells. Other highly expressed CFSs in cultured lymphocytes include those at 16q23 (FRA16D), 6q26 (FRA6E), 7q32.3 (FRA7H), and Xp22.3 (FRAXB). These and at least eight other CFSs have been molecularly characterized [3–14]. They share a number of characteristics that may contribute to their instability, such as long stretches of AT-rich sequence, including AT-repeats, late replication, and their presence within very large genes (reviewed in [15,16]). In addition, it has been shown that histone hypoacetylation is able to reduce the incidence of CFS breakage after APH treatment [17]. CFSs are normally stable in somatic cells *in vivo* but are often associated with chromosome rearrangements in tumor cells, particularly large, submicroscopic deletions or copy number alterations (reviewed in [18]), and we have recently shown that APH induces similar deletions at FRA3B and elsewhere in the genome in cultured cells [19,20]. CFSs may be among the earliest loci in the genome to be deleted during tumorigenesis in association with replication stress [21–23]. The presence of putative tumor suppressor genes at some CFSs, such as *FHIT* at FRA3B [24] and *WWOX* at FRA16D [25] (reviewed in [26]), suggests that CFS instability may lead to a selective growth advantage via inactivation of these genes in some cancers, while other CFS deletions may be neutral but act as signatures of replication stress.

We and others have identified a number of cell cycle checkpoint and DNA repair proteins that are important in maintaining CFS stability, including ATR, BRCA1, CHK1, FANCD2, RAD51, DNA-PKcs, Ligase IV, HUS1, and SMC1 [27–31]. The fact that CFS breakage occurs following modest levels of replication inhibition and that it is regulated by these checkpoint and repair pathways has led to the use of CFS breakage as a cytological assay in studies of the DNA damage response to replication stress.

While considerable progress has been made in identifying the cellular pathways required for maintenance of CFS stability, little is known about the mechanisms involved in the initial breakage events. CFS regions complete replication late in the cell cycle and contain AT-rich sequences that have the potential to form secondary structures that could further impede replication [5,32,33]. Current models for CFS breakage suggest that polymerase stalling and perhaps fork collapse caused by APH and certain other forms of

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replication stress lead to incomplete replication at these sites that can result in DNA double strand breaks [1].

Topoisomerase I (TopoI) unwinds positive supercoils in DNA created by the replicative helicase during replication [34] (reviewed in [35]). TopoI acts by transiently cleaving one strand of duplex DNA, unwinding the DNA, and religating the cleavage site. It has been shown that TopoI is part of the GINS–MCM replication complex and is recruited to replication origins, after which it moves with the replication fork [36]. Camptothecin (CPT) is a powerful chemotherapeutic agent used to treat multiple types of cancer (reviewed in [37]). CPT inhibits TopoI, its only known target, by blocking religation of the DNA, resulting in a dose-dependent reduction in replication [38–41]. At high doses, CPT results in large amounts of DNA double strand breaks, which are thought to be formed when the replication machinery proceeds to the site of the stabilized, TopoI-induced, single stranded nick [42]. At these high doses, this replication-dependant damage can be prevented by co-treating cells with high doses of APH, which stall the replication forks before they can collide with the stabilized TopoI cleavage complex [43–45]. Because of its critical role in replication dynamics, we hypothesize that TopoI plays a role in CFS instability.

To test this hypothesis, we treated normal human cells with APH and varying doses of CPT to partially inhibit TopoI and evaluated them for CFS breaks on metaphase chromosomes as well as for evidence of S-phase or G_2/M checkpoint activation. Our results show that treatment with low doses of CPT almost completely prevents APH-induced breaks at CFSs. We also demonstrate that betulinic acid, a TopoI inhibitor that prevents DNA cleavage also prevents APH-induced CFS breaks. In addition, CPT reduces the spontaneous CFS breakage that occurs in ATR-deficient cells. This reduction in CFS gaps and breaks on metaphase chromosomes is accompanied by a decrease in activation of CHK1, PCNA, and RPA2, consistent with reduced amounts of single stranded DNA (ssDNA) at stalled replication forks. These results from cultured human cells indicate that TopoI activity is required for CFS breakage, and are consistent with *in vitro* models of polymerase–helicase uncoupling and suggest that this uncoupling is an initial key event in CFS instability after replication perturbation.

2. Materials and methods

2.1. Cell culture and fragile site analysis

Normal UML-49 lymphoblastoid cells were grown in RPMI medium (Invitrogen) supplemented with 15% FBS. Primary blood lymphocytes were cultured immediately after venipuncture using conventional techniques. Aliquots of blood were transferred to RPMI (Invitrogen) supplemented with 10% FBS. Lymphocytes were stimulated with phytohemagglutinin (Sigma) for three days prior to experimental treatments. All cells were grown at 37 °C in a humidified atmosphere containing 5% CO_2.

Common fragile sites were induced by exposure of cells to 0.2–0.4 μM APH for 24 h prior to harvest, in the presence or absence of camptothecin (CPT, Sigma) or betulinic acid (BA, Sigma). Cells were harvested for chromosome preparation using standard conditions of 45 min of colcemid treatment (50 ng/ml) followed by an 18 min incubation in 0.075 M KCl at 37 °C and multiple changes of Carnoy fixative (3:1 methanol:glacial acetic acid). Cells were dropped onto slides and baked for 1 h at 60 °C before Giemsa banding or FISH protocols were carried out. Chromosome preparations and G-banding were done by standard methods.

YAC and BAC probes that map to fragile site regions were used for FISH analysis, following standard protocols [46]. YAC 850A6 was used to detect FRA3B and BAC 264L1 (RP-11) was used to detect FRA16D. Probes were labeled with biotin-14-dATP or digoxigenin-11-dUTP using a BioNick Translation Kit (Invitrogen). FISH signals were visualized by incubation with fluorescein isothiocyanate (FITC)-conjugated avidin-DCS and fluorescein-conjugated anti-avidin IgG (Vector Laboratories). Chromosomes were counterstained with 4′,6-diamidino-2-phenylindole (DAPI, Vector Laboratories). Since the FISH probes are smaller than CFSs, chromosome gaps or breaks with a FISH signal immediately proximal, distal, or crossing the break were scored as an induced CFS. FISH results were analyzed with a Zeiss Axioscope epifluorescence microscope and Quips PathVysion imaging software (Vysis Inc).

2.2. Immunocytochemistry

For the detection of RPA2 nuclear foci, cells were fixed in 4% (w/v) paraformaldehyde at 4 °C followed by permeabilization in 0.3% (v/v) Triton X-100 in PBS. Fixed cells were incubated with anti-RPA2 antibody (RPA34-19, Calbiochem) in 5% v/v goat serum, 0.1% v/v NP-40, in PBS for 2 h, washed three times in PBS and incubated for 1 h at room temperature with a mouse-specific fluorescein-conjugated secondary antibody (Molecular Probes). Cells were counterstained and mounted in vectashield plus 406-diamidine-2-phenylindole dihydrochloride (DAPI) (Vector Laboratories). Cells were visualized and images acquired using a Zeiss Axioscope epifluorescence microscope with Quips PathVysion imaging software (Vysis Inc.).

2.3. ATR depletion

HCT116 $ATR^{flox/-}$ cells, which allow for cre-lox mediated removal of ATR, were obtained from Dr. Stephen J. Elledge (Harvard Medical School, Boston, MA) and maintained in DMEM medium + 10% FBS + 200 μg/ml G418. Expression of cre recombinase in these cells was accomplished through infection with adenovirus AdCre1, which was obtained from Dr. Frank Graham (McMaster University, Hamilton, Ontario, Canada) as previously described [27,47].

2.4. Western blots

Cell lysates were prepared by resuspending cell pellets in SDS-lysis buffer followed by sonication. NuPAGE 4–12% Bis-Tris, 10% Bis-Tris, or 3–8% Tris-acetate gels were used to resolve proteins. Whole-cell lysate (25 μg) was loaded per lane. Gels were transferred to PVDF membrane (Millipore Inc.) using a Trans-Blot SD Semi-Dry Transfer system (Bio-Rad Laboratories). Antibody hybridization and chemiluminescence detection were performed according to standard protocols. CHK1 protein was detected with sc-8408 (Santa-Cruz Biotechnology Inc.), CHK2 protein was detected with ab8108 (Novus Biologicals), RPA2 protein was detected with RPA34-19 (EMD), CHK1 phosphorylation on Ser317 was detected with Phosho-Chk1 (Ser317) (Cell Signaling), CHK2 phosphorylation on Thr68 was detected with Phospho-Chk2 (Thr68) (Cell Signaling), and RPA2 phosphorylation on Ser4/8 was detected with BL647 (Bethyl Laboratories, Inc.). PCNA protein was detected with sc-56 (Santa-Cruz Biotechnology Inc.). ATR was detected with a rabbit anti-ATR antibody (Abcam, Inc.). HRP-conjugated anti-mouse and anti-rabbit antibodies were obtained from Amersham Biosciences, Piscataway, NJ, USA. Protein bands were quantitated using Scion Image software (Scion Corp.).

2.5. Cell cycle profiles

UML-49 lymphoblasts were grown for 24 h in medium containing 0.4 μM APH, 30 nM CPT, or both. After a 15 min treatment with 30 μM BrdU, cells were harvested, resuspended in PBS, fixed with