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Identification of RecQL1 as a Holliday junction processing enzyme in human cell lines

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ABSTRACT

Homologous recombination provides an effective way to repair DNA double-strand breaks (DSBs) and is required for genetic recombination. During the process of homologous recombination, a heteroduplex DNA structure, or a ‘Holliday junction’ (HJ), is formed. The movement, or branch migration, of this junction is necessary for recombination to proceed correctly. In prokaryotes, the RecQ protein or the RuvA/RuvB protein complex can promote ATP-dependent branch migration of Holliday junctions. Much less is known about the processing of Holliday junctions in eukaryotes. Here, we identify RecQL1 as a predominant ATP-dependent HJ branch migrator present in human nuclear extracts. A reduction in the level of RecQL1 induced by RNA interference in HeLa cells leads to an increase in sister chromatid exchange. We propose that RecQL1 is involved in the processing of Holliday junctions in human cells.

INTRODUCTION

DNA double-strand breaks (DSBs) arise as a result of ionizing radiation, DNA-damaging chemicals and as a product of blocked replication forks. Mechanisms that repair DNA DSBs are critical for the maintenance of genome integrity and cell viability. Homologous recombination provides an efficient way to repair DNA DSBs. Homologous recombination also occurs during meiosis providing an effective source of genetic variation. During the process of homologous recombination, complementary DNA strand exchange leads to the formation of Holliday junctions (HJs) (1). The initial strand invasion process is carried out by RecA in prokaryotes and the RecA homolog (RAD51) in conjunction with RAD54 in eukaryotes (2). In prokaryotes, the movement, or branch migration, of this junction is facilitated by the RuvA and RuvB proteins. RuvA is a Holliday junction specific binding protein that loads RuvB, an ATP-dependent unwinding protein onto the HJ. The RuvA and RuvB proteins cooperate with the highly specific HJ endonuclease, RuvC, which cuts junctions by symmetrically nicking opposing strands of like polarity. This reaction (termed resolution) produces ligatable products (3). Similar to RuvA, RuvC is a HJ structure specific binding protein. However, it cleaves HJs in a sequence-specific manner within the consensus sequence 5’-AATTG-3’ (4). Branch migration of the HJ by the RuvAB complex allows RuvC to scan for cleavable sequences (5).

The Escherichia coli RecQ protein is another prokaryotic enzyme that can facilitate ATP-dependent branch migration of HJs (6). RecQ belongs to the recF recombination pathway and has been shown to be involved in the resumption of DNA synthesis following DNA damage (7,8). RecQ has also been shown to suppress illegitimate recombination (9).

Much less is known about the processing of HJs in eukaryotes. Branch migration and resolution activities have been detected in mammalian cell free extracts. However, attempts to identify the proteins responsible were unsuccessful (10,11). Recently, it was reported that extracts prepared from cultured mammalian cell lines that have mutations in the RAD51 paralog RAD51C and XRCC3 have reduced levels of resolution activity. Furthermore, immunodepletion of RAD51C from fractionated mammalian extracts resulted in a loss of branch migration and resolution activity. However, neither recombinant protein was found to possess branch migration or resolution activity (12).

Bloom’s syndrome is a rare genetic disorder associated with a predisposition to cancer and genomic instability. Bloom’s syndrome is caused by a mutation in the gene encoding the BLM protein (13). Analysis of this protein established that it is a member of the RecQ family of helicases and is capable of binding to and inducing branch migration of HJs (14). Cultured Bloom’s syndrome cells exhibit a high incidence of spontaneous sister chromatid exchanges (SCEs) (15).

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Recent studies have demonstrated that BLM and topoisomerase III are part of a large complex in human cells, and BLM and topoisomerase III can catalyze the dissolution of Holliday junctions in vitro (16,17). Furthermore, genetic studies have revealed that the yeast homologs of BLM and topoisomerase III, Sgs1 and Top3, suppress the formation of crossover products arising from homologous recombination (18,19). The WRN protein is another member of the RecQ family that is responsible for Werner’s syndrome, a rare autosomal recessive disorder (20). Individuals with Werner’s syndrome manifest the clinical symptoms of premature aging and a predisposition to certain cancers. Cells cultured from Werner’s syndrome patients exhibit abnormal genomic rearrangements and large chromosomal deletions (21,22). Similar to BLM, the WRN protein is also capable of branch migrating HJs (23). Both BLM and WRN are members of a larger family of RecQ-related helicases in humans that also includes RecQL1, RecQ4 and RecQ5β (24). RecQL1 was first identified as an abundant DNA-dependent ATPase with DNA helicase activity in human cells (25) and through a fortuitous protein association (26), whereas other members of the family have been identified through homology. The precise functions of the different RecQ family proteins in maintaining the stability of the genome remain unresolved. Although both BLM and WRN are capable of branch migrating HJs, nuclear extracts prepared from BLM−/− and WRN−/− cell lines still possess comparable levels of ATP-dependent HJ branch migration activity which suggests that another protein may be performing this function (10). We therefore set out to identify the protein responsible for the predominant ATP-dependent HJ branch migration activity present in human cell lines.

**MATERIALS AND METHODS**

**Purification of RecQL1**

RecQL1 was purified from log phase growth HEK293 cell nuclei (2 x 10^10 cells) cultured in DMEM supplemented with 10% fetal calf serum (FCS) (GIBCO-BRL). Cells were lysed in 4 l hypotonic lysis buffer (10 mM Tris–HCl, pH 7.5, 1.5 mM MgCl₂, 0.25 mM Sucrose, 1 mM DTT and 0.5 mM phenylmethylsulfonyl fluoride). Nuclei were pelleted by centrifugation and the supernatant containing the cytosol was discarded. The nuclei were extracted with 100 ml of buffer F (10 mM Tris–HCl, pH 7.5, 250 mM NaCl, 30 mM Na₂PO₄, 50 mM NaF, 5 μM ZnCl₂, 0.25 M Sucrose, 1 mM DTT and 0.5 mM MgCl₂) and the bound proteins were eluted with 0.5 M fraction was diluted with BC0 to a final concentration of 150 mM KCl and loaded onto a 50 ml column containing ssDNA-Agarose (Sigma) equilibrated in BC100. The column was washed with 50 ml of BC100 and bound proteins were eluted with 500 ml linear gradient of BC100–BC500. Branch migration activity eluted at the KCl concentration of ~0.2 M. Fractions containing branch migration activity were pooled, dialyzed against BC100 and loaded onto a 1 ml column containing ssDNA-Agarose (Sigma) equilibrated in BC100. The column was washed with BC100 and the proteins were eluted with a 40 ml linear gradient of BC100–BC1000. Branch migration activity eluted at a KCl concentration of ~0.5 M. The active fractions were pooled and ~1/4 of this fraction was subjected to gel filtration on a 24 ml Superdex-200 (Pharmacia) FPLC column equilibrated and run in BC200. ATP-dependent branch migration activity elutes with a relative molecular mass of 150 kDa.

**Holliday junction substrates**

Synthetic Holliday junction substrate was prepared by annealing the following oligonucleotides in PCR buffer (Roche): oligonucleotide 1, (5’CCGCTACCACTGATCACAATGG-ATTGTGATACCTGTTGCGCCGCACTGCCGACGTTCGC-3’); oligonucleotide 2, (5’TGGGTGACCTGAGGTGGGC-AAAAGATGTCCTAGCATCCATTGCATTGTACAGTCGCAA-GCT3’); oligonucleotide 3, (5’GGACCTTACGCTCATAGA-CAATGAGTCTAGGACATCTTTGCGCTTTGCTAA-TATCGG-3’) and oligonucleotide 4, (5’TGGCCCATATT-GACAGACGCGCAAAGATGTCCTAGCATCCATTGG- TGATCATGGTACCCTG3’).

Annealing was carried out by heating the oligos to 95°C and then allowing them to cool to 25°C. 32P-labeled oligonucleotide 2 was annealed with a 2-fold excess of the other three oligonucleotides. Holliday junctions were then purified by gel filtration on a Sephadex G-200 column equilibrated and run in STE (10 mM Tris–HCl, pH 7.5, 1 mM EDTA and 50 mM NaCl).

**Holliday junction branch migration and resolution assays**

Approximately 5 ng of 32P-labeled synthetic Holliday junction was incubated in a 40 μl assay with 10 μl of each fraction (in BC50) and reaction buffer (60 mM Na₂HPO₄/NaH₂PO₄, pH 7.4, 6 mM MgCl₂, 1 mM DTT and 100 μg/ml BSA) in the presence or absence of 2 mM ATP when indicated. Assays performed for the first two chromatographic steps also contained sonicated salmon sperm DNA (Stratagene) 100 μg/ml to inhibit non-specific nucleases. The reactions were incubated for 45 min at 37°C. The reactions were then stopped and denatured with 200 μg/ml protease K and 0.1% SDS for 5 min at 37°C. DNA products were resolved on a 10% polyacrylamide neutral gel and visualized using X-ray film.

**Transfections and FLAG-immunoprecipitations**

HEK293 cells were cultured in DMEM supplemented with 10% FCS (GIBCO-BRL). Cells were transfected via the calcium phosphate method with 10 μg of vector that expresses the
indicated N-terminal FLAG-fusion protein. Nuclear extract was prepared as described above. For immunoprecipitations, nuclear extracts were incubated with anti-FLAG M2 Agarose beads (Sigma) for 4 h at 4°C. The beads were then washed extensively with buffer F and eluted with 2 bead volumes of buffer F containing 50 ng/ml of FLAG peptide (Sigma) for 12 h at 4°C.

Western blot
Proteins were resolved on 10% SDS–PAGE gels. Proteins were transferred to Immobilon-P (Millipore) probed with M2-FLAG monoclonal antibody in TBST (20 mM Tris–HCl, pH 7.5, 150 mM NaCl and 0.2% Tween-20). After extensive washing the blots were probed with anti-mouse secondary antibody coupled to horseradish peroxidase. The blots were washed and developed by chemiluminescence (Amersham Biosciences).

Mass spectrometry
The protein identification was carried out by nanoscale micro-capillary LC-MS/MS analysis method. An ULTIMATE capillary HPLC system with SWITCHOS II and FAMS autoinjector (Dionex, Sunnyvale, CA) and a LCQ DECA XP PLUS ion trap mass spectrometer (Thermo Finnigan, San Jose, CA) were used. A 10 cm length fritless 75 μm id fused-silica capillary column was packed with 5 μm, 100Å, C18 resin. The flow rate was 120 nl/min and the 0.1 M acetic acid buffer system was used. All MS/MS spectra were analyzed using SEQUEST program.

RNA interference (siRNA)
For RecQ1-siRNA, SMART pool® (siRNAs) were selected and produced by Dharmacon, Inc. HeLa cells grown in DMEM supplemented with 10% calf serum to ~30% confluence were transfected with RecQ1 siRNA. Twenty-four hours later the medium was replaced and the cells were transfected with RecQ1 siRNA for a second time. Forty-eight hours after the second transfection the cells were harvested for the SCE assay or RNA was isolated for RT–PCR. Transformations were preformed utilizing Oligofectamine™ following procedures recommended by the manufacturer (Invitrogen).

RT–PCR
RNA was extracted from cells with Trizol® (Sigma). For RT–PCR, a Superscript™ One-Step RT–PCR with Platinum® Taq kit was used, utilizing cDNA specific primer pairs, following procedures suggested by the manufacturer (Invitrogen). Products from the reactions were separated by agarose gel electrophoresis, stained with ethidium bromide, visualized by UV-illumination and photographed. Reactions contained the following primers: RecQ1 5′ primer 5-ATGGGCCTC-GTTCAGCTCTA-3, 3′ primer 5-TAACATCTATCAG-GCATCATCG-3; RAD54L 5′ primer 5-ATGAGGAGAGC-TGGGCTCCC-3, 3′ primer 5-ACCACACACCTGCTTGAT-TATCA-3; RAD51C 5′ primer 5-ATGCGCGGGAACAGCG- TTCCGC-3, 3′ primer 5-TTGAGATTTGTTTCTGGTTA-3

SCE assay
SCE assays were performed as described previously (27). Briefly, HeLa cells grown to ~60% confluency, 24 h after

RESULTS AND DISCUSSION
The factor(s) mediating the branch migration of HJs in human cells remains undefined since nuclear extracts prepared from BLM<sup>-/-</sup> and WRN<sup>-/-</sup> cell lines still possess comparable levels of ATP-dependent HJ branch migration activity (10). We therefore set out to identify the protein(s) responsible for the predominant ATP-dependent HJ branch migration activity present in human cell lines using biochemical purification. To accomplish this, we utilized an <em>in vitro</em> assay employing synthetic<sup>32</sup>P-labelled Holliday junctions (Figure 1A). The synthetic HJs contain a 26 bp homologous core flanked by regions of non-homology, as described previously (10). This assay detects both ATP-dependent HJ branch migration and HJ cleavage activity. ATP-dependent HJ branch migration leads to the formation of spliced DNAs whereas HJ cleavage produces nicked DNA products. Employing the <em>in vitro</em> assay, we followed ATP-dependent HJ branch migration activity through an extensive chromatographic fractionation of HEK293 cell nuclear extracts (Figure 1B). Owing to the presence of non-specific nucleases, the assays performed on early chromatography steps contained sonicated salmon sperm DNA in addition to the synthetic<sup>32</sup>P-labelled synthetic HJs. The nuclear extract was first subjected to phosphocellulose chromatography. The extract was loaded in a buffer containing 100 mM KCl and the bound proteins were eluted with sequential steps of buffer containing 350 mM, 600 mM and 1 M KCl. These fractions were then assayed for HJ processing activities. As depicted in Figure 1C, ATP-dependent HJ branch migration activity was detected in the 350 mM fraction, whereas the majority of HJ cleavage activity was present in the 600 mM fraction. We believe that a portion of this cleavage activity is the same as an activity previously described as Resolvase A, because after further fractionation an identical cleavage pattern was observed (data not shown) (28). The 350 mM fraction containing ATP-dependent HJ branch migration activity was then subjected to chromatographic fractionation on four more resins as summarized in Figure 1B. By the third column step (SP-Sepharose), all HJ cleavage activity was removed from the peak of branch migration activity. The final step of chromatography was a Superdex-200 gel filtration column. As depicted in Figure 2A, HJ branch migration activity eluted
Figure 1. (A) Schematic diagram of a synthetic Holliday Junction and the products of ATP-dependent branch migration and resolution. (B) Chromatographic scheme used to purify RecQL1. (C) Holliday junction branch migration and cleavage assay with HEK293 cell nuclear extract phosphocellulose fractions. Fractions were mixed with 32P-labelled synthetic Holliday junctions, with added ATP as indicated. 32P-labelled DNA products were separated by PAGE and analyzed by autoradiography.

Figure 2. (A) Holliday junction branch migration assay with fractions from the Superdex-200 gel filtration column. Fractions were mixed with 32P-labelled synthetic Holliday junctions, with added ATP as indicated. 32P-labelled DNA products were separated by PAGE and analyzed by autoradiography. (B) SDS–PAGE and silver staining of protein fractions from the Superdex-200 gel filtration column. Peptide sequencing by microcapillary HPLC-ion trap mass spectrometry identified the 70 kDa protein as RecQL1. Fractions were loaded as indicated and the positions of RecQL1 and molecular weight markers are indicated. (C) The purified RecQL1 protein exhibits ATP-dependent Holliday junction branch migration activity. Fraction 48 from the Superdex-200 gel filtration column was tested in the assay. The fraction and ATP were mixed with 32P-labelled synthetic Holliday junctions as indicated. 32P-labelled DNA products were separated by PAGE and analyzed by autoradiography.
Human RecQL1 is a member of the RecQ helicase family of proteins, which participate in maintaining genomic stability as a critical factor in Holliday junction (HJ) resolution. The frequency of SCE is reduced upon restoration of BLM expression (34). We therefore wanted to determine whether a reduction of RecQL1 levels in HeLa cells could have a similar effect on SCE. HeLa cells were chosen because they transfected well and form good chromosome spreads. Employing RNA interference (siRNA) we were able to achieve a >95% knockdown in RecQL1 mRNA levels (Figure 4A). SCEs were monitored using the SCE assay, which utilizes the differential staining of sister chromatids from cells that have replicated their DNA in the presence of bromodeoxyuridine (BrdU). The RecQL1-siRNA HeLa cells exhibited an ~3.5-fold increase in the number of SCEs per cell when compared with wild-type HeLa cells (Figure 4B and C). To assess if RecQL1 was also critical for DNA damage-associated SCE, we treated HeLa cells with mitomycin C (MMC), which is a DNA cross-linking agent that causes DNA replication fork arrest and increases the frequency of SCE events. We observed a similar 3-fold increase in MMC-induced SCEs in the RecQL1-siRNA HeLa cells compared with wild-type HeLa cells. Transfection of a control siRNA to GFP had no effect on the frequency of SCE with or without MMC (data not shown).

Here, we report the identification of RecQL1 as the protein responsible for a predominant ATP-dependent HJ branch migration activity present in nuclear extracts prepared from proliferating HEK293 and HeLa cells. Further support for RecQL1 as a critical factor in HJ resolution came from HeLa cells with reduced levels of RecQL1 mediated by siRNA, which exhibit a substantial increase in SCE that is further stimulated by MMC-induced DNA damage. It is important to consider our findings in light of a previous report that a genetic knockout of the RecQL1 gene in the chicken lymphocyte cell line DT40 yielded viable cells with no major defect in SCE (35).

RecQ family proteins are widely expressed in proliferating HEK293 and HeLa cells. Further support for RecQL1 as a critical factor in HJ resolution came from HeLa cells with reduced levels of RecQL1 mediated by siRNA, which exhibit a substantial increase in SCE that is further stimulated by MMC-induced DNA damage. It is important to consider our findings in light of a previous report that a genetic knockout of the RecQL1 gene in the chicken lymphocyte cell line DT40 yielded viable cells with no major defect in SCE (35).

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well as double-strand DNA repair (24,36,37). RecQ helicases are also proposed to function during DNA replication in restoring stalled or broken replication forks through homologous recombination (24). Inherited mutations in the genes encoding three members of this family, BLM, WRN and RecQL4, cause Bloom’s syndrome, Werner’s syndrome and Rothmund-Thomson syndrome, respectively. All three display genomic instability and a predisposition to cancer in humans or mouse models (24,38). Mutations in the RecQL1 gene have not been shown to be associated with any human disorders as of yet. Since it is likely that RecQL1 is a predominant HJ branch migration protein in proliferating cells, its loss may be too detrimental for survival. Alternately, this protein may serve other critical functions in development beyond an overlapping role with other RecQ family members in genomic stability.

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