DNA ligase III is the major high molecular weight DNA joining activity in SV40-transformed human fibroblasts: normal levels of DNA ligase III activity in Bloom syndrome cells

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ABSTRACT

The phenotypes of cultured cell lines established from individuals with Bloom syndrome (BLM), including an elevated spontaneous frequency of sister chromatid exchanges (SCEs), are consistent with a defect in DNA joining. We have investigated the levels of DNA ligase I and DNA ligase III in an SV40-transformed control and BLM fibroblast cell line, as well as clonal derivatives of the BLM cell line complemented or not for the elevated SCE phenotype. No differences in either DNA ligase I or DNA ligase III were detected in extracts from these cell lines. Furthermore, the data indicate that in dividing cultures of SV40-transformed fibroblasts, DNA ligase III contributes >85% of high molecular weight DNA joining activity. This observation contrasts with previous studies in which DNA ligase I was reported to be the major DNA joining activity in extracts from proliferating mammalian cells.

INTRODUCTION

Bloom syndrome (BLM) is a rare autosomal recessive human disease occurring at a significantly higher frequency within the Ashkenazi Jewish population (1). The clinical symptoms of BLM include short stature, sun sensitivity, immunodeficiency and a marked predisposition to malignancy (2). At the cellular level, the hallmark feature of this disease is chromosomal instability, in particular a marked increase in the frequency of spontaneous sister chromatid exchange (SCE) (3). BLM cell lines also exhibit increased rates of spontaneous mutation and somatic recombination (4,5) and a slightly increased sensitivity to the cytotoxic effects of several different DNA damaging agents (6,7). Studies on DNA replication in BLM cells have also revealed slower DNA chain growth and an abnormal profile of replication intermediates (8–10). Furthermore, *in vivo* joining of linear

plasmid DNA is reduced and error-prone in BLM cells (11). These abnormalities in DNA metabolism are similar to those observed in DNA ligase mutants of *E. coli* and *S. cerevisiae* (12,13) suggesting that BLM may be caused by a defect in DNA ligation.

In support of this hypothesis, alterations in high molecular weight DNA ligase activity, presumed to be DNA ligase I, have been observed in extracts from BLM cell lines. These include reduced levels of DNA ligase I, which was also anomalously temperature-sensitive, and forms of DNA ligase I with altered aggregation properties (14–16). However, the subsequent cloning and sequencing of the DNA ligase I gene failed to reveal mutations in BLM alleles (17,18).

There is increasing evidence that DNA joining is a complex function. Three distinct species of DNA ligase have been identified in mammalian cell extracts (19). The Stokes radius of the recently described DNA ligase III is similar to that of DNA ligase I (19,20) and therefore, after gel filtration, DNA ligase III contributes to high molecular weight DNA joining that was initially attributed solely to DNA ligase I. DNA ligase I activity is regulated by at least two different post-translational mechanisms. Phosphorylation of this enzyme in the N-terminal region is required for catalytic activity, suggesting that the non-phosphorylated N-terminal domain blocks the active site (21). A specific protein inhibitor of DNA ligase I has also been identified. This protein forms a complex with DNA ligase I and inhibits DNA joining but not formation of the enzyme-adenylate intermediate (22). Therefore, it is possible that the previously reported alterations in high molecular weight DNA ligase activity in BLM cell lines may have been caused by a defect in DNA ligase III or abnormal post-translational regulation of DNA ligase activity.

Cell fusion experiments between cell lines established from different BLM patients indicate that BLM is caused by a defect

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in a single gene (23). Microcell-mediated chromosome transfer has been used to demonstrate correction of the spontaneously elevated sister chromatid exchange phenotype and localize the BLM gene to human chromosome 15 (24). The cell line used in those experiments, GM08505, is the only SV40-transformed BLM fibroblast cell line available. It was reported previously that this cell line contains a reduced level of anomalously temperature sensitive DNA ligase I activity (16). In this study, we have determined the relative contributions of DNA ligase I and DNA ligase III to high molecular weight DNA joining activity in extracts from SV40-transformed human fibroblasts and examined the effect of phenotypic complementation in BLM cells on high molecular weight DNA ligase activity.

MATERIALS AND METHODS

Cell culture

GM00847 is an SV40-transformed fibroblast cell line established from an individual with Lesch Nyhan syndrome and has been used previously as a control cell line given that its growth characteristics and chromosome number are similar to that seen in GM08505 cells (24). GM08505 is an SV40-transformed fibroblast cell line established from a BLM patient. Microcell transfers of normal chromosome 15 to GM08505 was previously shown to yield multiple clones unambiguously complemented for the elevated SCE phenotype, including the subclone BSxA15-A3 used in the present studies (24). The chromosome transfers also resulted in rare subclones, such as BSxA15-H3, which acquired deleted derivatives of chromosome 15 and failed to demonstrate complementation (24).

DNA ligase assays

These were carried out as described by Tomkinson *et al.* (19,25). 1 unit of DNA ligase renders 1 pmol of 5' phosphate resistant to phosphatase in 15 min at 16°C.

Formation of DNA ligase-adenylate intermediate

Assays were carried out as described by Tomkinson *et al.* (25). After incubation with [α^{32} P]ATP, polypeptides were separated by denaturing polyacrylamide gel electrophoresis (26). Labeled enzyme-adenylate complexes were detected by autoradiography.

Partial purification of DNA ligase I and DNA ligase III

Cells were harvested from subconfluent monolayers, washed in isotonic phosphate buffered saline and frozen at -80°C. Cell pellets (2.3–3.7g) were resuspended in 20 mM Tris-HCl (pH 7.5), 1.5 mM MgCl₂, 1 mM DTT, 5 mM KCl, 1 mM PMSF containing 5 μ g/ml leupeptin, chymostatin, TLCK and pepstatin, and 1.9 μ g/ml aprotinin (Buffer A). After 20 min on ice, cells were lysed by Dounce homogenization. The solution was adjusted to 0.2 M NaCl by the addition of 5 M NaCl and the incubation was continued for 1 h. Cellular debris was removed by centrifugation at 10,000 rpm in a Beckman JA20. The pellets were re-extracted with buffer A containing 0.2 M NaCl and, after centrifugation, the supernatants were pooled. Proteins were fractionated by ammonium sulfate precipitation. Proteins precipitated by 35 to 67% ammonium sulfate were resuspended in 1 ml of 50 mM Tris-HCl (pH 7.5), 1 M NaCl, 1 mM EDTA and 0.5 mM DTT (Buffer B). After dialysis against buffer B, the dialysate was clarified by centrifugation and then applied to a 1 $\times$ 70 cm Ultrogel AcA34 column, pre-equilibrated with buffer B. Fractions were assayed for DNA ligase activity and active

fractions were pooled. An aliquot of this pool (Fraction I) was dialysed against 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 30 mM NaCl, 0.5 mM DTT, 50% glycerol (Buffer C) and stored at -20°C.

The remainder of the pool was further fractionated by hydroxylapatite chromatography. After the addition of KPO₄ (pH 7.5) to a final concentration of 1 mM, the sample was applied to a hydroxylapatite column that had been pre-equilibrated with 50 mM Tris-HCl (pH 7.5), 1 M NaCl, 0.5 mM DTT and 1 mM KPO₄. Proteins were eluted stepwise with 50 mM, 150 mM and 400 mM KPO₄ (pH 7.5) buffers containing 0.5 mM DTT. Fractions containing protein, measured by the method of Bradford (27) were pooled, dialysed against buffer C and stored at -20°C.

Immunoblots

Proteins were separated by denaturing polyacrylamide gel electrophoresis (26), transferred to nitrocellulose and detected by autoradiography following incubation with antiserum raised against homogeneous bovine DNA ligase I and [125 I] protein A as described previously (20).

RESULTS

Partial purification of high molecular weight DNA ligase activity

Extracts from a control cell line (GM00847, Fig. 1A), a BLM cell line (GM08505, Fig. 1B) and BLM non-complemented (BSxA15-H3, Fig. 1C) and complemented (BSxA15-A3, Fig. 1D) subclones were fractionated by ammonium sulfate precipitation and gel filtration column chromatography. Fractions from the column eluates were assayed for DNA ligase activity using the oligo (dT)/poly (dA) substrate. A single major peak of high molecular weight DNA joining activity was detected in all the cell lines. Under these fractionation conditions, DNA joining by the 72 kDa DNA ligase II is masked by contaminating nucleases (19). The control and BLM cell lines contained similar levels of the high molecular weight DNA ligase activity (Fig. 1A and 1B) and these levels were not altered by the presence of complementing and non-complementing versions of chromosome 15 (Fig. 1C and 1D). Furthermore, the relative molecular mass of the high molecular weight DNA ligase activity was similar for all the cell lines examined.

The high molecular weight DNA joining activity contains both DNA ligase I and DNA ligase III

A second high molecular weight DNA ligase activity, DNA ligase III, which can be distinguished from DNA ligase I by its ability to join oligo (dT) molecules hybridized to a poly (rA) template has been described recently (19). In assays with this substrate, we detected a peak of activity that co-eluted with the major peak of activity measured on the oligo (dT)/poly (dA) substrate in all the cell lines (Fig. 2A–D). The level of activity on the oligo (dT)/poly (rA) substrate was 7–15% of that measured on the oligo (dT)/poly (dA) substrate.

High molecular weight DNA ligase fractions were pooled and concentrated. In immunoblotting experiments with antiserum raised against homogeneous bovine DNA ligase I, we determined that these fractions from all the cell lines contained similar amounts of DNA ligase I protein per mg total protein. Assays of DNA joining by high molecular weight DNA ligase activity from the cell lines were linear over a 10-fold range of protein concentration. We were unable to detect any significant

Figure 1. Size fractionation of DNA joining activities measured with the oligo (dT)/poly (dA) substrate. Extracts from; **A**, GM00847; **B**, GM08505; **C**, BSxA15-H3; **D**, BSxA15-A3 were fractionated as described in Materials and Methods. Fractions (2 ml) from the gel filtration column were assayed for protein content (n) and DNA ligase activity (l).

differences in specific activity using either oligo (dT)/poly (dA) or oligo (dT)/poly (rA) substrates (data not shown).

When the high molecular weight DNA ligase fraction was incubated with [α^{32} P]ATP, we detected two labeled polypeptides with relative molecular masses of 125 and 100 kDa, corresponding to the sizes of the enzyme-adenylate intermediates formed by DNA ligase I (20) and DNA ligase III (19), respectively (Figure 3). This fraction appears to contain 2–3 fold more DNA ligase III molecules than DNA ligase I. However, the adenylation assay may not accurately reflect the relative amounts of these enzymes. The minor labeled polypeptide at 85 kDa may represent a proteolytic fragment of DNA ligase I and/or DNA ligase III since proteolytic cleavage of both these enzymes generates polypeptides of about 85 kDa that retain the catalytic activity and substrate specificity of the intact enzyme (19).

Separation of DNA ligase I and DNA ligase III by hydroxylapatite chromatography

In order to determine the relative contribution of DNA ligase I and DNA ligase III to the DNA joining measured in the high molecular weight DNA ligase fraction, we separated DNA ligase I and DNA ligase III by hydroxylapatite chromatography. Consistent with previous results, DNA ligase I was eluted from

the hydroxylapatite resin by 150 mM KPO_4 (20). Similar amounts of DNA ligase I were detected by formation of the 125 kDa enzyme-adenylate intermediate (Fig. 4) and by immunoblotting (Fig. 5) in the 150 mM KPO_4 eluates from the BLM parental cells, GM08505 and the complemented derivative of this line, BSxA15-A3. Although the 150 mM KPO_4 eluates contained significant amounts of DNA ligase I protein that was able to form the enzyme-adenylate intermediate (Fig. 4), these fractions contained surprisingly low levels of DNA joining activity (Table 1).

DNA ligase III, detected by formation of the labeled 100 kDa enzyme-adenylate intermediate, was present at similar levels in the 400 mM KPO_4 eluates from the BLM parental cells, GM08505 and the complemented derivative of this line, BSxA15-A3 (Fig. 4). In addition to the 100 kDa polypeptide, an 85 kDa labeled polypeptide was also detected. Both the 125 kDa form of DNA ligase I and the 100 kDa form of DNA ligase III can give rise to active fragments of about 85 kDa that retain catalytic activity (19,20). The majority of the labeled 85 kDa polypeptide in the 400 mM eluates appears to be derived from DNA ligase III as a cross-reaction was not detected at this relative molecular mass in the immunoblot with antiserum raised against homogeneous bovine DNA ligase I (Fig. 5). In DNA joining

Figure 2. Size fractionation of DNA joining activities measured with the oligo (dT)/poly (rA) substrate. Extracts from; A, GM00847; B, GM08505; C, BSxA15-H3; D, BSxA15-A3 were fractionated as described in Materials and Methods. Fractions (2 ml) from the gel filtration column were assayed for protein content (n) and DNA ligase activity (l).

assays, the 400 mM KPO₄ fractions from GM08505 and the complemented derivative BSxA15-A3 had similar levels of activity (Table 1).

The 400 mM KPO₄ eluates contained greater than 85% of the total DNA joining activity recovered after hydroxylapatite chromatography (Table 1). Therefore, we conclude that DNA ligase III is the predominant high molecular weight DNA joining activity in SV40-transformed human fibroblasts. This contrasts with the situation in extracts from bovine thymus glands and human lymphoblastoid cells where DNA ligase I constitutes the major activity (19).

DISCUSSION

The phenotypes of cell lines established from BLM patients are similar to those of microbial DNA ligase mutants, suggesting that this disease may be caused by a defect in DNA joining. There have been several reports of DNA ligase I with altered biochemical properties in BLM cells (14–16), but these observations remain controversial (28). Alterations in several other enzymes in BLM cells, including DNA topoisomerase II (29), uracil DNA glycosylase (30) and superoxide dismutase (31),

Figure 3. Formation of labeled enzyme-adenylate complexes by the high molecular weight DNA ligase fraction. Fractions from the gel filtration column containing DNA ligase activity were pooled and concentrated by dialysis as described in Materials and Methods. Reaction mixtures (20 µl) contained 60 mM Tris-HCl (pH 8.0), 10 mM MgCl₂, 50 mg/ml bovine serum albumin, 1 mCi α³²PATP were incubated for 15 min at room temperature with; lane 1, 1.4 mg BSxA15-H3; lane 2, 3.5 mg BSxA15-H3. Reactions were stopped by the addition of SDS sample buffer. After separation by denaturing gel electrophoresis, the gel was fixed, dried and labeled polypeptides detected by autoradiography. The positions of ¹⁴C-labeled molecular mass standards (Amersham) are indicated on the left. Similar results were obtained with the DNA ligase fractions from GM08505, GM00847 and BSxA15-A3.

Figure 4. Formation of DNA ligase-adenylate after hydroxylapatite chromatography. High molecular weight DNA joining activity from BSxA15-A3 and GM08505 were each fractionated by hydroxylapatite chromatography as described in Materials and Methods. Fractions (2 μ l) from the hydroxylapatite were assayed for formation of the enzyme-adenylate as described in the legend to Fig. 3. Lane 1, BSxA15-A3 50 mM KPO₄ eluate; lane 2, BSxA15-A3 150 mM KPO₄ eluate; lane 3, BSxA15-A3 400 mM KPO₄ eluate; lane 4, GM08505 50 mM KPO₄ eluate; lane 5, GM08505 150 mM KPO₄ eluate; lane 6, GM08505 400 mM KPO₄ eluate. The positions of ¹⁴C-labeled molecular mass standards (Amersham) are indicated on the left.

Table 1. Separation of high molecular weight DNA joining activity by hydroxylapatite chromatography

	Protein activity (mg)	DNA ligase activity (units)	Specific (units/ml)
BSxA15-A3; 150 mM KPO ₄	1.3	7.1	5.5
400 mM KPO ₄	1.1	53	48.2
GM08505; 150 mM KPO ₄	1.4	5.8	4.1
400 mM KPO ₄	1.3	45	34.6

High molecular weight DNA joining activity from BSxA15-A3 and GM08505 were each fractionated by hydroxylapatite chromatography as described in Materials and Methods. DNA joining activity was measured with the oligo (dT)/poly (dA) substrate.

have also been described. However, BLM appears to be caused by a defect in a single gene which has been mapped to chromosome 15 (23,24). The chromosomal location of the genes encoding the enzymes mentioned above have been determined and none of these genes reside on chromosome 15 (32).

The initial studies on DNA ligase activity were carried out prior to the discovery of a second high molecular weight DNA ligase, DNA ligase III (19). Furthermore, it is now known that DNA ligase I activity is regulated by phosphorylation (21) and by interaction with a specific protein inhibitor (22). This recent information about the complexity of DNA ligation has prompted a re-investigation of the biochemical properties of high molecular weight DNA ligase activity in extracts from SV40-transformed control and BLM fibroblasts. In addition, we have examined whether complementation of the spontaneously elevated SCE exchange phenotype of BLM by human chromosome 15 (24) alters high molecular weight DNA ligase activity.

After purification by ammonium sulfate fractionation and gel filtration, a single major peak of high molecular weight DNA ligase activity was detected. Within this peak, we identified DNA ligase I by immunoblotting and by formation of the 125 kDa enzyme-adenylate intermediate (20), and DNA ligase III by joining of the oligo (dT)/poly (rA) substrate and by formation of the 100 kDa enzyme-adenylate intermediate (19). Following

Figure 5. Detection of DNA ligase I by immunoblotting after hydroxylapatite chromatography. High molecular weight DNA joining activity from BSxA15-A3 and Gm08505 were each fractionated by hydroxylapatite chromatography as described in Materials and Methods. Proteins in the 150 mM and 400 mM KPO₄ eluates were separated by denaturing gel electrophoresis and transferred to nitrocellulose. The membrane was incubated with antiserum raised against bovine DNA ligase I. Antigen-antibody complexes were detected by incubation with [¹²⁵I]protein A (Amersham) followed by autoradiography. Lane 1, BSxA15-A3 2 μ g 150 mM KPO₄ eluate; lane 2, BSxA15-A3 2 μ g 400 mM KPO₄ eluate; lane 3, GM08505 2 μ g 150 mM KPO₄ eluate; lane 4, GM08505 2 μ g 400 mM KPO₄ eluate.

incubation of the high molecular weight DNA ligase fraction with [α -³²P]ATP, 2–3 times more labeled DNA ligase III-adenylate than labeled DNA ligase I-adenylate was formed. These results are comparable to those reported in the analysis of high molecular weight DNA ligase activity from the SV-40 transformed normal fibroblast MRCV (18).

After separation of DNA ligase I and DNA ligase III by hydroxylapatite chromatography, we determined that DNA ligase III contributed greater than 85% of the high molecular weight DNA joining activity. Indeed, this may be an underestimate because the ammonium sulfate fractionation conditions used were optimal for DNA ligase I and not for DNA ligase III (19). This was a surprising result since it had been reported previously that DNA ligase I was the major activity in proliferating cells (33). Studies utilizing bovine thymus glands or human lymphoblastoid cells demonstrated DNA ligase I to be the major activity (19,20).

In the only documented case of human DNA ligase I deficiency, the clinical symptoms of the patient included severe combined immunodeficiency, sun-sensitivity and growth retardation (18). DNA ligase I encoded by the mutant allele present in the SV40-transformed fibroblast cell line, 46BR.1G1, failed to form labeled DNA ligase I-adenylate although normal levels of labeled DNA ligase III-adenylate were formed (18). However, 46BR.1G1 extracts contained apparently normal levels of high molecular weight DNA joining activity. The data reported here, demonstrating that DNA ligase III is the predominant high molecular weight DNA ligase activity in SV40-transformed fibroblasts, appears to account for the previous paradoxical observations with 46BR.1G1 (18).

Considering the amount of DNA ligase I protein that retained at least partial activity as judged by enzyme-AMP formation, the level of DNA joining activity was unexpectedly small. In fact, the biochemical properties of the enzyme suggest that it may be in a complex with the recently described protein inhibitor of DNA ligase I that inhibits DNA joining but not adenylation (22). The level of this inhibitor, which was originally isolated from HeLa cells (22), may vary in different tissues and cell lines. It is possible that SV40-transformed fibroblasts contain high levels of the inhibitor but, at the present time, there are no specific molecular probes or immunological reagents for this protein.

Although BLM is not caused by a mutation in the DNA ligase I gene (17,18), alterations in DNA ligation may be due to a defect in DNA ligase III or abnormal post-translational modification of DNA ligase activity. We did not reproduce the previously reported reduction in high molecular weight DNA ligase activity in GM08505 cells (16) and found that this cell line contained similar levels of DNA ligase I protein compared with a control cell line. Furthermore, the level of high molecular weight DNA ligase activity was not altered in a derivative of GM08505, BSxA15-A3, in which spontaneous SCE frequency has been restored to normal levels. It was suggested by Mezzina *et al.* (28) that the reduction in high molecular weight DNA ligase activity in extracts from Ashkenazi Jewish BLM cell lines could be attributed to specific precipitation of BLM DNA ligase I by polmin P. However, this effect was not observed in immunoblotting experiments with DNA ligase I specific antiserum (33). Alternatively, it is possible that either BLM DNA ligase III or BLM DNA ligase I inhibitor may behave abnormally during polymyxin P precipitation. In contrast to the observations of Chan and Becker (34), we were unable to detect any difference in the ability of DNA ligase I from normal and BLM cells to form the enzyme-adenylate intermediate. This result indicates that there are no significant alterations in phosphorylation at the N terminal domain of BLM DNA ligase I, given that the absence of this modification blocks enzyme-adenylate formation (21).

In conclusion, we have shown that DNA ligase III is the major high molecular weight DNA joining activity in dividing SV40-transformed fibroblasts. We have been unable to detect any alterations in this enzyme or DNA ligase I from BLM cells. The unexpectedly low DNA joining activity of DNA ligase I in extracts from SV40-transformed fibroblasts suggests that the majority of this enzyme in this preparation may be complexed with the recently described DNA ligase I inhibitor (22). Previous to this report, DNA ligase III had been examined in thymocytes and a human B cell lymphoid line where it was found to be a minor activity relative to DNA ligase I (19). Since a defect in DNA ligase I appears to cause severe immunodeficiency (18), this enzyme may be present at elevated levels in cells of the immune system to complete immunoglobulin gene rearrangement. The high levels of DNA ligase III in dividing cells may indicate that this enzyme is involved in DNA replication or, alternatively, may be a consequence of SV-40 transformation. Further investigation of DNA ligase III and the DNA ligase I inhibitor in different cell lines and tissues may provide some clues as to the cellular role(s) of these proteins.

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